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PROLIFERATION AND ABILITY FOR EPIDERMAL AUTOREGENERATION IN PATIENTS WITH CHRONIC LOWER LEG VENOUS ULCERATIONS*

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The protein p63 plays a significant role in the development of animal epithelium. p63 is a regulator of differentiation, senescence and adhesion programs in numerous mature epithelial tissues. In patients with a healthy epidermis, p63 maintains cell progenitor potential – the ability for cellular division to occur using the delayed differentiation program. It is also responsible for the protecting the epithelial phenotype from depletion in migrating cells, thus resulting in invasion and infiltration after altering its endogenous expression.

The aim of the study was to compare the number of cells with p63 protein expression and the presence of Ki67 proliferation marker in the epidermis in patients with chronic venous ulcerations versus those with properly healing wounds.

Material and methods. Study materials were comprised of biopsy samples collected from healthy volunteers and patients treated for venous ulcerations. The specimens were subjected to immunohistochemical staining using available monoclonal antibodies and were analyzed with an imaging analysis program which evaluated the expression indices of both proteins in areas of intensified cellular division, i.e. wound edges.

Results. The number of cells displaying protein expression in patients with chronic venous ulcerations was significantly lower in comparison to the values observed in healthy volunteers. This was determined during the intermediary phase of wound healing, the most pronounced phase of cellular response to injury.

Conclusions. Decreased epidermal p63 expression in patients with venous ulcerations suggests insufficient protein production for the maintenance of autoregeneration and long-lasting division; both are required for the supplementation of migrating cells. The above-mentioned phenomenon suggests that there may be a role for p63 in regulation of the healing process and pathophysiology of chronic venous leg ulcerations.

Key words: p63 protein, proliferation, differentiation, keratinocytes, healing, chronic leg ulcerations

Trophic crural ulcerations that occur during chronic venous insufficiency constitute one example of improper wound healing. After many years of investigation, knowledge concerning the molecular etiology of the above-mentioned pathology is unknown. Damage attributed to increased venous pressure might be associated with venous capillary fibrosis and skin oxygenation disturbances (1), damaged capillaries and the activation of factors responsible for chronic inflammation (2), and increased vascular permeability for macromolecular "catching" of growth factors (3). These theories do not explain the characteristic features of chronic venous ulcerations and visible lower leg lesions. One may observe an epidermal wall localized at the margin of the wound which does not migrate towards the well-differentiated

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granulation tissue. The activity of keratinocytes is limited to the margins of the lesion (4). Granulation tissue fibroblasts, in spite of proper appearance, are also damaged and display limited mobility and cellular division (5).

Cellular migration and proliferation disturbances in the case of patients with ulcerations might be influenced by environmental factors (6). Venous hypertension, which is a primary etiological factor, directly affects the process of cellular senescence (7). The degree of differentiation and cellular senescence are responsible for healing (8). In a normal wound, the reserve of undifferentiated cells is continuously maintained and supplemented (9) which enables further transformation. p63 plays one of the most important roles in the maintenance of undifferentiation of cells and control of keratinocytic senescence. Its role in healing has not been investigated, although p63 expression changes in the migrating epidermis suggest a role for p63 in the control of epithelialization over granulation tissue (10). In a healthy epidermis, p63 maintains the undifferentiated potential of keratinocytes (11), regulates differentiation (12) and senescence (13), and the cellular adhesion program (14).

The expression of p63 in the basal layer of the epidermis is characterized by a patchy pattern appearance (11). Due to its physiological functions and limited number of cells, p63 is considered an important marker of epidermal and adnexal stem cells (15). It is, however, more feasible that p63 localization is evidence of the presence of partially differentiated and transiently amplifying cells (TIA) which are predominant and arranged in proliferative units. These units may correspond to the patchy protein expression. TIA cells, similar to stem cells, are subjected to epidermal transformation in chronic wound healing. In case of ulcerations, one may observe accelerated stem cell division which leads to local cellular depletion, limited capability of autoregeneration, and inhibited epidermal migration (16). TIAs might be associated with the effect of different proteins acting in synergy with p63 on the cellular cycle and regulating the proliferation of TIA cells during the healing process.

The comparison of p63 protein expression in cases of venous ulcers and healthy wounds demonstrate significant differences which might suggest the uneven ability of cells to maintain proliferation during both processes. The aim of the study was to determine the amount of p63 and Ki67 (proliferation marker) expression at the margins of depletion. p63 expression in epidermal ulcers is significantly lower relative to values observed in properly healing wounds; this might be evidence of reduced ability for autoregeneration of the ulcer cells, which is necessary during healing.

MATERIAL AND METHODS

The study group was comprised of 24 patients between 35 and 67 years of age with non-healing, crural venous ulcers without accompanying ischemia. Patients were qualified according to CEAP (Clinical Etiological and Anatomical-Pathophysiological) classification criteria with ulcerations described as C5-C6. For each patient, a small (4x5 mm) wedge-shaped skin sample from the edge of the wound or ulceration, which was comprised of undamaged epidermal fragments and adherent granulation tissue, was collected. Most of the specimen was subjected to histopathological evaluation to exclude neoplastic lesions. The remaining fragments were immersed in Jung's solution and immediately frozen in liquid nitrogen. The wounds were dressed and allowed to spontaneously heal.

For comparison, archival samples which were collected from properly healing wounds of 18 healthy volunteers were used. Samples were collected between one and 28 days from injury. The above-mentioned biopsy specimens were previously used in another study (17). Tissues surrounding the area of the planned biopsy were subjected to infiltration anesthesia (0.5% solution of lidocaine) at least 5 mm from the site of collection. For each volunteer, we collected a small sample of healthy tissue (2x2 mm) from the area below the axillary fossa. The wound was dressed and subjected to granulation healing. Between the first and 28th day after sample collection, the defects were completely excised, immersed in Jung's solution, and frozen in liquid nitrogen.

The following day all specimens were sectioned on a microtome and subjected to immunohistochemical staining. The study project was approved by the Bioethical Committee of Medical Center for Postgraduate Education.

Antibodies and immunohistochemical evaluation

Monoclonal mouse antibodies were obtained from Santa Cruz Biotechnology Inc. and No-

vocastra U.K. (4A4 anti-protein p63 and NCL-Ki67-MM1 anti-protein Ki67, respectively). In order to detect bound antibodies, the LSAB kit (DAKO, US) was used. Immunohistochemical staining was performed by the previously described protocol (17). Briefly, the specimens were prepared for staining by means of the peroxidase method, fixed in cold acetone, rinsed in phosphate buffered saline (PBS), and incubated in a 0.1% solution of hydrogen peroxide to block endogenous peroxidase activity. The specimens were then incubated with specific primary antibodies diluted in a PBS solution. After serial PBS washes, sections were incubated with secondary antibodies and streptavidine from the LSAB+ kit. The color reaction was performed using 3.3' diaminobenzidine (DAB) and hematoxylin. The correctness of the reaction was controlled for by replacing the primary antibody with non-specific mouse serum. All control investigations proved negative.

Calculations

The specimens were examined and photographed using a digital camera. Pictures were analyzed by means of specifically adapted picture analysis software (Mat Laboratory). The parameters of the program for the evaluation of the number of cells, color, and extent of the immunohistochemical reaction were calibrated in the same specimens in healthy skin outside the wound area.

Specimens of properly healing wounds were divided into three groups depending on the stage of healing. The first group was comprised of specimens collected between 1 and 4 days after trauma. The second group was comprised of specimens from the intermediary stage of healing (7-14 days after trauma), while the third group was comprised of specimens collected from scars in the early reorganization stage (20-28 days). Results were averaged in each group.

We evaluated the number of stained and non-stained cells in the area of thickened epidermis surrounding the margin of ulceration or the primary edge of the healthy wound. The number of cells demonstrating Ki67 and p63 expression was determined at the same site in subsequent slices of the same biopsy specimen. Results were presented as a protein expression index, i.e. the mean percentage of positive cells considering all epidermal cells in the investigated field of vision of 10 consecutive sections. The STATISTICA program was used for statistical analysis. Data distribution was evaluated by means of the Kołmogorow-Smirnow test and results were compared using the t-Student test. Non-parametric U-Mann-Whitney or Z-Wilcoxon tests were used for confirmation.

RESULTS

p63 and Ki67 protein expression in properly healing wounds

Between the first and second day after biopsy collection, we observed no significant changes as compared to the healthy skin with respect to the epidermis surrounding the defect and p63 or Ki67 expression. Significant changes in keratinocytic migration and rapid cellular stratification in the primary wound edge were observed three and four days after the injury. In this area, we observed an increased number of cells demonstrating Ki67 and p63 protein expression (fig. 1). The indices of protein expression in this stage were, however, similar to those of the healthy skin. Five days after the injury, the amount of cells positive

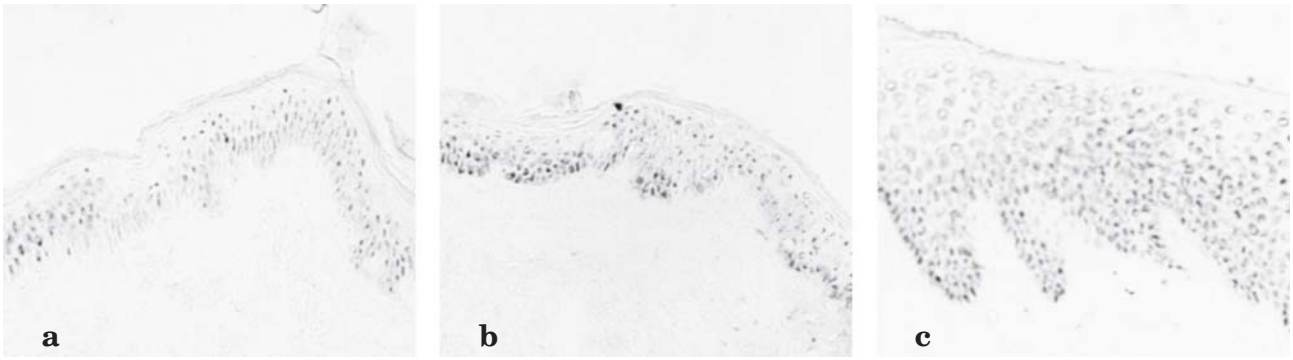

Fig. 1. p63 protein expression (a) in the healthy epidermis, and (b) in the margin of the wound, 3 days and (c) five days after injury. For specimen transparency, hematoxylin staining was not performed (magnification 400x)

for both proteins reached high values. Afterwards, the increase was non-significant.

During the period between the seventh and fourteenth day after injury, the migrating epidermis transversed the epidermis from the other side, covering the entire defect. The stratification of proliferating cells at the margin of the wound formed a visible thickening; Ki67 expression was detected in all cells of the basal layer, as well as numerous cells of the suprabasal layers. Simultaneously, cells demonstrating p63 expression in this area were predominant with respect to all cells in the healthy epidermis. The amount of Ki67 and p63 positive cells during the initial days of healing differed significantly from previously observed values (fig. 2). Biopsy specimens collected 20 days after the injury demonstrated a gradual decrease in Ki67 and p63 protein expression which approached values similar to those observed in healthy skin. Nevertheless, the above-mentioned values remained elevated 28 days after the injury (fig. 2).

p63 and Ki67 protein expression in patients with crural venous ulcerations

To determine proliferation and the potential for further division of keratinocytes in patients with venous ulcerations, we calculated p63 and Ki67 protein expression indices and compared them to normal healing wound indices. Comparisons with the early stage of healing (between 1-4 days) demonstrated no significant differences in the expression of Ki67 and p63 (fig. 3). This shows the limited ability of protein production in ulcer cells in comparison to early wound healing or healthy skin.

Comparison of ulcerated skin with the intermediate stage of proper healing when the cellular response is most pronounced (7-14 day) demonstrated a significant reduction of p63 and Ki67 indices (fig. 4). This is evidence of reduced mitotic activity and inhibition of the potential for further proliferation in patients with venous ulcerations (fig. 5). Comparison of ulcerated skin with expression during the early period of scar remodeling (20-28 day) demonstrated no differences in p63 protein expression. However, a decrease of the mean Ki67 value was observed; this value was below the proper value typical for scars (fig. 6).

DISCUSSION

Venous hypertension is the primary etiological factor responsible for skin ulceration during chronic venous insufficiency. It initiates the chain of pathological transformations leading to damage of the cells taking part in the healing process. It is well-known that ulcer fibroblasts display features of senescence and inhibition of proliferation (18) which is likely associated with the direct influence of venous hypertension (7). These cells are also characterized by a reduced response to the stimulating effect of TGF-β, which is connected with reduced receptor expression for this protein. As a result of phosphorylation disturbances of mitogen activated protein kinases (MAPK), growth regulation, differentiation, and apoptosis disturbances may

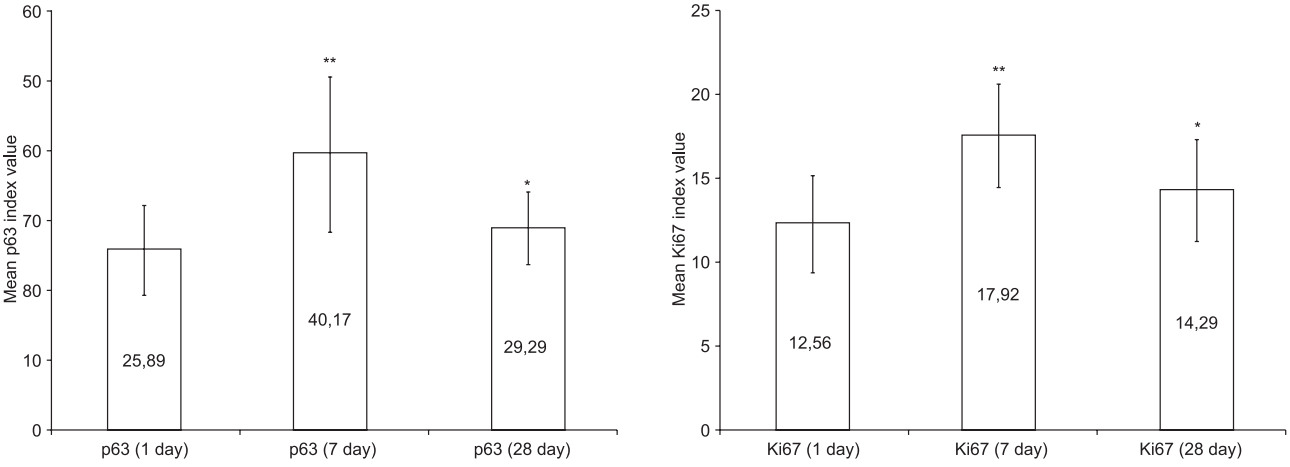

Fig. 2. Comparison of p63 and Ki67 expression indices during consecutive stages of proper healing. Statistically significant differences in the number of positive cells for both proteins are observed between 1-7 days (** $p<0.01$), and 7-28 days (* $p<0.05$)

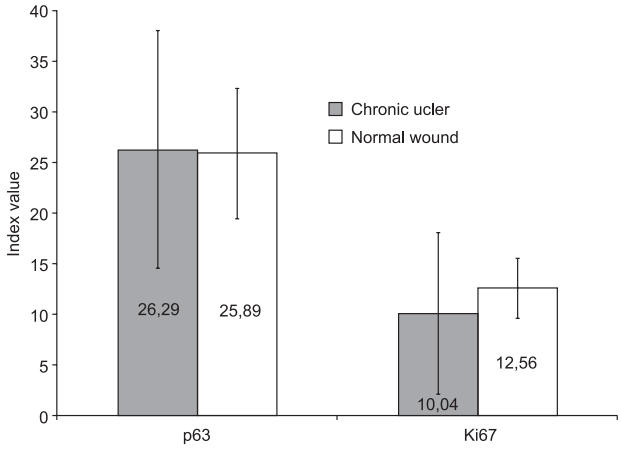

Fig. 3. Comparison of p63 and Ki67 expression indices in normal wounds and venous ulcers during the early stage of normal healing (1-4 days). Differences are statistically insignificant

appear (19). Apart from TGF-β, other proteins in the wound exudate might directly influence pathways regulating MAPK (5).

Relatively little is known about mechanisms resulting in keratinocyte migration disturbances. In spite of well-developed granulation tissue, keratinocytes accumulate at the margin of ulcers and are responsible for the absence of epithelialization. Increased proteolysis may inhibit the motility of these cells, thus influencing their ability to adhere to wound provisional matrix (20). Despite this, the physiological efficiency of ulcer keratinocytes remains unchanged (21). Since the expression of many proteins responsible for the regulation of proliferation, differentiation and apoptosis of ke-

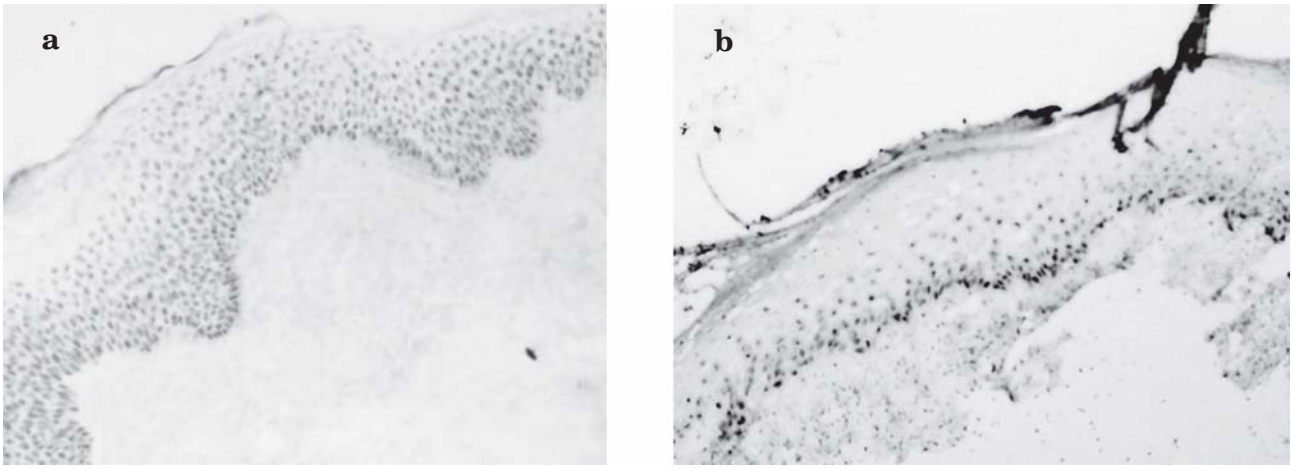

Fig. 4. p63 protein expression (a) in the margin of the normal wound 7 days after injury, and (b) in the ulcer edge. For specimen transparency, hematoxylin staining was not performed (magnification 400x)

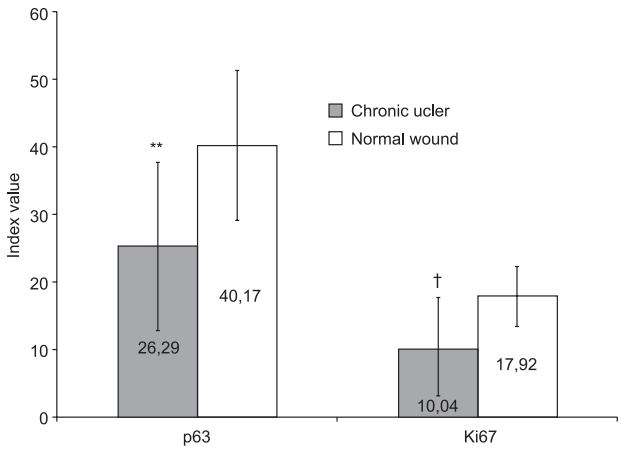

Fig. 5. Comparison of p63 and Ki67 expression indices in normal wounds and venous ulcers during the intermediate stage of normal healing (7-14 days). Differences in the number of positive cells are statistically significant for both protein p63 (** $p<0.01$) and Ki67 († $p<0.001$) expression

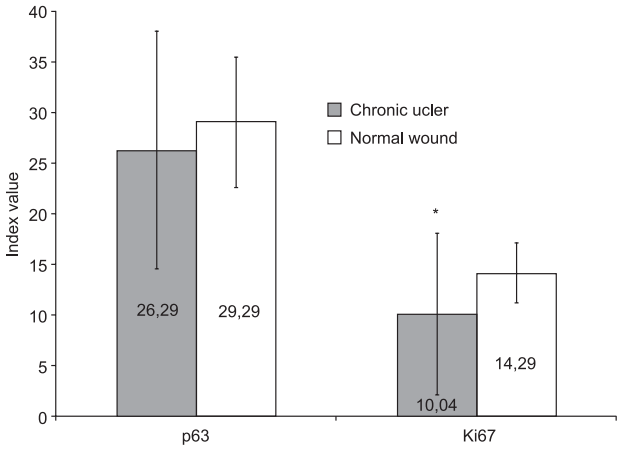

Fig. 6. Comparison of p63 and Ki67 expression indices in normal wounds and venous ulcers during the early stage of scar remodeling (28th day). Differences in the number of positive cells are statistically significant only for protein Ki67 (* $p<0.05$)

ratinocytes is also not decreased, some authors consider migration disturbances to be associated with abnormalities in the stroma of the wound such as inflammation or incomplete access to nutritional elements (22).

In the presented study, we observed a decreased number of proliferating and p63 positive keratinocytes at the margin of the ulcers; this may be a result of a limited cellular response to trauma. However, the response characteristic for chronic wounds might significantly differ from physiological reactions in acute wounds. The proper cellular response observed after injury consists of rapid metabolism changes in damaged keratinocytes which initiate the program of activation. During the initial hours they release interleukin-1 (IL-1) which prepares surrounding cells for the healing process. The differentiation program, appropriate for the undamaged epidermis, is inhibited at the margin of the defect as reflected by the appearance of p63 expression in the suprabasal layers (23). Keratinocytes initiate the protein production required for migration.

In chronic wounds, the program of activation does not proceed normally. The thick, cornifying marginal epidermis of such a defect displays features of parakeratosis, i.e. presence of nuclei in the corneal layer (24). The presence of nucleated keratinocytes in a layer where only dead cells reside suggests non-terminated or abnormal differentiation. It is possible that cells attempt to differentiate or initiate the activation program but due to unknown reasons, the transformations are interrupted and fail. The decreased number of cells displaying p63 expression may confirm the above-mentioned hypothesis. Reduced p63 activity inhibits the proliferating potential, that is the ability of cell division, at the cost of delayed differentiation. Thus, keratinocytes attempt differentiation which is also inhibited due to internal disturbances. In the epidermis of chronic wounds, one may observe the continuous activity of c-myc and the β-catenin pathway leading to the activation of stem cells until complete depletion (16). As a result, cells incapable of frequent division would initiate the differentiation program, although stimuli responsible for the activation pathway would also inhibit this reaction. Migration would be inhibited by the increased activity of β-catenin, a protein responsible for the inhibitory effect of glucocorticosteroids on the healing process (16). Interestingly, p63 also directly regulates the process of migration. Decreased protein expression is characteristic of a normally migrating epidermis (23). p63 regulates the process of epithelial adhesion (14) and the inhibition of its function stimulates cellular mobility and neoplastic infiltration (25).

As a result of the described disturbances, p63 expression in the ulcer epidermis might resemble healthy skin, where the proliferative potential and differentiation program are balanced. Only TIA cells divide, which is necessary for the regeneration of the exfoliating surface. The intensity of p63 staining in the ulcer epidermis was assessed semiquantitatively and only slightly differs from that observed in healthy skin (22). Abnormalities in protein expression may be observed in comparison with normal healing; p63 expression in the margin of the wound is increased.

However, this does not explain the reasons responsible for the fact that decreased expression of p63 and Ki67 proteins in ulcer cells may be disclosed only after comparison with the intermediate stage of normal wound healing, when the granulation surface is already epithelialized. As a matter of fact, similar observations concerning the expression of the Ki67 proliferating marker were previously observed by other authors (26). It may be associated with the metabolism of normal healing, when keratinocytic changes in the primary wound margin react to changes occurring in the migrating epidermis with some delay. Contact inhibition initially inverts changes in activated leading epidermis, but only in the center of the defect. After some time, the information reaches the primary margin of the wound which continued to supply the lesion with new cells.

Thus, the above-mentioned investigations suggest that there is insufficient expression of p63 in the epidermis of venous ulcers, which favors keratinocytic differentiation disturbances and long-term healing. However, further investigations are required to explain the role of p63 in the process of epidermal repair.

CONCLUSIONS

The proliferative potential (the ability for autorenewal and long-lasting division of the epidermis of venous ulcers) seems limited relative to the values observed in cases of properly healing wounds.

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