EGFR- tyrosine kinase inhibitors diminish transforming growth factor-alpha induced pulmonary fibrosis

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Running Title: EGFR tyrosine kinase inhibitors in fibrosis
Abstract

Transforming growth factor-alpha (TGFα) is a ligand for the epidermal growth factor receptor (EGFR). EGFR activation is associated with fibroproliferative processes in human lung disease and animal models of pulmonary fibrosis. We determined the effects of EGFR tyrosine kinase inhibitors gefitinib (Iressa®) and erlotinib (Tarceva®) on the development and progression of TGFα-induced pulmonary fibrosis. Using a doxycycline regulatable transgenic mouse model of lung-specific TGFα expression, we determined effects of treatment with gefitinib and erlotinib on changes in lung histology, total lung collagen, pulmonary mechanics, pulmonary hypertension and expression of genes associated with synthesis of extracellular matrix and vascular remodeling. Induction in the lung of TGFα caused progressive pulmonary fibrosis over an 8 week period. Daily administration of gefitinib or erlotinib prevented development of fibrosis, reduced accumulation of total lung collagen, prevented weight loss, and prevented changes in pulmonary mechanics. Treatment of mice with gefitinib 4 weeks after the induction of TGFα prevented further increases in and partially reversed total collagen levels and changes in pulmonary mechanics and pulmonary hypertension. Increases in expression of genes associated with synthesis of extracellular matrix as well as decreases of genes associated with vascular remodeling were also prevented or partially reversed. Administration of gefitinib or erlotinib did not cause interstitial fibrosis or increases in lavage cell counts. Administration of small molecule EGFR tyrosine kinase inhibitors prevented further increases in and partially reversed pulmonary fibrosis induced directly by EGFR activation without inducing inflammatory cell influx or additional lung injury.
Introduction

Interstitial lung diseases (ILD) are a heterogeneous group of disorders associated with over 130 distinct entities (6). Pulmonary fibrosis is characteristic of many forms of ILD, and idiopathic pulmonary fibrosis (IPF) affects over 90,000 people in the United States and more than 5 million patients worldwide (4, 27, 57). Currently there are no proven therapies that prevent or reverse pulmonary fibrogenesis, emphasizing the need to identify new molecular targets.

A number of signaling pathways and molecules regulate matrix deposition and fibroblast proliferation in the lung including tumor necrosis factor, platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor and transforming growth factor-β1 (TGFβ1) (5, 29). TGFα, along with epidermal growth factor (EGF) and amphiregulin are ligands for the epidermal growth factor receptor (EGFR). The EGFR is a membrane-bound receptor tyrosine kinase that belongs to a subfamily of four closely related receptors: HER1/EGFR/ERBB1, HER2/NEU/ERBB2, HER3/ERBB3, and HER4/ERBB4. Following ligand binding, these receptors form homo- and heterodimers leading to autophosphorylation of tyrosine residues in the cytosolic domains of the proteins. The phosphorylated tyrosine residues become docking sites for signaling molecules that activate cellular signaling pathways regulating a number of cellular processes, including proliferation and survival (38). A number of experimental studies support a role for EGFR activation in fibroproliferative processes. Madtes et. al. demonstrated increased TGFα and EGFR after bleomycin injury in the rat with the highest expression noted at times of cellular proliferation and collagen deposition (34). Van Winkle et al demonstrated increased TGFα and EGFR expression at sites of bronchiolar cell proliferation after naphthalene exposure (55). Similarly, EGFR and its ligands were increased in the lung following exposure to asbestosis and hyperoxia (32, 59).

We previously generated transgenic mice wherein lung-specific expression of TGFα was driven by the human surfactant protein C (SP-C) promoter (28). Expression of TGFα in transgenic mouse lungs
throughout pre and postnatal lung development caused extensive peribronchial, perivascular, pleural, and interstitial fibrosis without inflammation (13, 18, 28). The extent of fibrosis was directly related to the level of TGFα protein (9). Using a doxycycline (Dox) regulatable transgenic system in the adult lung, expression of TGFα caused progressive and extensive vascular adventitial, peribronchial, interstitial and pleural fibrosis that was independent of inflammatory or developmental influences (17). Gene expression profiles observed after expression of TGFα in the mouse lung were similar to those found in pulmonary fibrotic disease in humans (16).

Small molecule EGFR tyrosine kinase inhibitors (TKIs) act by competitive inhibition of ATP binding, which blocks intracellular autophosphorylation of EGFR tyrosine residues. EGFR TKIs are used in the treatment of tumors with activated EGFR including subtypes of non-small cell lung cancers. In a subgroup of lung cancer patients treated with the EGFR TKI, gefitinib, interstitial lung disease (ILD) was reported at a modestly increased frequency (22-24, 50, 54). Although placebo-controlled trials with the EGFR TKI erlotinib in lung cancer patients has not demonstrated pulmonary toxicity (2, 45), recent case reports have identified ILD in individual patients receiving erlotinib (1, 33, 36, 52).

Animal studies also suggest that EGFR TKIs may be useful in treating pulmonary fibrosis. Treatment with AG1478 prevented vanadium pentoxide-induced peribronchial fibrosis in rats, and prevented inflammation and ovalbumin-induced lung remodeling in mice (42, 56). Ishii et al. reported that gefitinib prevented bleomycin-induced fibrosis in mice (25). In contrast, Suzuki et al reported that gefitinib augmented intratracheal bleomycin-induced fibrosis in mice (49). Considering the controversy over the efficacy of EGFR inhibition in bleomycin-induced fibrosis and the possibility of pulmonary toxicity due to the use of EGFR TKIs, the present study was undertaken to determine whether gefitinib and erlotinib prevented or reversed pulmonary fibrosis directly mediated through EGFR signaling.
Methods

**Transgenic Mice:** CCSP-rtTA activator mice expressing the reverse tetracycline-responsive transactivator (rtTA) under control of the 2.3-kb rat Clara Cell Secretory Protein (CCSP), a.k.a. secretoglobin, family 1A, member 1 (\(Scgb1a1\)) gene promoter (53) were mated to conditional doxycycline (Dox) regulated transgenic mice containing the human TGF\(\alpha\) cDNA under the control of seven copies of the tetracycline operon ((TetO)\(_7\)-cmv TGF\(\alpha\)) plus a minimal CMV promoter (17). Single transgenic (CCSP-rtTA\(^{+/}\)) and bitransgenic CCSP-rtTA\(^{+/}\)/(TetO)\(_7\)-cmv TGF\(\alpha\)\(^{+/}\) mice were produced within the same litter by mating homozygous CCSP-rtTA\(^{+/}\) mice to hemizygous (TetO)\(_7\)-cmv TGF\(\alpha\)\(^{+/}\) mice. All mice were derived from the FVB/NJ inbred strain. Mice were maintained in virus-free containment and handled in accordance with the Institutional Animal Use and Care Committee of the Cincinnati Children’s Hospital Research Foundation. To induce TGF\(\alpha\) expression, Dox (Sigma, St. Louis, MO) was administered in the drinking water at a final concentration of 0.5 mg/ml and in food (62.5mg/kg). Water was replaced three times per week. Mice were genotyped as previously described (17).

**Administration of gefitinib and erlotinib:** Gefitinib (100mg/kg; AstraZeneca, Macclesfield, England) was diluted in sterile, deionized water (0.0125 mg/uL) and stirred (4 h; 4°C) prior to administration to mice. Erlotinib powder (25 or 100 mg/kg; OSI Pharmaceuticals Melville, NY) was suspended in 0.5% methyl cellulose (0.015mg/µL; 37°C; Colorcon, West Point, PA). Three hours prior to administration, food and water were removed from cages. Mice were then anesthetized (Isoflurane; Abbott Labs, Chicago, IL), and 150-250 µl sterile vehicle (water for gefitinib, methyl cellulose for erlotinib) or drug was administered by gavage using a 20 gauge feeding catheter (Harvard Apparatus, Holliston, MA). Drug dosage was based upon initial body weight and not adjusted with weight change during the study period. Mice were weighed at the beginning of the study and at weekly intervals. Statistical significance was determined by testing for normality and using a Kruskal-Wallis one way analysis of variance followed by an all pair-wise multiple comparison procedure (Dunn's Method).
Lung Histology, Immunohistochemistry and Morphometrics: Mice were killed with pentobarbital sodium (65 mg/ml) euthanasia solution (Fort Dodge Animal Health, Fort Dodge, IA), and lungs were inflation fixed using 4% paraformaldehyde at 25 cm H₂O of pressure, and then allowed to fix overnight at 4°C. Fixed lungs were then washed with phosphate-buffered saline (PBS), dehydrated through a graded series of ethanols, and processed for paraffin embedding. Sections (5 µm) were loaded onto polysine slides for immunostaining, hematoxylin and eosin (H&E) staining, or trichrome as previously described (10). A Nikon Microphot FXA EPI-FL3 microscope was used to acquire digital images, which were then analyzed in MetaMorph imaging software (v6.2; Universal Imaging). Pixel density for each x10 digital image was 0.266 µm/pixel. As previously described (13, 14) pleural thickness was performed on digital images of five randomly selected fields per animal from distal gas exchange regions (n = 5 per group). Pleural thickness was measured using the measured distance function of MetaMorph.

Pulmonary Mechanics: Lung mechanics were assessed on mice with a computerized Flexi Vent system (SCIREQ, Montreal, Canada), as previously described (21, 44). Briefly, mice were anesthetized with ketamine and xylazine, tracheostomized and then ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min and positive end-expiratory pressure (PEEP) of 2 cm H₂O computerized by the SCIREQ system thereby permitting analysis of dynamic lung compliance. The ventilation mode was changed to forced oscillatory signal (0.5-19.6 Hz), and respiratory impedance was measured. Tissue resistance or damping and tissue elastance was obtained for mice at 2 cmH₂O PEEP by fitting a model to each impedance spectrum. With this system, the calibration procedure removed the impedance of the equipment and tracheal tube.

Total Lung Collagen: Total lung collagen was determined by quantifying total soluble collagen (Sircol Collagen Assay, Biocolor, Ireland). The left lung was homogenized in 5 ml 0.5 M acetic acid
containing pepsin (1 mg/10 mg tissue; Sigma-Aldrich) and incubated (24 h; 24°C; with 240 rpm shaking). Sircol dye was added (1 ml/100 µl; 30 min), the sample centrifuged (12,000 rpm for 12 min), and the pellet was suspended (1 ml 0.5 M NaOH). The optical density measured with a spectrophotometer (540nm).

Pulmonary hypertension: Right ventricular hypertrophy (RVH) was assessed as an index of pulmonary hypertension as previously described (7, 30). Briefly, hearts were removed and dissected to isolate the free wall of the RV from the left ventricle and septum (LV + S). The ratios of RV weight to LV + S weight (RV/LV + S) were used as an index of RVH, which develops as a result of pulmonary hypertension.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): Fourteen transcripts previously identified by microarray to be altered in human idiopathic pulmonary fibrosis and in murine models of pulmonary fibrosis were measured by qRT-PCR (15). Total RNA was isolated from mouse lung (n=3-4 mice/group) with TRIzol reagent (Invitrogen, Carlsbad, CA) and quantity was assessed by absorbance at 260 and 280 nm (SmartSpec 3000, Bio-Rad, Hercules, CA). RNA (100 ng) was reverse transcribed (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA) and cDNA (10 µg) was used in each PCR reaction with primers and TaqMan Universal PCR Master Mix, (Applied Biosystems). Primers for procollagen, type I, alpha 1 (COL1A1) (Cat. No. Mm00801666_g1), procollagen, type III, alpha 1 (COL3A1) (Cat. No. Mm00802331_m1), endothelin receptor type B (EDNRB) (Cat. No.), frizzled homolog 1 (Drosophila) (FZD1) (Cat. No. Mm00445405_s1), midkine (MDK) (Cat. No. Mm00440279_m1), caveolin, caveolae protein 1 (CAV1) (Cat. No. Mm00483057_m1), platelet/endothelial cell adhesion molecule 1 (PECAM1) (Cat. No. Mm00476702_m1), kinase insert domain protein receptor (KDR) (Cat. No. Mm00486524_m1), forkhead box F1a (FOXF1A), (Cat. No. Mm00487497_m1), resistin like alpha (RETNLA) (Cat No. Mm00457862_m1) vascular endothelial growth factor A (VEGFA) (Cat. No. Mm00455174_m1) serine
(or cysteine) peptidase inhibitor, clade F, member 1 (SERPINF1) (Cat. No. Mm00465988_m1), superoxide dismutase 3, extracellular (SOD3) (Cat. No. Mm00448831_m1), and endothelial-specific receptor tyrosine kinase (TEK) (Cat. Mm00607939_s1). Analysis was performed with an Applied Biosystems 7900HT System (95°C 10 min; 40 cycles 95°C , 15 s; 60°C ,1 min). For relative quantization, 42 Days on Dox samples were compared to non-treated controls (No Dox) samples using the comparative C_T method (ΔΔC_T) normalizing each sample to beta-actin (ACTB).

**TGFα measurements:** TGFα concentrations in mouse lung with and without Dox induction was determined by ELISA (Oncogene Research Products, Cambridge, MA) as previously described (17, 18). Briefly, lungs were homogenized in 2 mL PBS (pH 7.4) containing protease inhibitors (Complete protease inhibitor cocktail; Roche, Indianapolis, IN), centrifuged (1,500g x 15 min) and the supernatant stored at -70°C.

**EGFR Western Blots:** EGFR activation was confirmed by assessing the levels of phosphorylated EGFR relative to total EGFR in control and bitransgenic mice after 1 day of Dox. Western blot analysis was performed in lung tissue from single-transgene controls (CCSP/-) and bitransgenic mice treated with and without 100 mg/kg erlotinib by using primary antibodies against phosphorylated EGFR (1:1,000, rabbit monoclonal, pY1086; Epitonic, Burlingame, CA), total EGFR (1:5,000, rabbit polyclonal; kind gift from Dr. Brad Warner, Washington University). Goat anti-rabbit secondary antibodies were used and chemiluminescence detection was performed using the ECL Plus system (Amersham Biosciences).

**BAL Cell Counts:** Following euthanization, the trachea was cannulated and the lungs lavaged 3 times with 1 mL Hank’s balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 4.2 mM NaHCO₃, and 5.6 mM glucose) as previously described (17, 19). Differential cell counts were performed on Diff-Quick-stained (Baxter Diagnostics, McGraw Park, IL) cytospin
(Cytospin 3, Shandon Scientific). Two hundred cells per slide were counted. The remaining BAL fluid was then centrifuged (150 g x 10 min), and cells were resuspended in PBS and 10 uL was mixed with 10 uL of trypan blue (0.4%) and counted on a hemocytometer.

Statistical Analysis: Data are means ± standard error (SE). Statistical comparisons were made using ANOVA followed by a Student-Newman-Keuls all pair-wise comparison to identify significant differences except as described above for bodyweight measurements. P < 0.05 was considered statistically significant.
Results

Prevention of TGFα-mediated fibrosis with gefitinib. CCSP-rtTA/otet-TGFα mice were treated with doxycycline (Dox) to induce TGFα expression and concomitantly gavaged daily with either sterile water or gefitinib for 4 weeks. As previously reported, induction of TGFα caused extensive fibrosis localized to the pleural surfaces, perivascular and peribronchial adventitia. Gefitinib-treated mice demonstrated reductions in pulmonary fibrosis (Figure 1), and mean pleural thickness (Table 1) and improvements in altered lung mechanics (Figure 2) caused by expression of TGFα. Gefitinib prevented increases in lung collagen content in mice caused by expression of TGFα (Figure 3). Total and differential cell counts in BAL were not altered by gefitinib (Table 2).

Partial reversal of TGFα-mediated fibrosis with gefitinib. After 4 weeks of TGFα expression lung histology revealed extensive fibrotic lesions associated with alterations in lung mechanics. To determine whether EGFR inhibition influences the progression of established fibrosis, mice were gavaged with gefitinib or water at the beginning of week 5 of Dox and treated daily during an additional 4 weeks of Dox induction (8 weeks total Dox). Gefitinib significantly improved pulmonary fibrosis assessed by histology, mean pleural thickness and total lung collagen content and prevented further declines in lung mechanics (Table 1 and Figures 2, 3 and 4). Gefitinib prevented the progressive weight loss caused by expression of TGFα in this model (Figure 5). Expression of TGFα for 8 weeks caused right ventricular hypertrophy that was diminished by treatment with gefitinib (Figure 6).

Effects of gefitinib on gene expression. To assess the effects of gefitinib on expression of mRNAs previously associated with pulmonary fibrosis, qRT-PCR analysis for a selected group of mRNAs induced by TGFα was determined (15). Gefitinib treatment reduced TGFα-induced changes in expression of MDK, SERPINF1, and FZD1 (Figure 7A) and a number of mRNAs associated with vascular development and remodeling (VEGFA, KDR, TEK, EDNRB, FOXF1A, PECAM1) (Figure
7B). Gefitinib treatment did not alter the levels of TGFα protein expressed in the lung of the Dox-treated mice (Table 1).

**Erlo tinib prevents TGFα-induced fibrosis.** CCSP-rtTA/otet-TGFα mice were placed on Dox and concomitantly gavaged with either sterile water or 25 or 100 mg/kg erlotinib daily for 4 weeks. Erlotinib treatment at 100 mg/kg/day, but not at 25 mg/kg/day, reduced total lung collagen compared with vehicle-treated mice (Figure 8A). Both doses of erlotinib prevented TGFα-induced abnormalities in pulmonary mechanics (Figure 8B) and prevented histological evidence of pulmonary fibrosis (data not shown). 100 mg/kg erlotinib prevented TGFα-induced phosphorylation of EGFR as measured by western blot of whole lung homogenates (Figure 8C).

**Effects of erlotinib on gene expression.** Treatment with erlotinib at 100 mg/kg/day prevented TGFα-mediated changes in expression of mRNAs (Figures 9A and 9B). Erlotinib at 25 mg/kg partially ameliorated TGFα-induced changes in gene expression.
Discussion

Using a transgenic mouse model of pulmonary fibrosis caused by lung specific expression of the EGFR ligand, TGF\(\alpha\), the present study demonstrates that administration of EGFR inhibitors prevents both the generation as well as the progression of established pulmonary fibrosis and associated alterations in lung mechanics. These findings, in the context of elevated TGF\(\alpha\) and EGFR detected in a number of human fibrotic lung diseases, support the concept of targeted EGFR inhibition for progressive fibrotic disease (12, 35, 39, 40, 47, 48, 51, 58).

In the present study neither gefitinib nor erlotinib induced chronic lung injury, nor did gefitinib exacerbate fibrosis or cause additional lung injury in mice with pre-existing fibrosis. The potential use of EGFR inhibitors for treatment of pulmonary fibrosis is complicated by reports of ILD in patients with lung cancer who were treated with gefitinib and erlotinib (33, 50). In greater than 185,000 cancer patients worldwide who have received gefitinib as of September 2004, the reported frequency of ILD is approximately 1% (54). The frequency is higher in Japan (approximately 2%) than in other countries (0.3%). Two studies from Japan identified preexisting pulmonary fibrosis as among the highest risk factors for developing ILD with gefitinib treatment (22, 23). In contrast, in a study of lung cancer patients where erlotinib was used after failure of first or second line chemotherapy, the incidence of pulmonary fibrosis, pulmonary infiltrates or pneumonitis did not differ between erlotinib treated and placebo control patients (2, 45). However, in recent case reports, ILD was detected in lung cancer patients treated with erlotinib including a patient with IPF (1, 33, 36, 52). Currently it is unclear what mechanisms lead to ILD in patients receiving gefitinib or erlotinib. Bleomycin injury is a frequently used experimental method of inducing pulmonary fibrosis in animal models. The benefit of pharmacologic EGFR inhibition in reducing bleomycin induced lung fibrosis is controversial. Suzuki et. al. (49) reported that mice receiving intratracheal bleomycin treated with gefitinib had increased fibrosis compared with mice receiving bleomycin alone. In contrast, Ishii et. al (25) reported a protective effect of gefitinib. Differences in mouse strains and doses of gefitinib may have contributed
to the different outcomes. Further studies are needed to determine if ILD detected in lung cancer trials is caused by the EGFR inhibition or complicated by radiation or chemotherapy. Considering the increased incidence in patients from Japan, ILD may also be a drug-specific response in a genetically susceptible subset of patients.

Induction of TGFα caused elevated right ventricular hypertrophy associated with extensive adventitial thickening surrounding pulmonary vessels. Pulmonary hypertension is a frequent complication of advanced interstitial diseases and is associated with reduced survival (9, 11, 31). In the current study TGFα induction of 4 and 8 weeks caused decreases in expression of several mRNA transcripts associated with vascular development and function including VEGF-A, its receptor KDR, and FOXF1, a transcription factor critical for pulmonary vascular genesis (26). TGFα increased MDK, SERPINF1 and FZD1 gene expression. Elevated MDK expression in the lung epithelium of transgenic mice caused increased muscularization of small pulmonary arteries and increased smooth muscle actin deposition (41). SERPINF1 is increased in the fibroblastic foci of IPF patients in addition to decreased VEGF-A (3). FZD1, an antagonist of the canonical Wnt/beta-catenin signaling pathway that is critical to several developmental processes, including lung airway and vascular development (46, 60). Attenuation of these alterations in gene expression following gefitinib treatment in association with reductions in right ventricular hypertrophy (Figure 6) suggests EGFR signaling induces a number of growth factors, receptors and transcription factors directly mediating pulmonary fibrosis and pulmonary hypertension. Furthermore, dose-dependent normalization of both transcription changes and total lung collagen with erlotinib underscore that higher doses of EGFR TKI may be necessary to modulate the fibrotic response. This observation is supported by tumor models which demonstrate dose-related antitumor effects with erlotinib (20).

Mice expressing a lung-specific dominant-negative mutation in ERBB3 receptor demonstrate reduced lung fibrosis following intratracheal bleomycin-associated with decreased ERBB2 phosphorylation
(37). A recent study in mice treated with a monoclonal antibody directed against ERBB2 which blocks ERBB2/ERBB3 signaling also revealed reduced lung fibrosis following bleomycin (8). Studies demonstrate that epithelial tumor cell lines and human tumor xenograft models overexpressing ERBB2 are sensitive to gefitinib and erlotinib (43), thus ERBB2 and ERBB3 may be involved in the induction of fibrosis in our model. However, we previously demonstrated that overexpression of a dominant negative mutant EGFR in the distal lung epithelium prevented TGFα induced pulmonary fibrosis and pulmonary hypertension suggesting that EGFR activation is the primary EGFR family member in the fibrotic response (14, 30). Nevertheless, signaling induced by other members of the Erb receptor family may contribute to lung fibrosis and targeted inhibition of other Erb family members may also be useful in treating lung fibrosis.

In summary the present study demonstrates that oral EGFR inhibitors prevent and inhibit progression of pulmonary fibrosis due to TGFα induction of EGFR signaling. Moreover, the use of the inhibitors did not cause inflammatory cell influx or acute lung injury. These findings support further studies to determine the role of EGFR activation in human lung fibrotic disease, which could be amenable to targeted therapy.
<table>
<thead>
<tr>
<th></th>
<th>Pleural Thickness (uM)</th>
<th>TGFα Levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Dox</td>
<td>8.9 + .5</td>
<td>236 + 12</td>
</tr>
<tr>
<td>4 wk Dox</td>
<td>100 + 7*</td>
<td>2,441 + 19*</td>
</tr>
<tr>
<td>4 wk Dox + 4 wk gefitinib</td>
<td>8.4 + .4</td>
<td>2,468 + 15*</td>
</tr>
<tr>
<td>8 wk Dox</td>
<td>106 + 9*</td>
<td>2,396 + 36*</td>
</tr>
<tr>
<td>8 wk Dox + 4 wk gefitinib</td>
<td>21.7 + 3*Ω</td>
<td>2,458 + 19*</td>
</tr>
</tbody>
</table>

**Table 1.** Median pleural thickness and lung homogenate TGFα protein levels from CCSP-rtTA/otet-TGFα mice with and without gefitinib treatment. Data derived from 5 mice in each group. * p<0.01 compared with No Dox. Ω p<0.01 compared with 4 wk Dox.
<table>
<thead>
<tr>
<th></th>
<th>Total Cells</th>
<th>Differential (% macrophages)</th>
<th>Differential (% neutrophils)</th>
</tr>
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<tbody>
<tr>
<td><strong>No Dox</strong></td>
<td>101,140 ± 24,400</td>
<td>97 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td><strong>4 wk Dox</strong></td>
<td>147,900 ± 19,1800</td>
<td>98 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td><strong>4 wk Dox + 4 wk gefit.</strong></td>
<td>153,350 ± 26,100</td>
<td>98 ± 2</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

**Table 2.** Cell counts and differential in BAL from CCSP-rtTA/otet-TGFα mice following pulmonary expression of TGFα. Data derived from 5 mice in each group.
Figure 1. Gefitinib prevents pulmonary fibrosis. CCSP-rtTA/otet-TGFα mice were treated with Dox to induce TGFα expression and concomitantly gavaged daily with either sterile water or 100 mg/kg gefitinib for 4 weeks. Lung sections were stained with hematoxylin and eosin and demonstrated extensive pleural, perivascular and peribronchial fibrosis in TGFα expressing mice which was reversed with gefitinib treatment. Photomicrographs are representative of lungs from 5 mice in each group.

Figure 2. Gefitinib prevents TGFα-dependent declines in lung function. Pulmonary mechanics were determined as described in Methods. TGFα expression for 4 (4 weeks Dox) and 8 weeks (8 weeks Dox) caused increased airway resistance, airway and tissue elastance, and decreased compliance. Gefitinib administered for 4 weeks at the time of TGFα-induction prevented changes in lung mechanics. Gefitinib administered 4 weeks after TGFα induction (8 weeks Dox, 4 weeks gefitinib) prevented further changes in lung mechanics. Data are derived from 7-10 mice per group. * p<0.001 compared with No Dox controls and mice receiving 4 wks Dox and gefitinib. ** p<0.001 compared with all groups.

Figure 3. Gefitinib prevents TGFα-dependent increases in lung collagen. Lung collagen content was determined from lungs of transgenic mice as described in Methods. Expression of TGFα for 4 (4 weeks Dox) and 8 (8 weeks Dox) weeks caused increased lung collagen. Gefitinib administered for 4 weeks at the time of TGFα-induction (4 weeks Dox, 4 weeks ZD1839) prevented increases in lung collagen. Gefitinib administered 4 weeks after TGFα induction (8 weeks Dox, 4 weeks ZD1839) prevented further accumulation of lung collagen. * p<0.001 compared with controls and mice receiving 4 wks Dox and gefitinib. ** p<0.01 compared with all groups. Data derived from 4-10 mice per group.
Figure 4. Gefitinib prevents progression of pulmonary fibrosis. Sections of lungs from transgenic mice with 4 weeks of Dox-induced TGFα expression were stained with the trichrome stain. Dox-induced TGFα for 8 weeks caused extensive fibrosis in the perivascular and peribronchial adventitia and along pleural surfaces (left panels). Gefitinib administered 4 weeks after TGFα induction prevented further lung fibrosis (right panel). Photomicrographs are representative of lungs from 5 mice in each group.

Figure 5. Gefitinib prevents progressive weight loss. Mice were weighed daily during treatments as described in Methods. Dox induced expression of TGFα for 8 weeks caused progressive weight loss (red line) while mice treated with gefitinib 4 weeks after TGFα induction did not have changes in body weight (green line). Data derived from 6-22 mice per group. * p<0.05 compared to gefitinib-treated and controls (blue line).

Figure 6. Gefitinib prevents right ventricular hypertrophy. Hearts were dissected and weighed as described in Methods. Dox induced expression of TGFα for 8 weeks caused an increase in the ratio of right ventricle to left ventricle plus septum (RV/LV+S) weights (8 weeks Dox). Gefitinib administered 4 weeks after TGFα induction reduced RV/LV+S weights (8 weeks Dox, 4 weeks gefitinib). Data were derived from 6-10 mice per group. * p<0.05 comparing bitransgenic mice on Dox only to both bitransgenic mice treated with gefitinib and controls.

Figure 7. Gefitinib attenuates changes in matrix and vascular mRNAs. Quantitative Real-Time Polymerase Chain Reaction was performed on RNA prepared from lungs as described in Methods. RNA from mice with TGFα expression for 8 weeks was compared with TGFα expressing mice treated with gefitinib (100 mg/kg) for 4 weeks beginning week 5. Compared with non-transgenic controls, TGFα expression increased selected transcripts associated with matrix production (A) and decreased transcripts associated with vascular development and remodeling (B). Gefitinib attenuated or blocked
changes in transcripts. *Significantly different from control; ** significantly different from 8 weeks Dox only as determined by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls all pair-wise multiple test (p<0.05). Data derived from 3-4 mice in each group.

Figure 8. Erlotinib prevents TGFα-dependent declines in lung function and TGFα induced collagen content. Pulmonary mechanics were determined as described in Methods. Erlotinib (100 mg/kg) administered daily for 4 weeks at the time of TGFα-induction prevented increases in lung collagen (A). Erlotinib (25 mg/kg and 100 mg/kg) administered for 4 weeks at the time of TGFα-induction prevented changes in lung function (B). Erlotinib (100 mg/kg) prevented TGFα-induced phosphorylation of EGFR measured by western blot of whole lung homogenates (C). * p<0.05 compared to controls.

Figure 9. Erlotinib alters TGFα induced changes in matrix and vascular mRNAs. Quantitative Real-Time Polymerase Chain Reaction was performed RNA prepared from lungs as described in Methods. RNA from mice with TGFα expression for 4 weeks was compared with TGFα expressing mice treated with daily erlotinib (25 mg/kg, cross hatched bar and 100 mg/kg, filled bar). Erlotinib (100 mg/kg) reduced TGFα-induced changes in transcripts associated with matrix production (A) and vascular development and remodeling (B). *Significantly different from control; + significantly different from 4 weeks Dox only as determined by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls all pair-wise multiple comparison (p<0.05). Data derived from 3-4 mice in each group.
REFERENCES


Figure 1

4 Weeks Dox

4 Weeks Dox & gefitinib
Figure 2

Airway Resistance (cmH2O*s/ml)

Airway Elastance (cmH2O/ml)

Tissue Elastance (cmH2O/ml)

Compliance (ml/cmH2O*kg)

No Dox 4 wks Dox 4 wks Dox 8 wks Dox 8 wks Dox
4 wks Gefit. 4 wks Gefit. 4 wks Gefit. 4 wks Gefit.

Figure 2
Figure 3

Total Collagen (µg/ml)

- No Dox
- 4 wks Dox
- 4 wks Gefit
- 8 wks Dox
- 8 wks Gefit

* Significantly different from No Dox
** Significantly different from 4 wks Dox
Figure 4

8 wks Dox

8 wks Dox + 4 wks gefitinib
Figure 5

- **CCSP/-**: 8 wks Dox
- **CCSP/TGF**: 8 wks Dox
- **CCSP/TGF**: 8 wks Dox, 4 wks gefitinib

Weeks On Dox

Body Weight (gm)

- \( \downarrow \) begin Dox
- \( \downarrow \) begin gefitinib

* * *
Figure 6

- CCSP/- 8 wk Dox
- CCSP/TGF 8 wk Dox
- CCSP/TGF 8 wk Dox 4 wk Gefit

RV/LV+S
Figure 7
Figure 8C
Figure 9