Activation of Epidermal Growth Factor Receptor Promotes Late Terminal Differentiation of Cell-Matrix Interaction-disrupted Keratinocytes*

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The biological effects of epidermal growth factor receptor (EGFR) activation may differ between epidermal suprabasal and basal keratinocytes, since growth factors are mitogenic in adherent cells only in the presence of cell-extracellular matrix (ECM) interaction. To investigate biological effects of EGFR activation on keratinocytes without cell-ECM interaction, we cultured normal human keratinocytes on polyhydroxyethylmethacrylate-coated plates, which disrupt cell-ECM but not cell-cell interaction. The cells initially expressed keratin 10 (K10) and then profilaggrin, mimicking sequential differentiation of epidermal suprabasal keratinocytes. The addition of EGF or transforming growth factor-α promoted late terminal differentiation (profilaggrin expression, type 1 transglutaminase expression and activity, and cornified envelope formation) of the suspended keratinocytes, while suppressing K10 expression, an early differentiation marker. These effects were attenuated by EGFR tyrosine kinase inhibitor PD153035 or an anti-EGFR monoclonal antibody, whereas protein kinase C inhibitors H7 and bisindolylmaleimide I or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD98059 abolished profilaggrin up-regulation but not K10 suppression. Since the antidermatization role of EGFR on cell-ECM interaction-conserved keratinocytes has been well documented, our results indicate that the biological effects of EGFR on keratinocytes are influenced by cell-ECM interaction and suggest that EGFR activation promotes rather than inhibits the terminal differentiation of suprabasal epidermal keratinocytes.

Extrinsic cues, which determine cell fates such as proliferation, differentiation, and apoptosis, include spatial cues such as cell-cell and cell-extracellular matrix (ECM) interaction (1), soluble factors such as growth factors, and chemophysical cues exemplified by ultraviolet. These cues coordinate, antagonistically, and interdependently evoke biologic effects. For example, growth factors could not stimulate proliferation of nontreated, adherent cells without cell-ECM interaction (2, 3). In normal epidermis, keratinocyte proliferation is confined to basal cells attached to the basement membrane; however, as the cells move to suprabasal layers, they cease proliferation and undergo terminal differentiation. Accordingly, integrins, which are primarily responsible for the biological function of cell-ECM interaction (2, 3), are mainly expressed in the basal layer (4). When cultured keratinocytes are placed in suspension, they also cease proliferation and terminally differentiate even in the presence of growth factors (5).

Epidermal growth factor receptor (EGFR) activated by its relevant ligands exerts diverse biologic actions in a variety of tissues including epidermis. Carpenter and Cohen (6) first demonstrated a vital role for EGF in maintaining epidermal homeostasis in vivo by showing that EGF promotes skin maturation and multilayered thickening. Targeted disruption of EGFR in mice results in a thin and immature epidermis (7, 8). On the other hand, transgenic mice expressing an EGFR dominant negative mutant selectively in the basal layer of epidermis shows marked epidermal hyperplasia (9). In cultured keratinocytes, EGF stimulates proliferation (10, 11) and inhibits the expression of differentiation markers such as keratin 10 (K10), type 1 transglutaminase (TGase 1) and filaggrin (10, 12–14). Conversely, inactivation of EGFR tyrosine kinase activity induces K10 expression (14). These in vitro proliferation-promoting and differentiation-suppressing effects of EGF on keratinocytes however, could not explain an in vivo role for EGF in maintaining the balance between proliferation and differentiation of epidermal keratinocytes.

Effects of EGFR activation have been primarily studied in vitro using keratinocytes in cell-ECM interaction-conserved 2-dimensional culture, suggesting that the effects reflect the action of EGF/EGF only on epidermal basal cells. In normal epidermis, however, most keratinocytes are situated in the suprabasal portion, in which keratinocytes interact with each other but not with ECM. Although EGFR expression is highest in the basal layer, suprabasal cells in normal epidermis also express EGFR (15), suggesting that EGF/EGF exerts a biological action on suprabasal keratinocytes, which may be distinct from that on basal keratinocytes. In fact, Sakai et al. (16) suggested a role of EGF in epidermal differentiation, based on their finding that EGF/EGF precursor was exclusively expressed in the granular layer of normal epidermis. As far as we are aware, however, direct biological effects of EGFR activation in suprabasal cells have not yet been demonstrated.

In the present study, we showed that activation of EGFR by EGF or transforming growth factor (TGF)-α promoted late terminal differentiation of cell-ECM interaction-disrupted keratinocytes. Effects of EGFR activation were apparently in contrast to those previously reported in attached keratinocytes (10–13). Our results suggest that EGFR activation in the su-
Experimental Procedures

Cell Culture and Reagents—Normal human keratinocytes derived from foreskins (Epipack, Clonetics Corp., San Diego, CA) were grown in serum-free, keratinocyte growth medium (Clonetics) containing 0.1 mM Ca\(^{2+}\) supplemented with 0.1 ng/ml EGF, 60 ng/ml whole bovine pituitary extract, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 50 μg/ml gentamicin, and 50 μg/ml amphotericin B in a humidified incubator with 5% CO\(_2\) in air at 37 °C.

Murine mAbs employed were as follows: LL002, to K14 (Cymbus Bioscience, Southampton, UK); LL025, to K16 (Medac, Hamburg, Germany); MAB1605, to K10 (Chemicon International Inc., Temecula, CA); B.C1, to keratinocyte-specific T\(\alpha\)ase 1 (Harbor Bio-Products, Norwood, MA); and ARH1, to human profilaggrin and filaggrin (Biomedical Technologies, Inc., Stoughton, MA). Fluorescein isothiocyanate-conjugated affinity-purified goat antibody directed against the mouse IgG F(ab\(^{'},\))\(_{2}\) was obtained from Cappel (Cochraville, PA). All reagents were obtained from Genzyme Co. (Boston, MA) except for TGF-β1, which was from Roche Molecular Biochemicals and keratinocyte growth factor from Upstate Biotechnology, Inc. (Lake Placid, NY). Inhibitors of signal transduction pathways used were protein kinase C (PKC) inhibitors including H\(\gamma\) (Seikakagu Co.; Tokyo, Japan) and bisindolylmaleimide I (Calbiochem), mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase (MEK) 1/2 inhibitor PD98059 (Biomol, Plymouth Meeting, PA), and the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (Wako; Osaka, Japan). EGFR function was inhibited by a monoclonal antibody (Ab-3, Calbiochem) or EGFR tyrosine kinase inhibitor PD153035 (Tocris Cookson, Bristol, UK). All other reagents, unless otherwise specified, were purchased from Sigma.

Suspension Culture—Suspension culture of poly(hydmethylacrylate) (poly-HEMA)-coated plates was performed according to the method of Frisch and Francis (3). In brief, cells were harvested with trypsin/EDTA and suspended at 5 × 10\(^{4}\) cells/ml in fresh keratinocyte growth medium. Two ml of cell suspension were incubated on 3.5-cm dishes coated with poly-HEMA in a humidified incubator at 37 °C, 1 mM dithiothreitol, and 2 mM EDTA. Five ml drops were immediately pipetted onto polylysine (0.1 mg/ml hydrocortisone, 10 μg/ml insulin, 50 ng/ml amphotericin B in a humidified incubator with 5% CO\(_2\) in air at 37 °C. After incubation, cells were washed with phosphate-buffered saline and then suspended in 2 ml of distilled water. Three μl drops were immediately pipetted onto polylysine (0.1 mg/ml) coated coverslips and vacuum-dried for immunostaining. The residual suspension was centrifuged, and the pellets were used for immunoblotting.

Cell Lysis and Immunoblotting—For detection of K10, K14, K16, T\(\alpha\)ase 1, and profilaggrin/filaggrin, cells were homogenized in 10 ml Tris, pH 8.0, containing 9.5 M urea, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM EDTA. Five μl of the lysate were used for determining protein concentrations using a DC protein assay (Bio-Rad). Residual samples were constituted with 2% SDS, 25 mM Tris, pH 8.0, 9.5 M urea, 2 mM phenylmethylsulfonyl fluoride, and 50 mM NaCl and then heated at 100 °C for 5 min. This procedure has been shown to optimize retention and detection of profilaggrin (17). Extracts were electrophoresed on SDS-polyacrylamide gel electrophoresis gradient gels (5–15%; Ready gels J, Bio-Rad). For detection of T\(\alpha\)ase 1 by B.C1, the gel was reanimated after electrophoresis as described previously (18). Proteins were then transferred to nitrocellulose membranes. Transfers were incubated sequentially with mAbs (1 μg/ml), biotinylated rabbit anti-mouse IgG (Dako, Glostrup, Denmark), and streptavidin-horseradish peroxidase conjugate (Life Technologies, Inc.). Membrane-bound peroxidase was detected by an ECL detection kit (Amersham Pharmacia Biotech). Densitometric analysis of the luminescence intensity was performed with a computer-assisted image analyzer (NIH Image).

Immunostaining—Indirect immunofluorescence staining was performed as described previously (19). In brief, keratinocytes on coverslips were sequentially permeabilized in cold methanol and fixed with 4% paraformaldehyde. After blocking with 10% normal goat serum, cells were incubated with antibodies followed by fluorescein isothiocyanate-conjugated anti-mouse IgG and 500 ng/ml propidium iodide (PI) for nuclear counter-staining.

Reverse-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from cultured keratinocytes by the single-step guanidinium thiocyanate method of Chomczynski and Sacchi (20). A GeneAmp RNA PCR kit (Perkin-Elmer) was used for RT-PCR, as has been previously described (19). In preliminary experiments, primers, reaction and PCR cycles were titrated to establish standard curves to determine linearity, which allowed semiquantitative analysis of signal strength. The sequence of the primer pairs, 5′ and 3′, respectively, were as follows: K14, TGGAGGAAACAGATTTGACCA (localization of bases; 575–594) and GTCTTGTTGGAAGAACCTGT (971–990); K10, ACTACAAACCACATCGTGTGC (2269–2288) and GCAAGTTGTTCTATATGGTTCTGCT (2651–2632); K16, ATCAAGACTAGCTCCTCTCTCCTG (956–975) and GATCTGCTGAGGAATCTGAC (1276–1295) and GTGCTTTAGAAGATGTTGTTGGTGTCTGA (1626–1607); profilaggrin, AGACTAACGAACTCGAGCTGTA (4153–4172) and ATGATGTTTCTCTTGAAGGAC (4674–4655); profilaggrin, TGGATTGCTGAGGAATCTGAC (1276–1295) and GTGCTTTAGAAGATGTTGTTGGTGTCTGA (1626–1607); profilaggrin, AGACTAACGAACTCGAGCTGTA (4153–4172) and ATGATGTTTCTCTTGAAGGAC (4674–4655); and β-actin, GGCCCAAGGACAGAGG (212–213) and GTGCTTACATGACCAGTCT (505–486). The product size was 416 bp for K14, 383 bp for K10, 476 bp for T\(\alpha\)ase 1, 521 bp for profilaggrin, and 294 bp for β-actin.

Cortified Envelope Content—Cortified envelope (CE) content of the cultures was determined using a method utilizing the envelope insolubility (21). Cells were suspension-cultured for various periods of time and harvested in 2% SDS solution. After light sonication (5 s), aliquots were taken for protein determination with the DC assay. Cells were dissolved in 2% SDS, 20 μM dithiothreitol and boiled for 1 h, and amounts of insoluble cross-linked envelopes were quantified by measuring the absorbance at 340 nm. CE content was then expressed as SOD/μg of cell protein of triplicate wells.

Transglutaminase Activity—Membrane-associated transglutaminase was isolated as outlined previously (22). To determine T\(\alpha\)ase activity in keratinocytes, we used a nonradioactive microtiter plate assay. A kinetic measurement of absorbance was determined at 30 s intervals for a period of 30 min using an EL 340 Bio Kinetic Reader (Bio-Tek Instruments Inc., Winooski, VM) and analyzed by Delta-Soft II software (BioMetallics Inc., Princeton, NJ). Transglutaminase activity was expressed as units of optical density (μOD/min).

Statistical Analysis—Data from triplicate experiments were analyzed with the Student’s t test. Two other independent experiments were performed and gave identical results (statistically significant differences). A p value of <0.05 was considered to be statistically significant.

Results

EGF Enhances T\(\alpha\)ase 1 and Profilaggrin Expression in Cell-ECM Interaction-disrupted Keratinocytes—To investigate the biological effects of EGF activation on cell-ECM interaction-disrupted keratinocytes, we employed a suspension culture on poly-HHEMA-coated culture plates, which inhibit cell-ECM but not cell-cell interaction. Although single cell suspension culture in methylcellulose has been commonly used for studying ECM disruption-induced keratinocyte differentiation, this method interrupts cell-cell interaction, which is preserved in in vivo suprabasal keratinocytes. The former method is therefore likely to maintain keratinocytes in conditions more closely resembling the in vivo physiological situation.

We initially prepared keratinocytes for suspension culture by detaching keratinocytes from plates with trypsin/EDTA solution. Both cell-ECM and cell-cell interaction were completely distracted by this procedure. Cells were then resuspended in keratinocyte growth medium with or without EGF and seeded onto poly-HEMA-coated plates. Ten ng/ml EGF was used, since this concentration has been shown to be optimal for proliferation of attached keratinocytes (10, 11). As shown in Fig. 1, at the beginning of suspension culture, only single cells were observed floating on the plate. Both in the absence and presence of EGF, cells had started to interact with each other by 6 h and had gathered into irregular clumps by 12 h. After 24 h in suspension, EGF-treated cells were more compactly aggregated than untreated cells. Large aggregations of cells were observed in EGF-ununtreated cells at 48 h, whereas EGF-treated cells continued to exhibit small, round aggregations. To become floating on the plate. Both in the absence and presence of EGF, cells had started to interact with each other by 6 h and had gathered into irregular clumps by 12 h. After 24 h in suspension, EGF-treated cells were more compactly aggregated than untreated cells. Large aggregations of cells were observed in EGF-ununtreated cells at 48 h, whereas EGF-treated cells continued to exhibit small, round aggregations. To become
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Fig. 1. Morphological changes in keratinocytes induced by suspension and modulation by EGF. Keratinocytes detached from culture plates by trypsin/EDTA solution were suspension-cultured on poly-HEMA-coated plates with or without 10 ng/ml EGF. Suspended cells were photographed with phase contrast microscopy after the indicated incubation time. All photographs except for 0 h with EGF are at the same magnification. Bars, 25 μm.

Fig. 2. EGF up-regulates TGase 1 and profilaggrin but suppresses K10 expression induced by suspension. a, differentiation marker expression in keratinocytes suspension-cultured for 48 h on poly-HEMA-coated plates was examined by immunoblotting as described under “Experimental Procedures.” For comparison, some cells were seeded on noncoated plates and incubated for 48 h (Attached keratinocytes [KC]). b, keratinocytes were suspension-cultured on poly-HEMA for indicated periods of time in the presence or absence of 10 ng/ml EGF. K10 and profilaggrin expression was then examined by immunoblotting.

plates instead of poly-HEMA-coated plates and incubated for 48 h (Attached cells in Figs.). K14 expression was not altered by suspension or EGF. K10 was not observed in attached cells but was strongly induced by suspension without EGF. However, K10 could be detected in attached cells when higher amounts of proteins were loaded (Fig. 2b). K10 induction by suspension was markedly attenuated by EGF. A weak TGase 1 expression was observed in attached keratinocytes and augmented by suspension. EGF augmented TGase 1 expression in suspended keratinocytes. Although the enhancement of TGase 1 expression by suspension or EGF was relatively weak, the effect was reproducible, and a significant difference was confirmed by densitometric analysis of signal intensity (data not shown). Profilaggrin expression was more clearly modulated by suspension and EGF. Although profilaggrin was not detected in attached keratinocytes, it was expressed by suspended keratinocytes, and EGF augmented the expression. However, filaggrin (Fig. 2a, size at open arrowhead) was not detected in suspended keratinocytes in the absence or presence of EGF, confirming the absence of filaggrin induction in submerged cultured human keratinocytes (24). Induction of K6/16, which are expressed instead of K1/10 in hyperproliferative suprabasal keratinocytes (25), has been demonstrated in cultured keratinocytes by EGF and TGF-α (26). Although suspension could induce K16 expression, EGF was ineffective in suspension-induced K16 expression.

Since EGF remarkably affected the expression of K10 and profilaggrin in suspended keratinocytes, we analyzed the time course of K10 and profilaggrin expression by suspension culture on poly-HEMA. Although K10 was detected from the suspended keratinocytes before incubation (Fig. 2b), K10 up-regulation was evident after 24 h of incubation. In this immunoblot data and Fig. 8, anti-K10 mAb detected two bands at 59 and 67 kDa. The former (Fig. 2, lower band) was identical in molecular weight to K10, whereas the latter (Fig. 2, upper band) had a molecular weight identical to K1. In addition, signal intensity of this band was enhanced by suspension. It is then highly likely that the band is K1. Profilaggrin expression was more delayed and detected 48 h after incubation commenced. These results showed that K10 and profilaggrin were sequentially expressed after the suspension. In addition, we simultaneously examined the time course of EGF-induced modulation of K10 and profilaggrin expression (Fig. 2b). EGF almost completely blocked K10 up-regulation, whereas (probable) K1 expression was increased 24 h after suspension despite the presence of EGF. However, K1 expression was not detected at 48 h. Although EGF up-regulated profilaggrin expression at 48 h, EGF could not shorten the period required for profilaggrin expression to become detectable by immunoblotting. However, when keratinocytes were cultured in suspension for 96 h, levels of profilaggrin expression were comparable between EGF-treated and untreated cells and higher than at 48 h of incubation (data not shown). Therefore, it is plausible that EGF promotes suspension-induced profilaggrin expression.

Enhancement of TGase 1 expression by EGF was weak compared with profilaggrin. However, detection of TGase 1 in immunoblots by the mAb B.C1 requires a specific technique of renaturation before protein transfer. This complexity might have obscured differences in TGase 1 expression. We then compared the expression of differentiation markers including TGase 1 more directly by immunofluorescence staining (Fig. 3), which also revealed the localization of markers. To accurately assess expression, cells were nuclear-stained with PI. Two days after incubation, K10 was detected in most aggregated cells, whereas some single cells did not express K10. TGase 1 was also predominantly expressed in cellular aggregations, but the fluorescence was weaker than for K10. Profilaggrin was detected in only a few cells located mainly at the central portion of large cell aggregates. When cells were suspension-cultured with EGF, K10 expression was almost negligible. In contrast, TGase 1 expression was markedly enhanced, and localization at cell-cell contacts was visible. Profilaggrin expression was also enhanced, and most of the cells in aggregates expressed profilaggrin. Because of the weak fluorescence of profilaggrin, relatively long exposure times were required to detect profilaggrin in EGF-untreated cells, resulting in more intense PI fluorescence than in EGF-treated cells, which showed intense green fluorescence for profilaggrin despite weak PI signals. EGF Enhances Suspension-induced CE Formation and TGase 1 Activation—In addition to differentiation marker protein expression, we examined the effects of EGF on other markers of differentiation including CE formation and TGase 1 activity.

CE formation was not detected in attached keratinocytes in
the absence or presence of EGF (Fig. 4a). When cells were cultured in suspension for 48 h, CE formation was detected in EGF-free conditions. The addition of EGF significantly up-regulated CE formation (p < 0.01).

Membrane-associated TGase activity, which is largely dependent on TGase 1 (27), was investigated 24 h after suspension (Fig. 4b). For comparison, membrane-associated TGase activity in attached keratinocytes cultured in high calcium (1.5 mM) for 48 h was analyzed. Attached keratinocytes, seeded on plates and incubated for 24 h, showed weak membrane-associated TGase activity that was not modulated by the presence of EGF. In contrast, attached keratinocytes cultured for 48 h at high calcium exhibited high TGase activity. When keratinocytes were suspension-cultured for 24 h without EGF, the TGase activity was up-regulated at levels comparable with attached keratinocytes in high calcium. The addition of EGF augmented TGase activity to levels in excess of those of attached keratinocytes in high calcium (p < 0.01).

Next, we examined the time course of terminal differentiation of suspended keratinocytes in response to EGF. Since CE is an end product of terminally differentiated keratinocyte in culture, we monitored the time course of CE formation (Fig. 4c). When keratinocytes were suspension-cultured without EGF, CE formation progressed up to 96 h incubation, whereas in the presence of EGF, the level of CE formation increased up to 72 h incubation and then reached a plateau. The final (maximal) level of CE formation was comparable between EGF-untreated and -treated keratinocytes. These results suggest that EGF accelerates the suspension-induced terminal differentiation of keratinocytes.

**EGF Modulates Steady-state mRNA Levels of Differentiation Markers in Suspended Keratinocytes**—To determine the mechanism of differentiation-modulating effects of EGF, we examined steady-state mRNA levels of differentiation markers in keratinocyte suspension cultured for 24 h by RT-PCR (Fig. 5). β-Actin, K14, and K16 mRNA levels (cDNA amplified for 25 cycles) were identical among suspended keratinocytes with and without EGF and attached keratinocytes. The K10 mRNA level was higher in suspended keratinocytes without EGF than in suspended keratinocytes with EGF or attached keratinocytes. In contrast, TGase 1 and profilaggrin mRNA levels were up-regulated in suspended keratinocytes with EGF compared with attached keratinocytes. However, suspended keratinocytes without EGF did not exhibit up-regulated mRNA levels of TGase 1 or profilaggrin.

**EGFR Activation Mediates Differentiation-modulating Effects of EGF**—We then confirmed the role of EGFR in the differentiation-modulating effects of EGF. Although profilaggrin was not detected in suspended keratinocytes (Fig. 6), EGF clearly up-regulated profilaggrin expression as detectable by immunoblotting. The anti-EGFR mAb, which inhibits binding of EGF to EGFR, suppressed both EGF-induced profilaggrin expression and K10 down-regulation. PD153035, which selectively inhibits protein-tyrosine kinase activity of EGFR, also suppressed the effects of EGF on K10 and profilaggrin expression. Moreover, PD153035 completely attenuated EGF-induced enhancement of CE formation and membrane-associated TGase activity in suspended keratinocytes (Fig. 7).

We also examined the response of keratinocytes to TGF-α,
which, like EGF, interacts with and activates EGFR and is one of the physiological ligands of EGFR in epidermal keratinocytes. Expression of K10 and profilaggrin was blocked and induced, respectively, in response to TGF-α, whereas TGF-β1, which inhibits both differentiation and proliferation of keratinocytes, had no effect on K10 and profilaggrin expression of suspended keratinocytes (Fig. 6). In addition, TGF-α enhanced CE formation and membrane-associated TGase activity in suspended keratinocytes, which were also reverted by PD153035 (Fig. 7). Furthermore, keratinocyte growth factor, a potent modulator of keratinocyte proliferation/differentiation, could not modulate K10 and profilaggrin expression (data not shown).

H-7 and PD98059 Selectively Suppressed Up-regulation of Profilaggrin Expression by EGF—To investigate intracellular signaling pathways mediating differentiation-modulating effects of EGFR activation, various inhibitors of intracellular signaling intermediates were cocultured with EGF in suspended keratinocytes (Fig. 8).

Since it has been revealed that PKC selectively participates in late terminal differentiation of keratinocytes (28, 29), the transition from spinous to granular layer, we first examined the role of PKC in EGF-induced profilaggrin expression, one of the most reliable markers of granular cell differentiation. Keratinocytes were suspension-cultured in the presence of EGF with or without H-7, a selective inhibitor of PKC activity. H-7 apparently inhibited EGF-induced profilaggrin expression. Although 12-O-tetradecanoylphorbol-13-acetate-induced activation of PKC could repress K1/10 mRNA expression in keratinocytes (28), the suppression of K10 expression by EGF was not abrogated by H-7. In addition, other PKC-specific inhibitor bisindolylmaleimide I (1 μM) also inhibited EGF-induced profilaggrin expression but not K10 suppression (data not shown).

The Ras/Raf/MEK/MAPK pathway and a phosphatidylinositol 3-kinase-mediated pathway have both been shown to be triggered by EGFR activation (30). We therefore inhibited these pathways using a MEK inhibitor (PD98059) and a phosphatidylinositol 3-kinase inhibitor (wortmannin), respectively. PD98059 alone did not modulate K10 or profilaggrin expression in suspended keratinocytes, whereas wortmannin alone enhanced suspension-induced profilaggrin expression without any effect on K10 expression. PD98059 inhibited EGF-induced profilaggrin up-regulation but not K10 suppression, whereas wortmannin could not modulate the effect of EGF on profilaggrin or K10 expression.
DISCUSSION

Our findings that EGF enhanced expression of late phase differentiation markers in suspended keratinocytes are in apparent contrast with previously reported effects of EGF on attached keratinocytes (10–13). Since growth factors are mitogenic only if the cell-ECM interaction is preserved (2, 3), biological effects of EGF appear to be drastically changed by disrupting cell-ECM interaction. It has been suggested that coexistence of other signals could change the biological activity of EGF from proliferative to differentiative. For example, EGF alone is mitogenic in PC12 cells, whereas the combination of cAMP and EGF induces neuronal differentiation in those cells (31). Tyrosine phosphorylation of the 80-kDa membrane-associated protein by disrupting α6β4 integrin-mediated anchorage of human keratinocytes to laminin 5 suggests that ECM disruption induces intracellular signaling (32). Conversely, the binding of α6β4 integrin to laminin 5 activates the Ras-MAPK pathway in keratinocytes (33). Therefore, EGF in combination with the signal(s) induced by loss of cell-ECM interaction or with the attenuation of signal(s) triggered by cell-ECM interaction might enhance late terminal differentiation of keratinocytes. In conjunction with the previous reports such as the exclusive immunoreactivity of EGF/EGF precursor in the granular layer of epidermis (16) and the ability of EGF to potentate the antiproliferative and differentiative activity of 1,25-dihydroxyvitamin D3 in cultured keratinocytes (34), our results suggest that EGF promotes the terminal differentiation of the keratinocytes, which has been already committed to undergo terminal differentiation process.

The effects of EGF on differentiation of suspended keratinocytes seem to reflect in vivo activation of EGFR in suprabasal keratinocytes, since (i) the suspended keratinocytes initially expressed K10 followed by profilaggrin expression, which mimics the characteristic gradual differentiation in epidermis, although the time course was shortened compared with normal epidermis, (ii) changes of extrinsic spatial cues in keratinocytes of our culture system imitated those observed during basal cells transit to suprabasal cells, and (iii) the effects of EGF were duplicated by TGF-α, one of the physiological ligands for EGFR in epidermal keratinocytes, and were inhibited by PD153035, a specific inhibitor of protein-tyrosine kinase activity of EGFR and by anti-EGFR mAb, which blocks the binding of EGF to EGFR. Therefore, EGFR activation in the suprabasal layer may promote late terminal differentiation of keratinocytes. The effect of EGFR activation appears to be compensatory for the proliferation-enhancing effects of EGFR activation in the basal layer to maintain homeostasis of the epidermal architecture, which has been suggested by in vivo studies using EGFR gene expression-manipulated mice (7–9).

Ligand-activated EGFR transduces diverse intracellular signaling cascades (30). Complete abolition of EGF-induced profilaggrin up-regulation by H7 and bisindolylmaleimide I indicates that PKC mediates profilaggrin up-regulation by EGF. This result is consistent with the selective requirement of PKC for granular cell differentiation in cultured keratinocytes (28, 29). Although EGFR activation in attached keratinocytes could not induce tyrosine phosphorylation of phospholipase C (35), which is crucial for EGFR-mediated PKC activation in other cell types (30, 36), TGF-α activates PKC in keratinocytes by increasing the release of arachidonic acid (35). However, PKC activation by TGF-α was weak compared with that by phorbol ester (35), which induces terminal differentiation of attached keratinocytes. Therefore, differentiation-suppressing signaling...
from the cell-ECM interaction might extinguish the differentiation-promoting signals from EGF. In fact, EGF could transiently elevate TГase 1 and involucrin mRNA levels in attached keratinocytes without inducing their protein synthesis (12). Moreover, since EGF could enhance cell-ECM interaction by augmenting integrin expression (37), EGF might suppress its own differentiation-promoting activity when cell-ECM interaction is conserved.

In addition to PKC suppression, inactivation of MEK also reverted EGF-induced profilaggrin up-regulation in suspended keratinocytes. In general, the Raf/MEK/MAPK cascade is positively regulated by PKC and could mediate the biologic action of PKC (38). In keratinocytes, PKC can activate both MAPK (39) and the AP-1 transcription factor family, which regulates the expression of late differentiation markers (40). Since EGF-activated MAPK can induce transactivation of AP-1 activity (41) and positively regulate the differentiation of various cell types (42, 43), cooperation of PKC and MAPK cascades might mediate EGF-induced profilaggrin up-regulation.

Although PKC activation by 12-O-tetradecanoylphorbol-13-acetate has been shown to suppress K10 expression in attached keratinocytes (28), neither H7 nor bisindolylmaleimide I reverted EGF-induced K10 suppression in suspended keratinocytes. This suggests that EGF inhibits K10 expression via signaling intermediates other than PKC or by H7- and bisindolylmaleimide I-insensitive PKC isoforms. Since K10 expression is also suppressed by EGF in attached keratinocytes (10) and the inactivation of EGFR induces K10 expression of the cells (14), cell-ECM interaction could not affect the EGFR signaling, which regulates K10 expression. However, the responsible signaling pathway is unknown. In suspended keratinocytes, the suppressive effect of EGF on K10 expression was not reverted by inhibition of MEK or phosphatidylinositol 3-kinase activities, suggesting that other signaling intermediates such as signal transducers and activators of transcription (STATs) (30) might be responsible for K10 suppression by EGF.

In addition to the loss of cell-ECM interaction, an increase in intracellular free calcium has been suggested to mediate suspension-induced keratinocyte differentiation (44). Since EGF elevates the levels of intracellular free calcium in keratinocytes (45), EGF might promote suspension-induced late terminal differentiation by enhancing calcium mobilization. However, it is difficult to confirm this speculation, since the chelation of intracellular calcium completely suppressed suspension-induced K10 and profilaggrin expression in our culture system (data not shown) and in others (44), and EGF-induced calcium mobilization could not be selectively inhibited.

It was very recently demonstrated that phospholipase C signaling pathway by EGF was largely dependent on the existence of EGFR on cell surface, compared with other EGF-induced signaling (46). Since suspension-induced drastic changes of cytoskeletal organization has the possibility to impair the internalization of ligand-bound EGFR, activated EGFR-mediated phospholipase C signaling and the resultant PKC activation might be augmented in suspended keratinocytes.

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