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Original Article

Histomorphological observation of surgical debridement combined with negative pressure therapy in treatment of diabetic foot

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ABSTRACT

Purpose: To further study the mechanism of epithelization on the fascia side of the flap after surgical incision and the treatment of the negative pressure therapy.
Methods: With the patients' informed consent, parts of tissue samples were obtained from a 51-year-old diabetic patient who was suffering lower extremity ulcers. The samples were processed with hematoxylin and eosin (HE) staining and Masson trichrome staining. The keratin 19, keratin 15 and carcinoembryonic antigen (CEA) were immunohistochemically detected.
Results: The results of HE staining showed that the specimen was divided into two regions, newborn area and original epithelial area. There were more inflammatory cells infiltrating in the dermis in the newborn epithelial area, compared with the original epithelial area. Cells in newborn epithelial area were more active and many dinuclear and polynuclear cells were observed in newborn epithelial area. But there were more cuticular layers and obvious rete pegs in original epithelial area. In addition, the cells with keratin 19 and CEA positive were found around hair follicle, while keratin 15 was negative. Masson trichrome staining showed that there was a lot of de novo collagen in newborn epithelial area.
Conclusion: Epidermal cells on the fascia side of the flap could be derived from the stem cells. Negative pressure wound therapy would attract not only cells but also other elements such as growth factors, cytokines, some nutrients and extracellular matrix. With the formation of the appropriate microenvironment after debridement, the migrated cells can grow, differentiate and spread, eventually leading to the epithelization on the fascia side of the flap in diabetic foot.

Introduction

Foot ulcer is one of the common clinical complications of diabetes. Patients with neuropathy and different degrees of peripheral vascular disease, often lead to wound infection, and (or) deep tissue damage. The treatment of lower limb ulcer needs a long time in wound healing and the recurrence of this disease is up to 50%. The clinical treatment on diabetic foot involves debridement, local negative pressure treatment and regular dressing change. Wound epithelization is regarded as a final important index to evaluate the wound healing. But the epithelization in diabetic foot is difficult in the process of treatment. One special case in our department, which had been reported previously,[1] caught our attention because we found the several scattered epithelial islands in the wound side of the fascia flap and the fast epithelization of the same site after the regular treatment. This phenomenon arose some new questions in our mind. Since these "newborn epithelial cells" are not spreading from surrounding healthy tissue, where are these epidermal cells derived from? How would they violate the cell polarity, and reversely grow to the fascia side? Would these epithelial cells derive from stem cells stored in normal basal lamina, or from the nucleated cells or adult stem cells in the circulating blood? As we all know, basal membrane of normal skin is enriched with epidermal stem cells, and hair follicle bulge contains lots of stem cells. In hairless areas such as palm of hand, sole of foot, epidermal stem cells are located in the basal layer connected with the top of stratum papillare dermidis. According to the general rules of wound healing, the keratinocytes at the wound margin are divided and migrated to cover the wound surface, so as to form a new epithelial layer.[2] But in this case, the newborn epithelial pieces sporadically grew out of central area of skin flap, with no contact with wound edge. Therefore, the newborn epithelial cells may be not derived from surrounding healthy

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tissue. Based on this phenomenon, we obtained tissue samples for further histopathological study after the patient gave the informed consent.

Materials and methods

Patients

A 51-year-old patient suffered lower extremity ulcers with 16 years history of diabetes. The patient was treated with urgent surgical incision to reduce tension and extensive debridement to remove the necrotic tissue after hospitalization. And then, the wound was washed with hydrogen peroxide and saline. In the meantime, negative pressure therapy was applied on the wound (7 days for each cycle). After a total of 4 courses of negative pressure therapy and 10 weeks of hospitalization, the wound completely healed. During the treatment, we were surprised to find that there were several scattered epithelial islands in the wound side of the fascia flap on the 7th day after surgical incision. These epithelial islands gradually expanded and converged (Fig. 1) a few days later. The tissue sample was obtained on the 12th day after surgical incision when written informed consent was obtained in order to further study this special phenomenon. A series of histopathological studies were carried on as follows.

Hematoxylin and eosin (HE) staining

The tissue samples were fixed in formalin for 24 h, regularly dehydrated by Shandon excelsior automation tissue dehydrating machine and were routinely embedded. Sections were deparaffinized and rehydrated with xylene, 100% alcohol, 95% alcohol and 75% alcohol. The sections were stained in Harris hematoxylin solution for 10 min, differentiated in 1% acid alcohol for 3–5 s until nucleus turned blue, then counterstained in eosin Y solution for 30 s, dehydrated in turn with 95% alcohol, 100% alcohol, xylene and mounted with neutral balsam.

Masson trichrome staining

Paraffin sections was deparaffinized to water. The sections were respectively stained in Harris hematoxylin solution for 10 min, ponceau 2R for 5 min, 5% phosphomolybdic acid for 20 min and 0.1% light green for 60 min.

Immunohistochemical staining of keratin 15, keratin 19 and carcinoembryonic antigen (CEA)

Paraffin sections (4 μm) were treated with 3% H_2O_2 and blocked with serum-blocking reagent for 10 min each at room temperature; sections were then incubated with primary antibody (keratin 15, 1:200 dilutions, Santa Cruz, sc-56520; keratin 19, 1:200 dilutions, Santa Cruz, sc-53258; CEA, 1:100 dilutions, Cell SIGNALING, #2383) overnight at 4 °C. Sections were washed with PBS and incubated with secondary antibody (1:100 dilutions, Santa Cruz) for 30 min at room temperature, washed with PBS, incubated with 100 μl GTVision ChemMate Envision HRP (DAKO) at room temperature for 30 min and developed with DAB substrate solution (DAKO KIT freshly made just before use) to reveal the color of antibody staining. All sections were then examined and photographed with Zeiss KS400 image analysis system (Carl Zeiss, Oberkochen, Germany).

Results

HE staining

After HE staining the specimen was divided into two regions, newborn epithelial area (A) and original epithelial area (B) (Fig. 2). Compared with area A, the original epithelial area (B) had more cuticular layers and obvious rete pegs (Fig. 3). Microscopic observation showed that the newborn region had a large number of cellular components compared with the original areas. There were a large number of inflammatory cells infiltrating in the dermis in area A (Fig. 4). Cells in newborn area (A) were more active, and many dinuclear and polynuclear cells were observed (Fig. 5).

Masson trichrome staining

The results showed that collagen fiber in newborn area (A) was almost de novo, thin and tightly arranged. But in original area (B), the collagen fiber showed progressive maturity from outer to inner layer, and the loose and dense collagen in dermal layer was arranged in an orderly manner (Fig. 6).

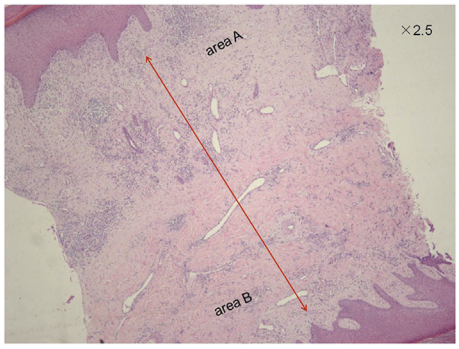

Fig. 2. HE staining on the 12th day after surgical incision showed that the specimen was divided into newborn epithelial area A and original epithelial area B.

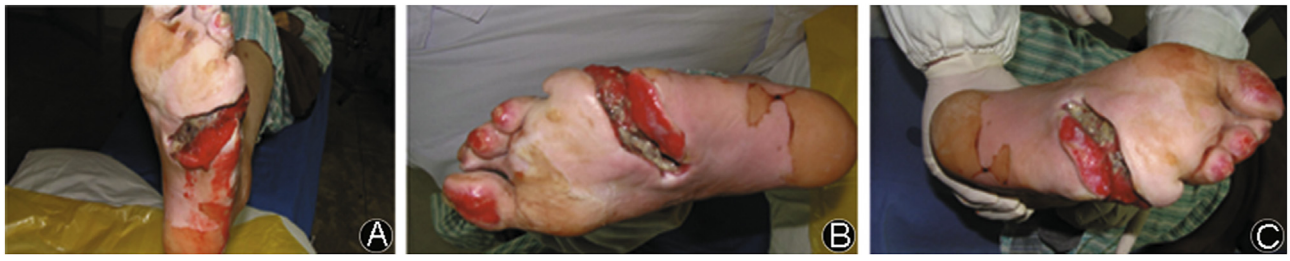

Fig. 1. **A**: On the 7th day after surgical incision, there were several scattered epithelial islands in the wound side of fascia flap. **B**: On the 8th day after surgical incision, epithelial islands were gradually broadened. **C**: On the 9th day after surgical incision, epithelial islands were almost confluent.

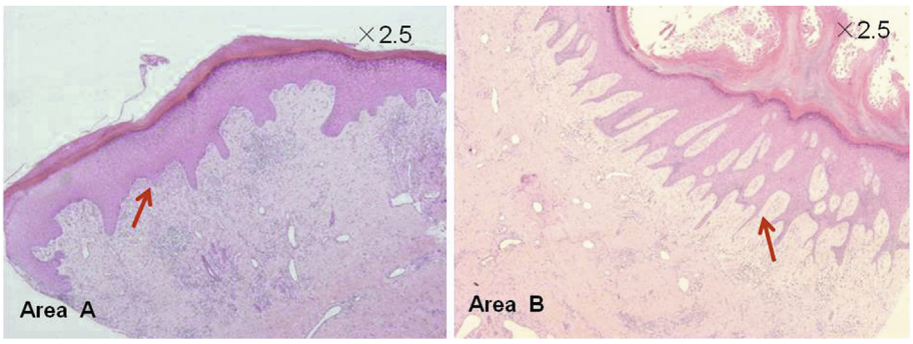

Fig. 3. HE staining on the 12th day after surgical incision showed that there were more cuticular layers and obvious rete pegs in the original epithelial area B compared with the newborn epithelial area A.

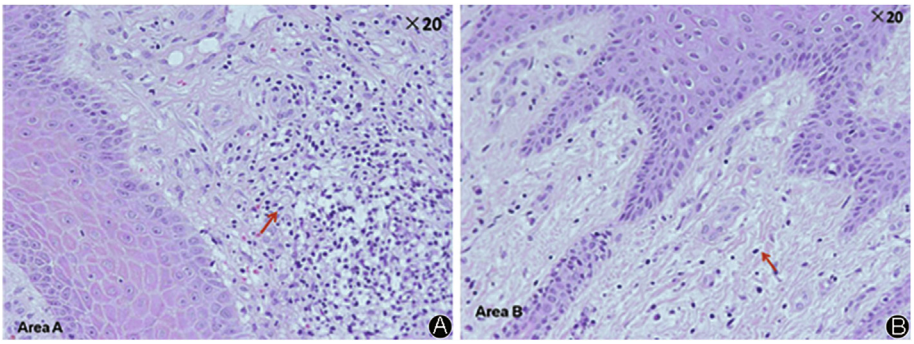

Fig. 4. HE staining on the 12th day after surgical incision showed that there were a large number of inflammatory cells infiltrating in the dermis in the newborn epithelial area (A) compared with the original epithelial area (B).

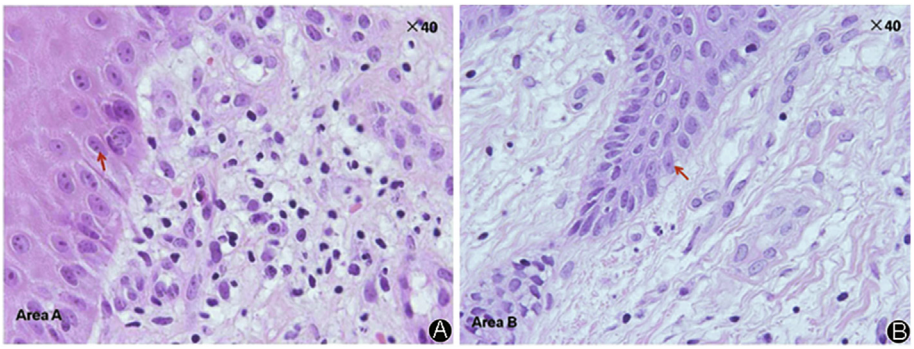

Fig. 5. HE staining on the 12th day after surgical incision showed that cells in newborn epithelial area (A) were more active and there were many dinuclear and polynuclear cells in area A.

Immunohistochemical staining of keratin 15, keratin 19, CEA

Keratin 19 positive and CEA weakly positive cells were specifically found in corium around hair follicle slightly near the original epithelial area (B) (Fig. 7). But keratin 15 positive cells were not observed in the same region (Fig. 8). CEA positive cells were also found scattered in the dermal layer of both the newborn and original areas (Fig. 9).

Discussion

The incidence of limb (especially the lower extremities) gangrene and ulcer in diabetic patients was about one hundred times higher than that in non-diabetic patients. Lower limb ulcer and gangrene were greatly increased, especially in patients with long duration of diabetes. Data showed that 15% of diabetic patients had ulcer during their life cycle. Once diabetic lower extremity ulcer occurred, it could develop rapidly and prolong the time of wound healing. Even 5%–10% of diabetic lower extremity ulcers required amputation. The treatment of refractory diabetic wound is challenging in clinic because the epithelialization of the wound site is always difficult. So how to promote wound epithelialization, and what is its mechanism are hot spots in researches.

In this diabetic foot case, the scattered epithelial pieces in the flap fascia side were found after conventional treatment of debridement, negative pressure therapy and dressing change. These epithelial pieces had a fast rate of expansion and fusion only in a few days. Further histological observation found that there were a larger number of inflammatory cells and dinuclear and polynuclear histiocytes. It showed the function of cell proliferation

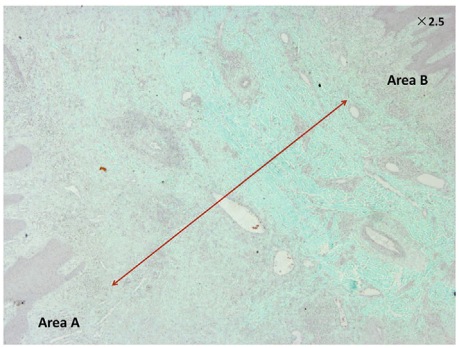

Fig. 6. Masson trichrome staining on the 12th day after surgical incision showed that there was much de novo collagen in area A and the collagen had progressive maturity from outer to inner layer in area B.

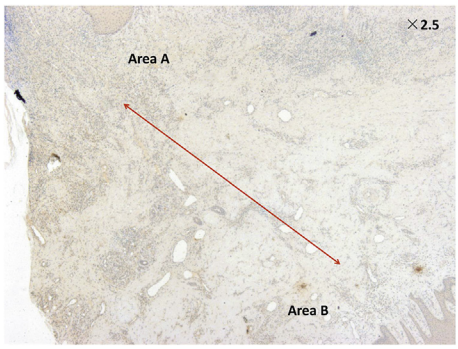

Fig. 8. Immunohistochemical staining showed negative expression of keratin 15 on the 12th day after surgical incision.

over newborn epithelial area. On the basis of immunohistochemistry results of this study, we found that there was keratin 19 and CEA positive expression around the hair follicle in the original epithelial side. But the expression of keratin 15 was negative in this part.

Keratin 15 and 19 are special skin epidermal stem cells markers, and CEA positive cells are considered to be embryonic stem cell-derived epidermal stem cells. With the gradual differentiation of stem cells, decreased expression of keratin 15 is earlier than that of keratin 19,[3] so the cells with keratin 15 negative and keratin 19 positive may be early transit amplifying cells (TACs). Stem cells are stored in a special microenvironment (or niche) under a resting state.[4] When this microenvironment changes, e.g. in trauma, these resting stem cells enter mitosis and become terminally-differentiated cells.[5] Based on cell division mechanism, stem cells are not directly differentiated into terminally-differentiated cells, but differentiated into TACs. After less than 10 times of postmitotic differentiation, TACs are further differentiated into postmitotic cells and terminally-differentiated cells. The regenerative capacity of the skin, including the continuous replacement of exfoliated cells and healing of injuries, relies on the epidermal stem cells and their immediate cell descendants. The relative contribution of hair follicle stem cells and interfollicular stem cells to dermal wound healing is still under active investigation. While hair follicle stem cells can contribute to the healing process by migrating into the wound field to help reestablishing the epithelial barrier.[6–9] The discovery of TACs around hair follicle in this study showed that stem cells in skin tissue of this area had initiated wound repair mode and entered the stage of differentiation. This provided strong evidences for our hypothesis that these epidermal cells might be differentiated from stem cells in the basal layer.

On the other hand, our result showed that newborn epidermal area contained a lot of binuclear and polynuclear histiocytes. Some scholars have found that embryonic stem cells, mononuclear cells or adult stem cells from peripheral blood such as marrow stroma cells (MSCs) have the function of multi-directional differentiation. For example, in inflammation period, they are locally migrated to the wound sites and transformed into macrophages; in the repair period, they can also be converted to repair cells.[10,11] Although the existing methods can not exactly confirm the types of dinuclear and polynuclear histiocytes, the positive expression of CEA can at least indicate that these cells have relatively primitive and strong proliferative activity.

Why did these epidermal cells not grow to the normal epidermis, but to the side of the fascia? We speculated that it might be closely related to negative pressure suction treatment. Vacuum-assisted therapy was applied to the wound by Argenta et al[12] and Fleischmann et al[13] since 1990s. It can not only reduce edema but also drain inflammatory cytokines and bacterial toxin.[14] Meanwhile, negative pressure creates a relative hypoxic environment for cells, which greatly promotes microvessel formation and inhibits the growth of aerobic bacteria.[15,16] Moreover, it enhances local blood supply and promotes granulation tissue formation.[1] Clinical practice has proved that local application of negative pressure treatment can promote the proliferation and migration of endothelial cells,[17] increase vascularization and oxygen tension, and reduce bacterial aggregation to promote wound healing.[12] Negative pressure suction is undoubtedly an external traction for tissues and cells because epithelial repair is related to cell polarity motion and cell polarity is essential for cell movement.[18] In this case, we concluded that negative pressure gravitation would change the movement polarity of stem cells or histiocytes and make the cell motion based on the physical strength, i.e., by cell protrusion force, the membrane can relax and retract to make the cell move forwards and migrate to fascial side of skin. At the same time, vacuum suction would induce the cells to migrate to the pressure side through the redistribution of elements such as growth factors, cytokines,

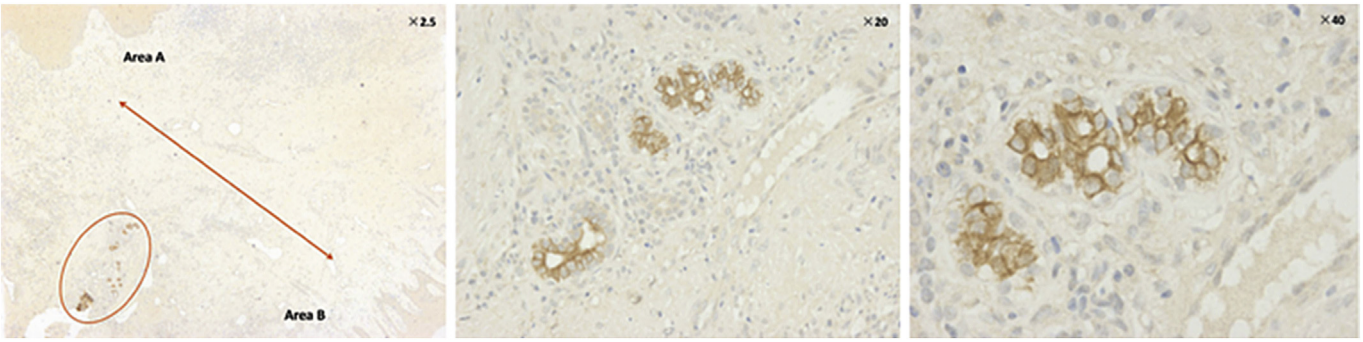

Fig. 7. Immunohistochemical staining on the 12th day after surgical incision showed that keratin 19 was a specific marker of hair follicle stem cells in corium slightly near area B.

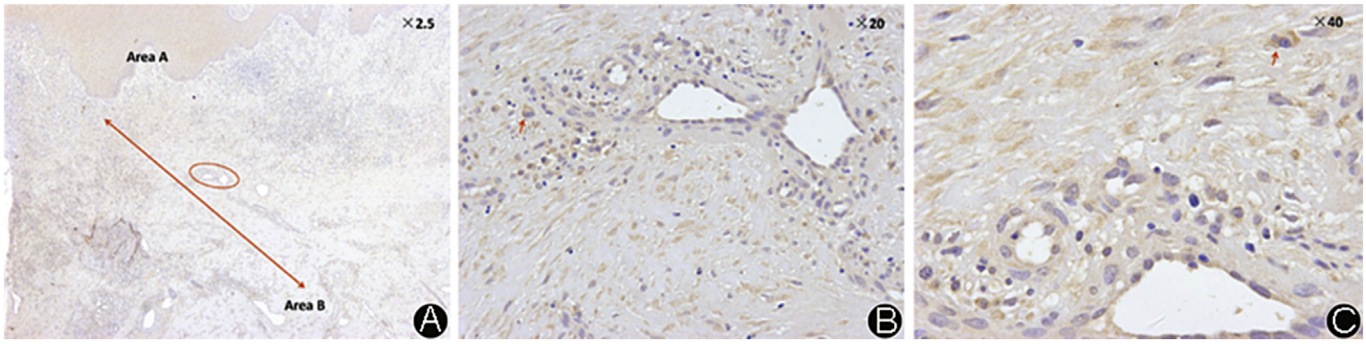

Fig. 9. Immunohistochemical staining on the 12th day after surgical incision showed that the cells with carcino-embryonic antigen weakly positive were found in corium around hair follicle in area B, but also scattered in both regions (A and B).

some nutrients and extracellular matrix which are necessary for the differentiation, proliferation and migration of cells.

During the treatment, other methods cannot be ignored such as debridement, changing dressing timely and washing with H_2O_2, which effectively remove the necrotic tissue in fascia side and provide beneficial base for the growth and attachment of cells.

To sum up, in this case, the patient's foot contained rich hair follicle stem cells, which became the good "niche" for stem cells to rest silently and could provide rich "seed cells" for epidermal reconstruction. The appropriate suction for skin tissue would provide directional traction for stem cell movement, change the "niche" of stem cells, prompt the stem cells to have mitosis, leave the "niche" after proliferation and differentiation and aggregate towards the negative pressure side and the wound area because of chemotaxis.[19] Besides, negative pressure therapy could attract cytokines, chemotactic factors and growth factors to the wound site in order to provide sufficient nutrients for the cells. In the appropriate microenvironment after debridement, the migrated cells could grow, differentiate and spread, eventually leading to epithelial coverage on fascia side of flap. In this study, we investigated the possible source of epidermal cells during wound healing process and validated the negative pressure treatment for wound healing. The mechanism of negative pressure suction on cell biology still needs further research.

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