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# Invited Review

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## Novel Insights into Wound Healing Sequence of Events

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### ABSTRACT

Effective wound healing leads to restoration of tissue integrity and occurs through a highly organized multistage process involving various cell types. Currently, methods for wound healing assessment lack a structured system for analysis of quantitative parameters. We have established a unique quantitative assessment strategy of wound healing stages based on histological criteria. Distinctive immunohistochemical parameters including re-epithelialization, epidermal differentiation, cell migration, proliferation, inflammatory response as well as dermal closure, matrix distribution, and skin remodelling were identified and followed during the timeline of wound healing progression. Assessment was based on various defined characteristics and each stage-specific parameter was independently quantified for complete wound closure. This analysis allowed a follow-up of wound healing dynamics and identified the contribution of critical and specific parameters to wound healing physiology and pathology. In this review we demonstrate our assessment strategy of crucial wound healing events and introduce a unique quantification system for each of the processes involved in wound repair. We believe that our unique method can be utilized as a diagnostic platform for standardizing assessment of wound healing progression as well as a screening tool for potential therapies.

*Keywords.* Skin; wound healing; quantitative assessment; reepithelialization; matrix.

### INTRODUCTION

The various stages of wound healing have been widely investigated over the years and several major events associated with healing have been discussed in the literature (Bertone, 1989; Kirsner and Eaglstein, 1993; Singer and Clark, 1999). Wound healing is a multistep process that involves a multitude of cells and events. Initial stages of wound healing involve the formation of a blood clot and inflammation. The inflammatory response is followed by proliferation and migration of dermal and epidermal cells, and matrix synthesis, in order to fill the wound gap and reestablish the skin barrier (Cotran et al., 1999; Hackam and Ford, 2002; Harding et al., 2005). Finally, tissue remodelling and differentiation enable full recovery of the skin tissue and restoration of skin aesthetics (Hackam and Ford, 2002; Diegelmann and Evans, 2004). The consensus in the literature is that the stepwise process of wound healing first strives toward immediate filling of the gap, followed by re-epithelialization and reestablishment of the skin barrier (Yamaguchi and Yoshikawa, 2001).

However, while the mechanisms underlying skin physiology and function have been extensively studied *in vitro*, an in-depth evaluation of the wound-healing process *in vivo* has been almost unattainable, due to the lack of suitable animal models. In addition, the lack of a consensus for monitoring as well as a standard wound model in which to evaluate the complex interactions involving a multitude of cells and processes in the *in vivo* milieu, poses a challenge to the systematic investigation of wound healing. In this review, we present a quantitative, repeatable and reliable method for *in vivo* evaluation of the progression of wound healing in acute and chronic wounds. The sequence of events in wound healing was followed longitudinally from 1 to 30 days postwounding.

Monitoring throughout the wound-healing process enabled a longitudinal assessment of the discrete steps that occur over a time continuum. Evaluation of individual components allowed us to study the contribution of overlapping, but distinct steps to the overall process of wound healing. Furthermore, the methodology we developed enabled an accurate assessment of the underlying equilibrium in normal wound healing, of the changes which lead to healing impairment in wound pathology, and the influence of potential treatments. The insights gained from this type of assessment are expected to facilitate the development of novel therapies by delineating their specific contribution to the progression of discrete healing steps as well as to the continuum of the wound-healing process in a time- and stage-specific manner.

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Abbreviations: K1, keratin 1; K6, keratin 6; K14, keratin 14; H&E, hematoxylin and eosin staining; PCNA, proliferating cell nuclear antigen; NOD, non-obese diabetic mice; STZ, streptozotocine; HGF, hepatocytes growth factor; KGF, keratinocytes growth factor; TGFbeta, transforming growth factor beta; PDGF, platelet derived growth factor; IGF1, insulin-like growth factor 1; IL1, interleukine 1; betaFGF, beta fibroblast growth factor.

### QUANTIFYING WOUND HEALING—AN OBJECTIVE ASSESSMENT

Our model of skin damage encompasses the activation and participation of multiple processes which involve all skin components including: epidermis, dermis, hypodermis, blood vessels, and connective tissue. These processes are assessed through the study of skin properties and changes in skin-cell physiology, including proliferation, migration, differentiation, matrix synthesis, and tissue remodelling. When any of these processes does not function properly and the skin's physiological properties are impaired, appropriate treatment can prevent further skin damage and induce timely tissue repair. We designed an *in vivo* model system that enables us to perform uniform, reproducible wound-healing experiments. Utilizing our methodology we have found that to attain statistical significance groups representing each time point or healing parameter should include at least 6 animals. Stratified randomization of animals into groups ensured even distribution of various characteristics, such as weight and age.

The procedure requires the infliction of a full-thickness skin incision and follow-up of the healing period on a longitudinal time scale (i.e., 6 to 10 animals per group, for each time point, all wounded on the same day). In addition, each experiment includes a quality-control group with well established wound-healing properties (when testing the effect of various treatments, this is the vehicle-treated group). This model allowed us to quantitatively examine the effects of various peptides, growth factors and other pharmaceuticals on the skin's regenerative properties.

Traditional wound-infliction techniques, which mimic chronic wound-healing models include punch biopsies and excisions, which tend to form nonuniform wounds (Escamez et al., 2004; Geer et al., 2004; Thami et al., 2004; Zcharia et al., 2005). Such wounds produce nonhomogeneous wound groups, which poses a major problem in terms of experimental reproducibility. After testing several surgical procedures for wounding, we established a surgical method that meets our standard criteria. In this technique, we introduce a full-thickness, 2 cm long incision along the spine at the upper back of the mouse. By 3 hours after wounding, due to skin elasticity and mouse behaviour, the longitudinal incision becomes an elliptical, tear-shaped wound through all layers of the skin. This specific wounding technique produces homogeneous groups: it is easy to perform and is highly reproducible (Figure 1). In addition it ensures the consistency of the wound length when 3 hours after wounding only the width varies.

We followed the wound-healing process over a period of 30 days, examining various healing parameters on days 1, 4, 7, 9, 12, 18, and 30. The experiments were performed with acute animal-model systems, each consisting of 6 to 10 mice per group. In order to characterize the various wound healing steps, we focused on several critical milestones of the healing process: inflammation, reepithelialization, dermal closure, epidermal differentiation, tissue remodelling, and scar formation.

Histological assessment was carried out on sections derived from the widest part of the wound. Thus, only the most completely disrupted part of the wound was considered for healing assessment. By implementing this strategy, we were

able to assess distinct changes in the wound-healing process and ensure reproducibility.

Grossly, wound-healing analysis was dissected into several stages with characteristic parameters:

- At wound infliction, 1 to 3 days postwounding: This stage includes blood-clot formation (primary clot), activation of epidermal edges, and early inflammatory response (characterized by abundance of neutrophils at the wound gap).
- 4 to 7 days postwounding: Morphologically, this stage is marked by scab formation. Histological analysis reveals migration of the epidermal edges, selective proliferation of the early granulation tissue, and inflammatory response (lymphocytes and macrophages present in abundance).
- 8 to 12 days postwounding: Morphologically, scab detachment is observed. Histological results exhibit the formation of new epidermis that becomes differentiated by day 12. In addition, dermal closure is initiated, concomitant to granulation-tissue formation. This stage is accompanied by attenuation of the inflammatory response.
- 12 to 30 days postwounding advanced healing stages: characterized by matrix remodelling, terminal differentiation of the newly formed epidermis, increased elastic-fiber content and increased wound strength.

This array of functional parameters and structural changes can be followed by studying distinct markers, summarized in Table 1.

In order to allow accurate assessment of the data, a binary grading system was designed to enable quantitative analysis of distinct stage specific parameters of wound healing. Quantification of healing progression was based on the percentage of healed versus nonhealed wounds. In order to score as positive for a given parameter or stage in the study, wounds were expected to meet strict goals assessed independently for each parameter. The list of parameters evaluated is described here:

- **Epidermal Closure**—K14 staining was used to assess basal-layer formation (epidermal closure). Epidermal closure was recognized only in wounds exhibiting complete K14 staining across the wound gap. In wounds that did not exhibit complete basal-layer formation, the gap was measured in millimeters using a ruler at magnification 50X or 32X (32X for extremely large wounds) and wound sizes were compared. The analysis of wound closure as complete or not (with no intermediate score), was found to statistically empower the results by avoiding measurements of partial closure. In cases where experimental wounds were extremely large, relative closure parameters were obtained by assessing the appearance of epidermal wound edges in a fixed, predetermined field (epidermal migration).
- **Granulation tissue-staining of proliferating cells (PCNA), collagen fibers (Masson Trichrome) and H&E staining** were used to assess granulation-tissue formation, as shown in Table 1. Granulation tissue was considered fully formed (100%) when the following parameters existed at the wound gap: (1) a continuous layer of granulation tissue formed across the entire wound gap; (2) the layer of granulation tissue filled the entire wound depth. Wounds exhibiting sporadic formation patterns were considered negative for granulation-tissue formation.

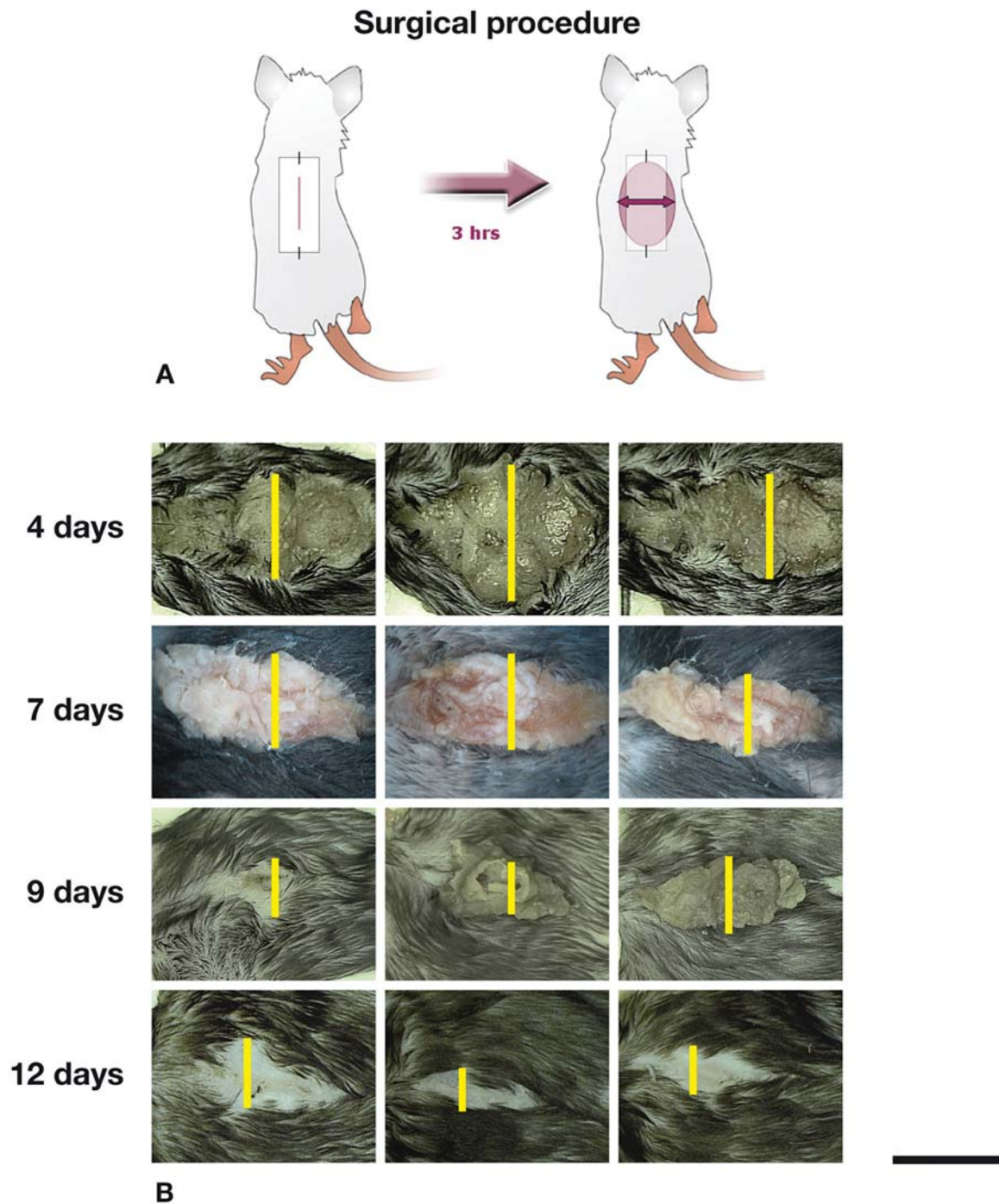


FIGURE 1.—(A) Wounding procedure: a 2-cm longitudinal incision is inflicted on the upper back of the mouse, after administering anaesthetic and shaving the fur. Following wounding, a sterile pad/surgical dressing (20 × 5 mm) is sutured to the mouse's skin with two stitches at the wound's top and bottom edges, 5 mm from each edge. When the mouse has fully recovered from anaesthesia, it begins to tamper with the wounded area, scratching it. Three hours after wounding, when the wound has taken on its final form, the homogeneity of the procedure is assessed by measuring the wound at its widest point (initial wound width). (B) Representative morphological results at days 4, 7, 9, and 12 postwounding are presented. At the end of each period, mice were sacrificed and the entire wound area, including the skin and tissues near the wound, were dissected and fixed in 4% paraformaldehyde. Pictures were then taken using a binocular. Scale bar in lower right-hand corner represents 1 cm. Following morphological assessment of all tissues, paraffin blocks were prepared and histological analysis performed only on the widest area of the wound (marked by yellow line).

- **Epidermal hyperplasia**—PCNA as an indicator of epidermal proliferation was determined per field of skin tissue. Total proliferation, as well as the contribution of supra basal proliferation to wound healing progression, was calculated at several time points during wound progression.
- **Inflammation**—H&E staining was used to assess the inflammatory response at the wound area. Three parameters were followed: (1) high leukocytosis at the wound gap (>200 cells in a fixed field, 200X magnification); (2) abundance of leukocytes in blood vessels at the wound gap;

TABLE 1.—Histological skin cell parameters for assessment of wound healing

Marker (abbreviation)	Healing Parameter	Assessment Parameter
Keratin-14 (K14)	Epidermal closure	Basal layer of the epidermis. To assess the newly formed epidermis
Keratin-1 (K1)	Epidermal differentiation	Spinous epidermal differentiation (early)
Filaggrin	Epidermal differentiation	Granular epidermal differentiation (late)
Keratin-6 (K6)	Epidermal migration	Migrating cells
Proliferating cell nuclear antigen (PCNA)	Granulation tissue formation	Epidermal hyperplasia
Masson Trichrome	Granulation tissue and matrix formation	Proliferating cells
Hematoxylin & Eosin (H&E)	Inflammation	Collagen fiber deposition
Elastic staining (Van Geisen)	Dermal closure	White blood cells, abscesses
	Late stage of matrix remodelling	Matrix remodelling
		Elastin fiber deposition

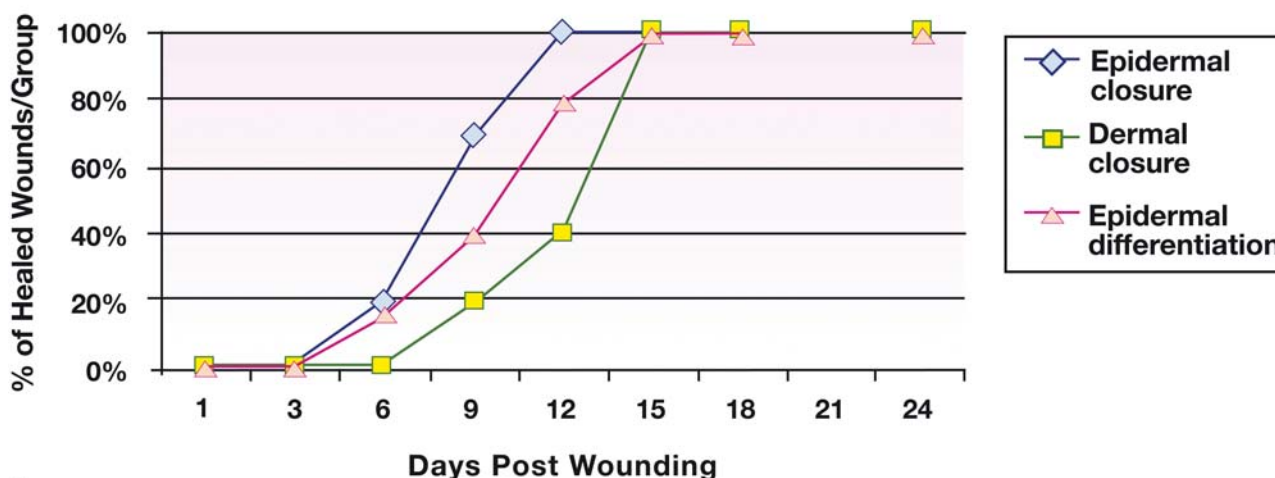
(3) abscesses at the wound gap. Wounds were considered severely inflamed when at least 2 of these parameters were present at the wound gap. In addition, specific cell types could be followed by staining for distinct inflammatory cell types such as neutrophils, macrophages, and mast cells.

- Dermal closure—H&E staining was used to evaluate dermal closure and remodelling. To assess dermal closure, the wound gap was measured in millimetres using a ruler under a binocular (Zeiss Stemi 200-C) at 50X and 32X magnification. Wounds exhibiting a gap of less than 2 mm between two dermal edges were considered closed.
- Granulation tissue formation—Masson-Trichrom staining for advanced granulation tissue formation as well as for tissue remodelling. The advancement of the collagen deposition was assessed by determination of the percentage of the wound gap covered completely with collagen. Advanced remodelling of the wound is defined by 100% collagen deposition through out the wound gap.
- Matrix Remodelling—Elastic fiber staining when present indicates late stage matrix remodelling. When elastic fibers

are observed the wound is considered to be at the final stages of matrix remodelling.

Through our extensive in vivo research of wound healing, we defined the longitudinal time course of healing encompassing all of these healing parameters. Figure 2 demonstrates the progression of several parameters towards healing. Thus, we demonstrate that epidermal closure precedes all other parameters and is fully achieved in all animals (100%) by day 12. However, matrix formation progresses considerably more slowly. For example, at 12 days following wounding, 40% of the wounds exhibit dermal closure. Only by day 18 have 100% of the wounds reached dermal closure; a week after epidermal closure has been completed. These results demonstrate that migration of the basal keratinocytes across the wound gap is an early step, initiating the wound-healing process. Similarly, by 18 days post-wounding, full differentiation of the newly formed epidermis is noted as well. The differentiation process appears to occur concomitantly with the dermal closure, but observing the scheme suggests that

Wound Healing Progression



A

FIGURE 2.—A Longitudinal Study of Selected Basic Wound-Healing Parameters. Summary of acute wound-healing experiments in C57BL/J mice (n = 140) by monitoring healing progression with specific markers: (1) Epidermal closure indicated by full distribution of K14 staining across the wound gap. (2) Dermal closure determined by measuring a gap smaller than 2 mm utilizing H&E staining. (3) Epidermal differentiation by demonstrating full distribution of K1 staining across the wound gap. The results are presented as % of total animals per experimental group. The graph represents the progression of wound healing as determined at various time points: 1, 4, 7, 9, 12, 18, 22, and 30 days.

on a longitudinal time scale, epidermal differentiation precedes dermal closure. As our quantitative assessment strictly scores, only full K1 staining of the formed epidermis, the end point of these two processes, is the same. Furthermore, sealing of the wound by formation of a new basal layer of epidermis is intimately linked to advancement of the wound-healing process, including dermal contraction and tissue remodelling.

This quantitative approach can be used to understand not only the various normal wound-healing events but also those occurring under pathological conditions. Thus, it can be used to pinpoint the specific stages that are impaired in distinct pathological conditions such as diabetes. In addition, development of effective novel therapies for wound healing can be incorporated into this assessment protocol to better understand their effect on the total wound-healing process. This quantitative approach to the development of new products will simplify the process while increasing its accuracy.

The manifest stages of wound healing:

**Reepithelialization:** Reepithelialization is widely accepted to be one of the major processes in wound healing that ensures successful repair (Escamez et al., 2004; Martin, 1997; Wysocki, 1999). Our wound-healing studies confirmed this by showing that at the very early stages of healing, keratinocytes at the wound edge begin to migrate across the wound gap. In contrast to the common assumption that keratinocytes are restricted to migration over newly formed matrix (i.e., granulation tissue), they can, in fact, migrate over any wound-related matrix, including clot-related debris. Thus, our studies showed that keratinocyte migration is independent of granulation-tissue formation and that it is, in fact, the first step in normal healing (Figure 3 panels B, F). Another interesting observation was that the hyperplastic epidermis at the wound edges, constitutes an essential component of the migrating cell pool which migrates to seal the wound gap. Our comprehensive longitudinal experiments demonstrated that this epidermal leading edge is composed mainly of cells with migratory properties rather than proliferating cells (Figure 3 panels B vs C). The proliferation rates of the cells in both the epidermis and the dermal gap peaks as reepithelialization and the synthesis of matrix components are established (Figure 4 panels B, D). Thus, migration of keratinocytes towards the gap of the wound in essence "jump starts" the wound-healing process and provides the basis for subsequent stages, such as fibroblast proliferation and migration, and granulation-tissue formation. Another common assumption is that differentiation of the epidermis follows formation of the full primary epidermal layer (Martin, 1997; Konstantinova et al., 1998). However, our observations indicated that although spinous differentiation of the epidermis (depicted by K1 staining) lags behind the migrating cell pool, differentiation is an ongoing process that continues until the epidermis covers the entire wound gap (Figures 3D and 4C). Epidermal differentiation is balanced by the proliferative capacity of keratinocytes. Thus, only nondifferentiating layers express this proliferative capacity, while in the differentiating epidermis, proliferation is attenuated and restricted to the basal layer (Figure 4E vs D). Thus our results show that initiation of healing is primarily dependent on epidermal migration which drives re-epithelialization; healing also requires differentiation and regulated prolifer-

ation processes occurring in a parallel and sequential manner.

**Inflammation.** Another major process involved in the early stages of healing is related to the inflammatory response (Lin et al., 2003; Jones et al., 2004). The initial inflammatory response involves the recruitment of cells that fight potential bacterial contamination of the wound and activate cytokine secretion to activate dermal and epidermal processes (Kirsner and Eaglstein, 1993). However, if inflammation increases beyond a certain level, it will lead to healing impairment, destruction of the early migratory effect and an arrest of the healing progression (Diegelmann and Evans, 2004). Furthermore, sustained chronic inflammatory response leads to ECM collapse and formation of necrotic centers.

**Matrix formation:** It is widely accepted that collagen formation is an initiating step of the wound-healing process (Kirsner and Eaglstein, 1993; Ruszczak, 2003). However, our longitudinal wound-healing data showed that the collagen formation characteristic of the early stages of wound healing is premature and non-structured, as indicated by the absence of specific staining at the wound gap (Figure 3F). It is our understanding that extensive proliferation of fibroblasts is the first manifestation of the early matrix, observed subsequent to attenuation of the initial inflammatory response. This extensive proliferation is closely linked to early granulation-tissue formation, in contrast to mature granulation tissue which is associated with deposition of collagen fibers at later stages of the wound-healing process (Richardson, 2004; Singer and Clark, 1999). Mature granulation is characteristic of a more advanced healing stage, and is apparent when a complete epidermis has already formed, granular differentiation is in progress and attenuation of fibroblast proliferation is observed (Figure 4A–4E). The formation of new matrix fibers, leads to a significant reduction in wound size (Figure 5A, 5B).

**Remodeling.** In the final stages of healing, wounds are fully reepithelialized and the final steps of dermal reorganization are underway (Figure 5 A, B). At this stage, the wounded skin regains its strength and elasticity, and proceeds through reorganization of the collagen and elastic fibers for final reconstruction of the dermis (Figure 5A). Normal dermal remodelling is also consistent with another healing stage, scar-tissue formation. Although there might not be any morphological traces of the wound, histological analysis always reveals a small section marking of the wounded area, identified by a gap in hair-follicle distribution.

This stage marks the termination of the healing process since all of the other assessed parameters have returned to pre-wounding levels and distribution. Final tissue remodelling is analyzed by measuring skin strength utilizing bursting chamber system (Seror et al., 2003).

#### *Use of the HealOr Assessment Strategy for Analyzing Diabetic Wound Impairment*

Diabetes mellitus is well known for its skin complications, usually leading to the formation of chronic debilitating ulcers (Levin, 1995; Cavanagh et al., 1998; Brem et al., 2003). Wound-healing impairment is characterized by the inability of the healing process to progress, thus leaving the

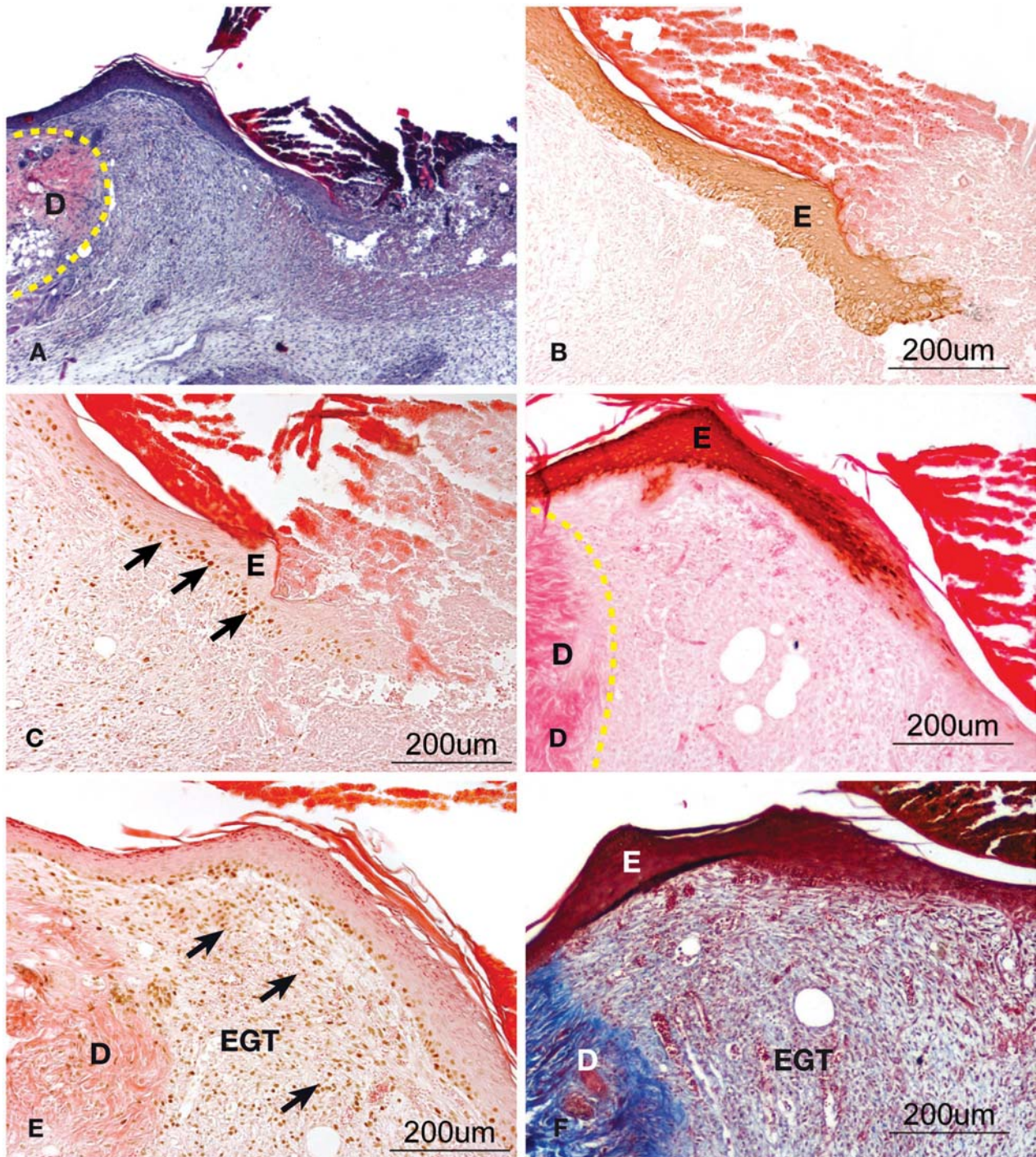


FIGURE 3.—Histological evaluation of wound healing on day 4 postwounding. Paraffin blocks were prepared from the wounded tissue; histological slides were obtained from the widest area of the wound and stained for several histological and immunohistochemical markers. A: H&E-stained wound, 40X magnification; B: anti-keratin 14 staining of migrating epidermis, 100X magnification; C: anti-proliferating cell nuclear antigen (PCNA) staining of migrating epidermis, 100X magnification; D: anti-keratin 1 staining of migrating epidermis near the wound edge, 100X magnification; E: anti-proliferating cell nuclear antigen (PCNA) staining of migrating epidermis and premature granulation tissue near the wound edge, 100X magnification; F: Masson Trichrome staining for collagen deposition at the wound gap, 100X magnification. Deposition of collagen fibers is stained in blue. E-epidermis; D-dermis; EGT-early granulation tissue; black arrows indicate PCNA positive cells; yellow lines indicate a border between normal dermis and wound gap.

wound susceptible to external infections as well as to deterioration of the underlying tissue, leading to morbidity and sometimes requires amputation (Brem et al., 2003; Freedman et al., 2004; Mousley, 2003; Wertheimer, 2004). We examined

the parameters that underlie wound-healing impairment in a chemically induced diabetes model system consisting of animals injected with STZ (streptozotocine). The STZ model is frequently used for type 1 diabetes and for the study of

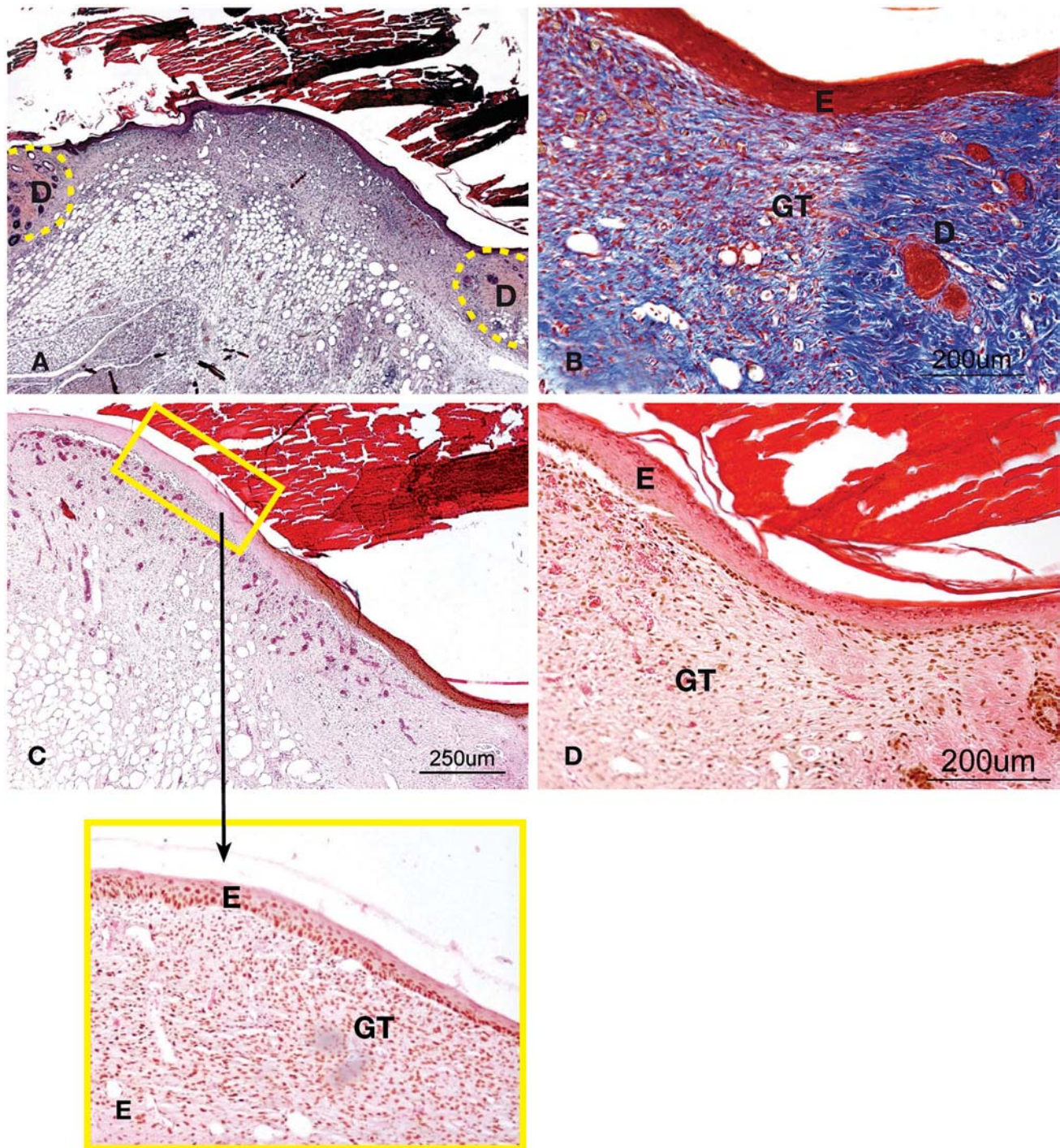


FIGURE 4.—Histological evaluation of wound healing on day 9 following wounding. Paraffin blocks were prepared from the wounded tissue; histological slides were obtained from the widest area of the wound and subjected to several histological and immunohistochemical staining procedures. A: H&E-stained wound, 20X magnification; B: Masson Trichrome staining for collagen deposition at the wound gap, 100X magnification; C: anti-keratin 1 staining of newly formed epidermis, 100X magnification; D: anti-proliferating cell nuclear antigen (PCNA) staining of newly formed epidermis near the wound edge, 100X magnification; E: anti-proliferating cell nuclear antigen (PCNA) staining of newly formed epidermis and granulation tissue at the wound gap, 400X magnification. E-epidermis; D-dermis; GT-granulation tissue; yellow lines indicate a border between normal dermis and wound gap.

various complications of this disease (Seifter et al., 1981; Franzen and Roberg, 1995; Schaffer et al., 1997; Perez et al., 2005).

We performed a comprehensive histological analysis of wounds of STZ-induced diabetic animals using the quanti-

tative assessment strategy described here. As summarized in the histological example presented in Figure 6, 9 days postwounding, diabetic wounds exhibit an overabundance of proliferating epidermis at the wound edges, but fail to induce keratinocyte migration and subsequent reepithelialization

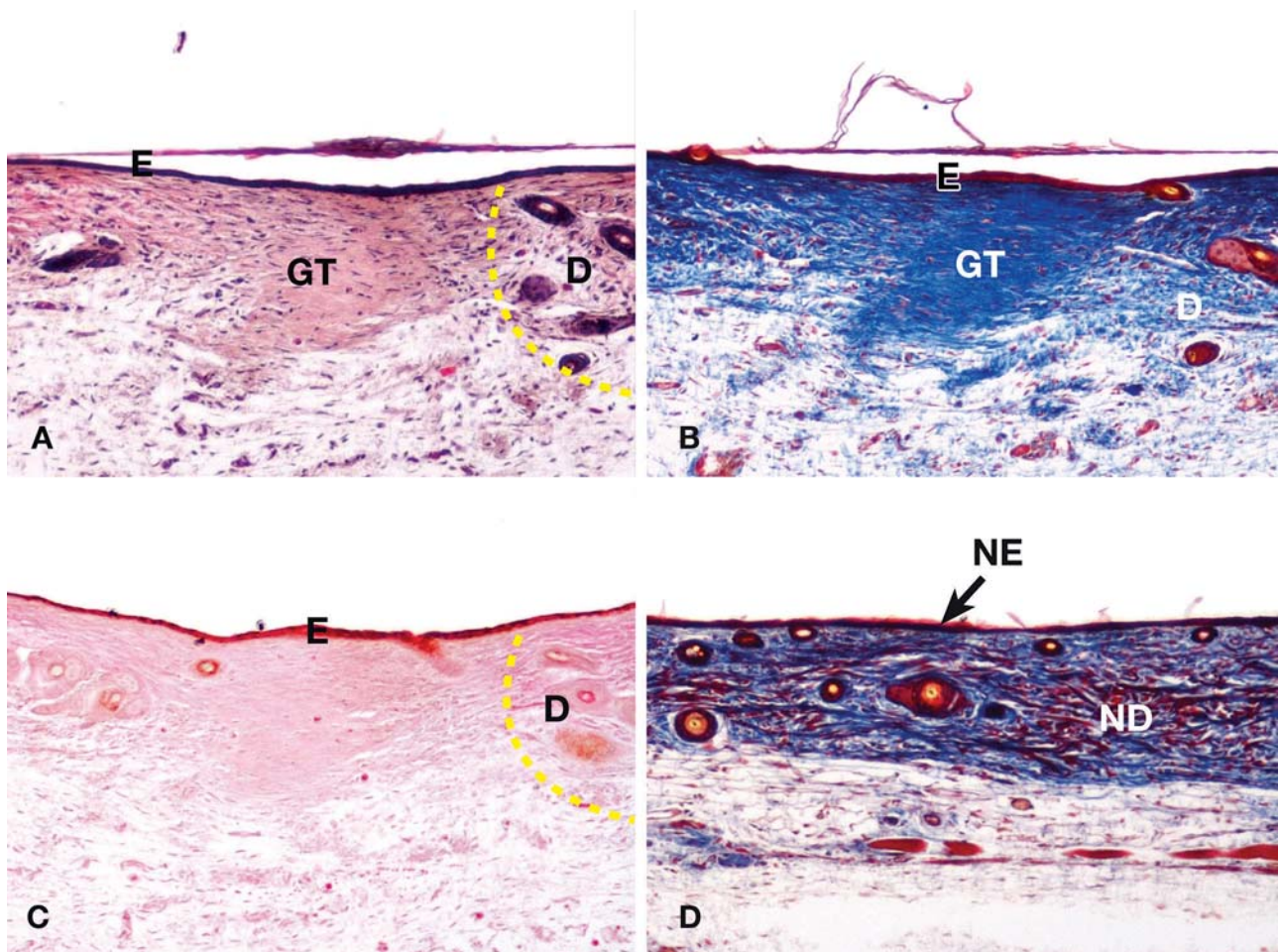


FIGURE 5.—Histological evaluation of final wound healing stages on day 18 postwounding (A,B,C) compared to unwounded skin (D). Paraffin blocks were prepared from wounded tissue; histological slides were obtained from the widest area of the wound and subjected to several histological and immunohistochemical staining procedures. A: H&E-stained wound, 40X magnifications; B: Masson Trichrom staining for collagen deposition at the wound gap, 40X magnification. C: anti-keratin 14 staining of the epidermis, 40X magnification; D: Masson Trichrome staining for collagen deposition of unwounded skin 40X magnification. E-epidermis; D-dermis; GT-granulation tissue; ND-normal dermis; NE-normal epidermis; black arrows indicate normal epidermis; yellow lines indicate a border between normal dermis and wound gap.

(Figure 6A epidermal closure). In addition, these wounds are characterized by a severe inflammatory response which probably contributes to the inhibition of healing progression. Another aspect of wound impairment is associated with formation of the granulation tissue (Figure 6A inflammation, granulation tissue). Although the wound gap is wide open and severe inflammation is apparent, collagen is distributed along the length of the wound gap (staining not shown). Granulation tissue analysis failed to show its formation in 60% of the animals (Figure 6A, 6B). Despite this extensive collagen distribution, sustained deposition of granulation tissue is delayed or suspended in wounds followed for up to 15 days. Therefore, the collagen deposition is not sufficient to establish a mature matrix that will provide support to the wound but rather, occurs as compensatory attempt by the granulation tissue to overcome the healing impairment. Moreover, as can be seen in our histological sections, the matrix filling the wound gap of diabetic wounds is disorganized and exhibits only initial deposition of filamentous fibers.

A clear confirmation of our matrix analysis is provided through wound strength testing. Wound strength was mea-

sured utilizing a bursting chamber which measures the amount of pressure required to rupture a tested tissue. Wound biopsies from diabetic animals are characterized by reduced strength, exhibiting lower bursting pressure compared to non diabetic controls (Figure 6C). These results confirm that the collagen distribution in diabetic wounds does not necessarily indicate functional recovery.

Thus, the quantitative approach to wound-healing analysis described here provides insight into the specific defects found at several stages and involving a variety of cells and pathways of the wound-healing process in STZ-induced diabetic mice (Figure 6 only depicts this assessment at 9 days postwounding). Similar results were obtained when examining wounds induced in other diabetic animal models such as NOD mice, a strain genetically prone to diabetes (results not shown).

Effects of growth factors on the wound-healing process:

The quantitative assessment strategy of wound-healing parameters allows us to screen the efficacy of various drugs, growth factors and cytokines in affecting distinct stages of the wound-healing process. Moreover, this type of assessment

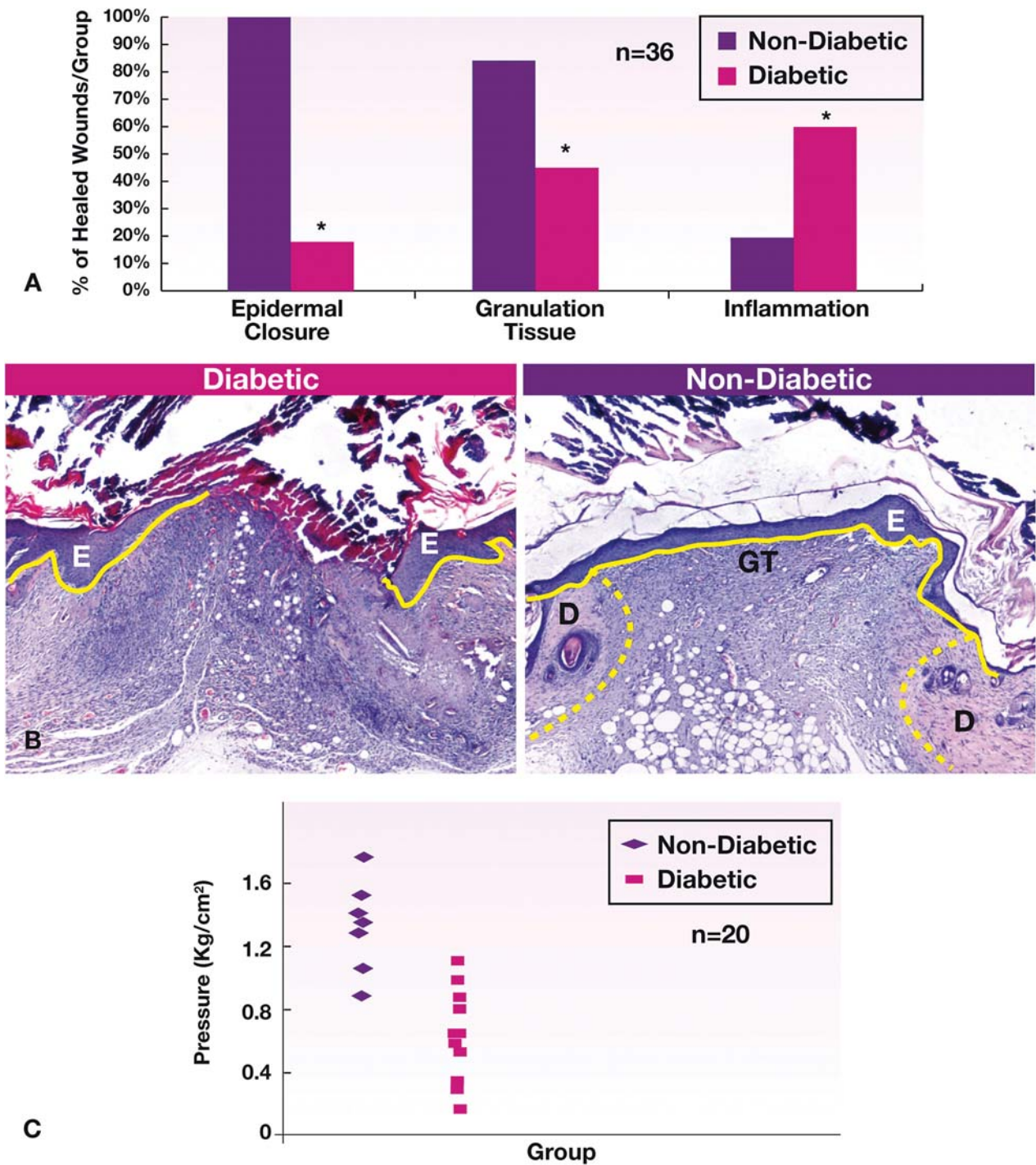


FIGURE 6.—Longitudinal full-thickness incisions were performed on the upper back of non-diabetic C57BL mice and those with STZ-induced diabetes. Tissues were collected 9 days postwounding. (A) Summary graph of histological sections presenting the quantitative assessment described herein. The wounds were analyzed for dermal closure (H&E), epidermal closure (K14) and severe inflammation according to HealOr’s parameters. Wounds were scored as positive only when each parameter was fully present. These were summarized and presented as percent of healed wounds in a group. \*  $p < 0.02$ . (B) H&E staining demonstrates the overall impairment of diabetic versus nondiabetic wounds. 100X magnification. E-epidermis; D-dermis; GT-granulation tissue; yellow speckled lines indicate a border between normal dermis and wound gap; yellow continues line indicate reepithelialization. (C) Wound-strength measurements 30 days following wounding of normal vs. diabetic rats. The data were collected utilizing the bursting chamber method. N = 10 animals per group; representative experiments of at least 3 repeats are shown.

tests the ability of these agents to overcome the various pathological manifestations of impaired wound healing. Possible approaches include studying the effects of various growth factors and their combinations as possible therapies for wound

healing in experimental and clinical studies (Blakytyn et al., 2000; Carrington and Boulton, 2005; Enoch et al., 2006; Rio et al., 1999). Several growth factors and cytokines, including platelet-derived growth factor (PDGF), transforming growth

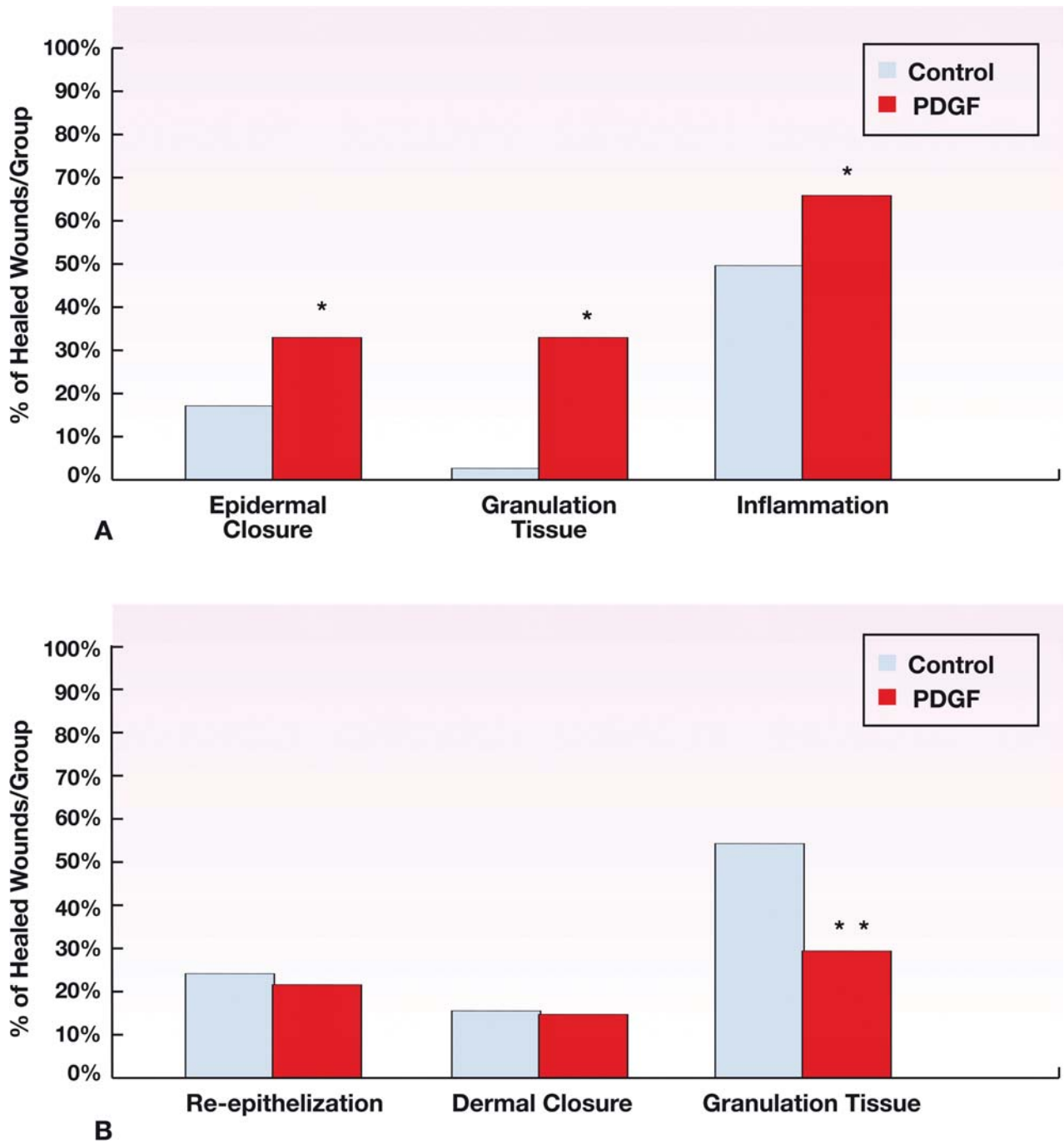


FIGURE 7.—Efficacy of PDGF ( $10^{-7}$  M) in parameters of wound healing 4 days (A) and 7 days postwounding (B). Comparison of the effects of PDGF on the following wound-healing parameters: epidermal closure, granulation-tissue formation, dermal closure and inflammation. Epidermal closure was assessed by K14 staining, granulation-tissue formation, dermal closure and angiogenesis were assessed by H&E staining as described. PDGF-platelet-derived growth factor (n = 36, 18 animals per group, pooled data from three separate experiments). \*  $p < 0.05$  \*\*  $p < 0.04$ .

factor  $\beta$  (TGF $\beta$ ), keratinocyte growth factor (KGF) and insulin, have demonstrated beneficial effects on the wound-healing process (Andresen et al., 1997; Pierre et al., 1998; Steiling and Werner, 2003; Werner and Grose, 2003). In the past few years, the availability of genetically modified mice has enabled an elucidation of the function of various genes in the healing process, and these studies have shed light on the role of growth factors, cytokines, and their downstream effec-

tors in wound repair (Grose and Werner, 2003). However, no comprehensive histological analysis of wounds treated with these factors has been performed. Furthermore, most of the studies demonstrating the role of growth factors in wound healing have focused on individual stages, rather than examining the diverse stages of wound healing as part of a continuum (Werner and Grose, 2003). One example of a prominent therapy for chronic debilitating wounds in recent years is

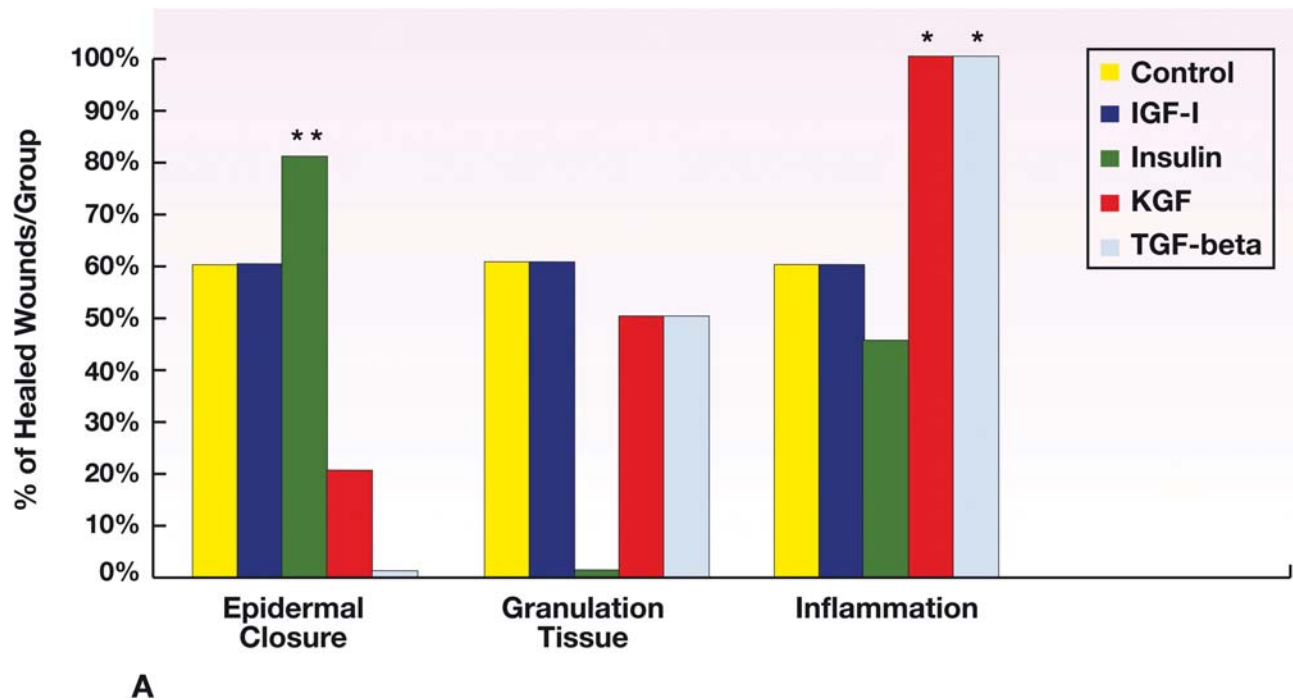


FIGURE 8.—Efficacy of growth factors in wound healing 7 days postwounding. Graph comparing the effects of various growth factors on the following wound-healing parameters: epidermal closure, granulation-tissue formation and inflammation at the wound gap. Epidermal closure was assessed by K14 staining; granulation-tissue formation and inflammation were assessed by H&E staining as described. IGF-insulin-like growth factor ( $10^{-7}$ M), KGF-keratinocyte growth factor ( $10^{-6}$ M), TGF- $\beta$  transforming growth factor  $\beta$  (5 ng/ml) insulin  $10^{-7}$  M n=50. \* $p < 0.01$  \*\* $p < 0.04$ .

the application of topical treatment with PDGF approved for clinical use. PDGF has been shown to enhance the migration of neutrophils, monocytes and fibroblasts into the wounded area. It was also reported to promote rapid cell proliferation, which can even result in the formation of hypertrophic scars (Gao et al., 2005; Nagai and Embil, 2002; Werner and Grose, 2003). A summary of wound-healing studies in various animal models suggests that its mechanism of action can be attributed to its positive effect on endothelial cell proliferation and the development of new blood vessels, i.e. angiogenesis, at the wound bed (Lee et al., 2005; Brown et al., 1994). Using our longitudinal studies comprising a quantitative histological assessment, we tested the effects of PDGF on wound healing (Figure 7), and found that it differentially affects various cell types and stages during healing. In the early stages, PDGF initiates the migration of keratinocytes and fibroblasts to the epidermal edges 4 days postwounding and promoted the formation of granulation tissue 4 days post-wounding, including induction of blood vessels formation (Figure 7A, B). However, these contributions are diminished in later stages of wound healing. At the same time, the prolonged inflammatory response and extensive cell proliferation prevent advancement of the migratory edges towards wound reepithelialization. Although the granulation tissue at this stage is quite advanced, the wound cannot form new epidermis and complete the healing process (Figure 7B). This example clearly demonstrates the importance of looking at wound healing from a longitudinal perspective. PDGF can accelerate some stages of healing in certain types of wounds when administered during specific stages of healing. However, prolonged treatment can undermine the granulation-tissue foundation that it has

just promoted because of excessive chronic inflammation induced at the wound site. Moreover, the excess proliferation arrests the migratory potential of epidermal cells at a crucial time point, also delaying the reepithelialization process.

We also tested other growth factors for their influence on the healing process of wounds including: insulin, insulin-like growth factor I (IGF-I), TGF $\beta$ , and KGF. As published in the literature, these factors are potential therapies for wound healing. Several studies have shown that TGF $\beta$  and KGF promote matrix formation and deposition of collagen fibers at very early stages of wound healing (Finch et al., 1989; Yang et al., 2001). However, as demonstrated in this review, filling the wound with collagen at early wound-healing stages does not necessarily promote efficient repair of the tissue (Figure 8). The natural longitudinal process of wound healing exhibits this type of deposition of collagen and matrix formation at more advanced stages (Figure 5). Promotion of this stage before migration of epidermal and dermal cells results in impairment of barrier reconstruction and granulation-tissue formation (Figure 8). Furthermore, treatment of wounds with KGF or TGF $\beta$  results in a sustained inflammatory response which, at this point, is damaging to the tissue-recovery process (Figure 8).

On the other hand, insulin dose exhibit some beneficial effects on distinct stages of wound healing. The graph in Figure 8 demonstrates insulin's ability to accelerate the initiation of epidermal migratory edges. Moreover, in advanced stages of the healing process, insulin augments epidermal reepithelialization and reconstruction (Figure 8). However, insulin cannot assist in the formation of granulation tissue nor in the deposition of matrix fibers to promote dermal

remodeling (Figure 8). Furthermore, like other growth factors, insulin also produces a white blood cell infiltrate as a result of prolonged inflammatory induction. Thus, even though specific growth factors can be an important treatment at certain stages of wound healing, they are clearly insufficient to promote the entire longitudinal process. These data therefore suggest combination treatments as the preferred approach in developing future therapies for wound healing.

Research in this direction is already being implemented by several groups, including the combination of  $\beta$ FGF and hepatocytes growth factor (HGF), HGF combined with cytokines such as IL-1 and interferon- $\gamma$ ,  $\beta$ FGF and IGF-I, among others (Brown et al., 1994; Werner and Grose, 2003; Bevan et al., 2004).

#### SUMMARY AND CONCLUSION

This study defines a methodology for a structured approach to wound healing assessment in the preclinical stages by utilizing compatible animal models and quantitative objective histological assessment. This approach allows the identification of stage specific pathologies in wound healing impairment. Furthermore, quantitative assessment criteria can serve as a screening platform to identify the contribution of stage specific factors based on mechanism of action by accelerating distinct biochemical and physiological pathways. Furthermore, this methodology can serve for selection of novel drugs to promote critical healing stages in wound healing pathology. Furthermore, combinational approach to drug intervention allows to achieve synergy, reduce the effective dose of each active compound therefore, minimizing adverse events and toxicological effects of the used pharmaceuticals. Promoting concomitantly several critical healing stages in parallel to achieve synergy in the healing process will advance the development of effective novel therapies for the treatment of debilitating, and currently untreatable ulcers.

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