Wound-induced ATP release and EGF receptor activation in epithelial cells

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Summary

We have shown previously that wounding of human corneal epithelial (HCE) cells resulted in epidermal growth factor receptor (EGFR) transactivation through ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF). However, the initial signal to trigger these signaling events in response to cell injury remains elusive. In the present study, we investigated the role of ATP released from the injured cells in EGFR transactivation in HCE cells as well as in BEAS 2B cells, a bronchial epithelial cell line. Wounding of epithelial monolayer resulted in the release of ATP into the culture medium. The wound-induced rapid activation of phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways in HCE cells was attenuated by eliminating extracellular ATP, ADP and adenosine. The nonhydrolyzable ATP analog

ATP-γ-S induced rapid and sustained EGFR activation that depended on HB-EGF shedding and ADAM (a disintegrin and metalloproteinase). Targeting pathways leading to HB-EGF shedding and EGFR activation attenuated ATP-γ-S-enhanced closure of small scratch wounds. The purinoceptor antagonist reactive blue 2 decreased wound closure and attenuated ATP-γ-S induced HB-EGF shedding. Taken together, our data suggest that ATP, released upon epithelial injury, acts as an early signal to trigger cell responses including an increase in HB-EGF shedding, subsequent EGFR transactivation and its downstream signaling, resulting in wound healing.

Key words: EGFR, ATP, Cell migration, Wound healing, HB-EGF, Purinoceptor

Introduction

Epithelial cells form a protective barrier for all tissues and are subjected to constant physical, chemical and biological insults, often resulting in a wound and loss of functions. Rapid healing of a wound is essential for an injured tissue to restore its homeostasis and function. The wound repair process involves cell adhesion, migration, proliferation, matrix deposition and tissue remodeling (Martin, 1997). Many of these biological processes are mediated by growth factors, cytokines and other mediators released in the injured tissues or cells (Werner and Grose, 2003). Studying the underlying mechanisms of wound healing contributed to clinical application of such factors. For instance, growth factors have been recognized as important mediators of proper wound repair (Imanishi et al., 2000) and the treatment using growth factors such as platelet-derived growth factor (PDGF)-BB and recombinant human epidermal growth factor (EGF) has been shown to be beneficial for patients with chronic pressure ulcers or nonhealing diabetic ulcers (Hong et al., 2006; Mustoe et al., 1994; Robson et al., 1992; Smiell et al., 1999). Corneal epithelium is a good model to study wound healing for its avascularity and extensive innervation. We and others have shown that epithelial wounding induces EGF receptor (EGFR) transactivation via ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF) in human corneal epithelial (HCE) cells, and this wound-induced activation of EGFR and its co-receptor erbB2 is required for epithelial migration and wound closure (Block et al., 2004; Mazie et al., 2006; Xu et al., 2004a; Xu et al., 2004b).

HB-EGF is synthesized as a type-1 transmembrane protein that can be cleaved enzymatically by sheddases to release a soluble 14-20 kDa growth factor; the process has been termed ectodomain shedding (Gechtman et al., 1999; Goishi et al., 1995; Raab et al., 1994). Whereas the transmembrane form of HB-EGF acts in a juxtacrine manner to signal to neighboring cells (Higashiyama et al., 1995), the soluble form of HB-EGF is a potent mitogen and chemoattractant for many types of cells, including keratinocytes and other epithelial cells (Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997). The released HB-EGF acts through the stimulation of specific cell-surface receptors (Hynes et al., 2001). Four related receptor tyrosine kinases have been identified as EGFR/erbB1/HER1, erbB2/HER2/neu, erbB3/HER3 and erbB4/HER4 (Hynes et al., 2001). Shed EGFR ligands such as HB-EGF act in an autocrine and/or paracrine fashion to stimulate its activation. Phosphorylation of EGFR creates docking sites for adaptor proteins such as Grb2, Shc and Gab and leads to activation (tyrosine phosphorylation) of effectors, such as phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (ERK), which have been shown to be involved in corneal epithelial wound healing (Chandrasekher and Bazan, 1999; Chandrasekher et al., 2001; Glading et al., 2000; Klemke et al., 1997; Zhang and Akhtar, 1996; Zhang and Akhtar, 1997).

Ectodomain shedding has emerged as an important post-translational mechanism to regulate the functions of various membrane proteins (Blobel, 2002; Schlondorff and Blobel,

1999). Metalloproteinases of the zinc-dependent ADAM (a disintegrin and metalloproteinase) family have been shown to be responsible for the cleavage of the majority of shed proteins, including EGFR ligands (Blobel, 2005; Sanderson et al., 2006). Ectodomain shedding is a highly regulated process (Blobel, 2005). However, the mechanism by which intracellular signaling pathways lead to an increase in sheddase activity has not been fully established. PKC or intracellular Ca^{2+} pathways have been shown to upregulate the activity of sheddases. In epithelial cells, cell injury results in the fast propagation of a Ca^{2+} wave that is believed to be generated, at least in part, by extracellular adenosine triphosphate (ATP) (Klepeis et al., 2001). ATP was first thought to be solely an intracellular energy source but later proved to be an important extracellular signaling molecule, like those released by neuronal cells as a neurotransmitter in the central and peripheral nervous systems (Khakh, 2001). Purinoceptors are divided into two classes, P1 or adenosine receptors, and P2 receptors that recognize extracellular ATP, ADP, UTP and UDP. P2 receptors are further categorized into two subclasses. P2X receptors are extracellular ATP-gated, Ca^{2+}-permeable, nonselective cation channels. P2Y receptors couple to heterotrimeric G proteins and phospholipases to raise the intracellular free Ca^{2+} concentration (Schwiebert and Zsembery, 2003). Several P2Y receptors have been found in corneal epithelial cells and inhibition of these receptors attenuated epithelial wound healing in vitro (Klepeis et al., 2004). Since injury of epithelial cells is likely to cause the release of cellular ATP, the released ATP can act as a 'cell (or tissue)-damage' signal to trigger cell signaling events including Ca^{2+} waves and G-protein-coupled-receptor activation, both of which transactivate EGFR and its downstream signaling pathways.

Here, we report that ATP is rapidly released into culture medium after wounding, and that the released ATP contributes to the activation of EGFR signaling and the regulation of epithelial wound closure. Our results indicate that ATP and its purinoceptors play an important role in the early stage of epithelial wound healing by transactivating EGFR signaling.

Results
Wounding increases ATP concentration in the culture medium of epithelial cells
To assess the release of ATP, THCE cells, an SV-40 immortalized HCE cell line, at confluence were extensively injured and ATP released into the medium was assessed by luciferase-luciferin ATP bioluminescent assay. Using ATP concentration standard, the amount of ATP released into the culture medium collected during the wounding process was calculated. The ATP concentration in the culture medium of resting cells was ~15 nM. Sham wounding caused no reduction in cell numbers and a threefold increase in ATP release. Extensive scratch wounding resulted in a loss of 52.5% of cells and an ATP concentration of 1.25 μM in the conditioned medium, representing 12.5×10^{-5} moles of ATP released from ~1.8×10^6 damaged cells. The volume of damaged cells was approximately 20 μl and the volume of culture medium in which the wounds were made was 2000 μl; therefore the concentration of extracellular ATP released from injured cells onto surrounding cells at the wound site can reach 125 μM at the moment of cell injury (Fig. 1A). Upon in vivo corneal wounding, cell debris and the released cellular content,

including ATP, are washed away by tears and blinking. To mimic this pathophysiological condition, injured cells in culture were rinsed once with PBS after which fresh medium was added. As shown in Fig. 1B, the luminescent reading increased slightly because of the mechanical stimulation induced by sham wounding and/or washing but eventually declined to the basal level in the non-wounded control cells. In extensively wounded THCE cells, the ATP concentration in the culture medium collected 2 minutes after wounding, was 8.8 times higher than that in the control-cell medium and remained elevated (five times higher than the control) 30 minutes after wounding (data not shown). Fig. 2C shows that ATP concentration in the culture
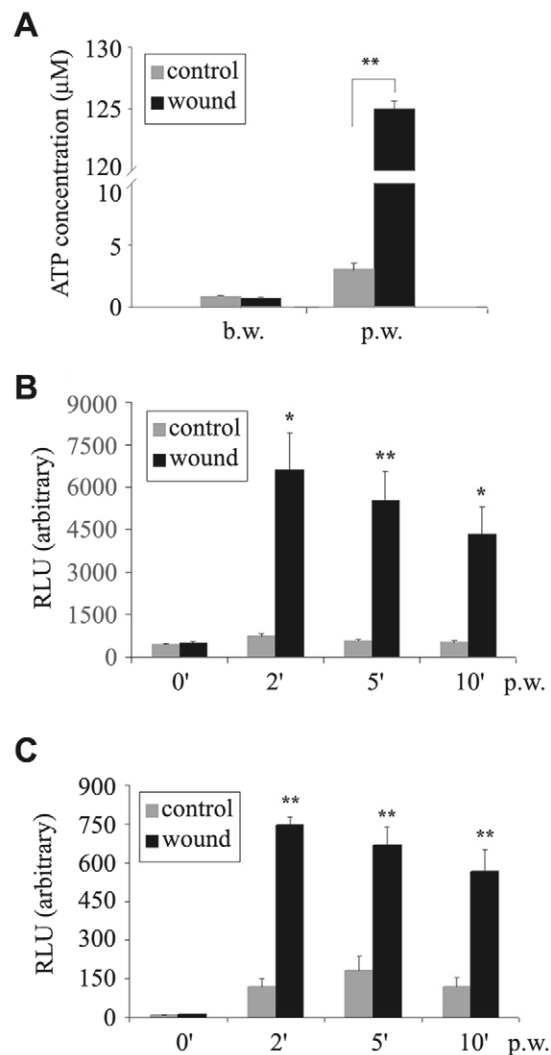

Fig. 1. Wounding increases ATP concentration in culture medium of corneal and airway epithelial cells. (A-C) THCE cells (A,B) or BEAS 2B cells (C) were seeded in 60-mm dishes and growth-factor- or serum-starved overnight. Cells were extensively wounded and ATP concentration in the culture medium was measured as described in Materials and Methods. Results are expressed as ATP concentration in μM (A) or relative light units (RLU) (B,C) and data are given as the mean ± s.e.m. (*n*=3). *$P<0.05$ and **$P<0.01$, significant increase of ATP concentration in culture medium after wounding compared with the control; b.w., before wounding, p.w., post wounding.

Fig. 2. Wounding-induced but not EGFR-ligand-induced AKT and ERK phosphorylation is ATP-dependent. (A) Growth factor-starved THCE cells were pretreated with a combination of apyrase and adenosine deaminase (5 units/ml each) for 30 minutes, and were then either extensively injured or stimulated with HB-EGF (50 ng/ml) as a positive control. After removing debris, cells were further incubated in fresh KBM containing the combination of apyrase and adenosine deaminase or HB-EGF for 2 minutes. Cell lysates were subjected to western blotting with antibodies against phosphorylated AKT (*P*-AKT), phosphorylated ERK 1/2 (*P*-ERK), AKT and ERK2. (B) Growth factor-starved THCE cells were pretreated with the apyrase and adenosine deaminase mix as in A and wounded with a 0.1-10 μl pipette tip. Cells were then fixed 15 minutes post wounding and incubated with antibody against *P*-ERK, followed by visualization with FITC-conjugated secondary antibody. Corresponding nuclear staining was performed with DAPI. Arrows indicate nuclear staining of *P*-ERK at the wound edge (arrowhead).
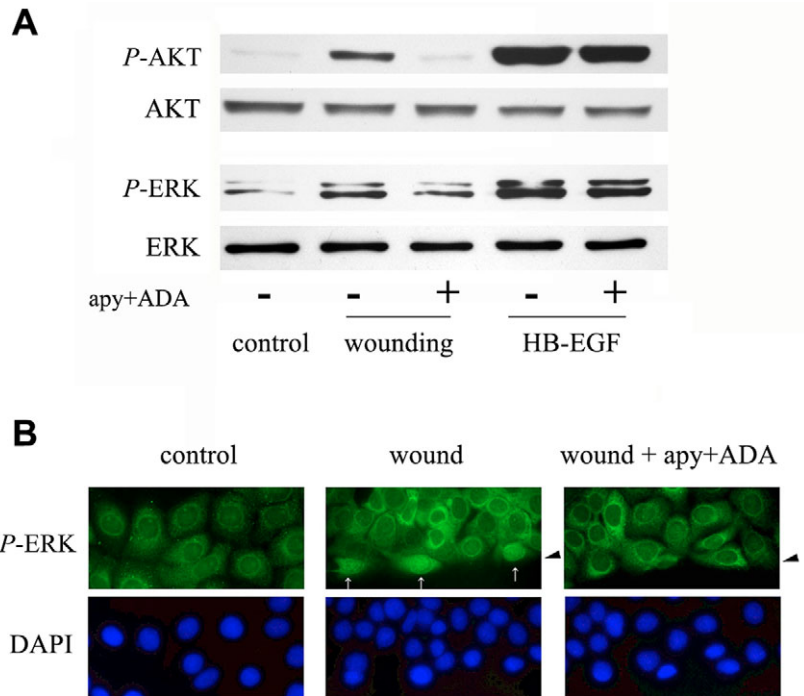
medium of extensively wounded bronchial epithelial (BEAS 2B) cells, collected 2, 5 and 10 minutes after wounding, was 6.2, 3.6 and 4.8 times, respectively, of that in the control-cell medium. Similar effects were also observed using intestinal epithelial cell Caco-2 cells (data not shown).

Wound-induced AKT and ERK activation is sensitive to the elimination of ATP, ADP and adenosine

We have previously shown that wounding of HCE cell monolayer triggers rapid phosphorylation of ERK1/2 and AKT (a major substrate and downstream effector of PI3K) (Xu et al., 2004a; Xu et al., 2004b). To determine the role of ATP in the early stage of cell activation in response to wounding, apyrase and adenosine deaminase were used to eliminate extracellular ATP, ADP and adenosine. Apyrase cleaves the terminal phosphate groups off ATP and ADP, and adenosine deaminase deaminates adenosine to inosine. The combination of these two enzymes, therefore, can be used to effectively scavenge adenosine purinoceptor agonists from culture medium (Ahmad et al., 2005). As expected, epithelial wounding induced rapid phosphorylation of AKT and ERK, observed as early as 2 minutes after wounding (Fig. 2A). This wound-induced phosphorylation of AKT and ERK was inhibited by treating cells with 5 units/ml apyrase and 5 units/ml adenosine deaminase. However, these enzymes exhibited minimal effects on HB-EGF-induced AKT and ERK activations, suggesting that ATP acts upstream to the release of EGFR ligand(s) and the activation of EGFR in HCE cells. ATP-stimulated ERK activation was further confirmed by immunostaining (Fig. 2B). Unwounded control cells exhibited low levels of phosphorylated (*P*-) ERK; however, there was a marked increase in the staining for *P*-ERK in the several rows of cells close to the wounding edge. Furthermore, cells at the wounding edge displayed nuclear translocation of *P*-ERK. In cells depleted of the purinoceptor agonist, there appeared to be less

– albeit above control – cytoplasmic *P*-ERK staining; no nuclear *P*-ERK staining was detected.

ATP-γ-S induces the activation of EGFR signaling

Since many cellular components including lipids and proteins are also known to activate cells around the site of injury, we next investigate whether wounding-induced release of ATP contributes to the activation of epithelial cells by challenging uninjured cells with ATP. We used the nonhydrolyzable ATP analog ATP-γ-S to mimic extracellular ATP released after wounding. In the initial preliminary experiments, THCE cells were found to respond to as little as 1 μM ATP-γ-S, and consistent maximal effects on ERK and AKT phosphorylation were observed at 100 μM ATP-γ-S (data not shown, also see Fig. 5A). Thus, 100 μM ATP-γ-S was used to examine the activation of EGFR and its downstream effectors, AKT and ERK in THCE cells (Fig. 3A), primary HCE cells (Fig. 3B), as well as BEAS 2B cells (Fig. 3C). In THCE cells, 100 μM ATP-γ-S induced phosphorylation of EGFR that occurred as early as 5 minutes post stimulation (the earliest time tested) and this elevated EGFR phosphorylation was still detectable 2 hours after wounding. Consistent with EGFR activation, AKT and ERK phosphorylation was also observed during the course of ATP-γ-S stimulation. Furthermore, ATP-γ-S also elicited a similar response in primary HCE cells and BEAS 2B cells, although its effect on signaling in BEAS 2B cells was more transient than that in THCE cells.

ATP-γ-S-induced AKT and ERK activation is Ca^{2+} and EGFR dependent

Klepeis et al. showed that mechanical injury induces an elevation in Ca^{2+} that was immediately initiated at the wound edge and traveled as a wave to neighboring cells; they predicted that the propagation of Ca^{2+} wave occurred via diffusion of an extracellular mediator, most probably a nucleotide (Klepeis et

Fig. 3. ATP-γ-S activates EGFR signaling in corneal and airway epithelial cells. (A-C) Growth-factor-starved THCE cells (A), primary HCE cells (B) or serum-starved BEAS 2B cells (C) were stimulated with 100 μM ATP-γ-S for the indicated times. Cells were then lysed. THCE cell lysates were immunoprecipitated with EGFR antibody, immunoblotted with anti-PY99 antibody (*P*-EGFR) and re-probed with anti-EGFR antibody (EGFR) to assess the amount of EGFR precipitated. Lysates of THCE, primary HCE and BEAS 2B cells were also subjected to western blotting with antibodies against phosphorylated AKT (*P*-AKT), phosphorylated ERK1/2 (*P*-ERK), EGFR Y1068, AKT and ERK2.
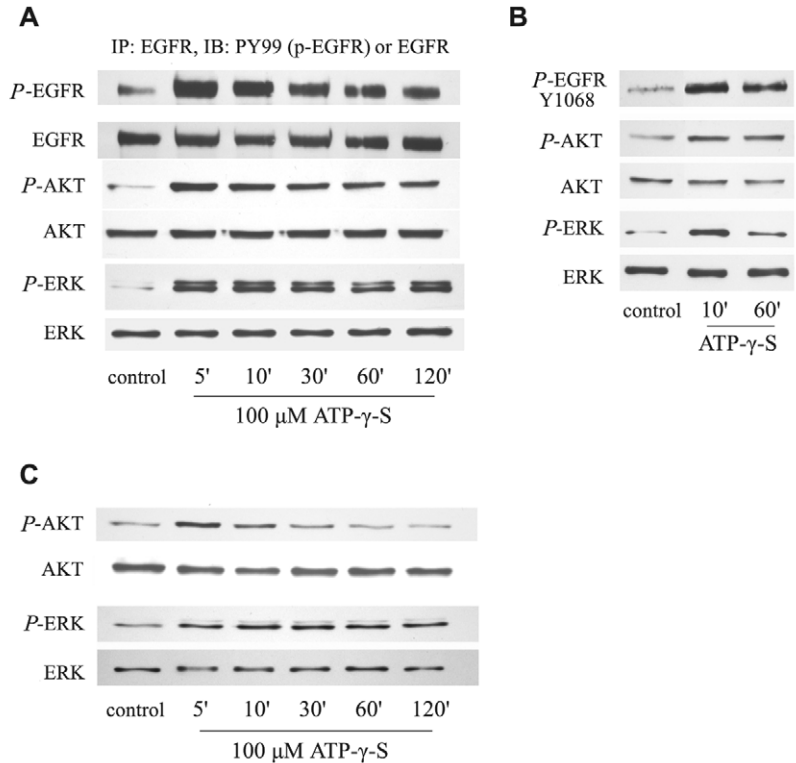
al., 2001). To examine whether ATP-γ-S-induced cell activation is Ca^{2+} dependent, we pretreated THCE cells with BAPTA-AM, a selective chelator of intracellular Ca^{2+}, and examined AKT and ERK activation. As shown in Fig. 4A, ATP-γ-S-induced AKT and ERK phosphorylation was abolished by BAPTA-AM, suggesting that the effect of ATP on cell activation is through the release of Ca^{2+} from intracellular storage. Moreover, presence of the selective EGFR inhibitor AG1478 in the medium attenuated ATP-γ-S-elicited AKT and ERK phosphorylation. Taken together, these results indicate that extracellular ATP-induced activation of PI3K and ERK is Ca^{2+} dependent and occurs via EGFR signaling network.

ATP-γ-S induces HB-EGF shedding and enhances the closure of small scratch wounds

Using epithelial wound model and a THCE cell line transfected with the expression plasmid HB-EGF-AP (Dethlefsen et al., 1998; Xu et al., 2004a), we previously showed that wounding elicited HB-EGF ectodomain shedding and the released HB-EGF acted as an endogenous agonist for EGFR activation in an autocrine and/or paracrine manner (Xu et al., 2004a). To determine whether wounding-induced ATP surge causes HB-EGF shedding, we stimulated the cells expressing HB-EGF-AP with ATP-γ-S and measured alkaline phosphatase (AP) activity in the culture medium (Fig. 5). Fig. 5A shows a dose response curve, indicating a linear increase in the release of AP in THCE cells stimulated with increased (1-100 μM) concentration of ATP-γ-S. At 100 μM, ATP-γ-S stimulation resulted in 3.5-fold, 8.1-fold and 8.7-fold increase in AP activity in the culture medium compared with the control at 10, 30 and 60 minutes, respectively, post stimulation, indicating that ATP-γ-S stimulates pro-HB-EGF shedding in HCE cells (Fig. 5B). Since ADP is also a P2-receptor agonist, we compared the effects of 100 μM ADP, ATP-γ-S and wounding on HB-EGF shedding (Fig. 5C). As shown before, extensive wounding induced a dramatic increase in AP activity (Xu et al., 2004a). ADP also increased HB-EGF shedding, although its effect was much less than that of ATP-γ-S.

To evaluate the effects of ADP and ATP-γ-S on wound healing, THCE monolayers were wounded with a tooth of a DNA-sequencing-gel comb to create a small scratch wound and were allowed to heal in keratinocyte basal medium (KBM) with or without 100 μM ADP or ATP-γ-S for 6 hours. Since

KBM does not contain any growth factors, the wound closure was termed 'spontaneous wound healing'. Whereas the control cells healed 18%, cells treated with 100 μM ATP-γ-S covered 51% of the original wound within 6 hours (Fig. 6). ADP, however, was unable to stimulate wound closure (15.5% wound area covered).

HB-EGF shedding and EGFR activation induced by ATP-γ-S are sensitive to ADAM inhibition

Shedding of EGFR ligands, such as HB-EGF and TGF-α, has been suggested to be mediated by ADAMs (Higashiyama and Nanba, 2005). To determine the involvement of ADAMs in extracellular ATP-induced HB-EGF shedding, cells expressing HB-EGF-AP were first treated with the broad-spectrum metalloproteinase inhibitor GM 6001 or its inactive analog (Fig. 7A). GM 6001, but not its inactive analog, greatly attenuated basal and ATP-γ-S-induced AP activity in the culture medium. To assure the involvement of ADAMs in the shedding of HB-EGF in HCE cells, the specific inhibitor of ADAM10 and ADAM17, GW280264X (Hundhausen et al., 2003), was used to treat cells expressing HB-EGF-AP. GW280264X attenuated basal as well as ATP-γ-S-induced HB-EGF shedding, suggesting that ADAM10 and/or ADAM17 participate in the process (Fig. 7B). Moreover, GM6001 and GW280264X attenuated ATP-γ-S-induced EGFR phosphorylation (Fig. 7C). CRM197, an HB-EGF antagonist, partially inhibited ATP-γ-S induced EGFR phosphorylation, indicating that HB-EGF is, at least in part, responsible for the signaling from ATP to EGFR.

ATP-γ-S enhanced epithelial wound closure is related to HB-EGF-mediated EGFR transactivation
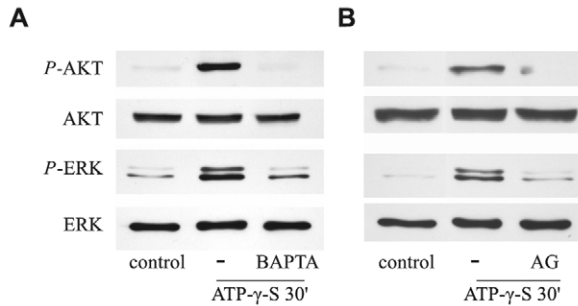
Having demonstrated that ATP-γ-S induces EGFR

Fig. 4. ATP-γ-S-induced AKT and ERK phosphorylation is Ca^{2+} and EGFR dependent. Growth-factor-starved THCE cells were pretreated with either 50 μM Ca^{2+} chelator BAPTA-AM (A) or 1 μM EGFR inhibitor AG 1478 (B) for 1 hour and then stimulated with 100 μM ATP-γ-S for 30 minutes. Cell lysates were subjected to western blotting with antibodies against phosphorylated AKT (*P*-AKT), phosphorylated ERK1/2 (*P*-ERK), AKT and ERK2.

transactivation through ADAM-mediated ectodomain shedding and enhances wound healing in HCE cells, we next sought to determine the effects of inhibitors, targeting different steps of the HB-EGF–EGFR–ERK–PI3K pathway on the healing of small scratch wound. As shown in Fig. 6, ATP-γ-S significantly accelerated wound closure in HCE cells. This ATP-γ-S-enhanced wound healing was attenuated by AG1478, CRM197, GM6001, GW280264X, U0126 (MEK inhibitor) and wortmannin (PI3K inhibitor) to levels similar to those of control cells, suggesting that HB-EGF shedding and subsequent activation of EGFR signaling pathways are required for ATP to carry out its wound-healing-enhancing function (Fig. 8).

The P2Y antagonist reactive blue 2 inhibits spontaneous wound healing and HB-EGF shedding

To determine the effects of purinoceptor blockade on spontaneous HCE cell wound healing, a monolayer of THCE cells was wounded with pipette tips and allowed to heal in KBM containing 100 μM reactive blue 2 (RB2) for 24 hours. RB2 is a P2Y purinoceptor antagonist and especially potent for ATP-activated channels. As shown in Fig. 9A,B, RB2 inhibited wound closure in HCE cells; in the presence of RB2 only 21.7% of the wound was covered, whereas 70.7% was healed in control cells Suramin, an inhibitor of both P2X and P2Y purinoceptors, and also a weak antagonist of growth factors, also attenuated ATP-γ-S-enhanced wound closure (data not shown). To determine whether ATP acts through an intracellular purinoceptor to trigger HB-EGF shedding, we pretreated cells expressing HB-EGF-AP with RB2, challenged with ATP-γ-S and measured the release of AP in the culture medium (Fig. 9C). RB2 significantly (*P*<0.001) inhibited ATP-γ-S induced, but not basal, shedding of HB-EGF in THCE cells, suggesting that an intracellular P2Y receptor is required for signaling to link ATP to HB-EGF shedding.

Discussion

We previously showed that EGFR signaling network is activated in response to wounding and plays a key role in mediating corneal epithelial wound healing. To identify
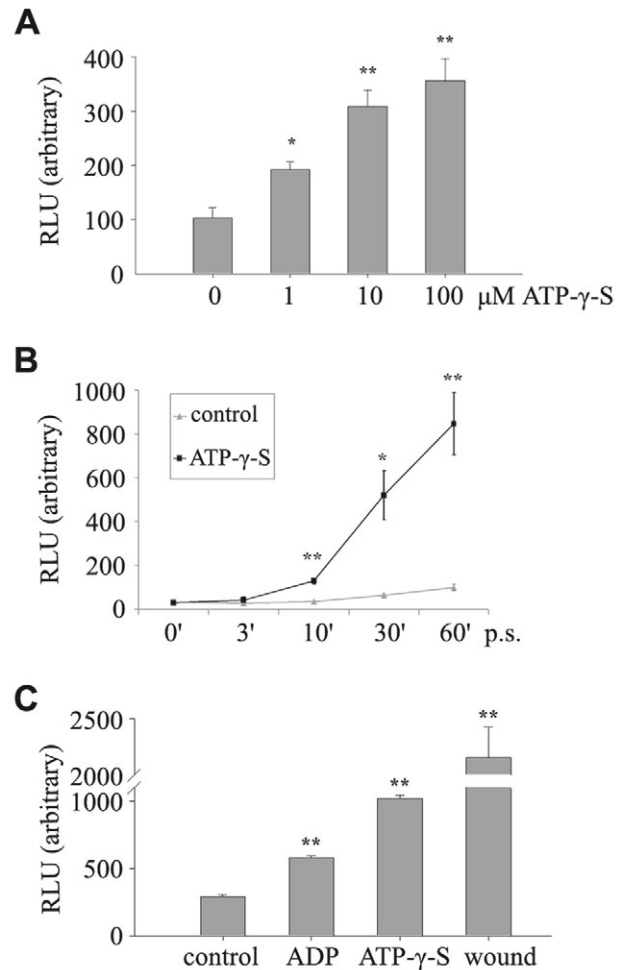

Fig. 5. ATP-γ-S induces HB-EGF release in THCE cells expressing HB-EGF-AP. (A) Cells were cultured in 6-well dishes and treated with different concentrations of ATP-γ-S (1-100 μM) for 20 minutes. AP released into the collected medium was measured as described in Materials and Methods and expressed as relative light units (RLU). (B) Cells were stimulated with 100 μM ATP-γ-S for indicated times post stimulation (p.s.); AP activity was measured and expressed as in A. (C) Cells were stimulated with 100 μM ADP, 100 μM ATP-γ-S, or were extensively wounded; AP activity was measured and expressed as in A. Each RLU represents the mean ± s.e.m. (*n*=3). *P<0.05 and **P<0.01, significant increase in AP released into the culture medium after ATP-γ-S treatment compared with the control.

extracellular mediator(s) that participate in the activation of EGFR signaling, we showed in this study that ATP is released upon cell injury and that the released ATP acts as an extracellular mediator of EGFR signaling in cultured HCE cells as well as in bronchial epithelial cells. Stimulation of THCE cells with nonhydrolyzable ATP-γ-S resulted in rapid and prolonged activation of EGFR and its downstream signaling pathways PI3K-AKT and ERK-MAPK. ATP-γ-S stimulated PI3K and ERK activation in a Ca^{2+}-dependent manner and through induction of HB-EGF ectodomain shedding and EGFR transactivation. Consistent with its ability to stimulate EGFR signaling, the presence of exogenous ATP-γ-S in the culture medium enhanced HCE cell wound closure, whereas blockade of the ATP-purinoceptor pathways with two
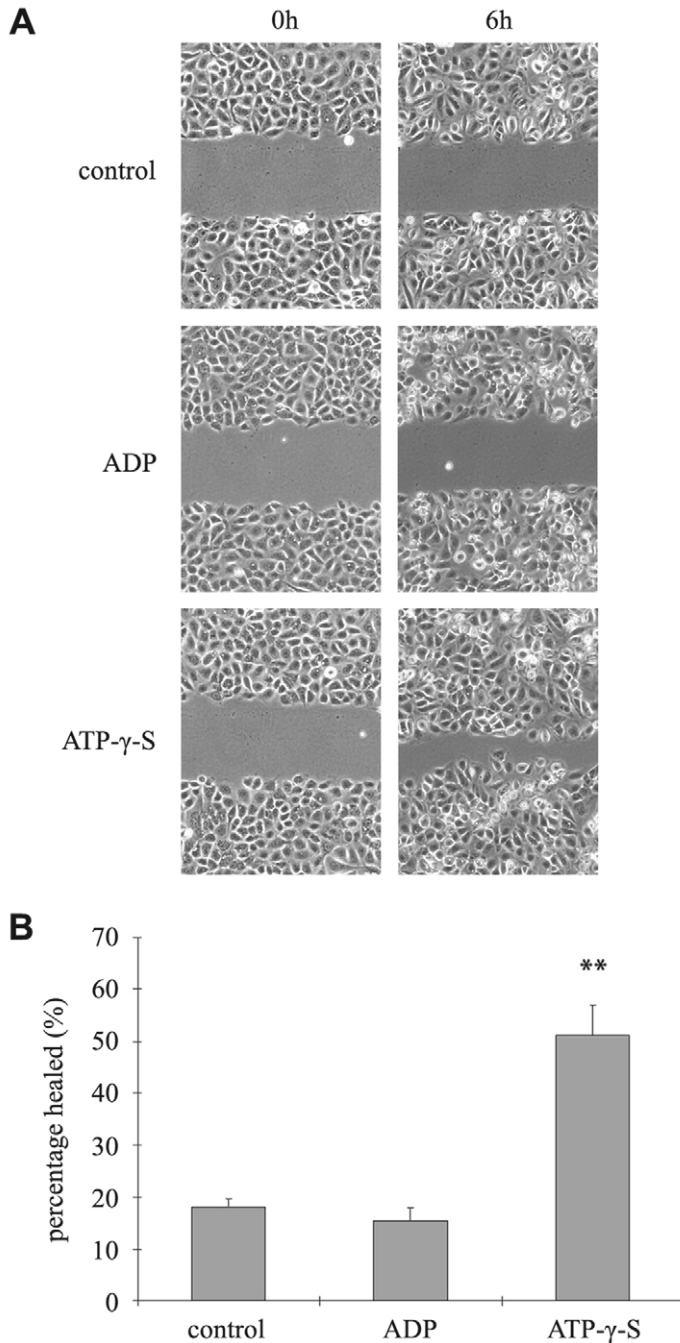
Fig. 6. ATP-γ-S, but not ADP, enhances epithelial wound closure. (A,B) Growth-factor-starved THCE cells were wounded with a single tooth of a 48-well sharkstooth comb for DNA sequencing gels. Cells were allowed to heal in KBM containing 100 μM ADP or 100 μM ATP-γ-S. Wound closure was photographed immediately after wounding (0 h) or 6 hours post wounding (6 h). Micrographs (A) represent one of three samples performed each time. Statistical analysis (B) indicates healing extent. Values are expressed as the mean ± s.e.m. (*n*=3), **P<0.01.

Fig. 7. ATP-γ-S-induced HB-EGF shedding and EGFR activation are sensitive to ADAM inhibition. (A,B) Cells expressing HB-EGF-AP were pretreated with (A) 50 μM GM6001 (GM) or its inactive analog (GM neg) or (B) 4 μM GW280264X (GW) for 1 hour and stimulated with 100 μM ATP-γ-S. AP activity was measured and expressed as in Fig. 5. **P<0.01, significant decrease in AP release by the inhibitors in the control and ATP-γ-S-treated cells. NT, no inhibitor treatment. (C) Growth-factor-starved THCE cells were pretreated with 10 μg/ml CRM197 (CRM), 4 μM GW280264X, 50 μM GM6001 or 50 μM GM6001 inactive analog for 1 hour and then stimulated with 100 μM ATP-γ-S for 10 minutes. Cells were then lysed and lysates were subjected to EGFR immunoprecipitation as described in Fig. 3A and phosphorylation determination.

different inhibitors retarded it. These findings provide new insights into the molecular mechanisms by which the EGFR signaling pathway is activated upon wounding, leading to the initiation of epithelial response to injury. The potential
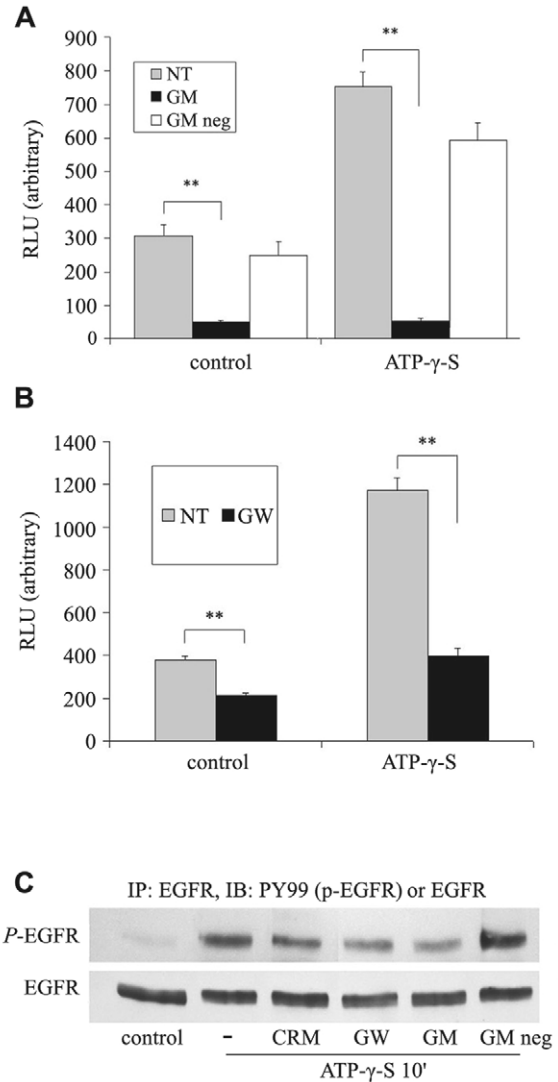
involvement of released ATP in EGFR transactivation and wound healing is summarized in Fig. 10.

The steady-state cytosolic concentration of ATP, the fuel for countless metabolic and enzymatic reactions, is 3-10 mM; whereas the steady-state extracellular ATP is approximately 10 nM under basal conditions (Schwiebert and Zsembery, 2003). Here, we directly measured the release of ATP in ATP bioluminescence assays, and observed ~15 nM ATP on resting cells – well below the range to activate purinoceptors such as
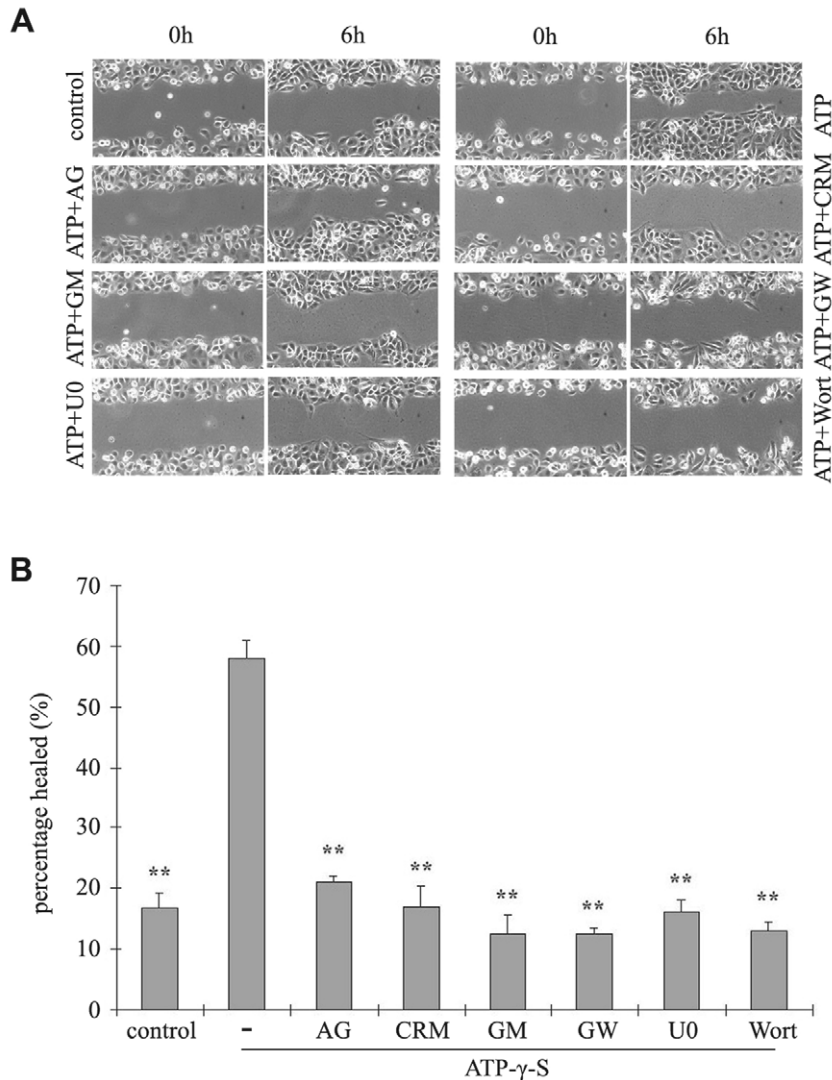
Fig. 8. ATP-γ-S enhances epithelial wound closure in an EGFR-, HB-EGF-, ADAM10-, ADAM17-, ERK- and PI3K-dependent manner. Growth-factor-starved THCE cells were pretreated with the following inhibitors 1 μM AG 1478 (AG), 10 μg/ml CRM197 (CRM), 50 μM GM6001 (GM), 4 μM GW280264X (GW), 10 μM U0126 (U0) or 100 nM wortmannin (Wort) for 1 hour, wounded with a single tooth of a 48-well sharkstooth comb for DNA sequencing gels and allowed to heal in KBM containing 100 μM ATP-γ-S in the presence of inhibitors. Wound closure was photographed immediately after wounding (0 h) or 6 hours p.w. (6 h). Micrographs in A represent one of three samples performed each time. Bar graph in B gives the statistical analysis of healing extent. Values are the mean ± s.e.m. (n=3), **P<0.01.

P2Y. Scratch wounds result in the loss of more than 50% of cells and a surge of ATP released from these damaged cells in the culture medium (1.25 μM of wounded cells vs 60 nM of sham-wounded cells). This suggests that, in vivo at the moment of cell injury, the extracellular ATP concentration reaches 125 μM or more around wound site in the cornea or other epithelial surface. Thus, cell injury causes a remarkable outflow of ATP that reaches the range for P2Y receptor activation (Lazarowski et al., 1995; Paradiso et al., 2001). After PBS washing that mimics tears and blinking, a significant amount of ATP was still present in wounded epithelial cell culture. We also demonstrated that extracellular ATP concentration was elevated after wounding in bronchial and intestinal epithelial cells (data not shown), indicating that injury-caused ATP release might be a common scenario among epithelial cells. Hence, we suggest that epithelial injury causes a release of ATP high enough to locally elicit intracellular cell signaling, such as a Ca^{2+} wave (Klepeis et al., 2001; Klepeis et al., 2004). Indeed, we showed that in the presence of apyrase, an ATPase and ADPase that is known to eliminate ATP and ADP from solutions rapidly, and adenosine deaminase, which converts adenosine into inert inosine in the medium, abolished early wound-induced AKT and ERK phosphorylation, as well

as nuclear translocation of P-ERK. Injury-induced ERK1/2 phosphorylation due to the activation of P2Y receptors by released ATP has been reported previously (Yang et al., 2004). Thus, our results showed that extracellular ATP released from the injured cells activates not only ERK but also the PI3K-AKT pathway in surrounding uninjured epithelial cells.

PI3K-AKT and ERK1/2 are two common effectors of receptor tyrosine kinase signaling pathways, including the EGFR pathway. Our previous studies showed that epithelial wounding resulted in rapid activation of these two pathways in an EGFR-dependent manner (Xu et al., 2004a). The observation that elimination of ATP from the medium attenuated wound-induced activation of ERK and PI3K suggests a potential role of extracellular ATP in EGFR transactivation. Using the nonhydrolyzable ATP analogue ATP-γ-S and cell lines expressing HB-EGF-AP, we observed that ATP-γ-S stimulated the release of AP in a concentration-dependent manner (1-100 μM tested), with 100 μM ATP-γ-S providing consistent and maximal effects. Thus, we used 100 μM ATP-μ-S in our study and observed that the presence of 100 μM ATP-γ-S in the culture medium stimulated rapid and strong phosphorylation of EGFR. As discussed above, the ATP concentration can reach 100 μM or higher in cells neighboring
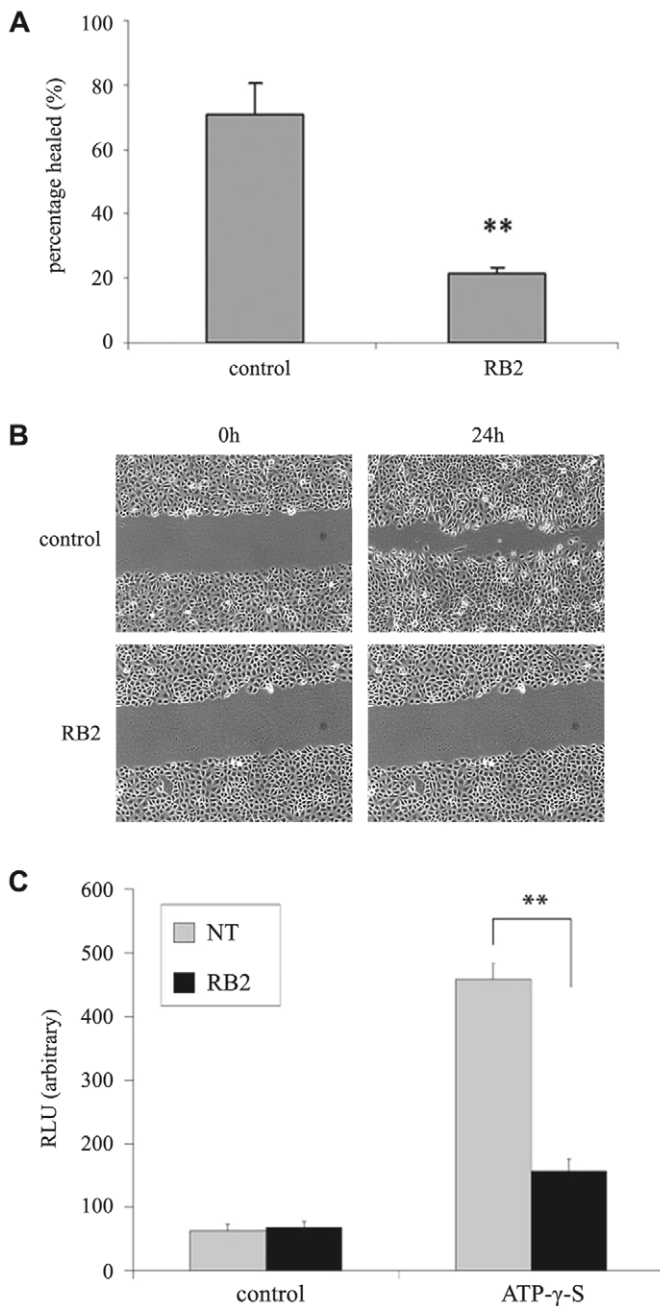
Fig. 9. The P2 antagonist RB2 attenuates epithelial wound closure and HB-EGF shedding. (A,B) Growth factor-starved THCE cells were pretreated with 100 µM RB2 for 1 hour and then wounded with a 0.1-10 µl pipette tip. Cells were allowed to heal in KBM with (RB2) or without (control) RB2 for 24 hours. Cell migration was monitored by photographing the wound immediately (0 h) or 24 hours post wounding (24 h). Micrographs in A represent one of three samples performed each time. Statistical analysis of healing extent is shown in B. Values are the mean ± s.e.m. (n=3), **P<0.01. (C) Cells expressing HB-EGF-AP were pretreated with (RB2) or without (NT) 10 µm RB2 and stimulated with 100 µM ATP-γ-S. AP activity was measured and expressed as RLU. **P<0.01, significant inhibition in AP release by RB2 in the control or ATP-γ-S-treated cells.

hydrolyzed by cytosolic esterases and trapped intracellularly as the active chelator BAPTA. Thus, release of Ca^{2+} from intracellular stores is an important link between extracellular ATP and EGFR transactivation. Furthermore, the study that used THCE cells expressing HB-EGF-AP revealed that ATP-γ-S elicited HB-EGF shedding, providing a line of evidence that HB-EGF is an EGFR ligand that is cleaved upon ATP stimulation.

Ectodomain shedding of EGFR ligands has been linked to the ADAM family of proteins of which ADAM10 and ADAM17/TACE are particularly important in the context of ectodomain generation (Sahin et al., 2004; Sanderson et al., 2006; Schlondorff and Blobel, 1999). Using the broad-spectrum MMP inhibitor GM6001, and the ADAM10- and ADAM17-specific inhibitor GW280264X, we showed that both ATP-induced release of HB-EGF and activation of EGFR are sensitive to these inhibitors, suggesting the involvement of ADAM10 and ADAM17 in extracellular ATP-mediated EGFR transactivation. It should be mentioned that GW280264X consistently inhibited ATP-γ-S-induced HB-EGF shedding and EGFR phosphorylation in several separated experiments; the effects of GI254023X, which preferentially blocks ADAM10, are inconclusive because the treatment is either partially inhibitory or not significantly different from the control in separated experiments (data not shown). The reason for the discrepancy among experiments using GI254023X is not clear. It might be because ADAM10 inhibits constitutive – but not induced – ectodomain shedding of HB-EGF, like that shown for CX3CL1 (Hundhausen et al., 2003). Furthermore, inhibition of P2Y receptors with RB2 significantly attenuated the ATP-γ-S-induced release of HB-EGF-AP in cultured HCE cells, suggesting the requirement of intracellular purinoceptor(s) for elevated extracellular ATP to trigger ADAM activation and HB-EGF shedding. Taken together, extracellular ATP, through activated purinoceptors, may function in HCE cells by generating an intracellular Ca^{2+} wave that, in turn, induces ADAM-mediated HB-EGF ectodomain shedding and EGFR transactivation, leading to the activation of PI3K-AKT and ERK-MAPK pathways. Similarly, activation of PI3K-AKT and ERK-MAPK pathways was also observed in bronchial epithelial cells challenged with ATP-γ-S, suggesting that ATP-mediated EGFR transactivation and activation of its downstream signaling pathways are a common phenomenon in epithelial cells.

Consistent with its ability to stimulate EGFR transactivation, ATP-γ-S enhanced HCE cell migration into the denuded region of a scratch wound. Of the two P2-receptor families, P2X

injured cells. In line with this, a recent study – using recombinant membrane-targeted luciferase – detected 100-200 µM ATP that was released locally in HEK293 cells after activation of $P2X_7$ (Pellegatti et al., 2005). Hence, the use of 100 µM ATP-γ-S is physiologically relevant. Consistent with activation of EGFR, ATP-γ-S also activated AKT and ERK in HCE and BEAS 2B cells, suggesting a common pathway from elevated extracellular ATP to EGFR signaling network in epithelial cells. Furthermore, our pharmacological studies revealed that the ATP-γ-S-stimulated activation of PI3K-AKT and MAPK was Ca^{2+} dependent and required the activity of EGFR kinase, because BAPTA-AM and AG1478 blocked ATP-γ-S-induced AKT and ERK1/2 phosphorylation in THCE cells. BAPTA-AM is a membrane-permeable form of the Ca^{2+}-chelating agent, which is freely taken up by cells, where it is
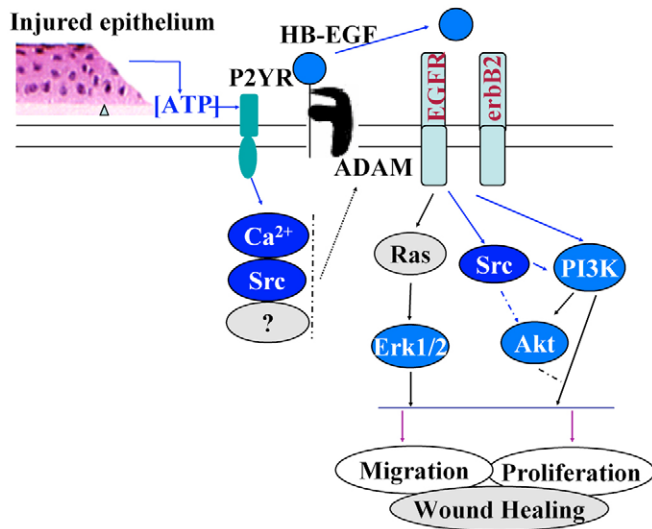
Fig. 10. Proposed extracellular ATP involvement in corneal epithelial wound healing that summarizes the pathway from ATP release caused by cell injury to wound closure, including P2Y activation, intracellular Ca^{2+} release and EGFR signaling. Wounding results in the release of ATP from damaged cells, and the released ATP – through its P2 receptors – triggers intracellular Ca^{2+} waves leading to activation of ADAM protein(s) in a neighboring cell that is not injured ($\triangle$). ADAM protein(s) cleaves pro-HB-EGF at the cell surface and HB-EGF in an autocrine and/or paracrine fashion binds EGFR, which transduces the signals into the intracellular signaling network. After EGFR activation, HCE cell migration, proliferation and wound healing are induced via PI3K, ERK and other intracellular signaling pathways.

receptors represent a class of ligand-gated ion channels with two predicted transmembrane domains, whereas P2Y receptors belong to the superfamily of rhodopsin-like G-protein-coupled receptors with seven transmembrane domains. To date, there is only one study reporting that exogenously added ATP enhances the release of neural cell-adhesion molecule from hippocampal neurons in culture (Hubschmann et al., 2005). Our study is the first to link extracellular ATP released upon injury to EGFR transactivation and wound healing. Furthermore, our data showed that ADP induces low-level HB-EGF shedding and has no effect on wound closure, a result consistent with a recent report (Mediero et al., 2006), suggesting that ATP, but not ADP, serves as one of the initial signals to trigger wound response in HCE cells. Previous studies, using various inhibitors, showed that P2Y but not P2X receptors are responsible for ATP-induced Ca^{2+} dynamics in human and rabbit corneal epithelium (Kimura et al., 1999; Klepeis et al., 2004). To assess the involvement of extracellular ATP in epithelial wound healing, we used two purinoceptor inhibitors suramin and RB2 to determine the effects of P2Y blockade on HCE cell wound healing. Both suramin and RB2 abrogated spontaneous wound closure effectively, indicating the potential importance of P2Y receptors in epithelial wound healing in the cornea. As several isoforms of P2Y were identified in human corneal epithelial cells (Klepeis et al., 2004), further studies targeting specific P2Y isoforms are warranted.

In conclusion, our results demonstrated that the release of endogenous ATP upon mechanical injury serves as one of the extracellular mediators that trigger EGFR transactivation and the activation of its downstream signaling pathways in corneal and bronchial epithelial cells. Thus, injury-induced release of ATP and its subsequent induction of Ca^{2+} waves may serve as an early response to stress including wounding and/or infection (Kaufmann et al., 2005), and provide a tissue damage signal to promote wound healing and functional recovery in epithelial cells. Because ATP and other P2Y-receptor agonists, such as diuridine tetraphosphate (diquafosol) and the uracil-cytosine dinucleotide denufosol, are already in clinical trials for certain diseases, including dry-eye disease, retinal detachment, upper respiratory tract symptoms, cystic fibrosis and lung cancer (Agteresch et al., 2000; Agteresch et al., 2002; Brunschweiger and Muller, 2006), these findings may eventually lead to applications in preventing and/or treating wound-healing-related diseases in the cornea and other tissues and organs.

Materials and Methods
Materials
Defined keratinocyte serum-free medium (SFM) was purchased from Invitrogen (Grand Island, NY). Keratinocyte basal medium (KBM) was from BioWhittaker (Walkersville, MD). Fibronectin-collagen coating mix (FNC) was from Biological Research Faculty and Facility (Ljamsville, MD). Human recombinant HB-EGF was obtained from R&D System (Minneapolis, MN). The Ca^{2+} EGFR inhibitor AG1478 chelator BAPTA-AM, MEK inhibitor U0126, GM6001, a hydroxamic acid matrix metalloproteinase (MMP) inhibitor, and its inactive analog were from Calbiochem (La Jolla, CA). Hydroxamaate GW280264X, capable of blocking ADAM10 and ADAM17, and also GI254023X, which preferentially blocks ADAM10 and not ADAM17, were kindly provided by Andreas Ludwig, Christian-Albrechts-University, Kiel, Germany (Hundhausen et al., 2003). Antibodies against human EGFR, ERK2 (p42 MAPK), *P*-ERK1/2 (p42/p44), phosphorylated tyrosine (PY99) and phosphorylated (*P*)-EGFR (tyrosine 1068) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against AKT and *P*-AKT were obtained from cell signaling (Beverly, MA). All other reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Cell culture
Three epithelial cell types were used in this study. THCE cells, an SV40-immortalized HCE cell line (Araki-Sasaki et al., 1995), were maintained in defined keratinocyte SFM as previously described (Xu et al., 2004b). Primary HCE cells were isolated from human donor corneas obtained from Michigan Eye Bank. The epithelial sheet was separated from underlying stroma after overnight dispase treatment at 4°C. The dissected epithelial sheet was trypsinized and cells were then collected by centrifugation (500 g, 5 minutes). Primary HCE cells were grown in defined keratinocyte SFM in a humidified 5% CO_2 incubator at 37°C and used at passage three. Normal immortalized bronchial epithelial cell line, BEAS 2B, was provided by Shijie Sheng (Wayne State University School of Medicine, Detroit, MI) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Bioluminescence detection of extracellular ATP
Cells grown on FNC-coated 60-mm dishes were cultured in basal medium overnight for growth factor starvation or serum starvation before experiments. Cells were extensively wounded by multiple linear scratches using a cut of a 48-well sharkstooth comb for DNA sequencing gel (Bio-Rad, Hercules, CA). Cells of the control group were sham-wounded in that the comb did not touch the cells. To measure ATP release upon wounding, the medium in which the scratch wounds were made (2 ml) was collected and cell debris removed by centrifugation. Cells were trypsinized, centrifuged at 500 g for 5 minutes and the volume of cell pellets was estimated. The cells were then resuspended in basal medium and the numbers of remaining cells were determined with Z1 Coulter particle counter (Beckman Coulter). The presence of ATP in the collected culture medium was measured using an ATP Bioluminescent Assay Kit following manufacturer's instruction (Sigma-Aldrich). Briefly, 100 μl culture medium was mixed with 100 μl ATP Assay Mix solution and luminescence was quantified on a GENios Fluorometer (Phenix Research Products, Candler, NC). ATP concentration was determined by comparing the luminescence of samples with an ATP-standard curve. To determine retention of the released ATP after wounding, cells were injured in the same fashion, washed once with phosphate buffered saline (PBS) and 2 ml fresh basal medium was added to the culture. At the indicated time points, 150 μl culture medium was collected into a chilled polypropylene tube. ATP concentration was measured as described above. At the end of sample collection, cells were lysed for protein determination, using bovine serum albumin as a

standard (BCA kit, Pierce, Rockford, IL). The readings, after subtracting background luminescence of basal medium, were normalized against culture medium volume and cellular protein concentration, and results were expressed as the mean of relative light units (RLUs) ± s.e.m. (*n*=3). Statistical parameters were ascertained by software (SigmaStat), with the Student's *t*-test and a value of *P*<0.05 indicating a significant difference.

Immunofluorescence staining of *P*-ERK

THCE cells grown on 8-well glass chamber slides were pretreated with a mix of 5 units/ml apyrase and 5 units/ml adenosine deaminase for 30 minutes, then wounded with a 0.1-10 µl pipette tip, and cultured for further 15 minutes. Cells were washed in PBS and fixed with methanol at –20°C for 20 minutes. Slides were blocked with PBS containing 10% goat serum at room temperature for 1 hour and then incubated with anti-*P*-ERK1/2 mouse monoclonal antibody (1:20) in PBS containing 4% bovine serum albumin (BSA), at 4°C overnight. Following the washes in PBS, cells were incubated with FITC-conjugated donkey anti-mouse IgG (1:100) in PBS containing 0.1% BSA, at room temperature for 1 hour. Slides were then washed in PBS and mounted with Vectashield mounting medium with DAPI, and examined under a Carl Zeiss fluorescence microscope Axioplan 2 equipped with an ApoTome digital camera (Carl Zeiss Microimaging. Inc.)

Determination of EGFR, AKT and ERK activation by western blotting

Cells were cultured to confluence on FNC-coated 100-mm dishes and growth-factor-starved in basal medium overnight. After different treatments, cells were lysed with RIPA buffer (150 mM NaCl, 100 mM Tris-HCl pH 7.5, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktails, and 0.1 mM phenylmethylsulfonyl fluoride). For each sample, 600 µg proteins were immunoprecipitated with antibody against EGFR (10 µg) and the precipitants were immunoblotted with antibody against PY99. Membrane was then stripped and reprobed with antibody against EGFR to evaluate the total amount of EGFR precipitated. Phosphorylation of EGFR at tyrosine (Y) 1068 was analyzed using specific antibody. Phosphorylation of ERK1/2 and AKT was determined using monoclonal antibodies against *P*-ERK1/2 and *P*-AKT; antibodies against ERK2 and AKT were used to detect equal protein loading of the respective phosphorylated proteins.

Measurement of HB-EGF shedding

A cell line expressing HB-EGF-AP fusion protein with AP inserted into the heparin binding region of HB-EGF was established by transfection of THCE cells with the expression plasmid pHB-EGF-AP (Xu et al., 2004a). Cells were cultured in 6-well plates and challenged with ADP, ATP-γ-S (Sigma) or by extensive wounding after growth factor starvation. At indicated time points, 100 µl culture medium was collected and AP activity in collected media was measured using Great EscAPe SEAP Chemiluminescence Detection Kit (BD Biosciences, Palo Alto, CA) following the manufacturer's instruction. Briefly, 15 µl of the collected culture medium was heated with dilution buffer at 65°C for 30 minutes in a 96-well plate, followed by addition of assay buffer and substrate. Chemiluminescence was quantified on a GENios Fluorometer. The readings, after subtracting background luminescence in KBM, were normalized against culture-medium volume and cellular protein concentration as measured with BCA kit and shown as RLUs. The results are presented as the mean ± s.e.m. (*n*=3). Statistical parameters were ascertained by software (SigmaStat) with Student's *t*-test and a value of *P*<0.05 was considered significantly different.

Wounding assays

To assess the effects of ADP and ATP signaling on wound closure, THCE cells were cultured to confluence on 12-well plates and starved overnight in KBM. Cells were pretreated with different inhibitors for 1 hour, then wounded by scratch with a single tooth of a 48-well DNA-sequencing gel comb (to create a small scratch) and further cultured for 6 hours in fresh KBM containing 100 µM ADP or 100 µM ATP-γ-S, with or without different inhibitors, for 6 hours. To study the effects of purinoceptor blockade on wound healing, growth-factor-starved THCE cells were pretreated with 100 µM P2Y antagonist reactive blue 2 (RB2, Sigma) for 1 hour, wounded by scratch with a sterile 0.1-10 µl pipet tip and further cultured in fresh KBM containing RB2 for 24 hours. The progress of migration was photographed immediately and 6 hours or 24 hours after wounding at the same location along the wound lines with an inverted microscope equipped with a SPOT digit camera. The extent of healing over time was defined as the ratio of denuded areas of the original wound and the wound after 6 or 24 hours.

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