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Brief treatment with heparin-binding EGF-like growth factor, but not with EGF, is sufficient to accelerate epithelial wound healing

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Abstract

Background—Heparin-binding EGF-like growth factor (HB-EGF) contains, in contrast to EGF, a domain that binds to negatively charged glycans on cell surfaces and in extracellular matrix. We speculated that a short exposure to HB-EGF induces prolonged biological effects such as healing of wounds after immobilization in tissues.

Methods—Epithelial cell sheets in tissue and corneas in organ culture were treated briefly with HB-EGF or EGF and binding of the growth factors, time course of activation of the EGF receptor, and healing of wounds were compared.

Results—Treating human corneal epithelial cells for two minutes with HB-EGF resulted in eight hours of detectable activation of the EGF receptor, but activation was much shorter after EGF treatment. A brief treatment with HB-EGF, but not with EGF, induced significant acceleration of healing in wounds in epithelial sheets in tissue and organ culture. Bound HB-EGF was detectable up to 16 hours after brief treatments. Neutralizing antibodies added after HB-EGF treatment blocked acceleration of healing, demonstrating the role of bound HB-EGF in accelerating healing.

Conclusions—A brief exposure to HB-EGF, but not to EGF, is sufficient to induce prolonged activation of the EGF receptor and to enhance healing.

General Significance—Bound HB-EGF can serve as a pool that induces prolonged activation of the EGF receptor. EGF has been used experimentally to treat poorly healing wounds, but the frequent applications that are necessary have hampered its use clinically. The findings imply that HB-EGF may be a useful long-acting alternative to EGF.

Keywords

EGF; heparin-binding EGF-like growth factor; wound healing; cornea

1. Introduction

Wound healing is a complex phenomenon that involves inflammation, cell migration and division, and extracellular matrix production and remodeling [1; 2]. Epithelia respond to wounding by migrating to cover denuded areas and restoring their functions as physical barriers. In many epithelia, including the gut, airway, epidermis, and cornea, activation of

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the EGF receptor (EGFR) is a key event that controls initiation of motility [3; 4; 5; 6; 7]. The receptor is activated by heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin which are generated by proteolysis of precursors in the cell membrane [8; 9]. Poorly-healing wounds and chronic ulcers are often difficult to treat and significant efforts are directed to develop improved therapies. One approach is to use growth factors to stimulate healing, and many reports have described the successful use of EGF (for references see Lozano *et al.* [10]). However, in epithelia that are covered with fluids, such as the corneal epithelium, one major difficulty is that EGF is rapidly washed out. Since continued movement of the epithelium requires persistent stimulation of the receptor [11], positive outcomes have required very frequent application of the growth factor or the use of some form of continuous-release device [12; 13]. This has impeded development of practical procedures for using EGF to treat ulcers in the cornea and other tissues in humans.

Seven high-affinity ligands for the EGFR have been identified [14; 15]. Amphiregulin and HB-EGF contain a domain that binds to negatively charged glycans on the cell surface and in extracellular matrix [16; 17]. Binding to the glycans relieves an intramolecular inhibition of the EGFR binding domain resulting in increased levels of EGFR activation [18; 19]. Binding of some growth factors, for instance fibroblast growth factor and transforming growth factor- β , to components of the cell surface and extracellular matrix produces a pool that can prolong activation of their respective receptors [20]. We speculated that bound HB-EGF might similarly produce a prolonged signal, and we therefore tested the duration of signaling induced by bound HB-EGF and its effect on wound healing.

2. Materials and Methods

2.1. Materials

Antibodies against the EGFR phosphorylated on tyrosine 1173 and against a C-terminal epitope (to detect total amounts of receptor), and antibodies against ERK1 and ERK1/2 phosphorylated on tyrosine 402 were from Santa Cruz Biotechnology (catalog numbers SC-12351-R, SC-03, and SC-7381, respectively). Antibodies against β -actin were from Sigma (catalog number A5216). EGF was from Invitrogen (catalog number 10450-013) and HB-EGF was from EMD Chemicals (catalog number PF078). Neutralizing antibodies to HB-EGF were from RD Systems (catalog number AF-259-NA). HCLE cells were kindly provided by Dr. Ilene Gipson. Fresh rabbit eyes were from Pel-Freez Biologicals (catalog number 41211-2). Cell culture reagents were from MediaTech, and all other supplies and reagents were from ISC BioExpress or ThermoFisher, except where indicated.

2.2. Cell culture, in vitro wounding assay, and immunoblotting

HCLE cells [21] were cultured in keratinocyte serum-free medium (Invitrogen, catalog number 17005-042) supplemented with 0.3 mM CaCl_2 , 25 $\mu\text{g/ml}$ bovine pituitary extract (supplied with the keratinocyte serum-free medium), 0.1 ng/ml human recombinant EGF, 50 IU/ml penicillin, and 50 mg/ml streptomycin. After reaching confluence, the cells were transferred to stratification medium (F12 Medium:Dulbecco's Modified Eagle's Medium (DMEM) 1:1 with 10% newborn calf serum) and incubated for two days. The cells were then cultured overnight in the same medium with 2% newborn calf serum before stimulation with growth factors. For wound healing assays, cells were grown to confluence in 12 well dishes, each well containing a single agarose strip [8], and they were induced to stratify as above. Where indicated, the cultures were incubated with EGF or HB-EGF and washed 4 times to remove unbound growth factor. The agarose strips were removed and the cultures were allowed to heal for 18 hours before fixation and staining with 0.05% Gentian Violet. Micrographs of cultures were obtained before and after wounding and wound areas were quantified using the region tracing utility in ImageJ software (National Institutes of Health,

USA). To test binding of HB-EGF to cell layers, the growth factor was bound in 1 ml medium per well in 12 well dishes for 2 minutes. After four washes, bound HB-EGF was eluted with 0.15 ml medium supplemented with 1 M NaCl for 10 minutes. The eluate from each well was diluted with 0.5 ml water and added to a new well containing 1 ml medium and wounded HCLE cells as described above. Healing was scored as above.

For immunoblotting, the cells were washed once in ice-cold phosphate buffered saline (PBS), and 200 μ l 1% SDS in water was added. After sonication and normalization for protein content, SDS-polyacrylamide gel electrophoresis and immunoblotting were performed using the Mini-Protean® electrophoresis system (Bio-Rad). Densitometry of autoradiographs was performed using Adobe® Photoshop®.

2.3. Organ culture and wounding assay

To inflict wounds in rabbit eyes, 7.5 mm diameter marks were made with a trephine, and the epithelium was removed with an Algerbrush II (Alger Equipment Co.). The wounded corneas were excised with 2–3 mm scleral rims and placed on supports in 12-well dishes. The wounds were briefly stained with 0.1% fluorescein solution and photographed with a digital camera under brief illumination provided by a hand-held long-wave UV light source. The corneas were incubated with medium (DMEM:F12, 1:1 with 30 μ g/ml gentamycin) containing appropriate growth factors for ½ hour, and washed 4 times with medium and growth factor as required, and subsequently incubated at 37°C on plastic supports such that the corneas and the limbal areas were raised just above the incubation medium. After 16 hours, the corneas were stained with 0.1% alizarin red in PBS, and photographed again. The stained areas were quantitated using ImageJ software as above.

3. Results and Discussion

We initially determined the time course of activation of the EGFR and its down-stream effectors extracellular signal-regulated kinases 1 and 2 (ERK1/2) after a brief treatment with either EGF or HB-EGF. For these experiments, we used human corneal limbal epithelial (HCLE) cells, which have been immortalized by abrogation of p16INK4A/Rb and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme [21]. The cells were treated with the growth factors for 2 minutes, the unbound factors were washed away, and the EGFR examined by immunoblotting cell extracts with an antibody that recognizes the receptor autophosphorylated on tyrosine 1173. As is seen in Fig. 1A, both HB-EGF and EGF produced a rapid and similar increase of activation of the EGFR, but whereas the activity of the EGFR reverted to background levels 2 hours after stimulation with EGF, the EGFR was still detectably activated 8 hours after stimulation with HB-EGF. Activation of ERK1/2 occurred more slowly, but was similarly significantly more prolonged with HB-EGF.

To compare the biological activities of EGF and HB-EGF, we used a previously described in vitro assay that measures the rates of closure of gaps in sheets of cells [8]. Healing was promoted to similar extents by the two growth factors when they were present continuously (Fig. 2). As previously reported, stimulation for only the initial 1 hour with EGF did not accelerate healing of wounds [11], but in sharp contrast, a 1 hour incubation with HB-EGF was sufficient to promote maximal rates of healing. To evaluate whether a very brief treatment with HB-EGF is sufficient to stimulate healing, we incubated the HCLE cells with HB-EGF for short times and assayed healing rates. As is seen in Fig. 3. even two minutes stimulation was sufficient to elicit near-maximal rates of wound closure.

HB-EGF is immobilized to glycans on the cell surface and in the extracellular matrix [16; 17]. To examine whether this might constitute a pool for continued stimulation of cell

migration, we first devised an assay to determine whether biologically significant amounts of HB-EGF are attached to the surfaces: HCLE cells were incubated with EGF or HB-EGF, washed, bound growth factor eluted with high salt [22], and added to new cultures of wounded cells. As is seen in Fig.4A, eluates from cells incubated with HB-EGF stimulated wound healing in the target cultures almost to the extent that is stimulated by continuous presence of the growth factor. In contrast, eluates from untreated cells or cells treated with EGF, did not stimulate healing. Eluates from cultures exposed briefly to HB-EGF and then incubated for 8 or 16 hours also enhanced cell migration significantly. The eluted activities were completely inhibited by a blocking antibody to HB-EGF (Fig 4B). Together, these experiments suggests that biologically significant amounts of HB-EGF are bound to the cultures and are present for an extended period of time

To directly confirm the role of bound HB-EGF in stimulating cell migration, we added a blocking antibody after unbound HB-EGF had been washed away. In agreement with previous observations, we found that anti-HB-EGF antibodies have a small effect on migration of HCLE cells in the absence of added growth factor [23], presumably due to blocking the effects of endogenous HB-EGF. Importantly, the antibody completely inhibited HB-EGF-induced acceleration of healing when added after a brief exposure to exogenous HB-EGF (Fig. 5). Together, data reported in Figs. 4 and 5 strongly support that immobilized HB-EGF drives the enhanced motility of the cells.

To test whether a short exposure to HB-EGF is sufficient to induce healing of wounds in conditions that more closely approximate in vivo conditions, we examined the effects of immobilized HB-EGF in organ culture. For these experiments, we inflicted epithelial wounds in commercially obtained rabbit eyes and cultured the excised corneas in an air/liquid interphase culture system. Similar to our observations in tissue culture, we found that EGF only stimulated healing significantly when present continuously, whereas HB-EGF stimulates healing even after the unbound fraction had been washed out after ½ hour incubation (Fig. 6).

The results presented here show that a short treatment with HB-EGF can provide the prolonged stimulus needed for extended EGFR activation and promote healing. It is unlikely that the differences in activation of the EGFR are determined by differences in rates of degradation of the EGFR because the degree of downregulation of the EGFR by the two ligands in HCLE cells is modest and appears similar (Fig. 1 and data not shown). The data presented in this paper are in accord with the presence of an immobilized pool of HB-EGF on the cell surface and extracellular matrix that serves as a source of continuous triggering of the receptor.

Poor healing of wounds is a major health problem [24; 25; 26], and there is consequently much interest in developing appropriate therapies. One major impediment to using EGF to promote healing is that it is rapidly washed out, especially in epithelia that are bathed in fluids such as the corneal and gut epithelium. Although experimental treatments of wounds in the cornea have been encouraging, very frequent applications have been required. In clinical trials, the use of EGF has been less impressive and the requirement for efficient delivery remains a major problem (for references see [12]). The results reported here suggest that HB-EGF may reduce the needed frequency of application since it may cause prolonged EGFR signaling when immobilized in wounds.

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Abbreviations

HB-EGF	heparin-binding EGF-like growth factor
EGFR	EGF receptor
HCLE	human corneal limbal epithelial
NI	non-immune

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Research Highlights

Heparin-binding EGF-like growth factor (HB-EGF), but not EGF, binds to glycans on the cell surface

Bound HB-EGF induces prolonged EGF receptor activation and healing of epithelial wounds

A brief treatment with HB-EGF is sufficient to induce epithelial healing

EGF rapidly washed out from tissues, and is not generally useful for treating wounds

HB-EGF may be more useful than EGF as a therapeutic agent to treat wounds

Fig 1.

Effects of brief treatments with EGF or HB-EGF on signaling. HCLE cells were treated with 4.3 nM EGF(A) or HB-EGF (B) for 2 minutes. Unbound ligand was then removed by washing, and the cells were incubated for the indicated periods of time. Top panels are immunoblots of extracts with the indicated phospho-specific antibodies. The blots were stripped and blotted with antibodies that recognize total EGFR or ERK1. Each individual sample was normalized for content of total EGFR or ERK1. The values in the graphs are means $\pm$ S.D. of quadruplicate or more determinations in this and the following figures, and all experiments were performed at least three times with similar results. At 8 hours, HB-EGF treatment resulted in significant increase in EGFR and ERK1/2 activation compared to controls according to Student's *t* test ($p < 0.001$, and $p < 0.01$, respectively).

Fig 2.

A brief treatment with HB-EGF induces a prolonged stimulus for cell migration. Differentiated sheets of HCLE cells were wounded and incubated with 4.3 nM EGF or HB-EGF for 1 hour and washed where indicated before allowing healing for an additional 16 hours. The EGF without washing and both HB-EGF treatments were significantly increased over control ($p < 0.01$).

Fig 3.

A very short treatment with HB-EGF is sufficient to promote healing in tissue culture. The HCLE cells were treated with 4.3 nM HB-EGF for the indicated periods of time, washed, and allowed to heal. Healing was significantly increased at each time point of treatment ($p < 0.01$).

Fig. 4.

HB-EGF, but not EGF, is bound to the surface of HCLE cells. (A) In the first three columns the growth factors were added directly to the target cells. For the remaining columns, 4.3 nM of the growth factors were first incubated on sheets of HCLE cells for 2 minutes, the cells were then washed and eluted and added to the target cells. 8h and 16h: the growth factors were bound to the HCLE cell sheets, and the cells were incubated for these periods of time before elution. Cells incubated with eluates from HB-EGF-treated cells induced significantly faster migration compared to controls ($p < 0.001$, each of the three last conditions). (B) Eluates from mock (0) or HB-EGF-treated cells were added to target cells in the presence of 20 $\mu\text{g/ml}$ non-immune IgG (NI) or neutralizing anti-HB-EGF antibodies (αHB). Healing was significantly reduced by the HB-EGF antibody ($p < 0.01$).

Fig. 5.

Bound HB-EGF causes accelerated wound healing. Cells were wounded, treated for 10 minutes with HB-EGF, and incubated with 20 $\mu\text{g/ml}$ non-immune IgG (NI) or neutralizing anti-HB-EGF antibodies (αHB). Whereas the eluates from HB-EGF significantly enhanced the rates of healing over controls ($p < 0.0001$), no significant difference was detected between the two anti-HB-EGF treated conditions.

Fig 6.

Bound HB-EGF stimulates healing of wounds in organ culture. Wounds were introduced in rabbit corneas and incubated in an air/liquid interphase organ culture system. The corneas were incubated with 0.86 nM EGF or HB-EGF for 30 minutes and, where indicated, washed four times to remove unbound growth factor before allowing healing for 16 hours. A. B. The “EGF”, “HB-EGF”, and “HB-EGF washed” were statistically different from the controls according to one-way ANOVA and the Bonferoni test for multiple comparisons ($P < 0.001$, 0.01, and 0.001, respectively). No significant differences were evident among these three conditions ($p > 0.05$). Also no difference was detected between the control and the EGF washed condition ($p > 0.05$).