



Effects of erlotinib on pulmonary function and airway remodeling after sensitization and repeated allergen challenge in Brown-Norway rats

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ABSTRACT

Erlotinib, an EGFR tyrosine kinase inhibitor, can inhibit the proliferation and survival of cancer cells. It has been widely used to treat non-small cell lung cancer. This study aimed to evaluate the effects of erlotinib on bronchial hyperresponsiveness, airway inflammation, and airway remodeling in sensitized, ovalbumin-challenged rats. Two experimental groups of Brown-Norway rats were sensitized and repeatedly challenged by breathing aerosolized ovalbumin. Since Day 1, one group was given oral erlotinib (OA-erlotinib group) while the other group was given only oral saline (OA-saline group). The control group was sensitized and challenged using saline. All were anesthetized and paralyzed, and pulmonary function tests conducted at baseline and after provocation with varying doses of acetylcholine. Lung tissues were examined for airway inflammation, airway remodeling, and Th2-related cytokine mRNA expression. Results showed that the OA-erlotinib group had better pulmonary function and less airway inflammation, Th2-related cytokines and their mRNA expression, and airway remodeling compared to the OA-saline group. In conclusion, erlotinib effectively prevents bronchial hyperreactivity, airway inflammation, Th2-related cytokine mRNA expression, and airway remodeling after sensitization and repeated allergen challenge in Brown-Norway rats.

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1. Introduction

Asthma is a combination of chronic airway inflammation and remodeling (Davies et al., 2003). Evidence has shown that the epithelium of an asthmatic patient is structurally and functionally abnormal, making it more susceptible to inhaled irritants (Bucchieri et al., 2002). Under normal conditions, the epithelium releases prostaglandin E2 and 15-hydroxyeicosatetraenoic acid to suppress the function of mesenchymal cells. Epithelial activation and/or injury decreases prostaglandin E2 and 15-hydroxyeicosatetraenoic acid production, with a concomitant increase in fibroproliferative and fibrogenic growth factors and greater expression of epidermal growth factor receptor (EGFR) in damaged areas (Davies et al., 2003; Puddicombe et al., 2000; Zhang et al., 1999). EGFR expression in asthma positively correlates with the thickness of the lamina reticularis, the extent of epithelial damage and activation, and the severity of asthma (Burgel and Nadel, 2004; Wong, 2005).

Due to abnormal epithelial injury and repair, growth arrest and prolonged repair enhance cell-to-cell communication within the

epithelial mesenchymal trophic unit, which leads to myofibroblast activation, enhanced proliferation, and increased collagen gene expression. This then leads to epithelial injury and dysfunctional epithelium, and subsequently, a chronic airway “wound” with disordered repair underlying the pathogenesis of airway remodeling in asthma (Davies et al., 2003).

In addition, EGFR may contribute to proinflammatory responses, higher IL-8 expression, and production of innate immune responses. This may contribute to airway neutrophilia as well as partially irreversible, accelerated, and progressive loss of lung function and incomplete response to corticosteroids in severe asthma patients (Hamilton et al., 2003; Puddicombe et al., 2003; Semlali et al., 2008).

Protein tyrosine kinases are essential for the activation and proliferation of inflammatory cells and airway-resident cells. Once ligand-binding EGFR occurs, EGFR homodimerise or heterodimerise, which then induce intrinsic protein tyrosine kinase activities that initiate multiple downstream intracellular signaling pathways. This leads to differentiation, survival, activation, proliferation, degranulation, and chemotaxis of inflammatory cell and resident airway cell, all of which contribute to asthma pathogenesis (Bogdan and Klämbt, 2001; Burgel and Nadel, 2004; Pinho et al., 2005). Activated EGFR also induces hyperplasia of airway goblet cell, enhances the expression of mucin genes, and increases mucin production in the airway epithelium (Deshmukh et al., 2005;

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Kitazaki et al., 2005; Nadel and Burgel, 2001). Not surprisingly, inhibitors of tyrosine kinase have already been studied as a novel therapeutic strategy for asthma treatment (Vargaftig and Singer, 2003).

Erlotinib, an EGFR tyrosine kinase inhibitor, can inhibit the proliferation and survival of cancer cells. It has been widely used to treat non-small cell lung cancer (Lyseng-Williamson, 2010). The purpose of this study is to evaluate the protective effect of erlotinib on airway inflammation, T-cell-related cytokine mRNA expression, and airway remodeling in rats sensitized and repeatedly challenged with ovalbumin.

2. Materials and methods

2.1. Animals

Thirty Brown-Norway rats were divided into three groups of 10 weight-matched male animals each. Two experimental groups were sensitized by breathing 2 mL of aerosolized 1% ovalbumin on Day 1. Fifteen days later, the animals were challenged by ovalbumin 6 times with 3-day intervals in-between. The control group breathed aerosolized saline instead of ovalbumin under the same conditions as the experimental groups. The 2 experimental groups were also treated orally from Days 1 to 31 with erlotinib 1.5 mg/kg/day (OA-erlotinib group) or normal saline (OA-saline group). When being challenged, the experimental groups were provoked by breathing 3 mL of 4% aerosolized ovalbumin delivered by a nebulizer. The control group underwent the same with aerosolized saline instead of ovalbumin. The last challenge was on Day 30. The study was performed on Day 31. This study was approved by our Institutional Animal Care and Use Committee.

2.2. Study protocol, procedure and pulmonary function tests

Pulmonary function and acetylcholine (ACh) provocation tests were carried out under general anesthesia (chloral hydrate, 400 mg/kg via intra-peritoneal injection), using whole-body plethysmograph 24 h after the final challenge, as previously described (Lin et al., 2008; Takezawa et al., 1980). The animals were then paralyzed with intravenous gallamine triethiodide (4 mg/kg) and artificially ventilated with a small animal ventilator at 6 mL/kg tidal volume and 60 breaths/min frequency. All of the animals were stable without spontaneous breathing 5 min after the gallamine injection.

Pulmonary function tests conducted at baseline included the following: (1) airway pressure “peak insufflation airway pressure during tidal breathing” under ventilator breathing; (2) total respiratory system compliance; and (3) maximal forced expiratory maneuver. Thereafter, 25, 50, 75, and 100 µg/kg of ACh was given intravenously at 30-min intervals. Pulmonary function tests were done 5 s after each ACh dose. Before each successive dose, airflow was returned to baseline.

For the maximal forced expiratory maneuver, the lungs were inflated to total lung capacity (lung volume at airway opening pressure of 30 cm H₂O) thrice, while a solenoid valve regulated the inflation. At peak volume during the third inflation, the valve was shut-off and another solenoid valve was immediately opened for deflation. The deflation valve was connected to a 20-L container at a pressure of −30 cm H₂O (sub-atmospheric), such that the negative pressure produced maximal expiratory flow.

Variables recorded included the flow volume loop, peak flow, MFEF 75%, MFEF 50%, and MFEF 25% (maximal forced expiratory flow rate at 75%, 50%, and 25% of total lung capacity, respectively). PD20 MFEF 50% ACh was the dose required to produce a −20% decrease in MFEF 50% from baseline. Bronchoalveolar lavage (BAL) was performed as previously described. The rats were then immediately

sacrificed and the trachea and lungs were removed for histologic examination (Lin et al., 2008; Takezawa et al., 1980).

2.3. Cytokine levels in lavage fluid

The concentrations of IL-10, IL-13, TGF-β1, and vascular endothelial growth factor (VEGF) in the BAL fluid were measured in duplicate and in blinded fashion using commercially available rat-specific TGF-β1 and VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA) and IL-10, IL-13 ELISA kits (Invitrogen Corporation, Camarillo, CA, USA), with a detection limit of <1.7 pg/mL for TGF-β1, <3.9 pg/mL for VEGF, <5 pg/mL for IL-10, and <1.5 pg/mL for IL-13.

Prior to analysis, latent TGF-β1 in the BAL fluid was converted to their active forms using 1 N HCl and the reported levels were the total of the latent and active forms. A micro-plate reader (Spectra MAX 190; Molecular Devices, Sunnyvale, CA, USA) measured absorbance at 450 nm, while total protein in the BAL fluid was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) (Lin et al., 2008).

2.4. Histologic examination

After BAL, the trachea and lungs from each animal were removed immediately and fixed by 10% buffered formalin. Sections of each lobe (5 per animal) were stained for microscopic examination with haematoxylin and eosin, Masson's trichrome, smooth muscle actin, and von Willebrand factor. Haematoxylin and eosin staining were used to evaluate the severity of the inflammation. Two pathologists (blinded) assigned a total inflammation score for each section of lung parenchyma: 0, normal; 1, inflammatory infiltration of eosinophils, lymphocytes, and neutrophils in <25% of the entire section; 2, inflammatory infiltrate in 25–50% of the entire section; 3, inflammatory infiltrate >50% of the entire section. The total inflammation score for each animal was calculated as the mean of the scores for the 5 lung sections.

Masson's trichrome was used to determine the area of collagen deposition in the lamina propria of the airway, defined as the area from the sub-epithelial basement membrane to the adventitial layer of each terminal bronchiole and the membranous portion of the trachea. Smooth muscle actin was used to measure the average thickness of the airway smooth muscle layer at four sites tangential to each terminal bronchioles and the membranous portion of the trachea. The von Willebrand factor was used to identify the number of blood vessels in the lamina propria of the terminal bronchiole and the membranous portion of the trachea. Morphometry was performed using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA), which randomly examined each minimum of 10 successive microscopic fields of terminal bronchioles throughout the sections of each lobe of the lung and trachea.

2.5. Immuno-histochemical staining of alpha smooth muscle actin and von Willebrand factor

Paraffin lung sections were de-waxed in xylene and rehydrated in a graded alcohol series. To detect the von Willebrand factor, antigen retrieval was done with protease 1 reagent (Ventana Medical Systems, Tucson, Arizona, USA) for 20 min at 37 °C. Alpha smooth muscle actin staining was performed without an antigen retrieval procedure. Sections were followed by rinsing in Phosphate Buffered Saline with Tween 20 (PBST) and incubated in Protein Block™ solution (Biogenex, San Ramon, CA, USA) for 20 min at room temperature. Primary antibody mouse anti-alpha smooth muscle actin (1:100; Dako, Dakopatts AB, Sweden), and rabbit anti-von Willebrand factor antibody (1:200; Dako, Hamburg, Germany) diluted with antibody diluent (Zymed Laboratories, San Francisco, CA, USA) were then applied for 1 h at room temperature.

Table 1
Pulmonary function tests in Brown-Norway rats at baseline.

Tests	OA-erlotinib (n = 10)	OA-saline (n = 10)	Control (n = 10)
Peak flow (mL/s)	104.6 ± 7.3	103.2 ± 6.5	106.1 ± 7.1
MFEF 75% (mL/s)	96.4 ± 5.8	96.9 ± 6.1	97.2 ± 6.4
MFEF 50% (mL/s)	84.7 ± 4.7 ^a	77.2 ± 5.1	85.4 ± 5.6 ^b
MFEF 25% (mL/s)	54.2 ± 4.5 ^a	45.2 ± 5.3	56.7 ± 4.8 ^b
Crs (mL/cm H ₂ O)	0.44 ± 0.07	0.42 ± 0.06	0.45 ± 0.09
Airway pressure (cm H ₂ O)	3.4 ± 0.5	3.5 ± 0.6	3.3 ± 0.6
TLC (mL)	13.2 ± 1.4	12.9 ± 1.8	13.4 ± 1.6

ANOVA and Scheffe tests were used.

The OA-erlotinib group was treated with 1.5 mg/kg/day of erlotinib; the OA-saline group was sensitized with ovalbumin but treated with saline only.

Abbreviations: TLC, total lung capacity; MFEF 75%, the maximal forced expiratory flow rate at 75% TLC; MFEF 50%, the maximal forced expiratory flow rate at 50% TLC; MFEF 25%, the maximal forced expiratory flow rate at 25% TLC; Crs, total respiratory system compliance; Airway pressure, peak insufflation airway pressure during tidal breathing.

^a Differences in MFEF 25% and MFEF 50% between the OA-erlotinib and the OA-saline groups were significant ($p < 0.05$).

^b Differences in MFEF 25% and MFEF 50% between the OA-saline and the control groups were significant ($p < 0.05$).

Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany) for 10 min at room temperature. The Super Sensitive™ Non-Biotin HRP Detection System (Biogenex) was used as secondary antibody/detection system. Briefly, the sections were rinsed in PBST, incubated with Super Enhancer™ reagent (Biogenex) for 20 min at room temperature, rinsed in PBST, and incubated in Polymer-HRP reagent (Biogenex) for 30 min at room temperature. Finally, color was developed using 3,3-diaminobenzidine (Biogenex) chromogen solution for 5 min at room temperature, followed by rinsing in PBST and counter-staining with haematoxylin (Kasper et al., 1996).

2.6. RNA isolation and real-time reverse transcription-polymerase chain reaction assay

Expression of IL-4, IL-5, IL-10, IL-13, TNF- α , inducible nitric oxide synthase (iNOS), TGF- β 1, VEGF-A and β -actin mRNA were examined using real-time reverse transcription-PCR assay. Total RNA was extracted from lung tissue samples using an RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). First strand cDNA was reverse-transcribed from RNA using Clontech's Advantage RT-for-PCR kit (Clontech-Takara Bio, Mountain View, CA, USA). Each 50 μ L of PCR reaction volume contained 25 μ L of 2 $\times$ TaqMan Universal PCR Master Mixes, 2.5 μ L of 20 $\times$ TaqMan probes (Applied Biosystems, Foster City, CA, USA), and 100 ng cDNA. Each PCR reaction was performed in triplicate using an ABI Prism 7000 sequence detection system (Applied Biosystems).

Fluorescence was quantified as Ct value, defined as the cycle number in which the detected fluorescence exceeded the threshold value. mRNA expression was reported as the ratio of target Ct over β -actin Ct. Data were presented as the fold change between the experimental and control groups (Lin et al., 2008).

2.7. Data analysis

Student's *t*-test and analysis of variance (ANOVA) were used for statistical analysis using the SPSS for Windows (Version 14.0; SPSS, Inc., Chicago, IL, USA) where appropriate. If there was statistical significance by ANOVA, Scheffe test was also performed. All values were expressed as the mean $\pm$ standard deviation with significance accepted at $p < 0.05$.

3. Results

3.1. Pulmonary function tests

Mean peak flow, MFEF 75%, airway pressure, total respiratory system compliance, and total lung capacity did not differ significantly among the three groups at baseline. The MFEF 50% and

MFEF 25% were significantly lower in the OA-saline group than in the other groups, but not significantly different between the OA-erlotinib and control groups (Table 1).

At doses of Ach tested (25–100 μ g/kg), the percent change in MFEF 50% was significantly lower and PD20 MFEF 50% Ach was significantly higher in the OA-erlotinib group than in the OA-saline group. At doses of Ach tested (50–100 μ g/kg), the percent change in MFEF 50% was significantly higher and in PD20 MFEF 50% Ach was significantly lower in the OA-erlotinib group than in the controls (Fig. 1).

3.2. Bronchoalveolar lavage

There was no difference in amount of BAL fluid recovered from the three groups (84.7 $\pm$ 3.1% vs. 85.2 $\pm$ 3.3% vs. 85.6 $\pm$ 2.9%). The control group had the lowest total cell and absolute counts for each cell-type among the three groups (Table 2). The OA-erlotinib group had lower total cell and absolute counts than the OA-saline group. The control group also had the lowest IL-10, IL-13, TGF- β 1, VEGF cytokine, and total protein levels in the BAL fluid among the three, and the OA-erlotinib group had lower levels in the BAL fluid than the OA-saline group (Table 3).

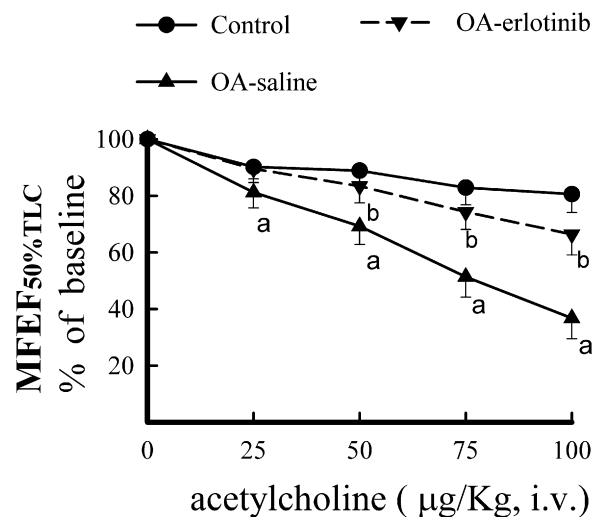


Fig. 1. Dose response curve of the maximal forced expiratory flow in the 3 groups of Brown-Norway rats. The percentage change in maximal forced expiratory flow rate at 50% total lung capacity from baseline (MFEF 50%) in the OA-saline group was higher than that of the OA-erlotinib group at tested doses of Ach (25–100 μ g/kg). The percentage change of MFEF 50% in the OA-erlotinib group was higher than that of the control group at tested doses of Ach (50–100 μ g/kg). ^a $p < 0.05$ comparison between the OA-erlotinib and the OA-saline group. ^b $p < 0.05$ comparison between the OA-erlotinib and the control groups.

Table 2

Total and differential cell counts in bronchoalveolar lavage fluid.

Cell counts	OA-erlotinib (n = 10)	OA-saline (n = 10)	Control (n = 10)
Total cell count ($\times 10^4$ /mL)	2.76 $\pm$ 0.71 ^a	9.52 $\pm$ 1.84	0.87 $\pm$ 0.21 ^b
Macrophages ($\times 10^4$ /mL)	2.15 $\pm$ 0.63 ^a	5.2 $\pm$ 1.12	0.78 $\pm$ 0.19 ^b
Lymphocytes ($\times 10^4$ /mL)	0.24 $\pm$ 0.12 ^a	1.83 $\pm$ 0.39	0.04 $\pm$ 0.02 ^b
Neutrophils ($\times 10^4$ /mL)	0.09 $\pm$ 0.03 ^a	0.41 $\pm$ 0.09	0.02 $\pm$ 0.01 ^b
Eosinophils ($\times 10^4$ /mL)	0.28 $\pm$ 0.09 ^a	2.08 $\pm$ 0.42	0.03 $\pm$ 0.01 ^b

ANOVA and Scheffe tests were used.

The OA-erlotinib group was treated with 1.5 mg/kg/day of erlotinib; the OA-saline group was sensitized with ovalbumin but treated with saline only.

^a Differences in the cell counts between the OA-erlotinib and the OA-saline groups were significant ($p < 0.05$).^b Differences in the cell counts between the OA-erlotinib and the control groups were significant ($p < 0.05$).

3.3. Lung tissue histology and remodeling

Lung tissue and trachea of the OA-saline group showed severe inflammation, characterized by hyperemia, interstitial edema, and inflammatory cell infiltration (especially lymphocytes and eosinophils) (Figs. 2A and 3A). In contrast, there was only mild hyperemia, interstitial edema, and inflammatory cell infiltration in the lung tissue and airways of the OA-erlotinib group (Figs. 2B and 3B), while the control group had no inflammation (Figs. 2C and 3C).

The mean inflammatory score of the terminal bronchioles in the OA-erlotinib group was lower than that of the OA-saline group (1.1 ± 0.3 and 2.1 ± 0.5 , respectively; $p < 0.05$). Mean inflammatory score of the trachea in the OA-erlotinib group was also lower than that of the OA-saline group (1.0 ± 0.2 and 2.0 ± 0.4 , respectively; $p < 0.05$).

The area of collagen deposition surrounding the airways was lowest in the control group and significantly lower in the OA-erlotinib than in the OA-saline group. The mean fold-change of the mean area of airway collagen deposits surrounding the terminal bronchioles relative to the control group was 1.7-fold in the OA-erlotinib group and 3.3-fold in the OA-saline group (Figs. 2D–F and 4). The mean fold-change of the mean area of airway collagen deposits surrounding the trachea relative to the control group was 1.5-fold in the OA-erlotinib group and 3.1-fold in the OA-saline group (Figs. 3D–F and 5).

The mean thickness of the smooth muscle layer surrounding the airways was lowest in the control group and was significantly lower in the OA-erlotinib than in the OA-saline group. The mean fold-change of the mean thickness of the smooth muscle layer surrounding the terminal bronchioles relative to the control group was 1.4-fold in the OA-erlotinib group and 2.2-fold in the OA-saline group (Figs. 2G–I and 4). The mean fold-change of the mean thickness of the smooth muscle layer surrounding the trachea relative to the control group was 1.4-fold and 2.0-fold in the OA-erlotinib and OA-saline groups, respectively (Figs. 3G–I and 5).

Mean number of blood vessels surrounding the airways was lowest in the control group and significantly lower in the OA-erlotinib than in the OA-saline group. The mean fold-change of the mean number of blood vessels surrounding the terminal bronchiole relative to the control group was 1.6-fold in the OA-erlotinib group

and 2.3-fold in the OA-saline group (Figs. 2J–L and 4). The mean fold-change of the mean number of blood vessels surrounding the trachea relative to the control group was 1.5-fold and 2.1-fold in the OA-erlotinib and OA-saline groups, respectively (Figs. 3J–L and 5).

3.4. Cytokine mRNA profile using real-time reverse transcription-polymerase chain reaction in lung tissue

Real-time reverse transcription-polymerase chain reaction of IL-4, IL-5, IL-10, IL-13, TNF- α , iNOS, TGF- β 1 and VEGF-A mRNA expression was consistently lowest in the control group, and significantly lower in the OA-erlotinib than in the OA-saline group (Figs. 6 and 7).

4. Discussion

This study demonstrates that Brown-Norway rats of the OA-saline group, after sensitization and repeated allergen challenge, have increased bronchial hyperreactivity, airway inflammation, Th2-related cytokine, TGF- β , VEGF and Th2-related cytokine mRNA expression, and airway remodeling than those in the control group. This study also shows that rats from the OA-erlotinib group have lower bronchial hyperreactivity, airway inflammation, IL-10, IL-13, TGF- β 1, VEGF cytokines level of BAL fluid and IL-4, IL-5, IL-10, IL-13, TGF- β 1, VEGF, TNF- α , and the iNOS mRNA expression than those of the OA-saline group.

Inhaled irritants can activate EGFR tyrosine kinase signaling through tyrosine kinase receptors, which exaggerates the innate and adaptive immune responses and leads to airway mucin production and neutrophil recruitment. This plays a critical role in the pathogenesis of allergic airway inflammation and airway remodeling (Burgel and Nadel, 2008). Selective inhibition of EGFR tyrosine kinase is anticipated for producing anti-inflammatory effects in asthma (Wong, 2005). Duan et al. (2003) have demonstrated that genistein (a broad-spectrum non-selective tyrosine kinase inhibitor) significantly reduces antigen-induced increases in total cell count, eosinophil count, eosinophil peroxidase activity recovered in BAL fluid, and acute bronchoconstriction in an *in vivo* guinea-pig model of asthma. Vargaftig and Singer (2003) have shown that intratracheal instillation of the EGFR inhibitor AG-1478 inhibited airway eosinophil infiltration, hyperresponsive-

Table 3

Cytokine and total protein levels in bronchoalveolar lavage fluid.

Cytokine and protein levels	OA-erlotinib (n = 10)	OA-saline (n = 10)	Control (n = 10)
IL-10 (pg/mL)	16.6 $\pm$ 5.3 ^a	38.2 $\pm$ 8.7	9.5 $\pm$ 2.7 ^b
IL-13 (pg/mL)	24.3 $\pm$ 6.8 ^a	42.5 $\pm$ 10.2	12.1 $\pm$ 3.7 ^b
TGF- β 1 (pg/mL)	10.5 $\pm$ 3.1 ^a	20.4 $\pm$ 5.5	5.7 $\pm$ 1.7 ^b
VEGF (pg/mL)	47.2 $\pm$ 12.4 ^a	80.3 $\pm$ 20.2	15.1 $\pm$ 6.1 ^b
Protein (μ g/mL)	168.7 $\pm$ 45.2 ^a	358.4 $\pm$ 78.7	97.8 $\pm$ 21.8 ^b

ANOVA and Scheffe test were used.

The OA-erlotinib group was treated with 1.5 mg/kg/day of erlotinib; the OA-saline group was sensitized with ovalbumin but treated with saline only.

^a Differences in cytokine and total protein levels between the OA-erlotinib and the OA-saline groups were significant ($p < 0.05$).^b Differences in cytokine and total protein levels between the OA-erlotinib and the control groups were significant ($p < 0.05$).

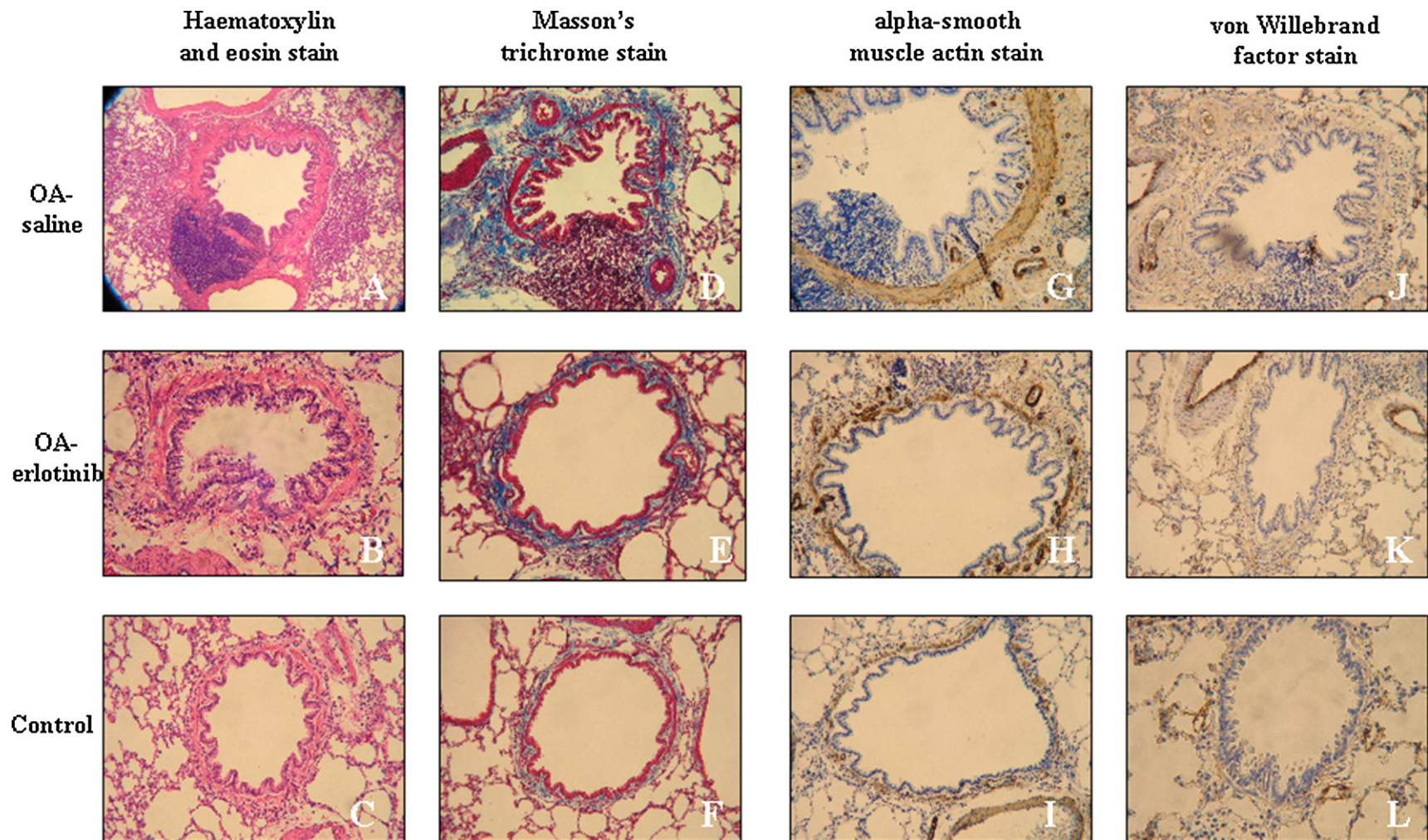


Fig. 2. Histopathology of airway remodeling (terminal bronchioles); photographs representing the 3 experiments: (A–C) haematoxylin and eosin stain, 100 $\times$; (D–F) Masson's trichrome stain, 100 $\times$; (G–I) alpha-smooth muscle actin stain, 100 $\times$; and (J–L) von Willebrand factor stain, 100 $\times$. Inflammation was highest in the (A) OA-saline group vs. the (B) OA-erlotinib and (C) control groups. Collagen deposits surrounding the airways were highest in the (D) OA-saline group than in the (E) OA-erlotinib and (F) control groups. Thickness of the smooth muscle layer surrounding the airways was greatest in the (G) OA-saline group than in the (H) OA-erlotinib and (I) control groups. Number of blood vessels surrounding the airways was highest in the (J) OA-saline group than in the (K) OA-erlotinib and (L) control groups.

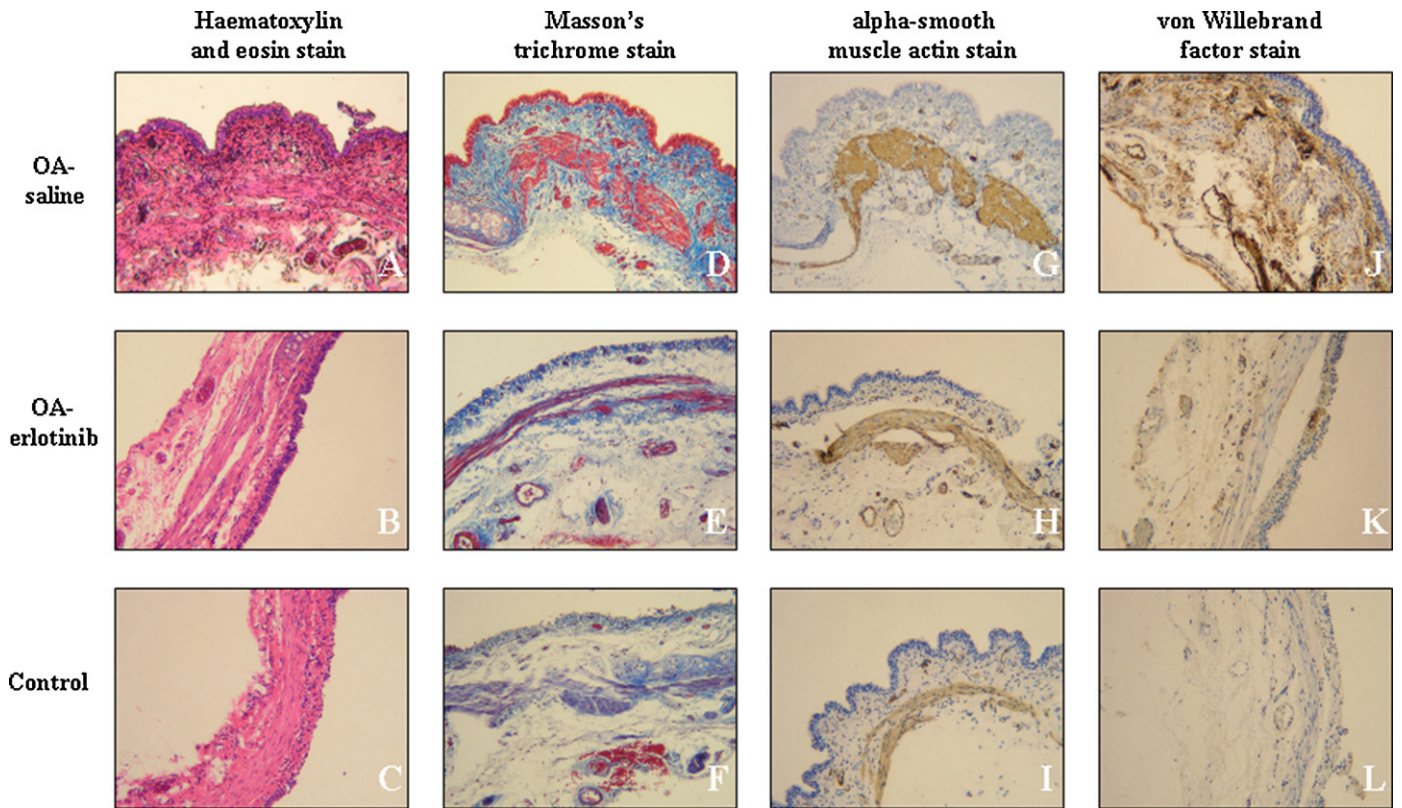


Fig. 3. Histopathology of airway remodeling (trachea); photographs representing the 3 experiments: (A–C) haematoxylin and eosin stain, 100 $\times$; (D–F) Masson's trichrome stain, 100 $\times$; (G–I) alpha-smooth muscle actin stain, 100 $\times$; and (J–L) von Willebrand factor stain, 100 $\times$. Inflammation was highest in the (A) OA-saline group than in the (B) OA-erlotinib and (C) control groups. Collagen deposits surrounding the airways were highest in the (D) OA-saline group than in the (E) OA-erlotinib and (F) control groups. Thickness of the smooth muscle layer surrounding the airways was greatest in the (G) OA-saline group compared to the (H) OA-erlotinib and (I) control groups. Number of blood vessels surrounding the airways was highest in the (J) OA-saline group compared to the (K) OA-erlotinib and (L) control groups.

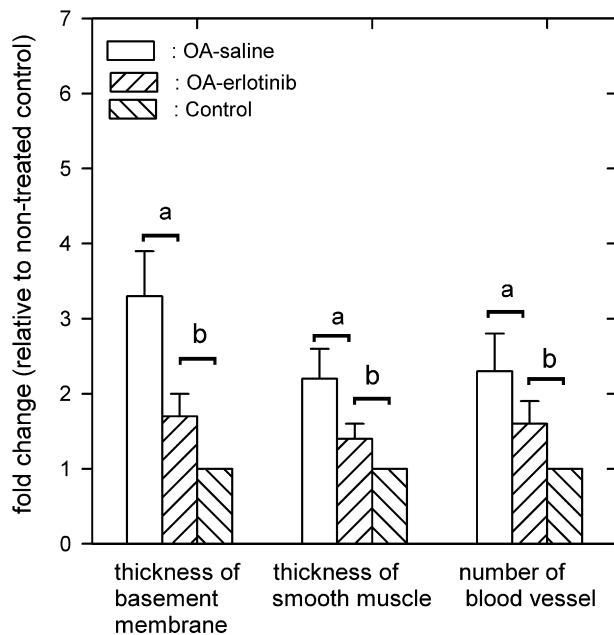


Fig. 4. The mean area of collagen deposition surrounding the airways (terminal bronchioles) was lower in the control group than in the OA-erlotinib group, which was significantly lower than in the OA-saline group. The mean thickness of the smooth muscle layer and the mean number of the blood vessels surrounding the airways were lower in the control group than in the OA-erlotinib group, which were significantly lower than in the OA-saline group. ^a $p < 0.05$ comparing the OA-saline and the OA-erlotinib groups; ^b $p < 0.05$ comparing the OA-erlotinib and control groups.

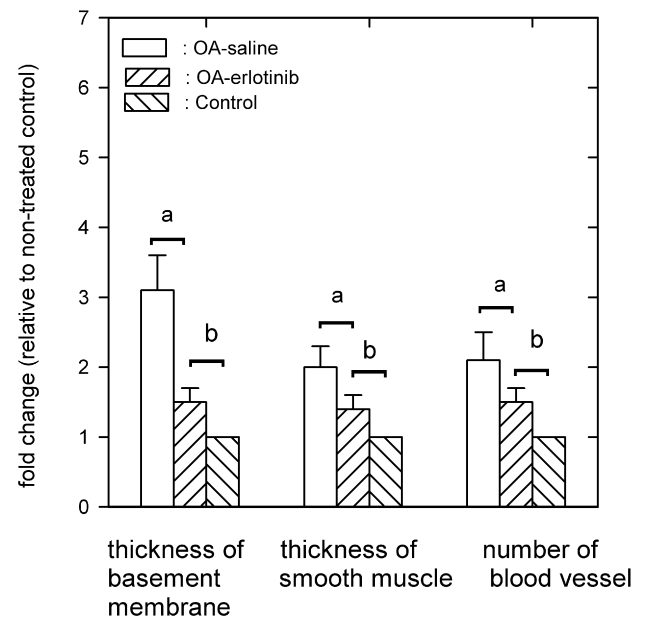


Fig. 5. The mean area of collagen deposition surrounding the airways (trachea) was lower in the control group than in the OA-erlotinib group, which was significantly lower than in the OA-saline group. The mean thickness of the smooth muscle layer and the mean number of the blood vessels surrounding the airways were lower in the control group than in the OA-erlotinib group and significantly lower in the OA-erlotinib group than in the OA-saline group. ^a $p < 0.05$ comparing the OA-saline and OA-erlotinib groups; ^b $p < 0.05$ comparing the OA-erlotinib and control groups.

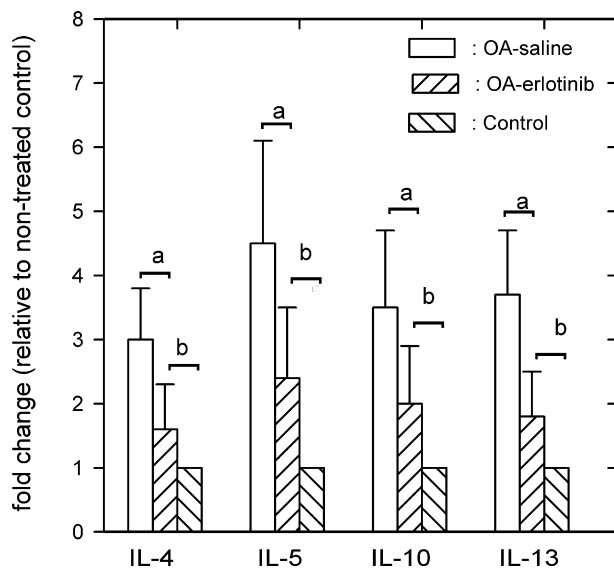


Fig. 6. Relative changes in IL-4, IL-5, IL-10 and IL-13 mRNA expressions in lung tissues of the OA-saline group were greater than those of the OA-erlotinib group, which were greater than those of the control group. ^a $p < 0.05$ comparison between the OA-saline and OA-erlotinib groups; ^b $p < 0.05$ comparison between the OA-erlotinib and control groups.

ness, mucus (and MUC5AC) production, and collagen deposits in a dose-dependent manner in a mouse model of asthma. Nath et al. (2005) have also shown that SP600125, an anthranyrazolone inhibitor of Jun N-terminal kinase, can suppress antigen-induced eosinophil infiltration in bronchial submucosa, goblet cell and airway smooth-muscle cell hyperplasia, and airway hyperresponsiveness in a chronic mouse asthma model, while Kassel et al. (2009) have found increased levels of epidermal growth factor and EGFR expression in airway smooth muscles in asthma, which may contribute to both hyperproliferation and hypercontractility.

Inhibition of EGFR blocks allergen-induced airway smooth muscle growth in a rat model of allergen-induced remodeling. Hur

et al. (2007) have shown that gefitinib dose-dependently reduces inflammatory cell counts and released cytokine concentrations (IL-4 and IL-13) in BAL fluid, as well as hampers BHR, mucus accumulation, and eosinophil recruitment in the lungs (Hur et al., 2007).

In the current animal model, inflammation and remodeling are constant features. They are present throughout the whole airway, including the trachea and small airways. Inflammatory cell infiltration and structural changes are similar for both the trachea and terminal bronchioles. This study also demonstrates that the OA-erlotinib group has decreased airway remodeling compared to those in the OA-saline group, as characterized by decreases in the area of collagen deposition, in thickness of the smooth muscle layer surrounding the airways, and in TGF- β and TGF- β mRNA expression. Much evidence shows that TGF- β is the most important cytokine related to remodeling (Davies et al., 2003). The effect on TGF- β is reduced by erlotinib, which may then reduce airway remodeling in ovalbumin-treated animals.

Even though the trachea and lungs of the rats have been immediately removed and fixed for histologic examination, and cytokine mRNA expression examined immediately after BAL fluid collection, one may argue that when BALF is drawn from the entire lung, severe hypoxia and hypotension may occur in these animals. These detrimental and traumatic effects of the lavage procedure may, in turn, affect certain results (e.g., hyperemia, interstitial edema, inflammatory cell infiltration) obtained in the study. However, physiologic data have been obtained before BAL and when BAL fluid was collected, the physiologic values and cytokine and total protein levels should not be affected by acute trauma from BAL. Lung tissue was removed and fixed immediately after completion of the BAL. Histologic changes of airway remodeling, such as collagen deposition, increased thickness of the smooth muscle layer of the airways, and increased numbers of blood vessels, did occur and cannot be simply explained by acute inflammatory changes during BAL.

Although the pathophysiology of airway remodeling in asthma is well understood, the relationship between airway remodeling and angiogenesis has only recently been studied. Angiogenesis is also associated with airway remodeling, even in early and mild bronchial asthma (Wilson and Hii, 2006). Minimal swelling and the effect of marginal submucosal thickening in the presence of maximal muscular contractions may cause severe airflow impairment (Lee et al., 2004). Multiple growth factors are implicated in airway angiogenesis, with VEGF among the most important factors. In this study, the submucosa of terminal bronchioles of rats in the OA-saline group exhibited a greater number of vessels than those in other groups. This study also shows that the OA-erlotinib group has lower VEGF cytokine levels, mRNA expression, and angiogenesis than the OA-saline group.

In conclusion, erlotinib effectively prevents bronchial hyperreactivity, inflammatory response, and IL-4, IL-5, IL-10, IL-13, TGF- β , iNOS and VEGF mRNA expressions, as well as airway remodeling, in Brown-Norway rats after sensitization and repeated allergen challenge.

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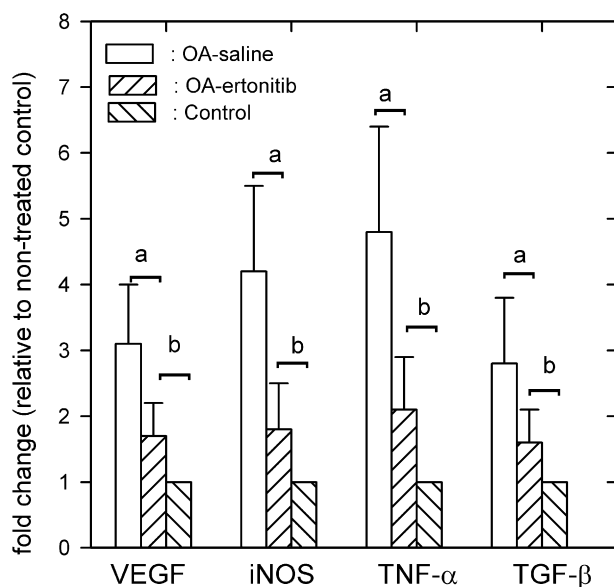


Fig. 7. Relative changes in VEGF, iNOS, TNF- α , and TGF- β 1 mRNA expressions in lung tissues of the OA-saline group were greater than those in the OA-erlotinib group, which were greater than those of the control group. ^a $p < 0.05$ comparison between the OA-saline and the OA-erlotinib groups; ^b $p < 0.05$ comparison between the OA-erlotinib and control groups.

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