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Wound healing from dermal grafts containing CD34+ cells is comparable to split-thickness skin micrografts

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Short running head: Dermal micrografts for full-thickness wound healing
Abstract

Background: Epidermal stem cells present in the skin appendages of the dermis might be crucial in wound healing. In this study we located these cells in the dermis and evaluated their contribution to full-thickness wound healing in a porcine model.

Methods: Four sequentially deeper 0.35mm thick skin grafts were harvested from the same donor site going down to 1.4 mm in depth (Layers 1-4). The layers were minced to 0.8 x 0.8 x 0.35 mm micrografts and transplanted (1:2) onto full-thickness porcine wounds. Healing was monitored up to 28 days and biopsies were collected on days 6 and 10. Multiple wound healing parameters were used to assess the quality of healing.

Results: Our results showed that wounds transplanted with Layer 2 (0.35-0.7 mm) and 3 (0.7-1.05 mm) micrografts demonstrated comparable re-epithelialization rate as split-thickness skin graft (Layer 1- 0.00-0.35 mm; STSG) at day 10. At day 28 dermal micrografts (Layers 2 and 3) showed comparable quality of healing to STSGs (Layer 1) in terms of wound contraction and scar elevation index. The amounts of epidermal stem cells (CD34+) and basal keratinocytes (KRT14) at each layer were quantified by immunohistochemistry.

Conclusions: The analysis evidenced that Layers 2 and 3 contained the most CD34 positive cells and Layer 1 was the richest in KRT14 positive cells. The immunohistochemistry also indicated that by day 6 CD34 positive cells had differentiated to basal keratinocytes (KRT14), which migrated from the grafts and contributed to the re-epithelialization of the wound.
Introduction

Skin has a strong regenerative ability. In a continuous process, new epidermis is constantly formed by epidermal stems cells (SCs) in the basal layer of the epidermis. The epidermal SCs in the basal layer commit to a terminal differentiation and begin their migration towards the surface of the skin [1]. As they leave the basal layer, the cells switch from expressing keratins (KRTs) 5 and 14 to expressing KRTs 1 and 10. They migrate through the different layers of epidermis, meanwhile the supply of nourishment decreases; cells accumulate keratin, and eventually die [2]. After about a month, the terminally differentiated, already dead cells reach the surface and are shed. The delicate balance between generation of new cells and desquamation of dead cells is crucial for skin to function normally [3].

Epidermal SCs are multipotent adult stem cells that reside in distinct stem cell niches within the skin. Besides in the basal layer of epidermis, epidermal SCs are also located in the bulge regions of the hair follicles and in the sebaceous glands of the dermis [4]. In the intact skin, the hair follicle stem cells are responsible of the uninterrupted renewal of hair follicles. First they differentiate into highly proliferative matrix cells, which further differentiate either into hair shaft cells or inner root sheath cells [5, 6]. Epidermal SCs in the sebaceous glands normally terminally differentiate to sebocytes, which produce lipids and sebum [7]. In their niches epidermal SCs are shown to express various stem cell markers such as cluster of differentiation 34 (CD34) until they respond to changes in their environment and commit to differentiation based on signals they receive [8-10]. Although cells in the hair follicle bulge regions and sebaceous glands are multipotent they only function in hair follicle and sebaceous gland homeostasis unless the skin is wounded. When the skin is wounded these stem cells activate, start migrating into the wound site, differentiating
into epidermal cells and finally contributing to the re-epithelialization of the wound [11]. It is believed that this contribution of epidermal SCs is crucial for wound healing. For instance, the important contribution of hair follicle stem cells for wound healing was shown in a study that demonstrated delayed re-epithelialization in mutant mice that lacked all hair follicle development [12]. Also in a mouse wound healing model, Lin et al (2014) increased the mobility of epidermal SCs with an immunostimulant and demonstrated accelerated time of complete healing of full-thickness wounds in comparison to non-treated controls [13].

The purpose of this study was to locate the epidermal SCs in the dermis by harvesting sequentially deeper dermal grafts and explore their potential to re-epithelialize a full-thickness wound. Four sequential 0.35mm thick skin grafts were harvested from the same donor site going down to 1.4 mm in depth (Layers 1-4). Subsequently the dermal grafts were minced to 0.8 x 0.8 x 0.35 mm micrografts and transplanted onto full-thickness porcine wounds. Wound healing was followed over time and various healing parameters were compared to those of split-thickness skin graft micrografts.

**Materials and Methods**

**Animals and anesthesia**

All the animal procedures were approved by the Harvard Medical Area Standing Committee on Animals. Three female Yorkshire pigs (Parson’s Farm, Hadley, Mass.) weighing 50-60 kg were used for this study. Pigs were allowed to acclimatize for 72 hours before the experiments. Anesthesia was induced with intramuscular administration of 4.4 mg/kg tiletamine and zolazepam (Telazol; Fort Dodge Veterinaria) and 2.5 mg/kg xylazine (Xyla-Ject; Phoenix) per protocol. General anesthesia was maintained with 1% to 3% isoflurane (Novaplus, Hospira) and oxygen via endo-tracheal intubation. Oxygen saturation and heart rate were routinely
measured intraoperatively with pulse oximeter ear sensors. In addition, respiratory rate and rectal temperature were monitored throughout the procedure. After the procedure, pigs were transferred back to the pen and monitored during recovery from anesthesia. A transdermal patch releasing 25 µg fentanyl per hour for 72 hours (Duralgesic, Janssen) was given for pain management during surgical recovery and buprenorphine 0.005 mg/kg was administered IM immediately after end of the procedure.

**Skin graft harvesting and mincing**

Before skin graft harvesting the skin was thoroughly disinfected with successive applications of 10% povidone-iodine scrub (Betadine; Purdue Products LP) and 70% isopropanol (Aaron Industries). Four sequential 0.35mm thick skin grafts were harvested on the buttock region of the pig from the same donor site going down to 1.4 mm in depth (Layers 1-4) with a pneumatic Zimmer dermatome (Zimmer Inc). The harvested skin grafts were washed twice in the Dulbecco’s Modified Eagle Medium (Sigma-Aldrich, Steinhein, Germany) before mincing. The mincing device consists of 24 parallel rotating cutting disks 0.8 mm apart (Xpansion Micrografting System). Using this device, the grafts from all 4 layers were cut twice, with the direction of the second cut perpendicular to the first, micrografts measuring 0.8 x 0.8 x 0.35 mm were obtained.

**Wound creation, transplantation and biopsy**

All together 14 wounds were created on each pig: three for each layer and two control wounds (dry and wet). Six identical control wounds (3 dry and 3 wet) were included from our previous to increase the amount of both control groups [14]. After marking 2.5 x 2.5 cm wounds in two parallel paraspinal stripes on the dorsum of the pig, the outlines were tattooed with black ink using an electric tattoo marker (Spaulding &
Rogers Mfg., Inc., Voorheesville, N.Y.). Full-thickness wounds down to fascia were excised. Wounds were separated by at least 4 cm of unwounded skin. Micrografts were transplanted and spread evenly over the wound bed without regard to the orientation with a 1:2 expansion ratio. A polyurethane wound chamber (Corium International, Grand Rapids, Mich.) was applied to cover each wound. The MGs were allowed to adhere for 30 minutes before 5 ml of keratinocyte medium (KSFM; Thermo Fisher Scientific, Waltham, MA; containing human keratinocyte growth supplement, penicillin and streptomycin) was added to the wound chamber through an injectable port. Healing was followed up to 28 days and biopsies were collected on days 6 and 10.

**Experimental groups**

The full-thickness wounds created on the dorsum of the pig were divided in four experimental groups (Layers 1-4) and two control groups (wet control, WC and dry control, DC). The experimental groups were placed in a randomized fashion (drawing pieces of paper from a box) to minimize the effect of wound location on healing. The remaining two wound groups served as control wounds. The first control group, the wet control (WC), was covered with the polyurethane wound chamber and filled with keratinocyte medium. The second control group, the dry control (DC), was covered with gauze and tape. Wounds were biopsied with a 0.5-cm margin of surrounding unwounded tissue at 6, 10 and 28 days after wounding. Tissue samples were fixed in 4% neutral buffered formalin (Sigma-Aldrich), embedded in paraffin and sectioned for staining.

**Analyses**

All the analyses were performed in a blinded fashion.
**Wound Contraction**

Wound contraction was measured at day 28 of the tattooed margins from macroscopic wound photos using Image J software [15]. The area of each wound was measured and expressed as a percentage of its original size on day 0.

**Scar elevation index**

The degree of scar hypertrophy was calculated using scar elevation index (SEI) that represents the ratio of the total wound area tissue height to the area of normal tissue below the hypertrophic scar. A SEI of 1 represents no scarring while higher number represents increased scarring [16]. The measurements were conducted using image analysis software [15].

**Histology and immunohistochemistry**

At the end of the experiment the wounds were excised, fixed in formalin, embedded in paraffin and sectioned. Hematoxylin-eosin (H&E) stained tissue sections were observed by light microscopy by 3 experienced, blinded observers. For morphometric analysis, slides were examined using an Eclipse E400 light microscope, and images captured using a DS-Fi1 camera (Nikon Corporation). Quantitative measurements were performed using NIS-Elements D3.0 digital image analysis software (Nikon Corporation). Re-epithelialization was defined as the sum of the new epithelium divided by the original wound length indicated by the tattoo. Epidermal thickness was measured in 5 representative areas of neoepidermis for each wound cross-section. The number of rete ridges per millimeter of neoepithelium was counted under the microscope from 5 standardized locations in each wound after 28 days of healing.

To locate the epidermal stem cells and basal keratinocytes in the harvested layers and in the wound tissue the slides were labeled with CD34+ and KRT14 antibodies (Abcam plc, Cambridge, MA).
Statistical Analysis

Statistical comparisons were performed using GraphPad Prism 6.0 (Graph Pad software, Inc. La Jolla, Calif.) Data is presented as mean ± SEM. All the tracked parameters were analyzed using a 2-way ANOVA test. Values of p <0.05 were considered statistically significant.

Results

Skin appendages in porcine skin

The harvested skin grafts from each layer and intact porcine skin biopsies were processed for histology. The depth of hair follicles and sebaceous glands were measured in H&E stained unwounded porcine skin. The average depth for hair follicle bulb was 1.6 (± 0.08) mm and for sebaceous glands 0.6 (±0.1) mm. The H&E staining from different layers showed that layer 1 (STSG, 0-0.35 mm) contains the whole epidermis and parts of dermis. The second (0.35-0.7 mm) and the third layer (0.7-1.05 mm) contain the skin appendages and layer 4 (1.05-1.4 mm) is mainly dermal tissue (Figure 1 A-B).

Donor site healing

The donor site wound was created by harvesting four sequential 0.35mm thick skin grafts on the buttock region of the pig going down to 1.4 mm in depth. All the donor site wounds healed well with minimal scarring and contraction and were completely re-epithelialized by day 10 after skin graft harvesting (Figure 1C).

Wound Healing

Re-epithelialization

Wound re-epithelialization for micrografts from different layers and the control wounds was studied. H&E stained slides demonstrated that by day 6, wounds transplanted with micrografts from layers 1, 2, 3 and 4 were re-epithelialized 55.5 (±
5.0) %, 47 (±21.9) %, 34 (±15.3) % and 37 (±19.1) % respectively as compared to the control wounds which demonstrated significantly (P<0.05) smaller re-epithelialization rates on day 6 (Wet control: 11.2 (±1.6) %; Dry control: 4.9 (±0.5) %). By day 10, wounds treated with layer 1 and 2 micrografts had healed more than 70% (L1: 74.6 (±23.7) %; L2: 72 (±13.1) %) while layer 3 and 4 micrografts demonstrated 64 (±26.3) % and 44 (±16.3) % re-epithelialization. Wet and dry control wounds were 42.3 (±3.3) % and 45.1 (±5.3) % re-epithelialized respectively. Interestingly, no statistically significant differences (P < 0.05) in the re-epithelialization rates between transplanted layers were observed on days 6 and 10. However, in comparison to controls all the transplanted wounds had significantly greater re-epithelialization rate on day 6 (P<0.05). On day 10 the Layers 1 and 2 showed significantly (P<0.05) greater re-epithelialization than the control wounds. All groups including both control groups had completely re-epithelialized by day 28 (Figure 2A).

Rete ridges
The number of rete ridges per linear millimeter has often been used as an indicator of the strength of the dermal-epidermal junction. By day 28, the amount rete ridges was lower in all transplant or control groups compared to the intact skin. No statistically significant differences were seen between different layers or controls (Figure 2B).

Epidermal thickness
The epidermal thickness of the intact skin on the dorsum of the Yorkshire pigs was measured (115 µm) and compared to the re-epithelialized wounds on day 28. By day 28, the epidermal thickness of wounds transplanted with layer 1 micrografts most closely approximated the intact skin (112 µm). In general, the epidermal thickness in all wounds with transplanted layers was slightly greater than in the control wounds but there were no statistically significant differences (Figure 2C).
**Quality of healing**

The quality of healing was assessed measuring wound contraction and scar elevation index (SEI) on day 28 postoperatively. The wound contraction was measured and expressed as a percentage of its original size on day 0. Layers 1 (57.5%), 2 (54.7%) and 4 (59.9%) had decreased contraction as compared to the control wounds (wet control: 54.8%; dry control: 67.6%) but these differences were not statistically significant (Figure 3A). SEI represents the ratio of total scar connective tissue area to the area of underlying dermis. A SEI of 1 represents no scarring while higher number represents increased scarring. The results showed that the SEI was very close to 1 in wounds transplanted with Layer 1 and 2 micrografts while it was more than 1.5 in control wounds. No statistically significant changes were observed between the transplanted layers. However, they all showed significantly (P<0.05) decreased SEI in comparison to controls (Figure 3B).

**Identification of epidermal SCs**

To provide further insight, all the grafted layers and the sectioned wound biopsies on days 0, 6 and 10 were labeled with CD34 and KRT14 antibodies. A quantitative analysis was done by comparing the percentage of positive cells in each layer and wound for both markers. On day 0 the cells in the grafted Layers 1, 2, 3 and 4 were respectively 89 (±1.2) %, 26 (±2.9) %, 15 (±1.4) % and 10 (±0.9) % positive for KRT14 and 25 (±2.2) %, 80 (±2.6) %, 66 (±6.9) %, 45 (±5.3) % positive for CD34. On day 6 the grafts from layers 1, 2, 3 and 4 contained 84 (±4.7) %, 76 (±4.2) %, 62 (±3.4) % and 9 (±1.2) % keratin positive cells respectively and 13 (±1.7) %, 18 (±1.7) %, 10 (±1.2) % and 6 (±1.0) % CD34 positive cells. The immunohistochemistry demonstrated that the high percentages of CD34+ cells on day 0 on Layers 2 and 3 had significantly decreased by day 6 and the cells were mainly positive for KRT14.
On day 10 the grafts had already reached the surface of the wound and the separation of cells from the grafts and from the wound edges was not possible anymore. (Figure 4).

**Discussion**

Deep and large burns require hospitalization and, without exception, surgical treatment [17]. Full-thickness wound healing requires both dermal and epidermal components in order to heal through regeneration instead of wound closure primarily through contraction that might result in fibrotic tissue and scarring [18]. The gold standard is to excise the necrotic, burnt tissue and replace it with split-thickness skin grafts (STSG) that contain the whole epidermis and some parts of dermis depending on the thickness of the graft. In this process, patient’s own healthy skin is harvested and subsequently transferred to the burnt site after resection of the dead tissue [19]. Although efficient, it is associated with substantial problems, such as poor quality skin, infections, scarring and lack of donor sites in large burns [20]. In order to address the problem of inadequate donor sites, we have previously shown that STSGs can be minced to small 0.3 x 0.3 mm grafts with preserved viability [14]. In addition, we have shown that minced grafts are very effective in wound repair and can provide 100-fold expansion of a skin graft [21].

The utility of dermal grafts as an alternative for STSGs has been explored previously. Ono et al. (1993) harvested full-thickness dermal grafts from the backs or thighs of adult patients. The dermis was later cut into 300 μm thick dermis slices and plated on a culture dish and incubated in DMEM, to which epidermal growth factor had been added. Their results showed that after a week in culture the dermal sheets had epithelialized from the skin appendages present in the dermal sheets [22]. Lindford et al. (2011) performed 16 dermis grafts in 9 burn patients and showed that dermis grafts
can provide an additional autologous option for permanent coverage in acute major burn wounds without increasing donor site size or morbidity [23]. Rubis et al. (2002) explored the use of the split-thickness dermal graft in a pig model. Two 305 μm thick dermal grafts were harvested from the same donor site and transplanted on full-thickness porcine wounds. The dermal grafts were able to epithelialize the wound in 4 weeks showing that they may provide definitive coverage of full-thickness skin deficits [24]. In another study Kogan and Govrin-Yehudain (2003) harvested dermal grafts from 8 patients. The grafts were meshed in the 1:1.5 ratio and placed on the area of a third-degree burn or on the open wounds. The graft take was good and their results showed that dermal graft epithelialization took on average for 13 days. They also reported good donor site healing in all patients [25]. Coruh and Yontar (2012) studied split thickness dermal grafts as a source of auto-skin grafting and transplanted 11 deep partial- and full-thickness burns with dermal grafts. The epithelization was completed generally 1 week later in comparison to split-thickness skin grafts. Also in their study the graft take was good and donor sites healed without problems [26]. Similarly, like in our study Chuenkongkaew (2004) harvested four layers of thin split-thickness skin grafts simultaneously in one stage. The most superficial layer was used to cover the donor site and the three only dermis layers were used for reconstruction. In all, 24 patients took part of the study. They reported that grafts covering the donor site took well within 6 days. The dermal grafts on recipient sites took in 8 days and re-epithelialization was completed in 19 days. No differences between the transplanted dermal layers were reported in the study [27].

In the current study, we introduce a novel option to expand skin grafts and minimize the donor sites by harvesting multiple skin grafts from the same donor site and mincing them to 0.8 x 0.8 mm micrografts. Our results indicated that dermal
micrografts contribute not only to the regeneration of dermis but also to the regeneration of epidermis due to the epidermal stem cell (SC) reservoirs in the skin appendages of the dermis. Interestingly, our results showed that quality of healing and rate of re-epithelialization using dermal micrografts (Layer 2 and 3) that contain epidermal SCs are comparable to STSG micrografts. Immunohistochemical analysis suggested that epidermal SCs present on day 0 in the micrografts differentiate to basal keratinocytes by day 6. Subsequently they migrate from the grafts and contribute to the re-epithelialization of the wound. However, due to the small sample size of this study more research and greater number of subjects are needed to confirm the correlation between the amount of CD34 positive cells and the wound healing outcomes.

Conclusions

Our results indicate that this method enables an opportunity to utilize the same donor site by harvesting multiple layers. Furthermore, these grafts can be minced to micrografts (0.8 x 0.8 x 0.35 mm) and expanded extensively. Our results indicate that wound healing using dermal grafts (Layer 2 and 3) is comparable to STSGs micrografts. In addition, the immunohistochemical analysis suggest that CD34 positive cells in the skin appendages contribute to wound closure by differentiating into basal keratinocytes during wound healing.
References


Legends to figures

Figure 1

A: Four sequential 0.35mm thick skin grafts were harvested on the buttock region of the pig from the same donor site going down to 1.4 mm in depth (Layers 1-4). Bar represents 100 µm. B: The H&E stainings from different layers showed that layer 1(STSG, 0-0.35 mm) contains the whole epidermis and parts of dermis. The second (0.35-0.7 mm) and the third layer (0.7-1.05 mm) contain the skin appendages and layer 4 (1.05-1.4 mm) is mainly dermal tissue. Bar represents 100 µm. C: All the donor site wounds healed well with minimal scarring and contraction and were completely re-epithelialized by day 10 after skin graft harvesting.

Figure 2

A: By day 6, wounds transplanted with micrografts from layers 1, 2, 3 and 4 were re-epithelialized 55.5 (± 5.0) %, 47 (±21.9) %, 34 (±15.3) % and 37 (±19.1) % respectively. As the empty control wounds demonstrated less than 10% healing on day 6. By day 10, wounds treated with layer 1 and 2 micrografts had healed more than 70% (L1: 74.6 (±23.7) %; L2: 72(±13.1) %) while layer 3 and 4 micrografts demonstrated (±26.3) % and 44 (±16.3) % re-epithelialization. Wet and dry control wounds were 42.3 (±3.3) % and 45.1(±5.3) % re-epithelialized respectively. No statistically significant differences (P<0.05) in the re-epithelialization rates between transplanted layers were observed. B: By day 28, the amount rete ridges was lower in all transplant or control groups compared to the intact skin. No statistically significant differences were seen between different layers or controls. C: The epidermal thickness of the intact skin on the dorsum of the pigs was measured (115 µm) and compared to the re-epithelialized wounds on day 28. By day 28, the epidermal
thickness of wounds transplanted with layer 1 micrografts most closely approximated the intact skin (112 µm). *p<0.05, **p<0.001.

**Figure 3**

A: The area of each wound was measured and expressed as a percentage of its original size on day 0. No statistically significant differences between groups were seen. The wounds transplanted with Layer 2 micrografts showed least contraction. B: A SEI of 1 represents no scarring while higher number represents increased scarring. SEI was very close to 1 in wounds transplanted with layer 1 and 2 micrografts while it was more than 1.5 in control wounds. *p<0.05.

**Figure 4**

A-C: A quantitative analysis was done to compare the % of positive cells in each layer for CD34 and pancytokeratin immunostains. On day 0 the cells in the grafted layers 1, 2, 3 and 4 were respectively 89 (±1.2) %, 26 (±2.9) %, 15 (±1.4) % and 10 (±0.9) % positive for pancytokeratin and 25 (±2.2) %, 80 (±2.6) %, 66 (±6.9) %, 45 (±5.3) % positive for CD34. On day 6 the grafts from layers 1, 2, 3 and 4 contained 84 (±4.7) %, 76 (±4.2) %, 62 (±3.4) % and 9 (±1.2) keratin positive cells respectively and 13 (±1.7) %, 18 (±1.7) %, 10 (±1.2) % and 6 (±1.0) % CD34 positive cells. *p<0.05, **p<0.001, ***p<0.0001.
Figure 1
Figure 2
Figure 3
Figure 4