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Potential use of an anticancer drug gefinitib, an EGFR inhibitor, on allergic airway inflammation

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Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; PI3K, phosphoinositol 3'-kinase

Abstract

The EGFR plays an essential role in goblet cell hyperplasia and mucus hypersecretion. EGFR has an intrinsic tyrosine kinase activity that, when activated, induces the production of MUC5AC through the signaling kinase cascade in the airway epithelium. We have investigated the effects of an EGFR tyrosine kinase inhibitor, gefitinib, on ovalbumin (OVA)-induced, allergic inflammation in airway epithelia of mice. OVA-sensitized mice were pretreated with gefitinib at two different doses (12.5 and 50 mg/kg) and then challenged with OVA. The OVA challenge increased the total cell count and eosinophil count in bronchoalveolar lavage fluid (BALF), as well as the concentrations of T-helper2 (Th2) cytokines, such as IL-4 and IL-13, overall eosinophil recruitment in the lung tissue and airway hyperresponsiveness (AHR). Pretreatment with gefitinib reduced the inflammatory cell counts and released cytokine concentrations (IL-4 and IL-13) in BALF, as well as eosinophil recruitment in the lungs and AHR, in a dose-dependent manner. This was associated with decreased EGFR and Akt phosphorylation. We showed that gefitnib inhibits EGFR and phosphoinositol 3'-kinase (PI3K)/Akt activation which were activated in OVA sensitized mice. These findings suggest that inhibitors of the EGFR cascade may have a role in the treatment of asthma.

Keywords: asthma; gefitinib; mucins; 1-phosphatidylinositol 3-kinase; proto-oncogene proteins c-akt; receptor, epidermal growth factor

Introduction

Bronchial asthma is a chronic inflammatory disease of airways characterized by eosinophilic infiltration and airway hyperrespensiveness (AHR). Pathological features of asthma include pulmonary infiltration by eosinophils, lymphocytes, and mast cells, and structural changes of the airways, including bronchial wall thickening, subepithelial fibrosis, and goblet cell and airway smooth muscle hyperplasia (Cohn *et al*., 2004). T-helper 2 (Th2) cells have been proposed to play an essential role in allergic airway inflammation, and there are many studies that examine the functional effects of Th2 cells in asthmatics (Robinson *et al*., 1992). To date, accumulating data from mice and humans have identified Th2 cytokines, such as IL-4, IL-5, and IL-13, as major contributors to allergy and asthma (Ngoc *et al*., 2005).

Protein tyrosine kinases are essential for the activation and proliferation of inflammatory cells and airway-resident cells. EGFR is a member of the receptor tyrosine kinase family. On ligand binding, EGFR homodimerise or heterodimerise, thereby inducing intrinsic kinase activities that initiate intracellular signal transduction cascades including the Ras/Raf/MAPK pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the STAT pathway (Bogdan and Klambt, 2001). The degree of EGFR expression reflects the epithelial damage and activation, and increases in proportion to asthma severity (Amishima *et al*., 1998; Puddicombe *et al*., 2000; Burgel and Nadel, 2004; Wong, 2005). Activated EGFR also has an essential role in mucin production in airway epithelium (Takeyama *et al*., 1999, 2001; Nadel, 2001; Nadel and Burgel, 2001). It induces airway goblet cell hyperplasia and enhances the expression of mucin genes, such as MUC5AC. When EGFR is activated, its ligands induce MUC5AC pro-

duction through the signaling tyrosine kinase cascade in the airway (Deshmukh *et al*., 2005). Therefore, inhibitors of tyrosine kinase have been widely studied as a novel therapeutic strategy for the treatment of asthma (Vargaftig and Singer, 2003). Recent studies suggest that PI3K may contribute to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells (Pinho *et al*., 2005). Gefitinib, an EGFR tyrosine kinase inhibitor, is an active agent in non- small cell lung cancer (Gandara *et al*., 2004). Gefitinib blocks signal kinase transduction through EGFR and inhibits the proliferation and survival of cancer cells. As mentioned above, EGFR regulates mucin accumulation in the goblet cells of airway epithelium. There are some reports of an inhibitory effect of gefitinib on MUC5AC synthesis in mucin-secreting, non-small cell lung cancer, which was determined by blocking the EGFR signaling cascade (Kitazaki *et al*., 2005a, b).

Here, we investigated the inhibitory effects of the EGFR tyrosine kinase inhibitor gefitinib on OVA-induced allergic inflammation in mice *in vivo*. We hypothesized that the EGFR tyrosine kinase inhibitor would inhibit allergic inflammation by blocking the tyrosine kinase signaling cascade.

Materials and Methods

All experimental procedures were conducted in accordance with the standard guidelines for animal experiments of College of Medicine, Korea University.

Animals, immunization, and materials

Male BALB/c mice (Orient Ltd., Korea), weighing about 15 g, were housed in a temperature- and humidity-controlled room; standard laboratory food and water were provided freely. The animals were assigned at random to a normal control group or to ovalbumin (OVA; Grade V ovalbumin, Sigma, St. Louis, MO)-induced control or treatment groups. Five animals were studied in each group. The mice in the OVA groups were immunized intraperitoneally with 10 μg of OVA and 4 mg of aluminum hydroxide (Sigma) in 0.1 ml of normal saline on days 0 and 7. On days 14 and 15, the mice were anesthetized with methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL), administered by inhalation, and 10 μg of OVA in 50 μl of PBS were administrated intranasally.

To evaluate the effects of the EGFR tyrosine kinase inhibitor, gefitinib on allergic inflammation in the OVA-sensitized mouse airway, the mice were treated with purified gefitinib (12.5 or 50 mg/kg, AstraZeneca, UK) administered by oral gavage for the duration of the experiment, beginning 1 day before the OVA challenge and continuing daily until the animals were sacrificed. Bronchoalveolar lavage (BAL) was performed in five mice from each group 72 h after the last OVA challenge. For histologic evaluation, five additional mice from each group were sacrificed 72 h after the last challenge.

BAL cell and cytokine analysis

The number of eosinophil and neutrophil in the BAL was quantitated at 24 h intervals after the last challenge. These cell's counts progressively increased in the lung lavage from pilot study. Eosinophil and neutrophil count started within 24 h after the last intranasal challenge and reached maximum levels at 72 h (data not shown). After 72 h, the number of eosinophil and neutrophil dropped but were still elevated compared with the 24 h time point. Based on this observation, mice were sacrificed 72 h after the last intranasal challenge, and the collected BALF was used for cytospin preparations. The slides were fixed and stained with Diff-Quick (Baxter Healthcare, McGaw Park, IL), and differential cell counts were obtained using light microscopic evaluation of 300 cells per slide. Total BAL cell counts were performed with a hemocytometer. After the removal of cells from the BALF by centrifugation, 100-μl aliquots were analyzed by ELISA for the presence of IL-4 and IL-13, using commercially available kits and following the manufacturer's instructions (Biosource, Camarillo, CA) (Kim *et al*., 2006).

Tissue preparation

Animals were euthanized with ketamine (5 mg/kg, intraperitoneal) 3 days after the last OVA challenge. Protein extracts isolated from the left lung of each mouse were used for Western blotting. The systemic circulation was perfused with 1% paraformaldehyde in diethylpyrocarbonate (Sigma)-treated PBS via the left ventricle. For paraffin sections, tissues were placed in 4% paraformaldehyde overnight, dehydrated with ethanol, and embedded in paraffin. The embedded tissues were cut as 4-μm-thick cross sections and placed on glass slides.

Quantification of goblet cell metaplasia

The areas positive for Alcian blue (AB) and periodic acid-Schiff (PAS) staining and the total epithelial area were measured, and the AB-PAS areas were expressed as a percentage of the total epithelial area. The stained slides were examined under a light microscope (Olympus BX51, Tokyo, Japan) connected to a video camera (Olympus DP 50, Tokyo, Japan), and the images were captured with a Viewfinder program (Viewfinder Lite v. 1.0; Pixera Co., Los. Gatos, CA). Images of the airway epithelium were recorded from

six consecutive high-power fields at × 400. The analysis was performed with the SigmaScan Pro program (SPSS Inc., Chicago, IL).

Immunohistochemistry of EGFR

The expression of activated EGFR was examined using immunohistochemical staining with an antibody specific for phospho-EGFR (P-EGFR, Cell Signaling Technology Inc., Beverly, MA). Images were viewed with an Olympus BX60 microscope and captured with a Viewfinder program. For each section, the extent of P-EGFR staining was graded on a scale of 0 to (2+), with 0 representing no detectable staining, (1+) positive staining in ≤ 50% of total bronchial epithelial area, and (2+) representing positive staining in > 50% of total bronchial epithelial area.

Immunoblotting for activated EGFR

Left lung tissues for Western blotting were scraped and lysed with whole lysis buffer (Mammalian protein extract, M-PER; Pierce, Rockford, IL), and a protease inhibitor cocktail (Pierce) was added. Total proteins were separated by SDS-PAGE in 8% Tris-glycine gels and transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were incubated in 5% fat-free milk in TBS containing 0.05% Tween 20 for 1 h and then incubated with P-EGFR antibody (1:1,000, Cell Signaling Technology Inc.), anti-P-44/42 MAPK (1:1,000, Cell Signaling Technology Inc.), anti-P-Akt antibody (1:1,000, Calbiochem) and β-actin (1:20,000, Sigma) at 4°C overnight. After washing, the membranes were incubated with anti-rabbit IgG HRP-linked secondary antibody (1:2,000, Vector Laboratories, Inc.). Bound antibody was visualized using SuperSignal West Pico chemiluminescent substrate (Pierce).

Measurement of airway hyperresponsiveness (AHR)

Enhanced pauses (Penh) of the airway were assessed for indices of AHR in unrestrained conscious mice at 24 h after the final OVA challenge by using a single-chamber whole-body plethysmograph (Allmedicus, Anyang, Korea). In the plethysmograph, mice were exposed for 3 min to nebulized PBS and subsequently to increasing concentrations of nebulized methacholine (Sigma) in PBS, using an aerosonic ultrasonic nebulizer (DeVilbiss, Somerset, PA). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and are expressed for each methacholine concentration (3-50 mg/ml) as a percentage of the baseline Penh value following PBS exposure.

Statistics

All data were expressed as the mean ± SEM. One-way ANOVA was used to determine statistically significant differences between groups. Scheffé's *F*-test was used to correct for multiple comparisons when statistical significances were identified by ANOVA. Differences were considered significant at the level of $P < 0.05$.

Results

Cell and cytokine levels in BALF of OVA-induced mice

OVA exposure increased the total cell count and the eosinophil count in the BALF compared with the counts in the BALF of the normal control group. The BALF of control mice contained few inflammatory cells, and OVA stimulation of the airways caused eosinophil recruitment. Pretreatment with gefitinib inhibited the recruitment of eosinophils in the BALF in a dose-dependent manner (Figure 1). The concentrations of IL-4 and IL-13 were significantly increased in the groups exposed to OVA. Both of these cytokines are consistent with a Th2 cytokine profile. In the gefitinib-treated group, the levels of both cytokines were decreased in a dose-dependent manner (Figure 2).

Effects of gefitinib on goblet cell production in mouse airway

In control animals, the airway epithelium showed sparse positive staining with AB/PAS. Exposure to

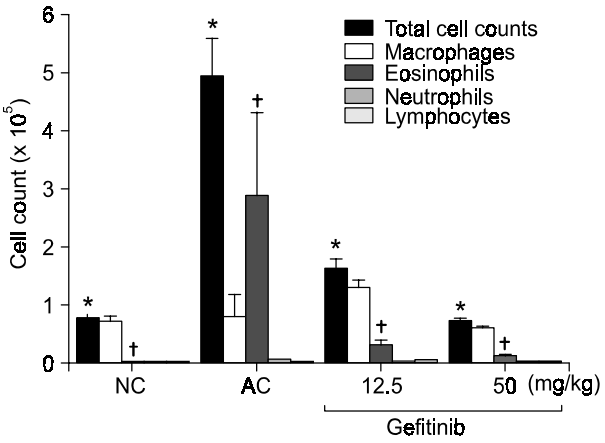

Figure 1. Gefitinib inhibits the recruitment of eosinophils in the BAL fluid. After OVA challenge and gefitinib pretreatment (12.5 mg/kg and 50 mg/kg), BALF were collected at 72 h after the last intranasal OVA challenge, and which were used for total BAL cell count and differential cell counts. **P* = 0.001, † *P* = 0.002. Values are reported as mean ± SEM. NC, normal control; AC , asthma control.

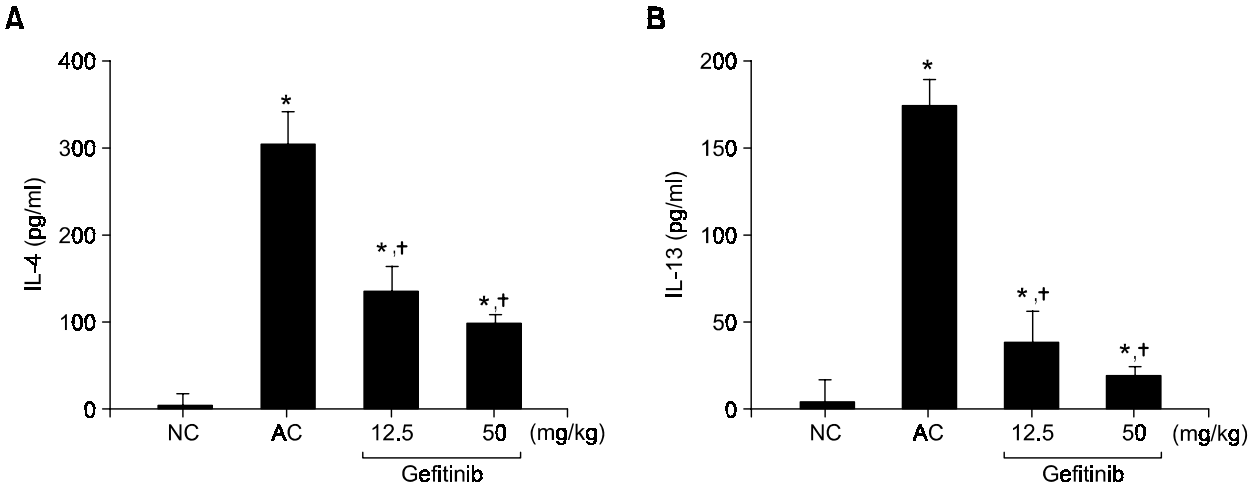

Figure 2. IL-4 (A) and IL-13 (B) levels in BALF supernatant of each group. After OVA challenge and gefitinib pretreatment (12.5 mg/kg and 50 mg/kg), BALF were collected at 72 h after the last intranasal OVA challenge, and their supernatants were collected after centrifuge for ELISA of IL-4 and IL-13. $^{*}P < 0.05$ compared with NC, $^{\dagger}P < 0.05$ compared with AC. Values are reported as mean ± SEM. NC, normal control; AC, asthma control.

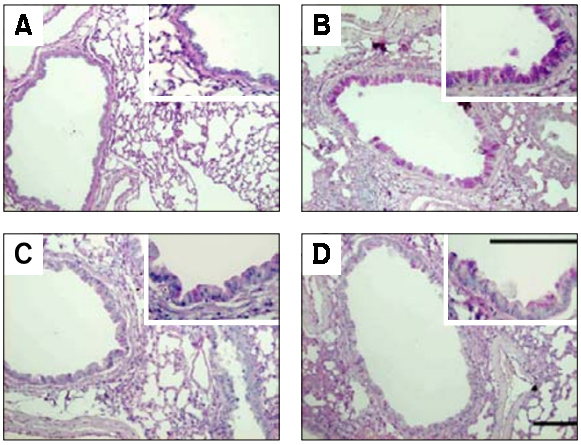

Figure 3. Photomicrographs of the effect of gefitinib on AB/PAS staining in bronchial epithelium in mice sensitized to OVA. (A) Control mice, (B) OVA-sensitized, and -challenged mice, (C) OVA-mice pretreated with gefitinib (12.5 mg/kg), and (D) OVA-mice pretreated with gefitinib (50 mg/kg) for 5 days were sacrificed, and their lung tissues were prepared for AB/PAS staining. Photomicrographs were shown at × 200 magnification; inserts show magnification at × 400 (bars = 100 μm).

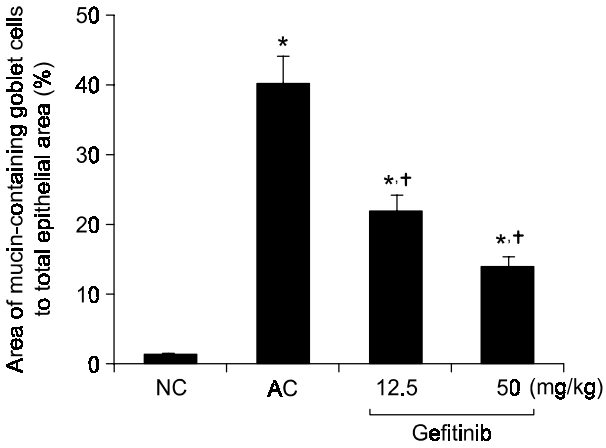

Figure 4. Gefitinib decreases the percentage of bronchial epithelium area stained with AB/PAS. After AB/PAS staining, the images of each group were captured and positively stained airway epithelial areas were calculated by the SigmaScan Pro Program at × 400 magnification. $^{*}P < 0.05$ compared with NC, $^{\dagger}P < 0.05$ compared with AC, $n = 5$ per group. Values are expressed as means ± SEM. NC, normal control; AC, asthma control.

OVA increased the AB/PAS-stained area (40.03 ± 7.65% of epithelium; $P < 0.05$). Pretreatment with gefitinib inhibited goblet cell metaplasia in a dose-dependent manner [21.91 ± 4.69% of epithelium in mice pretreated with 12.5 mg/kg gefitinib; 15.18 ± 4.17% of epithelium in mice pretreated with 50 mg/kg gefitinib; $P < 0.05$ compared with Asthma control group (AC)]; Figures 3 and 4. These results indicate that gefitinib decreases OVA-induced goblet cell metaplasia in the airway epithelium of mice.

Immunohistochemical expression of P-EGFR

There was strong P-EGFR staining in the epithelial cells of OVA-induced allergic airways. In contrast, pretreatment with gefitinib reduced the P-EGFR immunoreactivity in a dose-dependent manner. The level of P-EGFR immunoreactivity decreased in proportion to the AB/PAS-stained area in each group (Figure 5).

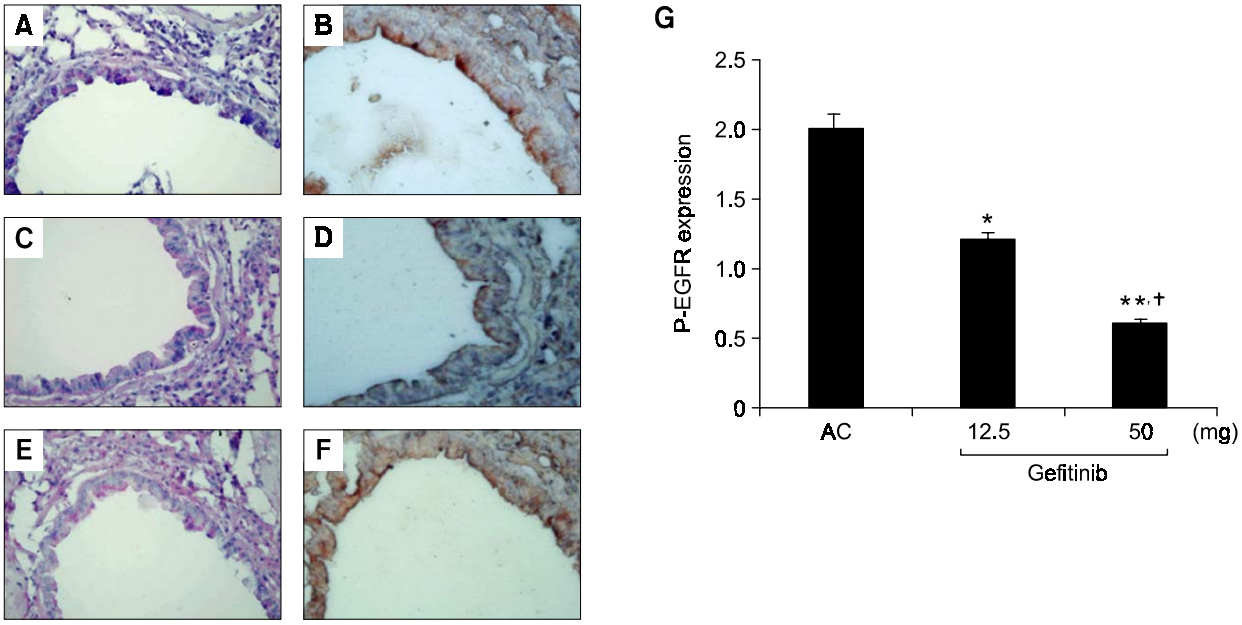

Figure 5. The comparison of expression of AB/PAS and P-EGFR immunohistochemical staining in mice airways. Tissues of OVA-sensitized, and -challenged mice were stained with (A) AB/PAS, and (B) an antibody specific for P-EGFR. Tissues of gefitinib (12.5 mg/kg) pretreated mice were stained with (C) AB/PAS, and (D) a P-EGFR specific antibody. Tissues of higher dose gefitinib (50 mg/kg) group were stained with (E) AB/PAS, and (F) a P-EGFR specific antibody. (G) P-EGFR expression was determined by calculating the P-EGFR stained area per microscopic field for each group. All images were shown and calculated at $\times$400 magnification. *$P < 0.05$; **$P < 0.01$ compared with control, $^{\dagger}P < 0.05$ compared with 12.5 mg/kg group, n = 5 per group.

Immunoblotting for activated EGFR

The immunoblot analysis of lung tissue identified the presence of activated-EGFR protein in each group. We performed Western blot analysis to detect P-EGFR, P-Akt and P-44/42 MAPK, because Akt and MAPK are downstream in the EGFR cascade. In OVA-sensitized mice, exposure to OVA increased the expression of P-EGFR, P-Akt and P-44/42 MAPK, and treatment with gefitinib inhibited the expression of them (Figure 6).

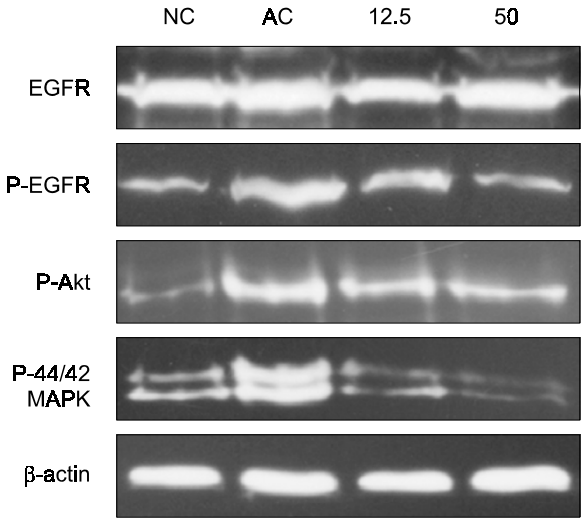

Figure 6. Effects of gefitinib on P-EGFR, P-Akt and P-44/42 MAPK expression in OVA-induced asthma model. Lung tissues of each group were lysed with whole lysis buffer, and a protease inhibitor cocktail was added. P-EGFR, P-Akt, and P-44/42 MAPK were identified by Western blot analysis using anti-P-EGFR, anti-P-Akt, and anti-P-44/42 MAPK antibodies. NC, normal control; AC, asthma control.

Effect of gefitinib on AHR

Mice sensitized to OVA and challenged with intranasal administration of OVA developed increases in airway responsiveness to methacholine compared with normal control mice ($P < 0.02$). Intragastric administration of gefitinib reduced airway responsiveness to methacholine in mice challenged with OVA compared with the responsiveness of untreated mice challenged with OVA ($P < 0.02$, Figure 7).

Discussion

In this study, we have investigated whether the EGFR tyrosine kinase inhibitor, gefitinib can reduce allergic inflammation in the airway epithelial cells of mice. Our results show that exposure to OVA induces the recruitment of eosinophils, the release of IL-4 and IL-13, and an increase in airway hyperresponsiveness in

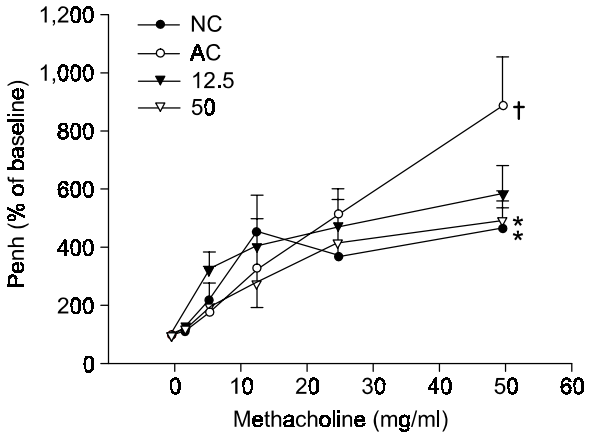

Figure 7. Gefitinib inhibited the developed AHR. Enhanced pauses (Penh) were measured at 24 h after the final OVA challenge using whole-body plethysmograph. Mice were exposed for 3 min subsequently to increasing concentrations of nebulized methacholine in PBS. The Penh values were expressed for each methacholine concentration (3-50 mg/ml) as a percentage of the baseline Penh value. $^{*}P < 0.02$ compared with NC, $^{\dagger}P < 0.02$ compared with AC, $n = 5$ per group Values are reported as mean ± SEM. NC, normal control; AC, asthma control; 12.5, gefitinib 12.5 mg/kg/day; 50, gefitinib 50 mg/kg/day.

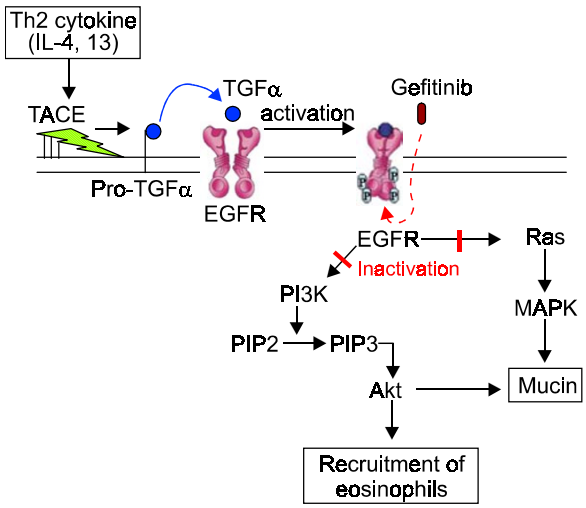

Figure 8. Model for the inhibition of mucin and eosinophils by gefitinib. This model combines others' previous results on mechanisms responsible for the ability of Th2 cytokines to activate TNF-α-converting enzyme (TACE) and cleavage TGFα from pro-TGFα with the present results. In asthma model, Th2 cytokines released from Th2 cells, especially IL-13, may induce release of the soluble form of TGFα from the bronchial epithelium, which in turn can transactivate EGFR to induce mucin gene expression and recruitment of eosinophils. When gefitinib binds with EGFR, PI3K and Ras pathway were blocked. Therefore, mucin production and recruitment of eosinophils were inhibited by gefitinib.

mice. Pretreatment with gefitinib inhibits the activation of EGFR and Akt, reduces the release of Th2 cytokines from airway epithelium, and decreases the recruitment of eosinophils into airway epithelium. In fact, EGFR expression is increased in bronchial epithelium in adult and childhood asthma and there is an increasing number of studies using EGFR to regulate airway inflammation in asthma (Amishima *et al*., 1998; Polosa *et al*., 2002; Fedorov *et al*., 2005). However, it is not well known the mechanisms that EGFR tyrosine kinase inhibitor inhibits allergic airway inflammation.

Protein tyrosine kinase signaling cascades have been shown to be important in the activation and proliferation of inflammatory cells and resident airway cells. For example, EGFR plays an essential role in airway inflammation, reflecting epithelial damage and activation, and its expression increases in proportion to the severity of the asthma (Amishima *et al*., 1998; Puddicombe *et al*., 2000; Burgel and Nadel, 2004; Wong, 2005). EGFR is a 170-kDa membrane glycoprotein which is activated by multiple ligands including EGF, TGF-α, heparin binding (HB)-EGF, amphiregulin, β-cellulin, and epiregulin. These protein ligands are synthesized as transmembrane precursors and are cleaved by metalloproteases (TNF-alpha converting enzyme, TACE) to release the mature soluble growth factor. In asthma model, Th2 cytokines released from Th2 cells, especially IL-4 and IL-13, may activate the TACE on bronchial epithelial cells, resulting in cleavage of TGFα, which in turn can transactivate EGFR tyrosine kinase signaling (Figure 8) (Lordan *et al*., 2002; King *et al*., 2004; Yoshisue and Hasegawa, 2004). Activation of tyrosine kinases invokes multiple down stream signaling pathway, including PI3K, MAPK and NF-κB, leading to cell differentiation, survival, proliferation, degranulation and chemotaxis (Burgel and Nadel, 2004).

In our study, we focused on the inhibitory effects of gefitinib in allergic airway inflammation. Gefitinib, an EGFR tyrosine kinase inhibitor, is a useful targeted therapeutic agent for non-small cell lung cancer (Gandara *et al*., 2004). EGFR bound its ligands, was dimerized and phosphorylated, which activates EGFR signaling cascade, leading to the induction of cell proliferation (Sako *et al*., 2000). Gefitinib blocks the signal transduction via EGFR by inhibiting its autophosphorylation and thus blocking downstream intracellular signaling. This results in the inhibition of cell proliferation and the reduced survival of cells (Ciardiello and Tortora, 2001). Recently, there have been reports that gefitinib could also reduce mucin production and suppress the MUC5AC gene in patients with bronchioloalveolar carcinoma who exhibit large amounts of mucus production (Kitazaki *et al*., 2005a,b). Since tyrosine kinase signaling cascades are involved in almost all aspects of asthma patho-

genesis, the possible therapeutic use of a tyrosine kinase inhibitor to treat asthma has been investigated in several studies (Duan *et al*., 2003, 2004; Vargaftig and Singer, 2003). Until now other studies have showed that EGFR tyrosine kinase inhibitors have anti-inflammatory effects in asthma model, but can not explain adequate mechanisms. In the current study, we tried to reveal mechanisms that is related with inhibitory effect of geftinib on allergic airway inflammation which included mucin production, eosinophilic infiltration and airway hyperresponsiveness. In other word, mucin production is related with EGFR phosphorylation and eosinophilic allergic inflammation is related with PI3K/ Akt which is EGFR subsignal enzyme. To reveal these relationships we chose gefitinib which inhibits EGFR phosphorylation, PI3K/ Akt and MAPK, simultaneously. Based on previous studies (Chue *et al*., 2004; Lee *et al*., 2006a), selective Akt/PI3K or MAPK inhibitors reduce airway inflammation. In that study, they showed that airway inflammation or hyperresponsiveness are related with selective inhibitors. However, airway inflammation or mucin productions are not only related with only one pathway, we chose the gefitinib that inhibits both pathways. And the gefitinib doses chosen for the *in vivo* study were based on the literatures. The high dose gefitinib (50 mg/kg body weight per day) was based on previously reported work that yielded minimal gefitinib's toxic dose (50 mg/kg) (Sirotnak *et al*., 2000). The doses of gefitinib used in their study were all below the dose that would cause lethal toxicity on the schedule of administration used. And in our preliminary study, we confirmed that gefitinib's bone marrow suppression was not developed at high dose gefitinib group (data not shown). Thus, we chose a high dose of 50 mg/kg to minimize toxicity for the present study.

Recently, the activation of EGFR was shown to induce goblet cell proliferation and to increase MUC5AC gene expression and protein synthesis of MUC5AC in asthmatic patients (Takeyama *et al*., 2001). Amishima *et al*. (1998) also reported increased expression of EGF and EGFR in asthmatic human airway epithelium compared with their levels in a control population, suggesting that EGF may contribute to the pathophysiology of bronchial asthma. EGF and eosinophil-derived TGF-α, another natural ligand for the EGFR, have been shown to stimulate MUC5AC mucin gene expression and protein synthesis in a human airway epithelial cell line, and goblet cell metaplasia (Burgel and Nadel, 2004). The expression and activation of EGFR causes goblet cell metaplasia from Clara cells by a process of cell differentiation (Nadel and Takeyama, 1999; Nadel, 2001; Nadel and Burgel, 2001; Lee *et al*., 2006b). These goblet cell metaplasia and increased mucin production are important factors contributing to disease pathogenesis. We examined the effects of gefitinib on goblet cell metaplasia. Our results show that gefitinib significantly and dose-dependently reduces goblet cell metaplasia, but does not totally inhibit it. For this result, the regulation of mucin expression (goblet cell metaplasia) is influenced by elaborate cross-link between signaling cascades instigated by products of the environment, immune cells, and epithelial cells themselves (Andrianifahanana *et al*., 2006). Because mucin production is not exclusively influenced by EGFR, OVA-induced goblet cell metaplasia is not totally inhibited by gefitinib.

Eosinophils are effector cells that play an important role in the pathophysiology of allergic disease. Eosinophil recruitment to sites of allergic inflammation depends on the concerted action of a variety of molecules, including chemokines, and activation of PI3K (Sotsios and Ward, 2000). IL-4 and IL-13 are chemotactic for eosinophils, which are principal elements in the pathogenesis of allergic inflammation (Rankin *et al*., 1996; Humbert *et al*., 1997). Eosinophil transmigration into the airways is a complex process that is coordinated by Th2 cytokines and several adhesion molecules (Lukacs, 2001). IL-4 regulates allergic inflammation and development of eosinophils by promoting Th2 cell differentiation, and mucus hypersecretion (Moser *et al*., 1992; Takeyama *et al*., 1999). IL-13 promotes B cell differentiation and is capable of inducing isotype-switching in B cells to produce IgE (Cocks *et al*., 1993). Recent studies suggest that PI3K plays a pivotal role in the recruitment and activation of eosinophils by induction of the Th2 response (Palframan *et al*., 1998; Myou *et al*., 2003; Lee *et al*., 2006a). PI3K is a signal transduction enzyme, which phosphorylates the D3 position of the inositol ring of phosphoinositide and its phosphorylated derivatives (Vanhaesebroeck and Waterfield, 1999). This phosphorylation then stimulates the catalytic activity of Akt, resulting in the subsequent phosphorylation of a host of other proteins which affect cell growth, cell cycle entry, and cell survival (Datta *et al*., 1997). Gefitinib is associated with the inhibition of EGFR and with two main downstream signaling pathways, those involving MAPK and PI3K/Akt. In recent years, a second pathway, downstream of PI3K/Akt, has become a focus of interest, and is currently receiving much attention as a primary regulator of mammalian cell proliferation and migration (Cantley, 2002; Pinho *et al*., 2005). Our results show that gefitinib reduces the level of Th2 cytokines (IL-4 and IL-13), the expression of P-Akt and the eosinophil count in BALF. These findings are in agreement with a recent report that tyrosine kinase signaling cascades are involved in almost all of the pathogenic processes of asthma (Vargaftig and Singer, 2003; Wong, 2005).

With regard to AHR, the exact mechanism has not been clearly understood until now. It is believed that the eosinophils infiltrating the asthmatic lung degranulate to release tissue-damaging granular proteins, such as the major basic protein and eosinophil peroxidase, as well as release oxygen free radicals. These mediators, acting in concert, may then cause airway epithelial damage, resulting in the development of AHR (Laitinen *et al*., 1985; Pretolani *et al*., 1994). We measured AHR to methacholine by an invasive method using body plethysmography (Cho *et al*., 2004). Our results show that gefitinib significantly and dose-dependently inhibits OVA-induced AHR to methacholine, possibly as a result of multiple mediators.

Bronchial asthma is a complex inflammatory lung disease and is multifactorial in origin, a pathogenic process mediated by multiple inflammatory cells and mediators. Protein tyrosine kinase signaling cascades are essential for the activation and proliferation of inflammatory cells and airway resident cells; in particular, the EGFR signaling cascade is critical to the process of allergic inflammation. Our results demonstrate that the EGFR tyrosine kinase inhibitor gefitinib can effectively reduce allergic inflammation by decreasing OVA-induced Th2 cytokine production, eosinophil recruitment, mucin production, and AHR in a murine asthma model. These findings suggest that the inhibition of the EGFR cascade may have therapeutic potential in the treatment of asthma.

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