Differentiation of human airway epithelia is dependent on erbB2

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Vermeer, Paola D., Lacey Panko, Philip Karp, John H. Lee, and Joseph Zabner. Differentiation of human airway epithelia is dependent on erbB2. Am J Physiol Lung Cell Mol Physiol 291: L175–L180, 2006.—A clinical case documented a reversible change in airway epithelial differentiation that coincided with the initiation and discontinuation of trastuzumab, an anti-erbB2 antibody. This prompted the investigation into whether blocking the erbB2 receptor alters differentiation of the airway epithelium. To test this hypothesis, we treated an in vitro model of well-differentiated human airway epithelia with trastuzumab or heregulin-α, an erbB ligand. In addition, coculturing with human lung fibroblasts tested whether in vivo subepithelial fibroblast function as an endogenous source of ligands able to activate erbB receptors expressed by the overlying epithelial cells. Epithelia were stained with hematoxylin and eosin and used for morphometric analysis. Trastuzumab treatment decreased the ciliated cell number by 49% and increased the metaplastic, flat cell number by 640%. Heregulin-α treatment increased epithelial height and decreased the number of metaplastic and nonciliated columnar cells, whereas it increased the goblet cell number. We found that normal human lung fibroblasts express transforming growth factor-α, heparin-binding epidermal-like growth factor, epiregulin, heregulin-α, and amphiregulin, all of which are erbB ligands. Cocultures of airway epithelia with primary fibroblasts increased epithelial height comparable to that achieved following heregulin-α treatment. These data show that erbB2 stimulation is required for maintaining epithelial differentiation. Furthermore, the mesenchyme underlying the airway epithelium secretes a variety of erbB ligands that may direct various pathways of epithelial differentiation.

trastuzumab; fibroblasts

The following case report describes a 56-year-old woman who underwent a left mastectomy with axillary node dissection. Pathological examination revealed an infiltrating ductal carcinoma grade II–III with involvement of the nipple. Immunohistochemistry revealed HER2/Neu-positive cells, suggesting it may respond to anti-erbB2 directed antibody therapy. A survey for metastasis was negative. After chemotherapy and radiation therapy, the patient elected to participate in a clinical research protocol studying the effects of weekly trastuzumab (Herceptin; Genentech, San Francisco, CA) on patients with breast cancer without metastasis at presentation (National Surgical Adjuvant Breast And Bowel Project clinical trial B31). She was started on trastuzumab (4 mg/kg iv), followed by 2 mg/kg weekly doses for 51 wk. Three months after trastuzumab was initiated, the patient developed a nonproductive cough that persisted despite several courses of antibiotics and got worse throughout the year of trastuzumab treatment. A flexible fiber-optic bronchoscopy revealed no endobronchial lesions; endobronchial biopsies from the carina and contralateral main bronchus revealed severe metaplasia of the bronchial and inflammation (Fig. 1, A and B). The patient’s cough improved 3 mo after trastuzumab was discontinued, and 6 mo later her nasal mucosa appeared normal on physical examination. A repeat flexible fibroptic bronchoscopy with endobronchial biopsies revealed normal airway epithelial morphology (Fig. 1, C and D).

This clinical presentation and course suggested a reversible change in airway epithelial differentiation that coincided with initiation and discontinuation of trastuzumab. Investigators in our group (30) demonstrated a novel mechanism of receptor-ligand interaction in airway epithelia that regulates injury and repair. In this model, the epithelium secretes heregulin-α into the apical airway surface liquid and restricts erbB receptors to the basolateral membrane. Compromising epithelial integrity results in immediate receptor-ligand interactions that enhance wound repair.

ErbB receptors belong to a family of tyrosine kinase receptors (6) whose prototype is epithelial growth factor receptor (EGFR; also known as erbB1). These receptors are implicated in a variety of cancers and are associated with poor prognoses, which has led to molecular targeting of erbB receptors in anti-cancer therapy (10, 21, 22). Trastuzumab, a humanized anti-erbB2 antibody, accelerates receptor internalization and degradation (5, 9) and has become the standard of care in patients with increased erbB2 breast cancer (3, 8, 11, 12, 19).

Importantly, 4.7% of patients experience cardiac dysfunction (27); however, the benefits from trastuzumab outweigh this potential risk (29). Although the mechanism of cardiac toxicity remains unclear, it has been hypothesized that erbB2 may be required for cardiac myocyte regeneration following injury.

This case presentation suggests that blocking erbB2 alters airway epithelial differentiation. If trastuzumab plays a causal role in dedifferentiating the patient’s airway epithelium, heregulin-α and/or other erbB ligands may preserve differentiation. Thus we hypothesized that a basolateral source of erbB ligands sustains a basal state of differentiation; disruption of signaling by trastuzumab dedifferentiates the epithelium.

METHODS

Primary human airway epithelial cell culture model. Airway epithelial cells were isolated from trachea and bronchi of donor lungs and cultured at the air-liquid interface as previously described (15, 26, 33, 34). Experiments were performed on epithelia from seven different donors.

Cocultures of primary human airway epithelia and normal human lung fibroblasts were grown on 6.5-mm clear Transwells (Corning...
Fig. 1. Hematoxylin- and eosin-stained sections of bronchial biopsies taken during treatment with trastuzumab (A and B, enlarged from inset in A) and 6 mo after termination of trastuzumab (C and D, enlarged from inset in C). A metaplastic epithelium lacking cilia is evident in biopsies obtained during treatment with trastuzumab. Ciliated cells were restored after termination of trastuzumab. Scale bars, 20 μm.

Costar, Corning, NY). One day before seeding, the epithelial sides of Transwells were coated with human placental collagen type IV (Sigma, St. Louis, MO) as previously described (7, 15, 17). The next day, the collagen was aspirated and Transwells were washed twice with phosphate-buffered saline. On the day of seeding, a mixture of 80% Vitrogen (bovine dermal collagen; Cohesion Technologies, Palo Alto, CA), 10% Dulbecco’s modified Eagle’s (DME)/F-12 medium, and 10% 0.1 N NaOH was titrated to a pH of 7.2. Passage 5 normal human lung fibroblasts (Cambrex, East Rutherford, NJ) were added to this working Vitrogen solution at a density of 2.0–3.8 × 10^6 fibroblasts per milliliter of gel. The underside of the Transwells was fitted with a plastic insert ring attachment holder (Jim’s Instruments, Iowa City, IA) onto which aliquots of fibroblasts and Vitrogen mixture were seeded. Inserts were maintained upside down in a 37°C incubator without CO₂ for at least 1 h to allow the Vitrogen matrix to gel. Once gelled, Transwells were flipped right side up and primary human airway epithelia were seeded on top. Cocultures were initially maintained in this medium; 2% serum added to USG had no appreciable effect on epithelial differentiation.

Control cultures were not treated.

Fibroblasts embedded in Vitrogen matrix were pulsed away from Transwells and incubated in 1% collagenase (Sigma) for 30 min at 37°C to digest the matrix. Fibroblasts were then harvested by centrifugation and washed with phosphate-buffered saline. RNA isolation was performed using the RNAqueous-4PCR kit (Ambion, Austin, TX) per manufacturer’s instructions. RNA was reverse transcribed with Thermoscript RT (Invitrogen, Carlsbad, CA). Controls included the omission of RT. The generated cDNA served as the template in PCR reactions using erbB ligand-specific forward and reverse primers for betacellulin, TGF-α, heparin-binding epithelial growth factor (HB-EGF), epiregulin, herregulin-α, and amphiregulin. The forward and reverse primers used were as follows: betacellulin,
Fig. 2. Primary cultures of human airway epithelia were treated basolaterally with 0 or 1 mg/ml trastuzumab for 2 wk. Cultures were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Loss of differentiation is evident in trastuzumab-treated cultures (B) compared with untreated controls (A). When analyzed using morphometry, trastuzumab treatment resulted in a decrease in the percentage of ciliated cells and an increase in metaplastic cells (C). *P < 0.01. Scale bar, 40 μm.

Fig. 3. Primary cultures of human airway epithelia were treated basolaterally with 0, 0.3, 3, or 30 nM of recombinant human heregulin-α for 2 wk. Cultures were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (A; scale bar, 40 μm). Two donors were analyzed per condition, and 6 epithelia were analyzed per donor. The height of the epithelia was then measured as described (see METHODS). Heregulin-α treatment increased epithelial height (B). Cell number was not affected by treatment resulting in a lower cell density per epithelium (C). The percentage of ciliated (D), goblet (E), nonciliated columnar (F), basal (G), and metaplastic cells (H) in control and heregulin-α-treated cultures was determined using morphometry. Heregulin-α treatment increased the number of goblet cells and decreased the number of nonciliated columnar cells. *P < 0.01.
GAA TAT GTC CCT GGG TGT GG and CTA CAA GCC AGG ACA CC; TGF-α, TAG GCA TTT CAG GCC AAA TC and GCA TTT CCT CAC ATA AGG AGT TT; HB-EGF, CTC TCC CTG CCA AGT CTC AG and TGA ACC AGG TTT GGA AAT ACA; epieregulin, CCA AGG AGC GAA AAT GCT TA and AAA GGG CAT AGT GCT TGC AT; heregulin-α, ATA TCC ACC ACT GGG ACA AGC CAT and TAT CAC GGG TGG AGA CAT TCC CGA; and amphiregulin, TGG ATT GGA CCT CAA TGA CA and CGT TCA CCG AAA TAT TCT TGC. After heat denaturation, DNA was amplified in an Eppendorf Mastercycler thermocycler (Westbury, NY) as follows: 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 40 cycles, followed by a 72°C postdwell for 2 min. Amplified DNA was separated by agarose gel electrophoresis and imaged (Polaroid, Cambridge, MA) under UV light.

RESULTS

Trastuzumab treatment causes dedifferentiation of human airway epithelium grown at air-liquid interface. Trastuzumab was added to an in vitro model of well-differentiated human airway epithelium (15). Hematoxylin and eosin-stained sections (Fig. 2, A and B) demonstrate that trastuzumab treatment decreased the number of ciliated cells by 49% and increased the number of metaplastic cells by 640% (Fig. 2C). These data suggest that erbB2 is important in airway epithelial differentiation and that receptor activation is required.

Heregulin-α stimulates human airway epithelia differentiation. Heregulin-α and erbB2 are important in epithelial differentiation and wound repair (30). The current data further suggest that when erbB2 is blocked by trastuzumab, the epithelium dedifferentiates. Whether trastuzumab treatment alters erbB2 receptor mass or leads to loss of receptor signaling without changing receptor mass is not clear from these studies. These data suggest that an erbB ligand, heregulin-α, might have the opposite effect of trastuzumab, which is to stimulate epithelial differentiation. To quantitatively examine the effects of heregulin-α on human airway epithelia, we added increasing concentrations of recombinant human heregulin-α to the basolateral medium. Figure 3A shows examples of hematoxylin and eosin-stained sections. In contrast to trastuzumab, heregulin-α treatment increased epithelial height, a characteristic of well-differentiated epithelial airway epithelia (24), and decreased the number of cells per area (Fig. 3, B and C). Interestingly, heregulin-α treatment appeared to make the cilia longer; however, this effect was not quantified.

Heregulin-α stimulates differentiation of airway epithelial cell types. We asked whether heregulin-α treatment had an effect on the cell types present in the epithelium. We found that the percentage of ciliated and basal cells was not affected (Fig. 3, D and G). However, there was a significant increase in the number of goblet cells and a decrease in the number of nonciliated columnar cells (Fig. 3, E and F). Interestingly, the number of metaplastic cells decreased at the highest dose of heregulin-α tested (Fig. 3H).

Lung fibroblasts express erbB ligands and stimulate human airway epithelial differentiation. Because the airway epithelial dedifferentiates when exposed to trastuzumab, erbB receptor stimulation may be required to maintain a basal level of differentiation in the epithelium. In vitro, erbB ligands may leak from the apical side of the epithelia or some ligands may be provided by the USG in the medium. However, in vivo, subepithelial lung fibroblasts are a likely source of ligand. Their localization basal to the epithelia would be optimal for direct and constitutive epithelial erbB receptor stimulation. Therefore, pulmonary fibroblasts were assayed for expression of erbB ligands by RT-PCR. Human lung fibroblasts express TGF-α, HB-EGF, epieregulin, heregulin-α, and amphiregulin, all erbB ligands (16) (Fig. 4A). Betacellulin was the only ligand tested that was not expressed. We then asked whether pulmonary fibroblasts could alter the differentiation of human airway epithelia in a manner similar to that of basolateral recombinant heregulin-α treatment. Qualitative changes are shown in epithelial morphology in cocultures (Fig. 4C) compared with untreated primary cultures of epithelia grown in isolation (Fig. 4B). These changes resemble those of adding recombinant heregulin-α to epithelial cultures in isolation (Fig. 3A). Epithelium grown in isolation reach a height of 33.7 μm with a standard deviation of 3.7 μm. However, when grown in the presence of pulmonary fibroblasts, epithelial height increased to 43.2 ± 8.8 μm, comparable to the epithelial height achieved after basolateral treatment with 3 nM heregulin-α (45.1 ± 4.9 μm). Figure 4D demonstrates Western blot analysis of erbB2...
receptor phosphorylation. In primary epithelial cultures, erbB2 is not phosphorylated. As expected, however, basolateral treatment of primary epithelial cultures with recombinant heregulin-α as well as epithelia grown in coculture with fibroblasts both resulted in erbB2 receptor phosphorylation. In addition, when epithelial-fibroblast cocultures were treated with trastuzumab, erbB receptor phosphorylation was inhibited (data not shown).

**DISCUSSION**

In the case study, we describe a patient with HER2/Neu3-positive infiltrating ductal carcinoma grade II–III on trastuzumab therapy. Three months after trastuzumab therapy was initiated, the patient developed a nonproductive cough that persisted despite several courses of antibiotics and worsened throughout the year of trastuzumab treatment. Although the presence of cough may be explained by a number of factors (a combination of chemotherapy, radiation therapy, and environmental or other causes), that it was concurrent with trastuzumab was noteworthy. Two of these monomers are uniquely handicapped: erbB2 and erbB4, resulting in a conformational change allowing for receptor homo- or heterodimer formation. Differential ligand affinity for monomers and the resulting receptor dimer combinations allow for a complex and promiscuous signaling capacity. Two of these monomers are uniquely handicapped: erbB2 is an orphan receptor (ligand for erbB2 has not yet been identified), and erbB3 has a dead kinase. However, these two monomers can conveniently come together and complement each other’s limitations. ErbB3 binds heregulin, resulting in dimerization with erbB2, which, once engaged, autophosphorylates and transphosphorylates erbB3. ErbB2 also can dimerize with erbB1 and erbB4, resulting in complex, coordinated, and specific signaling cascades in response to all known erbB ligands (EGF, amphiregulin, TGF-α, betacellulin, HB-EGF, and epieregulin).

In an in vitro model of human airway epithelia, incubation with trastuzumab led to epithelial dedifferentiation. The number of flat, metaplastic cells increased, a finding that closely resembled the patient’s airway biopsies. Although not tested in this study, trastuzumab treatment also may result in cell death, a possibility that cannot be excluded. Incubation with heregulin-α increased epithelial cell height and decreased the number of metaplastic cells. Interestingly, heregulin-α treatment also increased the number of goblet cells, whereas trastuzumab did not alter the goblet cell population. These data suggest that increasing concentrations of heregulin-α induce transdifferentiation of nonciliated columnar cells into goblet cells. Transdifferentiation is well documented (4). In addition, this paradox suggests that whereas heregulin-α stimulates epithelial differentiation, increasing goblet cell numbers, other ligands acting via erbB2-containing heterodimers must regulate other differentiation lineages. Alternately, altering erbB2 activation may result in Clara cell and goblet cell differentiation, rather than global epithelial dedifferentiation; the present study cannot distinguish between the two possibilities. Interestingly, whereas our data suggest that erbB2 activation stimulates both cellular proliferation and differentiation, others have demonstrated that activation of erbB1 leads to goblet cell hyperplasia without altering the total number of epithelial cells (28). These data suggest a role for erbB1 in differentiation but not in proliferation. Similarly, Shimizu et al. (25) found that inhaling endotoxin led to an increase in the goblet cell population without changing the total cell number. Together, these data suggest that multiple ligands activate a variety of erbB receptors and that this complexity likely maintains both epithelial cell numbers and differentiation status, factors critical for normal airway barrier function. This also is consistent with our data showing that pulmonary fibroblasts express all but one of the erbB ligands and that human airway epithelia express all four erbB receptors (1, 2, 20, 30). Importantly, Western blot analysis (Fig. 4D) demonstrated that epithelial-fibroblast cocultures resulted in erbB2 receptor activation. Such interactions between epithelia and subepithelial cells are not new. In fact, epithelial/mesenchymal communications have been widely studied, and their roles in development and carcinogenesis have been recognized for decades (13, 14, 18).

Despite being cultured in the absence of fibroblast, the in vitro model of human airway epithelia appears morphologically differentiated. One explanation for this is that apical ligand may constitutively diffuse basolaterally at low levels. Moreover, normal turnover of cells may allow airway surface liquid ligands to diffuse into the basolateral compartment and stimulate cellular differentiation in a precise temporal and localized fashion. Finally, an additional artificial source of ligand may be the serum substitute (Ultrorser G) present in the medium. Ultrorser G is a largely undefined serum substitute that Widdicombe and colleagues (23, 32) have shown to promote airway epithelial differentiation.

The data presented suggest a central but complex role for erbB receptors and ligands for epithelial differentiation. They further suggest that mesenchymal-epithelial communication moderates this process. Our initial studies focused on only one ligand, heregulin-α, and serve as proof of a principle from which to model future studies aimed at unraveling this complexity.

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