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ERK/p44p42 Mitogen-Activated Protein Kinase Mediates EGF-Stimulated Proliferation of Conjunctival Goblet Cells in Culture

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Abstract

Purpose—To determine whether activation of the ERK pathway by EGF leads to rat and human goblet cell proliferation.

Methods—The conjunctiva was removed from male Sprague-Dawley rats. Human conjunctiva was removed during ocular surgery. The tissue was minced and goblet cells were grown. The cells were stimulated with EGF (10^{-7} M) for 1 and 5 minutes and Western blot analysis was performed with an antibody against phosphorylated EGFR, to measure the activation of the EGF receptor (EGFR). The cells were incubated with EGF (10^{-7} M) for 24 hours, and cell proliferation was measured by WST-8. Inhibitors were added either 20 minutes before EGF or 2 hours after. The cells were stimulated with EGF (10^{-7} M) for 1 minute to 24 hours. The number of cells expressing phosphorylated ERK (pERK) in the nucleus and Ki-67 was determined by immunofluorescence.

Results—EGF increased the activation of EGFR in rat conjunctival goblet cells. EGF-stimulated proliferation was inhibited by the EGFR inhibitor AG1478 and the MEK inhibitor U0126 in rat and human cultured goblet cells. EGF caused the translocation of pERK to the nucleus in a biphasic manner. Inhibition of the second peak with U0126 prevented proliferation. EGF-stimulated goblet cells progressed through the cell cycle expressing pERK in the nucleus.

Conclusions—EGF stimulated human and rat conjunctival goblet cell proliferation by activating the EGFR. EGFR stimulated ERK causing its biphasic translocation to the nucleus. The second peak response is responsible for cell proliferation, but the role of the first peak is not known.

Goblet cells are polarized secretory cells that are located in the conjunctival epithelium. They synthesize and secrete the large soluble mucin, MUC5AC, into the tear film forming the inner mucous layer.¹ This tear film layer provides a physical and chemical barrier between the ocular surface (cornea and conjunctiva) and the external environment, thereby protecting it from the constantly changing external environment. The mucous layer is also critical for maintaining the health of the ocular surface. A decrease in the number of goblet cells in the conjunctiva or an inability of the goblet cells to produce mucin is associated with pathologic abnormalities of the ocular surface such as neurotrophic keratitis, dry eye syndromes, ocular cicatricial pemphigoid, vitamin A deficiency, and Stevens-Johnson syndrome.² On the other hand, an overproduction of mucus is associated with diseases such as atopy, mucus fishing syndrome, and seasonal allergic conjunctivitis.³ Both an increase and a decrease in goblet cell mucin production occurs with ocular surface disease implying that there is an optimal amount of mucin production and suggesting that goblet cell mucin production is tightly regulated.

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Four different processes contribute to goblet cell mucin production: (1) the rate and amount of mucin secretion, (2) the rate of mucin synthesis, (3) the number of goblet cells present in the conjunctiva, and (4) the rate of mucin degradation. There is almost no information about the regulation of MUC5AC synthesis or the rate of mucin degradation. In contrast, the regulation of goblet cell secretion and proliferation has been addressed in several studies. Measurement of conjunctival goblet cell mucin secretion in rats and humans indicates that cholinergic agonists using M₂ and M₃ muscarinic receptors stimulate goblet cell secretion.⁴ Activation of these receptors increases intracellular [Ca²⁺] and activates protein kinase C isoforms. Intracellular [Ca²⁺] and protein kinase C isoforms activate the nonreceptor tyrosine kinases PYK2 and Src to transactivate the EGF receptor (EGFR).⁵ This transactivation causes phosphorylation of the homo- and heterodimerized EGF family of receptors. The phosphorylated EGFR activates the extracellular-related kinase (ERK 1/2, also known as p44/p42 mitogen-activated protein kinase [MAPK]) pathway to induce secretion. The MAPK pathway includes attraction of the adapter proteins Grb2 and Shc to the EGFR that induce SOS. SOS is a guanine nucleotide exchange factor that activates Ras. Ras then stimulates Raf (MAPK kinase kinase) and then MEK (MAPK kinase) which phosphorylates ERK 1/2. To cause secretion, ERK 1/2 remains in the cytosol and interacts with as yet unidentified target proteins.

Recent studies have begun to address the complex regulation of conjunctival goblet cell proliferation. In vitro experiments using rat conjunctival goblet cells in culture demonstrated that EGF and its family members, transforming growth factor (TGF) β and heparin-binding (HB)EGF, but not heregulin, stimulate goblet cell proliferation.⁶ Growth factors that increase proliferation bind to the EGFR implicating its participation in proliferation as well. In vivo evidence also suggests that EGF regulates conjunctival goblet cell proliferation. In Sjögren's syndrome, the autoimmune form of dry eye, the EGF concentration in tears is decreased and correlates with a decrease in the number of conjunctival goblet cells.⁷ In addition to effects on the conjunctiva, it well established that EGF and its family members stimulate proliferation in a variety of cell types.⁸⁻¹⁰

Epithelial cells are the major source of EGF in tears and other body fluids. In almost all epithelia, except the submandibular gland, the EGF family members are membrane-spanning proteins. Membrane-spanning EGF is present as a 170-kDa proform that contains the 6-kDa EGF-like active fragment.¹¹ Similarly, the lacrimal gland acinar and duct cells as well as the stratified squamous cells of the cornea contain membrane-spanning EGF.^{12,13} This form of EGF can be released from cells, by the action of metalloproteinases, as a 150-kDa precursor form.¹⁴ Release of EGF by this mechanism is known as ectodomain shedding. The precursor form can be further cleaved to release the 6-kDa active motif. All three forms of EGF (170, 150, and 6 kDa) are potentially active. TGF α and HB-EGF are similarly released by ectodomain shedding.^{15,16}

The sources of EGF that could stimulate conjunctival goblet cell proliferation are the lacrimal gland, the conjunctival epithelium (stratified squamous and goblet cells), and the corneal epithelium. EGF was detected in both the basal and apical membranes of lacrimal gland acinar and duct cells.¹² EGF released from the apical membrane would enter the tear film and be available to interact with goblet cells. EGF mRNA is present in corneal epithelial cells as well as in the stratified squamous and goblet cells of the conjunctiva.^{3,6,17,18} It is not known whether these cells can release EGF. However, HB-EGF can be released from the cornea and plays a role in corneal wound healing.¹⁹

Binding of EGF to the EGFR leads to homo- and heterodimerization and subsequent phosphorylation of multiple tyrosine residues on the C-terminal domain. Phosphotyrosines serve as docking sites for adapter proteins that are in turn phosphorylated. There are five main autophosphorylation sites: Y992, which binds phospholipase C (PLC) γ 1; Y1046 which binds

Cbl; Y1068 and Y1086 which bind GRB2 and GAB1, respectively; Y1148 which binds SHC; and Y1173 which binds SHP1, SHC, and PLC γ 1.²⁰⁻²² Attraction of each adapter protein leads to activation of specific signaling pathways. As examples, PLC γ 1 uses the Ca²⁺ and PKC pathway, GRB2 and SHC activate the ERK signaling cascade, and the GRB2/GAB1 site stimulates the PI-3K/AKT pathway.²³ Stimulation of these signaling pathways involves translocation of a signaling component into the nucleus where they differentially activate transcription factors. EGF stimulation of these signaling pathways leads to proliferation, migration, differentiation, and tumor formation.²⁴

Using multiplex technology, we found that a 5-minute stimulation with EGF activated ERK, p38 MAPK, and JNK, but not AKT⁶ in conjunctival goblet cells. Thus, in the present study, we began characterizing the ERK pathway activated by EGF in cultured conjunctival goblet cells and found that EGF transactivates the EGFR to activate the ERK signaling cascade, which translocates ERK to the nucleus where it initiates goblet cell proliferation.

Materials and Methods

EGF was purchased from PeproTech, Inc. (Rocky Hill, NJ), U0126 from EMD Biosciences (San Diego, CA), cell proliferation reagent WST-8 from Dojindo Molecular Technologies (Gaithersburg, MD), and *Ulex europaeus* agglutinin (UEA-1) lectin conjugated to FITC from Pierce (Rockford, IL). All other reagents were obtained from Sigma-Aldrich (St. Louis MO).

Antibodies

To detect the EGFR, we used a rabbit polyclonal antibody to the human EGFR receptor in Western blot experiments (Santa Cruz Biotechnology, Santa Cruz, CA). To detect the phosphorylation of the EGFR, we used a rabbit polyclonal antibody that recognizes the phosphotyrosine Y1068, the docking site for GRB2 and GAB1. This antibody was used in conjunction with an antibody that recognizes the phosphorylated and nonphosphorylated forms (Cell Signaling Technology, Inc., Danvers, MA). Mouse monoclonal antibodies generated against ERK1 (p42 MAPK) and phosphorylated ERK1 and -2 (p42/p44 MAPK), which specifically reacts with Tyr-204 of phosphorylated ERK1 and -2, were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against phosphorylated ERK (pERK) was used in immunofluorescence microscopy experiments and was purchased from Cell Signaling Technology. Antibody to Ki67 was purchased from Vector Laboratories (Burlingame, CA). The secondary antibodies used for immunofluorescence microscopy were Cy2 or Cy3 conjugated to mouse or rabbit IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blot analysis were from Santa Cruz Biotechnology.

Animals

Male Sprague-Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY). The rats were anesthetized with CO₂ for 1 minute and decapitated, and the nictitating membranes and conjunctival fornix were removed from both eyes and minced. The procedure for removal of the conjunctiva was in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Schepens Eye Research Institute (SERI) Animal Care and Use Committee.

Isolation and Culture of Goblet Cells

Human conjunctival tissue was obtained from patients during ocular surgery in a protocol that adhered to the tenets of the Declaration of Helsinki and was approved by both the Massachusetts Eye and Ear Infirmary and the SERI Human Subjects Internal Review Board. The tissue, which is normally discarded during surgery, was donated by seven patients (five males, two females;

age range, 35-86 years; average age, 68 years), and removed after informed consent. The diagnoses for the patients were retinal detachment (four patients), vitrectomy for age-related macular degeneration with subretinal hemorrhage, vitrectomy for aqueous misdirection, and vitrectomy for nonclearing vitreous hemorrhage. The tissue was immediately placed in 1× PBS consisting of 3× (300 μ g/mL) penicillin-streptomycin.

Goblet cells from rat and human conjunctiva were grown in organ culture as described previously.^{25,26} In brief, minced pieces of conjunctiva were placed in culture with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 μ g/mL penicillin-streptomycin. After nodules of cells were observed, the tissue plug was removed, and goblet cells were allowed to grow from the nodules. As described previously,^{25,26} cells were identified as goblet cells by the following characteristics: (1) morphology as visualized by light microscopy, (2) positive staining with the lectin UEA-1 and antibody to cytokeratin 7 viewed by immunofluorescence microscopy, and (3) negative staining for the stratified squamous cell marker the lectin *Griffonia (Bandeiraea) simplicifolia* lectin I and antibody to cytokeratin 4 viewed by immunofluorescence microscopy. First-passage goblet cells were used in all experiments.

Western Blot Analysis of EGFR Protein

To measure the activation of the EGFR, the cells were stimulated by EGF (10^{-7} M) for 1 and 5 minutes. The incubation was terminated by addition of homogenization buffer (30 mM Tris-HCl [pH 7.5], 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose) containing protease inhibitors (phenylmethylsulfonyl fluoride 100 μ L/mL, aprotinin 30 μ m/mL, and sodium orthovanadate 100 nM). The cells were scraped, and the samples were sonicated and centrifuged at 2000g for 15 minutes at 4°C. The supernatant was then centrifuged at 100,000g for 1 hour at 4°C. Proteins in the pellet were resuspended in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA), containing protease inhibitors (phenylmethylsulfonyl fluoride 100 μ L/mL, aprotinin 30 μ m/mL, and sodium orthovanadate 100 nM) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked overnight at 4°C in 5% nonfat dried milk in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20 (TBST) and then incubated with the primary antibody for 1 hour at room temperature or overnight at 4°C followed by incubation with an HRP-conjugated secondary antibody. Immunoreactive bands were detected by the enhanced chemiluminescence method and analyzed by NIH ImageJ (available by ftp at zippy.nimh.nih.gov/ or at <http://rsb.info.nih.gov/nih-image>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Values for phosphorylated EGFR were normalized to the amount of total EGFR and were compared to the control value, which was set at 1.

Immunohistochemical Analysis of Goblet Cell pERK Location and Entry into the Cell Cycle

To detect cellular location of pERK and to compare the time dependency of pERK location and entry into the cell cycle, primary cultures of rat conjunctival goblet cells were trypsinized, seeded onto glass coverslips, and grown to 75% confluence in RPMI medium containing 10% FBS. The cells were serum starved for 18 hours in RPMI medium containing 0.5% BSA. EGF at 10^{-7} M was added at various times from 0 to 24 hours. FBS (10%) or 0.5% BSA in RPMI (basal) were added for 24 hours. Incubation was terminated by removal of media and immediately fixing the cells with ice cold 4% paraformaldehyde. Fixed cells were incubated for 1.5 to 2 hours with anti-pERK or anti-Ki-67 antibodies. Ki-67-labeling of nuclei indicates cells that have entered into the cell cycle. The cells were incubated for 1 hour with the secondary

antibodies, conjugated to either Cy2 or Cy3. Coverslips were mounted on glass slides by using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), which stains all cell nuclei independent of cell-cycle stage. For the negative control, the primary antibody was omitted. The cells were viewed with a microscope (Eclipse E80i; Nikon, Tokyo, Japan) with a digital camera (SPOT Diagnostic Instruments Inc., Sterling, MI). DAPI-stained nuclei were counted to determine the total number of cells. The number of Ki67-labeled cells or the number of cells containing pERK in the nucleus and the total number of cells were counted in five fields per chamber. The number of goblet cells having entered the cell cycle or having pERK present in the nucleus was expressed as a percentage of the total number of cells.

Cells stimulated with EGF for 16, 18, and 24 hours were further analyzed to determine the stage of the cell cycle based on the pattern of Ki-67 staining and the presence of pERK in the nucleus.^{27,28}

Effect of Inhibition of ERK on Goblet Cell Proliferation

Goblet cells were serum starved in RPMI-1640 medium supplemented with 0.5% BSA for 24 hours. To determine the effect of pERK on EGF-stimulated goblet cell entry into the cell cycle, U0126 (10^{-7} M) was added 20 minutes before addition of EGF (10^{-7} M) for 24 hours. FBS (10%) was the positive control and was added for 24 hours. In select experiments, U0126 (10^{-7} M) was added 2 hours after the start of stimulation. Incubation was terminated and the cells fixed by addition of cold 100% methanol. Fixed cells were incubated for 1.5 to 2 hours at room temperature with an anti-Ki67 antibody. Slides were mounted in mounting medium containing DAPI, and the total number of cells and the number of cells expressing Ki-67 was determined by counting cells in five fields.

Measurement of Goblet Cell Proliferation with a WST-8 Assay

Human and rat conjunctival goblet cells in primary culture were trypsinized and seeded on 96-well culture plates at a density of 200 cells per well. The cells were grown for 24 hours to subconfluence. After 24 hours of serum starvation, the inhibitors U0126 or AG1478 at 10^{-8} to 10^{-5} M, or no additives were added for 20 minutes followed by RPMI with 0.5% BSA (basal) or EGF (10^{-7} M) for 24 hours. Incubation was stopped by the removal of supernatant and cell proliferation was determined by the WST-8 assay, a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenases in viable cells. The absorbance was then read at 465 nm after 60 minutes of incubation at 37°C.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Data are analyzed by Student's *t*-test. $P < 0.005$ is considered statistically significant.

Results

Effect of EGF on EGFR Activity and Cell Proliferation in Rat Conjunctival Goblet Cells

Using Western blot analysis and fluorescence immunohistochemistry, we previously showed that the EGFR was present in rat goblet cells both in conjunctival tissue and in culture.⁶ To determine whether EGF activates the EGFR in cultured conjunctival goblet cells, these cells were stimulated with EGF (10^{-7} M) for 1 and 5 minutes, and the amount of phosphorylated EGFR was compared to total EGFR by using an antibody specific for Y1068, the docking site for GRB2 and GAB1. EGF significantly increased phosphorylation of the EGFR by 28.6 ± 7.6 - and 29.2 ± 3.2 -fold at 1 and 5 minutes, respectively (Fig. 1). Rapid EGF activation of the

EGFR, the initial step in the signaling pathway, is present and operative in conjunctival goblet cells.

The effect of EGFR activation on rat goblet cell proliferation was determined with the WST-8 assay by preincubating cultured cells with the EGFR antagonist AG1478 (10^{-7} M) for 20 minutes followed by addition of EGF (10^{-7} M) for 24 hours. EGF alone significantly stimulated goblet cell proliferation 4.9 ± 1.8 -fold (Fig. 2) compared with basal. AG1478 significantly inhibited the EGF response by $87\% \pm 8\%$. AG1478 alone did not significantly increase proliferation compared with the basal level. Thus, goblet cell proliferation is induced by EGFR activation.

Translocation of pERK to the Nuclei of Rat Conjunctival Goblet Cells

We had demonstrated that EGF activates ERK 1/2 in cultured rat conjunctival goblet cells in a concentration- and time-dependent manner.^{6,29} To determine whether EGF causes the translocation of pERK to the nucleus, where it would activate genes controlling proliferation, rat conjunctival goblet cells were stimulated with EGF for increasing times from 0 to 24 hours, and the number of cells with pERK present in the nucleus was quantified. In parallel, the number of cells that had entered the cell cycle, as indicated by Ki-67 staining, was also counted. FBS (10%) incubated for 24 hours served as the positive control. At time 0, pERK (shown in green) was detected in the cytoplasm of goblet cells (Fig. 3A). With 1 minute of EGF stimulation, pERK was detected in the cell nuclei of goblet cells (Fig. 3B). The number of goblet cells with pERK in the nucleus decreased at 5 minutes of stimulation (Fig. 3C). Despite the early activation of pERK, goblet cells did not begin to enter the cell cycle (shown in red) until 16 hours of stimulation at which time there was minimal pERK in cell nuclei (Fig. 3D). At 18 hours of stimulation, there was a second peak of pERK translocation to the nucleus that coincided with a peak in cellular proliferation (Fig. 3E) that was sustained at 24 hours (Fig. 3F). As a positive control FBS at 24 hours induced substantial goblet cell proliferation as well as pERK translocation to multiple cells (Fig. 3G). EGF induced pERK translocation to the nucleus and stimulation of entry into the cell cycle was quantified in six experiments (Fig. 4). EGF induced two peaks of pERK translocation to the nucleus: a major peak at 1 minute of stimulation that returned to almost basal levels and a second minor peak at 18 hours of stimulation. Goblet cell proliferation did not occur until approximately 16 hours of stimulation, peaked at 18 hours, and remained elevated at 24 hours. Thus, EGF translocates pERK to the nucleus with two peaks of activity: a rapid peak and a later sustained peak that corresponds with entry of cells into the cell cycle.

In parallel, cells were also stained with the lectin UEA-1 to ensure that the cells entering the cell cycle were indeed goblet cells. As shown in Figure 5, all cells were stained with UEA-1 (shown in green) and the majority of cells had entered the cell cycle, as determined by expression of Ki-67 (shown in red).

Comparison of the Number of Cells with Translocated pERK and Entry into Cell Cycle Stages

Rat conjunctival goblet cells were stimulated with EGF for 16, 18, and 24 hours, the times at which cell proliferation had just begun, was intermediate, or was near completion, respectively. The percentage of cells that had entered the specific phases of the cell cycle was determined. Subsequently, the percentage of cells in each phase of the cell cycle that contained pERK translocated to the nucleus was measured. At 16 hours, $55\% \pm 12\%$ of cells were in the G₁ phase with smaller percentages of cells in the S, G₂, and M phases (Fig. 6A). At this time $46\% \pm 23\%$ of cells in G₁ contained translocated pERK. As the cells progressed farther into the cell cycle, a smaller percentage of proliferating cells contained pERK, until at the M phase no proliferating cells contained pERK. At 18 hours of stimulation, more cells were in each stage of the cell cycle compared to 16 hours (Fig. 6B). A larger percentage of cells in the G₁ phase,

60% $\pm$ 21%, contained translocated pERK than at 16 hours. The percentage of cells in the M phase containing pERK in the nucleus increased to 9% $\pm$ 9% compared with 0% of cells at 16 hours. At 24 hours of stimulation, the percentage of cells in G₁ decreased to 27% $\pm$ 7%, whereas 18% $\pm$ 4%, 32% $\pm$ 2%, and 25% $\pm$ 4% of the cells were in S, G₂, and M, respectively. These levels indicate that the cells were progressing through the cell cycle and dividing (Fig. 6C). At this time, fewer cells in the G₁ and S phases contained translocated pERK (32% $\pm$ 9% and 31% $\pm$ 6%), but more cells in the G₂ and M phases (20% $\pm$ 6% and 17% $\pm$ 10%, respectively) contained translocated pERK compared with 16 and 18 hours. Thus, as cells enter the cell cycle, a larger portion of them contain pERK translocated to the nucleus and activated. As stimulation with EGF is prolonged, a larger percentage of cells with activated ERK in the nucleus is observed. These findings are consistent with a major role for nuclear, activated ERK in EGF stimulation of goblet cell proliferation.

Role of ERK in EGF-Stimulated Rat Goblet Cell Proliferation

We used the MEK inhibitor U0126 to determine whether EGF activation of ERK stimulates goblet cell proliferation. Rat conjunctival goblet cells were preincubated with U0126 for 20 minutes, and then stimulated with EGF (10⁻⁷ M) for 24 hours. Proliferation was measured by the WST-8 assay. Compared with basal conditions, EGF significantly stimulated goblet cell proliferation by 3.3 $\pm$ 0.9-fold above basal (data not shown). In the positive control cells, 10% FBS stimulated proliferation by 2.4 $\pm$ 0.2-fold (data not shown). Inhibition of ERK blocked EGF-induced goblet cell proliferation in a concentration-dependent manner with maximum inhibition of 76% $\pm$ 22% obtained with 10⁻⁶ M U0126. U0126 also significantly inhibited proliferation by 53% $\pm$ 17% and 34% $\pm$ 13% at 10⁻⁶ and 10⁻⁷ M, respectively (Fig. 7A). U0126 by itself at all three concentrations used did not alter cell proliferation (data not shown).

We next compared the effect of adding U0126 before and after the first peak of pERK translocation to the nucleus on EGF-stimulated proliferation. To ensure that U0126 inhibited the second peak of pERK translocation to the nucleus, U0126 (10⁻⁷ M) was added 2 hours after the addition of EGF (10⁻⁷ M) and the location of pERK was determined 24 hours later by immunofluorescence techniques. As shown in Figure 7B, pERK was located in the nucleus in cells treated with EGF. This translocation was inhibited by U0126 (Fig. 7B). We then used U0126 added before or 2 hours after EGF.

The Ki-67 method was used in these experiments as it gave substantial stimulation by EGF and confirmed the results obtained with the WST-8 method. EGF (10⁻⁷ M) significantly stimulated goblet cell entry into the cell cycle by 13 $\pm$ 4-fold over basal conditions (Fig. 8A). U0126 itself did not alter goblet cell entry into the cell cycle. U0126 added before EGF completely inhibited goblet cell entry into the cell cycle (97% $\pm$ 1%; Fig. 8A). The positive control (10% FBS) stimulated goblet cell proliferation 47 $\pm$ 7-fold (data not shown). In a separate set of experiments U0126 was added 2 hours after EGF allowing stimulation of the first peak of ERK activation, but before the second peak (Fig. 8B). U0126 itself did not alter the entry of goblet cells into the cell cycle. U0126 added after 2 hours of stimulation also completely blocked the effect of EGF on goblet cell proliferation (Fig. 8B). Inhibition of both peaks of ERK activation (Fig. 8A) or prevention of the second peak (Fig. 8B), completely blocked EGF stimulated entry of goblet cells into the cell cycle. The second peak of ERK activation is thus necessary for goblet cell proliferation. We could not block the first peak of ERK activation, leaving the second peak intact, as U0126 is an irreversible MEK inhibitor.

Role of ERK EGF-Stimulated Human Goblet Cell Proliferation

To determine whether EGF stimulates human goblet cell proliferation, activates the EGFR, and utilizes ERK, goblet cells were grown from pieces of conjunctiva removed at the time of surgery. EGF used at 10⁻⁷ M was present for 24 hours. Under these conditions, proliferation

was stimulated 1.9 ± 0.3 -fold compared with basal conditions (data not shown). This stimulation was similar to that obtained in rat goblet cells. The EGFR inhibitor AG1478 blocked proliferation by $69\% \pm 19\%$, $75\% \pm 15\%$, $85\% \pm 11\%$, and $93\% \pm 6\%$ at 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M, respectively (Fig. 9). Inhibition was significant at all concentrations of AG1478. AG1478 by itself did not significantly stimulate proliferation. In these experiments, the positive control FBS at 10% increased proliferation 3.9 ± 0.4 -fold (data not shown).

In similar experiments, proliferation of conjunctival goblet cells incubated with EGF (10^{-7} M) for 24 hours increased 3.3 ± 0.9 -fold compared with basal conditions (data not shown). The MEK inhibitor U0126 significantly blocked proliferation by $80\% \pm 17\%$, $50\% \pm 18\%$, $59\% \pm 15\%$, and $60\% \pm 20\%$ at 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M, respectively (Fig. 10). In these experiments the positive control FBS at 10% increased proliferation 2.0 ± 3.2 -fold (data not shown). EGF, as in rat goblet cells, stimulates human goblet cell proliferation by binding to and activating the EGFR and then stimulating MAPK activity.

Discussion

This report shows that EGF stimulates both human and rat conjunctival goblet cell proliferation by activating the EGFR and promoting sustained signaling through a pathway involving activation of ERK1 and -2 and their translocation to the nucleus. ERK-dependent EGF stimulation of cell proliferation occurred in both human and rat cultured goblet cells. In all our studies to date, the results obtained from goblet cells from both species were very similar,^{25, 29} which suggests that the effects measured were robust, as they occurred in both species and that rat goblet cells are an excellent model for the study of human goblet cell functions.

Goblet cells are present in the airways of the lung, the intestine and colon, and epithelium of the inner ear. As in the conjunctiva, the goblet cells in these tissues, synthesize and secrete MUC5AC as well as other glycoproteins. EGF plays a role in cell proliferation in each of these cell types. Compared with the conjunctiva, mucin production in the gastrointestinal tract and the lung has been more thoroughly studied. Evidence implicates EGF and its family members in the stimulation of goblet cell mucin production. In the lung an increase in goblet cell mucin secretion is associated with chronic obstructive pulmonary disease and asthma. In these diseases, goblet cell mucin synthesis and secretion and the number of goblet cells (indicating proliferation) are increased, leading to a hypersecretory and hyperplastic state in which there is an increase in mucin production.³⁰ Activation of the EGF receptor and its signaling pathways is believed to be one of the contributing factors to the hypersecretory and hyperproliferative state.³⁰⁻³³

The EGF receptor and the signaling pathways activated by it play important roles in many other tissues, such as the salivary and lacrimal glands, where they play a role in fluid and protein secretion,^{34,35} development of the eye,³⁶ and development and function of the heart.³⁷ Thus, the information about the EGF receptor and its pathways obtained from cultured goblet cells could have widespread implications.

In the colon, cell lines have been developed from adenocarcinomas,³⁸ one of which exhibited a goblet cell differentiated phenotype. This cell line expressed the EGF receptor and another member of the EGF receptor family, the ErbB-2 (HER2) receptor. Two of the members of the EGF family of growth factors, transforming growth factor (TGF)- α and heregulin, stimulated the growth of these cells.

In conjunctival goblet cells, EGF caused two peaks of ERK translocation to the nucleus. The first was rapid with the peak occurring at 5 minutes, the earliest time point measured. This peak, with approximately 80% of the cells expressing pERK in the nucleus was transient, as it had begun to decrease by 10 minutes after stimulation. The second peak occurred at

approximately 18 hours after addition of EGF and was sustained as ERK translocation was still occurring at 24 hours. In the second peak, only approximately 40% of the cells contained translocated pERK. When both peaks or the second peak of ERK translocation were blocked by an inhibitor of MEK, EGF-induced proliferation was completely inhibited. These results suggest that the second peak of ERK translocation is necessary for proliferation and the first peak alone is not enough. We were not able to determine whether inhibition of the first peak alone blocked EGF-stimulated proliferation, as there are no reversible inhibitors of ERK. Thus, sustained stimulation by EGF is necessary for induction of goblet cell proliferation.

Murphy and Blenis³⁹ have studied the functional results of spatial and temporal dynamics of ERK activity. They found that many growth factors and cytokines activate ERK and translocate it to the nucleus, but can have differing responses. They hypothesize that the ultimate functional response depends on how long ERK is activated and stays in the nucleus. In transient signaling, activated ERK phosphorylates proteins at the cell membrane and in the cytosol, including ribosomal S6 kinases (RSKs), and translocates to the nucleus along with RSKs. Together, these kinases phosphorylate transcriptional regulators such as Ets, STAT, and CREB that in turn activate immediate early genes (IEGs).³⁹ If the signal is transient, IEGs are degraded by proteosomes and long-term functions, such as proliferation do not occur. If the signal is sustained, IEG products are translated, ERK remains in the nucleus, and long-term functions occur. Thus, in conjunctival goblet cells, the sustained EGF signal results in the second peak of translocation which is critical for induction of cell proliferation.

Two different techniques were used to measure goblet cell proliferation, one that measures cell number (WST-8) and one that indicates entry into the cell cycle (antibody to Ki67). In both techniques, EGF stimulated goblet cell proliferation, which was inhibited by U0126, the MEK inhibitor. Stimulation by EGF was more extensive when entry into the cell cycle was measured, as almost no cells had entered the cell cycle under basal conditions after serum starvation. Another reason for the greater response to EGF found when measuring entry into the cell cycle is that the Ki-67 protein is expressed as soon as the cells enter the cell cycle, whereas the number of cells is measured only after 24 hours. It is possible that not all cells that enter the cell cycle complete it and divide. In addition, a population of cells could take longer than 24 hours to complete the cell cycle. This possibility is likely, as approximately $26\% \pm 7\%$, $18\% \pm 4\%$, and $32\% \pm 2\%$ of the cells were in the G₁, S, and G₂ phases of the cell cycle, respectively, after a 24-hour stimulation with EGF (Fig. 6C). Both techniques indicated that EGF stimulates goblet cells to enter the cell cycle and to proliferate using the ERK signaling pathway.

Although not all steps in the pathway used by EGF to stimulate proliferation were investigated in the present study, we found that the EGFR was phosphorylated on Y1068, which attracts the adapter protein SHC, a component of the ERK pathway. The additional components of this pathway (Grb2, SOS, Raf, and MEK) are well known and have been thoroughly investigated in multiple tissues.^{4,5,40} Stimulation of the EGFR can also activate several parallel pathways including the PLC γ , PI3-K/AKT, and p38 MAPK pathways, each of which can induce proliferation.²⁰⁻²² Experiments performed in the present study do not eliminate the possibility that stimulation of additional parallel or interacting pathways could contribute to an increase in goblet cell proliferation. We can conclude, however, that ERK plays a major role in EGF-stimulated goblet cell proliferation, as ERK is activated by EGF (as measured by phosphorylation of ERK) and EGF-stimulated proliferation is inhibited by U0126, a highly specific inhibitor of MEK.⁴¹

In conclusion, EGF stimulates both human and rat conjunctival goblet cells to enter the cell cycle and proliferate by activating the EGFR on Y1068 activating ERK and translocating it to the nuclei in two peaks: a rapid transient peak and a slower, sustained peak. Activation of the

second peak of ERK translocation is essential for proliferation, whereas the consequences of the first peak are unknown.

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Figure 1.

Effect of EGF on phosphorylation of EGFR. Cultured rat conjunctival goblet cells were incubated with EGF (10^{-7} M) for 1 or 5 minutes, and the amount of activated EGFR was determined by Western blot analysis with antibodies against phosphorylated and total EGFR. A representative blot is shown in (A). Blots from four independent experiments were analyzed, and the results are shown in (B). Data are the mean $\pm$ SEM. *Significant versus no addition.

Figure 2.

Effect of the EGFR inhibitor, AG1478, on cell proliferation. Cultured rat conjunctival goblet cells were preincubated with AG1478 (10^{-7} M) for 20 minutes before incubation with EGF (10^{-7} M) for 24 hours. The number of proliferating cells was determined by WST-8. Data are the mean $\pm$ SEM from seven independent experiments. *Significant versus no addition; #significant versus EGF.

Figure 3.

Effect of EGF on translocation of pERK. Cultured rat conjunctival goblet cells were incubated with EGF (10^{-7} M) for (A) 0, (B) 1 minute, (C) 5 minutes, (D) 16 hours, (E) 18 hours, and (F) 24 hours and (G) in FBS for 24 hours. The cells were stained with an antibody against pERK (*green*) and an antibody against Ki-67, a protein expressed when cells enter the cell cycle (*red*). Images are representative of three independent experiments. Magnification, $\times 200$.

Figure 4.

Comparison of the effect of EGF on ERK translocation and cell-cycle entry. Cultured rat conjunctival goblet cells were incubated with EGF (10^{-7} M) for 0 to 24 hours. The number of cells expressing translocated pERK (Erk activation) or Ki-67 (goblet cell entry into the cell cycle) were counted. Data are the mean $\pm$ SEM of results in six independent experiments.

Figure 5. Effect of EGF on goblet cell entry into the cell cycle. Cultured rat conjunctival goblet cells were incubated with EGF (10^{-7} M) for 24 hours. The cells were stained with the lectin UEA-1 (*green*) or an antibody against Ki-67, a protein expressed when cells enter the cell cycle (*red*). Image is representative of three independent experiments. Magnification, $\times 200$.

Figure 6.

Effect of EGF on the number of cells with translocated pERK and cell cycle phase (G1, S, G2, M). Cultured rat conjunctival goblet cells were incubated with EGF (10^{-7} M) for (A) 16, (B) 18, and (C) 24 hours. The number of cells expressing pERK in the nucleus and Ki-67 were counted, and the stage in the cell cycle was determined. Data are mean $\pm$ SEM from three independent experiments. *Solid lines*: cells expressing pERK in the nucleus; *dotted lines*: cells expressing Ki-67.

Figure 7.

Effect of the MEK inhibitor, U0126, on proliferation of cultured rat conjunctival goblet cells on nuclear location of pERK. Cultured conjunctival goblet cells were preincubated with U0126 (10^{-7} M) for 20 minutes before incubation with EGF (10^{-7} M) for 24 hours. **(A)** The number of proliferating cells was determined by WST-8. Data are the mean $\pm$ SEM of results in five independent experiments. *Significant versus EGF alone. **(B)** After treatment, the cells were stained with an antibody directed against pERK (*green*). Magnification, $\times 200$.

Figure 8.

Effect of U0126 on two peaks of proliferation of cultured rat conjunctival goblet cells. Cultured conjunctival goblet cells were either preincubated with U0126 (10^{-7} M) for 20 minutes before incubation with EGF (10^{-7} M) or with no addition for 24 hours (**A**), or U0126 (10^{-7} M) was added 2 hours after the addition of EGF or buffer (**B**). The number of proliferating goblet cells was determined by Ki-67 immunohistochemistry. Data are the mean $\pm$ SEM of results of four independent experiments. *Significant versus EGF alone; #significant versus EGF.

Figure 9.

Effect of AG1478 on proliferation of cultured human conjunctival goblet cells. Cultured conjunctival goblet cells were preincubated with AG1478 (10^{-8} – 10^{-6} M) for 20 minutes before incubation with EGF (10^{-7} M) for 24 hours. The number of proliferating cells was determined by WST-8. Data are the mean $\pm$ SEM of results in four independent experiments. *Significant versus EGF alone.

Figure 10.

Effect of U0126 on proliferation of cultured human conjunctival goblet cells. Cultured conjunctival goblet cells were preincubated with U0126 (10^{-7} M) for 20 minutes before incubation with EGF (10^{-7} M) for 24 hours. The number of proliferating cells was determined by WST-8. Data are the mean $\pm$ SEM of results in four independent experiments. *Significant versus EGF alone.