

## Human Keratinocyte Growth Factor Effects in a Porcine Model of Epidermal Wound Healing

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### Summary

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family (hence the alternative designation FGF-7). It is produced by stromal cells, but acts as a mitogen for epithelial cells. We examined the effects of topically applied KGF on healing of wounds in a porcine model. In partial-thickness wounds, KGF stimulated the rate of reepithelialization ( $p < 0.0002$ ), associated with a thickening of the epidermis ( $p < 0.0001$ ). Epidermis from KGF-treated full-thickness wound sites was significantly thicker ( $0.31 \pm 0.22$  mm) compared with mirror image control sites ( $0.18 \pm 0.12$  mm) ( $p < 0.0001$ ). Moreover, the majority (77%) of KGF-treated wounds exhibited epidermis with a deep rete ridge pattern as compared with control sites. These effects were observed as early as 14 d and persisted for at least 4 wk. KGF treatment also increased the number of serrated basal cells associated with increased deposition of collagen fibers in the superficial dermis adjacent to the acanthotic epidermis. Electron microscopy revealed better developed hemidesmosomes associated with thicker bundles of tonofilaments in the serrated cells. The pattern of epidermal thickening observed in KGF-treated wounds resembled psoriasis. Psoriasis is a disease associated with epidermal thickening, parakeratosis as well as hyperproliferation that extends beyond the basal layer. In striking contrast to psoriasis, KGF-treated wounds exhibited normal orthokeratotic maturation, and proliferation was localized to the basal cells. Our present findings have significant implications concerning the role of KGF as a paracrine modulator of epidermal proliferation and differentiation.

During the past two decades, the importance of growth factors in wound healing has become increasingly apparent as a cascade of growth factor-mediated events must be temporally orchestrated to achieve proper wound healing (1, 2). Healing is a complex and protracted process involving an initial inflammatory phase, a period of cellular proliferation and extracellular matrix (ECM)<sup>1</sup> synthesis, and eventually a phase of remodeling of the matrix (3). These events are both independently and coordinately influenced by numerous growth factors and/or cytokines secreted by inflammatory cells, as well as by other cells residing within, or at the edges of the wound bed. The rate at which wounds heal,

and the degree of "healing" achieved, are likely influenced by growth factor presentation and cellular response within the wound bed (4, 5). Recent studies have implicated a wide variety of polypeptide growth factors as critical elements in angiogenesis, fibroplasia, cellular proliferation, and reepithelialization of wounds. Epidermal growth factor (EGF) and/or TGF- $\alpha$  influence migration and proliferation of epidermal keratinocytes, which represent critical elements in the essential reepithelialization process of wound healing (6-10). TGF- $\beta$  induces fibroplasia, including synthesis of collagen and fibronectin, both of which are associated with the wound matrix (11-19). Platelet-derived growth factor (PDGF) fosters angiogenesis and granulation tissue formation in the wound (20-25). Recent studies in animal models and humans have indicated that application of exogenous growth factors to wounds accelerates healing (7-11, 16, 20-25, 26-29).

Members of the fibroblast growth factor (FGF) family have been implicated in the wound-healing process (23, 24, 30).

<sup>1</sup> Abbreviations used in this paper: ANOVA, analysis of variance; BMZ, basement membrane zone; ECM, extracellular matrix; EGF, epidermal growth factor; EM, electron microscopy; FGF, fibroblast growth factor; H&E, hematoxylin and eosin; KGF, keratinocyte growth factor.

Addition of bFGF to wounds modulates the dermal response to injury (23, 24). Acidic (a)FGF, basic (b)FGF, FGF-5, and FGF-7 (also known as keratinocyte growth factor [KGF]) are expressed in normal, unwounded skin, and their expression is upregulated early after full thickness injury (31). Of particular interest, however, are recent observations that KGF (FGF-7) expression was increased to a much greater extent than the other FGFs (>100-fold above baseline). Moreover, its expression remained elevated through the first 7 d after injury, implicating KGF as a potentially important physiologic mediator of wound repair (31).

KGF is a unique member of the FGF family (32, 33), in that studies thus far indicate that it operates solely as a paracrine growth factor, exhibiting epithelial cell-specific targeting (for a review see reference 34). Dermal fibroblasts produce KGF but do not express its receptor and do not respond to exogenous KGF (34). In contrast, epidermal keratinocytes do not synthesize KGF, but instead express the KGF receptor. Keratinocytes were stimulated to proliferate (35) and migrate (35a) in response to exogenous KGF. KGF was as potent as EGF in its ability to induce mitogenesis in cultured keratinocytes (35). A distinguishing feature of KGF from EGF, however, was its ability to facilitate expression of differentiation-related markers in human keratinocytes exposed to 1.0 mM calcium (35). In addition, unlike its better characterized family member bFGF, which does not contain a signal peptide, KGF has a signal sequence and is secreted from cells (34).

The epithelial-specific targeting of KGF (in vitro), combined with its demonstrated expression during wound healing, suggest that KGF may play a pivotal role in early wound-healing processes. The present studies were designed to assess whether exogenous KGF could modulate the wound-healing response in porcine skin. The results of these studies suggest that KGF increases the rate of reepithelialization of partial-thickness wounds. In full-thickness wounds KGF treatment elicits: (a) epidermal thickening; (b) serration of basal cells; (c) better developed tonofilaments and hemidesmosomes in basal keratinocytes; and (d) increased abundance of collagen in the papillary dermis.

## Materials and Methods

**Source of KGF.** Recombinant KGF protein was expressed in bacteria (36) using the T7 expression system (37). After sonication and clarification of the bacterial lysate, KGF was purified by sequential chromatography on heparin-Sepharose (Pharmacia, Piscataway, NJ) and Mono-S (Pharmacia) resins. The homogeneity of this preparation was confirmed by silver-stain analysis of protein electrophoresed in SDS-polyacrylamide gels and the yield determined by amino acid analysis. The recombinant protein demonstrated mitogenic activity for mouse, human, and swine keratinocytes.

**Animal Use.** All experiments were carried out with prior approval of the Cornell University Medical College Institutional Animal Care and Use Committee. A total of 11 domestic Yorkshire pigs (50–80 pounds) were used for these studies.

**Creation of Partial-thickness Wounds.** Animals were anesthetized before surgery and wounds were created using a dermatome set

at a 0.015 inches in depth (Padget, a division of Kansas City Assemblage Co., Kansas City, MO). A series of shallow, mirror image, partial-thickness wounds ( $\sim 1 \times 2$  in) were created on the lateral paravertebral skin of each pig in a cephalad-caudad orientation. Mirror image wounds were chosen so that each KGF-treated wound had its own vehicle-control site that would be at an anatomically similar position with regard to the location (i.e., cephalad or caudad) on the pig. This is important since skin thickness, wound contraction, and healing can vary depending upon the site from which the skin is taken. Use of mirror image wounds allowed comparison of equivalent skin for each set of control and KGF-treated wounds.

Two animals were used for these studies. Nine pairs of wounds were created on the first pig, whereas four pairs of wounds were created on the second animal. After creation of the wounds, the left or right side of each mirror image wound pair was randomly chosen to receive either KGF (1  $\mu$ g/wound in 50  $\mu$ l PBS) or vehicle (50  $\mu$ l PBS alone). All wounds were photographed at a fixed distance (12 in) from the wound bed with a sterile ruler next to the wound for size standardization. A ring flash was used to ensure uniform lighting. Each wound was then dressed with polyurethane dressing (Op-Site; Smith and Nephew Med. Ltd., Hull, UK) and covered with burn dressing (Intersorb; Sherwood Med., St. Louis, MO) and Spandage (Medi-Tech International, Brooklyn, NY).

On days 3–4 after surgery, histologic analysis of all wounds was performed. This time period was chosen since such shallow wounds routinely heal within  $\sim 6$  d. Histologic examination of the wounds allowed us to assess whether the healing process and/or the morphologic appearance of the healed (or healing) skin was altered by treatment with KGF. Elliptical biopsies were made through the center of each wound, including normal margins of unwounded skin, 5- $\mu$ m sections were stained with hematoxylin and eosin (H&E). Three criteria, namely, reepithelialization, epidermal thickness, and epidermal maturity, were used to quantitate the effects of KGF on partial-thickness wound healing.

The percent of reepithelialization across each wound site was measured by microscopy using a reticle within the microscope eyepiece to measure the proportion of each wound that was covered by epidermis in comparison to the entire wound length.

Epidermal thickness was defined as the distance (in millimeters) from the top of the granular layer (if present) to the bottom of the basal layer. If a granular layer was not present, then the top-most layer of keratinocytes was used as the upper boundary for such quantitation. Light microscopy using an ocular reticle was again used for these measurements. Approximately 30 individual measurements of epidermal thickness were made along the wound site for each histologic section and the mean thickness quantitated.

Finally, the presence or absence of a mature granular layer and/or a stratum corneum were considered criteria for a maturing epidermis.

All wound evaluations were carried out with the investigator blinded to the treatment group. After decoding of the data, each parameter (i.e., reepithelialization, epidermal thickness, and epidermal maturity) from an individual KGF-treated wound was compared with its mirror image control site.

**Creation of Full-thickness Wounds.** Nine separate animals were used for studies of KGF effects. Before creation of full-thickness wounds, a series of grids (8  $\times$  8 cm subdivided into 1-cm<sup>2</sup> boxes) were stamped and tattooed in mirror image array on the dorsal surface of each animal as above. Full-thickness wounds (4 cm<sup>2</sup>) were created by surgical excision of the four center boxes of each grid to the trapezius or latissimus dorsi fascia. The left or right side of each mirror image wound pair was then treated with either KGF (1  $\mu$ g/wound in 50  $\mu$ l PBS) or vehicle (50  $\mu$ l PBS alone) (see

above). All sites were photographed under standard conditions and the wounds dressed (as described above). On day 3 after surgery, KGF (or vehicle) was reapplied to wounds as before. All wound dressings were changed on day 7 and at weekly intervals thereafter. At each dressing change, all wounds were photographed at a standard distance (12 in). Wounds (KGF and mirror image controls) were evaluated at weekly intervals up to 4 wk after wounding using the parameters described below.

Wound contraction was quantitated by computerized morphometric image analysis of the photographic slides taken of each wound (JAVA Video Analysis Software System; Jandel Scientific, Corte Madera, CA). Standardized photography, combined with the presence of a ruler within each photograph allowed accurate quantitation of wound size by electronically tracing the grid that outlined each wound (38). Data analysis was carried out with the investigator blinded to the treatment group of the wounds. Wound contraction of each KGF-treated site was then compared with its mirror image control site as a function of time after surgery.

The histologic appearance of the healing skin during the course of each experiment was determined by examination of H&E-stained 5- $\mu$ m sections as described above. Separate histologic sections also were stained with Masson Trichrome to evaluate the extent of collagen bundle formation in KGF- and control-treated wounds. Epidermal thickness was defined as described above and quantitated for each wound.

The kinetic profile of keratinocyte proliferation during wound healing was quantitated by standard immunohistochemical techniques utilizing an anti-Ki-67 antibody (MIB-1; Amac, Inc., Westbrook, ME) (39). Biopsies from control and KGF-treated sites obtained 1, 2, and 3 wk after wounding were reacted with Ki-67. Normal unwounded pig skin and human skin from a psoriatic patient served as controls.

Expression of the basement membrane component laminin was determined using anti-laminin Abs (clone 2E10) obtained from Dr. Eva Engvall (La Jolla Cancer Research Institute, La Jolla, CA) and reacted with air-fixed cryostat sections of skin.

Finally, ultrastructural features of the control and KGF-treated sites were assessed by electron microscopy (EM) on tissue that was fixed in cacodylate-buffered 2% glutaraldehyde, post-fixed in OsO<sub>4</sub>, and embedded in Epon 812 (EM Sciences; Fort Washington, PA) after progressive dehydration. Silver sections were cut on a microtome (Ultracut E; Reichert-Jung, Vienna, Austria). Sections were stained with uranyl acetate and lead citrate before viewing on an electron microscope (model 100 cx; JEOL U.S.A. Inc., Peabody, MA).

**Statistical Evaluation.** For partial-thickness wounds, analysis of variance (ANOVA) for a crossover trial with replications was used to determine if a difference existed between the two treatments (PBS and KGF) with regard to percent reepithelialization of the wounds and epidermal thickness of the healed skin. This design took into account the different number of paired sites within an animal (otherwise called replications), as well as the fact that each animal received each of two treatments (hence the term crossover);  $p < 0.01$  was considered statistically significant.

For full-thickness wounds, ANOVA for a crossover trial was again employed to determine the difference in thickness between PBS- and KGF-treated mirror image wounds;  $p < 0.01$  was considered statistically significant (see above for design considerations).

For wound contraction studies, paired Student's *t* tests were performed at each of six time points (days 0, 3, 7, 14, 21, and 28) with each replication or site per animal treated as a separate record to determine whether there was a difference between the two treatments (KGF and PBS). The paired Student's *t* test was used because each animal received both treatments, i.e., animals were paired across treatments;  $p < 0.01$  was considered statistically significant.

## Results

**The Effects of KGF on Reepithelialization of Partial-thickness Wounds.** KGF was applied to dermatome-derived superficial, partial-thickness wounds in two animals. The degree of reepithelialization, thickness of the epidermis, and degree of epidermal maturation were assessed in control and KGF-treated wounds on the third day after treatment (Table 1). KGF increased the extent of reepithelialization in partial-thickness wounds compared with mirror image control wounds ( $82.5 \pm 21.8$  and  $51.7 \pm 22.5\%$  reepithelialization for KGF and control wounds, respectively;  $p < 0.0002$ ). H&E-stained sections of a representative set of wounds showed complete epithelialization of the KGF-treated site (Fig. 1 B). By contrast, incomplete epithelial coverage of the mirror image control site was observed (Fig. 1 A). Healed skin from KGF-treated wounds was also significantly thicker than healed skin from mirror image control wounds ( $0.07 \pm 0.02$  vs  $0.05 \pm 0.02$  mm thickness for KGF- and PBS-treated wounds, respectively;  $p < 0.0001$ ).

The percent epithelialization and epidermal thickness of

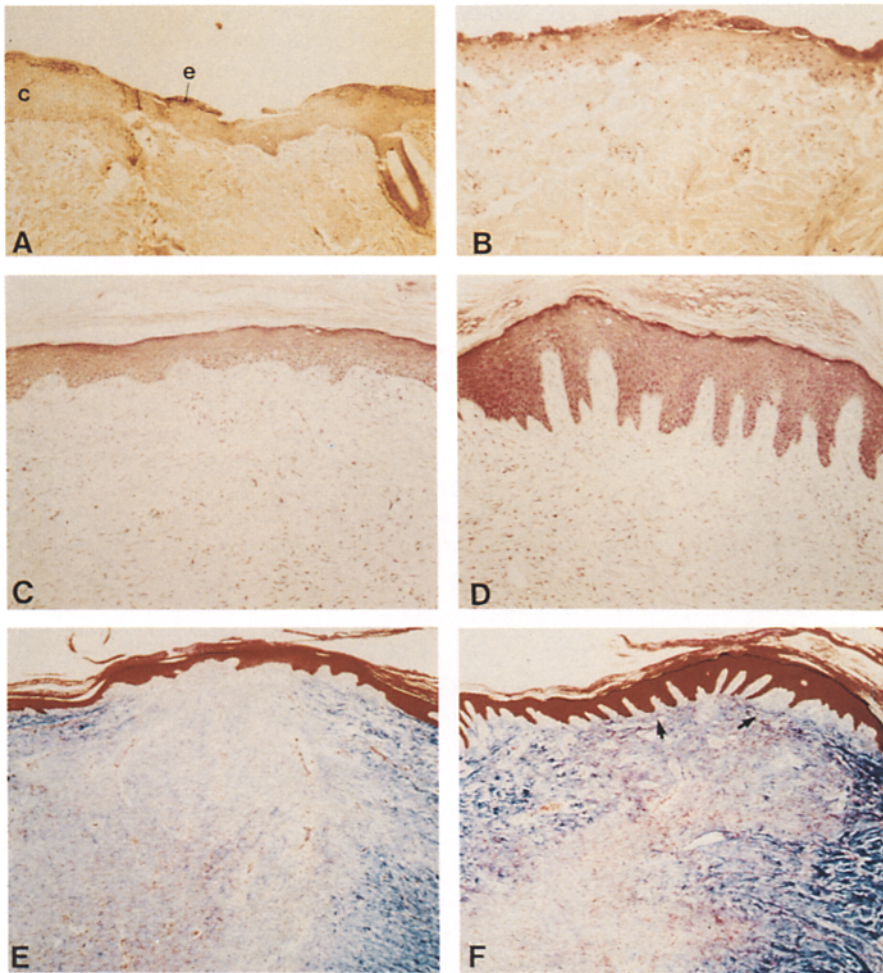
**Table 1.** Reepithelialization and Epidermal Thickness of Partial-thickness Wounds Treated with KGF or PBS

Expt.	Pairs of wounds	Reepithelialization (Mean % $\pm$ SD)		Epidermal thickness (mm $\pm$ SD)	
		PBS	KGF <sup>a</sup>	PBS	KGF <sup>b</sup>
1	9	41.1 $\pm$ 12.7	78.9 $\pm$ 24.2*	0.06 $\pm$ 0.02	0.08 $\pm$ 0.02
2	4	83.3 $\pm$ 11.6	93.3 $\pm$ 5.8	0.03 $\pm$ 0.02	0.06 $\pm$ 0.01
Total 2	13	51.7 $\pm$ 22.5	82.5 $\pm$ 21.8	0.05 $\pm$ 0.02	0.07 $\pm$ 0.02

Statistical analysis based upon ANOVA for a crossover trial design (see Materials and Methods).

<sup>a</sup> Significance =  $p < 0.0002$  for percent reepithelialization of PBS- vs. KGF-treated wounds.

<sup>b</sup> Significance =  $p < 0.0001$  for epidermal thickness of PBS- vs KGF-treated wounds.



**Figure 1.** The effects of KGF on wound healing in swine. (A and B) The effects of KGF on partial-thickness wounds. Mirror image wounds received either PBS (A) or KGF (B). A representative set of partial-thickness wounds revealed that KGF-treated wounds (B) (day 3 after surgery) exhibited more extensive reepithelialization compared with mirror image control sites (A). (A) (e) Edge of epidermis; (c) neutrophil-containing crust. (C-F) The effects of KGF on full-thickness wounds. Evaluation of H&E stained sections of KGF-treated wounds (D) showed a thickened epidermis with orthokeratotic maturation, compared with control wounds (C). Trichrome staining further revealed more extensive collagen deposition beneath KGF-treated wounds (arrows, F) compared with control wounds (E). (A-D)  $\times 95$ ; (E and F)  $\times 24$ .

the nine individual pairs of mirror image wounds from the first animal are shown in Fig. 2. Whereas four of nine KGF-treated wounds were completely covered by epidermis (mean percent reepithelialization,  $78.9 \pm 24.2\%$ ,  $n = 9$ ), none of the control wounds were 100% reepithelialized (mean percent reepithelialization,  $41.4 \pm 12.7\%$ ,  $n = 9$ ) (Fig. 2 A). Paired comparison of each mirror image set of wounds (shown as lines connecting each pair) revealed that in every case, KGF treatment accelerated epithelialization compared with its mirror image wound site. Besides a greater degree of epithelialization in KGF-treated wounds, paired analysis of epidermal thickness revealed significantly thicker epidermis in KGF-treated wounds compared with mirror image control sites ( $p < 0.0001$ ) (Fig. 2 B).

Examination of all the wounds ( $n = 26$  for the two animals) for epidermal maturity showed that only 4 of 26 wounds exhibited focal areas in which a well-developed granular layer was apparent. Of these four wounds, all were KGF-treated sites.

**Effects of KGF on the Healing of Full-thickness Wounds.** The effects of KGF on the healing of full-thickness wounds was evaluated in 31 pairs of mirror image wounds from nine sep-

arate animals (Table 2). Wounds were initially evaluated four wk after the first application of KGF. KGF treatment consistently induced significant epidermal thickening compared with mirror image control sites ( $0.18 \pm 0.12$  and  $0.31 \pm 0.22$  mm, epidermal thickness for control and KGF-treated sites, respectively;  $p < 0.0001$ ). Moreover, the epidermis in the majority (77%) of KGF-treated wounds possessed an epidermis that exhibited pronounced, deep rete ridges, such as those seen in psoriasis (Fig. 1 D). This epidermal morphology was unusual, since healing of full-thickness skin wounds is generally associated with a more flattened rete ridge pattern compared with normal skin (Fig. 1 C) (40). Indeed, all of the control wounds examined in the present study ( $n = 31$ ) had a flattened epidermis with few, if any rete ridges evident (Table 2, Fig. 1 C). KGF-treated wounds exhibited approximately six times as many rete ridges over the wound site compared with control wounds. Whereas the epidermis from KGF-treated wounds was acanthotic in appearance, keratinocyte maturation was orthokeratotic and few mitoses were apparent (Fig. 1 D). When similar wounds on the same animal were treated with either EGF or bFGF, neither uniform epidermal acanthosis nor rete elongation was observed (data not shown).





**Table 2.** Thickness of the Epidermis from Healed Full-thickness Wound Treated with KGF or PBS

Expt.	Pairs of wounds	Epidermal thickness* (mm ± SD)		Epidermal morphology (No. wounds)			
				PBS		KGF	
		PBS	KGF	Flat	Rete ridge	Flat	Rete ridge
1 <sup>†</sup>	5	0.10 ± 0.04	0.15 ± 0.04 <sup>‡</sup>	5 <sup>¶</sup>	0 <sup>¶</sup>	0	5
2	5	0.16 ± 0.05	0.24 ± 0.1	5	0	3	2
3	4	0.06 ± 0.02	0.13 ± 0.03	4	0	1	3
4	2	0.15 ± 0.07	0.35 ± 0.07	2	0	0	2
5	1	0.2	0.38	1	0	0	1
6	2	0.17 ± 0.0	0.43 ± 0.07	2	0	1	1
7	2	0.19 ± 0.03	0.29 ± 0.09	2	0	1	1
8	4	0.35 ± 0.16	0.45 ± 0.21	4	0	1	3
9	6	0.31 ± 0.12	0.51 ± 0.36	6	0	1	5
Total 9	31	0.18 ± 0.12	0.31 ± 0.22	31	0	7	24

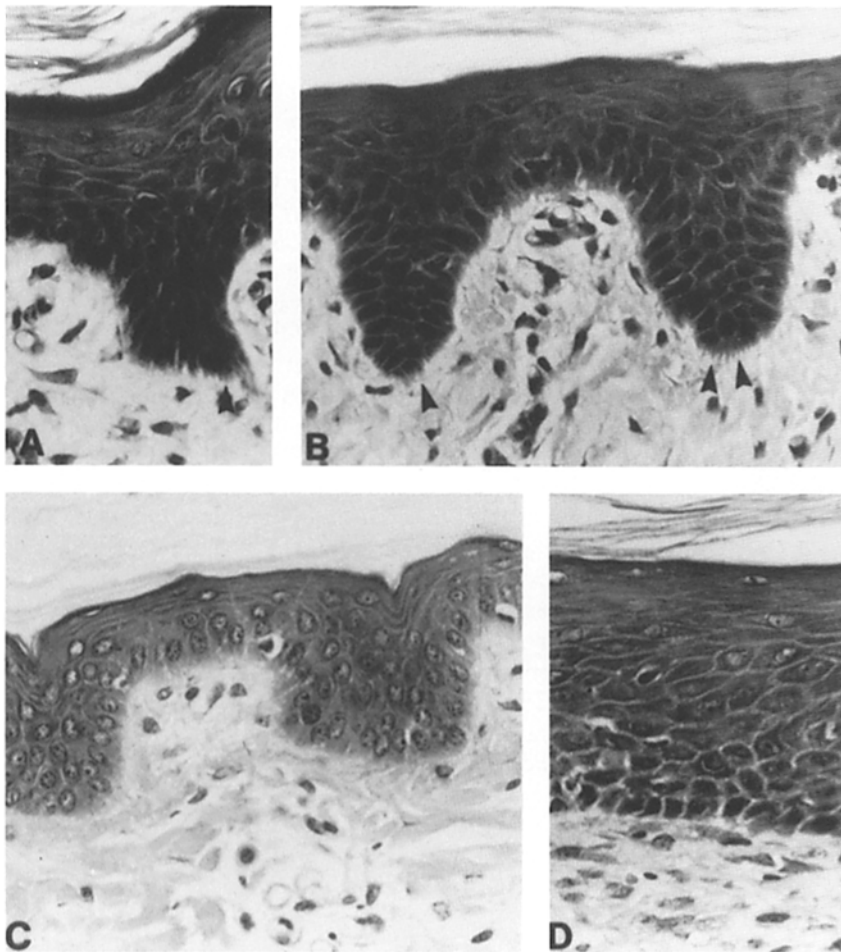
\* Epidermal thickness was measured by microscopy in vertical histologic sections as the distance (in millimeters) from the basal layer to the granular layer along the entire wound length. A minimum of 30 measurements was made for each histologic section. Where rete ridges were observed, measurements included the length along both the rete ridges and in the intervening flatter epidermis.

<sup>†</sup>Each experiment represents results from one individual pig.

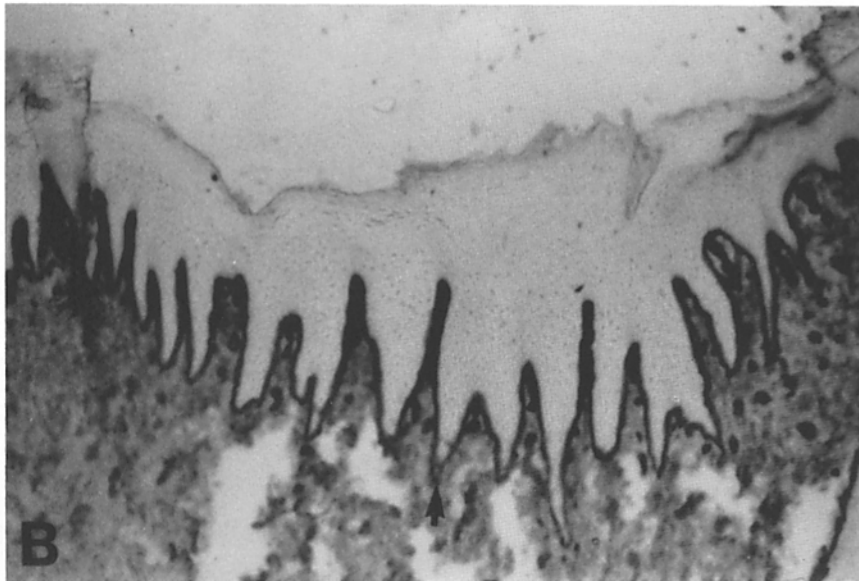
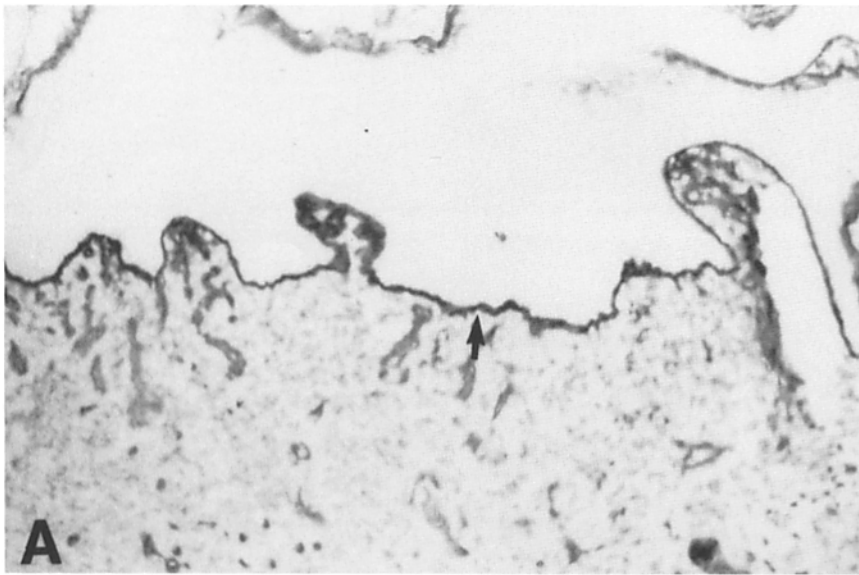
<sup>‡</sup>Difference between paired mirror image wounds ( $n = 31$  pairs for a total of 62 wounds) of KGF- and PBS-treated sites from nine different pigs;  $p < 0.0001$ .

<sup>¶</sup>Flat refers to the appearance of the basal side of the epidermis and represents an epidermis that essentially lacks rete ridges.

<sup>¶</sup>Morphologic appearance of the basal side of the epidermis exhibiting deep rete ridges.



**Figure 4.** The histologic appearance of basal layer epidermal cells from two representative KGF-treated wounds (A and B) shows rete elongation and a serrated basal cell phenotype with long processes that extended into the dermis (arrowheads). By contrast, the appearance of basal keratinocytes from normal skin adjacent to control wound sites (C) shows less rete elongation and basal cells with nonserrated surfaces. Finally the epidermis from PBS-treated wound sites (D) was considerably flatter than that of either nonwounded or KGF-treated sites. Basal cells from control wound sites also had a smooth cell surface with few processes extending into the dermis.  $\times 400$ .



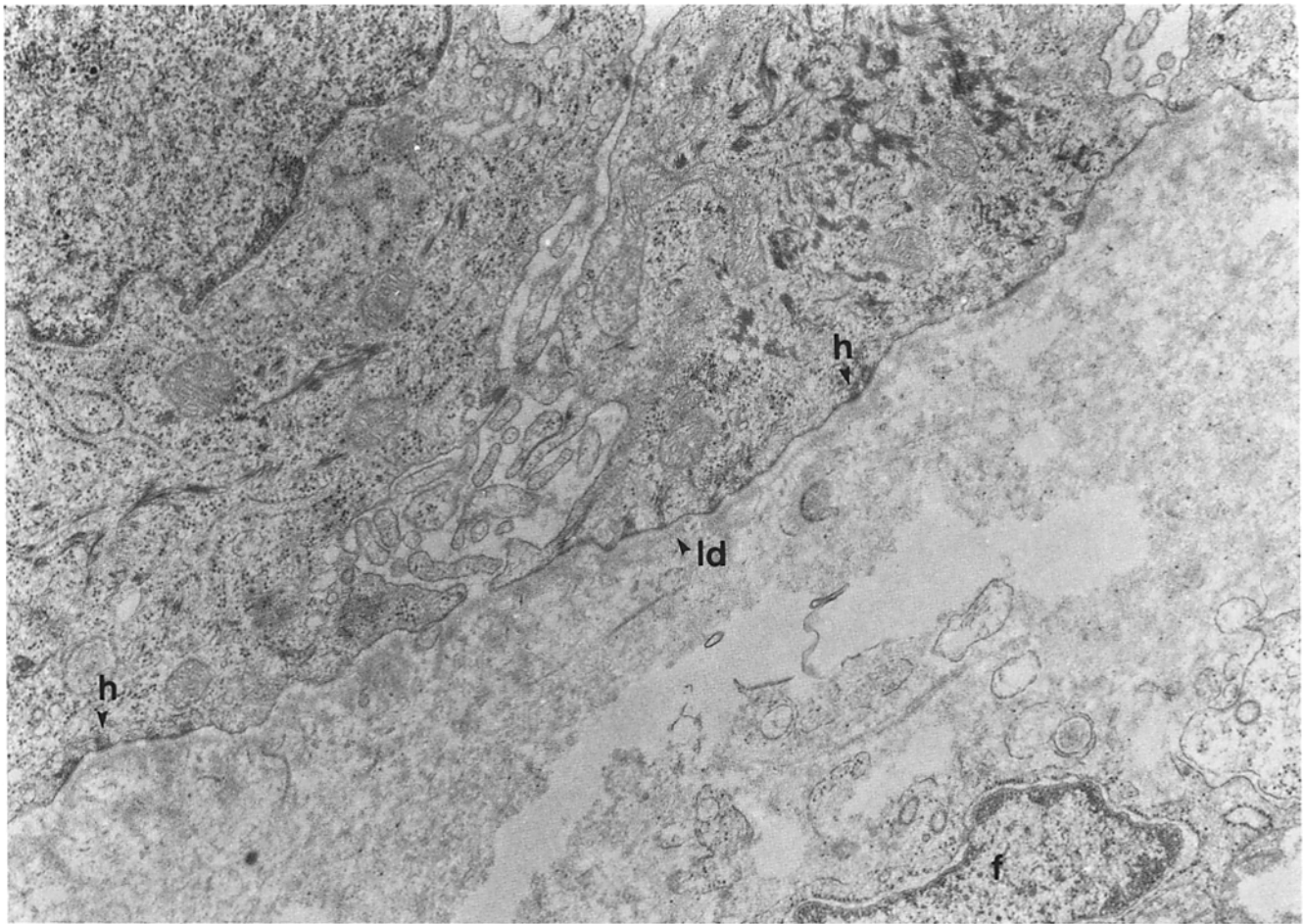
**Figure 5.** Immunolocalization of laminin (arrows) in biopsies (2 wk after surgery) from control (A) and KGF-treated (B) porcine wounds. Sections were reacted with anti-laminin Abs using standard immunoperoxidase techniques. A uniform layer of laminin (arrows) deposition was observed within the BMZ of both control (A) and KGF-treated (B) wounds.  $\times 200$ .

brane-associated, lower surface of the cell, but only a few well-developed tonofilaments were observed. The papillary dermis immediately below the keratinocyte was composed predominantly of a loose ECM with few collagen fibrils present.

In comparison, basal keratinocytes from KGF-treated wounds showed striking differences (Figs. 7 and 8). The surface of the basal keratinocyte was often heavily serrated (Fig. 7, arrowheads). The hemidesmosomes were better developed and thickened on the cytoplasmic side of the cell (Fig. 8, h). Thicker bundles of tonofilaments (Fig. 8, tf) were also abundant within the cytoplasm and in some areas the tonofilaments were observed in conjunction with the hemidesmosomes. Finally, collagen fibrils were more readily apparent in the papillary dermis. The appearance of synthetically active dermal fibroblasts with dilated endoplasmic reticulum

(Fig. 8, er), suggests that collagen fibers in KGF wounds might result from *in situ* synthesis. Well-formed anchoring fibrils were not observed in KGF-treated or control wounds. However, collagen fibers in KGF-treated wounds were often oriented perpendicular to the BMZ and appeared to connect with the BMZ near hemidesmosomes. The organization of collagen fibers adjacent to the BMZ suggests the possible existence of a functional tonofilament-hemidesmosome-collagen fiber unit or complex in 2-wk KGF-treated wounds.

*Kinetic Analysis of Rete Ridge Formation after KGF Treatment.* In the initial series of experiments, biopsies were obtained on day 28 after wounding. This day was chosen since it corresponded to a time at which all wounds (KGF and control) were healed and maximal contraction as assessed by computerized image analysis, was observed (see Fig. 3). To examine whether the response to KGF was an early or late



**Figure 6.** Representative electron micrograph of the dermal/epidermal junction of a control wound (2 wk after surgery). The lower surface of the basal keratinocyte is relatively flat with little interdigitation into the underlying tissue. Hemidesmosomes (*h*) are apparent and the lamina densa (*ld*) is indicated. The granulation tissue is composed primarily of loose ECM with few collagen fibers.  $\times 25,000$ .

event after wounding, a kinetic analysis of the morphologic appearance of KGF- and vehicle-treated full-thickness wounds was performed at weekly intervals.

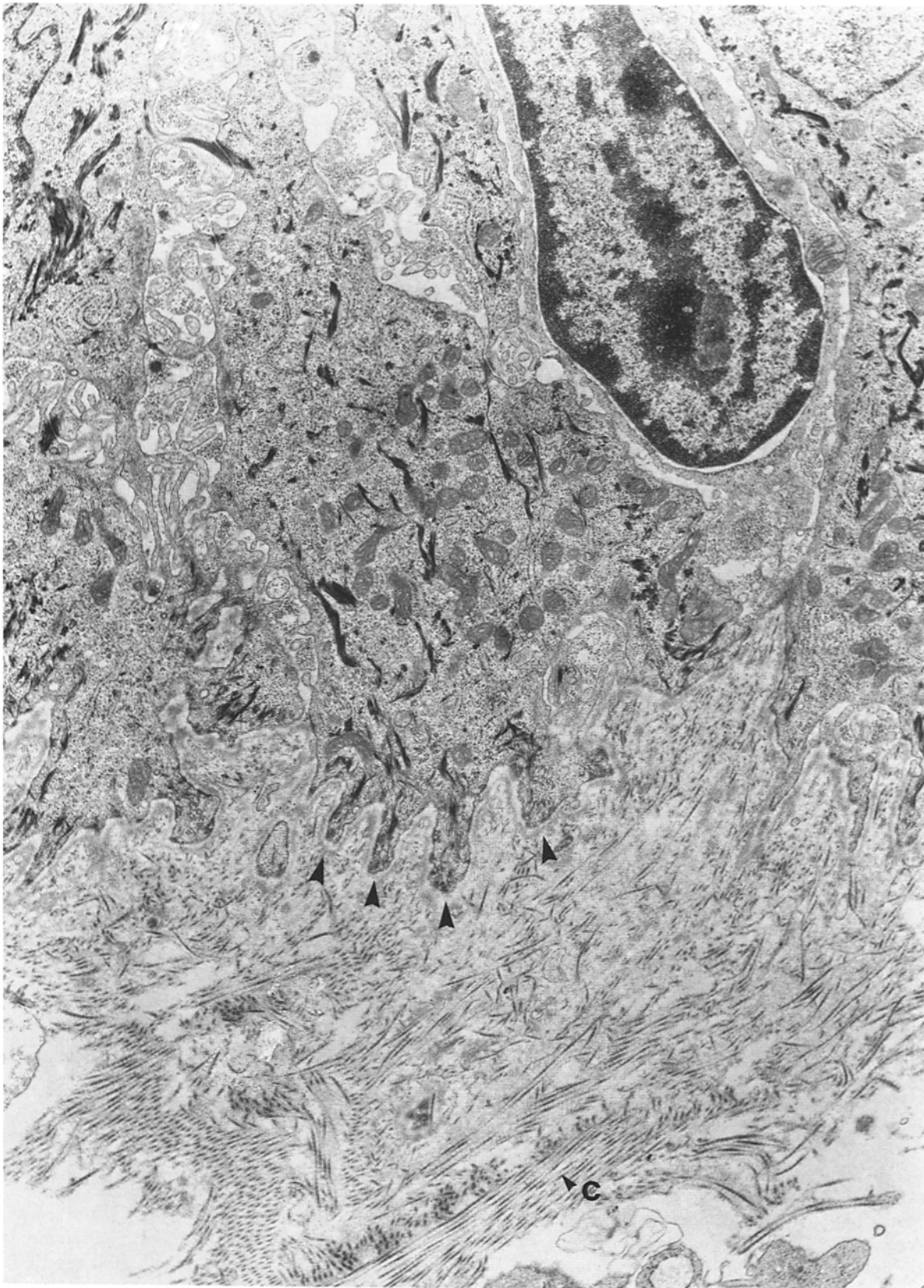
At 7 d after surgery, no significant closure was observed in control or KGF wounds, and crust covered the middle of the wounds (Fig. 9, *A* and *B*). Tongues of relatively undifferentiated epidermis, lacking a granular layer or stratum corneum, were observed at the wound edge (Fig. 9, *A* and *B*). In control wounds, the epidermis distal to the wound edge showed irregular acanthosis (Fig. 9 *A*, *open arrow*). In contrast, KGF-treated wounds already showed evidence of more uniform deep rete ridge pattern at this time (Fig. 9 *B*, *open arrow*). Examination of the wound bed on day 7 revealed loose granulation tissue with concomitant inflammatory cell infiltrates in both KGF and control wounds.

By day 14 after wounding, both control and KGF-treated wounds were generally reepithelialized (Fig. 9, *C* and *D*). In controls, the epidermis covering the original wound area displayed typical features associated with acute wound healing, that is, the epidermis was flat, parakeratotic in areas, and weakly

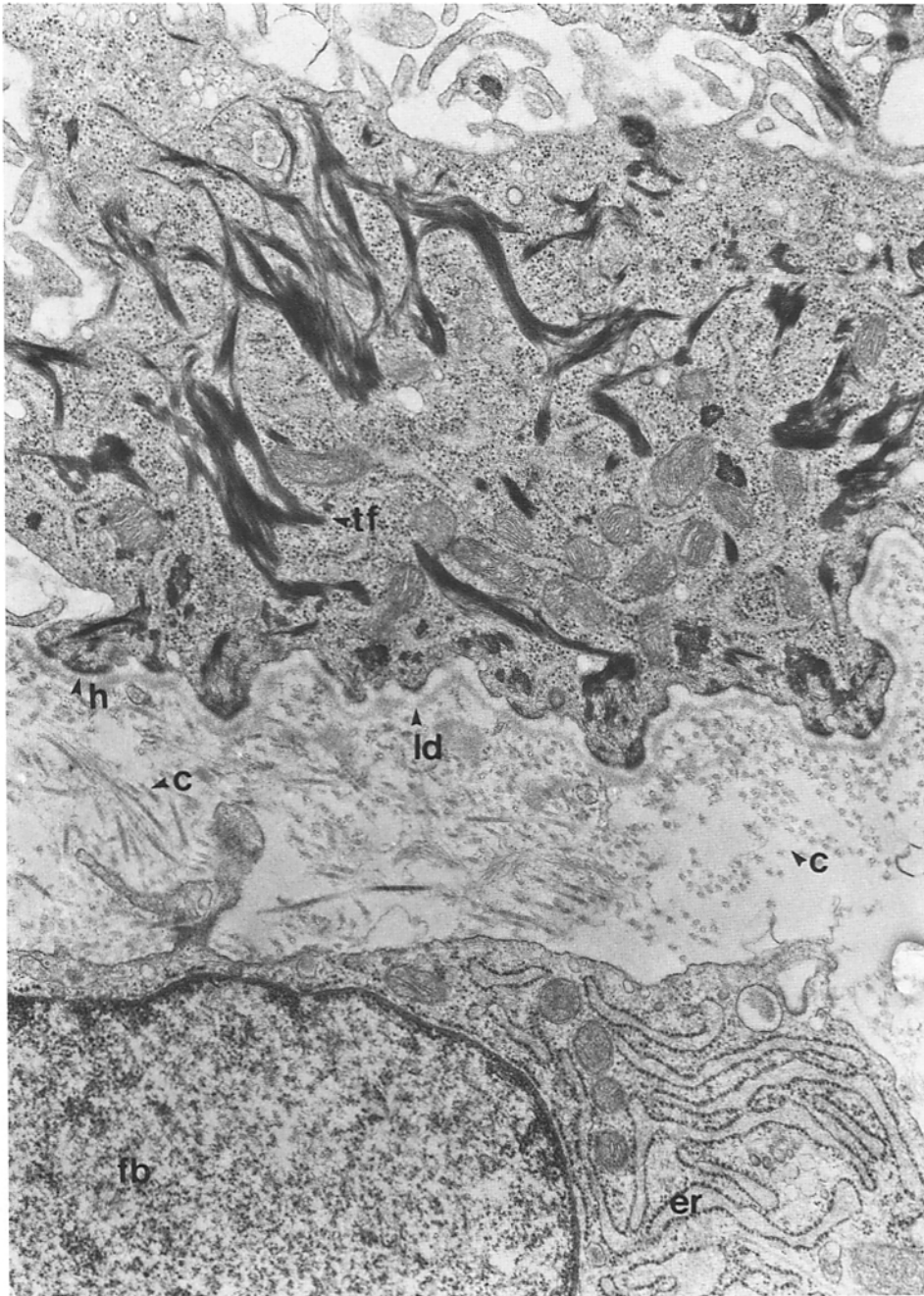
attached to the underlying granulation tissue (Fig. 9 *C*) (11, 12). By contrast, the newly formed epidermis covering the KGF-treated wound sites already exhibited pronounced rete ridge formation consistent with better attachment of the epidermis to the underlying wound bed (Fig. 9 *D*). As in control wounds, focal parakeratosis was observed in some KGF-treated wounds at this time. By 3 wk after wounding, complete differentiation of the epidermis was observed in both control (Fig. 9 *E*) and KGF-treated (Fig. 9 *F*) wounds. Again, KGF-treated wounds were thicker and more deeply interdigitated into the wound bed than the flattened epidermis of control wounds. These results suggest that the KGF induces an alteration in epidermal morphology early during the wound healing process which persists for at least several weeks after wound closure.

**Analysis of Proliferation Response in KGF-treated Wounds.** The histologic appearance of KGF-treated wounds superficially resembled the hyperproliferative epidermis seen in psoriasis and other dermatoses with psoriasiform hyperplasia (41). To determine whether, in fact, the epidermis from KGF-treated





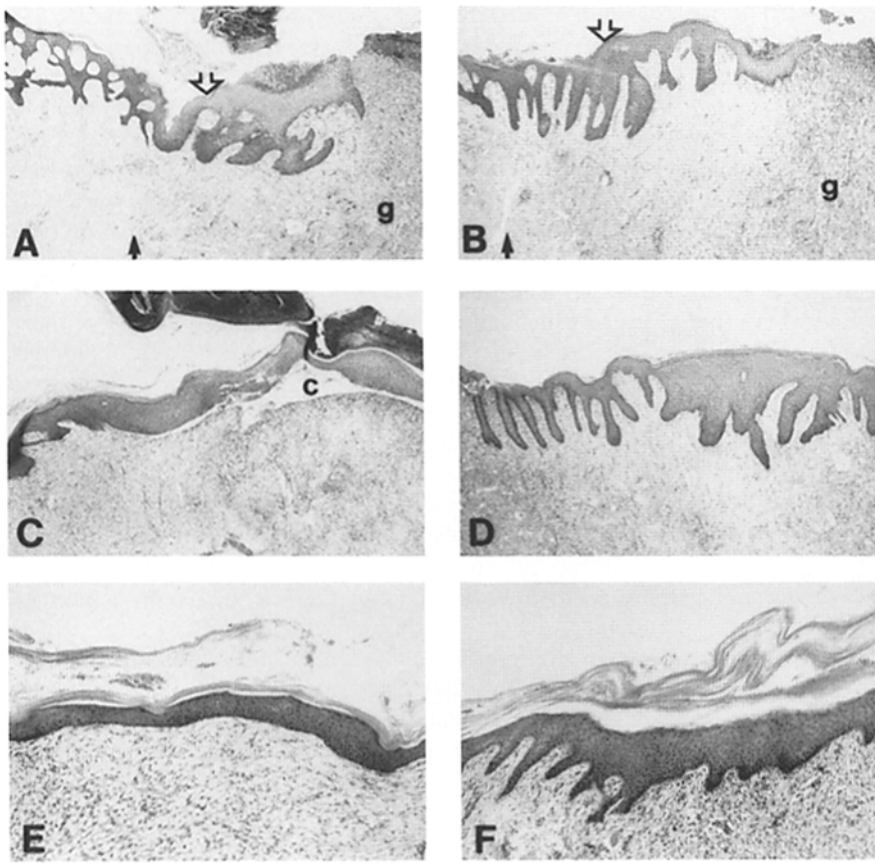
**Figure 7.** Representative electron micrograph of the dermal/epidermal junction of a KGF-treated wound (2 wk after surgery). Basal keratinocytes from KGF-treated wounds exhibited serrations (*arrowheads*) that extended down into the underlying wound bed. Numerous collagen fibrils (*c*) were apparent in the papillary dermis of such healed wounds.  $\times 12,500$ .



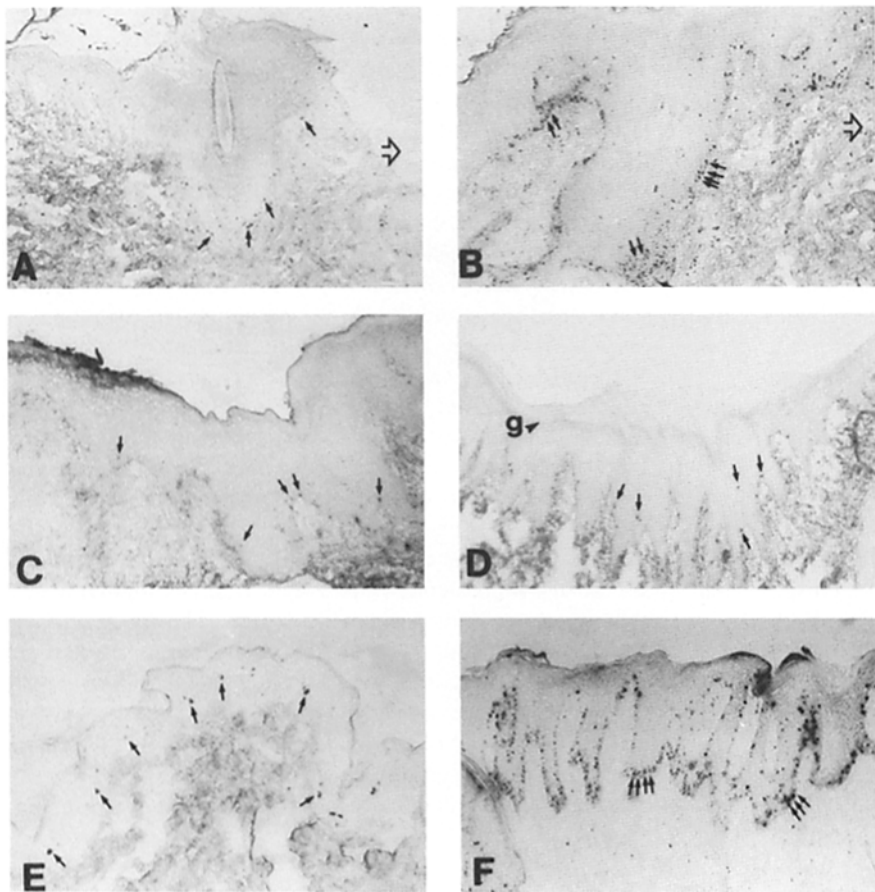
**Figure 8.** Representative electron micrograph (at higher magnification  $\times 25,000$ ) of the dermal/epidermal junction from a KGF-treated wound (2 wk after surgery). The cell surface of the basal keratinocyte exhibited processes that interdigitated into the underlying dermis. Hemidesmosomes (*h*) were apparent and thicker than those observed in control wounds (see Fig. 6). Moreover, thicker and more abundant tonofilaments (*tf*) were observed in KGF-treated wounds. Dermal fibroblasts (*fb*) contained abundant, dilated endoplasmic reticulum (*er*) suggestive of a metabolically active state.

wounds was hyperproliferative, a nuclear Ki-67-reactive Ab was used in frozen sections of full-thickness swine wounds (KGF and control) during the course of healing (Fig. 10). Cell proliferation was assessed by Ki-67 reactivity which is present only in actively proliferating cells (39). In normal (unwounded) pig epidermis, Ki-67 reactivity was localized focally within the basal layer of the epidermis, with  $\sim 9\%$  of basal cells being Ki-67<sup>+</sup> (Fig. 10 E; arrows). Within 1 wk

after wounding, an increase in the prevalence of Ki-67<sup>+</sup> epidermal cells was observed in both control (Fig. 10 A, arrows) and KGF-treated (Fig. 10 B, arrows) wounds. The magnitude of the proliferative response was, in general, greater in KGF-treated wounds (Fig. 10 B) compared with controls (Fig. 10 A). As described above, by 2 wk after wounding, the majority of KGF-treated wounds exhibited deep rete ridges (see Fig. 1). Examination of Ki-67 reactivity at this time showed that



**Figure 9.** Kinetic examination of the healing of full thickness wounds in response to KGF. Control (A, C, and E) or KGF-treated (B, D, and F) wounds were biopsied day 7 (A and B), 14 (C and D) or 21 (E and F) after surgery and their morphologic appearance assessed after H&E staining. At 1 wk after treatment, tongues of epidermis could be seen migrating over the wound beds (arrows indicate wound bed boundary). Whereas irregular acanthosis was observed at the edges of control wounds (A), a more uniform rete ridge pattern was observed in KGF-treated (B) wounds. By 14 d, both control (C) and KGF-treated (D) wounds had reepithelialized, although maturation of the epidermis was incomplete. The epidermis of control (C) wounds was flat with relatively poor attachment to the underlying wound bed (c shows a cleft between the epidermis and dermis). Equivalent KGF-treated wounds (D) exhibited deep rete ridges. By 21 d, orthokeratotic maturation was observed in both control (E) and KGF-treated (F) wounds. The epidermis from control (E) wounds remained flat and relatively thin, whereas the epidermis from KGF-treated (F) wounds retained its deep rete ridge appearance.  $\times 100$ .



**Figure 10.** Assessment of proliferative activity by Ki-67 reactivity in unwounded porcine epidermis (E), healing epidermis from control (A and C) and KGF-treated (B and D) wounds. Normal unwounded porcine epidermis (E) showed focal areas of nuclear reactivity with Ki-67 within the basal layer of the epidermis. At 7 d after wounding, an increase in Ki-67 reactivity was observed in both control (A) and KGF-treated (B) wounds at the migrating edges of the epidermis. By 2 wk, Ki-67 reactivity decreased in both control (C) and KGF-treated (D) wounds. Whereas 2 wk KGF-treated wounds exhibited a deep rete ridge pattern within the epidermis, the proliferative activity of such wounds could be clearly distinguished from that of psoriasis (F) which showed a similar morphologic appearance of the epidermis, but displayed significantly increased Ki-67 reactivity, not only within the basal layer of the epidermis, but also within suprabasal layers. (Thus the epidermis of KGF-treated wounds is not associated with persistent hyperplasia as seen in psoriasis.)  $\times 100$ .

the number Ki-67<sup>+</sup> cells decreased in both control (Fig. 10 C) and KGF-treated (Fig. 10 D) sites relative to 1-wk wounds.

Comparison of the epidermis from a 2-wk-old KGF-treated wound site (Fig. 10 D) and an active psoriatic lesion (Fig. 10 F) showed distinctly different patterns of Ki-67 reactivity. Characteristically, psoriatic epidermis was thickened with deep rete ridges (Fig. 10 F). The epidermis was parakeratotic and expressed high numbers of Ki-67<sup>+</sup> cells (~47%) within basal layer. Moreover, Ki-67 reactivity was not confined to the basal layer, but was apparent in suprabasal cell layers as well (Fig. 10 F; *arrows*); ~27% of cells one layer above the basal layer were reactive with Ki-67. In contrast, in epidermis from the KGF-treated site, Ki-67 reactivity was much less abundant and was confined to the single basal layer (~12% Ki-67<sup>+</sup> cells within the basal layer). These data suggest that KGF induces a transient early proliferative response with a persistent effect on epidermal morphology in the absence of sustained hyperplasia.

## Discussion

The results of the present study indicate that KGF has marked effects on the epithelialization and healing of partial- and full-thickness porcine wounds. Although KGF is a member of the FGF family, it interacts with an epithelial cell-specific receptor (34). The restricted expression of the KGF receptor presumably would limit the direct effect of KGF to epithelial tissues, unlike that of related family members aFGF or bFGF, which are mitogenic for fibroblasts, endothelial cells, and epithelial cells (23, 24, 42).

In the present experiments, KGF, as predicted, induced its predominant effects on the newly-formed epidermis. Epidermis from KGF-treated wounds was significantly thicker than epidermis from mirror image controls. In addition, 77% of KGF-treated full-thickness wounds exhibited deep rete ridges that extended into the dermis. The ultrastructural features of the epidermis revealed a more mature basal keratinocyte with an undulating cell surface and better developed tonofilaments and hemidesmosomes. Epidermal thickening similar to that exhibited by most healed KGF-treated full-thickness wounds, is also observed in human skin associated with psoriasis and some inflammatory dermatoses. However, the histologic appearance of KGF-treated wounds could be readily distinguished from these disorders by the normal pattern of orthokeratotic epidermal maturation, the presence of a granular layer, as well as by the absence of acute/chronic immune infiltration into affected tissue (above that of control wounds). Moreover, the Ki-67 staining revealed a proliferative pattern confined to the basal epidermal layer. This is distinctly different from psoriasis in which the basal layer and several suprabasal layers react intensely with Ki-67. Exogenous KGF, added early in wound repair appears to induce a transient proliferative response in keratinocytes that contrasts with the sustained epidermal hyperplasia in psoriasis.

The response of the epidermis to exogenous KGF is also different from that observed in response to either EGF or TGF- $\alpha$ . EGF, TGF- $\alpha$ , and related ligands have also been

shown to directly affect wound reepithelialization (26, 28). When injected intradermally, EGF produces marked thickening of associated epidermis, but does not cause rete elongation. In experimental wounds, EGF and TGF- $\alpha$  accelerate reepithelialization, but have minimal effects on overall epidermal structure. In skin wounds of transgenic mice that overexpress TGF- $\alpha$  in the adult epidermis, exophytic growths occur which resemble papillomas on histologic examination (43). KGF-treated wounds were macular or slightly depressed upon healing, and the epidermis was clearly different from that of the papillomatous epidermal hyperplasia in TGF- $\alpha$  transgenic mice (43). The difference in the histologic appearance of EGF- and KGF-treated wounds is consistent with observed differences in the ability of these molecules to modulate epidermal differentiation, even though both cytokines are mitogenic for keratinocytes (35).

Also of interest was the apparent effect of KGF on the formation of granulation tissue within full-thickness wounds. KGF induced an increased appearance of more mature collagen fibrils within the superficial dermis. Whereas this apparent change in collagen appearance could have resulted from local alterations in wound contraction, ultrastructural analysis suggests that the collagen apparent in the papillary dermis of KGF-treated wounds arises, at least in part, by de novo release of collagen from fibroblasts within the granulation tissue (Figs. 6 and 7). Since KGF seems to be epithelial specific in its action, the stimulation of collagen within the fibroblast would presumably occur via a secondary cytokine, perhaps released by the healing epidermis into the wound bed. Moreover, the effects of KGF on collagen bundle formation are clearly distinct from those produced by treatment of full-thickness wounds by TGF- $\beta$  which stimulates collagen deposition more uniformly throughout the wound (25).

The appearance of serrated basal layer epidermal cells (as assessed by light and electron microscopy) in conjunction with more mature collagen in the papillary dermis of KGF-treated wounds has significant implications for the strength and durability of such healed wounds. The presence of serrated and nonserrated basal cells has been documented since the 1950s, but initially their function was unknown (44). More recent studies of Lavker and Sun (45) have suggested that the serrated basal cells are a primary anchoring force for binding of the epidermis to the dermis (45). The presence of greater numbers of serrated basal cells within KGF-treated wounds compared with control wounds suggests that KGF might mediate better attachment of epidermis to the dermis, thereby providing an increased resistance to shear forces that can cause retraumatization of newly healed wounds. KGF might be a useful therapeutic agent in the treatment of some wounds associated with blistering (such as epidermolysis bullosa) which exhibit poor epidermal-dermal cohesion. The action of KGF appears to be unique in this regard compared with other established growth factors such as EGF or bFGF which induce an epidermal morphology in healing wounds that is similar to the PBS-treated control wounds presented in this study. These results strongly suggest that KGF not only affects ultrastructural features of basal keratinocyte differentiation, but



also modulates (perhaps via induction of secondary cytokines originating in the epidermis) the maturity and phenotype of the underlying wound bed. Thus KGF may exert impor-

tant effects on epidermal/dermal structure in its normal functions as a paracrine cytokine.

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