Transforming Growth Factor- α Enhances Corneal Epithelial Cell Migration by Promoting EGFR Recycling

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PURPOSE. The goal of this study was to determine the molecular mechanism by which transforming growth factor- α (TGF- α) is a more potent activator of epidermal growth factor receptor (EGFR)-mediated corneal wound healing than epidermal growth factor (EGF).

METHODS. Telomerase immortalized human corneal epithelial (hTCEpi) cells and primary human corneal epithelial cells were tested for their ability to migrate in response to EGF and TGF- α . In parallel, the endocytic trafficking of the EGFR in response to these same ligands was examined using indirect immunofluorescence, immunoblots, and radioligand binding.

RESULTS. TGF- α , compared with EGF, is a more potent activator of corneal epithelial cell migration. Although both TGF- α and EGF were able to induce EGFR internalization and phosphorylation, only those receptors that were stimulated with EGF progressed to lysosomal degradation. EGFRs stimulated with TGF- α recycled back to the plasma membrane, where they could be reactivated with ligand.

CONCLUSIONS. This study reveals that EGFR-mediated cell migration is limited by ligand-stimulated downregulation of the EGFR. This limitation can be overcome by treating cells with TGF- α because TGF- α stimulates EGFR endocytosis, but not degradation. After internalization of the TGF- α /EGFR complex, EGFR recycles back to the plasma membrane, where it can be restimulated. This sequence of events provides the receptor multiple opportunities for stimulation. Thus, stimulation with TGF- α prolongs EGFR signaling compared with EGF. (*Invest Ophthalmol Vis Sci.* 2010; 51:3455–3461) DOI:10.1167/iops.09-4386

The cornea is the clear, dome-shaped structure that covers the anterior of the eye. It has two main functions. First, the transparent layer of cells control and focus the entry of light into the eye. Second, it prevents external agents, such as particles, viruses, and bacteria, from entering the eye. A leading cause of blindness worldwide is the result of damage to and infection of the cornea.¹

The cornea's first line of defense is its most exterior layer, which is made up of epithelial cells. On wounding of the corneal epithelium, the epithelium immediately begins to re-establish its structural integrity. The three major cellular events in the re-formation of the corneal epithelium are the migration of cells from the surrounding basal epithelium to the wounded

area, the proliferation of these cells, and the differentiation of the cells into the stratified layers. Failure of these events to occur can result in painful corneal ulcerations and distorted vision.^{1,2}

A key regulator for maintaining a healthy cornea and promoting regrowth of the wounded cornea is the epidermal growth factor receptor (EGFR).³ The EGFR is the prototypical tyrosine kinase receptor localized to basal and differentiated epithelia in the cornea.⁴ It has been shown using an ex vivo organ system that activation of the EGFR can promote all three processes—proliferation, migration, and differentiation—involved in corneal epithelial wound healing.^{4–6} Further, patients taking EGFR inhibitors (e.g., Iressa [AstraZeneca Pharmaceuticals, Wilmington, DE] or Tarceva [Genentech, South San Francisco, CA]) therapeutically for the treatment of non-small cell lung carcinomas report an increased incidence of corneal ulcerations.^{7,8} Thus, the stimulation of the EGFR is necessary and sufficient for corneal wound healing.

Evidence from a rodent corneal wounding model is consistent with a ligand other than EGF as the primary mediator of wound healing.⁴ There are seven endogenous EGFR ligands.⁹ Four of these (EGF, TGF- α , HB-EGF, and amphiregulin) have been demonstrated to promote corneal wound healing.^{4,10} Further, TGF- α , but not EGF, is transcriptionally upregulated after corneal wounding.^{4,11}

A role for TGF- α in wound healing makes sense physiologically. TGF- α is more efficacious than EGF at promoting corneal wound healing,^{12,13} despite evidence that they stimulate the same receptor population and that they do so by way of the same molecular mechanism.¹⁴ The differences in efficacies are not a function of ligand binding; EGF and TGF- α bind the EGFR with comparable affinities (dissociation constant [K_d] = 5.7 nM and 4.6 nM, respectively).¹⁵ Thus, the mechanism of ligand activation of the receptor cannot account for the reported differences in signaling by these ligands.

In this study, we use primary and immortalized corneal epithelial cells to test the hypothesis that the differences in efficacies among EGFR ligands are a consequence of variations in cellular mechanisms for receptor inactivation. Ligand-mediated receptor desensitization is widely regarded as the primary method of EGFR inactivation.¹⁶ It has been well established that the EGF/EGFR complex is internalized by clathrin-coated pits on ligand binding. Once inside the cell, the EGF/EGFR moves through a series of defined endocytic compartments (early endosome to late endosome to lysosome) and ultimately results in degradation of the ligand and receptor.¹⁷ We postulated that in the human corneal epithelium, the endocytic trafficking of TGF- α /EGFR complex bypasses the degradation process. If this hypothesis is correct, after stimulation with TGF- α , the receptor would retain its activity longer or be able to undergo multiple rounds of stimulation. Identifying the molecular basis for the differences in ligand activity is the first step in developing strategies for accelerating and enhancing corneal wound healing.

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We found that, as has been reported in *ex vivo* models, TGF- α is a more potent activator of corneal epithelial cell migration than EGF. In addition, stimulation with EGF results in the internalization and degradation of the EGF/EGFR complex. In contrast, treatment with TGF- α promotes internalization of the EGFR but ultimately leads to recycling back to the plasma membrane. After treatment with TGF- α , the receptor can be restimulated with additional ligand. Therefore, the activity of the TGF- α -stimulated EGFR is enhanced because of the absence of receptor degradation and the ability of the receptor to be restimulated with ligand.

MATERIALS AND METHODS

Cell Lines

hTCEpi cells were obtained from Geron Corp. (Menlo Park, CA). Human corneal epithelial cells were immortalized by the stable transfection of human telomerase reverse transcriptase.¹⁸ Cells were grown in growth media (Defined Keratinocyte with growth supplement; Invitrogen, Carlsbad, CA) at 37°C and were maintained at 5% CO₂.

Primary human corneal epithelial cells were obtained from tissue that would otherwise have been discarded after corneal tissue transplantation from Donald Stone (Dean McGee Eye Institute, Oklahoma City, OK). Briefly, human limbal rims were rinsed in Dulbecco's-PBS (Invitrogen) containing 20 μ g/mL gentamicin (Invitrogen) for 2 to 3 minutes before they were cut into four pieces of equal size. This tissue was incubated in a dispase solution (25 caseinolytic U/mL dispase; Becton Dickinson Labware, Franklin Lakes, NJ) in Hanks' balanced salt solution with 5 μ g/mL gentamicin for 18 hours at 4°C. After incubation in the dispase solution, the epithelial layer was lifted from the stroma and digested in trypsin-EDTA solution (Invitrogen) for 5 minutes at 37°C. After 5 minutes, an equal volume of 10% FBS (Gemini Bio-Products, Inc., West Sacramento, CA) in PBS, pH 7.4, was added to neutralize the trypsin. Cells were pelleted at 1000 rpm for 5 minutes, the supernatant was removed, and the pellet was resuspended in growth media (Defined Keratinocyte Serum Free Media with growth supplement; Invitrogen) and was seeded onto a flask coated with serum-free tissue culture reagent (FNC Coating Mix; AthenaES, Baltimore, MD). Medium was changed every other day until cells reached 70% to 80% confluence.⁴ Cells were grown at 37°C and maintained at 5% CO₂. Use of human tissue adhered to the tenets of the Declaration of Helsinki.

Scratch Assay

Cell migration was assessed using a wound healing assay, as previously described.¹⁹ Briefly, hTCEpi cells were grown to confluence in 35-mm dishes that were marked to orient the region of interest. A 5-mm "wound" was made by displacing cells using a 1-mL pipette tip (TipOne; USA Scientific, Inc., Ocala, FL). Free cells were removed with two washes with PBS, and the media were replaced with either growth media or growth media supplemented with either EGF (10 ng/mL; ProSpec, Rehovot, Israel) or TGF- α (10 ng/mL; Biovision, Mountain View, CA). The wounded area was imaged using an inverted microscope (IX70; Olympus America, Inc., Center Valley, PA) and imaging software (QCapture Pro 6.0; QImaging, Surrey, BC, Canada) immediately after wounding and 24 hours later. Quantification of cell migration was determined by calculating the coverage of the wounded area with cells after 24 hours with or without ligand using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Transwell Migration Assays

Approximately 100,000 primary human corneal epithelial cells in serum-free media (Keratinocyte Defined Media without growth supplement, 100 μ L; Invitrogen) were plated in the upper chamber of an 8- μ m polycarbonate membrane, 6.5-mm insert (Corning, Inc., Corning,

NY). The lower chamber contained 600 μ L serum-free media containing the indicated concentrations of growth factor. Cells were allowed to migrate for 18 hours at 37°C in 5% CO₂. Migrated cells were determined by fixing the cells in methanol, staining in Giemsa, and counting the migrated cells under a microscope (TE-2000; Nikon, Tokyo, Japan) with a 60 $\times$ objective.²⁰

EGFR Degradation Assays

Confluent dishes of hTCEpi cells were treated with either EGF (10 ng/mL) or TGF- α (10 ng/mL) for the indicated amounts of time. At each time point, cell lysates were generated by washing the cells twice with PBS (pH 7.3), equilibrating the cells to 4°C, and solubilizing cells in lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0], 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethyl sulfonyl fluoride) on ice. Proteins were solubilized by rotating the lysis buffer/cell mix end over end for 10 minutes at 4°C; insoluble material was removed by microcentrifugation for 10 minutes at 4°C. The protein concentration of the solubilized protein was assessed by BCA assay (Pierce, Rockford, IL), and samples were diluted in SDS-sample buffer. Equivalent amounts of protein (indicated in the figure legends) were separated by SDS-PAGE, transferred to nitrocellulose, and detected using antibodies against the EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) or α -tubulin (Sigma-Aldrich, St. Louis, MO), followed by probing with a horseradish peroxidase-conjugated secondary antibody. Detected proteins were visualized by enhanced chemiluminescence using a UV Products (Carlsbad, NJ) imaging system.²¹

Radioligand Degradation/Secretion

Cells were incubated for 7.5 minutes with 1 ng/mL ¹²⁵I-EGF (Perkin Elmer, Waltham, MA) or ¹²⁵I-TGF- α (Bachem Americas, Inc., Torrance, CA) at 37°C in binding buffer (Dulbecco's modified Eagle's medium/20 mM HEPES/0.1% BSA, pH 7.3). Cells were washed four times in room temperature PBS to remove unbound radioligand. Prewarmed 37°C media were added to the cells, and they were returned to 37°C for the indicated periods of time. At each time point, the media were collected. Remaining cells were solubilized in 1% NP-40/20 mM Tris, pH 7.4. The radioactivity of each fraction was determined using a Beckman Coulter (Brea, CA) gamma counter.²² The percentage internalized was calculated as the amount of cell-associated radioactivity divided by the total (media and cell associated).

Confocal Fluorescence Microscopy

Cells were grown on coverslips, fixed in 4% p-formaldehyde/PBS (PBS, pH 7.4/0.5 mM CaCl₂, 0.5 mM MgCl₂), and permeabilized with 0.1% saponin/5% horse serum/PBS. After washing, cells were incubated for 1 hour with an anti-EGFR antibody (Ab-1; Calbiochem, Gibbstown, NJ). Cells were then incubated for 1 hour with an Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR), washed, and mounted on slides with reagent (Prolong Gold Antifade with DAPI; Molecular Probes, Eugene OR). Confocal images were collected using a confocal microscope (FV1000; Olympus America) and were analyzed using the appropriate software (Fluoview; Olympus America). Shown are representative images for each primary antibody scanned with identical confocal settings.²³

EGFR Restimulation Assays

hTCEpi cells were plated in a 60-mm dish containing five coverslips. Cells were treated with media supplemented with EGF (10 ng/mL) or TGF- α (10 ng/mL). Cells were incubated with ligand for 2 hours at 37°C. Cells were washed with PBS (pH 7.3) twice and returned to growth media (no ligands) at 37°C for an additional 4 hours. Cells were then restimulated with ligand for 15 minutes. At the indicated times, a coverslip was removed from the 60-mm dish, fixed, and processed for indirect immunofluorescence using an anti-EGFR antibody.

FIGURE 1. TGF- α is a more efficacious activator of primary corneal epithelial cell migration than EGF. Cell migration was assayed using a transwell assay. After incubation in the indicated amounts of ligand, primary human corneal epithelial cells were fixed and stained with Giemsa, and cells at the origin (top of membrane) were removed. The membrane was mounted on a microscope slide and visualized using light microscopy, and the number of migrated cells (bottom of membrane) was counted. Data are plotted as the average fold change in the number of migrated cells ($\pm$ SEM) for each ligand concentration ($n = 4$). * $P < 0.1$; ** $P < 0.05$ (paired Student's t -test).

RESULTS

It has been suggested that TGF- α is a more potent activator of corneal wound healing than EGF^{12,13}; the molecular mechanism that could account for this difference, however, has not been clear. Ex vivo models present a number of technical limitations that preclude dissection of any details. To overcome these obstacles, we turned to tissue culture models of the cornea that allowed us to examine the EGFR in individual cells and in whole populations of cells.

Our studies were initiated in primary human corneal epithelial cells. As a tissue culture model system, these cells have the least potential for undergoing modification. The limitations of primary cultures are that they are a scarce commodity and do not provide much material. Primary human corneal epithelial cells were obtained from tissue, using a protocol that has been described previously, that otherwise would have been discarded after corneal transplantation.⁴

We first wanted to know whether these cells responded to TGF- α and EGF, as had been reported in ex vivo models. We examined EGFR-mediated cell migration using a modified Boyden chamber assay in response to varying concentrations of TGF- α or EGF. Migration of the basement epithelial cells is the first step of corneal wound healing and is regarded by many as the rate-limiting step.

Stimulation with both EGF and TGF- α promotes cell migration (Fig. 1), with TGF- α stimulating cell migration at all concentrations to a greater extent than EGF. Cell migration occurs with a bell shaped dose-response curve that peaks at a concentration of 10 ng/mL for each ligand, which is an approximately 10-fold increase in what has been reported by others.¹² For all subsequent experiments, a ligand concentration of 10 ng/mL was used.

As a secondary approach and to overcome restrictions on the amounts of primary tissue we could obtain, we turned to an immortalized corneal epithelial cell line. Human corneal epithelial cells were stably transfected with the human telomerase reverse transcriptase (hTERT), which allows cells to maintain adequate telomere length through multiple rounds of

cell division. These immortalized cells, referred to as hTCEpi cells, have been extensively characterized to have a normal karyotype and cell cycle kinetics.¹⁸ These cells were used because they could provide sufficient material for the biochemical and cell biological analysis of EGFR signaling.

We used the scratch assay, a common in vitro assay to measure corneal epithelial cell migration.²⁴ To monitor cell migration, hTCEpi cells were grown to confluence and were wounded by dragging a 1-mL pipette tip through the confluent monolayer of cells. After removing any unattached cells by washing with PBS, the cells were incubated in media alone or in media supplemented with either EGF (10 ng/mL) or TGF- α (10 ng/mL). The wounded area was imaged immediately after wounding and again 24 hours later (Fig. 2A). The percentage of the original wound that was covered with cells after 24 hours was calculated for every condition. Compared with untreated cells, EGF produced a 50% increase in coverage of the wounded area, whereas TGF- α treatment yielded a 110% increase (Fig. 2B). Although this assay does not distinguish between the contribution of cell migration and that of proliferation, it does provide additional evidence that TGF- α is more efficacious than EGF in promoting at least one of the EGFR-mediated events in corneal epithelial cells.

EGF, but Not TGF- α , Causes EGFR Degradation

We next wanted to know the molecular basis for the magnitude in the EGF and TGF- α responses. It is well established that both EGF and TGF- α activate the EGFR by the same mechanism¹⁴ and that both ligands bind the receptor with comparable affinities.¹⁵ Therefore, we hypothesized that the differences in receptor signaling may be attributed to distinct mechanisms of EGFR inactivation. To test this hypothesis, we treated hTCEpi cells with either EGF or TGF- α for varying amounts of time (0–24 hours) and monitored the rate of EGFR degradation over time by immunoblot analysis (Fig. 3A). In both EGF- and TGF- α -treated cells, there is a slower migration of the EGFR, consistent with receptor phosphorylation after ligand binding. After treatment with EGF, there is a time-dependent decrease in the amount of detectable EGFR. This is most evident 6 and 24 hours after stimulation (Fig. 3B). In contrast, treatment with TGF- α over the same period does not show appreciable changes in the level of detectable EGFR.

This experiment was conducted in the absence of protein synthesis inhibitors to accurately mimic the naturally occurring conditions of the cornea. Therefore, because of the continual synthesis of new receptors, the rate of TGF- α -mediated EGFR degradation may be underestimated. Nevertheless, over the course of 24 hours, the rate of EGFR degradation in hTCEpi cells is much faster after treatment with EGF than with TGF- α .

TGF- α Causes the EGFR to Internalize and Recycle Back to the Plasma Membrane

As a secondary approach for analyzing the endocytic trafficking of the EGFR, we monitored the movement of the ligand/receptor complex using radioligands. By incubating cells with either ¹²⁵I-EGF or ¹²⁵I-TGF- α , we were able to assess the amount of EGF/EGFR and TGF- α /EGFR inside the cell. If the rates of ligand internalization, degradation, and recycling were similar, that would be consistent with identical trafficking itineraries. Alternatively, differences in the amount of time the radioligand remained in the cell would provide additional evidence of an altered route of trafficking.

After incubation with either ¹²⁵I-EGF or ¹²⁵I-TGF- α , a comparable percentage of cell-associated radioligand was intracellular (Fig. 4). When cells were incubated with ¹²⁵I-EGF, 50% of the internalized radioligand remained associated with the cell after 100 minutes. In contrast, when the cells were treated

FIGURE 2. TGF- α is a more efficacious activator of in vitro hTCEpi cell wound healing than EGF. (A) Confluent dishes of hTCEpi cells were wounded with a 1-mL pipette, washed with PBS, and incubated in either growth medium alone or growth medium supplemented with EGF (10 ng/mL) or TGF- α (10 ng/mL). Images were collected at 0 hours and 24 hours. (B) Quantification of cell migration from experiments as in (A). Data are presented as the percentage of coverage of the initially wounded area (average $\pm$ SEM; $n = 3$ each experiment performed in quadruplicate). All three conditions are statistically different from one another ($P < 0.0001$) when analyzed by repeated-measures ANOVA.

with ^{125}I -TGF- α , within 50 minutes only 50% of the internalized radioligand remained intracellular. The more rapid removal of radioligand from the cell and the slowed rate of receptor degradation are consistent with the notion that TGF- α promotes EGFR recycling and that EGF causes EGFR degradation.

To more clearly examine the EGFR endocytic trafficking itinerary in individual cells, we used indirect immunofluorescence to monitor the intracellular localization of the EGFR (Fig. 5). Primary corneal epithelial cells were treated with either

FIGURE 3. Stimulation of hTCEpi cells with EGF, but not TGF- α , promotes EGFR degradation. Dishes of hTCEpi cells were treated with either EGF (10 ng/mL) or TGF- α (10 ng/mL) for the indicated periods of time. At each time point, cell lysates (55 $\mu\text{g}/\text{lane}$) were prepared and resolved by 7.5% SDS-PAGE and were immunoblotted with antibodies against EGFR or α -tubulin as a loading control. (A) Shown is a representative experiment repeated three times. (B) Quantification of three experiments as shown in (A). Data were normalized to EGFR levels at time 0 minutes. Data are plotted as the average $\pm$ SEM. $**P < 0.05$ (paired Student's *t*-test).

EGF or TGF- α , and the subcellular distribution of the EGFR was monitored by indirect immunofluorescence. Shown are confocal micrographs collected from the center (along the *z*-axis) of each section from primary corneal epithelial cells (Fig. 5). Similar results were observed when the hTCEpi cells were used (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/51/7/3455/DC1>).

FIGURE 4. ^{125}I -TGF- α /EGFR and ^{125}I -EGF/EGFR have different kinetics of trafficking. hTCEpi cells were incubated with either ^{125}I -EGF or ^{125}I -TGF- α for 7.5 minutes and were chased with radioligand-free media for the indicated times. At each time point, the media and solubilized cells were collected and assayed for the associated radioactivity. Data are plotted as the percentage of ^{125}I that is intracellular at each time point (average $\pm$ SEM; $n = 3$). $**P < 0.05$ (Student's *t*-test).

Primary Human Corneal Epithelial Cells IF: EGFR

FIGURE 5. TGF- α promotes receptor recycling. Primary human corneal epithelial cells were incubated with either EGF (10 ng/mL) or TGF- α (10 ng/mL) for the indicated amounts of time. Cells were fixed and subjected to immunofluorescence with an anti-EGFR antibody and Alexa 488-conjugated goat anti-mouse secondary antibody (green). Nuclei were stained with DAPI (blue). Images were collected on a confocal microscope (FV1000; Olympus America) and were analyzed with software (Fluoview; Olympus America). Scale bar, 10 μ m. Shown are representative images of an experiment repeated three times.

In cells that have not been stimulated (with either ligand), the distribution of the EGFR (shown in green) is along the plasma membrane of the cell. Once either EGF or TGF- α has been added for 15 minutes, there is a dramatic redistribution of the receptor from the cell surface to intracellular vesicles. In cells that have been treated with EGF, the staining of the EGFR remains punctate and becomes increasingly perinuclear over time (Fig. 5, upper panels). With time, the intensity of EGFR staining decreases and is consistent with lysosomal degradation of the EGFR.

In cells that have been treated with TGF- α , after the initial localization of the receptor to endosomes, there is a time-dependent appearance of the EGFR back to the plasma membrane (Fig. 5, lower panels). The amount of plasma membrane staining of the EGFR increases over time. Twenty-four hours after treatment with TGF- α , the distribution of EGFRs is indistinguishable from that of untreated cells.

It is important to note that though, in principle, this experiment was similar to the immunoblot-based analysis of EGFR degradation, the assay provided different readouts that precluded their direct comparison. The more sensitive immunofluorescence assay used in Figure 5 indicates the distribution of the immunoreactive protein (the EGFR) present in the cell; the assay does not distinguish whether the immunoreactive protein is the native full-length receptor or a partially degraded fragment. In contrast, the immunoblot assay used in Figure 3 indicates whether there is a 180-kDa immunoreactive protein. As a result, the loss of the immunoreactive band in Figure 3 does not kinetically overlap with the diminished immunofluorescent signaling seen in Figure 5. Both sets of data indicate that stimulation with EGF promotes more rapid degradation than TGF- α .

After Recycling to Plasma Membrane, EGFRs Can Be Restimulated

Finally, we wanted to know whether the EGFR that reappears at the cell surface after TGF- α -mediated internalization is capable of restimulation. If so, this would provide strong data that there is a physiological consequence to EGFR recycling. To test this idea, we monitored the localization of the EGFR after pulsing the cells with TGF- α .

Cells were incubated in media alone or in media supplemented with TGF- α (10 ng/mL) for 2 hours to induce internalization. External ligand was removed from the media by a series of washes, and the cells were incubated in media without ligand. After 4 more hours (6 hours total), cells were reincubated with TGF- α (10 ng/mL) for 15 minutes (Fig. 6A). Cells were collected at various times during this treatment, and the localization of the EGFR was monitored by indirect immunofluorescence (Fig. 6B). As predicted by our previous data, the EGFR internalized and recycled back to the plasma membrane after TGF- α treatment. When those cells were retreated with TGF- α , the EGFR became internalized in endosomes (Fig. 6; 6.25 hours). In cells that were pulsed with EGF, the EGFR remained intracellular after washout, and restimulation with EGF had no effect on EGFR distribution (data not shown).

Using this system of introducing pulses of TGF- α to the cells, we were able to monitor EGFR trafficking. These data are consistent with the ability of TGF- α to stimulate EGFRs multiple times, whereas cells treated with EGF were only able to be activated once because the receptor was retained in the cell and then degraded.

DISCUSSION

Corneal wound healing and tissue homeostasis are critical to the maintenance of a healthy eye. It has been established using *in vivo* models that TGF- α is a potent ligand for promoting EGFR-mediated corneal epithelial growth after wounding of the tissue.^{4,10,13} Here we show, using tissue culture models, that the critical first step in corneal wound healing—cell migration—is more potently activated by TGF- α than EGF. In these studies, we identify the endocytic trafficking itinerary as the critical regulatory process that confers this difference. As indicated by the radioligand trafficking data, receptor degradation studies, and indirect immunofluorescence, stimulation with EGF promotes EGFR degradation. In contrast, when cells are stimulated with TGF- α , the ligand/receptor complex internalizes but recycles to the plasma membrane. At the plasma membrane, the EGFR can bind ligand and be activated. Thus, the difference in ligand efficacy can be accounted for by the duration and frequency of receptor stimulation.

A. Schematic of Treatment Schedule

B. IF: EGFR

Although the exact mechanism by which these ligands confer different routes of EGFR endocytic trafficking is unknown, one strong candidate rests in the binding properties of EGF compared with those of TGF-α. It has been shown in other systems that dissociation of TGF-α from the EGFR is more sensitive to pH than EGF dissociation. The pH at which 50% of the maximally bound TGF-α dissociates from the receptor has been reported to be 6.83; for EGF, 50% of the ligand is dissociated at pH 5.89.²⁵ Given that early endosomes have a pH of approximately 6.8,²⁶ it is likely that TGF-α dissociates from the EGFR. On ligand dissociation, the receptor's kinase becomes inactive and the receptor no longer is able to sustain tyrosine phosphorylation and communication with downstream effectors. These dephosphorylated receptors recycle back to the plasma membrane²⁷ (Fig. 7).

Although the model that TGF-α promotes multiple rounds of EGFR stimulation is attractive, one cannot rule out other

models. For instance, it has been shown in other cell lines that the endocytic pathway also provides spatial regulation of EGFR signaling.^{28,29} If the model of spatial regulation is correct, enhanced signaling by the TGF-α/EGFR complex would be the result of the liganded EGFR in the early endosome rather than of the EGF/EGFR complex. If true, this model also supports a role for the ligand-mediated itinerary of endocytic trafficking as a key determinant in ligand efficacy.

These data support the notion that manipulation of the endocytic pathway may be an effective mechanism for enhancing EGFR-mediated corneal wound healing. The use of ligands that cannot internalize or undergo degradation may provide a therapeutic strategy for reepithelialization of a wounded corneal epithelium. Alternatively, if the activities of proteins (rab5, tsg101, rab7) that regulate movement of the EGFR through the endocytic pathway are blocked, wound healing may occur more rapidly. However, as mentioned, the endocytic location

FIGURE 7. Model for the differences in endocytic trafficking with EGF and TGF-α. After stimulation with either EGF or TGF-α, the ligand receptor internalizes by way of clathrin-coated pits. Once inside the cell, the clathrin-coated vesicle sheds its clathrin, and the cargo is delivered to the early endosome. In the more acidic early endosome, TGF-α more readily dissociates from the EGFR than does EGF, thus providing the basis for differential trafficking. The unliganded receptor recycles back to the plasma membrane, where it can be restimulated. In contrast, the EGF/EGFR complex remains intact in the early endosome and continues to traffic through late endosomes/multivesicular bodies and to the lysosome for degradation.

of the EGFR may provide the necessary specificity for coupling to and activating effectors. Thus, simply disrupting the trafficking of the receptor may not be sufficient to promote the necessary signals for wound healing. Deciphering whether EGFR signaling is enhanced by spatial placement of the receptor or prolonging receptor activity is challenging. However, making these distinctions will prove to be important for designing therapeutic strategies that promote corneal wound healing.

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