Large induction of keratinocyte growth factor expression in the dermis during wound healing

(fibroblast growth factors/fibroblast growth factor receptors/gene expression/epidermis)

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ABSTRACT Recent studies have shown that application of basic fibroblast growth factor (basic FGF) to a wound has a beneficial effect. However, it has not been assessed whether endogenous FGF also plays a role in tissue repair. In this study we found a 160-fold induction of mRNA encoding keratinocyte growth factor (KGF) 1 day after skin injury. This large induction was unique within the family of FGFs, since mRNA levels of acidic FGF, basic FGF, and FGF-5 were only slightly induced (2- to 10-fold) during wound healing, and there was no expression of FGF-3, FGF-4, and FGF-6 detected in normal and wounded skin. High levels of FGF receptor 1 and FGF receptor 2 mRNA and low levels of FGF receptor 3 mRNA were found in both normal and wounded skin. No change in the levels of these transcripts was detected during wound healing. *In situ* hybridization studies revealed highest levels of KGF mRNA expression in the dermis at the wound edge and in the hypodermis below the wound. In contrast, mRNA encoding the receptor of this growth factor (a splice variant of FGF receptor 2) was predominantly expressed in the epidermis. These results suggest that basal keratinocytes are stimulated by dermally derived KGF during wound healing and implicate a unique role of this member of the FGF family in wound repair.

Cutaneous injury initiates a complex series of biological processes that are involved in wound healing. These processes involve many different cell types in migration, proliferation and differentiation, removal of damaged tissue, and production of extracellular matrices (1). Extensive histological studies have provided descriptive information on the cellular events involved in inflammation and tissue repair; however, little is known about the mediators that initiate and sustain wound repair.

The local application of platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) to wounds has been shown to accelerate dermal as well as epidermal wound healing (2–8). Therefore, it seemed likely that endogenous PDGF and fibroblast growth factor (FGF) are also involved in tissue repair. Whereas the induction of PDGF and PDGF receptor expression in wounds has been demonstrated (9), nothing is known about the role of endogenous FGF in wound healing. To gain insight into the contribution of FGFs *in vivo* to the healing of full-thickness wounds, we have investigated the mRNA expression of the seven members of the FGF family as well as their receptors (FGFR1–FGFR3) in normal skin and during wound healing. Our data show that acidic FGF (aFGF), bFGF, FGF-5, and particularly keratinocyte growth factor (KGF) are expressed in normal skin and induced upon injury. Whereas KGF was predominantly expressed in stromal cells below the wound and at the wound

edge, receptors for KGF (KGFR) were detected in the epidermis. This suggests that a KGF-mediated paracrine interaction may be important for the migration and proliferation of epidermal keratinocytes seen during wound healing. These data provide a molecular basis for understanding the mechanisms that contribute to tissue repair and imply an important role of KGF in wound healing.

MATERIALS AND METHODS

Animal Care. Mice were housed and fed according to the guidelines set forth by the Committee on Animal Research of the University of California, San Francisco.

Wounding and Preparation of Wound Tissue. Full-thickness excisional wounds were created along the backs of 50 adult BALB/c F₁ mice. Six wounds were created on each animal, and skin biopsy specimens from 10 animals were obtained at each of the following times: 12 hr, 1 day, 5 days, and 7 days after wounding. Biopsy of the 60 wounds from the 10 animals resulted in ≈4 cm² of tissue. A similar amount of skin was removed from the back of unwounded control animals. Tissue taken for RNA isolation was immediately frozen in liquid nitrogen. Tissue for *in situ* hybridization was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline and subsequently frozen in OCT compound (Miles).

RNA Isolation and RNase Protection Assay. For RNA isolation, fresh skin biopsy specimens were frozen in liquid nitrogen and used for RNA isolation as described (10). For RNase protection mapping of FGF and FGFR transcripts, DNA probes were cloned into the transcription vector pBlue-script II KS(+) (Stratagene) and linearized. An antisense transcript was synthesized *in vitro* by using T3 or T7 RNA polymerase and [³²P]rUTP (800 Ci/mmol, Amersham; 1 Ci = 37 GBq). Samples of 50 μg of total cellular RNA were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested for 40 min at 30°C with RNases A and T1 as described (11). Protected fragments were separated on 5% polyacrylamide/8 M urea gels and analyzed by autoradiography. The same RNA preparations were used for all protection assays. The increase in FGF mRNA levels was quantitated by laser scanning densitometry of the autoradiograms.

DNA Templates. FGFs were as follows: 227-base-pair (bp) fragment corresponding to nucleotides 242–469 of the murine aFGF cDNA (12); 239-bp fragment corresponding to nucleotides 175–414 of the murine bFGF cDNA (12); 276-bp fragment corresponding to nucleotides 1021–1297 of the murine Int-2 (mammary tumor integration site 2; FGF-3) cDNA (13); 343-bp fragment corresponding to nucleotides 267–610 of the

murine Hst (human stomach cancer; FGF-4) cDNA (12); 303-bp fragment corresponding to nucleotides 1–303 of the murine FGF-5 cDNA (12); 304-bp fragment corresponding to nucleotides 406–710 of the murine FGF-6 cDNA (14); 201-bp fragment corresponding to nucleotides 23–224 of the murine KGF cDNA (F.F.-P. and C. Dickson, unpublished data).

FGF receptors were as follows: 361-bp fragment corresponding to nucleotides 793–1154 of the murine FGFR1 cDNA (15). This fragment encodes the complete extracellular third immunoglobulin-like domain of the published murine FGFR1 sequence (15); 298-bp fragment corresponding to nucleotides 2140–2438 of murine FGFR2 cDNA (16). This fragment encodes the amino-terminal end of the first kinase domain and the kinase insert; 430-bp fragment corresponding to nucleotides 1233–1663 of the murine FGFR3 cDNA (D. Ornitz and P. Leder; personal communication). This fragment encodes sequences from the middle of the third immunoglobulin-like domain of murine FGFR3 to the beginning of the first kinase domain.

In Situ Hybridization. Antisense riboprobes were made by *in vitro* transcription with T3 and T7 RNA polymerases and ³⁵S-labeled UTP as described (11). Plasmids encoding the transmembrane region of FGFR1, the kinase I/kinase insert region of FGFR2, and the coding sequences of KGF (see above) were used as templates. For *in situ* hybridization, skin and wound tissues were prepared as described (17). *In situ* hybridization was performed on frozen sections as described (18). After hybridization, sections were coated with NTB2 nuclear emulsion (Kodak) and exposed in the dark at 4°C for 18 days. After development, the sections were counterstained with hematoxylin/eosin.

RESULTS

mRNA Expression of FGFRs in Normal and Wounded Skin.

To investigate the mRNA expression of FGFRs in normal and wounded skin, we isolated RNA from excisional wounds at different intervals after wounding and performed RNase protection assays. For each time point, 60 wounds from 10 mice were excised and used for RNA isolation. Normal skin from nonwounded mice was used as a control. mRNA encoding three different FGF receptors (FGFR1, FGFR2, and FGFR3) was detected in normal and wounded skin (Fig. 1). Whereas FGFR1 and FGFR2 were expressed at high levels, only low levels of FGFR3 mRNA were found. This is based on the finding that the protection assays with the FGFR1 and FGFR2 probes were exposed for 16 hr, whereas the protection assay with the FGFR3 probe was exposed for 4 days. No change in the mRNA levels of FGFR1 and FGFR2 was detected during the process of wound healing. FGFR3 mRNA was slightly induced within 5 days after injury.

mRNA Expression of FGFs in Normal and Wounded Skin.

To investigate the mRNA expression of different FGFs in normal and wounded skin, we performed RNase protection assays with the same RNA preparations that had been used for the expression analysis of FGFR transcripts (see above). Consistent with recent findings (19), mRNA encoding KGF was detected in normal skin (Fig. 2). During wound healing the expression of this mRNA was considerably increased (Fig. 2); the expression level of KGF transcripts was induced 9-fold within 12 hr after wounding and 160-fold within 24 hr (Fig. 3). In 7-day wounds, KGF mRNA levels were still 100-fold higher compared with the unwounded basal level.

Relative to KGF expression, much lower levels of aFGF, bFGF, and FGF-5 were found in normal and wounded skin (see Fig. 3 and autoradiogram exposure times in the legend of Fig. 2). Expression of aFGF and FGF-5 was induced 10-fold and 2-fold, respectively, within 24 hr after wounding (Fig. 3). Expression of bFGF peaked at day 5 after wounding, and expression levels were 4-fold higher after this period compared with the basal level. In contrast to the prolonged

FIG. 1. Expression of FGFR mRNAs in normal and wounded skin. Total cellular RNA (50 μ g) from normal and wounded mouse back skin was analyzed by RNase protection assay by using probes that hybridize to mRNA encoding FGFR1 (Top), FGFR2 (Middle), and FGFR3 (Bottom). Hybridization was performed under high-stringency conditions to avoid cross-hybridization with other FGFR mRNAs. The 361-nucleotide FGFR1 probe was complementary to the coding sequences of the complete third immunoglobulin-like domain of the published murine FGFR1 (15). The 298-nucleotide FGFR2 probe was complementary to sequences encoding the first kinase domain and the kinase insert of murine FGFR2 (16). The 430-nucleotide FGFR3 probe was complementary to sequences encoding the amino-terminal end of the third immunoglobulin-like domain, the transmembrane region, and the extracellular and intracellular juxtamembrane regions. The FGFR1 and FGFR2 gels were exposed for 16 hr, and the FGFR3 gel was exposed for 4 days. The time in days (d; 1, 3, 5, and 7) after injury is indicated at the top of each lane. Each hybridization probe (200–2000 cpm) was added to the lanes labeled "probe." They were used as a size reference and do not reflect the amounts of probe (100,000 cpm) that were added to all hybridization mixtures.

induction of KGF expression, aFGF, bFGF, and FGF-5 transcripts returned to the basal value within 7 days after wounding (Fig. 3). No expression of FGF-3 (Int-2), FGF-4 (Hst), and FGF-6 mRNA was detected in normal or wounded skin (data not shown). These results show that several members of the FGF family are expressed during wound healing and that KGF is the predominant FGF induced during this process.

In Situ Hybridization of KGF and FGFR2 mRNA. To determine the site of expression of KGF and FGFR2 during wound healing, we performed *in situ* hybridization on normal skin and on 1-day-old wounds. Consistent with the protection assay data, a significant induction of KGF mRNA expression was observed within 1 day after wounding, and transcripts encoding this growth factor were expressed at high levels in some cells below the wound (Fig. 4D) and at the wound edge (not shown). The observation that only certain cells in the dermis and hypodermis express high levels of KGF suggests that these cells represent a distinct cell population that might have an important function in wound healing.

The receptor for KGF was recently shown to be a splice variant of FGFR2 (20). Using a probe that detects all membrane-spanning variants of FGFR2, including the KGFR, we found mRNA encoding FGFR2 predominantly in the epidermis (Fig. 4C). By RNase protection assay we found that the specific splice variant of FGFR2 that is known to bind KGF is the predominant form of FGFR2 in the skin (data not shown). Therefore, the FGFR2 transcripts that we detected in the epidermis predominantly encode KGFRs. The same expression pattern of KGF and KGFR was observed at later

FIG. 2. mRNA expression of FGFs in normal and wounded skin. Total cellular RNA (50 μ g) from normal and wounded back skin was analyzed by RNase protection assay with RNA hybridization probes complementary to mRNA encoding (from top to bottom) KGF, aFGF, bFGF, and FGF-5. Hybridizations were performed under high-stringency conditions to avoid cross-hybridizations with other FGF mRNAs. The same RNA preparations were used for all hybridizations of Fig. 1 and Fig. 2. The gels were exposed for 12 hr (KGF), 3 days (FGF-5), or 5 days (aFGF and bFGF). The time after injury is indicated on top of each lane: 12h, 12 hours; 1d, 3d, and 7d, 1, 3, and 7 days.

stages of wound healing (days 3–7 after injury), with KGF being expressed in the dermis and KGFR in proliferating keratinocytes which are in close proximity to the KGF-producing cells (data not shown). This expression pattern of KGF and FGFR2 in skin suggests that KGF produced in the dermis acts in a paracrine manner on epidermal keratinocytes.

DISCUSSION

FGFs comprise a family of polypeptide mitogens including aFGF (21), bFGF (22), FGF-3 (Int-2) (23), FGF-4 (Hst) (24, 25), FGF-5 (26), FGF-6 (27), and KGF (28). They are potent mitogens and chemotactic agents *in vitro* for many cells of mesenchymal and epithelial origin and, therefore, have the properties expected of wound cytokines. Furthermore, the

FIG. 3. Induction of aFGF, bFGF, and KGF mRNA expression after injury. The induction of aFGF (■), bFGF (▲), FGF-5 (○), and KGF (□) mRNA expression after injury is shown schematically. The mRNA induction compared with the basal level is shown as a function of time after injury. The increase in FGF mRNA levels was quantified by laser scanning densitometry of the autoradiograms. No expression of FGF-3, FGF-4, and FGF-6 was found in normal skin and within 7 days after injury.

direct application of bFGF to wounds has been shown to have a beneficial effect on accelerating wound healing (2–5).

To investigate the role of endogenous FGFs and FGFRs in wound healing, we have analyzed the mRNA expression of these growth factors in normal skin and during the process of wound healing. Our results show that several members of the FGF family are expressed in normal skin and are induced upon injury. In addition we show that at least three different FGFRs are expressed in normal and wounded skin. These data suggest that multiple FGF-mediated autocrine and paracrine stimulations contribute to the healing of wounds.

Our most striking finding is the extraordinary induction of transcripts encoding KGF within 24 hr after injury. Since KGF had been shown to be a specific and potent growth factor for epithelial cells (28), one might suggest that the induction of its expression during wound healing underlies the migration and proliferation of epithelial cells during this process. This hypothesis is supported by our finding that the induction of KGF expression precedes the onset of epithelial cell proliferation. Furthermore, it persists during the first 7 days after injury, which are characterized by significant migration and proliferation of epithelial cells (29). By *in situ* hybridization we detected expression of KGF mRNA predominantly in some cells below and at the edges of 1-day-old wounds (Fig. 4) and also in later stages of wound healing (days 3–7 after injury; data not shown). These cells most likely represent a distinct population of fibroblasts that might have an important role in wound healing. The detection of KGF transcripts in the dermis of normal and wounded skin is consistent with previous Northern blot results from other investigators who found KGF expression in the dermis but not in the epidermis of normal skin (19). A high-affinity receptor for KGF was recently identified on cultured keratinocytes but not on dermal fibroblasts (20). Therefore, it was suggested that KGF stimulates epithelial cell growth in a paracrine manner.

The receptor for KGF had been shown to be a splice variant of FGFR2, which binds KGF and aFGF with high affinity but bFGF only with low affinity (20, 30). In contrast, other known

FIG. 4. Localization of FGFR2 and KGF mRNA in normal and wounded skin by *in situ* hybridization. (A) A hematoxylin/eosin stain of half of the wound. The arrowhead on the right indicates the region of normal skin where the photographs in B and C were taken. The arrow in the middle of the wound (left side of A) indicates the area where the photographs in D and E were taken. In A, the letters D, E, Es, and H indicate dermis, epidermis, eschar, and hair follicle, respectively. (B–D) (B and C) Sections from normal mouse back skin. (D and E) Sections from a 1-day-old wound. Sections were hybridized with ^{35}S -labeled riboprobes specific for KGF (B and D) and ^{35}S -labeled riboprobes specific for FGFR2 (C and E). The probes used for hybridization are described in *Materials and Methods*. The silver grains produced by the radioactive probe appear as white dots. They are indicated with arrows in C and D. In B and C the large bright nongranular areas represent artifacts of the autoradiography. Sections were counterstained with hematoxylin/eosin after hybridization. (A, $\times 45$, B–E, $\times 170$.)

FGFR2 splice variants bind aFGF and bFGF with high affinity but do not represent a receptor for KGF (30, 31). Our protection assay data demonstrate that the KGFR form is the

predominant FGFR2 splice variant in skin (data not shown). Therefore, the FGFR2 transcripts which we detected in the epidermis of normal skin and wounds predominantly encode

receptors for KGF which should enable a paracrine stimulation of epidermal fibroblasts by dermally derived KGF.

In contrast to FGFR2, which is found predominantly in the epidermis, FGFR1 mRNA was expressed at highest levels in the dermis of normal and wounded skin (data not shown). A similar pattern of expression of FGFR1 and FGFR2 was recently observed in embryonic skin (32). The differential expression of the two FGFR genes suggests that the encoded proteins mediate different functions. We recently demonstrated that the predominant form of FGFR1 in the skin binds acidic and basic FGF with high affinity but does not represent a receptor for KGF (33). This shows that the major FGFR variants found in the dermis and epidermis have different ligand-binding specificities.

In addition to KGF, we also found induction of aFGF, bFGF, and FGF-5 mRNA expression during wound healing. However, these factors were only expressed at low levels and were induced to a much lesser extent compared with KGF. Among this group of factors expressed at low level, aFGF was induced to the greatest extent. This is of particular interest, since aFGF is the only FGF besides KGF that binds with high affinity to the KGFR (30). The induced expression of aFGF might further enhance the proliferation of keratinocytes during wound healing.

Expression of bFGF in skin is consistent with findings of other authors that show the encoded protein in the epidermis (34). In contrast, expression of aFGF and FGF-5 in skin has not been demonstrated so far. The mechanism of action of bFGF and aFGF in normal and wounded skin is presently unclear, since these growth factors lack a signal sequence and, therefore, are not secreted. One might speculate that these mitogens are released by damaged cells upon injury and subsequently act in an autocrine or paracrine manner. In contrast with aFGF and bFGF, the other members of the FGF family, including KGF, are clearly secreted mitogens.

With the exception of bFGF, induction of FGF gene expression seems to be an early event in wound healing, since highest levels of KGF, aFGF, and FGF-5 mRNA were found within 24 hr after injury. Expression of all FGFs subsequently declined, demonstrating the reversibility of the process. Therefore, it seems likely that specific control mechanisms initiate gene expression after injury and subsequently turn off expression after healing. A defect in FGF gene induction might likely be associated with impaired wound healing, whereas a defect in gene suppression could lead to the formation of hypertrophic scars or keloids. In addition, a prolonged induction of FGF gene expression might cause pathologic uncontrolled cell growth which might lead to tumor formation.

In summary, we have provided evidence that FGFs are expressed in normal skin and that their expression is induced during wound healing. The tremendous induction of KGF expression suggests that this mitogen is not only important in the physiological renewal of the epidermis but is of particular importance in the controlled epidermal cell proliferation seen in wound healing.

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