Epiregulin, a Novel Member of the Epidermal Growth Factor Family, Is an Autocrine Growth Factor in Normal Human Keratinocytes*

(Received for publication, June 11, 1999, and in revised form, December 1, 1999)

Yuji Shirakata‡, Toshi Komurasakiš, Hitoshi Toyodaš, Yasushi Hanakawa, Kenshi Yamasaki, Sho Tokumaru, Koji Sayama, and Koji Hashimoto

From the Department of Dermatology, Ehime University School of Medicine, Shitsukawa, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan and the §Molecular Biology Laboratory, Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 403, Yoshino-cho, Omiyashi, Saitama 330-8530, Japan

Epiregulin is a new member of the epidermal growth factor (EGF) family purified from conditioned medium of NIH-3T3 clone T7. Some EGF family growth factors play essential roles in human keratinocytes in an autocrine manner. We show here that epiregulin is another autocrine growth factor for human keratinocytes. Epiregulin stimulated human keratinocyte proliferation under both subconfluent and confluent culture conditions in the absence of exogenous EGF family growth factors. Immunoprecipitation of [35S]methionine-labeled conditioned medium revealed a 5-kDa band corresponding to epiregulin. Northern blot analysis detected a 4.8-kilobase transcript of epiregulin, and the addition of epiregulin up-regulated epiregulin mRNA synthesis. Furthermore, an anti-epiregulin blocking antibody reduced DNA synthesis by 25%. Epiregulin up-regulated the mRNA levels of heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, and TGF-α. In turn, the addition of EGF, HB-EGF, amphiregulin, and TGF-α increased epiregulin mRNA levels. These results demonstrate that epiregulin acts as an autocrine growth factor in human epidermal keratinocytes and is part of autocrine and paracrine mechanisms involving HB-EGF, amphiregulin, and TGF-α. The mRNA expression profile resulting from induction of differentiation with high calcium and fetal calf serum revealed the differential expression of epiregulin, HB-EGF, amphiregulin, and TGF-α in keratinocytes. This indicates that these four growth factors have distinct, non-redundant biological functions.

Epiregulin is a novel member of the epidermal growth factor (EGF) family initially purified from conditioned medium of the mouse fibroblast-derived tumor cell line NIH-3T3 clone T7 (1). The coding sequence of human epiregulin cDNA predicts a 46-amino acid single-chain polypeptide, exhibiting 24–50% homology with the sequences of other EGF receptor (EGFR)-ligands. It binds to ErbB1 (EGFR) and is a potent mitogen for rat primary hepatocytes. Epiregulin exhibits a bifunctional regulatory property in that it inhibits the growth of several epithelial cell lines and stimulates the growth of fibroblasts and various other cell types (1–3).

The EGF family consists of EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), neuregulin 1, neuregulin 2, and neuregulin 3 (4–11). All members of the EGF family share six similarly spaced, conserved cysteine residues, have one or more EGF domains, and also have a transmembrane domain. The membrane-anchored precursor of the EGF family is enzymatically processed externally to release a mature soluble form that acts as a paracrine growth factor. Some members of the EGF family act as a juxtacrine growth factor in the membrane-anchored form. EGF-related peptides trigger an intracellular signaling cascade upon binding to their receptors, causing changes within the target cells. There are four types of EGF receptors: ErbB1, also referred to as EGFR or Her1, ErbB2 (Neu/Her2), ErbB3 (Her3), and ErbB4 (Her4) (12–15). The members of the EGF family are divided into two groups based on their receptor affinities. One group includes EGF, HB-EGF, TGF-α, AR, BTC, and epiregulin, which all bind ErbB1. HB-EGF, epiregulin, and BTC are known to bind to ErbB4 as well as ErbB1 (16–18). The other group includes the neuregulins, which are ligands for ErbB3 and ErbB4. Recent studies have explored the in vivo function of the EGF receptor-ligand signaling system in mice by disrupting the genes encoding the EGF receptor-ligands and their receptors, ErbB1, ErbB2, ErbB3, and ErbB4 (19–27). Knockout studies revealed that ErbB1 is essential for epithelial development in the skin, lung, and gastrointestinal tract. In contrast, neuregulins and their receptors, ErbB2, ErbB3, and ErbB4, are crucial for the development of cardiac muscle and the central nervous system.

Previous reports have shown that normal human keratinocytes are capable of producing a large number of cytokines and growth factors (28–32). Keratinocytes produce several growth factors that act as either positive or negative mediators for their growth (32). The most important growth factors for normal human keratinocyte proliferation are members of the EGF family, including TGF-α, HB-EGF, and AR. These three EGF family members are produced and secreted by keratinocytes and act as autocrine growth factors (33–35).

Because epiregulin binds to ErbB1, it is expected that it is the most recently described autocrine growth factor of the EGF family for normal human keratinocytes. However, there has been no study on the role of epiregulin in keratinocyte proliferation. In this study, for the first time, we demonstrate that
Epiregulin is an autocrine growth factor in normal human keratinocytes and that, along with HB-EGF, AR, and TGF-α, epieregulin forms auto- and cross-induction regulatory mechanisms during normal human keratinocyte growth. Furthermore, we demonstrate that the mRNA expression profile upon induction of differentiation reveals the differential expression of epieregulin, HB-EGF, AR, and TGF-α in keratinocytes. This indicates that the function of epieregulin is not redundant; it has its own distinct biological function like HB-EGF, AR, and TGF-α.

EXPERIMENTAL PROCEDURES

Materials—Recombinant EGF and TGF-α were generous gifts from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Recombinant AR was purchased from R & D Systems (Minneapolis, MN). Recombinant HB-EGF, HB-EGF cDNA, AR cDNA, and TGF-α cDNA were kindly provided by Shigeki Higashiyama (Osaka University, Osaka, Japan). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was purchased from CLONTECH.

Cell Culture—Normal human skin obtained from plastic surgery was cut into 3–5-mm strips and incubated with 250 units/ml disperse in Dulbecco’s modified Eagle’s medium overnight at 4 °C. After separation of the epidermis from the dermis using forceps, the epidermal sheets were rinsed with PBS (−), incubated in a 0.25% trypsin solution for 10 min at 37 °C, and teased with forceps. Epidermal cells were rinsed and collected by centrifugation and then resuspended in MCDB153 medium supplemented with insulin (5 μg/ml), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), and bovine thyrotropic hormone extract (0.5 μg/ml). Third or fourth passage cells were used in this study.

Measurement of Bromodeoxyuridine (BrdUrd) Uptake—Keratinocytes were seeded on 24-well plates at a density of 5 × 10⁴ cells/well. After reaching subconfluence, the cells were re-fed with medium lacking bovine hypothalamic extract. The following day, the cells were re-fed again with the same medium containing various concentrations of recombinant epieregulin and incubated for 24 h. The cells were then incubated with medium containing BrdUrd for 2 h. Incorporation of BrdUrd was determined using a cell proliferating enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. The absorbance at 450 nm was measured using a spectrophotometer (Amersham Pharmacia Biotech).

Metabolic Labeling with [35S]Methionine and Immunoprecipitation of Epieregulin—Keratinocytes were seeded at a density of 2 × 10⁶ cells/dish. After 24 h, the cells were washed with methionine-free medium. Then, 5 ml of MCDB153 containing 0.5 mCi of [35S]methionine was added to each sample and rocked for 1 h at 4 °C. After centrifugation, the resins were washed five times with 1 ml of radioimmunoprotein assay buffer. The precipitates were suspended in 30 μl of protein G-Sepharose (Pharmacia) and aliquots containing equal activity were transferred to a nitrocellulose membrane. The membrane was soaked with a 1:1,000 dilution of anti-phosphotyrosine antibody (Upstate Biotechnology) followed by addition of the fluorescent substrate Attophos (Amersham Pharmacia Biotech). The membrane was scanned on FluorImager (Molecular Dynamics Inc., Sunnyvale, CA).

RESULTS

Recombinant Human Epieregulin Stimulates Proliferation of Normal Human Epidermal Keratinocytes—We first tested recombinant epieregulin for its ability to stimulate normal human epidermal keratinocyte (NHEK) growth in the absence of other exogenous EGF family members under subconfluent, serum-free conditions. Epieregulin stimulated NHEK growth in a dose-dependent manner. 1 ng/ml epieregulin resulted in optimal proliferation (3.0-fold increase in cell number compared with control) (Fig. 1A). A profile cycle consisted of 1 min at 94 °C for denaturation, 1.5 min at 60 °C for annealing, and primer extension. 5 μl of each reaction mixture was electrophoresed on a 2% agarose gel containing ethidium bromide to evaluate amplification.

Growth Inhibition of NHEK by Anti-epieregulin Antibody—To examine the autocrine action of epieregulin in NHEKs, we determined the effect of anti-epieregulin blocking antibody on NHEK growth. Subconfluent NHEKs were re-fed with fresh medium containing anti-epieregulin antibody (10 μg/ml) or normal rabbit IgG (10 μg/ml). After 24 h, DNA synthesis was examined by measuring BrdUrd incorporation. In contrast to subconfluent conditions, epieregulin stimulated NHEK proliferation in a dose-dependent fashion up to 20 ng/ml. Maximum stimulation was observed at 10 ng/ml (1.9-fold) (Fig. 1B). These proliferative effects are dependent on cell density, and the effective dose is higher in confluent conditions than in subconfluent conditions.

Growth Inhibition of NHEK by Anti-epieregulin Antibody—To examine the autocrine action of epieregulin in NHEKs, we determined the effect of anti-epieregulin blocking antibody on NHEK growth. Subconfluent NHEKs were re-fed with fresh medium containing anti-epieregulin antibody (10 μg/ml) or normal rabbit IgG (10 μg/ml). After 24 h, DNA synthesis was examined by measuring BrdUrd incorporation. The blocking antibody reduced DNA synthesis by 75% of the level observed using normal rabbit IgG after a 24-h incubation period (p < 0.05, data not shown). This result indicates the autocrine nature of epieregulin in NHEKs.

Phosphorylation of ErbB1 by Epieregulin—Subconfluent keratinocytes were treated with 100 ng/ml epieregulin or EGF for 5 min. The cells were washed with cold PBS (−) and then harvested on ice with a cell scraper. The cells were centrifuged and re-suspended in 200 μl of lysis buffer containing proteinase inhibitors. After centrifugation, the cytosolates were immunoprecipitated with 1 μg of anti-EGFR (Ab-3, Oncogene Science) and 10 μl of protein G-Sepharose for 1 h at 4 °C. The pellet was washed with lysis buffer five times and then boiled with sample buffer. The extract was separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, the membrane was soaked with a 1:1,000 dilution of anti-phospho-tyrosine antibody (PY20, Transduction Lab., Lexington, KY). After washing, the membrane was incubated with 1:2,500 fluorescein-labeled goat anti-mouse IgG for 1 h. The signal was amplified with an anti-fluorescein alkaline phosphatase conjugate followed by addition of the fluorescent substrate Attphos (Amersham Pharmacia Biotech). The membrane was scanned on FluorImager (Molecular Dynamics Inc., Sunnyvale, CA).

Northern Blot Analysis—Total RNA from cultured human keratinocytes was prepared with Isogen (Nippon Gene, Tokyo, Japan). 20 μg of total RNA/lane was loaded and separated by electrophoresis on a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Hybond N−, Amersham Pharmacia Biotech) in 20× SSC (150 mM sodium chloride, 15 mM sodium citrate) according to standard procedure. Pre-hybridization was carried out in a roller bottle for 1 h at 68 °C in a hybridization buffer (Quick-Hyb, Stratagene). The membrane was probed for 2 h at 68 °C with 32P-labeled (Amersham Pharmacia Biotech) cDNA. The hybridized membranes were washed in 2× SSC, 0.1% SDS twice at room temperature and in 0.1× SSC, 0.1% SDS twice at 60 °C. Hybridization signals were detected either by autoradiography (Kodak BioMax MS) or by BAS 3000.

RT-PCR Analysis—Specific primers for human epieregulin, HB-EGF, TGF-α, AR, and GAPDH were made by selecting specific nucleotide sequences. The primers were as follows: epieregulin-TGCGGCCTCGCCATCGGCCG and GGTTCCACATATTCTCTGTG; HB-EGF-CCACACCATGCGGCTACCGGGG, and ATGGAGAAGCCCCACGATGAC; TGF-α-GAGGCTGAGTGTCGAACGCGGCGGGCCTGGG, and CCAGAGGATCCGATGTCACACCAGAGCCCGGGCCTGGG; AR-CAAAAAACAGCGGAGAAGTTGA and AGAGTACACGAGCAGACATGAGG, and GAPDH-ACCAGGCATTCCGATTCCTAC and TCCACCAC-CTGTTGCTGTA. The RT-PCR was performed using RT-PCR High Plus (Toyobo Co., Ltd., Osaka, Japan) according to the instructions of the manufacturer. cDNA was reverse-transcribed from total RNA for 30 min at 90 °C and heated to 94 °C for 2 min. Amplification was performed using a DNA thermal cycler (Astee, Fukushima, Japan) for 25 cycles. A profile cycle consisted of 1 min at 94 °C for denaturation, 1.5 min at 60 °C for annealing, and primer extension. 5 μl of each reaction mixture was electrophoresed on a 2.0% agarose gel containing ethidium bromide to evaluate amplification.

Phosphorylation of ErbB1 by Epieregulin—As epieregulin is known to bind to ErbB1 (1), we investigated whether the tyrosine residue of ErbB1 in NHEKs was phosphorylated by epieregulin stimulation. Subconfluent NHEKs were treated with 100
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A 32P-labeled epiregulin cDNA probe hybridized to a single 4.8-kilobase transcript of epiregulin. The size of the transcript is consistent with those found in human placenta, peripheral blood leukocytes, or other epithelial cell lines. We also studied whether epiregulin could up-regulate transcription of its own mRNA. Because 10 ng/ml epiregulin stimulated DNA synthesis, we decided to examine whether this concentration of epiregulin was capable of increasing epiregulin mRNA levels. Total RNA was isolated at 0, 1, 3, 6, 12, and 24 h after the addition of epiregulin. Epiregulin did increase its own mRNA production; the optimum 3.6-fold induction occurred 1 h after the addition of epiregulin (Fig. 3).

Cross-induction of Other EGF Family by Epiregulin—We examined the cross-induction ability of epiregulin in NHEKs because previous reports have shown that EGF-related autocrine growth factors are capable of inducing other EGF growth factors. Northern analysis showed that epiregulin addition increased the mRNA levels of AR, TGF-α, and HB-EGF, 2.9-fold, 3.4-fold, and 5.6-fold, respectively, after 1 h (Fig. 4).

Enhancement of TGF-α Production by Epiregulin—The addition of epiregulin increased the level of TGF-α mRNA, so we then measured the production of TGF-α in the culture medium. NHEKs were cultured in the presence of epiregulin (10 ng/ml), EGF (10 ng/ml), or HB-EGF (10 ng/ml) for a 24-h incubation period. Epiregulin increased TGF-α protein production by 8.5-fold compared with the no-treatment control. The increase was greater than that induced by HB-EGF (2.2-fold increase) but weaker than induction by EGF (20-fold increase) (Fig. 5).

Epiregulin mRNA Induction by EGF Family Growth Factors—Because epiregulin up-regulated the mRNA levels of HB-EGF, AR, and TGF-α, we investigated whether expression of epiregulin is enhanced by other EGF family growth factors. Two ng EGF optimally increased the level of epiregulin mRNA 4.5-fold at 1 h, and HB-EGF increased epiregulin mRNA synthesis 4.0-fold at 1 h. AR increased the epiregulin mRNA level 4.2-fold at 1 h, while TGF-α caused a 4.8-fold increase at 3 h (Fig. 6).

Enhancement of Epiregulin Protein Production by EGF and HB-EGF—NHEKs were treated with 20 ng/ml EGF or HB-EGF for 48 h and labeled with [35S]methionine; the labeled materials were then immunoprecipitated. A band approximately 5 kDa in size corresponding to epiregulin was up-regulated in the culture medium supplemented with EGF or HB-EGF (Fig. 7).

Differential Expression of EGF-related Autocrine Growth Factors Induced by Keratinocyte Differentiation—The combination of high calcium and FCS is a potent inducer of keratinocyte differentiation (36). We used RT-PCR to study the mRNA levels of TGF-α, AR, HB-EGF, and epiregulin following such a treatment. Total RNA was harvested at 0, 1, 3, 6, 12, 24, 48, and 72 h.
after the addition of MCDB153 medium containing 1.8 mM calcium and 10% FCS. Epiregulin and HB-EGF mRNA levels increased and declined in a similar manner. The greatest increase was observed at 6 h, and both epiregulin and HB-EGF declined to baseline levels at 24 h. The increase in the level of TGF-α mRNA was delayed compared with epiregulin and HB-EGF. TGF-α started to increase at 3 h and decreased gradually at 48 h; the largest increase was observed at 12 h. AR mRNA was not affected until 6 h, after which its level gradually decreased (Fig. 8).

**DISCUSSION**

Human keratinocytes are the main component cells of the epidermis and their growth is regulated by both positive and negative mediators (32). Of all these, the most important mechanism for keratinocyte growth is the EGF receptor-ligand system. Epiregulin is a recently described member of the EGF family, which also includes EGF, TGF-α, AR, HB-EGF, BTC, and neuregulins (4–11). This study directly demonstrates that epiregulin is one of the most recently described autocrine growth factors of the EGF family found in normal human keratinocytes. This was determined by the following findings: (i) epiregulin is produced by keratinocytes, (ii) epiregulin stimulates keratinocyte growth, (iii) anti-epiregulin blocking antibody reduces DNA synthesis, and (iv) epiregulin up-regulated its own mRNA production. The addition of epiregulin enhanced tyrosine phosphorylation of ErbB1, so the autocrine stimulation of keratinocytes by epiregulin is mediated mainly through its interaction with ErbB1 expressed by these cells.

Previous publications have shown that EGF-related autocrine growth factors are capable of inducing other EGF growth factors in keratinocytes (34, 37). We have shown here that epiregulin increased the levels of TGF-α, HB-EGF, and AR mRNA, and enhanced TGF-α protein production in the culture medium. In turn, epiregulin mRNA synthesis was enhanced by
other EGF family members including EGF, TGF-α, AR, and HB-EGF. Furthermore, EGF and HB-EGF both enhanced epiregulin protein production in the culture medium. These results indicate that other members of the EGF family are potent cross-inducers of epiregulin. These findings, taken together, indicate that epiregulin, HB-EGF, AR, and TGF-α interact with each other by a cross-induction mechanism as well as by autocrine induction. The combination of these two mechanisms is very effective in keratinocyte proliferation.

The extent of cross induction differs among the four different autocrine growth factors. The greatest induction of epiregulin mRNA synthesis by HB-EGF, AR, and EGF occurred as early as 1 h after their addition, whereas induction by TGF-α was greatest at 3 h. Previous reports have shown that HB-EGF and EGF optimally induce HB-EGF mRNA synthesis at 1 h, whereas TGF-α-induced HB-EGF mRNA synthesis peaks at 6 h (34). Moreover, induction of differentiation in keratinocytes results in three distinct EGFR ligand expression profiles. Epiregulin and HB-EGF expression levels are increased by 1 h, with their peak observed at 6 h and both declining to base line level at 24 h. The increase in TGF-α mRNA levels is delayed compared with epiregulin and HB-EGF. In contrast, AR mRNA levels remain constant until 6 h and then gradually decrease. The differential expression profiles seen in cross-induction and differentiation suggest that these factors have distinct, non-redundant biological functions.

Recent findings in other tissues also indicate that these four growth factors each have their own distinct biological functions. Tissue distribution, molecular characteristics, receptor binding, a preference for dimerization, and receptor affinity all differ among the four keratinocyte autocrine growth factors. Among the EGF family members, the structure of epiregulin is most similar to that of TGF-α on the basis of the length of each segment and the location of a single potential N-glycosylation site (1). HB-EGF and AR share structural characteristics and both bind heparin; they also both have a juxtacrine as well as paracrine function (38). However, the tissue distribution of epiregulin mRNA is not identical to that of TGF-α (3). Northern blot analysis showed that epiregulin mRNA was found predominantly in the placenta and peripheral blood leukocytes and to a lesser extent in the heart. Very faint expression was detected in normal adult bone marrow, ovary, small intestine, colon, lung, and liver. On the other hand, TGF-α mRNA was predominantly expressed in the kidney, pancreas, brain, lung, liver, and placenta (3). TGF-α and HB-EGF mature protein were both detected in normal epidermis, and TGF-α mRNA was up-regulated in psoriatic epidermis, which is a hyper-proliferative disorder (39–41). Expression of mature AR protein was undetectable in adult epidermis (42), whereas it was detected in developing fetal skin and overexpressed in psoriatic epidermis (43). These observations suggest that the expression of individual growth factors is quite different and that these EGF-related peptides are involved in different stages of proliferation, differentiation, and development. In terms of receptor binding and dimerization, TGF-α and AR bind to ErbB1 only, whereas epiregulin and HB-EGF are ligands for not only ErbB1 but for ErbB4 as well (16, 17). All the EGF-related peptides, except AR, were able to induce tyrosine phosphorylation of ErbB2 by forming a heterodimer with ErbB1 (44). The ability to induce tyrosine phosphorylation of ErbB3 varied among the four EGF-related peptides. Recent reports have shown that TGF-α/ErbB1-mediated autocrine growth of transformed epithelial cells is dependent on activation of Stat3 (45), whereas EGF-induced growth inhibition of A431 cells requires Stat1 activation (46). Moreover, epiregulin stimulates the growth of rat primary hepatocytes and A31 fibroblasts, but inhibits the growth of several epithelial tumor cells (1). These findings suggest the activation of different signal transduction pathways following receptor binding by the four autocrine growth factors.

Another interesting point is that epiregulin has the broadest specificity of the EGF-related ligands; it not only stimulates homodimers of both ErbB1 and ErbB4, but also activates all possible heterodimeric ErbB complexes (47, 48). The binding of epiregulin to most receptors results in the transmission of a more potent mitogenic signal than does the binding of EGF, and this discrepancy is the result of a mechanism that prevents receptor down-regulation, and results in a weak, but prolonged, state of receptor activation. In our experiment on tyrosine phosphorylation of ErbB1 by epiregulin and EGF, the induction of phosphorylation by epiregulin was lower than that by EGF. These findings suggest that epiregulin may have a distinct, non-redundant biological function in normal human keratinocyte proliferation, as do other autocrine growth factors.

In conclusion, epiregulin is an autocrine growth factor in normal human keratinocytes and organizes epidermal structure by regulating keratinocyte proliferation and differentiation as well as the expression of TGF-α, HB-EGF, and AR.

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J. Biol. Chem. 2000, 275:5748-5753.
doi: 10.1074/jbc.275.8.5748

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