Keratinocyte Growth Factor Stimulates Migration and Hyaluronan Synthesis in the Epidermis by Activation of Keratinocyte Hyaluronan Synthases 2 and 3

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Keratinocyte growth factor (KGF) activates keratinocyte migration and stimulates wound healing. Hyaluronan, an extracellular matrix glycosaminoglycan that accumulates in wounded epidermis, is known to promote cell migration, suggesting that increased synthesis of hyaluronan might be associated with the KGF response in keratinocytes. Treatment of monolayer cultures of rat epidermal keratinocytes led to an elongated and lifted cell shape, increased filopodial protrusions, enhanced cell migration, accumulation of intermediate size hyaluronan in the culture medium and within keratinocytes, and a rapid increase of hyaluronan synthase 2 (Has2) mRNA, suggesting a direct influence on this gene. In stratified, organotypic cultures of the same cell line, both Has2 and Has3 with the hyaluronan receptor CD44 were up-regulated and hyaluronan accumulated in the epidermis, the spinous cell layer in particular. At the same time the expression of the early differentiation marker keratin 10 was inhibited, whereas filaggrin expression and epidermal permeability were less affected.

The data indicate that Has2 and Has3 belong to the targets of KGF in keratinocytes, and support the idea that enhanced hyaluronan synthesis acts as an effector for the migratory response of keratinocytes in wound healing, whereas it may delay keratinocyte terminal differentiation.

Keratinocyte growth factor (KGF),1 a member of the fibroblast growth factor family (FGF-7), is a powerful paracrine agent, secreted by stromal fibroblasts and targeted to the epithelial cells, especially keratinocytes, which themselves are unable to produce it (1). KGF binds to a specific tyrosine kinase receptor, KGFR, a splice variant of fibroblast growth factor receptor 2, only expressed in epithelial cells (2). In the epidermis, KGFR is most abundant in the spinous cells, a layer which determines the differentiation rate of the keratinocytes (3). Some KGFR expression occurs also in the basal cell layer, but not in the granular or clear cell layers (3). KGF enhances keratinocyte proliferation (4), and migration (5), but delays differentiation (6, 7). The expression of KGF is strongly up-regulated in a wounded dermis (8, 9), and KGF enhances the wound closure (10, 11), suggesting its importance in the wound healing process.

Hyaluronan is a large glycosaminoglycan present in most extracellular matrices, including that between the vital cells of the epidermis (12). It forms a hydrophilic, viscous matrix that enhances cell migration by opening free space with its swelling pressure (13), but also by interacting with specific receptors, such as CD44 and receptor for hyaluronan-associated motility, which activate intracellular locomotory signals (14). We have recently shown that hyaluronan synthesis is up-regulated in cultured keratinocytes stimulated to migrate with EGF (15). Furthermore, transfection and overexpression of a hyaluronan synthase (Has2) into keratinocytes enhanced their migration, whereas an antisense construct of Has2 reduced keratinocyte migration (16), suggesting that hyaluronan synthesis may be one of the mediators of keratinocyte motility.

Revealing the target genes of KGF in keratinocytes to learn its mechanism of action has received considerable interest (for a review, see Ref. 17). Among the genes identified so far are nonselenium glutathione peroxidase to protect against oxygen radical attack (18), the matrix metalloproteinases collagenase-1 (19) and stromelysin-2 (20) to regulate invasion and migration, c-myc and a set of other genes to enhance proliferation (17), and vascular endothelial growth factor to stimulate subepithelial vascularization (21). Here we show that Has2 and Has3, enzymes synthesizing the hyaluronan matrix between keratinocytes and emerging as novel effectors in the migration, wound healing, and differentiation response of keratinocytes, belong to the target genes of KGF.

EXPERIMENTAL PROCEDURES

Cell Culture—A newborn rat epidermal keratinocyte (REK) cell line developed by MacCallum and Lillie (22), and originally isolated by Baden and Kubius (23), was used in all experiments. The cells were routinely cultured in Dulbecco’s minimal essential medium (low glucose, Invitrogen, Paisley, Scotland, United Kingdom (UK)) supplemented with 4 mm l-glutamine, and penicillin and streptomycin (50 units/ml and 50 μg/ml, respectively; Sigma) and 5% fetal bovine serum (EuroClone, Wethenby, UK) at 37 °C. Cells were passaged twice a week at a 1:5 split ratio using 0.05% trypsin (w/v), 0.02% EDTA (w/v) (Biochrom, Berlin, Germany) in phosphate-buffered saline (PBS).

Organotypic Cultures—Organotypic cultures were prepared as described previously (24, 25). Rat tail type I collagen (Becton Dickinson

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1 The abbreviations used are: KGF, keratinocyte growth factor; bHABC, biotinylated hyaluronan-binding complex; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DAB, 3,3-diaminobenzidine; EGF, epidermal growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; HBSS, Hank’s balanced salt solution; KGFR, keratinocyte growth factor receptor; PB, phosphate buffer; PBS, phosphate-buffered saline; REK, rat epidermal keratinocyte; RT, reverse transcription.
Labware, Bedford, MA; 3.83 ng/ml) was mixed with Earle’s balanced salt solution (10× EBSI, Invitrogen), 7.5% sodium bicarbonate (Invitrogen), and 1 mM sodium hydrosxide solution, at a volume ratio of 8:1.0:3.0:2.0, respectively, and allowed to solidify on 24-mm diameter culture inserts (3.0-μm pore size; Transwell®, Costar, Cambridge, MA). Recently confluent cultures of REKs were trypsinized, suspended in Dulbecco’s minimum essential medium (high glucose) as above except with 10% fetal bovine serum (HyClone, Logan, UT), applied on the collagen gels, and grown for 3 days with culture medium present both beneath and above the insert. The upper medium was then removed to facilitate differentiation at the air-liquid interface.

**KGF Treatment—**KGF (Sigma) was used at 0.1–100 ng/ml final concentrations in complete medium. In the monolayer cultures, treatment times of 0, 3 h, 72 h, and 144 h were used as described above. In the multiculture, KGF (2 and 20 ng/ml) was added to the culture medium on the 4th culture day, and thereafter with each fresh medium (every other day for the first week and then daily). To study the early effects of KGF on intracellular hyaluronan, some of the monolayer cultures were incubated for 20 min at room temperature in 10% turbidity reducing units/ml Streptomyces hyaluronidase (Seikagaku, Tokyo, Japan), washed with HBSS, and incubation continued in new medium with KGF and 10 units/ml hyaluronidase at 37 °C for 120 min.

**Staining of Hyaluronan in Monolayer Cultures—**REKs grown in 8-well chamber slides were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB), for 20 min, washed with PB, and permeabilized with 0.3% Triton X-100 in 1% BSA-PB for 30 min. In a part of the slides, extracellular hyaluronan was enzymatically digested with Streptomyces hyaluronidase (10 turbidity reducing units/ml, 10 min at 37 °C) prior to the permeabilization. After permeabilization, the cells were incubated overnight at 4 °C with a biotinylated complex of hyaluronan binding region of bovine articular cartilage aggrecan G1 domain and link protein (bHABC) (28), diluted to 3–5 ng/ml in 1% BSA. The bound probe was visualized by incubation with avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA) for 1 h and with 0.05% 3,3′-diaminobenzidine (DAB, Sigma) and 0.03% H₂O₂ for 5 min (26).

**Electron Microscopy—**REKs were fixed as described above and permeabilized with 0.5% saponin in 2% BSA-PB for 10 min on an ice bath. The staining for hyaluronan was done as above, except that all the incubation solutions and washing buffers contained 0.05% saponin, and the incubations were done at 4 °C. The samples were postfixed with 1% reduced osmium tetroxide for 15 min, dehydrated in graded ethanol, and embedded in Spurr’s resin. Thin sections were cut on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and viewed in a type 1200 EX microscope from JEOL (Tokyo, Japan).

**Confocal Microscopy—**The cells were fixed with 2% paraformaldehyde for 20 min, washed, and treated with Streptomyces hyaluronidase as described above. After permeabilization with 0.1% Triton X-100 in 1% BSA-PB for 10 min, the cells were incubated with HABC (5 μg/ml) and antibodies (anti-filaggrin [OX50, 1:50], followed by 1-h incubation with avidin biotin-peroxidase complex) for 1 h, washed with PBS, and incubated with 0.5 ml of 0.05% trypsin solution and the trypsin wash were combined and designated as the trypsin wash. The trypsin solution and the cell layer were incubated for 1 h with a horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA; 1:2,000 for filaggrin, and with an anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:20,000 for keratin 10) antibody. After washing in blocking buffer, the membranes were incubated for 1 h with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100). After washing in blocking buffer, the membranes were incubated for 1 h with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100). After washing in blocking buffer, the membranes were incubated for 1 h with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100).

**Western Blotting—**The epidermis from organotypic REK cultures was rinsed with ice-cold PBS and homogenized in 8 μm urea, 50 mM Tris-HCl, pH 7.6, 100 mM diithothiurea, 0.13 mM 2-mercaptoethanol, 100 μg/ml phenylmethylsulfonyl fluoride, and 100 μg/ml apronin as described before (24, 29). Equal amounts of soluble protein (30) from the extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon™ NC membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by 5% defatted milk powder and 0.2% Tween 20 in 10 mM Tris, 150 mM NaCl, pH 7.4 (blocking buffer), overnight at 4 °C (24). The membranes were incubated with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100). After washing in blocking buffer, the membranes were incubated for 1 h with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100). After washing in blocking buffer, the membranes were incubated for 1 h with the anti-filaggrin antibody (1:9,000 dilution in the blocking buffer) or the anti-keratin 10 antibody (1:100).

**In Vitro Hyaluronan Synthase Assay—**The assay was done essentially as described (31). Subconfluent REK cells (16–24×10⁶) were incubated for 14 h with 0–100 ng/ml KGF, membrane fraction isolated and incubated with 0.5 mM UDP-GlcNAc and 0.05 mM UDP-GalNAc (both from Sigma), the latter containing 2.5 μCi of UDP-[14C]GlcNAc (PerkinElmer Life Sciences), for 2 h at 37 °C. The samples were boiled with 1% SDS, incorporated activity separated by paper chromatography, quantified by liquid scintillation counting, and expressed as picomoles of GlcA incorporated/mg of protein in the membrane fraction (31).

**Metabolic Labeling Assay—**REKs were seeded into 6-well plates at 290,000 cells/well, and grown until subconfluent (2 days). Fresh medium (20 mM HEPES, 20 g/ml glucose, 0.2% BSA, 0.1% penicillin, and 0.1% streptomycin) (Amersham Biosciences, Little Chalfont, UK), and the appropriate amounts of KGF (0, 1, 10, and 100 ng/ml) were added to the cells and incubated for 6 or 18 h. The medium and two 0.3-ml HBSS (EuroClone) washes of the cell layer were combined and designated “medium.” Cell surface-associated hyaluronan was detached with 0.5 mM of 0.05% trypsin solution (10 min at 37 °C) and the term “trypsin wash” will be used to denote the pellet washed with 250 μl of HBSS. The trypsin solution and the HBSS wash were combined and designated “pericellular,” whereas the cell pellet was designated as the “intracellular” hyaluronan pool. Hyaluronan and other glycosaminoglycans were purified and quantitated from the different cellular compartments after determination of the radiolabeled activity of the hexosamines as described in detail previously (25, 27).

**Hyaluronan Disaccharide Analysis with Electrophoresis—**Medium samples (400 μl) were boiled for 10 min and digested with 40 μl of proteinase K (Sigma, 600 μg/ml in 100 mM ammonium acetate, pH 6.5) and 5.0 units/ml of trypsin (Sigma, 600 μg/ml in 100 mM ammonium acetate, pH 7.4) for 2 h at 37 °C. The digests were immediately analyzed by SDSPAGE with a precast 12.5% gel (Bio-Rad) and stained with Coomassie blue as before.
for 1.5 h at 60 °C. After proteinase K inactivation by boiling for 10 min, 50 μl of 50% trichloroacetic acid was added to precipitate proteins by centrifugation (15 min, 13,000 × g). Dialyzed supernatants were evaporated, dissolved in 100 μl ammonium acetate, pH 6.5, and digested for 3 h at 37 °C with 2 millilitres of Streptococcus hyaluronidase (Seikagaku), dried, and derivatized overnight at 37 °C in 5 μl of 0.1% 2-aminoacridone (Lambda Fluoreszenztechnologie GmbH, Graz, Austria) in 3:17 (v/v) acetic acid:dimethyl sulfoxide, and 5 μl of 1 μM NaBH₄CN. The 2-aminoacridone-derivatized disaccharides were stored at −20 °C until electrospray as described (16, 32), with the following modifications: 30% polyacrylamide gels were cast in the laboratory in 100 mM Tris dihydrochloride (Sigma) and 0.03% H₂O₂ in 0.1 M phosphate citrate °C for 3 h at °C. The void volume of the Sephacryl S-1000 column (Amersham Biosciences) was determined spectrophotometrically (BioDoc™ video documentation system (Biometra, Göttingen, Germany) and quantitated by ethidium bromide fluorescence by using the NIH Image software.

KGF Increases Epidermal Hyaluronan

Table I

| Primers, annealing temperatures, and cycle numbers used in the RT-PCR reactions |
|-----------------------------------------------|
| Has1 | 5′-GCT CTA TGG GGC GTC CCT C-3′ (left) | 57 °C | 35-37 |
| Has2 | 5′-TGG GAA CCA CAC TTG GAG TG-3′ | 62 °C | 33-35 |
| Has3 | 5′-CCA GTT AGT GAC TGA TTT GCC TTC C-3′ | 66 °C | 33 |
| CD44 | 5′-TTG GAG ACT ACT TCT CTT CG-3′ | 55 °C | 33 |
| Profilagrin | 5′-CTC AGG GCA TCG CTC GTC A-3′ | 64 °C | 34 |
| GAPDH | 5′-TGA TGC TGG TGC TGA TTG-3′ | 60 °C | 32 |

Hyaluronan Enzyme-linked Immunosorbent Assay—Organotypic cultures were changed into 1.5 ml of serum-free medium with KGF (0, 20, and 20 ng/ml) and continued for 24 h. The medium, epidermis, and culture medium, trypsin supernatant, and cell extract were subjected to gel filtration on a 1.5 ml wide lanes.

KGF Changes the Morphology of Keratinocytes Grown as Monolayers, and Stimulates Migration—The REK cells represent an exceptional keratinocyte cell line with a capacity for stratification and keratinization, but can also be maintained as monolayers if passaged before overt confluency (22, 23, 27). When cultured on plastic, REK cells of untreated control cultures had a flattened, epithelial morphology, whereas cells treated with KGF often showed a rounded (lift-up) appearance, with an elongated shape (Fig. 1a and b), typical for migrating cells. Electron microscopy of such cells revealed numerous microvilli on the upper cell surface (Fig. 1g). The migratory phenotype of the KGF-treated cells was confirmed by an in vitro wounding test in which a standardized area in confluent REK cultures was scraped free of cells from −1000-μm-wide lanes.
KGF showed a dose-dependent stimulation of REK migration from the wound edges to the cleared area (Table II). On the other hand, cell proliferation, measured in similar (but non-wounded) cultures by cell counting, was not significantly affected (Table II), suggesting that migration is a major biological function regulated by KGF in these cells.

**Hyaluronan Synthesis and Has2 Expression Are Rapidly Up-regulated by KGF**—Hyaluronan, isolated from the growth medium and assayed as fluorotagged disaccharides, increased in the presence of KGF in a dose-dependent way, and was more than doubled with the higher concentration, thus paralleling the enhanced migratory activity (Table II). The accumulation of hyaluronan in the culture medium was associated with a dose-dependent, 2–3-fold increase in Has activity, assayed in *vitro* from membrane preparations of REK cells treated with KGF (Fig. 2a). This suggested that the activity of one or more of the Has isoforms produced the higher level of hyaluronan in the REK cultures.

Isolation and analysis by RT-PCR of mRNA from cultures treated with KGF indicated that all three Has isoforms were expressed in the REK monolayer cultures (Fig. 2b). The change of fresh medium alone increased the expression of Has2 and Has3, whereas the signal of Has1 was not markedly affected, as indicated by comparison of the 0- and 3-h controls (Fig. 2b). The levels of Has1 and Has3 were not markedly affected by inclusion of KGF in the fresh medium, but Has2 levels were elevated at all time points (Fig. 2b). The up-regulation was confirmed in additional experiments, showing that Has2 mRNA levels started to increase within 3 h after introduction of KGF, and lasted at least for 24 h (Fig. 2c). This suggested that up-regulation of Has2 was mainly responsible for the induction of Has activity and increased concentration of hyaluronan. That hyaluronan synthesis was one of the early targets of KGF in keratinocytes was also supported by quantitation of the newly synthesized hyaluronan after brief (6-h) metabolic labeling (Fig. 2d).
KGF Increases Intermediate Size Hyaluronan—Because the biological functions of hyaluronan depend on its size, we analyzed the molecular mass of the radiolabeled hyaluronan in the different cellular compartments (Fig. 3). In control cultures, the hyaluronan released in the culture medium and present on the cell surface was largely excluded from the Sephacryl S-1000 gel filtration resin, indicating molecular mass at or above ~10^6 Da. In contrast, the hyaluronan extracted from the intracellular sources of control cells contained a relatively low proportion of high molecular mass hyaluronan, most of the intracellular hyaluronan being small fragments, apparently below 90 kDa, as reported previously (28).

In the growth medium, KGF specifically increased hyaluronan chains in the intermediate size range, ~0.4–2 × 10^6 Da, as estimated using the calibration curves supplied by the resin manufacturer. KGF showed no marked influence on the size of hyaluronan in control cultures.

### Table II

Influence of KGF on proliferation, migration, and hyaluronan secretion in REK monolayer cultures

| KGF (ng/ml) | Migration (μm) | Cell number × 10^-3 | Hyaluronan ng/10,000 cells |
|------------|---------------|----------------------|---------------------------|
| 0          | 164 ± 17      | 48 ± 7               | 1.46                      |
| 1          | ND            | 55 ± 9               | 1.94                      |
| 10         | 195 ± 18**    | 52 ± 7               | 2.65                      |
| 100        | 215 ± 23**    | 51 ± 7               | 3.47                      |

**Fig. 2.** Up-regulation of Has activity, Has mRNA levels, and hyaluronan synthesis in REK monolayer cultures treated with KGF. *a*, membranes were prepared from subconfluent REK cultures treated with 0–100 ng/ml KGF and determined for hyaluronan synthase activity as described under “Experimental Procedures.” The error bars indicate the range of two separate experiments. *b* and *c*, 3–24 h after change into a fresh culture medium with 100 ng/ml KGF, mRNA was isolated from the cultures, reverse transcribed, and amplified by PCR for Has1, Has2, Has3, and GAPDH (see Table I). In *c*, Has2/GAPDH pixel density ratios (mean ± S.E. of three experiments) are shown. In *d* and *e*, REKs were plated at 25,000 cells/cm², cultured for 48 h, and then incubated for 6 h (*d*) and 24 h (*e*) with [3H]glucosamine and [35S]sulfate in the presence of KGF in the concentrations indicated. In the 6-h experiment (*d*), the total newly synthesized hyaluronan in the cell layer and medium of KGF-treated cultures was combined and expressed as percentage of control cultures; the bar in controls shows the range of two replicate cultures. In the 24-h experiment (*e*), hyaluronan contents in the different compartments were analyzed separately. Mean and range of two experiments are shown.
Utilizing the metabolic double labeling with \([\text{\textsuperscript{3}H}]\)glucosamine and \([\text{\textsuperscript{35}S}]\)sulfate enabled the assay of newly synthesized hyaluronan. The content of intracellular hyaluronan increased both in the basal and suprabasal cell layers (Fig. 4, e and f). CD44, the main hyaluronan receptor in keratinocytes, was up-regulated by KGF in parallel with the synthesis of hyaluronan and was also increased both in the basal and suprabasal cell layers (Fig. 4, e and f).

In the organotypic cultures, RT-PCR showed not only an increased expression of Has2, but also a marked induction of Has3 (Fig. 4, h and i). Another finding specific for the organotypic cultures was the almost non-existent expression of Has1, whether KGF was present or not (Fig. 4h). In line with the

**Fig. 3.** Size distribution of newly synthesized hyaluronan in the different culture compartments of KGF-treated REK monolayer cultures. Radiolabeled culture medium, trypsin released material (pericellular), and cell extract (intracellular) from REK cultures treated for 24 h with 0 and 100 ng/ml KGF were chromatographed on an 1 × 30-cm column of Sephacryl S-1000, eluted at 0.4 ml/min with 0.15 M sodium acetate, 0.1% CHAPS, pH 6.8. Hyaluronan was analyzed in each fraction based on its susceptibility to Streptomyces hyaluronidase, as described under "Experimental Procedures." The void volume of the column (\(V_0\)) at fraction 18 and the total volume of the column (\(V_t\)) at fraction 47 are indicated.
histochemical findings, CD44 mRNA level was up-regulated by KGF, together with Has2 and Has3 (Fig. 4, h and i).

Influence of KGF on Epidermal Growth and Differentiation—KGF did not significantly change the bromodeoxyuridine labeling of the organotypic cultures, suggesting that there was a relatively minor stimulation of cell proliferation in the organotypic cultures (Table III), a finding in line with the lack of increase in cell numbers in monolayer cultures (Table II). Accordingly, whereas there was a trend for increased height of the basal cells, and increased thickness of the whole vital epidermis in organotypic cultures, the influence of KGF was not statistically significant (Table III).

A marked reduction in the expression of the early epidermal differentiation marker keratin 10 was found in KGF-treated cultures, as indicated by the immunostaining of the tissue sections (Fig. 5, a and b), and Western blots (Fig. 5c). The expression of the late differentiation marker filaggrin showed less reduction than keratin 10 (Fig. 5, c–e), suggesting that KGF retarded but did not inhibit the terminal differentiation. The level of profilaggrin mRNA was also unchanged as ana-
lyzed by RT-PCR (data not shown). In the organotypic cultures the epidermal permeability for corticosterone, a tracer often used in testing epidermal barrier function, showed a tendency to increase (not statistically significant) in the KGF-treated cultures (Fig. 5f).

**DISCUSSION**

The present study established that KGF enhances hyaluronan synthesis in keratinocytes, and that the increased hyaluronan synthesis is associated with an increased expression of the hyaluronan synthases Has2 and Has3. Stimulation of hyaluronan synthesis by KGF was seen both in monolayer cultures containing mainly proliferating and migrating basal cells, and in lifted organotypic cultures, which contain both proliferating basal cells and differentiating spinous and granular cells. The fact that the induction of hyaluronan synthesis was not dependent on the cellular environment or state of differentiation suggests that hyaluronan synthesis is an important target, perhaps one of the primary targets, of KGF. This idea is supported by the rapid up-regulation and maintenance of the elevated level of Has2 mRNA following KGF administration.

One of the main functions of KGF is its contribution to wound healing. The expression of KGF by dermal fibroblasts is rapidly and strongly up-regulated in wounded tissues (9), and the expression of KGF is associated with enhanced wound closure (10, 11). Accordingly, animals missing the KGFR function in epidermis show retarded wound healing (33). Our data show that KGF has a strong effect on keratinocyte migration, leading to a 30% faster closure of the epithelium in an in vitro wounding assay, a result in line with experiments on normal human keratinocytes (5). The enhanced migratory response correlated with increased hyaluronan synthesis, suggesting that hyaluronan plays an essential role in the KGF-stimulated motility of keratinocytes. A similar association exists between stimulated hyaluronan synthesis and migration in keratinocytes treated with EGF (15). Furthermore, keratinocytes overexpressing Has2 migrate faster, whereas antisense inhibition of Has2 retards keratinocyte migration (16). Although similar findings with transfected Has genes have been done in other cell types (34, 35), the influence of hyaluronan synthesis on motility depends on the cell type (36, 37). Nevertheless, in

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**TABLE III**

| KGF | Labeling index | Vital epidermis height | Basal cell height | Stratum corneum height |
|-----|----------------|------------------------|------------------|------------------------|
| ng/ml | % of control | μm | μm | μm |
| 0 | 100 (n = 6) | 26.3 ± 7.8 (n = 6) | 10.0 ± 2.5 (n = 6) | 19.1 ± 3.8 (n = 6) |
| 2 | 167 ± 29 (n = 4) | 35.8 ± 7.8 (n = 6) | 12.1 ± 1.6 (n = 6) | 17.2 ± 5.1 (n = 6) |
| 20 | 117 ± 18 (n = 10) | 35.8 ± 7.8 (n = 6) | 12.1 ± 1.6 (n = 6) | 17.2 ± 5.1 (n = 6) |

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**Fig. 5.** Influence of KGF on the indicators of differentiation in organotypic cultures. Organotypic REK cultures were grown for 2 weeks in the presence (b and d) or absence (a and c) of 20 ng/ml KGF and processed for histology. Staining for keratin 10 is shown in a and b and filaggrin in c and d. Magnification bar, 50 μm. In panel e, proteins extracted from KGF-treated (20 ng/ml) and control cultures were analyzed by Western blotting using antibodies against filaggrin and keratin 10. In f the diffusion of [3H]corticosterone through the organotypic epidermis was measured as described under “Experimental Procedures.” The data represent means and S.D. of 7 control and 10 KGF-treated cultures. The control and KGF-treated cultures were not statistically significantly different (p > 0.05, Mann-Whitney U test).
keratinocytes it is obvious that hyaluronan is an important determinant of the migratory activity, and KGF a major trigger of this response.

The most sensitive cellular targets of KGF in the organotypic cultures were the spinous cells, showing a very intense signal for hyaluronan. The stronger responsiveness of the spinous rather than basal cells is in line with the fact that the level of the KGF receptors is highest in the spinous cell layer (9). However, more facile diffusion into the collagen support from the basal cell layer cannot be ruled out as a contributor to the apparently greater augmentation of the hyaluronan signal in the spinous layer. Concomitantly with the higher level of hyaluronan in the spinous cell layer, KGF-treated cultures showed a lower expression of an early differentiation marker (keratin 10), whereas expression of the late differentiation marker filaggrin and the diffusion barrier were less affected. These findings closely correspond to those in human keratinocyte organotypic cultures (6), confirming the general validity of the present culture model.

An inverse correlation between the content of hyaluronan in the spinous cell layer and the indicators of epidermal differentiation has also been noted with other effectors like vitamin A (38) and EGF (39), both of which stimulate hyaluronan synthesis and inhibit differentiation. Conversely, we have found that pharmacological concentrations of hydrocortisone enhance differentiation but inhibit hyaluronan synthesis (40) and Has2 expression. This tight correlation between the status of epidermal differentiation and hyaluronan synthesis was noted with other effectors like vitamin A (39), interferon γ (41), and transforming growth factor β (38, 46). The present study indicates that the type of cellular interactions or stage of differentiation clearly modifies the regulation of the Has3 gene.

Altogether, the present study shows that KGF, a growth factor highly induced in most wounds and an important mediator of re-epithelialization and healing, increases the synthesis of hyaluronan in the epidermis through increased expression of Has2 and Has3 genes. This connection further emphasizes the role of hyaluronan in the physiological regulation of keratinocytes and in the challenges to epidermal homeostasis.

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