Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects

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Background: The c-erbB family of receptor tyrosine kinases act in a combinatorial fashion to regulate cell behavior. Disturbances in this system have been associated with neoplastic and inflammatory diseases.
Objectives: Although expression of the epidermal growth factor receptor (EGFR; c-erbB1) is increased in the bronchial epithelium in asthma, there is no information on expression of other members of the c-erbB receptor and ligand family that can modulate EGFR function.
Methods: Immunohistochemistry was used to compare expression of EGFR, c-erbB2, c-erbB3, epidermal growth factor, heparin-binding epidermal growth factor–like growth factor, and transforming growth factor α in bronchial biopsy specimens from normal and asthmatic subjects. Scrape-wounded monolayers of 16HBE 14o⁻ cells were used as an in vitro model of damage and repair. Changes in EGFR, c-erbB2, and c-erbB3 distribution were measured by means of immunocytochemistry, whereas tyrosine phosphorylation was measured by means of immunoprecipitation and Western blotting.
Results: Although epithelial staining for the EGFR was significantly increased in asthmatic epithelium ($P < .001$), there was no difference in staining for the other receptors and ligands studied. In scrape-wounded epithelial monolayers, tyrosine phosphorylation of EGFR, c-erbB2, and c-erbB3 occurred immediately after damage; however, only EGFR showed a change in expression in response to damage.
Conclusions: Even though EGFR levels are increased in asthma, this is not linked to changes in expression of its activating ligands or other c-erbB receptors. Because bronchial epithelial cells respond to physical damage through activation of several c-erbB family members, the shift in favor of increased EGFR levels in asthma may lead to altered epithelial function by influencing the number and type of heterodimeric signaling complexes, assuming sufficient ligand availability. (J Allergy Clin Immunol 2002;109:75-81.)

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Shedding of the bronchial epithelium with selective loss of columnar epithelial cells from their attachment to basal cells is a characteristic feature of all types of asthma.[1,2] The causes of this damage are unclear, but inflammatory cell products and environmental agents have been proposed to have pathogenetic roles.[3] Damage elicits structural changes and alters the epithelial phenotype, reflecting increased stress and repair responses. As a consequence, the airway epithelium becomes an important source of autacoid mediators, cytokines, chemokines, and growth factors and exhibits an altered profile of adhesion-molecule expression.[3]

Epithelial repair consists of an initial phase of epithelial spreading and migration into the wound, followed by proliferation and differentiation to replace the columnar cells that have been lost.[4] These processes are spatially and temporally regulated by local signals provided by a plethora of growth factors and adhesion molecules. Among the signals that regulate epithelial repair, the epidermal growth factor (EGF) family of growth factors—EGF, heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor α (TGF-α)—are particularly important. They stimulate cell migration, proliferation, and differentiation[5] and are known to enhance several phases of epithelial restitution. A direct role for EGF, HB-EGF, and TGF-α in cutaneous wound healing is already established,[6] and recent cell-culture experiments with guinea pig[7] or human[8] airway epithelial cells indicate that EGF and related factors significantly enhance wound closure.

EGF, HB-EGF, and TGF-α exert their biologic effects by binding to the EGF receptor (EGFR; c-erbB1, human EGF receptor 1 [HER1]), a transmembrane receptor with intrinsic tyrosine kinase activity.[5,9] Binding of cognate ligand stabilizes the EGFR in an activated dimeric form comprising either EGFR homodimers or EGFR/c-erbB heterodimers involving another member of the structurally homologous c-erbB family (ie, c-erbB2 [HER2], c-erbB3 [HER3], and c-erbB4 [HER4]).[9] Because each receptor has a kinase with its own reaction kinetics and substrate specificity, recruits different second messengers, and has a different rate of internalization, het-

Abbreviations used
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
HB-EGF: Heparin-binding EGF-like growth factor
HER: Human EGF receptor
SFM: Serum-free medium
TGF-α: Transforming growth factor α

erodimerization facilitates the initiation of a more diverse range of intracellular signals than those generated by EGFR homodimers alone.[5,9] c-erbB2 appears to be the preferred partner for heterodimerization, and its presence is critical for activation of c-erbB receptors by means of lateral signal transduction.[10] As a result, variations in the c-erbB receptor complement of different cell types will influence the pattern of heterodimerization, with consequent effects on the extent of activation of different intracellular signal transduction pathways and downstream functional responses, such as survival, proliferation, transformation, and differentiation.[11] Such a complex combinatorial relationship within the c-erbB receptor family offers the potential to finely regulate the molecular and cellular processes that need to be coordinated to effect complex processes, such as tissue repair.

We have recently demonstrated expression of EGFR, c-erbB2, c-erbB3, EGF, HB-EGF, TGF-α, and amphiregulin in normal human bronchial epithelial cells.[12] This colocalization of c-erbB receptors and ligands suggests productive c-erbB receptor interactions within the bronchial epithelium. We have also shown that expression of the EGFR is increased in asthmatic bronchial epithelium,[8] yet there is no evidence of increased proliferation,[13] even though EGF is a known mitogen. Because EGFR function can be modulated by heterodimerization, we now report quantitation of bronchial epithelial expression of c-erbB2 and c-erbB3, as well as EGFR, in asthmatic and nonasthmatic control subjects. Furthermore, because the availability of c-erbB ligands may be important for receptor activation, we also analyzed EGF, HB-EGF, and TGF-α. To determine the involvement of c-erbB receptors in epithelial repair, we investigated changes in c-erbB receptor expression and activation in monolayers of human bronchial epithelial cells subjected to mechanical injury in vitro.

METHODS

Patient characterization and fiberoptic bronchoscopy

Ten control subjects without asthma (8 men) with a mean ± SEM age of 26.0 ± 1.8 years and 13 subjects with mild-to-severe asthma (9 men) with a mean ± SEM age of 37.2 ± 2.4 years participated in this study. Asthmatic subjects had a mean ± SEM percent predicted FEV_1 of 76.9% ± 6.3%, and their geometric mean PC_{20} histamine was 0.94 mg/mL (range, 0.12-8.33 mg/mL). All were taking inhaled bronchodilators, and 9 of them were taking steroids. All subjects studied were nonsmokers and had not experienced recent symptoms of upper respiratory tract infection. The study was approved by the Southampton Joint University and Hospitals Ethics Committee, and all subjects gave their written consent after being fully informed about the nature and purpose of the study.

Measurements of baseline FEV_1 and bronchial reactivity to inhaled histamine were performed 1 to 4 weeks before bronchoscopy. Atopy was determined by means of skin prick testing with a panel of common aeroallergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, mixed grass pollen, dog allergen, mixed tree pollen, and cat allergen (Miles Inc, Hollister Stier). Fiberoptic bronchoscopy and bronchial biopsy were performed as previously described[14] and in accordance with current National Heart, Lung, and Blood Institute recommendations.[15] Biopsy specimens were fixed in ice-cold acetone containing iodoacetamide (20 mmol/L) and phenylmethylsulfonyl fluoride (2 mmol/L). After overnight fixation at –20°C, specimens were embedded in glycol methacrylate resin (Park Scientific), as previously described.[16]

Immunohistochemistry

The following antibodies were used: a sheep anti-EGFR polyclonal antibody (an IgG fraction of immune serum raised against affinity-purified EGFRs); a mouse mAb against c-erbB2 and a rabbit polyclonal antibody against c-erbB3 (Transduction Laboratories, supplied by Affiniti Research Products Ltd); an anti-EGF mAb (clone 3D3, a gift from Professor K. Nishikawa, Kanazawa Medical University); a TGF-α antibody (clone Ab-20; Cambridge BioScience); and a chicken anti-HB-EGF raised against the intracellular domain of HB-EGF (a gift from Dr R. Adam, Childrens Hospital, Boston). For the antibodies used, their specificity and lack of cross-reactivity were demonstrated in our previous studies.[12] Immunohistochemistry was performed with 2-μm sections of glycol methacrylate–embedded biopsy specimens, as previously described.[16] Immunostaining was quantified with computer-assisted image analysis (Colourvision 1.7.6, Improvision). For each biopsy specimen, the entire intact epithelium in 2 nonserial sections was systematically assessed on the basis of red-green-blue color balance. The image analysis system was standardized with a section of bronchial mucosa stained for c-erbB receptors by the same method to ensure reproducibility of analysis, as previously described.[8] Data are expressed as percentages of the total epithelial area. All measurements were performed by an observer who was unaware of the clinical group from which the biopsy specimen was derived.

Scrape wounding of bronchial epithelial cell monolayers

16HBE 14o$^-$ cells[17] were grown in 10% FBS-modified Eagle medium containing 50 IU/mL penicillin, 50 μg/mL streptomycin, and 2 mmol/L L-glutamine with either 4-well glass-chamber slides (Life Technologies) or 57-cm^2 petri dishes. At 90% confluence, the cells were placed in Ultraculture serum-free medium (SFM; BioWhittaker) for 24 hours, and the cell monolayer was washed in fresh SFM before scrape wounding. Cells were grown in 4-well chamber slides, as described above, to assess c-erbB receptor immunoreactivity. A series of 12 parallel wounds were made in each monolayer with a sterile comb, and the wounded cells were allowed to repair in fresh SFM. Slides were fixed in ice-cold methanol at 0, 3, 6, and 9 hours, and c-erbB receptors were detected by means of immunoperoxidase staining with anti-EGFR, anti-c-erbB2, and anti-c-erbB3 antibodies before visualization with 0.5 mg/mL diaminobenzidine in TRIS-HCl buffer containing 0.01% H_2O_2. Slides were counterstained with Mayer's hematoxylin and mounted in p-xylene-bis-pyridium bromide. Tyrosine phosphorylated proteins were immunoprecipitated from monolayers of cells into which multiple scrape wounds had been created with a sterile plastic comb to measure c-erbB receptor phosphorylation, as previously

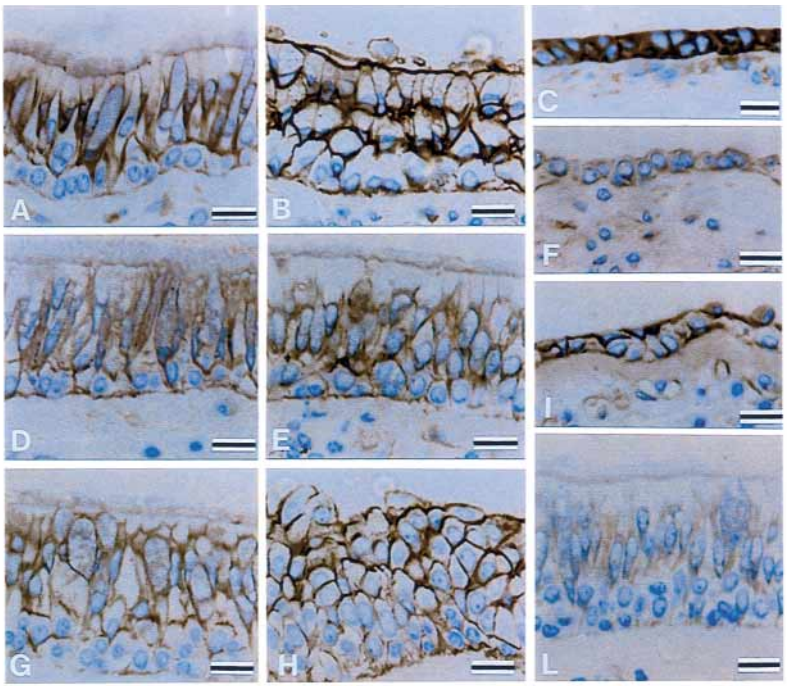


FIG 1. Bronchial biopsy specimens were stained for the presence of EGFR (**A-C**), c-erbB2 (**D-F**), and c-erbB3 (**G-I**). The plates show typical immunostaining in bronchial epithelium from a normal individual (**A**, **D**, and **G**) and morphologically intact (**B**, **E**, and **H**) or damaged (**C**, **F**, and **I**) epithelium from an asthmatic patient. **L** shows a control section in which the anti-EGFR antibody was preadsorbed with EGFRs. The sections are orientated such that the lumen is at the top and the mucosa at the bottom. *Scale bars* = 20 μm.

described.[8] C-erbB receptors were detected in the immunoprecipitates derived from 0.5 mg of total protein per condition by means of Western blotting with appropriate antibodies and controls.

Statistical analyses

Data for c-erbB receptor and ligand immunoreactivity were expressed as medians and ranges. Comparisons between groups were made by using the Mann-Whitney test. Correlations were sought with the Pearson and Spearman tests for normally and non-normally distributed data, respectively. A P value of less than .05 was regarded as statistically significant. Statistical analysis was performed with SPSS version 8.0 software for Windows.

RESULTS

Analysis of c-erbB receptor and ligand expression in asthmatic epithelium

c-erbB receptor and ligand expression was analyzed in sections of bronchial biopsy specimens that were obtained by means of fiberoptic bronchoscopy from normal (n = 10) or asthmatic subjects (n = 13). Immunohistochemical analysis of biopsy specimens of normal airway mucosa revealed positive epithelial staining for EGFR, c-erbB2, and c-erbB3 receptors. This was associated with cell membranes and confined to the lateral junctions between columnar epithelial cells and their junctions with basal cells; no staining was evident on the luminal surface of the epithelium (Fig 1, *A*, *D*, and *G*), as reported previously.[12] A similar distribution of staining within the bronchial mucosa was observed with anti-EGF, anti-HB-EGF, and anti-TGF-α antibodies (data not shown).

No significant differences in immunostaining of the bronchial epithelium were observed between normal and asthmatic subjects after staining with anti-c-erbB2 (epithelial staining comparing normal versus asthmatic subjects, respectively; 5.5% [range, 3.2%-12.7%] vs 7.4% [range, 3.3%-18.3%]; Fig 1, E, vs Fig 1, D; Fig 2), anti-c-erbB3 (9.0% [range, 4.7%-18.1%] vs 12.4% (range, 7.0%-24.5%]; Fig 1, H, vs Fig 1, G; Fig 2), anti-EGF (7.1% [range, 4.0%-17.8%] vs 9.5% [range, 5.1%-19.4%]), anti-HB-EGF (9.6% [range, 5.8%-16.3%] vs 11.6% [range, 4.8%-19.2%]), and anti-TGF-a (13.8% [range, 5.8%-20.2%] vs 13.3% [range, 7.2%-20.3%]; Fig 2). In contrast, in sections from the same subjects, EGFR immunostaining was markedly increased in the asthmatic biopsy specimens and occurred throughout the epithelial layer (Fig 1, B, vs Fig 1, A), as we have previously reported.8 Image analysis of the tissue sections confirmed that the extent of EGFR immunostaining in asthmatic epithelium was significantly ($P < .001$) higher than in normal epithelium. The median percentage of bronchial epithelium staining positively for the EGFR was 19.4% (range, 9.3%-31.8%) and 9.4% (range, 4.1%-20.4%) in asthmatic and normal subjects, respectively (Fig 2). When the asthmatic group was divided according to disease severity, there was a significant difference between EGFR expression in the subjects with mild (18.8% [range, 9.3%-28.9%], $P = .003$) or severe (step 4 according to the GINA guidelines; 25.4% [range, 19.9%-31.8%], $P < .001$) asthma compared with control subjects (9.4% [range, 4.1%-20.4%), as well as between subjects

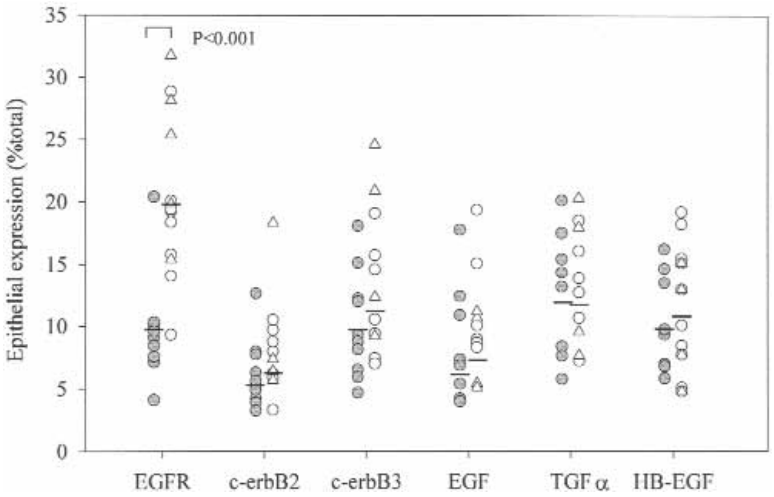


FIG 2. Comparison of bronchial epithelial c-erbB receptor and ligand immunoreactivity in biopsy specimens from healthy control subjects (n = 10) and subjects with asthma (n = 13). The statistical significance of the differences between groups was determined with 1-way ANOVA. The symbols indicate normal (*shaded circles*), mild *(open circles),* or severe *(triangles)* asthma.

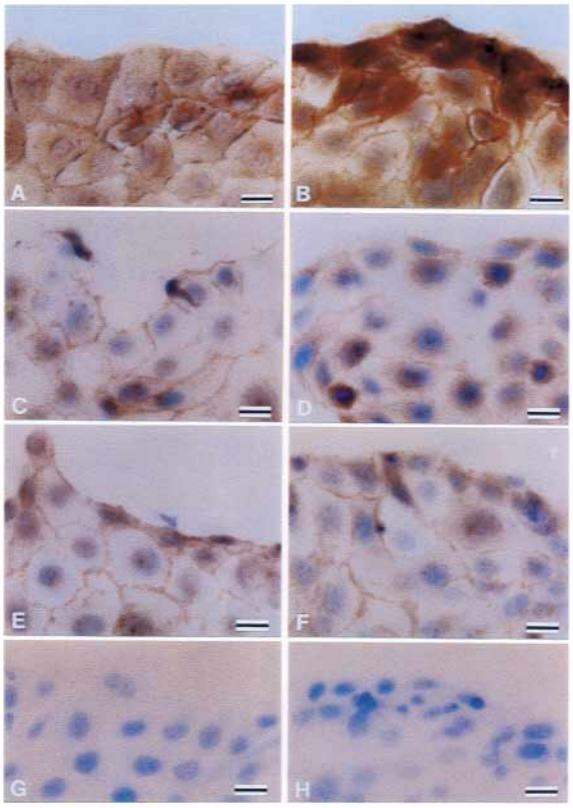


FIG 3. Confluent monolayers of 16HBE 14o⁻ cells were scrape wounded and allowed to repair in SFM. After 6 hours, cells were fixed and stained by means of immunocytochemistry for the presence of EGFR (**A** and **B**), c-erbB2 (**C** and **D**), and c-erbB3 (**E** and **F**). The plates show staining of the monolayer immediately after scrape wounding (**A**, **C**, **E**, and **G**) and 6 hours later (**B**, **D**, **F**, and **H**). **G** and **H** show the appearance of control monolayers after omitting the primary antibodies from the staining protocol. Comparable staining patterns were observed in 3 independent experiments. *Scale bars* = 40 μm.

with mild and severe asthma ($P = .04$), which is consistent with our previous observations.8 In contrast, there was no significant difference between the groups with mild and severe asthma in respect to c-erbB2, c-erbB3, EGF, TGF-a, and HB-EGF expression. In addition, a significant positive correlation between EGFR and c-erbB3 expression was found in the asthmatic subjects ($rs = 0.62$, $P < .05$).

Examination of the biopsy specimens revealed that a significant proportion of the bronchial epithelium from the asthmatic subjects was damaged and lacked columnar epithelial cells, as found previously.[1] In these areas membrane staining for the EGFR was particularly intense on all surfaces, including the apical surface of the epithelium, and marked cytoplasmic immunostaining was evident (Fig 1, *C*, vs Fig 1, *B*). In general, no local increase in immunostaining was observed in damaged areas with antibodies directed against c-erbB2 (Fig 1, *F*), c-erbB3 (Fig 1, *I*), EGF, HB-EGF, and TGF-α. However, in a few asthmatic subjects c-erbB3 staining in some areas of the damaged epithelium was modestly increased.

Effect of scrape wounding on c-erbB receptor expression and activation in vitro

Immunocytochemical staining of monolayers of 16HBE 14o⁻ cells showed strong membrane staining for EGFR (Fig 3, *A*) and c-erbB3 (Fig 3, *E*), whereas staining for c-erbB2 (Fig 3, *C*) was weaker and found scattered irregularly throughout the cell monolayer. After scrape wounding of the monolayers, the intensity of EGFR immunostaining increased at the wound edge between 3 and 9 hours after mechanical damage. Under high magnification, EGFR immunoreactivity appeared to be locally increased at the edges of the damaged monolayers with intense cytoplasmic staining (Fig 3, *B*), as observed in bronchial biopsy specimens. No local increase in immunostaining was observed with antibod-

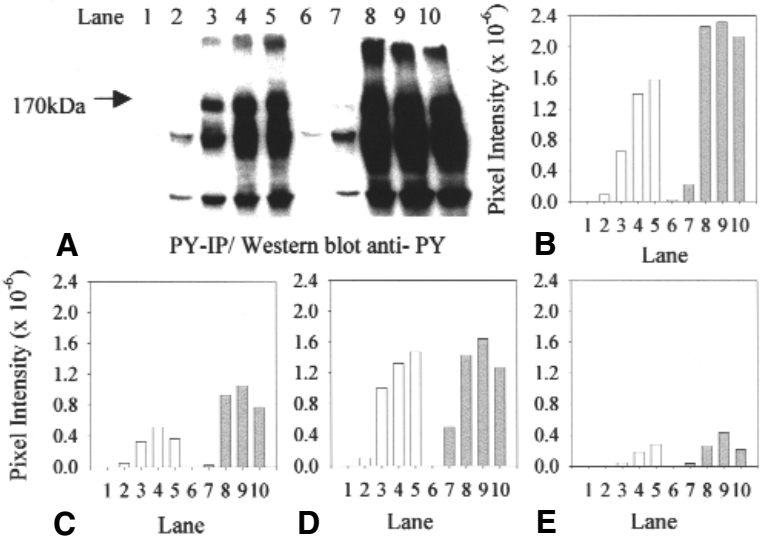


FIG 4. EGFR, c-erbB2, and c-erbB3 phosphorylation was analyzed in unwounded *(lanes 1-5)* or wounded *(lanes 6-10)* epithelial cultures at 0 (*lanes 1* and *6*), 1 (*lanes 2* and *7*), 5 (*lanes 3* and *8*), 10 (*lanes 4* and *9*), and 15 (*lanes 5* and *10*) minutes by means of immunoprecipitation of phosphotyrosine-containing proteins, followed by Western blotting and probing with antibodies toward phosphotyrosine (**A** and **B**), EGFR (**C**), c-erbB2 (**D**), and c-erbB3 (**E**). In **B** through **E**, changes in phosphorylation were quantified by means of densitometry; data are representative of 2 individual experiments.

ies directed against c-erbB2 (Fig 3, *D*) and c-erbB3 (Fig 3, *F*) at any of the time points studied. However, c-erbB2 staining in proximity of the edges was found to be more diffuse and cytoplasmic at 6 hours after wounding (Fig 3, *D*) than at baseline (Fig 3, *C*). In all of these experiments, no labeling was observed when the primary antibodies were omitted from the immunocytochemical protocol (Fig 3, *G* and *H*). Although the levels of c-erbB2 and c-erbB3 did not change after epithelial injury, their involvement in EGFR-mediated signal transduction was suggested by the rapid increase in their phosphorylation state within 1 to 5 minutes of scrape wounding (Fig 4, *D* and *E*, *lane 2* vs *lane 7* and *lane 3* vs *lane 8*). This was associated with a longer term increase in total tyrosine phosphorylation (Fig 4, *A* and *B*) and EGFR phosphorylation (Fig 4, *C*).

DISCUSSION

Combinatorial c-erbB receptor interactions leading to signal diversification and modulation of cellular responses is the focus of intense research activity in many areas of biology.[9] Because EGFR, c-erbB2, and c-erbB3 are coexpressed in the bronchial epithelium, we postulated that differential expression of these receptors regulates responses to stress or injury in asthma. Using a model of mechanical damage to human bronchial epithelial cells in vitro, which may reflect some of the physical disruption seen in asthmatic epithelium, we have previously shown that activation of the EGFR is required for epithelial repair.[8] We now extend this observation by demonstrating that activation of c-erbB2 and, to a lesser extent, c-erbB3 also occurs rapidly after mechanical damage. Because phosphorylation occurred in the absence of exogenous ligand, it seems likely that autocrine ligands are rapidly released by the damaged epithelial cells and contribute to epithelial repair. Because there is no known ligand for c-erbB2, it is probable that its phosphorylation results from heterodimerization with the EGFR rather than with c-erbB3, which has a defective tyrosine kinase domain. Consistent with this proposal, we have found that the EGFR-selective tyrosine kinase inhibitor tyrphostin AG1478 blocks both EGFR and c-erbB2 phosphorylation in EGF-stimulated bronchial epithelial cells (data not shown). Because c-erbB2 has been demonstrated to be involved in cell migration[18] and proliferation,[19] it seems likely that it functions together with the EGFR to bring about the appropriate responses required for epithelial repair.

The occurrence of epithelial damage in asthmatic airways remains controversial and has been attributed to biopsy-induced artifact.[20] However, the latter study was based purely on morphologic features of denudation without any assessment of markers of epithelial injury, which enable in vivo damage to be distinguished from that caused during the biopsy procedure. Increased EGFR expression has been reported in a number of conditions in which airway epithelial damage has been elicited in experimental settings. For example, expression of EGFR is increased in rodent bronchiolar epithelial cells after bleomycin-and naphthalene-induced lung injury[21,22] and in virgin mice after ovalbumin challenge.[23] Thus our previous finding that EGFR expression is increased in asthma[8] is consistent with epithelial damage occurring in vivo.

In asthmatic bronchial biopsy specimens, expression of c-erbB2 and c-erbB3 in the bronchial epithelium was unchanged by comparison with normal epithelium, even

though EGFR expression was increased in the same biopsy specimens, as previously reported.[8] We have previously demonstrated that EGFR expression is unaffected by treatment with inhaled corticosteroids,[8] and there did not appear to be a clear effect of steroids on the expression of the other family members. It has been shown that both EGFR and c-erbB2 expression is induced in type II alveolar cells and is associated with proliferation after endotoxin instillation into the rat lung.[24] This finding is at odds with the present study, in which no change in c-erbB2 was observed in asthmatic bronchial epithelium or in scrape-wounded bronchial epithelial cells in vitro. This suggests that there may be differences in the regulation of receptor expression in bronchial and alveolar epithelial cells. Alternatively, it may be due to a species-related difference or the type of insult used.

In dermal wounds,[25] as well as in carcinomas[26] and psoriasis,[27] increased EGFR expression drives epithelial proliferation through induction of autocrine ligand. Therefore to determine whether c-erbB receptors could be functionally active within the bronchial epithelium, we also examined the coexpression of 3 related EGF-like peptides. As with c-erbB2 and c-erbB3, there was no difference in immunostaining of normal or asthmatic bronchial epithelium with EGF, HB-EGF, or TGF-α antibodies. These findings extend and confirm a previous immunohistochemical study of EGF expression in bronchial epithelium of asthmatic patients[28] but contrast with studies in skin[6] and the gastrointestinal tract,[29] where wounding not only caused induction of epithelial EGFR expression but also HB-EGF and TGF-α expression. Because the asthmatic bronchial epithelium shows little evidence of proliferation, as demonstrated by the low level of proliferating cell nuclear antigen expression in the basal cells,[13] failure to detect any changes in EGFR ligand expression in asthmatic epithelium might account for the poor proliferative response of asthmatic epithelium. This contrasts with the hyperproliferative state of the bronchial epithelium in chronic obstructive pulmonary disease.[13]

Epithelial EGFR expression is increased in smokers,[30] whereas overexpression of EGFR and c-erbB2 is observed in lung carcinomas,[26] suggesting a link between costimulation of EGFR and c-erbB2 with increased proliferation. In contrast, there is no change in EGFR expression in the airway epithelial cells of patients with cystic fibrosis, although TGF-α levels are elevated.[31] Similarly, in idiopathic pulmonary fibrosis there is only a small change in EGFR levels, but levels of TGF-α are elevated in vascular endothelial cells.[32] This suggests that the relative levels of all erbB receptors (and their ligands) may be a characteristic feature of a particular disease and may link epithelial phenotype to altered function.

In general, signaling by homodimeric complexes is relatively weak, whereas heterodimers, and especially those containing c-erbB2, are more potent transmitters of signals.[33] Although the number and distribution of homodimers, heterodimers, or both in healthy and asthmatic bronchial epithelium is not known, it is clear that injury, through altering the proportion of EGFRs relative to c-erbB2 and c-erbB3, will affect the populations of homodimeric and heterodimeric complexes through mass action. This perturbation would be expected to lead to activation of a different subset of signaling intermediates with distinct effector responses. Further studies are now required to determine whether different diseases are characterized by distinct patterns of erbB receptor expression and how the altered c-erbB expression profile influences the individual stages of epithelial repair and their associated phenotypes.

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