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

K.S. Lee^{*,†}, S.J. Park^{*,†}, S.R. Kim^{*,†}, 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^{*}

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

A murine model of allergic airway disease was used 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 signalling regulates TGF- β_1 expression in allergic airway disease.

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 vascular endothelial growth factor attenuates peribronchial fibrosis, at least when mediated by regulation of transforming growth factor- β_1 expression through phosphoinositide 3-kinase/Akt pathway in a murine model of allergic airway disease.

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

Bronchial asthma is a chronic inflammatory disease characterised by airway wall remodelling. Airway remodelling is due, at least in part, to an excess of extracellular matrix deposition in the airway wall, which leads to subepithelial collagen deposition [1]. It has been speculated that this airway remodelling is an irreversible airway obstruction and is one of the factors that make asthma patients difficult to treat [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]. It has been suggested that these

vascular alterations 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 remodelling 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

AFFILIATIONS

^{*}Dept of Internal Medicine, Airway Remodeling Laboratory,

[#]Dept of Biochemistry, Chonbuk National University Medical School, Jeonju,

[†]Dept of Pediatrics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea.

[†]These authors contributed equally to the present work.

CORRESPONDENCE

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

Fax: 82 632541609

E-mail: leeyc@chonbuk.ac.kr

Received:

September 22 2007

Accepted after revision:

November 15 2007

SUPPORT STATEMENT

Y.C. Lee's work was supported by the Korea Science and Engineering Foundation through the National Research Lab. Programme funded by the Ministry of Science and Technology (ROA-2005-000-10052-0(2007)), by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund KRF-2005-201-E100014), by a grant of the Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea (A060169), and also by a grant of the Korea Health 21 R&D Project (0412-CR03-0704-0001).

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

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 remodelling and act as potent inhibitors of proliferation for most cell types [6], as well as show pro-inflammatory effects in various settings of inflammation. It has been well established that TGF- β_1 plays an important role in the 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 that catalyses the phosphorylation of phosphatidylinositol (4,5)-bispophosphate 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 interleukin (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 signalling, including p110 δ isoform, is associated with the regulation of VEGF expression and activation [15, 16]. Along the same lines, PI3K has also been shown to play an important role in VEGF-mediated signalling, 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, the potential role of VEGF on TGF- β_1 expression and the signal pathways involved in these processes in subepithelial fibrosis of allergic airway disease were investigated.

MATERIALS AND METHODS

Animals and experimental protocol

Female C57BL/6 mice, aged 8–10 weeks and free of murine-specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, South Korea), housed throughout the experiments in a laminar flow cabinet and maintained on standard laboratory chow *ad libitum*. All experimental animals used in the present study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University (Jeonju, South Korea). Standard guidelines

for laboratory animal care were followed [20]. Mice were sensitised on days 1 and 14 by intraperitoneal injection of 20 μ g ovalbumin (OVA; Sigma-Aldrich, St Louis, MO, USA) emulsified in 1 mg of aluminium hydroxide (Pierce Chemical Co., Rockford, IL, USA) in a total volume of 200 μ L, as previously described, with some modifications [13, 21, 22]. On days 20, 21, 22, 23, 24 and 25 after the initial sensitisation, 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 nebuliser (NE-U12; Omron, Tokyo, Japan). Bronchoalveolar lavage (BAL) was performed 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; half maximal inhibitory concentration (IC₅₀) 1.2 μ M, 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; IC₅₀ 700 nM, Flk-1/KDR; IC₅₀ 1.3 μ M, D-Phe-Pro(79–93); Calbiochem) were used to inhibit VEGF activity. SU5614 (2.5 mg·kg⁻¹ body weight·day⁻¹), CBO-P11 (2 mg·kg⁻¹ body weight·day⁻¹), or vehicle control (0.05% dimethyl sulphoxide (DMSO)) diluted with 0.9% NaCl was administered intraperitoneally six times at 24-h intervals, beginning 1 h before the first challenge with OVA, as previously described, with some modifications [23]. Wortmannin (100 μ g·kg⁻¹ body weight·day⁻¹; Calbiochem), LY-294002 (1.5 mg·kg⁻¹ body weight·day⁻¹; 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 μ L, as described previously, with some modifications [13]. Wortmannin or LY-294002 was administered intratracheally three times to each treated animal at 48-h intervals, beginning 1 h before the first challenge.

Western blot analysis

Protein expression levels were analysed 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 (anti-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, foetal 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 $100 \times g$ 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% CO₂ 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·mL⁻¹ penicillin, and 100 µg·mL⁻¹ streptomycin in a humidified 5% CO₂ 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, deparaffinised 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 immunoglobulin (Ig)G (Santa Cruz Biotechnology) overnight at 4°C and were incubated with pre-diluted biotinylated pan-specific IgG for 10 min.

Quantification 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 10 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, UK).

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed as a change in airway function after challenge with aerosolised methacholine *via* the airways, as described elsewhere [16]. Anaesthesia was achieved with 80 mg·kg⁻¹ of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomised and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montreal, QC, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 mg·kg⁻¹ at a frequency of 150 breaths·min⁻¹ and a positive end-expiratory pressure of 2 cmH₂O (0.2 kPa) 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 nebuliser 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 (2.5–50 mg·mL⁻¹ in saline). After each methacholine challenge, the data of respiratory system resistance (*R*_s) were continuously collected. Maximum values of *R*_s were selected to express changes in airway function, which was represented as a per cent change from baseline after saline aerosol.

Densitometric analyses and statistics

All immunoreactive and phosphorylative signals were analysed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc., Hercules, CA, USA). Data are expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Significant differences between two groups were determined using unpaired 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-sensitised 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 these 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-sensitised and -challenged mice

OVA-sensitised and -challenged mice (figs 2b, 2e and 2f) had a significant increase in 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

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 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. Data are presented as mean $\pm$ SEM from eight mice per group. Enzyme immunoassay of TGF- β_1 in BAL fluids was not determined for the SAL+SAL group. *: $p < 0.05$ versus OVA+SAL; #: $p < 0.05$ versus SAL+SAL.

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-sensitised and -challenged mice

To support the contention that the increased VEGF protein in allergen-induced airway inflammation occurred through the PI3K pathway, Western blotting and PIP3 competition enzyme immunoassay were performed in order to determine Akt phosphorylation and to measure PI3K enzyme activity. Levels of p-Akt protein in the lung tissues were significantly increased 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 groups 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 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-sensitised 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 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 per cent increase of R_{rs} in response to increasing doses of methacholine. In OVA-sensitised and -challenged mice, the dose-response curve of per cent R_{rs} shifted to the left compared with that of control mice (data not shown). In addition, the per cent R_{rs} produced by methacholine administration (at doses 10–50 mg·mL⁻¹) increased significantly in the OVA-sensitised and -challenged mice, compared with the controls. OVA-sensitised and -challenged mice treated with SU5614 or CBO-P11 showed a dose-response curve of per cent R_{rs} 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.

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 7 days after the last challenge in a) saline-inhaled mice administered drug vehicle (SAL+VEH), b) ovalbumin (OVA)-inhaled mice administered drug vehicle (OVA+VEH), c) OVA-inhaled mice administered SU5614 (OVA+SU5614) and d) OVA-inhaled mice administered CBO-P11 (OVA+CBO-P11). The blue colour indicates peribronchial and perivascular trichrome staining collagen deposition/fibrosis. Scale bars=50 μm . e) Quantification of peribronchial fibrosis. f) Total lung collagen content. Sampling was performed 7 days after the last challenge in all groups. Data are presented as mean $\pm$ SEM from eight mice per group. *: $p < 0.05$ versus OVA+VEH; #: $p < 0.05$ versus SAL+VEH.

Localisation of immunoreactive TGF- β_1 in BAL cells and in epithelial cells of OVA-sensitised and -challenged mice

Immunocytochemical analysis showed localisation 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- β_1 production by VEGF and effect of PI3K inhibitor on VEGF-induced TGF- β_1 production in A549 cells

It was initially 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 $\text{ng} \cdot \text{mL}^{-1}$) at 16 h of incubation and that the maximal levels of TGF- β_1 were detected at 20 $\text{ng} \cdot \text{mL}^{-1}$ of VEGF (fig. 7). To support that VEGF signalling induces TGF- β_1 production via the PI3K/Akt pathway, lung epithelial cells were treated with wortmannin. Treatment of lung epithelial cells with wortmannin (500 nM) significantly reduced the production of TGF- β_1 induced by VEGF (20 $\text{ng} \cdot \text{mL}^{-1}$).

DISCUSSION

Fibrosis is an important cause of morbidity and mortality in the lung. This is illustrated in airway disorders such as asthma, which is characterised by chronic inflammation and

subepithelial/airway fibrosis [24]. TGF- β_1 is a multifunctional cytokine that plays pivotal roles in diverse biological processes, including tissue remodelling 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 asthma patients seems to correlate with disease severity and the degree of subepithelial fibrosis [27]. Although some Th2-inflammatory mediators, such as IL-13 and IL-11, are known to have the ability to enhance TGF- β_1 expression, the exact mechanisms for the 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. The present study involving 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

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 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 (PIP3) generation by PI3Ks in lung tissue extracts. Data are presented as mean $\pm$ SEM from eight mice per group. *: $p < 0.05$ versus OVA+SAL; #: $p < 0.05$ versus SAL+SAL.

fibrosis by preventing TGF- β_1 production in allergic airway disease.

VEGF is a potent stimulator of inflammation, airway remodeling and physiological dysregulation that augments antigen sensitisation 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

FIGURE 4. Effects of wortmannin or LY-294002 on transforming growth factor (TGF)- β_1 protein expression in lung tissues. Sampling was performed 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. Data are presented as mean $\pm$ SEM from eight mice per group. *: $p < 0.05$ versus OVA+SAL; #: $p < 0.05$ versus SAL+SAL.

shown that overproduction of VEGF causes airway inflammation and bronchial hyperresponsiveness [4]. Consistent with these observations, the 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 found to be a mediator of airway fibrosis that is a feature of airway remodelling [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 also reported that VEGF expression is associated with subepithelial fibrosis in humans. Consistent with these observations, the present study found that VEGF expression was upregulated and peribronchial fibrosis was also increased in OVA-induced allergic airway disease. Intriguingly, administration of the VEGF inhibitors SU5614 or CBO P-11

FIGURE 5. Effects of wortmannin or LY-294002 on collagen deposition in lung tissues. Sampling was performed 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). Data are presented as mean $\pm$ SEM from eight mice per group. *: $p < 0.05$ versus OVA+SAL; #: $p < 0.05$ versus SAL+SAL.

significantly reduced the increase of peribronchial fibrosis induced by OVA inhalation. Taken together, these findings suggest that VEGF may contribute to airway wall remodelling

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 reorganisation 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 with these observations, the present results have shown that p-Akt protein and PI3K enzyme activity were significantly increased after OVA inhalation and the increased levels of activity were substantially reduced by treatment with 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, the current authors have found that inhibition of VEGF decreased the increased expression of TGF- β_1 in the lungs after OVA inhalation. Using highly specific PI3K inhibitors, wortmannin or LY-294002, it was possible to elucidate whether

FIGURE 6. Localisation of immunoreactive transforming growth factor (TGF)- β_1 in bronchoalveolar lavage (BAL) fluids and in tracheal epithelial cells of ovalbumin (OVA)-sensitised 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). A brown colour indicates TGF- β_1 -positive cells. Scale bars=10 µm (a–c) or 50 µm (d–f).

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 dimethylsulphoxide (DMSO). Data are presented as mean $\pm$ SEM from five independent experiments. *: $p < 0.05$.

VEGF regulates the TGF- β_1 expression through the PI3K signalling pathway in a murine model of allergic airway disease. It has been shown that administration of PI3K inhibitors significantly reduced the increase of TGF- β_1 levels in lungs after OVA inhalation. These findings are consistent with previous evidence that activation of the PI3K/Akt pathway is important for TGF- β_1 expression [18, 19]. In addition, the present authors 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 significantly reduced 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 the 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 the 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-1 α subunit and a constitutively expressed HIF-1 β subunit [35]. In addition to the oxygen-dependent regulation of HIF-1 α 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-1 α and HIF-1 target genes [36]. These cytokines and growth factors induce HIF-1 α expression *via* the activation of phosphorylated signalling pathways, including the PI3K/Akt pathway [37]. In addition, several studies have demonstrated that activation of the PI3K/Akt pathway causes the increase of HIF-1 α protein

levels [38]. Consistent with these observations, determination of HIF-1 α protein levels in nuclear extracts has revealed that this protein level was substantially increased in the present mouse model of allergic airway disease and that the increased HIF-1 α activity was significantly reduced by the administration of wortmannin or LY-294002 (data not shown). Therefore, these findings imply that the HIF-1 α activation after OVA inhalation may depend on PI3K signalling, which is induced by VEGF in allergic airway disease. Moreover, the current authors have found that the administration of VEGF inhibitors reduced the OVA-induced increased VEGF expression itself, as well as PI3K activity, and have previously reported that the increased HIF-1 α activity *via* PI3K/Akt signalling activation increased the expression of VEGF in a murine model of allergic airway disease [15, 16]. Taken together, the present authors suggest that a positive feedback loop between HIF-1 α and VEGF exists in allergic airway disease.

In summary, the present data strongly indicate that the inhibition of vascular endothelial growth factor signalling is potentially powerful therapeutic strategy for subepithelial fibrosis of allergic airway disease, partly mediated by regulation of transforming growth factor- β_1 expression through the phosphoinositide 3-kinase/Akt pathway. Therefore, these findings provide an important mechanism for the use of vascular endothelial growth factor inhibitors to prevent and/or treat subepithelial fibrosis in allergic airway disease.

ACKNOWLEDGEMENTS

The authors would like to thank M-J. Im (Dept of Biochemistry, Chonbuk National University Medical School, Jeju, South Korea) for careful reading of the manuscript.

REFERENCES

- 1 Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 1: 520–524.
- 2 Lee YC, Lee HK. Vascular endothelial growth factor in patients with acute asthma. *J Allergy Clin Immunol* 2001; 107: 1106.
- 3 Kanazawa H, Nomura S, Yoshikawa J. Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med* 2004; 169: 1125–1130.
- 4 Lee YC, Kwak YG, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2002; 168: 3595–3600.
- 5 Lee CG, Link H, Baluk P, *et al.* Vascular endothelial growth factor (VEGF) induces remodeling and enhances T_H2-mediated sensitization and inflammation in the lung. *Nat Med* 2004; 10: 1095–1103.
- 6 Massague J, Blain SW, Lo RS. TGF- β signaling in growth control, cancer, and heritable disorders. *Cell* 2000; 103: 295–309.
- 7 Holgate ST, Lackie PM, Howarth PH, *et al.* Invited lecture: activation of the epithelial mesenchymal trophic unit in the pathogenesis of asthma. *Int Arch Allergy Immunol* 2001; 124: 253–258.

- 8 Kenyon NJ, Ward RW, McGrew G, Last JA. TGF- β_1 causes airway fibrosis and increased collagen I and III mRNA in mice. *Thorax* 2003; 58: 772–777.
- 9 Fukao T, Tanabe M, Terauchi Y, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol* 2002; 3: 875–881.
- 10 Palframan RT, Collins PD, Severs NJ, Rothery S, Williams TJ, Rankin SM. Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. *J Exp Med* 1998; 188: 1621–1632.
- 11 Dunzendorfer S, Meierhofer C, Wiedermann CJ. Signaling in neuropeptide-induced migration of human eosinophils. *J Leukoc Biol* 1998; 64: 828–834.
- 12 Bracke M, van de Graaf E, Lammers JW, Coffey PJ, Koenderman L. *In vivo* priming of Fc ϵ R functioning on eosinophils of allergic asthmatics. *J Leukoc Biol* 2000; 68: 655–661.
- 13 Kwak YG, Song CH, Yi HK, et al. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003; 111: 1083–1092.
- 14 Duan W, Aguinaldo Datiles AM, Leung BP, Vlahos CJ, Wong WS. An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model. *Int Immunopharmacol* 2005; 5: 495–502.
- 15 Lee KS, Kim SR, Park SJ, et al. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) reduces vascular endothelial growth factor expression in allergen-induced airway inflammation. *Mol Pharmacol* 2006; 69: 1829–1839.
- 16 Lee KS, Park SJ, Kim SR, et al. Phosphoinositide 3-kinase- δ inhibitor reduces vascular permeability in a murine model of asthma. *J Allergy Clin Immunol* 2006; 118: 403–409.
- 17 Thakker GD, Hajjar DP, Muller WA, Rosengart TK. The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. *J Biol Chem* 1999; 274: 10002–10007.
- 18 Li ZD, Bork JP, Krueger B, et al. VEGF induces proliferation, migration, and TGF- β_1 expression in mouse glomerular endothelial cells *via* mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Biochem Biophys Res Commun* 2005; 334: 1049–1060.
- 19 Lee SJ, Yang EK, Kim SG. Peroxisome proliferator-activated receptor- γ and retinoic acid X receptor α represses the TGF β_1 gene *via* PTEN-mediated p70 ribosomal S6 kinase-1 inhibition: role for Zf9 dephosphorylation. *Mol Pharmacol* 2006; 70: 415–425.
- 20 Institute of Laboratory Animal Research, Commission of Life Sciences, National Research Council. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, 1996.
- 21 Lee KS, Kim SR, Park HS, et al. Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice. *J Allergy Clin Immunol* 2007; 119: 141–149.
- 22 Locke NR, Royce SG, Wainwright GS, Samuel CS, Tang ML. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol* 2007; 36: 625–632.
- 23 Lee KS, Min KH, Kim SR, et al. Vascular endothelial growth factor modulates matrix metalloproteinase-9 expression in asthma. *Am J Respir Crit Care Med* 2006; 174: 161–170.
- 24 Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999; 104: 1001–1006.
- 25 Lee CG, Kang HR, Homer RJ, Chupp G, Elias JA. Transgenic modeling of transforming growth factor- β_1 : role of apoptosis in fibrosis and alveolar remodeling. *Proc Am Thorac Soc* 2006; 3: 418–423.
- 26 Chu HW, Trudeau JB, Balzar S, Wenzel SE. Peripheral blood and airway tissue expression of transforming growth factor β by neutrophils in asthmatic subjects and normal control subjects. *J Allergy Clin Immunol* 2000; 106: 1115–1123.
- 27 Minshall EM, Leung DY, Martin RJ, et al. Eosinophil-associated TGF- β_1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997; 17: 326–333.
- 28 Suzuma K, Naruse K, Suzuma I, et al. Vascular endothelial growth factor induces expression of connective growth factor *via* KDR, Flt1, and phosphatidylinositol 3-kinase-Akt-dependent pathways in retinal vascular cells. *J Biol Chem* 2000; 275: 40725–40731.
- 29 Kazi AS, Lotfi S, Goncharova EA, et al. Vascular endothelial growth factor-induced secretion of fibronectin is ERK dependent. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L539–L545.
- 30 Chetta A, Zanini A, Foresi A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodeling in asthma. *Clin Exp Allergy* 2005; 35: 1437–1442.
- 31 Whitman M, Downes CP, Keeler M, Keller T, Cantley L. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* 1998; 332: 644–646.
- 32 Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002; 296: 1655–1657.
- 33 Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM. The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. *FASEB J* 2006; 20: 1185–1187.
- 34 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; 3: 721–732.
- 35 Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O₂ tension. *Am J Physiol* 1996; 271: C1172–C1180.
- 36 Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1 α and insulin-like growth factor 2. *Cancer Res* 1999; 59: 3915–3918.
- 37 Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem* 2002; 277: 27975–27981.
- 38 Chan DA, Sutphin PD, Denko NC, Giaccia AJ. Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1 α . *J Biol Chem* 2003; 277: 40112–40117.