Keratinocyte growth factor (KGF) exhibits paracrine action on numerous epithelia, including skin. We have found that cultures of normal human keratinocytes must attain confluence before KGF promotes an increase in cell number relative to untreated controls. In postconfluent cultures, treatment with KGF promoted tight packing of keratinocytes with a small basal cell morphology. Based on these observations, we hypothesized that KGF increased cell number in postconfluent cultures by affecting the ability of normal keratinocytes to undergo terminal differentiation and/or programmed cell death. In support of this hypothesis, keratinocytes treated with KGF produced fewer cross-linked envelopes and exhibited reduced membrane-associated transglutaminase activity relative to cells treated with epidermal growth factor or untreated controls. We also found that nucleosomal fragmentation was reduced in postconfluent cultures of KGF-treated keratinocytes. Furthermore, KGF-treated keratinocytes were more resistant to suspension-induced nucleosomal fragmentation than control or epidermal growth factor-treated cultures. Therefore, it appears that KGF modulates aspects of keratinocyte terminal differentiation which share features with programmed cell death. We propose that stromally-derived KGF may act as a paracrine survival factor in skin and perhaps other renewal tissues.

Keratinocyte growth factor (KGF or FGF-7), synthesized and secreted by stromally-derived cells, is a member of the fibroblast growth factor (FGF) superfamily (Rubin et al., 1989). The FGFs are a family of structurally related polypeptide growth factors found to modulate proliferation and differentiation in a variety of cell types (reviewed by Basilico and Vlodavsky, 1991; Miki et al., 1991; Pierce et al., 1991; Werner et al., 1991). The expression pattern of KGF and KGFR, as well as the known biological activities of KGF, suggest that it functions as a paracrine mediator of epithelial cell growth in numerous tissues (reviewed by Rubin et al. (1995)). KGF-mediated mesenchymal-epithelial communication has been shown to play a role in regeneration of epidermis during wound healing (Werner et al., 1992; Tsuboi et al., 1993; Brauchle et al., 1994; Staiano-Coico et al., 1993; Pierce et al., 1994) and appears to be critical for the establishment of normal skin architecture during development (Guo et al., 1993; Werner et al., 1994; Finch et al., 1995). In developing mouse skin, KGF expression targeted to the basal layer by the keratin 14 promoter results in hyper-thickening of the epidermis and a pronounced increase in cell density of inner cell layers (Guo et al., 1993). Conversely, blocking KGFR function by targeted expression of a dominant-negative KGFR to basal keratinocytes of transgenic mice results in severe atrophy of the epidermis, with basal cells becoming flattened and exhibiting pyknotic nuclei (Werner et al., 1994).

The epidermis is a multilayered renewal tissue composed primarily of keratinocytes. Differentiated keratinocytes are continuously lost from the surface and replaced by the proliferation of basal keratinocytes. The rate at which a basal cell initiates and completes its differentiation program appears to be tightly regulated, although the molecular controls responsible for such regulation are ill-defined (reviewed by Fuchs (1993)). In vivo, the final stages of the terminal differentiation process are characterized by numerous changes including filagrin-mediated keratin intermediate filament bundling (Dale et al., 1978), release of lipids from membrane-coating granules into the intercellular space (reviewed by Schurer et al. (1991)), and formation of the cornified envelope which consists of several proteins that are covalently cross-linked by isodipeptide bonds catalyzed by the action of calcium-dependent transglutaminases found in keratinocytes (reviewed by Aeschlimann and Paulsson (1994) and Reichert et al. (1993)). Ultimately, keratinocytes lose intracellular organelles and enucleate in the upper layers of the epidermis. Molecular mechanisms which govern keratinocyte enucleation and terminal differentiation are poorly understood. Recent studies in our laboratory and those of others (reviewed by Fesus et al. (1991), Haake and

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1 The abbreviations used are: KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; FGF, fibroblast growth factor; EGF, epidermal growth factor; CE, cross-linked envelope factor; BSA, bovine serum albumin; TGF-α, transforming growth factor-α; PBS, phospate-buffered saline; TGase, transglutaminase; BrdUrd, 5-bromo-2-deoxyuridine; ELISA, enzyme-linked immunosorbent assay.
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Polakowska (1993), and Polakowska et al. (1994)) suggest that terminal differentiation in keratinocytes may constitute a specialized form of apoptosis. Although several studies have suggested a link between specific mediators of apoptosis and inherited or induced dermatopathologies in humans (Biandri et al., 1994; Nakagawa et al., 1994; reviewed by Haake and Polakowska (1993), Paus et al. (1993), and Polakowska and Hake (1994)) it has only recently been demonstrated that apoptosis is a naturally occurring process in normal human skin (Polakowska et al., 1994).

In the present study, we have investigated the effects of KGF on terminal differentiation and features of apoptosis in cultured epidermal keratinocytes. We examined the effect of KGF on several aspects of terminal differentiation in vitro, including cross-linked envelope (CE) formation and membrane-associated transglutaminase activity, as well as nucleosomal DNA fragmentation, a classical marker of apoptosis. Our studies suggest that KGF can forestall the ability of normal human keratinocytes to both initiate terminal differentiation and undergo features of apoptosis in vitro.

MATERIALS AND METHODS

Reagents— Recombinant human KGF was a generous gift from Drs. Stuart Aaronson (Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, NY) and Jeffrey Rubin (National Cancer Institute, Bethesda) or purchased from Promega Corp. Recombinant human KGF was obtained from Amgen Systems (Minneapolis, MN).

Cell Culture— Normal human keratinocytes were isolated from neonatal foreskin as described previously (Allen-Hoffmann and Rheinwald, 1984). Primary keratinocyte cultures (strain MH-B-Ep) were established using mitomycin-C treated Swiss mouse 3T3 fibroblast feeder cells. Complete growth medium was composed of 3 parts Ham's F-12 plus 1 part Dulbecco's modified Eagle's medium (0.66 mM calcium final concentration) containing the following supplements: 2.5% fetal calf serum or additives. Cells were recovered from suspension by repeated treatment with 0.5 mM EDTA, 0.1% trypsin as described above and rinsed with PBS (pH 7.2). Each treatment group contained 3 replicate plates. Cells from each plate were counted and resuspended in triplicate at 2.0 x 10^6 cells/ml in PBS (pH 7.2) containing 10% SDS and 20 mM dithiothreitol. For suspension experiments, cells were recovered as described previously and triplicate samples assayed. Samples were boiled for 2 min in a sand heating block (Model 137925, Sybron/Thermolyne Systems, Dubuque, IA) and cooled to room temperature. DNase (1 mg/ml) was added and CE's counted using a hemacytometer. CE formation was calculated as a percentage of input cells.

Transglutaminase Activity— Membrane-associated transglutaminase activity was assayed as outlined (Jetten et al., 1992). The procedure specifically measures TGF activity using an enzyme-linked immunosorbent assay in high binding microtiter plates (Costar, Cambridge, MA) coated with N,N'-dimethylcysteine (Sigma). The amine groups of 5-(bromoindol-3-yl)methylamine were blocked to N,N'-dimethylcysteine in the presence of calcium and active TGF. The biotinylated product was detected by streptavidin-alkaline phosphatase (Sigma) followed by addition of p-nitrophenyl phosphate to yield a colorimetric product whose absorbance was measured at 405 nm following a 30-min incubation at 37°C. Triplicate samples were analyzed for each treatment. The lower limit of detection for this assay is 5 activity units (OD/µg of protein/h) using ~0.05 µg/ml TGFase on the standard curve. Guinea pig liver transglutaminase (Sigma) was used to generate a standard activity curve for each experiment. The protein concentration of the membrane-associated TGFase fraction was determined by the Pierce BCA assay (Pierce). Each sample was assayed in triplicate. Sample means were corrected for protein concentration.

Analysis of Nucleosomal DNA Fragmentation— DNA fragments were recovered from genomic DNA samples using a modified protocol for the QIAamp Blood Kit (QIAGEN Inc., Chatsworth, CA). Twenty-three µl of a 20 mg/ml Proteinase K solution and 200 µl of reagent AL (QIAGEN) were added to the cell lysate prepared in SLS buffer. Following a 10-min incubation at 70°C, lysates were treated with 1 mg/ml DNase-free RNase at room temperature for 15 min. Subsequent steps were followed as specified in the manufacturer's instructions.

Intact and fragmented DNA were end labeled with [32P]dATP using a terminal deoxyxynucleotidyl transferase procedure as described (Tilly and Hsueh, 1993). Each reaction mixture was comprised of 1 x reaction buffer (0.2 mM potassium cacodylate, 0.25 mM bovine serum albumin, 25 mM Tris-HCl, pH 6.5, 25 mM sodium chloroacetate, terminal deoxynucleotidyl transferase enzyme (from calf thymus), 25 units; Boehringer Mannheim), 4.25 pmol of [32P]dATP (specific activity 3,000 Ci/mmol,
A suprabasal position due to stratification of the culture (Fig. 1). This occurred only after cultures attained confluence (Fig. 1). Keratinocytes that became postconfluent in serum-containing control cultures (data not shown), but did not promote accumulation of keratinocytes in postconfluent cultures (data not shown), but did not promote accumulation of keratinocytes in postconfluent cultures. These findings suggest that KGF does not simply act as a mitogen but may affect the balance between proliferation and differentiation once cells have achieved confluence.

Cross-linked Envelope Production in Keratinocytes Is Attenuated by KGF—We next investigated whether KGF promotes maintenance of a basal cell morphology and accumulation of keratinocytes in postconfluent cultures by altering the ability of the cells to terminally differentiate. Culture confluence in-
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KGF induces commitment to terminal differentiation which is accompanied by loss of proliferative potential and the induction of early differentiation markers such as keratins 1 and 10 (Poumay and Pittelkow, 1995). We monitored a separate marker of squamous differentiation, the formation of CE. In cultured human keratinocytes, early stages of CE assembly result in the formation of an immature cornified envelope composed of involucrin, cystatin-α, and other proteins, which represent the innermost third of the mature cornified envelope (Rice and Green, 1979; Steven and Steinert, 1994; Steinert, 1995; reviewed by Steinert and Marekov (1995)). We examined CE formation in control, KGF-treated, and EGF-treated keratinocyte cultures at different stages of confluence. In control keratinocyte cultures, the percentage of cells producing a CE increased 3-fold following confluence (Fig. 3). At confluence, keratinocytes produced comparable percentages of CE regardless of treatment group. However, in postconfluent cultures, both KGF- and EGF-treated cultures produced fewer envelopes than control cultures. In the KGF-treated culture, the reduction in CE production is sustained during the entire 5-day postconfluent period, unlike EGF-treated cultures.

To confirm that KGF specifically affects CE formation, preconfluent cultures of keratinocytes cultured with or without equimolar concentrations of KGF or EGF were triggered to undergo differentiation by suspension for 18 h in medium made semi-solid with methylcellulose. Many aspects of terminal differentiation, including differential expression of keratins (Drozdoff and Pledger, 1993) and CE formation (Green, 1977), can be triggered in vitro by loss of keratinocyte cell-cell and cell-substratum adhesion. We found that regardless of treatment group, <5% of all adherent preconfluent keratinocytes produce a CE (Table I). Following 18 h of suspension, control cultures readily produced CEs. However, keratinocytes cultured with KGF and to a lesser extent, with EGF, exhibited an attenuated ability to form CE following suspension. These findings are consistent with our observations that postconfluent keratinocytes cultured in the presence of KGF produced fewer CE when compared to control or EGF-treated postconfluent cultures.

KGF Modulates Membrane-associated TGase Activity in Postconfluent Keratinocytes—Because CE formation, both in vivo and in vitro, is tightly controlled by the enzymatic action of one or more of three calcium-dependent TGases found in the epidermis (reviewed by Aeschlimann and Paulsson (1994) and Reichert et al. (1993)), we determined if KGF treatment alters membrane-associated TGase activity in keratinocytes. Membrane-bound TGase 1 is localized exclusively to stratified squamous epithelium and is expressed in the suprabasal layers of skin (Thatcher and Rice, 1985; Eckert et al., 1993; Kim et al., 1995). TGase 1 is the most abundant TGase in skin (Kim et al., 1993) and its expression is associated with squamous differentiation (Greenberg et al., 1991; Thatcher and Rice, 1985). Membrane-associated TGase was isolated from control cells and cultures cultivated in the presence of KGF or EGF. All treatment groups were harvested at the indicated stages of confluence and enzyme activity measured. In control cultures, TGase activity increased approximately 10-fold as cultures attained confluence (Fig. 4). Postconfluent control cultures exhibited a 14- and 23-fold greater TGase activity at 8 and 10 days, respectively, than did preconfluent control cells. Cultivation with KGF did not alter the level of membrane-associated TGase activity in preconfluent or confluent cultures (Fig. 4). However, postconfluent KGF cultures possessed approximately half the activity found in postconfluent control cultures. Cultivation with EGF did not alter membrane-associated TGase activity in preconfluent or confluent cultures and was not as effective as KGF in maintaining low levels of TGase in postconfluent cultures, even at higher concentrations (data not shown). These data are consistent with our findings that cultivation with KGF
results in attenuation of CE production and accumulation of cells with a basal phenotype.

KGF Attenuates Suspension-induced Internucleosomal DNA Fragmentation—Taken together, our findings indicated that KGF may be functioning more as a survival factor than a mitogen for normal human keratinocytes. We next examined the effect of KGF on keratinocyte apoptosis by assaying internucleosomal cleavage of DNA. Specific DNA cleavage into oligonucleosomal fragments is a hallmark of apoptosis (Arends et al., 1990; Wyllie, 1980). Cells were cultured with or without equimolar concentrations of KGF or EGF and assayed for nucleosomal fragmentation patterns when preconfluent, newly confluent, or postconfluent (Fig. 5A). DNA from each treatment group was end-labeled with [a-32P]dATP. Preconfluent cultures exhibited little nucleosomal fragmentation regardless of treatment (Fig. 5A, lanes 1–3). At confluence, similar amounts of nucleosomal fragmentation were observed in all treatment groups (Fig. 5A, lanes 4–6). In contrast, KGF treatment forestalled nucleosomal cleavage in postconfluent cultures of keratinocytes relative to untreated controls or EGF-treated cultures (Fig. 5A, lanes 7–9). We also assessed the ability of keratinocytes cultured in KGF or control medium to undergo nucleosomal fragmentation following suspension. Cultured keratinocytes undergo morphological and biochemical features of apoptosis when deprived of cell-cell and cell-substratum attachment by suspension in semi-solid medium.2 Preconfluent keratinocyte treated with KGF or BSA for 3 days were suspended in the appropriate serum-free, semi-solid medium containing KGF or BSA for 20 h. Adherent cells were treated for an identical time with serum-free, additive-free medium supplemented with KGF or BSA. Adherent, preconfluent cultures in both treatment groups exhibited no detectable DNA fragmentation (Fig. 5B, lanes 1 and 2). Control keratinocytes suspended in semi-solid medium exhibited DNA fragmentation (Fig. 5B, lane 3). However, keratinocytes cultivated in KGF-containing medium exhibited less suspension-induced nucleosomal fragmentation compared to controls cells (Fig. 5B, lane 4).

DNA fragmentation was also assayed by a quantitative sandwich enzyme immunoassay using mouse monoclonal antibodies directed against DNA and BrdUrd. Following BrdUrd incorporation, keratinocytes from treatment groups remained adherent or were suspended in serum-free, additive-free semi-solid medium containing 0.5 mM KGF, 0.5 mM EGF, or BSA for 18 h. Adherent cells were treated with serum-free medium containing KGF, EGF, or BSA for the identical time. Similar to the results obtained with 32P end labeling, adherent cultures exhibited little fragmented DNA regardless of the presence or absence of growth factors (Fig. 6). However, keratinocytes cultivated in KGF-containing medium and suspended in semi-solid medium exhibited substantially less DNA fragmentation compared to suspended control cells. Cultivation in an equimolar concentration of EGF resulted in a modest attenuation of DNA fragmentation in this assay. Taken together, these findings suggest that a novel paracrine effect of KGF is to forestall keratinocyte nucleosomal fragmentation following confluence or suspension-induced differentiation.

DISCUSSION

We have found that cultivation with KGF sustains proliferation of human keratinocytes after cultures have achieved confluence. Following confluence, KGF-treated cultures exhibit both a decreased capacity to form CE's and reduced membrane-associated TGase activity compared to control or EGF-treated cultures. Furthermore, internucleosomal DNA fragmentation of keratinocyte DNA, a hallmark of apoptosis, is reduced by cultivation with KGF. Our findings suggest that KGF plays an important role in modulating features of terminal differentiation that may be related to programmed cell death.

Little is known about the specific biological activities of KGF in human keratinocytes. It is generally accepted that KGF, which is produced by dermal fibroblasts, acts in a paracrine manner on keratinocytes in the overlying epidermis (Finch et al., 1989). It has been previously shown that KGF is a potent mitogen for differentiation-defective murine keratinocytes, BALB/MK, inducing up to a 500-1000-fold increase in thymidine incorporation (Rubin et al., 1989). In normal murine keratinocytes, KGF has been shown to increase cell number approximately 2-fold following 7 days of treatment (Dlugosz et al.,...
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Figure 6. KGF decreases DNA fragmentation in suspended epidermal keratinocytes. Keratinocyte cultures were treated with standard medium supplemented with BSA, 0.5 nM EGF, or 0.5 nM KGF for 3 days (preconfluent). Cells in each treatment group were then labeled for 6 h with 10 μM BrdUrd. Labeled cells were suspended in their corresponding serum-free, additive-free semi-solid media supplemented with BSA (open bar), 0.5 nM EGF (hatched bar), or 0.5 nM KGF (closed bar) for 18 h. Identical adherent cultures were also labeled and incubated in serum-free, additive-free medium containing the indicated growth regulatory agents. Adherent and suspended normal human keratinocytes were lysed and supernatants containing labeled DNA fragments were quantified using the Cellular DNA Fragmentation ELISA. Values represent the mean of triplicate samples with standard deviations indicated by bars.

Marchese and co-workers (1990) also reported that KGF produced increases in human keratinocyte proliferation as measured by cumulative cell number. In both reports, the cell growth experiments were plated at high cell densities which likely resulted in cultures reaching confluence early during the treatment periods. We found that neither KGF nor EGF enhanced cell proliferation in low density cultures. Prior to confluence, there was no difference in cumulative cell numbers between control, EGF-treated, and KGF-treated keratinocytes. Following confluence, only KGF-treated cultures continued to accumulate cells while cell numbers in EGF-treated or control cultures appeared to plateau. Our findings with cultured human keratinocytes are consistent with a recent report on skin abnormalities exhibited by transgenic mice overexpressing KGF targeted to basal cells by the human keratin 14 promoter (Guo et al., 1993). Specifically, KGF expression resulted in an unusually high density of tightly packed keratinocytes in the epidermis. These mice had an increased thickness of the epidermis, primarily the result of an increase of cells in the spinous and granular layers. Taken together, these data suggest that KGF promotes accumulation of keratinocytes that have retained proliferative potential.

We observed that EGF did not affect the rate of cell growth or increase the accumulation of keratinocytes into tightly packed cell monolayers after confluence. Early experiments by Rheinwald and Green (1977) and subsequent studies by Barrand and Green (1987) also found that multiplication of small colonies of keratinocytes undergoing exponential growth was unaffected by EGF or TGF-α. They concluded that the effects of EGF and TGF-α on multiplication are dependent upon stimulation of migration. More recently, Dlugosz and co-workers (1994) demonstrated that KGF increases steady state TGF-α mRNA levels and secretion of TGF-α in primary cultures of keratinocytes isolated from newborn BALB/c mice. Given these data, it is reasonable to postulate that KGF-induced TGF-α expression may contribute to the accumulation of keratinocytes we have observed in postconfluent cultures by activating EGF receptor signaling. However, our studies show that EGF treatment does not promote tightly packed monolayers in postconfluent cultures as does KGF treatment. Furthermore, mice overexpressing TGF-α driven by the keratin 14 promoter also do not exhibit the increase in cell density or cell packing observed in KGF transgenic mice (Vassar and Fuchs, 1991; Guo et al., 1993). Given the divergent patterns of cell density and cell packing exhibited by KGF and TGF-α transgenic mice and our similar findings with KGF and EGF-treated human keratinocyte cultures, it is unlikely that KGF action is mediated solely through increased production of keratinocyte TGF-α.

The present study shows that KGF treatment reduces nucleosomal fragmentation in postconfluent cultures of keratinocytes. Furthermore, we found that KGF-treated preconfluent cultures exhibit decreased nucleosomal fragmentation following loss of cell-cell and cell-substratum adhesion. It has been well documented that keratinocytes from all species studied to date can undergo apoptosis both in vivo and in vitro (Marthi-nuss et al., 1995a, 1995b; McCall and Cohen, 1991; Bianchi et al., 1994; Frisch and Francis, 1994; Sayama et al., 1994; Schwarz et al., 1995). Epidermal keratinocytes are destined to undergo apoptosis and lose metabolic activity as part of their differentiation pathway. As basal keratinocytes of intact skin proliferate, cells which leave the basal compartment progress upwards through a program of terminal differentiation. Fully differentiated cells are eventually shed from the skin surface. A balance between basal cell proliferation and cell loss is required for the maintenance of normal epidermal architecture. Budtz and Spies (1989) studied the natural deletion of skin cells in the African toad, Bufo bufo, and demonstrated that apoptosis was one of the mechanisms by which surplus cells were lost. Using TUNEL analysis, Polakowska and co-workers (1994) demonstrated that intact tissue from developing fetal and adult human skin contains cells with DNA breaks in their nuclei. Keratinocytes that exhibited nuclear changes associated with apoptosis were located primarily in the upper granular layer and only occasionally in the spinous layer of epidermis. Because KGF-treated postconfluent cultures exhibited reduced nucleosomal fragmentation, it is reasonable to postulate that KGF promotes accumulation of cells by inhibiting basal cell loss through cell death pathways, such as apoptosis.

Many parallels exist between keratinocyte terminal differentiation and apoptosis. For example, mature cornified envelopes and apoptotic bodies are transglutaminase cross-linked end-products of terminal differentiation and programmed cell death pathways, respectively. The amino acid composition of cornified envelopes from human skin and apoptotic bodies from liver are very similar and show almost identical peptide mapping patterns (Tarcsa et al., 1992). It has been suggested that terminal differentiation of keratinocytes may actually be a specialized form of apoptosis (Polakowska and Hake, 1994; Polakowska et al., 1994; Fesus et al., 1991; Paus et al., 1993; Alison and Sarraf, 1992). We found that KGF reduced CE and membrane-associated TGase activity in postconfluent but not subconfluent cultures. These findings may, in part, explain the basal-like, tightly packed morphology of KGF-treated postconfluent cultures in contrast to the large, flattened cells observed in control and EGF-treated cultures. Equimolar concentrations of EGF were not as effective as KGF in reducing CE formation and membrane-associated TGase activity in postconfluent keratinocyte cultures. Marchese and co-workers (1990) reported that EGF, but not KGF, forestalls expression of the differentiation-specific keratin K1 as well as filaggrin in response to calcium-induced differentiation. This report, in conjunction with the phenotypes exhibited by TGF-α and KGF transgenic mice suggest that these two growth regulatory factors most
likely have differing biological activities in keratinocytes. Werner and co-workers (1994) recently reported that mice expressing a dominant-negative KGF receptor transgene targeted to basal epidermal keratinocytes exhibited severe atrophy of the epidermis. Specifically, the transgenic basal keratinocytes contained pyknotic nuclei and premature onset of cornification. It was concluded that addition of the dominant-negative KGF receptor disturbed the balance between proliferation and differentiation. Our findings of reduced nucleosomal fragmentation, CE formation, and membrane-associated TGase activity in postconfluent or suspended keratinocyte cultures suggest that KGF, and to a lesser extent EGF, promotes survival of keratinocytes signalled to differentiate. We propose that KGF may be functioning, in part, as a survival factor, thus affecting those aspects of keratinocyte differentiation which share features with cell death, such as enucleation and formation of CE (cornification).

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