Modulation of Keratinocyte Growth Factor and its Receptor in Reepithelializing Human Skin

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Summary

We investigated the expression and distribution of keratinocyte growth factor (KGF) (FGF-7) and its receptor (KGFR) during reepithelialization of human skin. KGF mRNA levels increased rapidly by 8–10-fold and remained elevated for several days. In contrast, KGFR transcript levels decreased early but were significantly elevated by 8–9 d. A KGF–immunoglobulin G fusion protein (KGF-HFc), which specifically and sensitively detects the KGFR, localized the receptor to differentiating keratinocytes of control epidermis, but revealed a striking decrease in receptor protein expression during the intermediate period of reepithelization. Suramin, which blocked KGF binding and stripped already bound KGF from its receptor, failed to unmask KGFRs in tissue sections from the intermediate phase of wound repair. The absence of KGFR protein despite increased KGFR transcript levels implies functional receptor downregulation in the presence of increased KGF. This temporal modulation of KGF and KGFRs provides strong evidence for the functional involvement of KGF in human skin reepithelialization.

The interactions between growth factors and their receptors play critical roles in normal development as well as in host responses to infection and tissue injury. In epithelial tissues, keratinocyte growth factor (KGF)[1] (also designated FGF-7) appears to be one such important mediator (1). KGF is expressed by stromal cells and acts specifically on epithelial cells in a variety of tissues, including skin, lung, and gastrointestinal tract, as well as in male and female reproductive organs (2–6). The actions of KGF have been most well characterized with respect to keratinocytes in vitro (4) and in vivo (7, 8). The growth factor is a potent mitogen for human keratinocytes in culture and promotes the normal differentiation program (4).

Evidence that KGF plays an important role in wound healing derives from recent findings in animal models. In mouse skin, the KGF transcript increased rapidly and to high levels relative to those of several other fibroblast growth factor (FGF) family members analyzed in response to full-thickness wounding (9). In the porcine model, topical application of KGF to both split and full-thickness wounds resulted in an increased rate of reepithelialization (10). Based on these findings and the known effects of KGF on human keratinocytes in vitro, we sought to characterize the in vivo modulation of KGF and its receptor in human skin during the normal wound repair process. For this study, we took advantage of a new approach for KGF receptor immunodetection by means of a chimeric KGF ligand fused to the HFc portion of the IgG molecule (11).

Materials and Methods

Tissue Preparation. Patients admitted to the Plastic Surgery Unit of the University of Rome "La Sapienza" were selected for the absence of any hyperproliferative skin disease or dysmetabolic or immunosuppressive disorder, as well as for any other pathology affecting the healing process. Patients ranged from 20 to 50 yr of age and required split-thickness skin grafts for unrelated conditions. Informed consent was obtained according to procedures approved by the Institutional Review Board of the university. The donor area was dressed with saline-soaked gauze. Three

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[1]*Abbreviations used in this paper:* EGF, FGF, KGF, and PDGF, epidermal, fibroblast, keratinocyte, and platelet-derived growth factor, respectively; HFc, IgG, heavy chain constant region; K1, keratin 1; KGFR, KGF receptor.

full-thickness biopsies (of ~16 mm²) were obtained from the same area. The first was harvested at the time of surgery under general anesthesia, that is, on day 0, whereas the remaining biopsies were taken under local anesthesia (1.5 ml carbocaine, mepivacaine, with 2% adrenaline) at varying intervals during the postoperative period. The biopsy donor sites were repaired with two 5/0 nylon simple stitches. Specimens were frozen in liquid nitrogen for further analysis.

125I-KGF–binding Analysis. KGF binding was performed using ^{125}I-KGF labeled by the chloramine T method as previously described (12).

Immunohistochemistry. Sections (5 μm) were prepared in the cryostat and fixed in a mixture of absolute ethanol, 1% acetone, 1.0 mM tetracetic acid/ethanol for 2 min, then transferred to 100% ethanol followed by 50% ethanol and PBS, pH 7.4, and then placed on chromium–potassium sulfate dodecahydrate gel–coated slides. After preincubation at room temperature in 5% dry milk, 0.1% Tween 20, and 0.05% thimerosal for 1 h, the milk was blotted from the slides, and the specimens were incubated either in the presence of the KGF-HFc chimera (11) or the control HFc IgG₁ for 1 h at room temperature in a humidified chamber. In some experiments, tissue sections were incubated with a mouse mAb reactive with the Ki67 nuclear antigen (Immunotech, Westbrook, ME) at 1:100 in 2% BSA/PBS or mouse IgG (control). After incubation, tissues were rinsed several times in PBS and further incubated in a three-step immunoperoxidase procedure. Immunoreactivity was visualized using 3,3-diamino benzidine tetrachloride (Pierce Chemical Co., Rockford, IL) as chromogen according to the manufacturer's protocol. In some experiments, tissue sections were incubated with 100 μM suramin, generously provided by the Drug Development Branch, National Cancer Institute, as described in Results.

Double Immunofluorescence. For double immunofluorescence, tissue sections, prepared as described above, were incubated with KGF-HFc for 2 h at 4°C in a humidified chamber, fixed in 4% paraformaldehyde for 30 min, and washed three times in PBS, followed by exposure to FITC-conjugated secondary antibody (affinity-purified goat anti–mouse IgG) (Cappel Laboratories, Cochranville, PA). The same sections were then incubated overnight with polyclonal guinea pig anti–human K1 serum (kindly provided by Dr. Dennis Roop, Baylor Medical School, Houston, TX) followed by biotinylated anti–guinea pig IgG (Vector Laboratories, Inc., Burlingame, CA) and subsequently Texas red–conjugated streptavidin (GIBCO BRL, Gaithersburg, MD).

RNA Preparation and Northern Blot Analysis. Tissue samples were pulverized in the presence of liquid nitrogen and homogenized in RNAzol (TelTest). Total RNA was precipitated with cold isopropanol (50% vol/vol), washed in 75% ethanol, and resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA). 20-μg samples of RNA were electrophoresed on 1% formaldehyde agarose gels and transferred to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH). To evaluate the integrity of the RNA, gels were stained with ethidium bromide. After cross-linking of the RNA to the membrane, filters were prehybridized for 2 h at 42°C in Hybrisol (50% formamide, 10% dextran sulfate, 1% SDS, 6× SSC, and blocking agents) (Oncor, Inc., Gaithersburg, MD) and were hybridized for 20 h in the same solution to which [³²P]dCTP-labeled cDNA probes were added. Filters were washed twice (30 min each time) at room temperature in 2× SSC, 0.1% SDS, twice at 45°C in 0.1× SSC, 0.1% SDS, and exposed to x-ray film (Eastman Kodak Co., Rochester, NY). Densitometric analysis was performed with a scanner densitometer (Bio Rad Laboratories, Richmond, CA).

Probes included cDNAs corresponding to exon 1 of the human KGF gene (13), a 110-bp fragment that detects the KGF receptor (KGFR) alternative exon (14), and the full coding sequence of the human vimentin gene (12).

Measurement of KGF Protein. Tissue samples were thawed and homogenized with a tissue disrupter (Polytron; Brinkmann Instruments, Inc., Westbury, NY) in a solution (2 ml/g wet wt) consisting of 1.0 M NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Samples were sonicated (3 pulses of 30 s, power setting = 10) (Heat Systems; Misonix, Farmingdale, NY) and cleared by centrifugation at 40,000 g for 30 min at 4°C. The protein content in the supernatants was measured, and all samples were adjusted to a uniform concentration before the assay. Supernatants were analyzed for KGF using a two-site ELISA. Briefly, 96-well polyvinyl microtiter plates (no. 3912; Falcon Labware, Oxnard, CA) were precoated overnight with 50 μl per well of a KGF mAb, 1G4 (8 μg/ml), and subsequently blocked with 4% BSA. Serial dilutions of tissue extracts (protein concentrations <11 μg/ml) were added to wells (50 μl per well) and incubated for 5 h. Wells were washed extensively with 0.05% Tween, 0.02% sodium azide in PBS, and further incubated overnight with a rabbit polyclonal antibody (designated 9492) raised against human recombinant KGF. After extensive washing, alkaline phosphatase–conjugated goat anti–rabbit IgG (Tago, Inc., Burlingame, CA) (1:15,000 dilution) was added to the wells. After 2 h, the wells were again washed and p-nitrophenyl phosphatate (2 mg/ml) was added. OD was measured at 405 nm with an ELISA scanner (Bio Rad Laboratories). The concentration of the recombinant human KGF standard (3) was based on amino acid analysis and extinction coefficient.

Results

Modulation of KGF and KGFR Transcript Levels during Human Skin Reepithelization. In an effort to investigate the modulation of KGF and its receptor during normal human wound repair, we initially measured transcript levels of each in tissue samples at various times after split-thickness grafting. The KGF exon I sequence was used as a cDNA probe because this exon is present at single copy number in the human genome, whereas KGF exons 2 and 3 are represented at multiple copies (13). A KGFR-specific cDNA probe was derived from the alternative exon that specifies KGF high affinity binding (14). Each served as an internal control for the other in hybridizations performed on the same tissue RNA sample.

Fig. 1 shows results obtained with two series of tissue samples from different patients during the course of the wound repair process. In each case, KGF transcript levels increased substantially compared with the control at early times (1 and 3 d) and remained elevated, but to a lesser extent, after 7–8 d. When standardized relative to vimentin transcript levels, the increase in KGF RNA was as much as 8–10-fold at early time points. These results implied a major and rapid upregulation in KGF RNA expression in wounded human epithelium. When the same tissue RNA samples were hybridized instead with the KGFR-specific probe, we observed decreased KGFR transcript levels at early time points (1 and 3 d), with a subsequent elevation
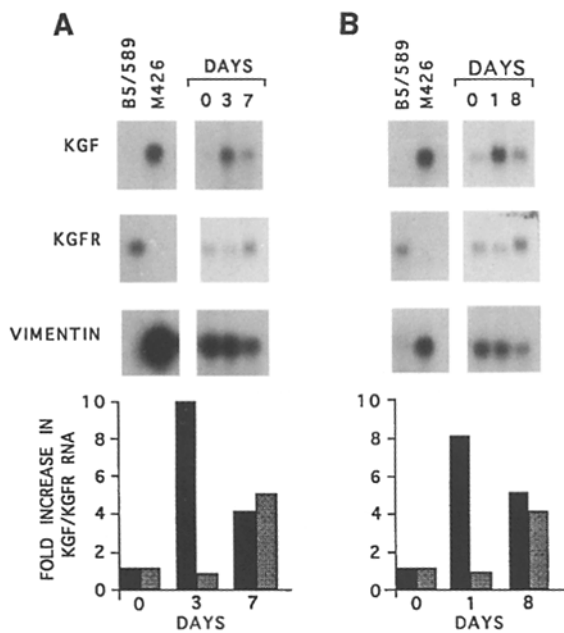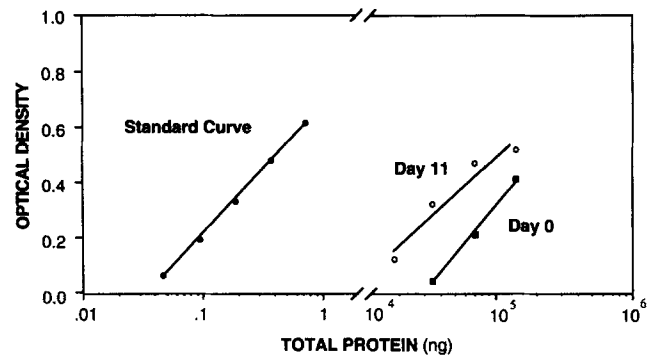
Figure 1. Modulation of KGF and KGFR RNAs after injury. Total RNA was extracted from tissue samples at different periods after surgery and analyzed for KGF, KGFR, and vimentin expression by Northern blot analysis. *A* and *B* represent samples obtained from two different patients. Autoradiograms were quantitated by densitometry, and the induction in KGF and KGFR transcripts is graphically represented at the bottom of each panel. Filled bars correspond to KGF RNA, and stippled bars correspond to KGFR RNA. In both cases, results were standardized relative to vimentin transcript levels.

Figure 2. ELISA analysis of KGF protein in wound-healing biopsies. KGF protein was extracted from tissue samples obtained at the time of surgery and 11 d after surgery. Serial dilutions of each sample were assayed along with dilutions of a recombinant human KGF standard. Each data point represents the mean value of duplicate measurements.

of 4–5-fold above control levels after 7–8 d. The early decrease could reflect the loss of epithelial cells (see below), which normally express the KGFR transcript (14–16).

Increased KGF Protein Expression in Response to Split-Thickness Wounding. To establish whether increased KGF transcript levels reflected elevated KGF protein expression in reepithelializing skin, we took advantage of an ELISA, which sensitively and specifically detects the KGF product (6). As shown in Fig. 2, tissue extracts from normal skin contained readily measurable KGF immunoreactivity. However, tissue samples from the same patient taken 11 d after wounding showed KGF immunoreactivity at increased levels, corresponding to a sustained two- to threefold elevation in growth factor concentration. Similar findings were obtained with other paired samples from control versus reepithelializing skin (data not shown). Thus, increased KGF transcript levels correlated with elevated tissue levels of the growth factor.

Immunohistochemical Localization of KGFRs during Tissue Repair. We recently developed a molecular approach to generate a chimeric protein encompassing the KGF coding sequence fused to the IgG HFc domain. This molecule can be secreted efficiently from mammalian cell transfectants, and it combines the receptor-binding properties of the growth factor and the convenient detection properties of an Ig. Moreover, we demonstrated the specificity of this monoclonal-like growth factor–Ig fusion protein in the immunodetection of KGFRs by FACS®, as well as in tissue

sections (11). The expression and distribution of KGFRs were evaluated on sections of healing wounds using the KGF-HFc chimera.

In control skin, KGFRs were specifically localized to keratinocytes throughout the stratum spinosum. The pattern of staining was uniform around the cell surface. Little staining was detected in either the basal layer or in the granulosum or corneum strata (Fig. 3 *A*). This pattern of staining was consistent with our recent findings of the distribution of KGFRs in normal skin (11). 3 d after surgery, there was a marked decrease in receptor expression (Fig. 3 *B*), and its lack or greatly diminished expression persisted through the intermediate healing period (9 d, Fig. 3 *C*). During this latter time, the suprabasal epithelium was visibly hypertrophic, with larger cells as well as an increased number of cell layers (Fig. 3 *C*). The striking absence of detectable KGFRs during this phase of the healing process resulted in a reasonably well-demarcated junction between the hypertrophic region undergoing reepithelialization and noninvolved areas, which expressed KGFRs (Fig. 3 *D*). Of note, the absence of detectable suprabasal KGFR expression occurred during a time which the epithelium was substantially renewed and expressed KGFR transcript levels four- to fivefold higher than control skin (Fig. 1). At late stages in the process of reepithelialization (15 d), KGFRs remained undetectable in proliferating keratinocytes of the basal layer, whereas receptor immunoreactivity had returned to the pattern of suprabasal distribution observed in the control (Fig. 3 *E*).

Absence of Immunodetectable KGFRs in Reepithelializing Keratinocytes Reflects Receptor Downmodulation. The absence of KGFR immunostaining during the intermediate stage of reepithelialization despite elevated KGF and KGFR transcript levels could reflect receptors occupied by increased levels of ligand and, thus, not available for reaction with the KGF-HFc probe. Alternatively, the lack of immunostaining could be caused by functional receptor downmodulation. To differentiate between these alternatives, we took advantage of the knowledge that suramin, a highly anionic naphthalene sulfonic acid derivative (17), can interfere with
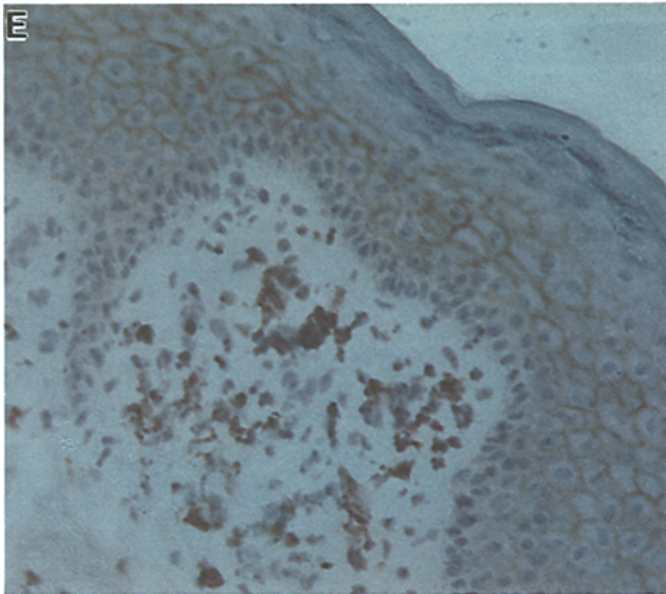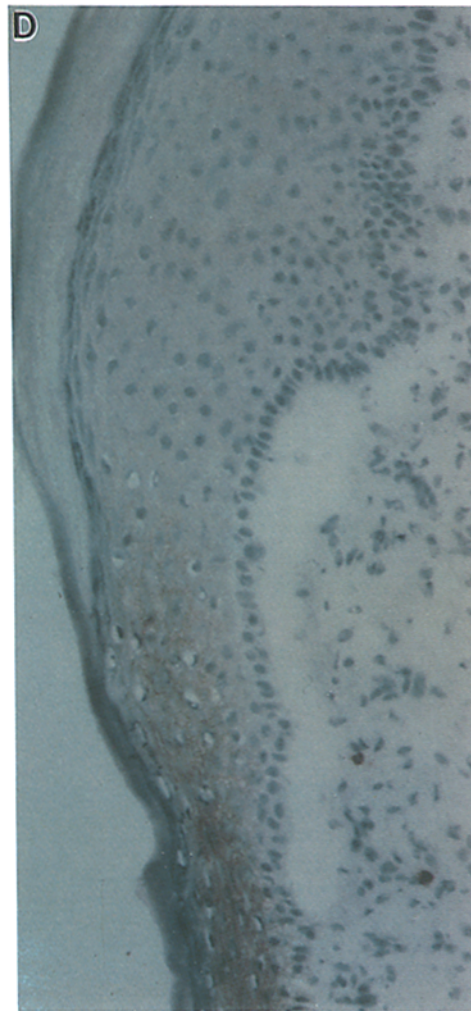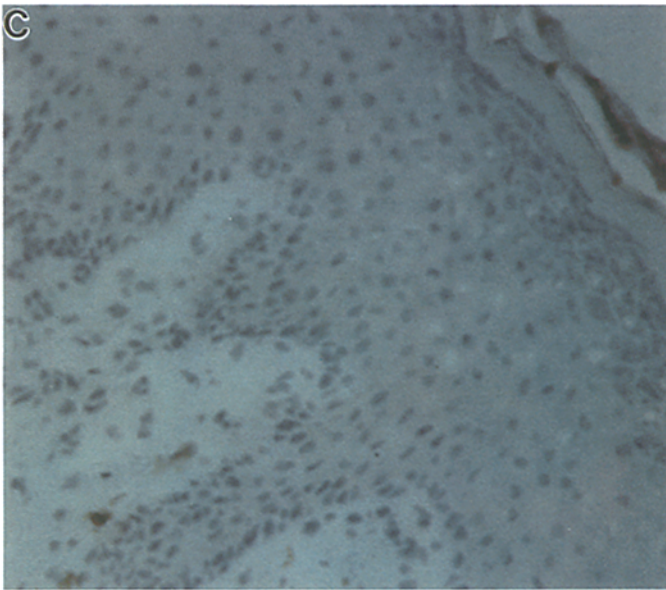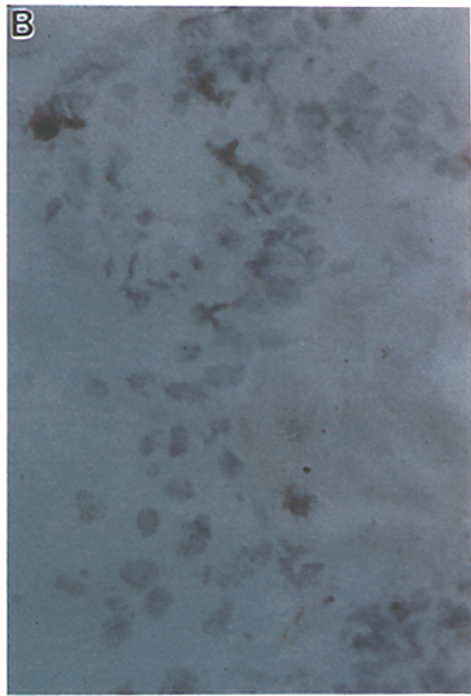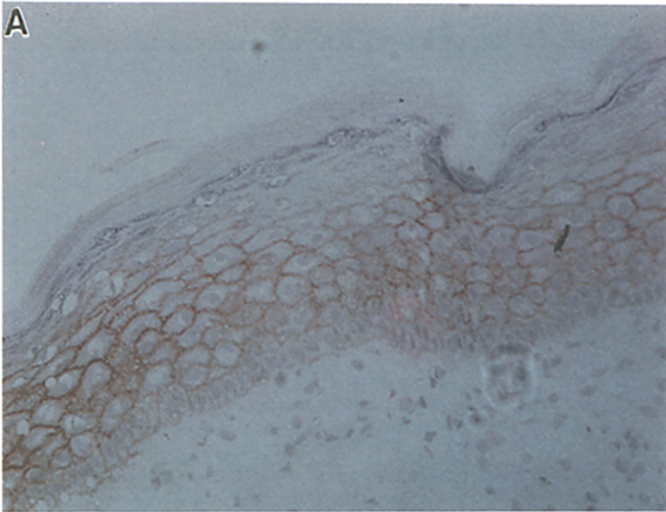
Table 1. *Suramin Inhibition of Specific ^{125}I-KGF Binding to KGFR-expressing Cells*

Treatment	Specific ^{125}I-KGF bound
^{125}I-KGF	10,814 ± 200
^{125}I-KGF + suramin	240 ± 35
^{125}I-KGF $\xrightarrow{\text{wash}}$ suramin	2,000 ± 50
suramin $\xrightarrow{\text{wash}}$ ^{125}I-KGF	10,209 ± 220

^{125}I-KGF binding was performed as described in Materials and Methods. Incubation with ^{125}I-KGF and/or suramin (100 μM) was as indicated. Results represent the mean values of experiments performed in duplicate.

Table 2. *Effects of Suramin on KGFR Detection in Human Skin Sections*

Exposure before the immunoperoxidase reaction	Control	Intermediate wound repair
KGF-HFc $\xrightarrow{\text{wash}}$	Positive	Negative
KGF-HFc + suramin $\xrightarrow{\text{wash}}$	Negative	Negative
KGF-HFc $\xrightarrow{\text{wash}}$ suramin $\xrightarrow{\text{wash}}$	Negative	Negative
suramin $\xrightarrow{\text{wash}}$ KGF-HFc	Positive	Negative

KGFR immunostaining was performed as described in Materials and Methods, according to the protocol as indicated for incubation with KGF-HFc and/or suramin (100 μM).

binding by certain growth factors (18). Table 1 shows that suramin dramatically inhibited specific ^{125}I-KGF binding to its receptor (Table 1). Moreover, suramin treatment was capable of efficiently removing already bound ^{125}I-KGF from cells (Table 1). Of note, the effects of suramin were completely reversible. Thus, washing of suramin-treated cells before ligand exposure was associated with ^{125}I-KGF–specific binding levels comparable to that of untreated cells (Table 1).

We next investigated the effects of suramin exposure on KGFR detectability in tissue sections of control skin. As summarized in Table 2, the KGF-HFc demonstrated the expected staining of the stratum spinosum. Simultaneous exposure to suramin effectively blocked KGF-HFc binding, but immunostaining was readily observed when tissue sections were first exposed to suramin and washed before immunostaining with KGF-HFc (Table 2). These results established that suramin treatment did not irreversibly impair the ability of the KGF-HFc to detect KGFRs. To determine whether suramin was also capable of stripping ligand already bound to receptors, tissue sections were incubated with KGF-HFc, washed, and then exposed to suramin. After a second series of washes, immunoperoxidase staining was performed. Under these conditions, no KGFR immunostaining was observed (Table 2), establishing that suramin was also capable of removing KGF-HFc already bound to KGFRs in the tissue section. In tissue sections from the intermediate stage of wound reepethelialization, which lacked detectable KGFR immunostaining, receptor immunoreactivity was not unmasked by previous exposure to suramin (Table 2). All of these results provide strong evidence that the absence of immunoreactive KGFRs during a time in wound repair when KGF and KGFR transcript levels were both markedly elevated reflects functional receptor downmodulation.

KGFR Downregulation Is Associated with a Differentiating Keratinocyte Population. The striking lack of correspondence between KGFR RNA and protein expression during the intermediate phase of reepithelialization was consistent with the emergence during this period of a suprabasal population whose phenotype resembled that of the KGFR-negative progenitor localized to the basal layer of normal skin. Alternatively, the cells might represent differentiating keratinocytes whose KGFRs were downregulated because of increased levels of KGF. In the normal epidermis, the expression of human keratin 1 (K1) is associated with the commitment of basal cells to terminal differentiation and the loss of proliferative capacity (19, 20). In an attempt to distinguish between these possibilities, we compared the localization of KGFRs with the distribution of K1 in wound-healing samples.

In normal skin, we observed colocalization of KGFRs and K1 in cells of the stratum spinosum (Fig. 4, A and B). Whereas KGFRs were localized to the plasma membrane, K1 was present in the cytoplasm. At 3 d, the few keratinocytes observed in tissue sections lacked both KGFR and K1 protein expression, consistent with a progenitor phenotype (data not shown). By 9 d, however, the pattern was very different (Fig. 4, C and D). Despite the absence of KGFRs, the same suprabasal cells were invariably K1 positive compared with basal cells in the same sections that lacked detectable K1 expression. By day 15, wounds were reepithelialized (Fig. 4, E and F), and the newly formed epidermis exhibited a normal pattern of differentiation markers, with colocalization of KGFRs and K1 in cells of the stratum spinosum (Fig. 1, E and F). Ki67 immunostaining confirmed that suprabasal cells during the intermediate wound-healing phase were nondividing. This marker of cells undergoing DNA synthesis (21) was localized to basal cells in control skin and was more prominently observed

Figure 3. Immunoperoxidase localization of KGFRs within skin samples during reepithelializaton. Immunostaining was performed as described in Materials and Methods. (A) In normal skin, KGFR staining localized to the cell surface throughout the stratum spinosum. (B) At 3 d, no detectable staining of KGFRs is observed in the wound area. (C) At 9 d, during the intermediate period of healing, the reepithelializing hyperthrophic epidermis appears virtually unstained. (D) On the same day, epithelial margins adjacent to a hypertrophic region undergoing reepithelialization show marked staining for KGFRs. (E) At 15 d, the epidermis is totally reepithelialized, showing a pattern of staining comparable to normal skin; immunostaining with the HFc control was negative under the same conditions (data not shown). A and C–E, ×400; B, ×800.
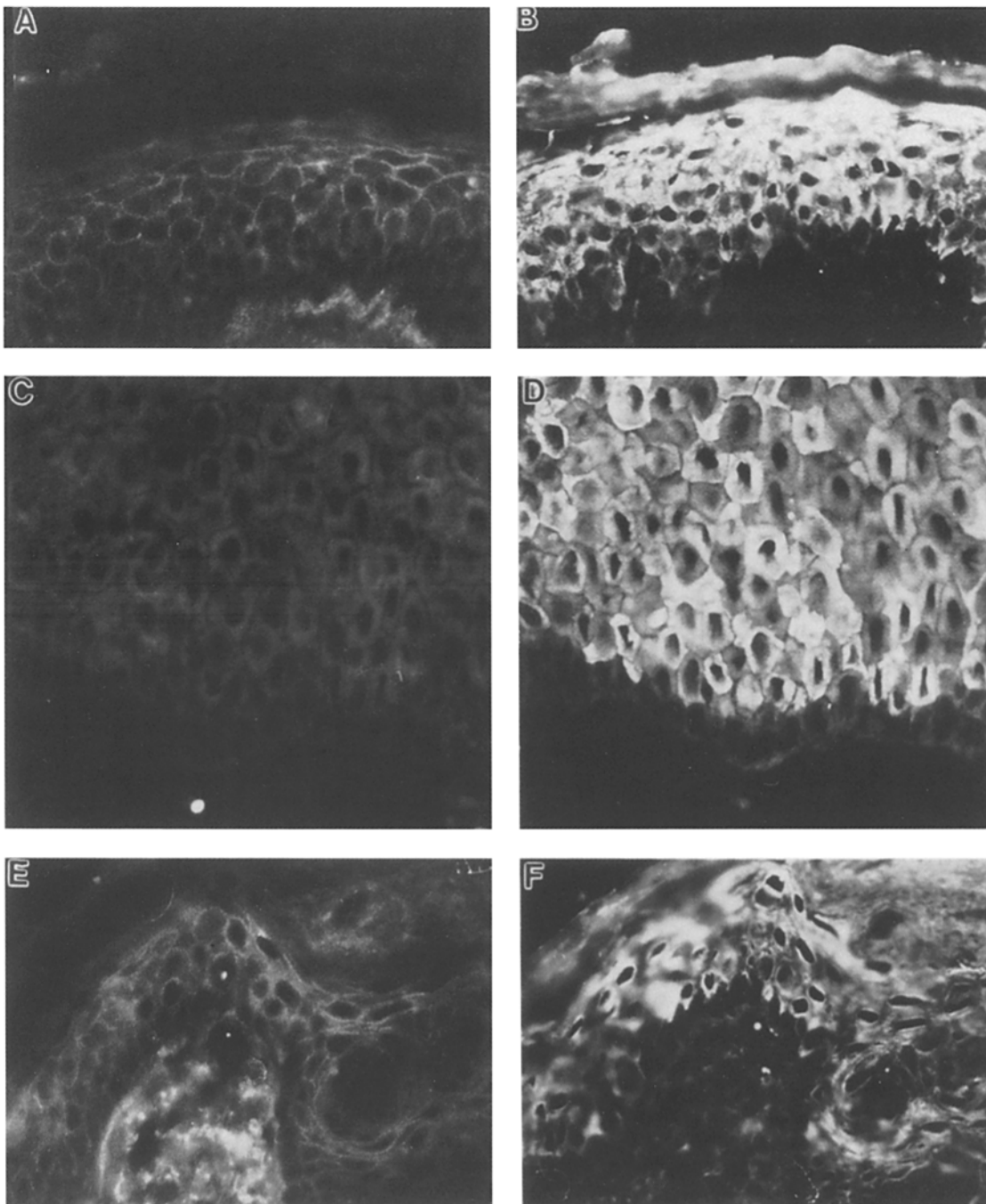
Figure 4. Localization of KGFR and K1 on normal and wounded skin. Double immunofluorescence staining with FITC for KGFRs, using chimeric HGF-HFc (*A, C,* and *E*), and TRITC for K1, using anti–human K1 polyclonal antibody (*B, D,* and *F*), on normal and wounded skin sections. Immunostaining was performed as described in Materials and Methods. Colocalization of KGFRs in the plasma membranes and K1 in the cytoplasm are shown in the cells of the stratum spinosum of normal skin. In the same section, the basal layer remains unlabeled for both KGFR and K1 (*A* and *B*). At 9 d of healing, the cells lack detectable expression of KGFRs, whereas in the same suprabasal cells, the signal for K1 is positive and irregularly distributed (*C* and *D*). At 15 d, tissue samples again show reactivity in cells of the suprabasal layer for both KGFRs and K1 (*E* and *F*). ×400.

in cells of the basal layer of keratinocytes during the intermediate wound-healing phase. However, Ki67-positive cells were not observed in hypertropic suprabasal cells of the same tissue (data not shown). Thus, the absence of detectable KGFRs in suprabasal K1-expressing cells during the intermediate period reflects KGFR downregulation in the nondividing, differentiating keratinocyte population. As such, these results imply that KGF plays a functional role in wound repair and may help to regulate the commitment to keratinocyte differentiation.

Discussion

In these studies, we observed increased expression of KGF, an epithelial cell–specific paracrine growth factor, during reepithelialization of normal human skin. KGF transcript levels increased as much as 8–10-fold during the early postwound period, accompanied by an elevation of 2–3-fold in growth factor protein expression in the same tissues. Elevated KGF transcript levels of even higher magnitude have been recently reported in a mouse wound-healing model (9). Studies in tissue culture have shown that a number of growth factors, including platelet-derived growth factor (PDGF) and TGF-α, increase KGF transcript levels in human fibroblasts (22). Moreover, the pro-inflammatory cytokine, IL-1α, was shown to induce the KGF transcript by a mechanism involving increased gene transcription (22). Because a number of these factors may be elevated during wound repair, any or all may be implicated in the in vivo activation of KGF gene expression.

There was also striking modulation of KGFR expression. KGFR transcript levels increased several-fold over basal levels in control skin during the intermediate period of wound repair. Whereas control epithelium expressed readily detectable receptor protein throughout the stratum spinosum, KGFR protein expression was low or absent in the hypertrophic suprabasal keratinocytes of renewing skin. The lack of detectable receptors during the intermediate phase of wound repair might reflect competition for receptor binding by overexpressed KGF. However, we showed that suramin, which effectively stripped bound ligand from KGFRs, was unable to unmask cell surface KGFRs in reepithelializing skin. It is well established that acute or chronic exposure to growth factors such as epidermal growth factor (EGF), PDGF, or FGF cause receptor activation and their internalization, which leads to a marked reduction in the number of cell surface receptors (23). Thus, we conclude that the loss of immunodetectable KGFRs in suprabasal keratinocytes of reepithelizing human skin, under conditions in which transcript levels of both KGF and the KGFR were increased, reflects activation and downregulation of the KGFR. As such, KGF must contribute functionally to the reepithelialization process.

Previous studies in tissue culture have shown that TGF-α or EGF acting through the EGF receptor can block Ca^{2+}-induced terminal differentiation of human keratinocytes, whereas KGF allows this differentiation process to proceed (4). Moreover, in a porcine wound repair model, exogenous KGF increased rete ridge formation, as well as the thickness of the superbasal layers of reepithelialized skin (10). In the rabbit ear wound repair model, KGF exposure was associated with accelerated epithelialization with evidence of specific stimulation of the proliferation and differentiation of progenitors within the hair follicles and sebaceous glands, in addition to the epidermis (24). Our results with normal human skin showed that suprabasal cells of regenerating epithelium expressed K1, a marker of keratinocyte differentiation, and lacked evidence of proliferation, supporting the concept that KGF functions in human wound repair in vivo to promote the keratinocyte differentiation process. KGFR protein expression was also low or undetectable in the basal keratinocytes during wound repair, where the index of cell proliferation was increased several-fold over that of basal cells of control skin. Thus, KGF may also play a role in the functional downmodulation of KGFRs in progenitor cells, which have the capacity to proliferate as well as differentiate.

Independent evidence for the effects of KGF in keratinocyte proliferation/differentiation in vivo derives from recent studies in transgenic mice in which KGF (25) or a dominant-negative KGF receptor (26) was targeted by the K14 promoter for overexpression in undifferentiated basal keratinocytes. The former was associated with hyperthickening of the epidermis (25), whereas the latter was associated with epidermal atrophy, a reduced steady-state proliferation rate of the basal epithelial cell layer, and reduced epithelialization after wounding (26). Wound healing involves a complex series of interactions involving many different cell types and the release of growth factors and cytokines (27–30). In this study, the elevation in KGF production in association with the downregulation of its receptor in keratinocytes during normal wound healing suggests a key role for this growth factor in maintaining the balance between proliferation and differentiation in the human regenerating epithelium.

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