Epidermal Growth Factor Stimulation of Phosphatidylinositol 3-Kinase During Wound Closure in Rabbit Corneal Epithelial Cells

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Purpose. To determine whether there is an association between epidermal growth factor (EGF)-induced activation of phosphatidylinositol 3-kinase (PI 3-kinase) and stimulation of wound closure in rabbit corneal epithelial cells.

Methods. Immortalized rabbit corneal epithelial cells were cultured in 24-well plates until they became confluent. Circular wounds were created in confluent cultures by cell denudation and then incubated in the absence and presence of EGF for varying intervals. Wound closure was monitored by staining the cells with Giemsa and quantifying the wound area with SigmaScan computer program. Cell proliferation during wound repair was estimated by measuring the incorporation of [3H]thymidine into nuclear DNA. Changes in PI 3-kinase activity were assessed by measuring the production of phosphatidylinositol 3,4,5-triphosphate [PI (3,4,5) P3] in 32 P-labeled cells as well as by immunoprecipitating and assaying PI 3-kinase activity with phosphatidylinositol 4,5-bisphosphate and [γ-32P]ATP as substrates. The enzyme product, PIP3, was analyzed by a combination of thin-layer and high-pressure liquid chromatography.

Results. Addition of 10 ng/ml EGF to the wounded corneal epithelial cells stimulated wound closure in a time-dependent manner, and the wound closed completely within 48 hours. The effect of EGF was dose dependent, and maximal wound closure occurred at 10 ng/ml EGF. As the epithelial cells were undergoing EGF-stimulated wound closure, there was a time-dependent increase in PI 3-kinase activity. The enzyme activity increased maximally at 24 hours and then decreased gradually as the incubation was continued to 48 hours. When the cells were treated with wortmannin, a PI 3-kinase inhibitor, the EGF-stimulated PIP3 formation as well as the wound closure were inhibited significantly. Treatment of the cells with genistein or tyrphostin B42 also decreased both EGF-stimulated PIP3 formation and wound closure in a dose-dependent manner. Concomitant with stimulation of wound repair, the growth factor increased [3H]thymidine incorporation into nuclear DNA, and this effect was inhibited by pretreatment of the cell with wortmannin.

Conclusions. The data suggest a close correlation between EGF-stimulated wound closure and activation of PI 3-kinase in corneal epithelial cells. It can be concluded that PI 3-kinase might be an important component in signal transduction cascade initiated by EGF-receptor interaction, which leads to mitosis and cell proliferation during wound closure in corneal epithelial cells. Invest Ophthalmol Vis Sci. 1997;38:1139–1148.

Corneal epithelial wound healing is a highly ordered and coordinated process that involves reorganization, migration, and proliferation of the epithelial cells.1–5 An important modulator of wound repair is the epidermal growth factor (EGF), which is produced by several cell types present in the wound. Additionally, the corneal epithelium is bathed continuously in tear fluid that contains EGF.4 Several studies have shown that treatment of the corneal epithelial cells with EGF, both in vivo and in vitro, accelerate wound closure by increased migration and mitosis of the epithelial cells.5–7 Although EGF receptors have been localized in the corneal epithelium,8 there is little known about the steps involved between the binding of EGF to its
In the current study, we have used cultured rabbit corneal epithelial cells, immortalized with adenovirus SV40, as an in vitro model of wound closure. These cells were supplied by Dr. Araki-Sasaki of Japan. To initiate subculture, the confluent cells were washed with calcium-magnesium-free Hanks balanced salt solution and treated with 0.1% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. Then, Dulbecco’s minimum essential medium—F₁₂ containing 15% fetal bovine serum was added and the cell suspension centrifuged at 300g for 5 minutes. The pelleted cells were resuspended and seeded in 24-well plates at a density of 1 × 10⁴/cm². The cultures were maintained by changing the medium every other day until the cells became confluent. At this time, the cells were starved for 6 hours in serum-free Dulbecco’s minimum essential medium—F₁₂ containing 2 mg/ml bovine serum albumin. Before a wound was created, the medium was aspirated, and a circular area 8 mm wide was marked in each well with a trephine. The wound was created by scraping the cells carefully in the marked area with a rubber scalpel. Any loose cells in the wound area were removed by washing the cultures twice with fresh medium. After this, the cells were allowed to grow and close the wound in a serum-free medium containing 2 mg/ml bovine serum albumin with or without EGF. When the effects of inhibitors on PI 3-kinase or tyrosine kinase were to be investigated, these agents were added to the cells 15 minutes before the addition of EGF. At specified intervals, the cells were washed once with Hanks solution, fixed in 10% formaldehyde solution for 2 hours and then stained with Giemsa. The wound area was digitized with an optical scanner (ScanJet 3C; Hewlett-Packard, Hopkins, MN) and quantified with a computer program, SigmaScan (Jandel Scientific, San Rafael, CA). All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Immunoprecipitation and Assay of PI 3-Kinase Activity During the Course of Wound Closure**

To assess PI 3-kinase activity during wound closure, confluent epithelial cells were wounded and then cultured in the presence and absence of EGF as described in the previous section. At specified intervals, the medium was aspirated and the cells washed in calcium-magnesium-containing PBS. Next, the cells were processed for immunoprecipitating PI 3-kinase as de-
scribed previously. Briefly, the cells were washed twice in buffer A that contained 137 mM sodium chloride (NaCl), 20 mM Tris (pH 7.4), 1 mM calcium chloride, 1 mM magnesium chloride, and 0.2 mM Na3VO4. Next, 0.3 ml ice-cold lysis buffer (buffer A containing 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, and 50 µM leupeptin) was added to each well. After keeping the plate on ice for 20 minutes, the cell lysates from three to four wells were pooled and centrifuged at 12,000g for 10 minutes. The cleared supernatant was incubated with 5 µl of anti-PI 3-kinase polyclonal antibody at 4°C overnight. Next, 40 µl of protein-A sepharose slurry was added, the sample incubated for 2 hours, and then centrifuged. The standard protocol for washing immunoprecipitate was three times in PBS/1% NP-40, three times in 100 mM Tris (pH 7.4)/5 mM lithium chloride, and two times in 10 mM Tris (pH 7.4)/150 mM NaCl/5 mM EDTA. Sodium orthovanadate (0.2 mM) was included in all wash solutions. During each of the washes, the immunoprecipitate was vortexed thoroughly, centrifuged at 10,000g for 10 seconds, and maintained on ice. The washed immunoprecipitate was used for PI 3-kinase activity assay as described below.

The PI 3-kinase assay was done in a final volume of 50 µl that contained 20 µl of the immunoprecipitate in 10 mM Tris (pH 7.4)/150 mM NaCl/5 mM EDTA/0.2 mM Na3VO4, 5 µl of sonicated PI(4,5)P2 (4 mg/ml in 10 mM Hepes, pH 7.5, containing 1 mM EGTA and 1 mg/ml phosphatidylinerine) and 25 µl of 0.1 mM [γ-32P]ATP (specific activity 4 Ci/mmol) in 40 mM Hepes (pH 7.5)/20 mM magnesium chloride. The incubations were conducted for 20 minutes at room temperature. The reaction was terminated by adding 40 µl of 4 N hydrochloric acid and vortexing the sample. The lipids were extracted by the method of Bligh and Dyer. The lipid extract was analyzed by one-dimensional thin-layer chromatography; the reaction products were detected by autoradiography and quantified by liquid scintillation counting.

**Analysis of PI(3,4,5)P3 in Epithelial Cells Labeled With 35Pi**

The effects of wortmannin, tyrphostin, or genistein on PI 3-kinase activity during wound closure were monitored by assessing changes in PI(3,4,5)P3 in intact cells. Shortly after creating the wound, the cells were treated with various concentrations of the inhibitors for 15 minutes. Next, the cells were incubated in a serum-free medium that contained 20 µCi [35P]Pi/ml for 24 hours in the absence and presence of 10 to 50 ng/ml EGF. The incubations were terminated by aspirating the medium and washing the cells in a nonradioactive medium. Then, 0.5 ml ice-cold acidified methanol was added to each well and the cells scraped and transferred to glass tubes. Generally, the cells from four to six wells were pooled to analyze for PI(3,4,5)Ps. Each experiment consisted of incubations sufficient to yield three independent pooled samples for each concentration of the inhibitor used. The phospholipids were extracted and analyzed by thin-layer chromatography using oxalate-impregnated silica gel plates. The developing solvent used was chloroform/acetonemethanol/acetic acid/water (80:30:26:24:14, by volume). The labeled lipids were visualized by autoradiography, identified by comparison with known standards, and quantified by liquid scintillation counting.

**Incorporation of [3H]Thymidine Into DNA**

The cells were seeded in 24-well plates at a density of 1 × 104 cells/cm2 overnight. The medium was removed and the cells washed once with fresh medium and then incubated in a serum-free medium (Dulbecco's minimum essential medium-F12 3:1 containing bovine serum albumin 2 mg/ml) with or without wortmannin (0.3 µM) for 12 hours. Next, [3H]thymidine (2 µCi; specific activity, 2 Ci/mmol) and EGF (10 ng/ml) were added and incubation continued for different intervals. The medium was removed and the cells washed in 2 ml ice-cold Hanks solution followed by three careful washes in 10% trichloroacetic acid. The precipitate was dissolved in 1 ml 1% sodium dodecyl sulfate–0.3 N sodium hydroxide solution and counted for radioactivity.

**Data Analysis**

Each experiment consisted of incubations that, when pooled, yielded three independent samples for each data point. All experiments were repeated at least twice. The results were expressed as mean ± standard error of the mean, and statistical analysis was performed using analysis of variance and Student’s t-test for nonpaired data.

**RESULTS**

**Effect of Epidermal Growth Factor on Wound Closure in Corneal Epithelial Cells**

Figure 1 shows the time course of wound closure in cultured corneal epithelial cells. In the absence of externally added EGF, the wound closed slowly: >60% of the original wound remained after 24 hours, and continued incubation to 48 hours did not result in significant further increase in wound closure. Addition of EGF (10 ng/ml) accelerated the rate of wound closure. In the presence of EGF, the wound areas were significantly smaller at each timepoint as compared with those of the untreated control specimen. By 48 hours, all wounds in the EGF-treated cultures were closed completely. As shown in Figure 2, the stimula-
Control

EGF (10 ng/ml)

Time (hrs) 0 12 24 36 48

FIGURE 1. Time course of the effect of epithelial growth factor (EGF) on wound closure in rabbit corneal epithelial cells. The rabbit corneal epithelial cells, grown in 24-well plates, were incubated in a serum-free medium for 6 hours. After the wounds were created by cell denudation, the cells were allowed to grow for 48 hours in a medium with or without EGF (10 ng/ml). At prescribed intervals, the cells were fixed in 10% formaldehyde solution and then stained by Giemsa. (upper) Photograph showing the effect of EGF on wound closure, (lower) Curves showing the effect of EGF on wound closure. After calculating the wound area by SigmaScan Computer program (Jandel Scientific), the data were expressed as the ratio of the repaired area to the wounded area made at zero time. Data represent the mean ± SEM of two experiments each conducted in triplicate.

tory effect of EGF was dose dependent. As low as 0.1 ng/ml EGF caused a significant increase in wound closure, and a maximal effect was observed at 10 ng/ml EGF.

Effect of Epidermal Growth Factor on PI 3-Kinase Activity During Wound Closure

To investigate whether EGF exerts any effect on PI 3-kinase activity in corneal epithelial cells undergoing wound closure, the PI 3-kinase assays were performed in parallel with the wound closure determinations. As shown in Figure 3, in untreated cells, the PI 3-kinase activity was increased by approximately 15% at 12 hours and by 40% at 24 hours postwounding. Increasing culture time further led to a decrease in PI 3-kinase activity, which returned to the basal level by 48 hours postwounding. When the wounds were treated with EGF (50 ng/ml), there was a much larger stimula-

FIGURE 2. Dose-response effect of epithelial growth factor on wound closure in rabbit corneal epithelial cells. After the wounds were created, the corneal epithelial cells were cultured for 48 hours in the absence and presence of various concentrations of epithelial growth factor. Data represent the mean ± SEM of two experiments each conducted in triplicate.

FIGURE 3. Time course of the effect of epithelial growth factor on phosphatidylinositol (PI) 3-kinase activity in rabbit corneal epithelial cells. After the wounds were created, the corneal epithelial cells were cultured in a medium in the absence or presence of epithelial growth factor (50 ng/ml). At prescribed intervals, the cells were lysed and the cell lysates from three to four wells were pooled and treated with anti-PI 3-kinase antibody. The immunoprecipitates were collected and assayed for PI 3-kinase activity as described in Materials and Methods. The data presented are mean ± SEM of two experiments. Each experiment was conducted with cells sufficient to yield three independent pooled samples for every data point.
PI 3-Kinase and Corneal Epithelial Wound Closure

FIGURE 4. Dose-response effect of epithelial growth factor on phosphatidylinositol 3-kinase activity in rabbit corneal epithelial cells. After the wounds were created, the corneal epithelial cells were cultured for 24 hours in the absence and presence of various concentrations of epithelial growth factor. The immunoprecipitable activity in the cell lysates was assayed as described in Materials and Methods. Data represent the mean ± SEM of six to eight independent samples from two experiments.

FIGURE 5. Dose-response effect of wortmannin on epithelial growth factor-stimulated formation of [32P]PIP₃ in corneal epithelial cells. After the wounds were created, the corneal epithelial cells were treated with various concentrations of wortmannin for 15 minutes. Next, the cells were cultured in a serum-free medium containing 20 μCi [32P]Pi/ml in the absence or presence of epithelial growth factor (50 ng/ml) for 24 hours. At the end of the incubation, the phospholipids were extracted and analyzed for radioactivity as described in Materials and Methods. The data on [32P]PIP₃ represent the mean ± SEM of two separate experiments with three independent samples for each data point.

Effect of Wortmannin on Epidermal Growth Factor-Induced Wound Closure and PI(3,4,5)P₃ Formation

To further investigate whether there was a causal relation between EGF-induced stimulation of PI 3-kinase and wound closure, the effects of wortmannin on the two responses were determined. Wortmannin, at low concentrations, has been shown to specifically inhibit PI 3-kinase activity in several tissues. As shown in Figure 5, addition of EGF to epithelial cells during wound closure resulted in a large increase in PI(3,4,5)P₃ formation, indicating activation of PI 3-kinase. When the cells were treated with wortmannin, the EGF-stimulated PIP₃ formation was reduced greatly. The effect of wortmannin was dose dependent, and at 1 μM, it caused complete inhibition of EGF-stimulated PIP₃ formation. Under similar experimental conditions, wortmannin also inhibited, although less potently, the EGF-stimulated wound closure (Fig. 6). Again, the inhibitory effect of wortmannin was dose dependent, and at 1 μM, it abolished the EGF effect on wound closure completely.

Effects of Tyrosine Kinase Inhibitors on Epidermal Growth Factor-Induced Wound Closure and PI(3,4,5)P₃ Formation

Because PI 3-kinase has been shown to lie downstream of receptor tyrosine kinase in other tissues, we used tyrosine kinase inhibitors, genistein and tyrphostin, to further confirm the involvement of PI 3-kinase in EGF-induced wound closure. As shown in Figure 7, genistein, which inhibits both receptor and nonreceptor tyrosine kinases, caused a dose-dependent decrease in wound closure.

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in EGF-stimulated \( \text{PI}(3,4,5)\text{P}_3 \) formation. A complete inhibition of \( \text{PI}(3,4,5)\text{P}_3 \) formation was observed when the genistein concentration was increased to 30 \( \mu \text{M} \). Treatment of the cells with tyrphostin B42, which selectively inhibits EGF receptor tyrosine kinase, also decreased the EGF-induced \( \text{PI}(3,4,5)\text{P}_3 \) formation. The inhibitory effect of tyrphostin B42 was slightly greater than that of genistein. In parallel experiments, genistein and tyrphostin also were found to inhibit EGF-induced wound closure (Fig. 8). Again, at 30 \( \mu \text{M} \), the inhibitors abolished the EGF effect on wound closure completely.

**Effect of Wortmannin on Epidermal Growth Factor-Induced \( [3\text{H}] \)Thymidine Incorporation Into DNA**

The purpose of these experiments was to determine whether EGF-induced wound closure involved proliferation of corneal epithelial cells and also to determine whether wortmannin inhibited the wound closure by inhibiting DNA synthesis. As shown in Figure 9, EGF increased \( [3\text{H}] \)thymidine incorporation into nuclear DNA in a time-dependent manner. When the cells were treated with wortmannin, there was a significant decrease in EGF-induced incorporation of \( [3\text{H}] \)thymidine.

**DISCUSSION**

Injured corneal epithelium heals by a number of biologic processes that include cell migration, cell division, and synthesis of extracellular matrix proteins.\(^5\) When applied topically, EGF promotes reepithelialization by stimulating both cell migration and cell division.\(^5\) The exact biochemical events that follow EGF-receptor interaction and lead to DNA synthesis

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**FIGURE 7.** Dose-response effect of genistein and tyrphostin B42 on epithelial growth factor-stimulated formation of \( [3\text{P}]\text{PIP}_2 \) in corneal epithelial cells. After the wounds were created, the corneal epithelial cells were treated with various concentrations of genistein or tyrphostin B42 for 15 minutes. Next, the cells were cultured in a serum-free medium containing 20 \( \mu \text{Ci} \) [\( ^{3}\text{P} \)]Pi/ml in the absence and presence of EGF (50 ng/ml) for 24 hours. At the end of the incubation, the phospholipids were extracted and analyzed for radioactivity as described in Materials and Methods. Data represent the mean ± SEM of two experiments with three independent samples for each data point.

**FIGURE 8.** Dose-response effect of genistein and tyrphostin B42 on epithelial growth factor-stimulated wound closure in corneal epithelial cells. After the wounds were created, the epithelial cells were treated with various concentrations of genistein or tyrphostin B42 for 15 minutes. At this time, epithelial growth factor (10 ng/ml) was added and the cells cultured in a serum-free medium for 48 hours. The data represent mean ± SEM of six independent samples from two separate experiments.

**FIGURE 9.** Effect of wortmannin on epithelial growth factor-induced \( [3\text{H}] \)thymidine incorporation into DNA in corneal epithelial cells. The semiconfluent cells were incubated in a serum-free medium with or without wortmannin (0.3 \( \mu \text{M} \)) for 12 hours. Next, 2 \( \mu \text{Ci} \) \( [3\text{H}] \)thymidine and epithelial growth factor (10 ng/ml) were added and incubation conducted for different intervals. The cultures then were processed for determination of radioactivity incorporated in the acid-insoluble material. Data represent the mean ± SEM of six determinations from two separate experiments. \(^*P < 0.05\) compared to the untreated control specimens. \( \Delta P < 0.05\) compared to the epithelial growth factor-treated cells.
and cell proliferation are not clear. It is known, however, that distinct signaling pathways exist for mediating mitogenic signals from EGF receptors to the cell nucleus. An early step in this signal transduction pathway involves tyrosine phosphorylation of specific proteins that contain SH2 and SH3 domains. For example, subsequent to EGF-receptor interaction, PLCγ1, ras GTPase-activating protein, and 85-kDa regulatory subunit of PI 3-kinase become associated with the EGF receptor and are phosphorylated by its intrinsic tyrosine kinase activity. It has been reported that tyrosine phosphorylation of the regulatory subunit of PI 3-kinase results in stimulation of its enzyme activity, producing PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3. These 3-phosphoinositides have been suggested to function as second-messenger molecules during cell growth and proliferation.

In the current study, we have used rabbit corneal epithelial cells to examine the effects of EGF on PI 3-kinase activity under conditions when the cells were actively dividing and, therefore, facilitating the wound closure. These SV40-immortalized cells previously were shown to propagate for many passages and, when compared to primary cultured epithelial cells, were able to maintain their morphologic and biochemical characteristics. Furthermore, like intact corneal epithelium, these cells elicited increased PIP_2 hydrolysis when stimulated with carbachol. The effect of carbachol was inhibited significantly by isoproterenol, suggesting that the immortalized cells were suitable for investigating the effects of agonists on phosphoinositide metabolism. Recently, we have shown that confluent cultures of these cells contain PI 3-kinase, which is stimulated by EGF. In the current study, we have used these cells as a model of corneal epithelial wound healing. This in vitro model system had the advantage that the degree of wound closure could be measured precisely, and possible influence from neighboring tissues could be eliminated. The changes in PI 3-kinase activity in response to EGF were determined by two assay methods: one by assaying PI 3-kinase activity in vitro using anti-PI 3-kinase immunoprecipitable protein in the cell lysates and the other by measuring PI 3-kinase product, PI(3,4,5)P_3, in intact 32P-labeled cells. Both methods provided reliable measure of the enzyme activity in cells treated with or without EGF.

The transformation of cells by adenovirus SV40 is a complex process that involves several cellular and genetic changes. Although the exact mechanisms underlying the transformation process are not clear, evidence suggests both activation of autocrine growth pathway and the inactivation of negative growth-regulating proteins. Thus, cells transformed by SV40 show secretion of growth-promoting substances and reduced requirement for exogenous growth factors. In the current work, when a wound was created in SV40-transformed corneal epithelial monolayer, the cells began proliferating and covering the wound area, suggesting that autocrine growth pathway probably is stimulated in these cells. By 24 to 36 hours, the wound had closed to approximately 40% of the original size (Fig. 1). Allowing the cell culture for 48 hours did not result in significant increase in wound closure. The reason for decreased cell proliferation at longer periods is not clear. It is possible, however, that at prolonged incubation periods, the autocrine secretion of growth factors probably is diminished, which could result in decreased cell proliferation. At 48 hours post-wounding, the cells were still metabolically healthy because the addition of serum to the culture medium led to renewed cell proliferation and wound closure (Zhang and Akhtar, unpublished data, 1996). When EGF was included in the culture medium, the wound closed at a much faster rate, and complete wound closure occurred by 48 hours. It is known that immortalization with SV40 T antigen releases the cells from many constraints over cell proliferation. However, the data from the current studies show that transformed corneal epithelial cells can still respond to EGF stimulation. This is in accord with the data on SV40-immortalized uterine, prostate, and keratinocyte cells in which EGF was found to exert a stimulatory effect on cell proliferation.

It also is clear from the data that as the cells were undergoing rapid mitosis and cell proliferation, the PI 3-kinase activity began to increase at a rate comparable with that of the wound closure. After reaching maximum at 24 hours, the PI 3-kinase activity declined gradually and reached the basal level by 48 hours (Fig. 3). The decrease in PI 3-kinase activity at later intervals probably was caused by a decline in autocrine secretion of growth factors. When stimulated with EGF, the profile for PI 3-kinase activity was identical to that of the unstimulated cells, except that the magnitude of enzyme activation was much higher in the presence of EGF. Again, after reaching maximum at 24 hours, the enzyme activity decreased to a level significantly higher than that for the basal value. The decrease in PI 3-kinase activity corresponded to low mitotic rate in wounds that were approaching maximal closure. Previously, we found that EGF stimulation of confluent corneal epithelial cells for 5 minutes caused increased activation of PI 3-kinase, primarily because of tyrosine phosphorylation of the existing enzyme. Therefore, the gradual increase observed in enzyme activity in a 24-hour period in the EGF-treated cells probably resulted from an increased synthesis of PI 3-kinase. Additional experiments showed that EGF increased both PI 3-kinase and wound closure in a dose-dependent manner (Figs. 2 and 4). However, maximal wound closure occurred at a lower EGF concentration than that required for maximal increase in PI 3-kinase activity.
activity. This could suggest that a small rise in PI 3-kinase activity is probably sufficient to cause a large increase in mitotic activity necessary for wound closure. The data on time-course and dose-response effects of EGF strongly suggest a correlation between activation of PI 3-kinase and progression of wound closure in the corneal epithelial cells. Recently, PI 3-kinase has been identified in intact rabbit corneal epithelium, and its activity was found to be increased 48 hours after wounding. 24

Three lines of evidence indicate that PI 3-kinase probably plays an important role in growth regulation and transformation. First, all mutants of polyoma virus middle T antigen, which either fail to associate with PI 3-kinase or are unable to elevate the levels of PI 3-kinase products in vivo, result in a transformation-defective phenotype. 25,26 Similarly, point mutation in the PI 3-kinase binding sites of the PDGF receptor impairs the receptor’s ability to initiate DNA synthesis. 27 Second, recent work has shown that micro injection of antibodies specific for the p110 subunit of the PI 3-kinase into quiescent fibroblasts inhibits PDGF-induced DNA synthesis. 28 Finally, when phosphoinositide metabolism was examined in dividing HT29 colonic epithelial cells, a large increase in the level PI(3)P was found only in cells in mitotic phase. 29

Additional support for the idea that EGF-induced activation of PI 3-kinase is related causally to the EGF effect on wound closure was provided by the data obtained from cells treated with wortmannin. At low (1 to 10 mM) concentrations, this agent has been shown to inhibit PI 3-kinase in purified preparations and cytosolic fractions. 30,31 We also found that wortmannin can inhibit the immunoprecipitated PI 3-kinase from corneal epithelial cells with an IC50 of 1 nM. 17 However, several studies have reported that in intact cells, relatively higher concentrations (0.1 to 1 µM) of wortmannin are required to inhibit the enzyme effectually. 31,32 In the current work, wortmannin produced a dose-dependent inhibition of EGF-stimulated PI 3-kinase with complete inhibition observed at 1 µM wortmannin (Fig. 5). Concomitantly, 1 µM wortmannin inhibited the EGF-stimulated wound closure completely (Fig. 6). At lower concentrations, wortmannin was less potent in inhibiting EGF-stimulated wound closure when compared to its effect on PI 3-kinase. For example, 0.3 µM wortmannin inhibited EGF-stimulated wound closure only by 25% when the PI 3-kinase was inhibited by 80%. The reason for the differential effect of wortmannin is not clear. It may be suggested, however, that any residual PI 3-kinase activity in the wortmannin-treated cells is probably sufficient to induce DNA synthesis in response to EGF. Additionally, the data could suggest that factors other than activation of PI 3-kinase also contribute to the events leading to EGF-induced DNA synthesis during wound closure. Separate experiments showed that wortmannin inhibition of wound closure was accompanied by reduced EGF-stimulated DNA synthesis in corneal epithelial cells (Fig. 9). Taken together, the data suggest that activation of PI 3-kinase plays an important role in epithelial cells proliferation and probably is required for stimulation of DNA synthesis. Our data on wortmannin are consistent with a previous report in which this agent inhibited the serum-induced activation of PI 3-kinase and cell proliferation in CHRF-288 cells. 33 In another study, when intact ST3-L1 adipocytes were treated with LY294002, a specific inhibitor for PI 3-kinase, the insulin- and serum-stimulated PI 3-kinase, and DNA synthesis were inhibited effectively. 34

A unique feature of activated growth factor receptors is their ability to phosphorylate cellular proteins at tyrosine residues. Because tyrosine phosphorylation of PI 3-kinase is believed to be responsible for its activation, 13 the inhibitors of tyrosine kinase were considered useful in delineating the role of PI 3-kinase in cell proliferation during corneal epithelial wound closure. We used genistein, which inhibits both receptor and nonreceptor tyrosine kinases, and tyrphostin B42, which inhibits EGF receptor tyrosine kinase only. When the cells were treated with these agents, there was a dose-dependent decrease in EGF-stimulated PI 3-kinase activity (Fig. 7). In parallel, genistein and tyrphostin B42 also caused a dose-dependent inhibition of EGF-stimulated wound closure (Fig. 8), providing additional evidence that PI 3-kinase activation probably is involved in EGF-stimulated DNA synthesis and cell proliferation during corneal epithelial wound closure.

The results of the current study show, for the first time, that activation of PI 3-kinase is associated with wound closure in corneal epithelial cells. However, our data do not provide any information regarding the mechanisms by which PI 3-kinase or its products might participate in biochemical reactions leading to EGF-stimulated DNA synthesis in corneal epithelial cells. It has been reported that Ras (p21ras) can interact directly with the catalytic subunit of PI 3-kinase (p110 subunit), raising the possibility that PI 3-kinase may serve as an effector of Ras. 35 Other studies have shown a link between PI 3-kinase and the small G proteins Rac and Rho, suggesting that the enzyme also may play a role in regulating cell shape. 36 More recently, it has been reported that 85-kDa subunit of PI 3-kinase interacts directly with the adapter protein Grb2, both in vitro and in vivo. 37 Grb2 binds phosphotyrosine motifs on activated growth factor receptors by way of its SH2 domain, whereas the two SH3 domains bind the guanine nucleotide exchange protein Sos. Signaling through PI 3-kinase, therefore, may in-
volve the ubiquitous adapter Grb2, which serves as a convergence point for multiple pathways.

Another possibility is that PIP₃, the product of PI 3-kinase, may have a second-messenger function in DNA synthesis, especially because the 3-phosphoinositides are not substrates for PLC and, therefore, are not precursors of second-messenger molecules. It has been reported that PI(3,4,5)P₃ can activate PKCα in vitro. Recent studies also have shown that PKCζ isoform is stimulated by PIP₃. This isoform differs from all the other PKC isoforms in that it is not stimulated by phorbol esters or DAG. It will be interesting to see whether PKCζ is activated in vivo in response to EGF in the corneal epithelial cells.

In summary, we have shown that PI 3-kinase is activated in mitotically dividing corneal epithelial cells involved in wound closure. Addition of EGF further stimulates PI 3-kinase activity, which corresponds to stimulation of wound closure. Wortmannin treatment of the cells causes inhibition of PI 3-kinase with concomitant inhibition of the wound closure. When treated with genistein or tyrphostin, both PI 3-kinase and wound closure are attenuated. It can be concluded from these data that PI 3-kinase probably is a key component of tyrosine kinase-regulated signaling pathway in corneal epithelial cells and probably plays an important role in EGF-mediated reepithelialization of the wounded corneal epithelium.

Key Words
corneal epithelial cells, epidermal growth factor, genistein, phosphatidylinositol 3-kinase, tyrphostin, wortmannin, wound closure

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