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Inhibition of VEGF blocks TGF-β_1 production through a PI3K/Akt signaling pathway

K.S. Lee[*,1], S.J. Park[*,1], S.R. Kim[*,1], K.H. Min[*], K.Y. Lee[*], Y.H. Choe[*], S.H. Hong[*], Y.R. Lee[#], J.S. Kim[#], S.J. Hong[¶], and Y.C. Lee[*]

AFFILIATIONS

[*]Department of Internal Medicine, Airway Remodeling Laboratory, and

[#]Department of Biochemistry, Chonbuk National University Medical School, Jeonju, South Korea.

[¶]Department of Pediatrics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

CORRESPONDENCES

Y.C. Lee

Department of Internal Medicine, Chonbuk National University Medical School, San 2-20 Geumam-dong, Deokjin-gu, Jeonju, Jeonbuk 561-180, South Korea.

Fax: 82-63-254-1609; E-mail: leeyc@chonbuk.ac.kr

[1]These authors contributed equally to this work.

SHORT TITLE: VEGF MODULATES TGF-β_1 IN ALLERGIC AIRWAY DISEASE

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a mediator of airway inflammation and remodeling in asthma. Transforming growth factor (TGF)-β_1 plays pivotal roles in diverse biologic processes including tissue remodeling and repair in a number of chronic lung diseases. However, there are little studies elucidating the interactions between VEGF and TGF-β_1 in allergic airway disease.

We used a murine model of allergic airway disease to define the mechanism by which VEGF induces subepithelial fibrosis and to investigate a potential relationship between VEGF and TGF-β_1 and the mechanisms by which VEGF signaling regulates TGF-β_1 expression in allergic airway disease.

This study with the ovalbumin (OVA)-inhaled murine model revealed the following typical pathophysiological features of allergic airway disease in the lungs: increased numbers of inflammatory cells of the airways**,** airway hyperresponsiveness, increased peribronchial fibrosis**,** and increased levels of VEGF and TGF-β_1. Administration of VEGF inhibitors reduced the pathophysiological signs of allergic airway disease and decreased the increased TGF-β_1 levels and peribronchial fibrosis, including phosphoinositide 3-kinase (PI3K) activity after OVA inhalation. In addition, the increased TGF-β_1 levels and collagen deposition after OVA inhalation were decreased by administration of PI3K inhibitors.

These results suggest that inhibition of VEGF attenuates peribronchial fibrosis, at least mediated by regulation of TGF-β_1 expression through PI3K/Akt pathway in a murine model of allergic airway disease.

KEYWORDS: Airway remodeling, phosphoinositide 3-kinase, subepithelial fibrosis, transforming growth factor-β_1, vascular endothelial growth factor

INTRODUCTION

Bronchial asthma is a chronic inflammatory disease characterized by airway wall remodelling. Airway remodeling is due, at least in part, to an excess of extracellular matrix deposition in the airway wall, which leads to subepithelial collagen deposition [1]. This airway remodeling has been speculated to be irreversible airway obstruction and one of the factors that make difficult to treat asthma patients [1]. The histological characteristics of chronic inflammation include angiogenesis, increased connective tissue deposition, and cellular proliferation of myofibroblasts. An increase in vessel size, vessel number, and vascular surface area and the exaggerated expression of vascular endothelial growth factor (VEGF) are documented in the asthmatic airway [2]. These vascular alterations have been suggested to contribute to the airway obstruction and/or airway hyperresponsiveness [3]. VEGF is an endothelial cell-specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis. VEGF also increases vascular permeability so that plasma proteins including inflammatory mediators and inflammatory cells can leak into the extravascular space to allow the migration of inflammatory cells into the airways [4]. VEGF is a mediator of vascular and extravascular remodeling and inflammation and thus the inhibition of VEGF may be a good therapeutic strategy [4, 5]. Although the mechanism by which VEGF could induce subepithelial fibrosis in asthmatic patients is not yet defined, it has been recently shown that VEGF exhibits a critical role in enhancing chronic T-helper type 2 cell (Th2)-

mediated inflammation and transforming growth factor (TGF)-β_1 production [5], which in turn may result in airway subepithelial fibrosis.

TGF-β_1 family proteins are influential regulators of tissue remodeling and act as a potent inhibitor of proliferation for most cell types [6], as well as show proinflammatory effects in various settings of inflammation. It has been well established that TGF-β_1 plays the important role in pathogenesis of structural changes including fibrosis in a number of chronic lung diseases [7]. Furthermore, TGF-β_1 administration to mice has been shown to promote peribronchial collagen deposition [8]. However, the interactions between VEGF and TGF-β_1 in subepithelial fibrosis of allergic airway disease are poorly understood.

Phosphoinositide 3-kinase (PI3K) is a signal transduction enzyme, which catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in response to activation of either receptor tyrosine kinase, G-protein coupled receptors, or cytokine receptors, which ultimately regulate cell growth, differentiation, survival, proliferation, migration, and cytokine production. Previous studies have suggested that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells [9-11]. PI3K plays a key role in induction of the Th2 response [9-11]. This enzyme is also essential for IL-5-induced eosinophil release from bone marrow [10] and migration of eosinophils caused by a number of chemoattractants [11]. Enhanced basal activity of PI3K has been reported in eosinophils

derived from allergic asthmatics [12]. Some studies have reported that PI3K inhibition reduces Th2 cytokine production, pulmonary eosinophilia, and airway inflammation and hyperresponsiveness in a mouse model of asthma [13, 14]. Additionally, PI3K signaling, including p110δ isoform, is associated with the regulation of VEGF expression and activation [15, 16]. In the same lines, PI3K has also been shown to play an important role in VEGF-mediated signaling and VEGF-induced PI3K activation has been linked to biologically diverse roles of VEGF, such as cell migration, vascular permeability, cell survival, and cell proliferation [17]. Moreover, recent studies have revealed that PI3K plays a pivotal role in regulation of the TGF-β_1 expression [18, 19].

In the present study, we investigated the potential role of VEGF on TGF-β_1 expression and the signal pathways involved these processes in subepithelial fibrosis of allergic airway disease.

MATERIALS AND METHODS

Animals and experimental protocol

Female C57BL/6 mice, 8-10 wk of age and free of murine specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, Korea), were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University. Standard guidelines for laboratory animal care were followed [20]. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μg ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) in a total volume of 200 μL, as previously described with some modifications [13, 21, 22]. On days 20, 21, 22, 23, 24, and 25 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (weight/volume) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed at 72 h or 7 days after the last challenge.

Administration of SU5614, CBO-P11, wortmannin, or LY-294002

An inhibitor of VEGF receptor tyrosine kinase, SU5614 (Flk-1; IC_{50} = 1.2 μM, 5-Chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone, Calbiochem, San Diego, CA, USA) and

cyclopeptidic vascular endothelial growth inhibitor, CBO-P11 (Flt-1; IC_{50} = 700 nM, Flk-1/KDR; IC_{50} = 1.3 μM, *D*-Phe-Pro(79-93), Calbiochem) were used to inhibit VEGF activity. SU5614 (2.5 $mg \cdot kg^{-1}$ body weight/day), CBO-P11 (2 $mg \cdot kg^{-1}$ body weight/day), or vehicle control (0.05% dimethyl sulfoxide (DMSO)) diluted with 0.9% NaCl was administered intraperitoneally six times at 24 h-interval, beginning at 1 h before the first challenge with OVA, as previously described with some modifications [23]. Wortmannin (100 $\mu g \cdot kg^{-1}$ body weight/day; Calbiochem), LY-294002 (1.5 $mg \cdot kg^{-1}$ body weight/day; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA), or vehicle control (0.05% DMSO) diluted with 0.9% NaCl was administered in a volume of 50 μL, as described previously with some modifications [13]. Wortmannin or LY-294002 was administered intratracheally three times to each treated animal at 48 h-interval, beginning at 1 h before the first challenge.

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis, as described previously [13]. The blots were incubated with an anti-TGF-β_1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Akt antibody (Cell Signaling Technology Inc., Beverly, MA, USA), or anti-phosphorylated Akt (p-Akt) antibody (Cell Signaling Technology Inc.).

Measurement of TGF-β_1

Levels of TGF-β_1 were quantified in the supernatants of BAL fluids by enzyme immunoassays (R&D Systems, Inc., Minneapolis, MN, USA).

Measurement of PI3K enzyme activity in lung tissues

PI3K enzyme activity in lung tissues was measured as described previously [16]. The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays (Echelon, Inc., Salt Lake City, UT, USA).

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as previously described [13]. The trachea proximal to the bronchial bifurcation was excised and adherent adipose tissue was removed. The trachea was opened longitudinally and cut into three pieces. The isolated tracheas were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% protease overnight at 4°C. Following tissue digestion, fetal bovine serum (FBS; 10% final concentration) was added to the medium to deactivate enzymes, undigested fragments of tissue were removed, and tracheal epithelial cells were harvested by centrifugation at 100 g for 5 min. The epithelial cells were seeded onto 35-mm collagen-coated dishes for submerged culture. The growth medium DMEM/F-12 (Sigma-Aldrich)

containing 10% FBS, penicillin, streptomycin, and amphotericin B was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and bovine serum albumin. The cells were maintained in a humidified 5% CO_2 incubator at 37°C until they adhered.

Cell culture of A549 and measurement of TGF-β_1

A549 cells, a human lung epithelial cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in modified F-12K medium supplemented with 5% FBS, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin in a humidified 5% CO_2 incubator at 37°C. After reaching confluence, cells were harvested and then seeded onto 12-well plates for enzyme immunoassay. Cells were treated with various concentrations of recombinant human VEGF (R&D systems, Inc) with or without wortmannin. Levels of TGF-β_1 were quantified in the supernatants of A549 cells by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc.).

Histology and immunocytochemistry

For histological examination, 4-μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with Masson's trichrome stain. For

immunocytochemistry of TGF-β_1, the cytocentrifuge preparations of BAL cells or of tracheal epithelial cells were incubated sequentially in accordance with the RTU Vectastain Universal Quick kit from Vector Laboratories Inc. (Burlingame, CA, USA). The slides were probed with an affinity-purified rabbit polyclonal TGF-β_1 IgG (Santa Cruz Biotechnology) overnight at 4°C, and were incubated with prediluted biotinylated pan-specific IgG for 10 min.

Quantitation of peribronchial fibrosis

Two methods (Masson's trichrome staining and total lung collagen content) were used to quantify peribronchial fibrosis.

Peribronchial trichrome staining. The area of peribronchial trichrome staining in a paraffin-embedded lung was outlined and quantified using a light microscope (Leica DM LB; Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) attached to an image-analysis system (analySIS Pro version 3.2; Soft Imaging System GmbH, Münster, Germany). Results were expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150-200 μm of internal diameter. At least ten bronchioles were counted in each slide.

Determination of total lung collagen content. The total lung collagen content was determined using the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, Northern Ireland).

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere [16]. Anesthesia was achieved with 80 mg·kg^{-1} of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 mg·kg^{-1} at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Before methacholine challenge, an aerosol of saline was given to obtain baseline of airway responsiveness in each group. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5 to 50 mg·mL^{-1} in saline). After each methacholine challenge, the data of respiratory system resistance (Rrs) was continuously collected. Maximum values of Rrs were selected to express changes in airway function which was represented as a percentage change from baseline after saline aerosol.

Densitometric analyses and statistics

All immunoreactive and phosphorylative signals were analyzed by densitometric scanning (Gel Doc XR, Bio-Rad Laboratories Inc., Hercules, CA, USA). Data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Significant differences between two groups were determined using *t* test. Statistical significance was set at $p<0.05$.

RESULTS

Effects of VEGF inhibitors on TGF-β_1 levels in lung tissues and BAL fluids of OVA-sensitized and -challenged mice

Western blot analysis revealed that levels of TGF-β_1 protein in lung tissues were significantly increased at 7 days after the last inhalation of OVA compared with the levels in the control mice (figs 1a and 1b). The increased TGF-β_1 levels after OVA inhalation were decreased significantly by the administration of SU5614 or CBO P-11. Consistent with the results, enzyme immunoassay revealed that the increased TGF-β_1 levels after the last OVA inhalation were decreased significantly by the administration of SU5614 or CBO-P11 (fig. 1c).

Effects of VEGF inhibitors on peribronchial fibrosis in lung tissues of OVA-sensitized and -challenged mice

OVA-sensitized and -challenged mice (figs 2b, 2e, and 2f) had a significant increase in the levels of peribronchial and perivascular fibrosis compared with control mice (figs 2a, 2e, and 2f) as assessed by trichrome staining and determination of total lung collagen content. The increased trichrome staining and collagen deposition after OVA inhalation was significantly decreased by the administration of SU5614 (figs 2c, 2e, and 2f) or CBO-P11 (figs 2d, 2e, and 2f) compared with that of untreated mice.

Effects of VEGF inhibitors on Akt phosphorylation and PI3K enzyme activity in lung tissues of OVA-sensitized and -challenged mice

To support the contention that the increased VEGF protein in allergen-induced airway inflammation functioned through the PI3K pathway, we performed Western blotting to determine Akt phosphorylation and PIP3 competition enzyme immunoassay to measure PI3K enzyme activity. Levels of p-Akt protein in the lung tissues were significantly increased at 7 days after OVA inhalation compared with the levels in the control mice (figs 3a and 3b). However, no significant changes in Akt protein levels were observed in any of the group tested. The increased p-Akt, but not Akt, protein levels in the lung tissues after OVA inhalation were significantly reduced by the administration of SU5614 or CBO-P11. The PI3K activity in the lung tissues was increased at 7 days after OVA inhalation compared with that in the control mice (fig. 3c). The increased PI3K activity in the lung tissues after OVA inhalation was significantly decreased by the administration of SU5614 or CBO-P11.

Effects of PI3K inhibitors on TGF-β_1 levels in lung tissues

Western blot analysis revealed that levels of TGF-β_1 protein in lung tissues were significantly increased at 7 days after the last inhalation of OVA compared with the levels in the control mice (fig. 4). The increased TGF-β_1 levels after OVA inhalation were decreased significantly by the administration of LY-294002 or wortmannin.

Effects of PI3K inhibitors on total lung collagen deposition

OVA-sensitized and -challenged mice had a significant increase in the levels of lung collagen deposition compared with control mice as assessed by determination of total lung collagen content (fig. 5). The increased collagen deposition after OVA inhalation was significantly decreased by the administration of wortmannin or LY-294002 compared with that of untreated mice.

Effects of VEGF inhibitors on cellular changes in BAL fluids

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 72 h after OVA inhalation compared with the numbers after saline inhalation (data not shown). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of SU5614 or CBO-P11.

Effects of VEGF inhibitors on airway hyperresponsiveness

Airway responsiveness was assessed as a percent increase of Rrs in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Rrs shifted to the left compared with that of control mice (data not shown). In addition, the percent Rrs produced by methacholine administration (at doses from 10 $mg \cdot mL^{-}$

^{1} to 50 mg·mL^{-1}) increased significantly in the OVA-sensitized and -challenged mice compared with the controls. OVA-sensitized and -challenged mice treated with SU5614 or CBO-P11 showed a dose-response curve of percent Rrs that shifted to the right compared with that of untreated mice. These results indicate that administration of SU5614 or CBO-P11 reduces OVA-induced airway hyperresponsiveness.

Localization of Immunoreactive TGF-β_1 in BAL cells and in epithelial cells of OVA-sensitized and -challenged mice

Immunocytological analysis showed localization of immunoreactive TGF-β_1 in both the BAL cells (fig. 6b) and the tracheal epithelial cells (fig. 6e) from OVA-exposed mice. However, immunoreactive TGF-β_1 was markedly reduced in BAL cells (fig. 6c) and in tracheal epithelial cells (fig. 6f) from OVA-exposed mice treated with CBO-P11.

Induction of TGF-β1 production by VEGF and effect of PI3K inhibitor on VEGF-induced TGF-β1 production in A549 cells

We first examined whether VEGF stimulates TGF-β_1 protein production in A549 lung epithelial cells. Enzyme immunoassays revealed that levels of TGF-β_1 protein in A549 cells were increased significantly by treatment with VEGF (10, 20, or 50 ng·mL^{-1}) at 16 h of incubation and that the maximal levels of TGF-β_1 were detected at 20 ng·mL^{-1} of VEGF (fig.

7). To support that VEGF signaling induces TGF-β_1 production via PI3K/Akt pathway, lung epithelial cells were treated with wortmannin. Treatment of lung epithelial cells with wortmannin (500 nM) reduced significantly the production of TGF-β_1 induced by VEGF (20 $ng \cdot mL^{-1}$).

DISCUSSION

Fibrosis is an important cause of morbidity and mortality in the lung. This is illustrated in airway disorders such as asthma which is characterized by chronic inflammation and subepithelial/airway fibrosis [24]. TGF-β_1 is a multifunctional cytokine that plays pivotal roles in diverse biologic processes, including tissue remodeling and repair [25]. In human chronic lung diseases like asthma, TGF-β_1 expression has been shown to be increased [26]. Moreover, TGF-β_1 administration promotes peribronchial collagen deposition [8] and the increase of TGF-β_1 expression in patients of asthma seems to correlation with disease severity and degree of subspithelial fibrosis [27]. Although some Th2-inflammatory mediators such as IL-13 and IL-11 are known to have ability to enhance the TGF-β_1 expression, the exact mechanisms for regulation of TGF-β_1 expression remain to be elucidated. A recent study has demonstrated that VEGF enhances TGF-β_1 production [5, 18], which in turn may result in airway subepithelial fibrosis. However, the mechanism by which VEGF induces subepithelial fibrosis, specifically the relationship between VEGF and TGF-β_1, in allergic airway disease is not yet defined. This study with the OVA-induced model revealed the following typical pathophysiological features of allergic airway disease in the lungs: increased numbers of airway inflammatory cells**,** bronchial hyperresponsiveness, increased peribronchial fibrosis**,** and increased levels of VEGF and TGF-β_1. Administration of VEGF inhibitors reduced these pathophysiological signs of allergic airway disease and

decreased the increase of TGF-β_1 levels and peribronchial fibrosis after OVA inhalation. These findings suggest that inhibition of VEGF attenuates peribronchial fibrosis by preventing TGF-β_1 production in allergic airway disease.

VEGF is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and Th2 inflammation [5]. VEGF can be produced by a wide variety of cells including macrophages, neutrophils, eosinophils, and lymphocytes [4]. Several studies have also shown that overproduction of VEGF causes airway inflammation and bronchial hyperresponsiveness [4]. Consistent with these observations, our present results have revealed that airway inflammatory cells and bronchial hyperresponsiveness after OVA inhalation were substantially increased and that the administration of VEGF inhibitors reduced the increase of airway inflammation and bronchial hyperresponsiveness. Recently, VEGF has been revealed as a mediator of airway fibrosis which is a feature of airway remodeling [28, 29]. VEGF can increase the expression of connective tissue growth factor, a growth factor that acts on fibroblast proliferation and matrix production [28], and stimulate fibronectin secretion from human airway muscle cells [29]. In addition to these in vitro studies, Lee et al. [5] using VEGF transgenic mice have clearly demonstrated that VEGF is a potent inducer both of angiogenesis and subepithelial fibrosis. Chetta et al. [30] have reported that VEGF expression is associated with subepithelial fibrosis in humans as well. Consistent with these observations, we have found

that VEGF expression was up-regulated and peribronchial fibrosis was also increased in OVA-induced allergic airway disease. Intriguingly, administration of VEGF inhibitors, SU5614 or CBO P-11, significantly reduced the increase of peribronchial fibrosis induced by OVA inhalation. Taken together, these findings suggest that VEGF may contribute to airway wall remodeling in allergic airway disease by affecting the extracellular matrix composition and by stimulating fibrotic changes.

Since PI3K was first identified as an activity associated with various oncoproteins and growth factor receptor [31], accumulating evidence has indicated that PI3Ks can provide a critical signal for cell proliferation, cell survival, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, and superoxide production as well as actin reorganization, and chemotaxis [32]. PI3K activity is also stimulated after antigen challenge in a murine model of allergic asthma and administration of wortmannin or LY-294002, two broad-spectrum inhibitors of PI3Ks, attenuates inflammation and airway hyperresponsiveness [13, 14]. Moreover, several studies have demonstrated that PI3K is involved in mediating the various biological functions of VEGF [17, 33]. Consistent these observations, our present results have shown that p-Akt protein and PI3K enzyme activity were significantly increased after OVA inhalation and the increased levels of them were substantially reduced by the treatment of VEGF inhibitors, SU5614 or CBO-P11.

A recent study has revealed that VEGF induces TGF-β_1 mRNA expression and protein production [18]. In keeping with these results, we have found that inhibition of VEGF decreased the increased expression of TGF-β_1 in lungs after OVA inhalation. Using highly specific PI3K inhibitors, wortmannin or LY-294002, we were able to elucidate whether VEGF regulates the TGF-β_1 expression through the PI3K signaling pathway in a murine model of allergic airway disease. We have shown that administration of PI3K inhibitors reduced significantly the increase of TGF-β_1 levels in lungs after OVA inhalation. These findings are consistent with previous evidence that activation of PI3K/Akt pathway is important for TGF-β_1 expression [18, 19]. In addition, we have found that TGF-β_1 production in human lung epithelial cells were induced by VEGF and that treatment of lung epithelial cells with wortmannin reduced significantly the production of TGF-β_1 induced by VEGF. Taken together, these observations suggest that inhibition of VEGF attenuates increased peribronchial fibrosis, at least through reduction of TGF-β_1 expression mediated by PI3K/Akt pathway in allergic airway disease.

Hypoxia-inducible factor (HIF)-1 is a transcriptional factor that functions as a master regulator of oxygen homeostasis. HIF-1 has been shown to regulate expression of dozens of target genes including VEGF, the protein products of which play important roles in angiogenesis, erythropoiesis, energy metabolism, and cell survival [34]. HIF-1 is a heterodimer composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed

HIF-1β subunit [35]. In addition to the oxygen-dependent regulation of HIF-1α expression and activity, an oxygen-independent regulatory pathway has been identified through which a variety of cytokines and growth factors have been shown to induce the expression of HIF-1α and HIF-1 target genes [36]. These cytokines and growth factors induce HIF-1α expression via the activation of phosphorylated signalings including PI3K/Akt pathway [37]. In addition, several studies have demonstrated that activation of the PI3K/Akt pathway causes the increase of HIF-1α protein levels [38]. Consistent with these observations, determination of HIF-1α protein level in nuclear extracts has revealed that this protein level was substantially increased in our present mouse model of allergic airway disease and that the increased HIF-1α activity was significantly reduced by the administration of wortmannin or LY-294002 (data not shown). Therefore, these findings imply that the HIF-1α activation after OVA inhalation may depend on PI3K signaling which is induced by VEGF in allergic airway disease. Moreover, we have found that the administration of VEGF inhibitors reduced the OVA-induced increased VEGF expression itself as well as PI3K activity, and we have previously reported that the increased HIF-1α activity by PI3K/Akt signaling activation increased the expression of VEGF in a murine model of allergic airway disease [15, 16]. Taken together, we suggest that a positive feedback loop between HIF-1α and VEGF exists in allergic airway disease.

In summary, our data strongly indicate that the inhibition of VEGF signaling is potentially powerful therapeutic strategy for subepithelial fibrosis of allergic airway disease, partly mediated by regulation of TGF-β_1 expression through PI3K/Akt pathway. Thus, these findings provide an important mechanism for the use of VEGF inhibitors to prevent and/or treat subepithelial fibrosis in allergic airway disease.

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FIGURE LEGENDS

FIGURE 1. Effects of SU5614 or CBO-P11 on transforming growth factor (TGF)-β_1 protein expression in lung tissues and in bronchoalveolar lavage (BAL) fluids. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin (OVA)-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered SU5614 (OVA+SU5614), and OVA-inhaled mice administered CBO-P11 (OVA+CBO-P11). a) Western blot analysis of TGF-β_1. b) Densitometric analyses are presented as the relative ratio of TGF-β_1 to actin. The relative ratio of TGF-β_1 in the lung tissues of SAL+SAL is arbitrarily presented as 1. *C*, Enzyme immunoassay of TGF-β_1 in BAL fluids. Bars represent mean ± SEM from 8 mice per group. #, $p<0.05$ versus SAL+SAL; *, $p<0.05$ versus OVA+SAL; N.D., not determined.

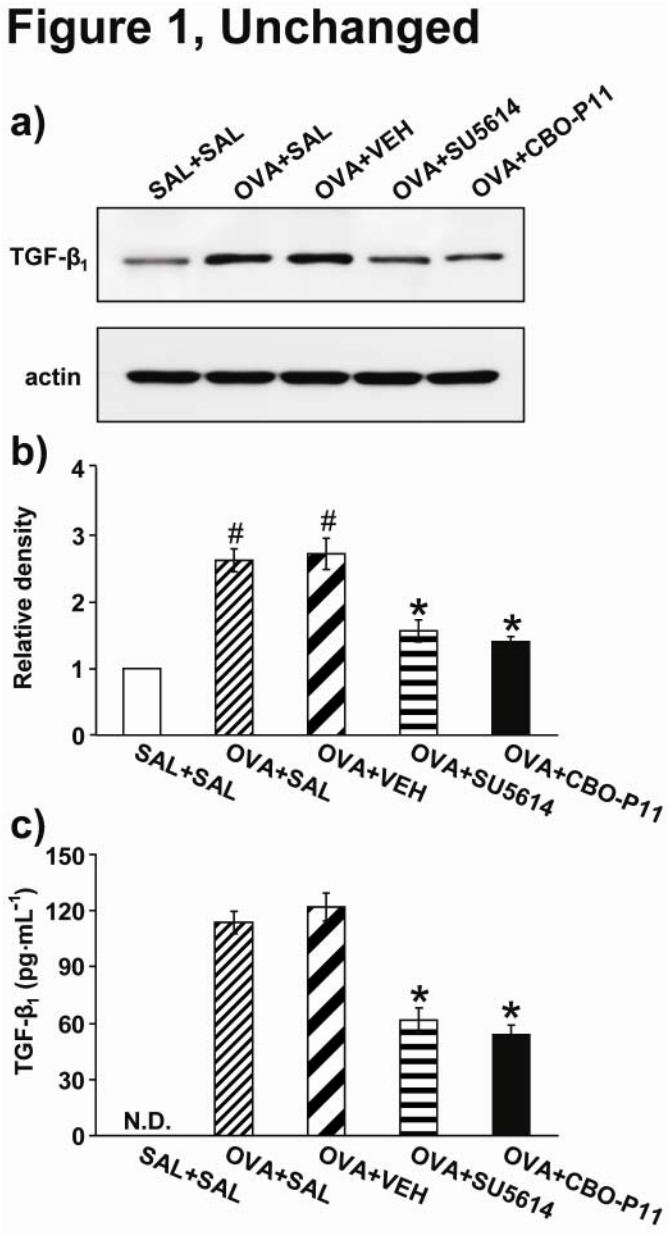

FIGURE 2. Effects of SU5614 or CBO-P11 on peribronchial fibrosis in lung tissues. a-d) Representative peribronchial and perivascular trichrome stained sections of the lungs. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered drug vehicle (a), ovalbumin (OVA)-inhaled mice administered drug vehicle (b), OVA-inhaled mice administered SU5614 (c), and OVA-inhaled mice administered CBO-P11

(d). The blue color indicates peribronchial and perivascular trichrome staining collagen deposition/fibrosis. Bars indicate 50 μm. e) Quantitation of peribronchial fibrosis. f) Total lung collagen content. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered SU5614 (OVA+SU5614), and OVA-inhaled mice administered CBO-P11 (OVA+CBO-P11). Bars represent mean ± SEM from 8 mice per group. #, $p<0.05$ versus SAL+VEH; *, $p<0.05$ versus OVA+VEH.

Figure 2, Unchanged

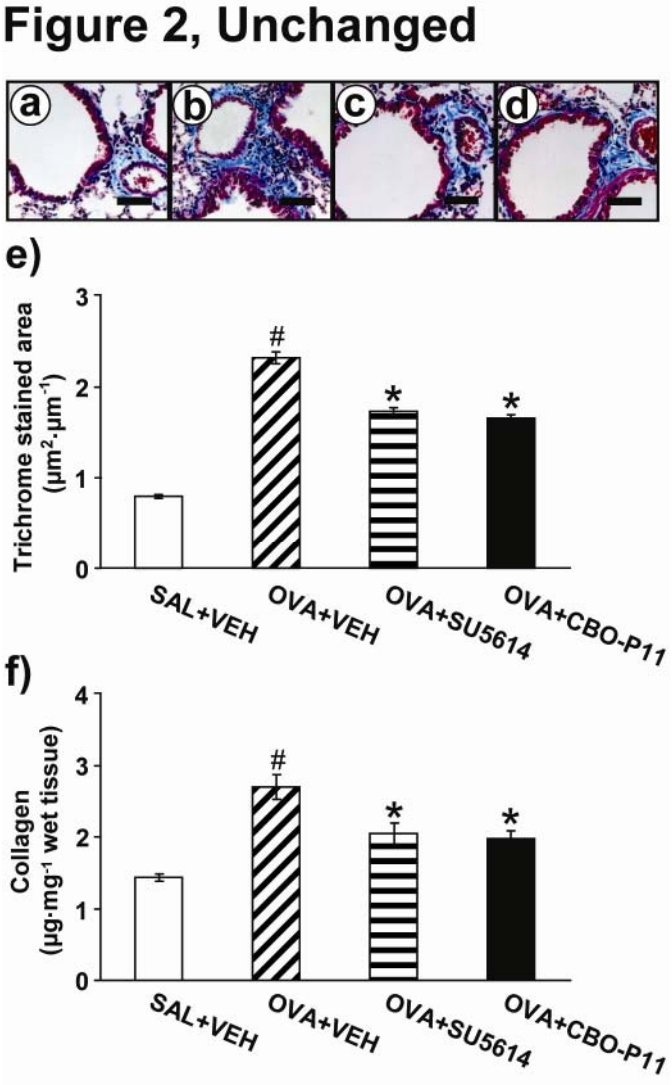

FIGURE 3. Effect of SU5614 or CBO-P11 on phosphorylated Akt (p-Akt), Akt protein levels, and phosphoinositide 3-kinase (PI3K) enzyme activity in lung tissues. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin (OVA)-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered SU5614 (OVA+SU5614), and OVA-inhaled mice administered CBO-P11 (OVA+CBO-P11). a) Western blotting of p-Akt and Akt in lung tissues. b) Densitometric analyses are presented as the relative ratio of p-Akt to Akt. The relative ratio of p-Akt in the lung tissues of SAL+SAL is arbitrarily presented as 1. c) Enzyme immunoassay of phosphatidylinositol (3,4,5)-trisphosphate generation by PI3Ks in lung tissue extracts. Bars represent mean ± SEM from 8 mice per group. #, $p<0.05$ versus SAL+SAL; *, $p<0.05$ versus OVA+SAL.

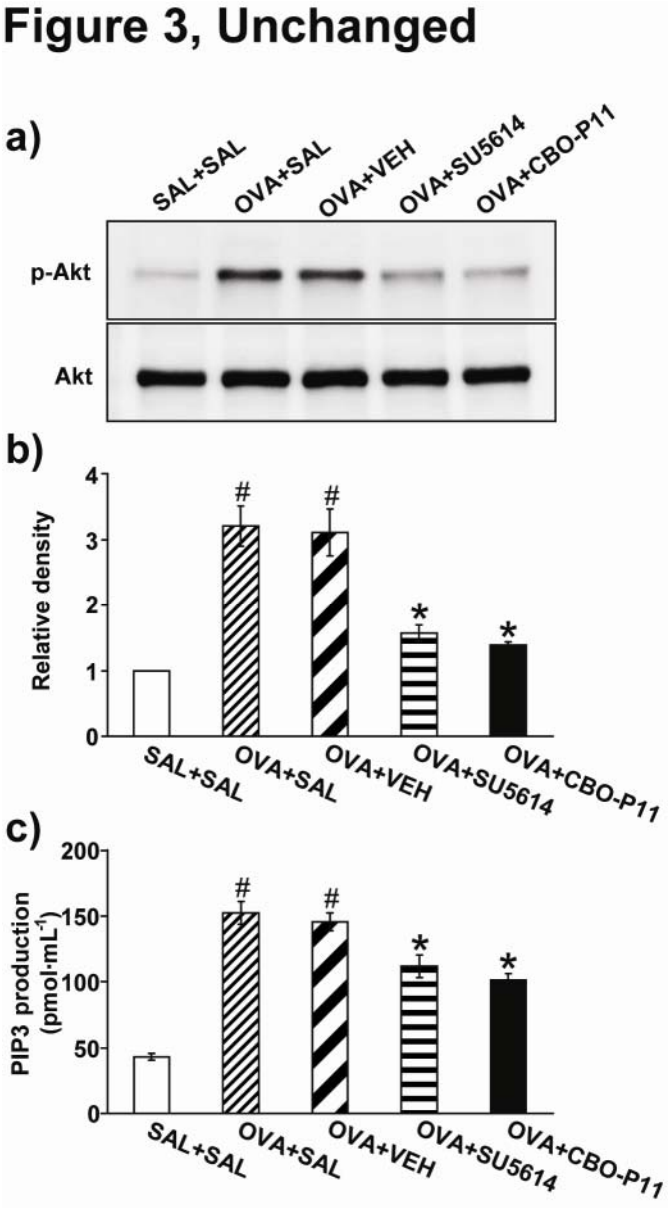

FIGURE 4. Effects of wortmannin or LY-294002 on transforming growth factor (TGF)-β_1 protein expression in lung tissues. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin (OVA)-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered wortmannin (OVA+wortmannin), and OVA-

inhaled mice administered LY-294002 (OVA+LY294002). a) Western blot analysis of TGF-β_1. b) Densitometric analyses are presented as the relative ratio of TGF-β_1 to actin. The relative ratio of TGF-β_1 in the lung tissues of SAL+SAL is arbitrarily presented as 1. Bars represent mean ± SEM from 8 mice per group. #, $p<0.05$ versus SAL+SAL; *, $p<0.05$ versus OVA+SAL.

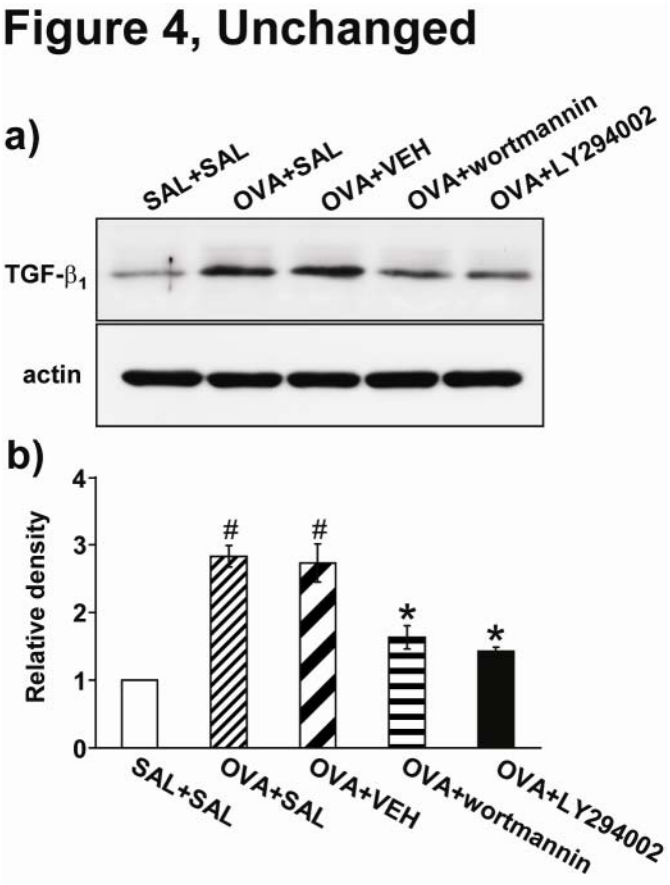

FIGURE 5. Effects of wortmannin or LY-294002 on collagen deposition in lung tissues. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin (OVA)-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled

mice administered wortmannin (OVA+wortmannin), and OVA-inhaled mice administered LY-294002 (OVA+LY294002). Bars represent mean ± SEM from 8 mice per group. #, $p<0.05$ versus SAL+SAL; *, $p<0.05$ versus OVA+SAL.

Figure 5, Unchanged

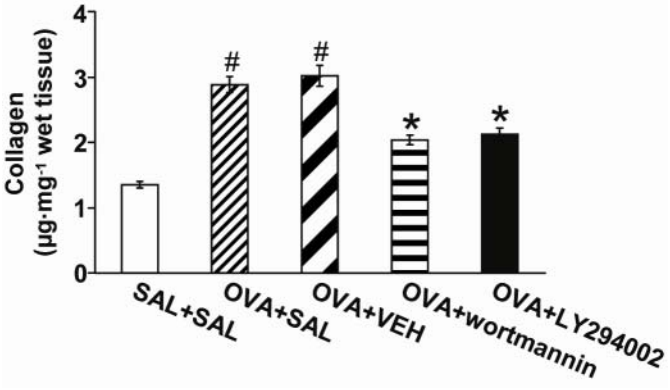

FIGURE 6. Localization of immunoreactive transforming growth factor (TGF)-β_1 in bronchoalveolar lavage (BAL) fluids and in tracheal epithelial cells of ovalbumin (OVA)-sensitized and -challenged mice. The BAL fluids and tracheal epithelial cells were from saline-inhaled mice administered saline (a and d), from OVA-inhaled mice administered saline (b and e), and from OVA-inhaled mice administered CBO-P11 (c and f). Representative light microscopy shows TGF-β_1-positive cells in the BAL cells (a-c) and in the tracheal epithelial cells (d-f). Brown color indicates TGF-β_1-positive cells. Bars indicate scale of 10 µm (a-c) or 50 µm (d-f).

Figure 6, New

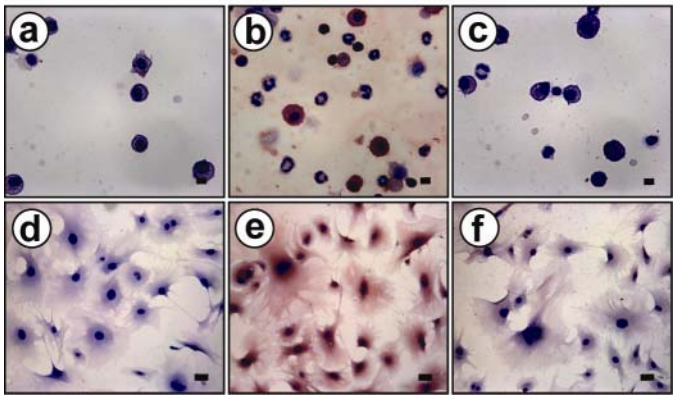

FIGURE 7. Effect of vascular endothelial growth factor (VEGF) on transforming growth factor (TGF)-β_1 production by human lung epithelial cells. TGF-β_1 levels were determined in epithelial cells stimulated with various concentrations of VEGF or treated with VEGF (20 ng·mL^{-1}) in the presence of wortmannin (500 nM) or an equivalent dose of the solvent dimethyl sulfoxide (DMSO). Data represent mean ± SEM from five independent experiments. #, $p<0.05$.

Figure 7, New