Secretion of IL-13 by Airway Epithelial Cells Enhances Epithelial Repair via HB-EGF

Sima Allahverdian*[1], Norihiro Harada*[1,2], Gurpreet K. Singhera[1], Darryl A. Knight[1], and Delbert R. Dorscheid[1]

[1]The James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, Faculty of Medicine, St. Paul's Hospital, University of British Columbia, Vancouver, British Columbia, Canada; and [2]Department of Respiratory Medicine, Juntendo University School of Medicine, Tokyo, Japan

Inappropriate repair after injury to the epithelium generates persistent activation, which may contribute to airway remodeling. In the present study we hypothesized that IL-13 is a normal mediator of airway epithelial repair. Mechanical injury of confluent airway epithelial cell (AEC) monolayers induced expression and release of IL-13 in a time-dependent manner coordinate with repair. Neutralizing of IL-13 secreted from injured epithelial cells by shIL-13Rα2.FC significantly reduced epithelial repair. Moreover, exogenous IL-13 enhanced epithelial repair and induced epidermal growth factor receptor (EGFR) phosphorylation. We examined secretion of two EGFR ligands, epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF), after mechanical injury. Our data showed a sequential release of the EGF and HB-EGF by AEC after injury. Interestingly, we found that IL-13 induces HB-EGF, but not EGF, synthesis and release from AEC. IL-13–induced EGFR phosphorylation and the IL-13–reparative effect on AEC are mediated via HB-EGF. Finally, we demonstrated that inhibition of EGFR tyrosine kinase activity by tyrphostin AG1478 increases IL-13 release after injury, suggesting negative feedback between EGFR and IL-13 during repair. Our data, for the first time, showed that IL-13 plays an important role in epithelial repair, and that its effect is mediated through the autocrine release of HB-EGF and activation of EGFR. Dysregulation of EGFR phosphorylation may contribute to a persistent repair phenotype and chronically increased IL-13 release, and in turn result in airway remodeling.

Keywords: asthma; bronchial epithelium; epithelial repair; epidermal growth factor; interleukin-13

The epithelial layer of airways is continuously exposed to gaseous and particulate components of the inhaled air and therefore is frequently damaged. Rapid regeneration after injury is crucial for restoring epithelial function to its normal state and involves an orderly progression of events. Bronchial epithelial cells can produce a diverse array of pro-inflammatory mediators, growth factors, and cytokines in response to environmental challenges (1–6), and are actively involved in different stages of epithelial repair. Among these, the ligands for the epidermal growth factor receptor (EGFR) are particularly important. Although the effect of exogenous epidermal growth factor (EGF) in acceleration of airway epithelial repair has been demonstrated (7, 8), the role for endogenous ligand(s), released by damaged or adjacent epithelium, in activation of EGFR and their role in epithelial repair remains to be determined.

CLINICAL RELEVANCE

The mechanisms for airway remodeling in asthma remain unknown. Elevated IL-13 and persistent airway epithelial damage are demonstrated in asthma. The presentation of IL-13–enhanced repair suggests that a defect in the process of repair may contribute to remodeling.

IL-13 is a Th2-like cytokine that has been considered as a central mediator of airway remodeling in asthma. Bronchial biopsy specimens and bronchoalveolar lavage (BAL) cells from allergic individuals with asthma show elevated expression of IL-13 compared with control subjects (9, 10). Several studies have shown some degree of relationship between IL-13 and EGFR pathway in which EGFR activation is necessary for IL-13–mediated mucin production and goblet cell metaplasia in airway epithelium (11, 12). IL-13 has also been shown to indirectly activate EGFR via production of TGF-α (13). While the majority of studies have focused on the role of IL-13 in mediating different features of asthma, the role of IL-13 in normal airway epithelial repair and its relationship with EGFR in this context needs to be determined.

In the present study we used a culture model of epithelial injury and repair to investigate the response of airway epithelial cells (AEC) to mechanical injury. Our hypothesis was that IL-13 contributes to the normal reparative response of AEC. Our data, for the first time, showed that AEC produce and release significant quantities of IL-13 in response to mechanical injury, which is necessary for epithelial repair. We also found a temporal release of EGF and heparin-binding EGF (HB-EGF) by AEC after mechanical injury. We showed that IL-13 increases EGFR phosphorylation and enhances epithelial repair through autocrine/paracrine release of HB-EGF. Interestingly, AEC release more IL-13 when EGFR phosphorylation is blocked. It is possible that the pathologic effects of IL-13 occur as a result of persistent excessive IL-13 release in response to incomplete repair. These findings have important implications for understanding basic mechanisms of epithelial repair and remodeling.

MATERIALS AND METHODS

Cell Culture

1HAEo$^-$ cells are an SV40-transformed normal human airway epithelial cells that have been characterized previously (14). Well-differentiated human bronchial epithelial cell cultures (EpiAirway, air–liquid interface [ALI]) were supplied by MatTek Co. (Ashland, MA).

RNA Isolation and RT-PCR

RNA was extracted from 1HAEo$^-$ cells using the TRIzol reagent (GIBCO BRL, Burlington, ON, Canada), according to the manufacturer's protocol.

(*Received in original form May 16, 2007 and in final form July 13, 2007*)

*These authors contributed equally to this project and are considered co–first authors.

This work was supported in part by the Canadian Institutes for Health Research/Allergen CIHR79632 and NHP CIHR78381 (D.R.D.), BC Lung Association (D.R.D), Michael Smith Foundation for Health Research (Scholar) (D.R.D), the National Institutes of Health (NIH 66026) (D. R. D.), and the Cordula and Gunter Paetzold Fellowship, University of British Columbia (S.A.).

Correspondence and requests for reprints should be addressed to Delbert R. Dorscheid, M.D., Ph.D., The James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, St. Paul's Hospital, Room 166, 1081 Burrard Street, Vancouver, BC, V6Z 1Y6 Canada. E-mail: ddorscheid@mrl.ubc.ca

Am J Respir Cell Mol Biol Vol 38. pp 153–160, 2008
Originally Published in Press as DOI: 10.1165/rcmb.2007-0173OC on August 23, 2007
Internet address: www.atsjournals.org

Conventional PCR was performed using primers specific for IL-13 and β-actin and 2 μl of the synthesized cDNA strand. Specific primers for IL-13 were synthesized by Sigma-Genosys according to published sequences (15): sense, 5′-CTC CTC AAT CCT CTC CTG TT-3′; antisense, 5′-GTT GAA CCG TCC CTC GCG AAA-3′. The samples were amplified in a thermal cycler for 40 cycles, consisting of 1 minute of denaturation at 95°C, 1 minute of annealing at 59°C, and 1 minute of extension at 72°C.

Monolayer Wound Repair Assay

We have established this method previously (8, 16). Briefly, 1HAEo⁻ cells were grown in 6-well plates and then placed in the serum-free medium (SFM) upon confluence. Circular wounds (~2.0 mm^2) were made in the confluent monolayer using a rubber stylet (four wounds per well). In each experiment, one well was used as a negative control with no treatment. Wounds were imaged 0, 8, and 24 hours after wound creation using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Coolpix E995 (Nikon, El Segundo, CA). Corresponding wound areas were determined using ImagePro Plus (Media Cybernetics, Silver Spring, MD) and the remaining wound areas calculated as a percentage of area at time 0.

Preparation of Protein Extracts and Immunoblotting

To determine protein expression of HB-EGF and IL-13 by bronchial epithelial cells after mechanical injury, confluent monolayers of 1HAEo⁻ cells, were subjected to multiple linear injuries (7 × 7 linear scratches in each well) using a rubber stylet. Monolayers with no scratch wounds were used as the control. Protein cell lysates were collected at different time points after injury. In other experiments, confluent monolayers of 1HAEo⁻ cells were treated with IL-13 (10 ng/ml) and protein lysates were collected.

Enzyme-Linked Immunosorbent Assay

Confluent monolayers of 1HAEo⁻ cells were mechanically injured as described previously. Injured monolayers were washed to remove cell debris and the medium was replaced with fresh SFM. Supernatants were collected at different time points after injury, centrifuged to remove cell debris, and frozen before analysis. HB-EGF, IL-13, and EGF levels were measured using a modified indirect enzyme-linked immunosorbent assay (ELISA) method developed in our laboratory. Briefly, serial dilutions of human recombinant HB-EGF, EGF, and IL-13 and the supernatants were coated onto the 96-well Immulon 2HB plates (Thermo Labsystems, Franklin, MA) and incubated overnight at 4°C. After blocking with 1% bovine serum albumin in PBS+0.05% Tween-20, monoclonal anti-human HB-EGF, EGF, and IL-13 (1 μg/ml) (Catalog # AF-259-NA, MAB236, MAB2131 respectively; R&D Systems, Minneapolis, MN) was added to each well and incubated overnight at 4°C. After washing with 0.05% Tween-20 in PBS, the secondary horseradish peroxidase–conjugated anti-goat or anti-mouse were added and incubated for 60 minutes at 37°C. A color reaction was then developed with tetramethylbenzidine (TMB) for 10 minutes at room temperature. After the addition of stop solution, absorbance was measured (450 nm test wavelength, 595 nm reference wavelength) on a microplate reader (SLT-Rainbow, MTX Lab Systems, Vienna, VA). Standard curves ranged from 500 to 0.05 ng/ml. Supernatants from each time point were assayed in duplicate.

Immunofluorescence Staining of ALI

ALI were fixed with 10% formalin and paraffin embedded. Immunofluorescence staining was performed using standard techniques and serial sections were stained with hematoxylin and eosin (H&E). ALI sections were incubated with anti–IL-13 (10 μg/ml) antibody followed by Alexa 546–conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342. All images were obtained using a Leica AOBS SP2 confocal microscope (Leica, Allendale, NJ) and analyzed by Velocity software.

Statistical Analysis

Comparisons between multiple groups were made by ANOVA; when significant differences were found, further comparisons were made by Student's *t* test.

RESULTS

AEC Synthesize and Release IL-13 in Response to Mechanical Injury

Given the persistent epithelial damage in asthma, we hypothesized that the elevated IL-13 may reflect as a part of the normal AEC response to injury. mRNA expression of IL-13 increased after mechanical injury in 1HAEo⁻ cells (Figure 1A). In cell lysates, IL-13 expression increased rapidly after injury and remained elevated for at least 24 hours (Figure 1B). As shown in Figure 1C, IL-13 is rapidly released after injury, with quantifiable levels of the cytokine detected in conditioned media (CM) as early as 30 minutes after injury. Expression of IL-13 by ALI after mechanical injury was also examined (Figure 1D). In noninjured ALI, IL-13 expression was restricted to the apical surface of columnar cells (Figure 1D, *panel a*). Mechanical injury induced expression of IL-13 in both basal and columnar cells at the wound edge 1 minute (*panel b*) and 30 minutes (*panel c*) after injury.

IL-13 Mediates Airway Epithelial Repair in an *In Vitro* Model of Epithelial Repair

AEC release of IL-13 in response to injury generates the question of what role this cytokine has in epithelial repair. To address this question, we used a recombinant soluble form of IL-13Rα2 (shIL-13Rα2.FC; R&D Systems) to neutralize the IL-13 released by injured AEC. This component has previously been shown to attenuate the effects of IL-13 in fibroblasts (17). Different concentrations of shIL-13Rα2.FC were added to the monolayers of 1HAEo⁻ cells immediately after injury. Figure 2A shows that addition of 10 μg/ml of shIL-13Rα2 significantly reduced epithelial repair 24 hours after mechanical injury ($^*P < 0.05$). These data demonstrate that the endogenous release of IL-13 is important in epithelial repair. Next, we tested whether exogenous IL-13 can also enhance epithelial repair. Injured monolayers of 1HAEo⁻ cells were treated with different concentrations of IL-13 (1–100 ng/ml). As shown in Figure 2B, addition of IL-13 at 10, 30, and 100 ng/ml significantly stimulated epithelial repair ($^‡P < 0.01$). This range of IL-13 was similar to the levels of endogenous IL-13 released by AEC during repair of monolayer wounds.

AEC Release Soluble EGFR Ligands in Response to Mechanical Injury

A rapid, damage-induced phosphorylation of the EGFR in epithelial cells has already been shown in many systems including airway epithelial cells (3). Activation of EGFR after mechanical injury in the absence of exogenous ligand suggests that activation is occurring through the release of endogenous mediators. To test whether bronchial epithelial cells produced soluble EGFR ligand(s) after mechanical injury, CM were collected from injured and noninjured monolayers of 1HAEo⁻ cells. These CM were added to intact 1HAEo⁻ monolayers. Phosphorylation of EGFR as induced by CM was assessed. Our data confirmed that mechanically injured 1HAEo⁻ cells release mediator(s) that phosphorylate and activate EGFR (Figure 3A). Concurrent treatment of confluent monolayers with CM and a neutralizing anti-EGF antibody (0.1 μg/ml) decreased CM-mediated EGFR phosphorylation by 50% (Figure 3B). Parallel confluent 1HAEo⁻ monolayers were treated with CM and neutralizing antibodies for EGF (0.1 μg/ml) and HB-EGF (3 μg/ml). As shown in Figure 3C, phosphorylation of EGFR was further decreased (8%, 30%, and 64% by 30 minutes, 2 hours, and 6 hours of CM, respectively) by the addition of anti–HB-EGF.

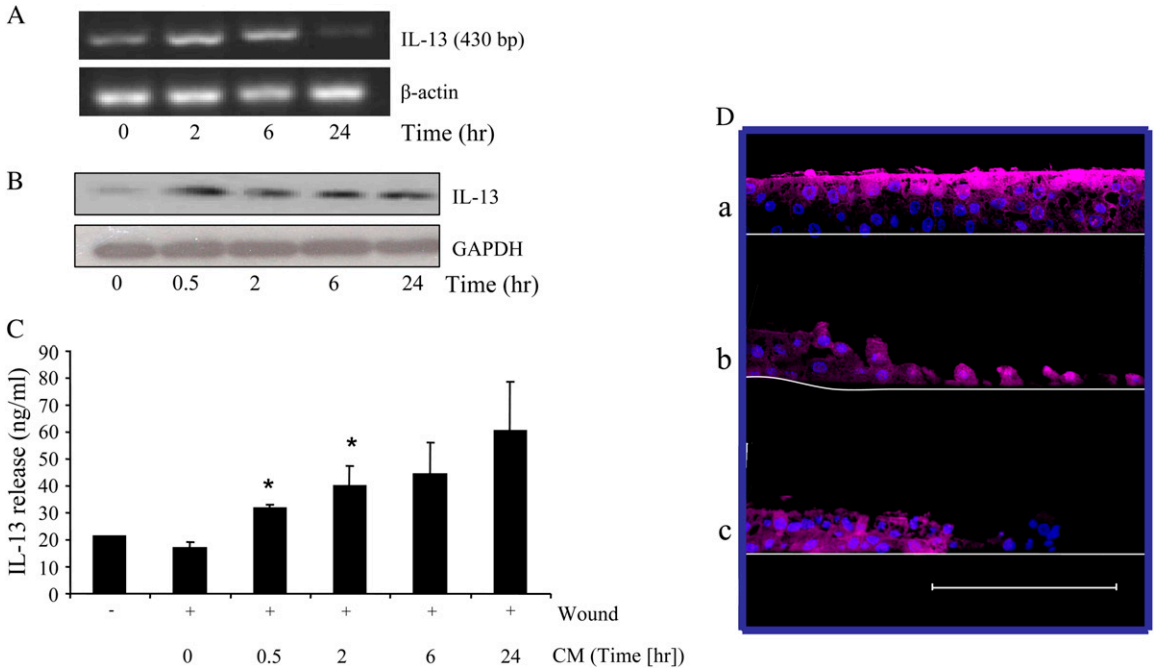

Figure 1. Airway epithelial cells synthesize and release IL-13 in response to mechanical injury. Multiple linear wounds were made in confluent monolayers of 1HAEo⁻ cells. Total RNA, protein lysates, and conditioned media (CM) were collected at indicated times. mRNA expression (*A*), protein synthesis (*B*), and release (*C*) of IL-13 after mechanical injury were examined by RT-PCR, Western blotting, and ELISA. Two linear wounds are created on air–liquid interface (ALI) and followed for 1 minute and 30 minutes and then fixed with 10% formalin. Sectioned ALI were stained for IL-13 using standard protocols. Representative images are noted in *D* with (*a*) confluent ALI, (*b*) ALI 1 minute, and (*c*) ALI 30 minutes after injury. *Scale bar* = 40 μm.

AEC Release EGF and HB-EGF in Response to Epithelial Injury

We directly examined release of EGFR ligands by AEC in response to mechanical injury. Multiple linear wounds were made on confluent monolayers of 1HAEo⁻ cells, and CM was collected at indicated times after wounding. As shown in Figure 4A, epithelial injury leads to a rapid release of EGF from 1HAEo⁻ cells (*$P < 0.05$). No further release or accumulation of EGF was detected in CM beyond 2 hours. We examined the level of HB-EGF protein expression and release in total cell lysates and CM collected from injured monolayers

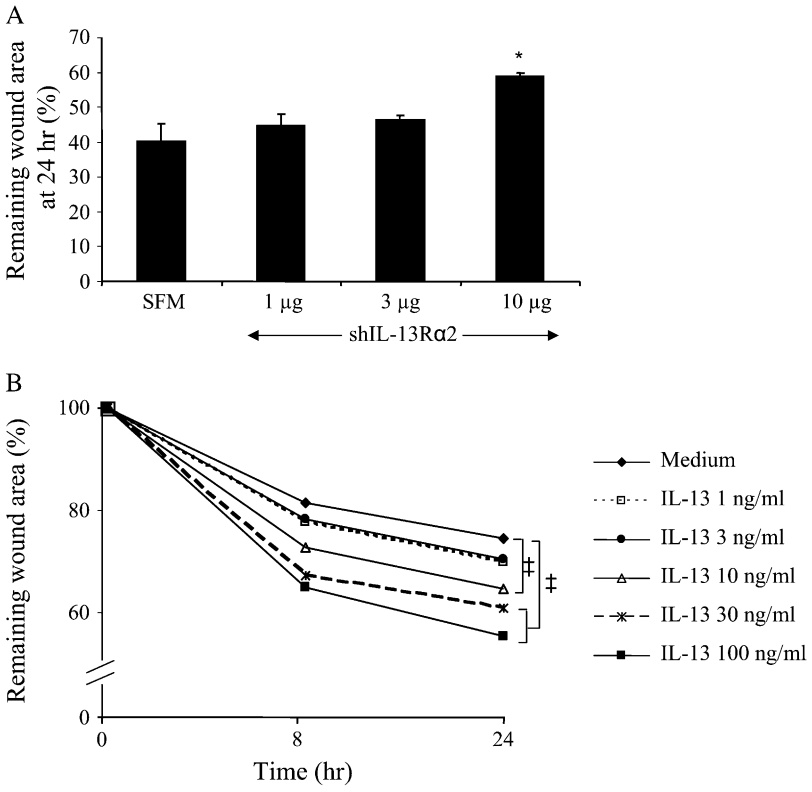

Figure 2. IL-13 mediates airway epithelial repair. Injured monolayers of 1HAEo⁻ cells were treated with different concentration of (*A*) shIL-13Rα2.FC (1–10 μg/ml) or (*B*) IL-13 (1–100 ng/ml) immediately after injury or kept in serum-free medium, and corresponding wound areas were determined. Data are mean ± SEM for eight wounds in each group. *$P < 0.05$, ‡$P < 0.01$.

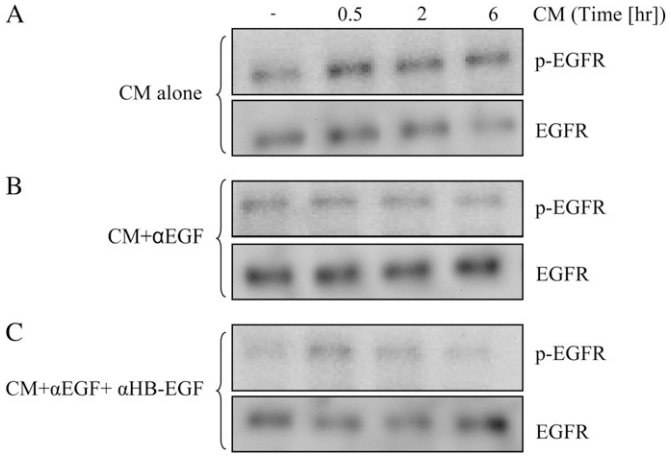

Figure 3. Airway epithelial cells release soluble epidermal growth factor (EGF) receptor (EGFR) ligands in response to epithelial injury. CM were collected from injured 1HAEo- monolayers at different time points. Confluent monolayers of 1HAEo- cells were treated with CM in the presence or absence of anti-EGF (0.1 μg/ml) or in combination with anti–heparin-binding EGF (HB-EGF) (3 μg/ml). Protein lysates were collected from the treated monolayers after 1 hour of CM exposure, and phosphorylation of EGFR was determined by Western blot.

of AEC. Levels of HB-EGF increased gradually after injury, with a maximal expression observed at 8 hours in SFM or at 4 hours when cells grown in the presence of 10% fetal calf serum (FCS) (Figure 4B). Injured monolayers of 1HAEo- cells in SFM conditions secrete HB-EGF into the supernatant as early as 30 minutes after injury with maximum secretion between 2 and 8 hours after injury (5-fold compared with injured monolayers at T0) ($*P < 0.05$ and $‡P < 0.01$) (Figure 4C).

Release of HB-EGF by Injured Epithelium Is Necessary for Epithelial Repair

To examine whether release of HB-EGF by injured epithelium is necessary for repair, injured monolayers of 1HAEo- cells were treated with a neutralizing anti–HB-EGF antibody, the diphtheria toxin analog, CRM197, and the metalloproteinase inhibitor, GM6001. As pro–HB-EGF is bound by diphtheria toxin, this analog inhibits only HB-EGF–induced EGFR activation (18). Addition of the neutralizing anti–HB-EGF significantly reduced epithelial repair compared with medium-alone monolayers ($*P < 0.05$) and the monolayers stimulated with HB-EGF ($‡P < 0.001$). Moreover, addition of anti–HB-EGF to HB-EGF, abrogates the effect of HB-EGF ($§P < 0.01$) (Figure 5A). Activation of HB-EGF is dependent upon protease cleavage of the pro–HB-EGF form (19). As shown in Figure 5B, addition of GM6001 significantly reduced the basal and HB-EGF–stimulated epithelial repair ($*P < 0.05$ and $‡P < 0.001$, respectively), supporting the hypothesis that cleavage of pro–HB-EGF is necessary for epithelial repair. Addition of HB-EGF to GM6001-treated monolayers significantly improved the rate of epithelial repair, indicating that the inhibition of repair was not due to direct effect of the protease inhibitor on receptor function or some non-specific toxic effect of the inhibitor on the cells. Addition of CRM197 similarly inhibited repair of the injured 1HAEo- monolayers compared to medium alone and HB-EGF treated monolayers ($*P < 0.05$ and $‡P < 0.001$, respectively) (Figure 5C).

IL-13 Induces the Production of HB-EGF, but Not EGF, by AEC

Our previous data demonstrated an essential role for IL-13 and HB-EGF in epithelial repair. It has been shown that IL-13 can produce EGFR ligands and transactivate EGFR (11, 12). Next, we asked whether IL-13 has a role in release of EGFR ligands in AEC. To address this question, we investigated whether IL-13 could induce expression of HB-EGF in bronchial epithelial cells. Confluent monolayers of 1HAEo- cells were treated with 10 ng/ml of IL-13 and expression of HB-EGF and EGF were measured at various time points. As shown in Figures 6A and 6B, expression and release of HB-EGF significantly increased after IL-13 exposure ($*P < 0.05$). However, IL-13 showed no effect on EGF release (Figure 6B).

IL-13 Enhances EGFR Phosphorylation and Stimulates Epithelial Repair via HB-EGF

Our previous data showed that IL-13 increases HB-EGF production and release (Figures 6A and 6B). HB-EGF is a known ligand for EGFR; therefore, IL-13 should be able to increase EGFR phosphorylation. Confluent monolayers of 1HAEo- cells were treated with 10 ng/ml of IL-13 and phosphorylation of EGFR was detected using anti-pEGFR (pY^{845}). IL-13 stimulated EGFR phosphorylation of 1HAEo- cells 1 hour after exposure. IL-13–induced EGFR phosphorylation was prevented when the cells were treated with both IL-13 and the anti–HB-EGF antibody (Figure 7A). To test whether the stimulatory effect of IL-13 on epithelial repair is mediated via EGFR and its ligand, HB-EGF, injured monolayers of 1HAEo- were treated with IL-13 with or without AG1478, anti–HB-EGF, and GM6001. As Figure 7B shows, IL-13 significantly stimulated epithelial repair ($*P < 0.05$), and addition of AG1478, anti–HB-EGF neutralizing antibody, or GM6001 to IL-13–treated monolayers suppressed this effect ($‡P < 0.01$). Prevention of EGFR phosphorylation and HB-EGF activity inhibited the IL-13 effects on wound repair. Altogether these data show that IL-13–stimulated EGFR phosphorylation and epithelial repair is mediated through HB-EGF.

Inhibition of EGFR Tyrosine Kinase Activity Enhances IL-13 Production from AEC

Our previous experiments showed that IL-13 is a mediator of normal epithelial repair (Figures 1 and 2). Overexpression of IL-13, as seen in asthma, may result from persistent secretion in an effort to affect the incomplete epithelial repair. This incomplete repair may result from the lack of EGFR function. To test this hypothesis, we examined IL-13 release from AEC when EGFR tyrosine kinase activity was inhibited by AG1478. Multiple linear wounds were created on confluent monolayers of AEC. Wounded monolayers were treated with tyrphostin AG1478 (1 μM) or kept in SFM. Corresponding wound areas were determined and CM were collected at indicated times after wounding. Percent of epithelial repair 24 hours after injury was significantly lower (15.4 ± 1.5) in monolayers treated with AG1478 compared with nontreated monolayers (23.3 ± 2.5) ($P < 0.05$). As shown in Figure 8, AEC release significantly more IL-13 in the presence of AG1478 ($*P < 0.05$). This effect of tyrphostin AG1478 on IL-13 release in response to mechanical injury could not be attributed to any nonspecific effect, as AG1478 when added to confluent monolayers without injury had no effect on IL-13 production.

DISCUSSION

Airway remodeling, which includes goblet cell hyperplasia, mucus hypersecretion, subepithelial fibrosis, and epithelial damage, is a characteristic feature of chronic asthma. The extent to which the epithelial abnormality is the result of inappropriate or incomplete repair remains to be described. IL-13 is known as a Th2 cytokine produced by T helper type 2 cells and other cells

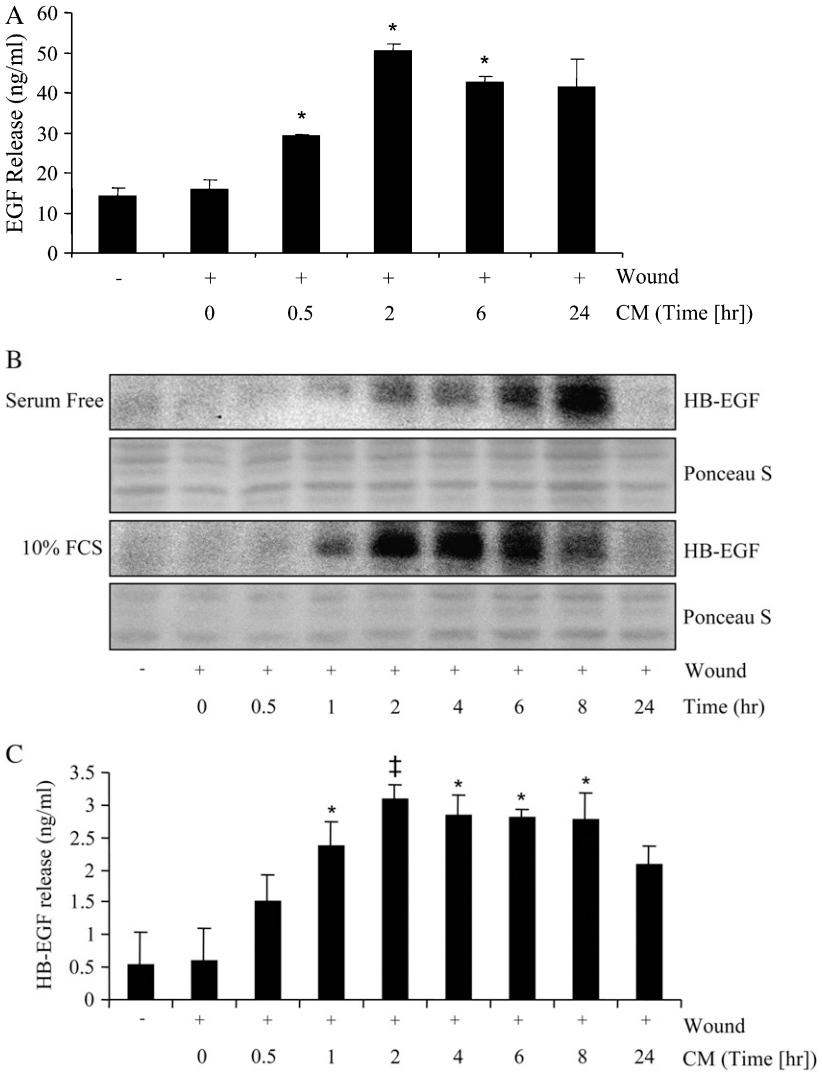

Figure 4. Airway epithelial cells release EGF and HB-EGF in response to mechanical injury. Total protein lysates and CM were collected from injured monolayers of 1HAEo$^-$ cells at indicated times. (*A*) Release of EGF into CM was measured by ELISA. (*B*) Synthesis and (*C*) release of HB-EGF after mechanical injury were examined by Western blotting and ELISA. All membranes were stained with Ponceau S to confirm loading. Synthesis of HB-EGF after injury was studied in both serum-free medium (SFM) and in the presence of 10% FCS (*B*), while release was examined only in serum-free conditions (*C*). $^*P < 0.05$, $^‡P < 0.01$.

recruited to the lung during allergic responses, which has been described to play a key role in many aspects of airway remodeling (20–22). In the present study we hypothesized that IL-13 is a part of the normal response to injury. Overproduction of IL-13 in response to inadequate epithelial repair could act on both epithelium and subepithelial elements leading to airway remodeling. We found that mechanical epithelial injury causes increased production and release of IL-13 by both AEC monolayers and cells grown in ALI. We also showed that this increased production of IL-13 augments epithelial repair that is mediated by EGFR activation. A previous study by Pourazar and colleagues showed an over expression of IL-13 by bronchial epithelial cells in response to exposure to diesel exhaust using an *in vivo* model (2). However, our investigation, to our knowledge, is the first study to show that IL-13 is a repair mediator released by airway epithelial cells in response to injury. We also demonstrated a mechanism by which IL-13 facilitates the repair, HB-EGF secretion.

Epithelial repair consists of a complex cascade of events that starts immediately after injury and leads to effective and normal repopulation of the epithelium. Persistent epithelial damage might be as a result of incomplete repair where any component of this cascade does not work properly. Autocrine activation of EGFR, at least in part, plays an essential role in mediating the key events during epithelial wound healing (23–25). Puddicombe and coworkers showed a rapid, damage-induced phosphorylation of the EGFR in AEC grown in monolayer, irrespective of the presence of exogenous ligand (3). Activation of EGFR after mechanical injury in the absence of exogenous ligand suggests that activation is occurring through the release of endogenous mediators. Bronchial epithelial cells produce several ligands for EGFR, including EGF, TGF-α, HB-EGF, and amphiregulin (26). An induction in the expression and release of EGFR ligands by AEC in response to different stimuli such as cigarette smoke extract (4), compressive stress (1), and oxidative stress has been demonstrated (6).

In the present study we found that bronchial epithelial cells release EGF and HB-EGF in response to mechanical injury. Our data also showed an early release of EGF and a late release of HB-EGF by AEC after mechanical injury. We further examined the role of endogenous HB-EGF in epithelial repair. Addition of a neutralizing antibody for HB-EGF as well as CRM197 significantly reduced the rate of epithelial repair. Members of the EGF family are synthesized as membrane-anchored forms and are then processed by proteolytic cleavage to give bioactive soluble forms (19). Addition of GM6001, a broad-spectrum metalloproteinase inhibitor, attenuated epithelial wound closure in our model even when no exogenous HB-EGF was added. These data showed that proteolytic release of HB-EGF is essential for complete airway epithelial repair.

Unlike the majority of the studies which have focused on the role of a single mediator in epithelial repair, we studied the role

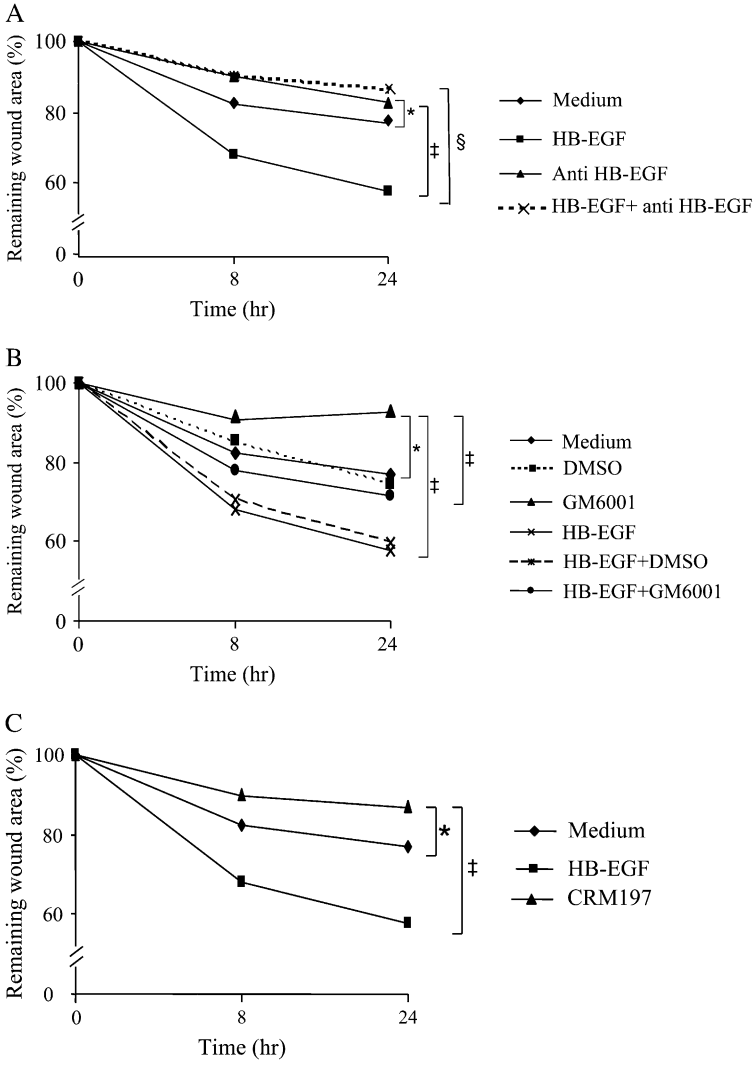

Figure 5. Release of HB-EGF by injured epithelium is necessary for epithelial repair. After mechanical injury monolayers of 1HAEo$^-$ cells were exposed to a neutralizing anti–HB-EGF antibody (3 μg/ml) or GM6001 (50 μM) in the absence or presence of HB-EGF (20 ng/ml) (*A* and *B*, respectively), or 10 μg/ml of CRM197 (*C*). Corresponding wound areas were determined 0, 8, and 24 hours after wound creation using time-lapse videomicroscopy. Data are mean ± SEM for eight wounds in each group. Error bars are omitted for clarity. $^*P < 0.05$, $^‡P < 0.001$, $^§P < 0.01$.

of IL-13 and two EGFR ligands. Our data demonstrated a rapid increase in EGF and IL-13 secretion by AEC after injury, followed by a later response by HB-EGF release. Up-regulation of multiple EGFR ligands has been observed in a few experimental models of epithelial stress. Chu and colleagues showed a sustained up-regulation of the EGFR ligands HB-EGF, epiregulin, and amphiregulin, but not TGF-α after compressive stress of bronchial epithelial cells (1). Similarly, in healing skin

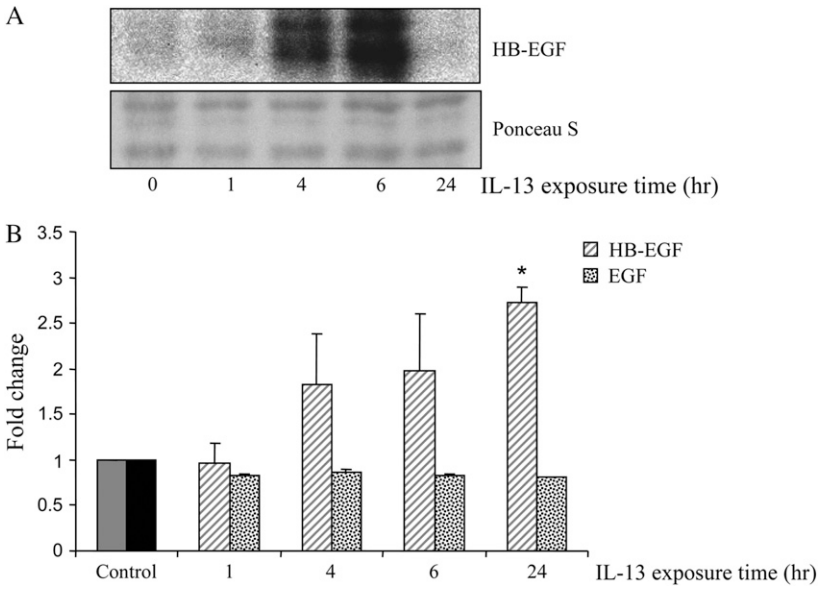

Figure 6. IL-13 enhances production and release of HB-EGF in a culture model of airway epithelium. Confluent monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) and total protein lysates and CM were collected. (*A*) Synthesis and (*B*) release of HB-EGF and release of EGF were examined by western blotting and ELISA. In each experiment, monolayers with no treatment were considered as control. Data in *B* are expressed as fold increase relative to the control monolayers with no treatment (*gray* and *black bars*). IL-13 induced (*A*) synthesis and (*B*) release of HB-EGF from 1HAEo$^-$ cells in a time-dependent manner. IL-13 had no effect on release of EGF by 1HAEo$^-$ cells (*B*).

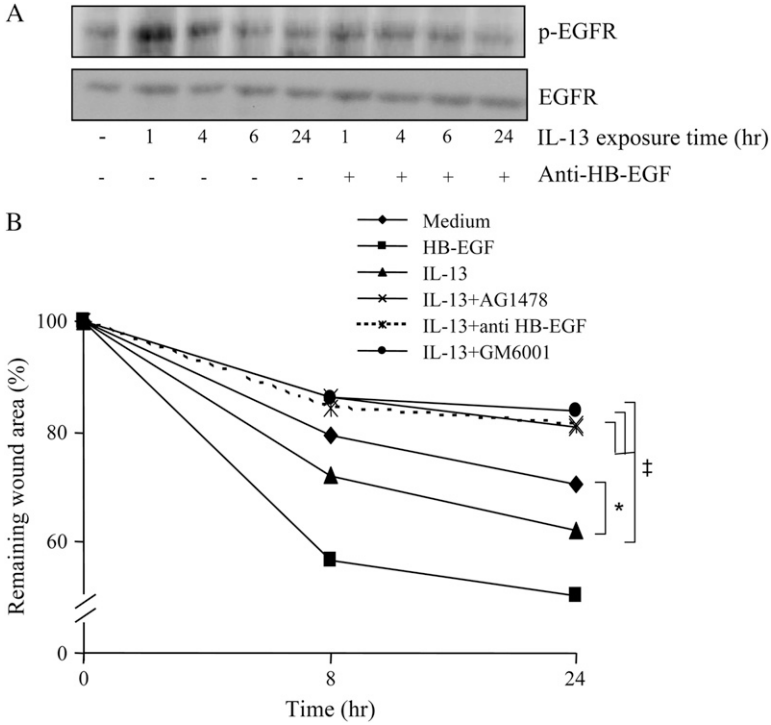

Figure 7. IL-13 induces EGFR phosphorylation and enhances airway epithelial repair via HB-EGF. Confluent monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) with or without concurrent treatment of anti–HB-EGF (3 μg/ml). (*A*) Total protein lysates were collected and phosphorylation of EGFR was examined by Western blotting. Injured monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) with and without AG1478 (1 μM), anti–HB-EGF (3 μg/ml), and GM6001 (50 μM) (*B*). Corresponding wound areas were determined at 0, 8, and 24 hours after injury. Data are mean ± SEM for eight wounds in each group. *$P < 0.05$, ‡$P < 0.01$.

wounds both HB-EGF and amphiregulin mRNA, have been shown to be up-regulated (27). Given the diverse binding specificities and signaling networks associated with EGFR ligands, the sequential expression of multiple ligands may serve to diversify the autocrine and paracrine responses to mechanical perturbation.

Many effects of IL-13 on airway epithelium are mediated through transactivation of EGFR (11, 12). A recent study has demonstrated that IL-13 and EGFR are complementary pathways in chronic goblet cell metaplasia (28). IL-13 has also been shown to induce proliferation of bronchial epithelial cells through production of TGF-α and activation of EGFR (13). Our data showed that IL-13 enhances epithelial repair and induces EGFR phosphorylation. We also demonstrated that IL-13 increases the expression and release of HB-EGF, but not EGF. Finally, we demonstrated that both IL-13–induced EGFR phosphorylation and epithelial repair are mediated through HB-EGF.

Impaired epithelial repair may contribute to airway remodeling as a result of prolonged presence and/or overproduction of inflammatory mediators and growth factors. In the present study we examined whether inhibition of EGFR-mediated epithelial repair has any effect on IL-13 production by AEC. We showed that AEC markedly increase the amount of IL-13 secreted when EGFR activity is inhibited and epithelial repair is prevented. In the response to injury, normal expression of IL-13 contributes to epithelial repair. However, excessive or prolonged release of this cytokine in an attempt to affect repair would have additional adverse effects on both epithelial and subepithelial cells and structures. Puddicombe and colleagues found that disruption of EGFR-mediated epithelial repair is paralleled by enhanced release of TGF-β2 by bronchial epithelial cells (3).

One limitation of our study was that we used monolayers of 1HAEo$^-$ cells in our injury-repair model. These cells show characteristics of nondifferentiated basal human airway epithelial cells. However, we have confirmed production of IL-13 by ALIs, which mimic many aspects of fully differentiated airway epithelium *in vivo.* Another concern is that the present study is not a complete investigation of the EGFR activation after epithelial injury by multiple ligands, but demonstrates the sequential involvement of at least two ligands for normal repair.

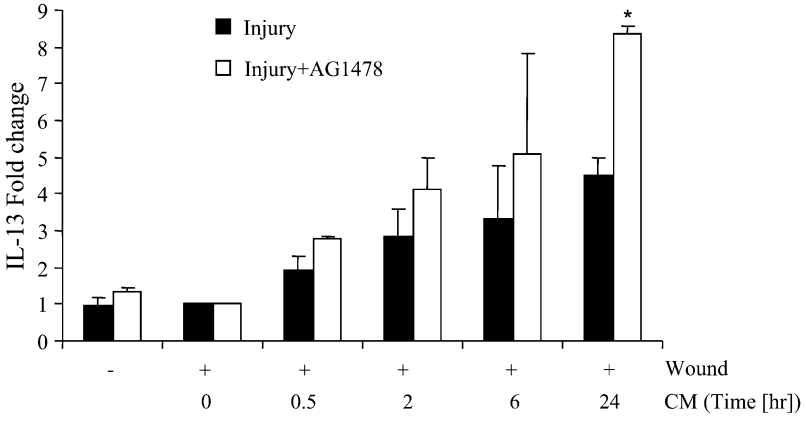

Figure 8. Disruption of EGFR Tyrosine kinase activity enhanced IL-13 release from airway epithelial cells. Wounded monolayers of 1HAEo$^-$ cells were treated with tyrphostin AG1478 (1 μM) or kept in SFM. CM were collected at indicated times and release of IL-13 was evaluated by ELISA. A significant increase in IL-13 release is induced in wounded monolayers when EGFR phosphorylation is inhibited by tyrphostin AG1478 (*$P < 0.05$).

In conclusion, we investigated a basic mechanism of epithelial repair in an *in vitro* model of airway epithelial injury and repair. We used both AEC monolayer and well-differentiated epithelial cells (ALI), which are similar in structure and function to human airway epithelium *in vivo*. We found that AEC release in a sequential manner—EGF, IL-13, and HB-EGF—in response to mechanical injury. We found that IL-13 is a mediator of normal epithelial repair that acts via the EGFR pathway. We also demonstrated an essential role for HB-EGF in epithelial repair. The results of this study highlight an important interaction between EGFR and IL-13 in the airway epithelium. Defective repair, not solely the presence of elevated cytokines, may be a major contributor to the chronic airway changes.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Chu EK, Foley JS, Cheng J, Patel AS, Drazen JM, Tschumperlin DJ. Bronchial epithelial compression regulates epidermal growth factor receptor family ligand expression in an autocrine manner. *Am J Respir Cell Mol Biol* 2005;32:373–380.
2. Pourazar J, Frew AJ, Blomberg A, Helleday R, Kelly FJ, Wilson S, Sandstrom T. Diesel exhaust exposure enhances the expression of il-13 in the bronchial epithelium of healthy subjects. *Respir Med* 2004; 98:821–825.
3. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000;14:1362–1374.
4. Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djukanovic R, Davies DE, Chu EK, Foley JS, Cheng J, *et al.* Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. *Am J Respir Cell Mol Biol* 2002;27:85–90.
5. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. *Respirology* 2003;8:432–446.
6. Zhang L, Rice AB, Adler K, Sannes P, Martin L, Gladwell W, Koo JS, Gray TE, Bonner JC. Vanadium stimulates human bronchial epithelial cells to produce heparin-binding epidermal growth factor-like growth factor: A mitogen for lung fibroblasts. *Am J Respir Cell Mol Biol* 2001;24:123–131.
7. Barrow RE, Wang CZ, Evans MJ, Herndon DN. Growth factors accelerate epithelial repair in sheep trachea. *Lung* 1993;171:335–344.
8. Dorscheid DR, Wojcik KR, Yule K, White SR. Role of cell surface glycosylation in mediating repair of human airway epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L982–L992.
9. Humbert M, Durham SR, Kimmitt P, Powell N, Assoufi B, Pfister R, Menz G, Kay AB, Corrigan CJ. Elevated expression of messenger ribonucleic acid encoding il-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol* 1997;99:657–665.
10. Ying S, Meng Q, Barata LT, Robinson DS, Durham SR, Kay AB. Associations between il-13 and il-4 (mrna and protein), vascular cell adhesion molecule-1 expression, and the infiltration of eosinophils, macrophages, and t cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Immunol* 1997;158:5050–5057.
11. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, Nadel JA. Il-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L134–L140.
12. Kim S, Shim JJ, Burgel PR, Ueki IF, Dao-Pick T, Tam DC, Nadel JA, Dabbagh K, Takeyama K. Il-13-induced clara cell secretory protein expression in airway epithelium: role of egfr signaling pathway. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L67–L75.
13. Booth BW, Adler KB, Bonner JC, Tournier F, Martin LD. Interleukin-13 induces proliferation of human airway epithelial cells in vitro via a mechanism mediated by transforming growth factor-alpha. *Am J Respir Cell Mol Biol* 2001;25:739–743.
14. Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol* 1995;268: L347–L360.
15. Hajoui O, Janani R, Tulic M, Joubert P, Ronis T, Hamid Q, Zheng H, Mazer BD. Synthesis of IL-13 by human B lymphocytes: regulation and role in IgE production. *J Allergy Clin Immunol* 2004;114:657–663.
16. Allahverdian S, Wojcik KR, Dorscheid DR. Airway epithelial wound repair: role of carbohydrate sialyl lewis x. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L828–L836.
17. Andrews AL, Nasir T, Bucchieri F, Holloway JW, Holgate ST, Davies DE. IL-13 receptor alpha 2: A regulator of il-13 and il-4 signal transduction in primary human fibroblasts. *J Allergy Clin Immunol* 2006; 118:858–865.
18. Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E. Diphtheria toxin binds to the epidermal growth factor (egf)-like domain of human heparin-binding egf-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J Biol Chem* 1995;270:1015–1019.
19. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr Relat Cancer* 2001;8:11–31.
20. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779–788.
21. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
22. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. Il-13 signaling through the il-13alpha2 receptor is involved in induction of tgf-beta1 production and fibrosis. *Nat Med* 2006;12:99–106.
23. Zieske JD, Takahashi H, Hutcheon AE, Dalbone AC. Activation of epidermal growth factor receptor during corneal epithelial migration. *Invest Ophthalmol Vis Sci* 2000;41:1346–1355.
24. Nakamura Y, Sotozono C, Kinoshita S. The epidermal growth factor receptor (egfr): Role in corneal wound healing and homeostasis. *Exp Eye Res* 2001;72:511–517.
25. Egan LJ, de Lecea A, Lehrman ED, Myhre GM, Eckmann L, Kagnoff MF. Nuclear factor-kappa b activation promotes restitution of wounded intestinal epithelial monolayers. *Am J Physiol Cell Physiol* 2003;285: C1028–C1035.
26. Polosa R, Prosperini G, Leir SH, Holgate ST, Lackie PM, Davies DE. Expression of c-erbb receptors and ligands in human bronchial mucosa. *Am J Respir Cell Mol Biol* 1999;20:914–923.
27. Stoll S, Garner W, Elder J. Heparin-binding ligands mediate autocrine epidermal growth factor receptor activation in skin organ culture. *J Clin Invest* 1997;100:1271–1281.
28. Tyner JW, Kim EY, Ide K, Pelletier MR, Roswit WT, Morton JD, Battaile JT, Patel AC, Patterson GA, Castro M, *et al.* Blocking airway mucous cell metaplasia by inhibiting egfr antiapoptosis and il-13 transdifferentiation signals. *J Clin Invest* 2006;116:309–321.