

Epidermal Proliferation Is Not Impaired in Chronic Venous Ulcers

MONIQUE P.M. ANDRIESEN¹, BERT H. VAN BERGEN¹, KARIN I.J. SPRUIJT¹, I. HAN GO²,
JOOST SCHALKWIJK¹ and PETER C.M. VAN DE KERKHOFF¹

¹Department of Dermatology, University Hospital Nijmegen and ²Department of Dermatology, Maria Hospital Tilburg, The Netherlands

In this study we have investigated epidermal growth and differentiation during wound healing in human skin. The studies were performed in excisional wounds in normal skin and in chronic venous ulcers. Tissues were analyzed by immunohistochemical staining for proliferation-associated nuclear antigens (PCNA and Ki-67 antigen) and cytokeratin 16. Healing of excisional wounds was studied from day 2 to 14. Recruitment of resting (G_0) epidermal cells started within 2 days after wounding; the number of cycling cells was maximal at day 4 and continued to be increased (compared to baseline levels in normal skin) after wound closure (7–14 days). Cytokeratin 16, a proliferation-associated keratin, was induced within 48 h and was expressed in the suprabasal keratinocytes of the wound edge. Cytokeratin 16 expression was maximal at day 4 and was still present in the neo-epidermis after restoration of epidermal continuity (7–14 days). Surprisingly, in chronic venous ulcers, cycling cells were present in the wound edges of all stages of the leg ulcers studied. Both the number and localization of cycling cells were similar to those in normal wound healing. Cytokeratin 16 was strongly expressed in all these ulcers. Our *in vivo* data demonstrate that recruitment of G_0 -cells into the cell cycle is not impaired in venous ulcers, which suggests that epidermal proliferation is not a limiting factor in the healing process of chronic venous ulcers. **Key words:** wound healing; leg ulcer; epidermis; Ki-67; cytokeratin 16.

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M. Andriessen, Department of Dermatology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Wounding of skin induces a complex sequence of events which includes inflammation, increased synthesis and remodelling of connective tissue and activation of keratinocytes (for reviews see 1–4). The transition of keratinocytes from a sedentary to migratory lifestyle is an early event and is crucial for rapid wound closure. The extracellular matrix molecules and their cognate receptors involved in keratinocyte migration have been extensively studied over the past years (5–7). Skin injury was shown to have profound effects on keratinocyte differentiation, as shown by the induction of the hyperproliferation-associated cytokeratins 6 and 16 (8, 9), and the induction of endogenous anti-inflammatory mechanisms, as we described previously (10, 11). However, the kinetics and topology of keratinocyte proliferation during wound healing in human skin have not been described in detail. Most of our knowledge of cellular proliferation *in vivo* is based on studies of epidermal regeneration in experimental animals, using ³H-thymidine incorporation, such as mice (12) and guinea pigs (13). These and other studies (14) suggested that migration is an early event that precedes increased mitotic activity. In normal skin wounds, healing and re-epithelialization proceed in a highly coordinated fashion. In chronic venous ulcers the coordination

is lost and various stages of wound healing exist within one ulcer. In addition to the unknown pathogenetic factors that impair re-epithelialization in chronic ulcers, ingrowth of keratinocytes is limited to the wound margins because adnexal structures that normally contribute to resurfacing are often absent in the ulcer bed. Recently, application of recombinant growth factors has been suggested as a therapeutic modality in normal and impaired wound healing (15, 16). In order to assess whether application of these factors can affect epidermal proliferation and differentiation, or whether they act at the level of epidermal migration and dermal interactions, it is mandatory to obtain information on epidermal hyperproliferation in normal and impaired wound healing. We therefore studied the kinetics of recruitment of keratinocytes into the cell cycle during normal healing of excisional wounds, and at various stages of chronic venous ulcers. We determined the localization and the number of cycling cells using proliferating cell nuclear antigen (PCNA) and Ki-67 staining. In addition we studied keratinocyte differentiation by cytokeratin 16 staining. Both the localization and the numbers of cycling cells in chronic venous ulcers were found to be similar to normal skin wounds.

MATERIAL AND METHODS

Chemicals and antisera

A monoclonal antibody (Ks8.12) against cytokeratin 16 was obtained from Sigma Immuno Chemicals (St. Louis, MO., USA). Ks8.12 also recognizes cytokeratin 13, which is not present in normal and injured skin; hence positive staining was interpreted as cytokeratin 16 staining. A monoclonal mouse antibody to human Ki-67 antigen (MIB-1) was obtained from Immunotech S.A., Marseille, France. PCNA and rabbit-anti-mouse immunoglobulin conjugated with horseradish peroxidase (RAMPO) were obtained from Dakopatts, Glostrup, Denmark. The Vectastain Elite ABC-kit was obtained from Vector Laboratories Inc., USA and the metal enhanced DAB substrate kit was obtained from Pierce, Rockford, Illinois, U.S.A.

Skin samples

To study impaired wound healing, biopsies were taken under local anaesthesia from the margins of 8 chronic venous or mixed venous/arteriolar ulcers. The diagnosis of venous and mixed type ulcer was based on history, physical examination and Doppler investigations. Permission from the local medical-ethical committee was obtained prior to the experiments. Patients were between 29 to 70 years of age, with a mean age of 60.7 years. A wedge of tissue, approximately 10 mm long and 3 mm wide, was excised to include the surrounding intact skin, the ulcer edge and the ulcer base. The biopsies were taken at clinically different stages of wound healing: 2 biopsies were taken in the necrotic phase, 3 in the necrotic/granulating and 3 in the re-epithelialization phase. The peri-ulcer skin of all patients appeared normal, except for two that showed clinical signs of lipodermatosclerosis. All patients received local wound care and ambulant compression therapy prior to and following the biopsy. The biopsy sites healed within 1 to 2 weeks, without complications.

To study wound healing in normal skin, the following model was applied in 8 normal subjects aged 22–43, mean 33.8 years. In each

subject, 4 wounds were made under local anaesthesia on the outside of the upper arm at day 0, using a biopsy punch of 3 mm diameter. The wound depth was 1 mm. At days 2, 4, 7 and 14, respectively, punch biopsies of 4 mm diameter were taken comprising the previously made wounds and some surrounding tissue. The wound depth was approximately 5 mm.

Immunohistology

Each biopsy was rinsed in phosphate-buffered saline (PBS) and subsequently fixed in 4% buffered formaldehyde. After 24 h, fixed tissues were embedded in paraffine wax, sectioned at 5 µm and mounted on 3-aminoalkyltriethoxysilane coated slides. Sections were deparaffinized, rehydrated and, before staining cytokeratin 16, preincubated with normal rabbit serum. The sections were incubated with monoclonal anti-cytokeratin 16 and PCNA, respectively. After incubation with RAMPO, the sections were developed with aminoethylcarbazole as the chromogenic substrate. Before staining Ki-67, sections were pretreated in citrate buffer pH 6.0, heated in a microwave oven (17) and preincubated with normal horse serum. The sections were incubated with MIB-1 followed by incubation with biotinylated anti-mouse IgG. To enhance the staining signal the sections were incubated with an avidin-biotin-complex (ABC), after which they were developed with metal enhanced DAB as the chromogenic substrate. The sections were counterstained with haematoxylin. Scoring of positive cells was performed by determination of the average percentage of positive cells per 50 counted cells.

RESULTS

Histopathology of the wounds

Normal wound healing. Routine H&E staining of biopsies taken over a 14-day period showed the well-known features of unimpaired wound healing. A dense infiltrate of inflammatory cells was present shortly after injury, and the wound bed was filled with fibrinous material. After 4 days all wounds were covered with a scab. Immunohistochemical staining for leukocyte elastase confirmed the presence of large amounts of PMN (polymorphonuclear leukocytes) in the superficial zone of the wound bed (not shown). The margin zone of this

cellular infiltrate was located just beneath the wound edge. After only 2 days re-epithelialization had started, and a sheet of ingrowing keratinocytes was visible. Up to 4 days after injury, PMN were prominently present. After 1 week re-epithelialization was nearly complete and only a few scattered PMN remained in the original wound bed. After 2 weeks the keratinocytes had resumed their normal stratification. However, the epidermis still remained acanthotic, and rete ridges were lacking. The cellular infiltrate consisted of mononuclear cells, fibroblasts and endothelial cells.

Chronic ulcers. On histological examination three zones could be identified within each biopsy: the ulcer base, the ulcer margin and the surrounding intact skin. The surrounding skin appeared to be normal in most biopsies, except that it was clearly acanthotic. The ulcer margin was covered by epidermis that migrated over, within or under the fibrinous exudate. The ulcer base varied from a fibrinous exudate to granulating tissue. In all cases many infiltrating cells were visible. At all the ulcer stages studied, PMN and mononuclear cells were found within the ulcer base.

Epidermal hyperproliferation

Normal wound healing. In normal human epidermis only a small proportion (5–10%) of the keratinocytes are actively cycling, as determined by staining for the proliferation-associated nuclear antigen Ki-67 (18). As shown in Fig. 1, the Ki-67-positive cells are the putative transit amplifying cells that are present in the basal and first suprabasal layer. Normally, the majority of the epidermal cells are in the G₀-phase of the cell cycle, from which they can be recruited following tapestripping or during skin diseases as psoriasis (9). At day 2 after experimental wounding of normal human skin, an area of more than 90% Ki-67-positive cells was observed in the adjacent epidermis approximately 10 to 15 cells from the wound edge. In the wound edge, only 10–20% Ki-67-positive cells were observed. The outermost, leading

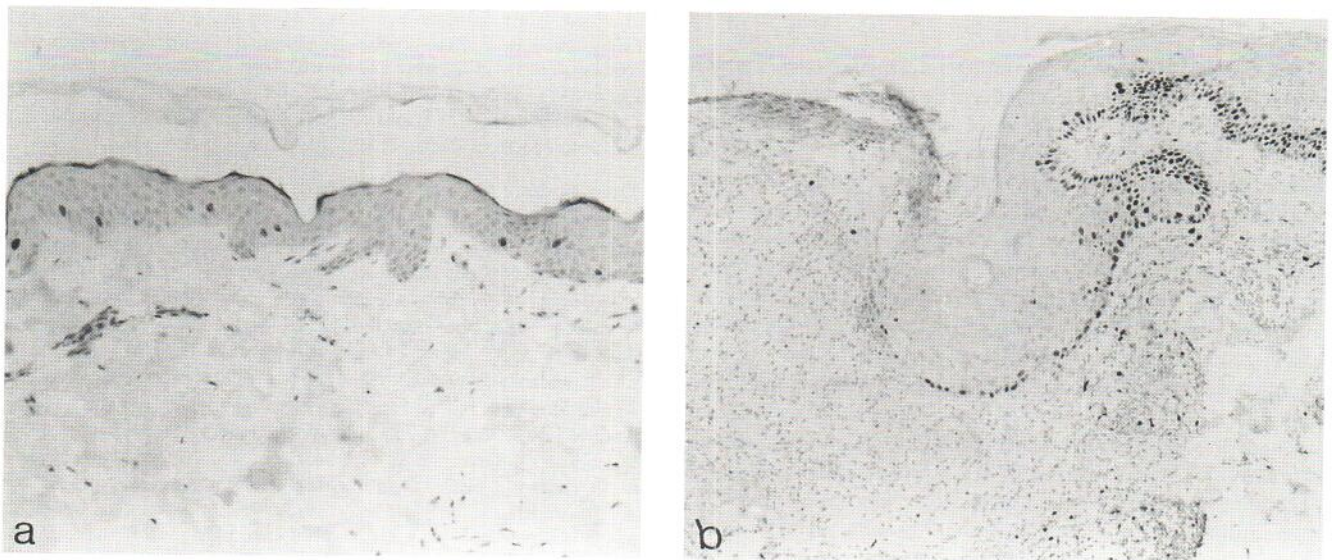


Fig. 1. (a) Histology of normal epidermis of a healthy volunteer, immunoperoxidase stained for Ki-67. Magnification 180×. Note the presence of Ki-67-positive nuclei in the basal and suprabasal layer. (b) Histology of a 4-day old wound, immunoperoxidase stained for Ki-67. Magnification 90×. Note the presence of a hyperproliferative zone of cells with pronounced nuclear staining, approximately 50 cells from the leading edge of the migrating epithelium.

edge of migrating keratinocytes with a flattened morphology was always negative for Ki-67. A steep increase in the number of positive cells was found going from the wound edge to the normal skin. At day 4 a similar hyperproliferative zone of more than 90% Ki-67-positive cells was observed at a distance of approximately 50 cells from the leading edge of the migrating epithelium (Fig. 1). One week after wounding complete re-epithelialization had taken place in most wounds. A hyperproliferative area (100% Ki-67-positive cells) was now observed directly under the original wound edge. The percentage of proliferating cells gradually diminished to approximately 20% in the direction of the centre of the wound. After 2 weeks a hyperproliferative basal layer was found under the entire neo-epidermis and the original wound edges (Fig. 2). All sections were additionally stained for PCNA, which is another marker for cycling cells and can be used to assess the growth fraction. Essentially the same results were found as for Ki-67 staining (data not shown).

Chronic ulcers. We studied several types of chronic ulcers within the spectrum of necrotic to re-epithelializing ulcers. In most ulcers a few Ki-67-positive cells were present at the distal part of the migrating epithelial sheet. Then, an intermediate zone was present at the wound edge, with 25–50% Ki-67-positive basal cells. As in normal wound healing, a steep increase to 75–100% positive cells was seen going towards the intact skin. This rise was seen approximately at a distance of 50 to 150 cells from the wound edge. Necrotic, necrotic/granulating and re-epithelializing ulcers all showed these areas with high numbers of Ki-67-positive basal cells (Fig. 3).

Epidermal differentiation during wound healing

Normal wound healing. At day 2 and 4, expression of cytokeratin 16 was seen in all cell layers of the migrating sheet of epidermal cells except for the most basal cell layer. This suprabasal cytoplasmic staining declined rapidly in the normal epidermis adjacent to the wound. One week after the initial injury half of the wounds were closed and half were nearly closed. In the latter the distal part of the ingrowing



Fig. 2. Histology of a 14-day-old wound, immunoperoxidase-stained for Ki-67. Magnification 45 \times . Note the presence of the hyperproliferative zone of Ki-67-positive cells under the entire neo-epidermis.



Fig. 3. Histology of a chronic ulcer, immunoperoxidase-stained for Ki-67. Magnification 90 \times . Note the abundance of Ki-67-positive cells in the basal layer of the epidermis adjacent to the wound edge (wound edge indicated by an arrow). The dotted line marks the dermo-epidermal junction.

epidermis consisted of 1 to 3 cell layers without distinct cytoplasmic staining for cytokeratin 16. In the remainder of the biopsies the newly formed epidermis consisted of 3 or more cell layers in which strong cytokeratin 16 staining was present (Fig. 4). Two weeks after injury the aspect of the epidermis was nearly normal except for acanthosis and the absence of rete ridges. Cytokeratin 16 staining remained positive in the suprabasal keratinocytes of the neo-epidermis and the former wound edges.

Chronic ulcers. The expression of cytokeratin 16 was identical in necrotic-, necrotic/granulating- and in re-epithelializing ulcers: a positive suprabasal staining of the ulcer margin and 1–3 positive suprabasal layers of the migrating sheet. Towards the intact skin keratin 16 staining gradually diminished (Fig. 5).

DISCUSSION

Re-epithelialization is an early event in the healing process of wounds. Within hours after injury keratinocytes migrate from

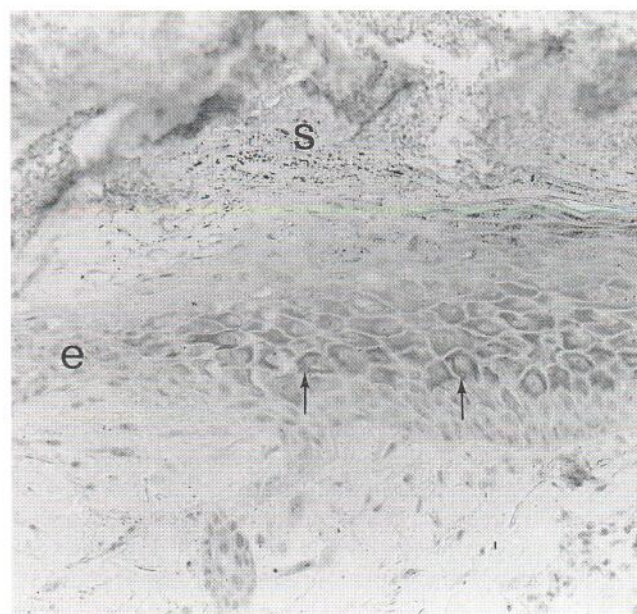


Fig. 4. Histology of a 7-day-old wound in the upper arm of a healthy volunteer, immunoperoxidase stained for cytokeratin 16. Magnification 180 \times . The ingrowing sheet of epidermis (e) is located beneath the scab (s). Note the suprabasal epidermal cytoplasmic staining of cytokeratin 16 (arrows).



Fig. 5. Histology of chronic leg ulcer, immunoperoxidase-stained for cytokeratin 16. Magnification $45\times$. The partly torn sheet of ingrowing epidermis is indicated by an asterisk. Note suprabasal epidermal cytoplasmic staining of cytokeratin 16 (arrows).

the wound edges and from the adnexal remnants over the wound bed (19). Another process which contributes to resurfacing the denuded area is cellular proliferation, which appears to be independent of migration and starts much later (19). In this study we have investigated the kinetics of keratinocyte proliferation after standardized full thickness wounding of normal human skin. It is shown that initially cellular proliferation is limited to a pool of cells some distance (50–100 cells) from the leading edge. At a later stage the entire neo-epidermis is engaged in active cellular proliferation. The localization of the proliferating pool of cells is similar to what was found in healing of wounded guinea pig skin, using ^3H -thymidine incorporation (13). Here we used the expression of proliferation-associated nuclear antigens to measure the growth fraction of the basal keratinocytes. Our findings are in contrast with the kinetics of cellular proliferation in mouse skin, where cell migration appears to be sufficient to cover the initial wound, and increased mitotic rates are only found after restoration of epidermal continuity (14). The induction of cytokeratin 16 in healing skin wounds is similar to findings in other model systems that we and others have described previously, such as tape stripping (9) and suction blisters (8).

To our knowledge, little information is available on keratinocyte proliferation and differentiation in chronic venous ulcers. The pathogenesis of chronic ulcers based on venous insufficiency is largely unknown and could be due to a host of different mechanisms. The reason that many of these ulcers fail to re-epithelialize properly could be due to disturbance of adhesion factors, migratory properties of the keratinocytes, or impaired cellular proliferation. Here we show that recruitment of basal cells from the G_0 -population into the cell cycle is very similar both in number and in topology to that found in normal wound healing. We therefore conclude that the perpetuation of chronic ulcers is not caused by a defect in cellular proliferation. Therefore, the therapeutic application of recombinant cyto-

kines that primarily promote keratinocyte proliferation appears to be redundant. Therapeutic strategies are possibly more effective when they promote the migrational behaviour of the epidermal cells, either directly or indirectly by enhancing the quality of the wound bed as a substrate for cell motility.

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