

## OCCASIONAL REVIEW

## Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium

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The epithelial cells lining the airways serve protective functions. The "barrier function" of the epithelium protects the individual from damage by inhaled irritants. The epithelium produces mucins which become hydrated and form a viscoelastic gel which spreads over the epithelial surface. In healthy individuals inhaled foreign materials become entrapped in the mucus and are cleared by mucociliary transport and by coughing. In many chronic inflammatory airway diseases, however, excessive mucus is produced and is inadequately cleared, leading to mucous obstruction and infection. At present there is no specific treatment for hypersecretion. However, the discovery that an epidermal growth factor receptor (EGFR) cascade is involved in mucin production by a wide variety of stimuli suggests that blockade may provide specific treatment for hypersecretory diseases. EGFR pathways have also been implicated in the repair of damaged airway epithelium. The roles of EGFR in airway epithelial cell hypersecretion and epithelial damage and repair are reviewed and future potential treatments are suggested.

leading to mucous obstruction and infection. Airway mucus hypersecretion and plugging have been related to death in acute asthma and to proliferation of *Pseudomonas aeruginosa* in cystic fibrosis. Furthermore, in both asthmatic patients and those with chronic obstructive pulmonary disease (COPD), chronic excessive sputum production is independently associated with an accelerated rate of decline in maximal expiratory flow.<sup>8</sup>

At present there is no specific treatment for hypersecretion. However, the discovery that an epidermal growth factor receptor (EGFR) cascade is involved in mucin production by a wide variety of stimuli suggests that blockade may provide specific treatment for hypersecretory diseases. EGFR pathways have also been implicated in the repair of damaged airway epithelium. This paper reviews the roles of EGFR in airway epithelial cell hypersecretion and epithelial damage and repair and suggests future potential treatments.

EGF was discovered by Cohen and subsequently his group expanded our understanding of EGF and its receptor EGFR.<sup>9</sup> EGFR is a 170 kDa membrane glycoprotein which is activated by multiple ligands including EGF, transforming growth factor (TGF)- $\alpha$ , heparin binding (HB)-EGF, amphiregulin,  $\beta$ -cellulin and epiregulin, all of which are synthesised as transmembrane precursors (proligands) that are inserted in the cell surface and cleaved by proteases to release the mature soluble growth factor.<sup>10</sup> The EGFR signalling pathway has been known to be involved in a variety of physiological responses including proliferation, differentiation, motility, and survival.<sup>11 12</sup> Because of the potential importance of the EGFR pathway in airway diseases and potentially in treatment, this paper focuses on mechanisms involved in mucus hypersecretion and in epithelial damage and repair.

#### ROLE OF EGFR IN MUCIN SYNTHESIS AND GOBLET CELL METAPLASIA

EGFR activation has been shown to induce epithelial cell proliferation in lung cancer cells under some circumstances such as sparse cultures. Takeyama *et al*<sup>13</sup> reasoned that epithelial cells under other circumstances (such as dense cultures) could stimulate cell differentiation and mucin production. They showed that dense cultures of airway epithelial (NCI-H292) cells

The epithelial cells lining the airways serve protective functions. The "barrier function" of the epithelium serves to protect the individual from damage by inhaled irritants, including bacteria, viruses, particulates and vapour phase irritants. Normally, mucus secretion plays a protective role. The epithelium produces mucins which are highly glycosylated proteins synthesised by surface epithelial (goblet) cells and by submucosal gland (mucous) cells. When mucins are released from cells (by degranulation) they become hydrated and form a viscoelastic gel which spreads over the epithelial surface. At least 19 mucin genes (MUC) have been cloned. There are two types of respiratory mucins—membrane-associated and gel-forming (secreted).<sup>1</sup> Three secreted mucins appear to be prominent in inflammatory airway diseases: MUC5AC (in airway epithelial goblet cells),<sup>2 3</sup> MUC5B in submucosal glands,<sup>4</sup> and MUC2 (in goblet cells and glands).<sup>5-7</sup> In healthy individuals, inhaled foreign materials become entrapped in the mucus and are cleared by mucociliary transport and by coughing. However, in many chronic inflammatory airway diseases excessive mucus is produced and is inadequately cleared,

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resulted in MUC5AC mucin gene expression and protein production. They also showed that addition of EGFR ligands (EGF and TGF- $\alpha$ ) to the densely cultured epithelial cells upregulates MUC5AC gene and protein expression in airway epithelial cells *in vitro*. Using selective inhibitors of EGFR tyrosine kinase phosphorylation, they reported that EGFR ligand induced mucin MUC5AC synthesis is dependent on EGFR activation.<sup>13</sup> Perrais *et al*<sup>14</sup> confirmed that EGF and TGF- $\alpha$  induce MUC5AC and MUC2 mucin synthesis in airway epithelial cells.

Takeyama *et al*<sup>13</sup> extended these findings by studying mechanisms of goblet cell metaplasia in rat trachea *in vivo*. Tumour necrosis factor- $\alpha$  induces EGFR expression in airway epithelial cells. Subsequent administration of an EGFR ligand (EGF, TGF- $\alpha$ ) caused EGFR activation of a downstream cascade, leading to mucin production (fig 1). From these studies, the authors concluded that activation of airway EGFR causes mucin production. After these original studies, several reports identified EGFR as a key target leading to mucin synthesis and/or goblet cell metaplasia in response to various stimuli (box 1).

### ROLE OF EGFR IN AIRWAY EPITHELIAL REPAIR

The role of EGFR activation in mediating epithelial repair has been shown in various cell types *in vitro* including keratinocytes,<sup>26</sup> mammary epithelial cells,<sup>27</sup> and alveolar epithelial cells.<sup>28</sup> In 1993 Barrow *et al*<sup>29</sup> hypothesised that EGFR ligands and other growth factors mediate bronchial epithelial repair. They showed that administration of aerosolised EGF plus PDGF for 2 weeks enhanced repair of sheep tracheal epithelium after cotton smoke injury *in vivo*. It was suggested that cell proliferation and differentiation were responsible for accelerated epithelial restitution, but the cellular mechanisms were not identified. Kim *et al*<sup>30</sup> hypothesised that EGF accelerates wound closure in airway epithelial

cells independently of cell proliferation. Culturing guinea pig airway epithelial cells *in vitro*, they found that EGF accelerates closure of small wounds in confluent epithelial monolayers over 24 hours and that EGF elicits migration of airway epithelial cells, suggesting that early events in EGF mediated wound closure involve cell migration. Subsequent work in cultured human airway (16HBE 14o-) epithelial cells showed that EGF induced epithelial repair occurs within 18 hours and that cell proliferation does not occur at this time,<sup>31</sup> establishing that EGF promotes airway epithelial repair via cell migration in this model. However, it is likely that cell proliferation is implicated in the repair of larger wounds in the airway epithelium. Puddicombe *et al*<sup>32</sup> showed that both EGF and another EGFR ligand, heparin binding EGF (HB-EGF), promote wound closure in airway epithelial cells. Using a selective EGFR tyrosine kinase inhibitor (AG 1478), they implicated EGFR activation in ligand induced wound closure. Because inhibition of EGFR tyrosine kinase phosphorylation also inhibited wound closure under basal conditions (serum free culture medium), the authors suggested that autocrine mechanisms are involved in EGFR mediated repair mechanisms in airway epithelial cells. Figure 2 shows the suggested steps leading to airway epithelial repair after injury.

### MECHANISMS OF EGFR EXPRESSION AND ACTIVATION BY DIFFERENT STIMULI

The expression of EGFR is sparse in the airway epithelium of pathogen free rodents (rats, mice) and in the upper and lower airways of normal humans.<sup>3 33</sup> However, EGFR expression in airway epithelium is increased in response to various inflammatory stimuli including TNF- $\alpha$ ,<sup>13</sup> mechanical damage,<sup>24 32</sup> cigarette smoke,<sup>18</sup> and inhalation of noxious products such as naphthalene<sup>34</sup> or bleomycin.<sup>35</sup>

EGFR activation may involve two different pathways—ligand dependent and ligand independent EGFR tyrosine phosphorylation. In ligand dependent EGFR tyrosine phosphorylation, EGFR ligands bind to EGF receptors in the extracellular domain and activate them (fig 3, left side) while, in ligand independent EGFR tyrosine phosphorylation, EGFR tyrosine phosphorylation occurs in the absence of exogenous EGFR ligands (fig 3, right side). Ligand independent EGFR phosphorylation is reported in response to oxidative stress<sup>36</sup> that can be produced by cigarette smoke<sup>18</sup> and by activated neutrophils.<sup>19</sup> However, it was later realised that airway epithelial cells, in addition to expressing EGFR, express EGFR proligands on their surface. As will be seen below, some of these stimuli (such as cigarette smoke) can induce shedding of EGFR proligands from the epithelial cell surface, leading to ligand binding to the receptor and ligand dependent EGFR activation.<sup>37</sup> Neutrophils are present in the airways of patients with hypersecretory diseases such as COPD, acute severe asthma, and cystic fibrosis and could promote ligand independent EGFR activation and mucin synthesis via the release of oxygen free radicals. Other inflammatory cells (such as macrophages and eosinophils) recruited to the airway epithelium in inflammatory respiratory diseases express EGFR ligands,<sup>38 39</sup> raising the possibility that interactions between these cells and epithelial cells could result in ligand dependent activation of EGFR signalling cascades and mucin production. Borchers *et al*<sup>40</sup> showed that exposure of mice to acrolein, a product of cigarette smoke, results in goblet cell metaplasia. They suggested that the effect was due to macrophage elastase. Kim *et al*<sup>41</sup> have recently shown that macrophages induce mucin production in cultured airway epithelial cells. Burgel *et al*<sup>20</sup> showed that isolated human eosinophils, when activated, induce mucin synthesis in cultured airway epithelial cells by EGFR activation. Soluble TGF- $\alpha$  was increased in cell culture

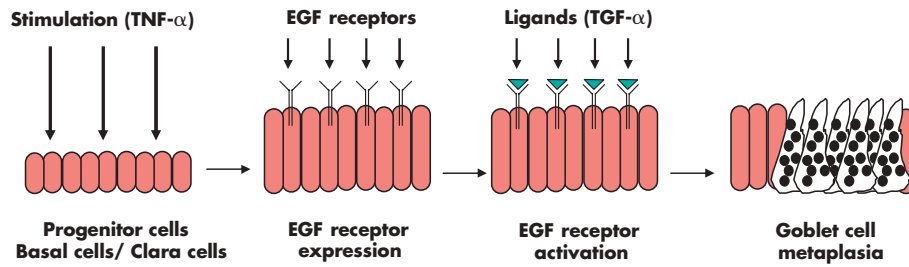
#### Box 1 Examples of stimuli that induce mucin synthesis *in vitro* or *in vivo* by EGFR activation in airways

##### In vitro experiments

- Bacterial products:
  - *P aeruginosa* supernatant<sup>15</sup>
  - Lipopolysaccharide (LPS)<sup>15 16</sup>
  - Lipoteichoic acid (LTA)<sup>17</sup>
- Phorbol 12-myristate 13-acetate (PMA)<sup>16</sup>
- Cigarette smoke<sup>18</sup>
- Inflammatory cells:
  - Neutrophils<sup>19</sup>
  - Eosinophils<sup>20</sup>
- Serine proteases:
  - Human neutrophil elastase<sup>21</sup>
  - Human airway trypsin-like protease<sup>22</sup>

##### In vivo experiments

- Th2 cells
  - Antigen (ovalbumin)<sup>13</sup>
  - IL-13<sup>23</sup>
- Mechanical damage of epithelium<sup>24</sup>
- Cigarette smoke<sup>18</sup>
- Leukotrienes<sup>25</sup>



**Figure 1** Epidermal growth factor (EGF) receptor expression and activation in goblet cell mucin production. Airway epithelial cells of pathogen free rats and lower airways of healthy humans show sparse expression of EGF receptors. Stimulation by tumour necrosis factor (TNF)- $\alpha$  induces EGF receptor expression in progenitor cells in airway epithelium but not mucin production. Release of exogenous EGF ligands (such as transforming growth factor (TGF)- $\alpha$ ) results in ligand binding to EGFR, EGFR activation, and subsequent mucin production in goblet cells (shown by dark granules in light cells).

medium of epithelial cells stimulated with activated eosinophils, and a blocking antibody to TGF- $\alpha$  reduced mucin synthesis. These results implicated an EGFR cascade and suggested that TGF- $\alpha$  is involved in the response.

Airway epithelial cells express several EGFR ligands—for example, EGF, TGF- $\alpha$ , HB-EGF, and amphiregulin.<sup>42</sup> Various stimuli have been reported to increase the expression of selected ligands in experimental models in vitro and in vivo, but mechanisms of inducing this expression are unknown. Among these stimuli are those that induce mucin production—for example, IL-13,<sup>43</sup> cigarette smoke,<sup>44</sup> and acrolein<sup>1</sup>—or stimuli used in studies of airway remodelling and repair—for example, vanadium,<sup>45</sup> bleomycin,<sup>35</sup> naphthalene,<sup>34</sup> and compression of bronchial epithelial cells in vitro.<sup>46</sup>

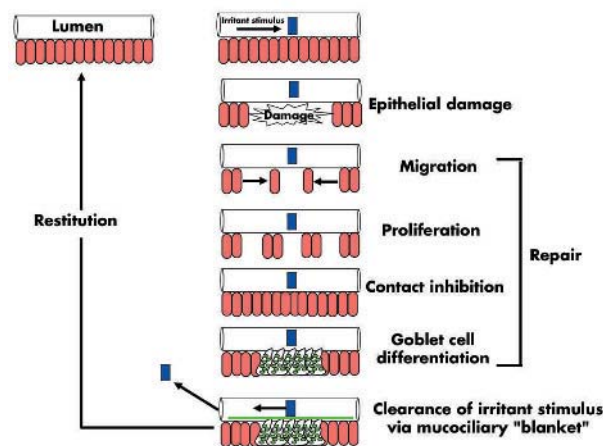
In the epithelium, EGFR proligands are synthesised as membrane anchored molecules that are cleaved by proteases to become activated.<sup>10</sup> Metalloproteases cleave EGFR proli-

gands in response to activation by G-protein agonists in mammary epithelial cells.<sup>47</sup> Lemjabbar *et al* showed that lipoteichoic acid (LTA), a component of Gram positive bacterial cell walls, induces mucin synthesis by activating EGFR in airway epithelial cells.<sup>17</sup> Mechanisms of EGFR activation in this model are reported to involve recognition of LTA by platelet activating factor receptor (PAFR), a G-protein coupled receptor that activates a membrane anchored metalloprotease, ADAM 10, resulting in cleavage of proHB-EGF, EGFR activation, and mucin synthesis. Shao *et al*<sup>16</sup> showed that TNF- $\alpha$  converting enzyme (TACE/ADAM 17), another member of “a disintegrin and metalloprotease” (ADAM) family, is an important regulator of EGFR activation leading to mucin synthesis in airways. Using cultured human airway epithelial cells, these authors showed that phorbol 12-myristate 13-acetate (PMA), an activator of TACE, and pathological stimuli (such as lipopolysaccharide (LPS), supernatant of the Gram negative bacteria *P aeruginosa*, and cigarette smoke) induce mucin synthesis.<sup>16, 37</sup> Importantly, knockdown of TACE by specific small interfering RNA prevented EGFR activation and mucin synthesis by these stimuli. Mechanisms involved are cleavage of epithelial membrane anchored proTGF- $\alpha$  by TACE, binding of soluble TGF- $\alpha$  to EGFR, and subsequent phosphorylation of EGFR leading to mucin synthesis. Thus, bacterial products of Gram positive and Gram negative bacteria induce mucin synthesis in airway epithelial cells in vitro by shedding of EGFR proligands leading to autocrine activation of an EGFR cascade. Cleavage of epithelial proligands and autocrine activation of EGFR can also be promoted by neutrophil proteases: Kohri *et al*<sup>21</sup> reported that induction of mucin synthesis by the serine protease human neutrophil elastase (HNE) causes EGFR activation: HNE causes the cleavage of membrane anchored proTGF- $\alpha$  from the epithelial surface resulting in the release of mature TGF- $\alpha$  which binds to EGFR, causing EGFR activation and mucin synthesis. An EGFR blocking antibody inhibited the response to elastase, implicating a ligand dependent process. Voynow *et al*<sup>48</sup> reported that the increase in MUC5AC mRNA following exposure to human neutrophil elastase could be due to increased mRNA stability.

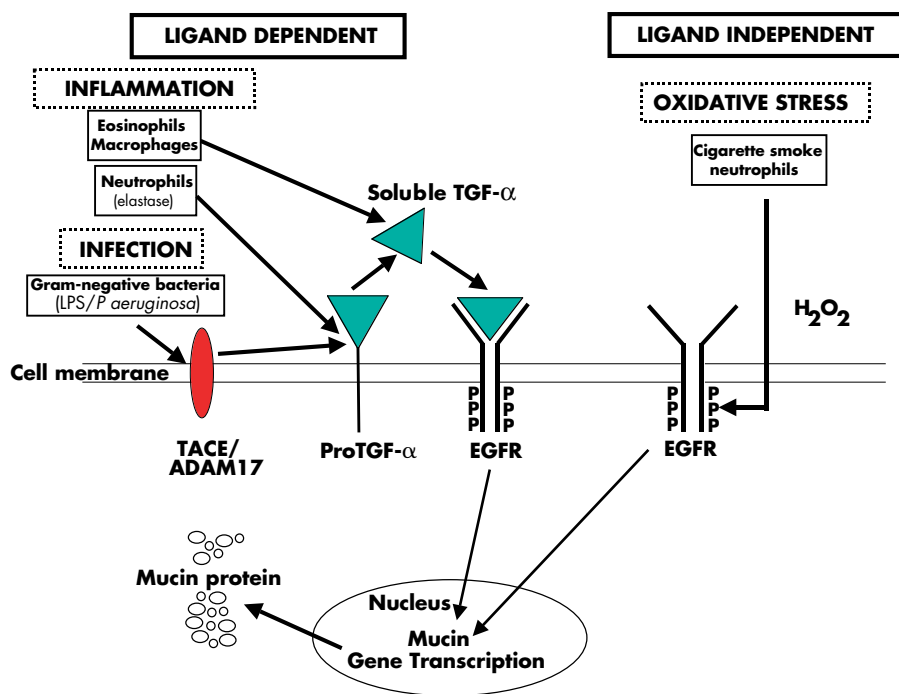
These results implicate EGFR activation by a wide variety of stimuli, and various paracrine interactions among cells and molecules are responsible for the effects on the airway epithelium.

**FUTURE CLINICAL STUDIES**

Mucus hypersecretion contributes to morbidity and mortality in various airway inflammatory diseases (such as asthma, COPD, cystic fibrosis, and nasal polyposis), but no treatment to prevent hypersecretion currently exists. Airway mucus



**Figure 2** Suggested steps of airway epithelial repair after epithelial damage by irritant stimulus. Normal airway epithelium contains few mucin producing (goblet) cells. Inhalation of foreign particles or noxious agents (dark rectangles) may induce loss of surface airway epithelial cells (epithelial damage) followed by a step by step repair process: (1) migration of epithelial cells from the borders of the wound followed by (2) cell proliferation; (3) when newly formed epithelial cells become confluent they undergo contact inhibition followed by (4) cell differentiation into mucin producing cells. Of course, goblet cell differentiation does not necessarily require prior epithelial damage. Mucus release in the lumen assists in clearance of foreign materials via mucociliary clearance and cough actions on the mucociliary “blanket”. The mucus cells then gradually disappear and the epithelial structure returns to normal (epithelial restitution).



**Figure 3** Examples of ligand dependent and ligand independent EGFR activation leading to mucin synthesis. Binding of EGFR ligands (triangles) in the extracellular domain in airway epithelial cells is followed by phosphorylation of tyrosine residues (P) in the intracellular domain (ligand dependent activation). Active (soluble) ligands (exemplified by TGF- $\alpha$ ) may be released by recruited inflammatory cells (such as eosinophils and macrophages) or produced by cleavage of membrane anchored proligand (exemplified by proTGF- $\alpha$ ). The proligand may be cleaved by neutrophil proteases (such as human neutrophil elastase) or by epithelial membrane anchored proteases (such as “a disintegrin and metalloprotease” (ADAM) family exemplified by ADAM 17/TNF- $\alpha$  converting enzyme) in response to stimuli such as *P aeruginosa* bacteria. Alternatively, activation of EGFR may occur in the absence of ligand binding (ligand independent activation) by phosphorylation of tyrosine residues in the intracellular domain directly (no ligand) in response to stimuli (such as cigarette smoke, activated neutrophils producing oxidative stress). Oxygen free radicals have also been shown to activate shedding of EGFR proligands in epithelial cells resulting in EGFR activation. Regardless of the mechanisms (ligand dependent or ligand independent) of EGFR activation, phosphorylation of tyrosine residues in the intracellular domain triggers a downstream cascade leading to mucin gene and protein synthesis.

hypersecretion can occur with limited clinical symptoms (especially in peripheral airways where cough receptors are absent and where extensive mucous plugging may be undetected by pulmonary function tests), and reproducible biological measurement of mucus production in airway secretions (for example, sputum and bronchoalveolar lavage fluid) in human diseases is difficult. Determining the outcomes in clinical studies of treatments that target mucus hypersecretion in humans is therefore complicated. In a recent study we assessed the effects of an intranasal corticosteroid on mucus production in nasal polyps.<sup>49</sup> One nasal polyp was removed surgically before treatment and another was removed after 8 weeks of treatment with intranasal fluticasone (400  $\mu$ g/day) in nine subjects. The polyp tissues were examined morphometrically. Evaluation of alcian blue (AB)/PAS staining for mucus glycoconjugates and staining with a monoclonal antibody to MUC5AC mucin in the epithelium showed that steroids did not affect mucin protein expression. Similarly, MUC5AC mRNA, assessed by in situ hybridisation, was expressed in epithelium before and after treatment, suggesting that intranasal corticosteroids do not reduce mucus production in nasal polyps. We suggest that, because of their location and accessibility, nasal polyps provide a convenient “model” for evaluating various treatments in the suppression of mucin production in the respiratory system.

The finding that various pathophysiological stimuli converge in the EGFR pathway to induce mucin production and goblet cell metaplasia provides new therapeutic opportunities, using treatment targeting mechanisms of EGFR expression or EGFR activation. TNF- $\alpha$  is increased in airways

in hypersecretory diseases and may contribute to EGFR expression.<sup>13</sup> Inhibitors of TNF- $\alpha$  or TNF- $\alpha$  receptors are in clinical use for rheumatoid arthritis and should be evaluated for treating hypersecretion. Small molecules inhibiting EGFR tyrosine kinase phosphorylation or monoclonal antibody to EGFR are undergoing clinical trials in patients with non-small cell lung cancer with minimal toxicity.<sup>50</sup> Clinical studies using molecules targeting EGFR activation in hypersecretory diseases will be of interest. Because various proteases (such as neutrophil elastase and members of the ADAM family of metalloproteases) have been implicated in the cleavage of EGFR proligands and in EGFR activation (see above), it is conceivable that treatments which inhibit these molecules might prevent mucus hypersecretion.

Damage to the airway epithelium has been described in asthma.<sup>51</sup> EGFR expression is increased in asthmatic epithelium and activation of EGFR contributes to airway epithelial repair.<sup>52</sup> Because recombinant EGF has been reported to have beneficial effects in ulcerative colitis,<sup>52</sup> a recent review suggests that the use of recombinant EGF may be beneficial in the treatment of asthma.<sup>53</sup> In processes where the predominant abnormality is epithelial damage, activation of EGFR may therefore result in improved wound healing. However, in diseases where mucin hypersecretion predominates, inhibition of EGFR phosphorylation could result in reversal of the pathophysiological process.

In conclusion, there is increasing evidence that EGFR is an important player in regulating mucus production in airway epithelium and in the repair of epithelium after injury. Studies performed in recent years have contributed to a better understanding of cellular and molecular mechanisms

involved in EGFR expression and activation leading to mucin production in response to noxious stimuli. It is suggested that disrupting the EGFR cascade that leads to mucus production is beneficial in airway inflammatory (hypersecretory) diseases. Proof of concept requires clinical trials evaluating new therapeutic opportunities opened by these discoveries.

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