



Administration of Pigment Epithelium-Derived Factor Inhibits Airway Inflammation and Remodeling in Chronic OVA-Induced Mice via VEGF Suppression

Wangjian Zha,¹ Mei Su,¹ Mao Huang,¹ Jiankang Cai,² Qiang Du^{2*}

¹Department of Respiratory Medicine, The First Affiliated Hospital, Nanjing Medical University, Nanjing, China

²Department of Respiratory Medicine, The Second Affiliated Hospital, Nanjing Medical University, Nanjing, China

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Purpose: Pigment epithelium-derived factor (PEDF) is a recently discovered antiangiogenesis protein. PEDF possesses powerful anti-inflammatory, antioxidative, antiangiogenic, and antifibrosis properties. It has been reported that PEDF can regulate vascular endothelial growth factor (VEGF) expression. This study aimed to evaluate whether recombinant PEDF protein could attenuate allergic airway inflammation and airway remodeling via the negative regulation of VEGF using a murine model of chronic ovalbumin (OVA)-induced asthma and BEAS-2B human bronchial epithelial cells.

Methods: In an *in vivo* experiment, mice sensitized with OVA were chronically airway challenged with aerosolized 1% OVA solution for 8 weeks. Treated mice were given injections of recombinant PEDF protein (50 or 100 µg/kg body weight) via the tail vein. In an *in vitro* experiment, we investigated the effects of recombinant PEDF protein on VEGF release levels in BEAS-2B cells stimulated with IL-1β. **Results:** Recombinant PEDF protein significantly inhibited eosinophilic airway inflammation, airway hyperresponsiveness, and airway remodeling, including goblet cell hyperplasia, subepithelial collagen deposition, and airway smooth muscle hypertrophy. In addition, recombinant PEDF protein suppressed the enhanced expression of VEGF protein in lung tissue and bronchoalveolar lavage fluid (BALF) in OVA-challenged chronically allergic mice. In the *in vitro* experiment, VEGF expression was increased after IL-1β stimulation. Pretreatment with 50 and 100 ng/mL of recombinant PEDF protein significantly attenuated the increase in VEGF release levels in a concentration-dependent manner in BEAS-2B cells stimulated by IL-1β. **Conclusions:** These results suggest that recombinant PEDF protein may abolish the development of characteristic features of chronic allergic asthma via VEGF suppression, providing a potential treatment option for chronic airway inflammation diseases such as asthma.

Key Words: Asthma; airway inflammation; airway remodeling; PEDF; VEGF

INTRODUCTION

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein with multiple functions, including anti-inflammation, antiangiogenic and antifibrosis properties.¹⁻³ PEDF has been found to inhibit NF-κB activation and the expression of several proinflammatory genes such as intercellular cell adhesion molecule-1 (ICAM-1), tumor necrosis factor alpha (TNF-α) and matrix metalloproteinases.⁴⁻⁶ PEDF has also been shown to participate in the pathogenesis of allergic rhinitis.⁷ These functions suggest that PEDF may be a potential target in the prevention and treatment of allergic asthma.

Bronchial asthma is a chronic airway inflammatory disease associated with airway hyperresponsiveness (AHR) and airway remodeling. Airway remodeling refers to structural changes, including mucous gland hyperplasia, smooth muscle prolifera-

tion, subepithelial fibrosis, and vascular changes.^{8,9} Vascular endothelial growth factor (VEGF) is believed to be involved in the pathogenesis of allergic asthma, particularly airway remodeling.¹⁰ VEGF expression is regulated by several stimuli, including TNF-α, interleukin (IL)-1β, and lipopolysaccharides (LPS).¹¹ Recent studies have shown that PEDF can negatively regulate VEGF expression.^{12,13} We hypothesize that PEDF treatment may down-regulate VEGF expression and may lead to the inhibition

Correspondence to: Qiang Du, PhD, Department of Respiratory Medicine, The Second Affiliated Hospital, Nanjing Medical University, 121 Jiangjiayuan Road, Nanjing 210011, P.R. China.

Tel: +86-189-5187-2628; Fax: +86-25-58509994; E-mail: jingshuyue@163.com
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of airway inflammation and airway remodeling.

The purpose of this study was to define the effects of PEDF on allergic airway inflammation and airway remodeling in BALB/c mice and in BEAS-2B human bronchial epithelial cells with *in vivo* and *in vitro* experiments, respectively. Our results clearly show that PEDF inhibits allergic airway inflammation and airway remodeling, at least in part, by suppressing VEGF expression.

MATERIALS AND METHODS

Animals

Six- to eight-week-old female BALB/c mice (each weighing approximately 20 g) were purchased from Shanghai Laboratory Animal Inc. (Shanghai, China). All experimental animals were utilized under protocols approved by the Institutional Animal Care and Use Committee of Nanjing Medical University and the institutional animal ethics committee (Nanjing, China).

Antigen sensitization, challenge, and treatment

Thirty two mice were randomly divided into the control, OVA, PEDF low and PEDF high groups. On Days 0 and 14, the mice in the OVA, PEDF low, and PEDF high groups were immunized by intraperitoneal injection of 100 μ g of chicken egg ovalbumin (OVA, Grade V; Sigma, St Louis, MO, USA) emulsified in 100 μ L of aluminum hydroxide gel (InvivoGen, San Diego, CA, USA). On day 21, the mice were placed in a Plexiglas box (29 \times 22 \times 18 cm) and were airway challenged with 1% aerosolized OVA for 30 minutes per day, 3 days per week, for a period of 8 weeks. The mice in the PEDF low and high groups were given injections via the tail vein with 50 or 100 μ g/kg body weight of recombinant PEDF protein¹³ (Peprotech, Rocky Hill, NJ, USA) before each OVA challenge. The mice in the control group received sensitization and airway challenge with phosphate-buffered saline (PBS) instead of OVA.

Airway hyperreactivity measurement

Airway responsiveness to acetylcholine chloride (ACh) was measured 24 hours after the last OVA challenge with an AniRes 2005 animal lung function analysis system (SYNOL High-Tech, Beijing, China) as previously described.¹⁶ Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (70 mg/kg). The trachea was then surgically exposed, and a plastic tube with an internal diameter of 4 mm was inserted into the trachea connected to a computer-controlled ventilator. A 27-gauge needle was inserted into the tail vein for ACh administration. The respiratory rate and the tidal volume were preset at 90 breaths/min and 6 mL/kg, respectively. Progressively increasing doses of ACh (10, 30, 90, and 270 μ g/kg) were administered intravenously with a microinfusion pump (36 mL/min) via the caudalis vein. Data were obtained, and the maximum values of lung resistance (RL) were used to express changes in airway hyperreactivity.⁹⁻¹¹

Analysis of bronchoalveolar lavage fluid and serum

After measurement of airway hyperreactivity, the retro-orbital puncture method was used to collect blood samples. Serum samples were collected after centrifuging at 1,000 g at 4°C for 15 minutes, and plasma was stored at -70°C until analysis. Airway lumina were washed 3 times with 0.5 mL volumes of saline. Bronchoalveolar lavage fluid (BALF) was centrifuged at 1,000 g at 4°C for 15 minutes, and upper fluid samples were collected for detection by enzyme-linked immunosorbent assay (ELISA). The cell pellets were suspended for total inflammatory cell counts with a hemocytometer. The smears of BALF cells were stained with Wright's stain for differential cell counts. The cells in the BALF were counted by 2 independent investigators in a single-blind study analyzing at least 200 cells each from 4 different random locations using a microscope. Commercially available ELISA kits were used to evaluate the levels of the Th2 cytokines IL-4 (Jingmei Biotech, Shanghai, China), IL-5, IL-13, transforming growth factor-beta1 (TGF- β 1), and VEGF (R&D Systems, Minneapolis, MN, USA) in BALF and OVA-specific serum IgE (Shibayagi, Gunma, Japan).

Lung histology and immunohistochemistry

The left lung was fixed in 4% paraformaldehyde overnight at 4°C and then embedded in paraffin. Paraffin sections (5 μ m) were stained with hematoxylin and eosin (H&E) to detect eosinophilic infiltration, periodic acid-Schiff (PAS) to assess mucus-secreting goblet cells, and Masson's trichrome stain to evaluate the peribronchiolar collagen layer or processed for α -smooth muscle actin (SMA) immunohistochemistry to estimate airway smooth muscle hypertrophy. Histological analyses were performed by pathologists blinded to the treatment groups. Scoring for mucus production was quantified as previously described¹⁵: 0, no goblet cells; 1, <25% of the epithelium; 2, 25%-50% of the epithelium; 3, 50%-75% of the epithelium; and 4, >75% of the epithelium. The area of peribronchial trichrome staining in the mouse lung is expressed as the area of trichrome staining per μ m length of the basement membrane (BM) of bronchioles. At least 10 bronchioles with internal diameters of 150-200 μ m in each slide were counted.

Lung sections were treated in xylene and rehydrated in graduated alcohol solutions. Endogenous peroxidase activity was blocked with 3% H₂O₂. Specimens were flooded with 5% normal goat serum to prevent the nonspecific absorption of immunoglobulin; then, specimens were incubated with anti- α -SMA monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1:50 for 90 minutes. A substitution of each primary antibody with PBS was used as a negative control. The slides were incubated overnight at 4°C, rinsed 3 times with PBS, and then incubated with peroxidase-labeled secondary antibody (DAKO, Glostrup, Denmark) for 30 minutes at room temperature. The sections were washed again with PBS, followed by diaminobenzidine staining. The sections were then counterstained with

hematoxylin, dehydrated, and observed. Data are expressed as the area of α -SMA immunostaining per μm length of the BM of bronchioles with internal diameters of 150-200 μm .

Measurement of hydroxyproline content

A hydroxyproline assay was used to determine the total collagen content of the right lungs using a commercially available hydroxyproline detection kit (Nanjing Jiancheng Biotechnology, Nanjing, China). Briefly, frozen right lung tissues were homogenized in saline containing 0.1 mol/L phenylmethylsulfonyl fluoride. Then, the homogenized lung tissues were hydrolyzed in 6 mol/L HCl. The hydroxyproline concentration was measured as previously described.¹⁷

Cell culture and treatment

BEAS-2B human bronchial epithelial cells were cultured in 6-well round-bottom plates supplemented with 4 mM L-glutamine, 20 $\mu\text{g}/\text{mL}$ streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum (all from HyClone, Logan, UT, USA). After starvation, the cells were cultured with 500 ng/mL IL-1 β (R&D Systems, Minneapolis, MN, USA) in the presence or absence of different doses of recombinant PEDF protein (50,100 ng/mL, Peprotech, USA).^{18,19} The supernatants were collected after 48 hours, and VEGF levels were assayed using ELISA kits.

Western blot analysis of VEGF in the lung tissue

The right lung tissues from each group were washed with ice-cold PBS and homogenized, and the lysates were prepared in RIPA lysis buffer and centrifuged at 12,000 g at 4°C for 15 minutes. Equal amounts (30 μg) of proteins were loaded onto 8%

SDS-PAGE gels. Subsequently, the proteins were electrotransferred (100 V for 2 hours). The membrane was blocked for 1 hour with Tris-buffered saline containing 0.05% Tween 20 (TBST) plus 5% skim milk and then incubated with 1:1,000 anti-VEGF (Abcam, Cambridge, UK) or 1:1,000 anti-GAPDH (Cell Signaling Technology Inc., Beverly, MA, USA) overnight at 4°C. The membrane was washed 3 times with TBST and then incubated with HRP-conjugated goat antirabbit IgG for 1 hour at room temperature. The membrane was washed 3 times with TBST again and detected with an ECL detection system (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

All data were assessed using a 1-way ANOVA followed by Dunnett's post hoc test with SPSS 17 software (Chicago, IL, USA). Differences were considered statistically significant if $P < 0.05$. Data are expressed as means \pm SEM.

RESULTS

PEDF decreases AHR in chronic experimental asthma

To determine the effects of PEDF on airway reactivity, lung resistance (LR) was evaluated in anesthetized mice by invasive whole-body plethysmography. There were no significant differences in baseline airway resistance between the 4 groups. The LR generated by the administration of Ach at doses from 30 to 270 $\mu\text{g}/\text{kg}$ was dramatically increased in the OVA group compared to the control group. RL was significantly decreased in the recombinant PEDF treated group (50 or 100 $\mu\text{g}/\text{kg}$ body weight) compared to the OVA group, implying that *in vivo* in-

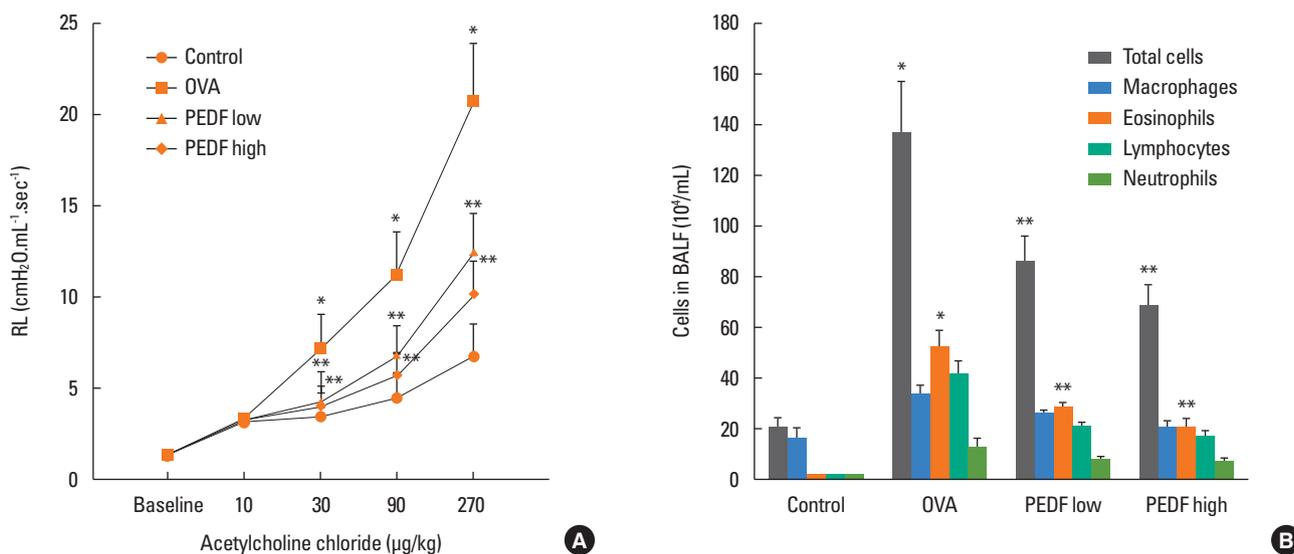


Fig. 1. PEDF suppresses (A) airway hyperreactivity (AHR) and (B) inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF) in a murine model of asthma. (A) PEDF decreased AHR compared to ovalbumin (OVA)-sensitized and challenged mice. (B) PEDF decreased the accumulation of total inflammatory cells, as well as eosinophils, compared to the OVA group. Data are presented as means \pm SEM ($n=8$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the OVA group.

flammation-mediated airway pathology was alleviated. Recombinant PEDF reduced AHR in a dose-dependent manner (Fig. 1A).

PEDF attenuates chronic airway inflammation

To determine the effects of PEDF on airway inflammation, the amount and classification of inflammatory cells in the BALF were evaluated. Few inflammatory cells were detected in saline-sensitized and saline-challenged control mice. The mice in the OVA group displayed a marked increase in the number of total inflammatory cells and eosinophils in the BALF. Treatment with PEDF significantly decreased the numbers of eosinophils and total inflammatory cells in BALF in a dose-dependent manner ($P < 0.05$, Fig. 1B). In addition, HE staining showed a marked reduction in the number of total inflammatory cells around the airway and the blood vessels in PEDF-treated mice compared to the OVA group (Fig. 2).

PEDF reduces Th2 cytokines and TGF- β 1 levels in BALF

To determine the effects of PEDF on Th2 cytokines and TGF- β 1, we measured the levels of T-helper type 2 (Th2) cytokines, such as IL-4, IL-5, IL-13, and TGF- β 1 in BALF. IL-4, IL-5, IL-13, and TGF- β 1 in BALF were dramatically increased in the OVA group compared to the control group ($P < 0.05$, Fig. 3). Administration of PEDF significantly decreased Th2 cytokine and TGF- β 1 levels in BALF in a dose-dependent manner compared to the OVA group ($P < 0.05$, Fig. 3).

PEDF decreases OVA-specific IgE in serum

To evaluate the effect of PEDF on IgE in serum, we also measured the level of OVA-specific IgE in serum. OVA-specific IgE in serum was obviously enhanced in the OVA group compared to the control group ($P < 0.05$, Fig. 3), treatment with PEDF significantly inhibited the level of OVA-specific IgE in a dose-dependent manner ($P < 0.05$, Fig. 3).

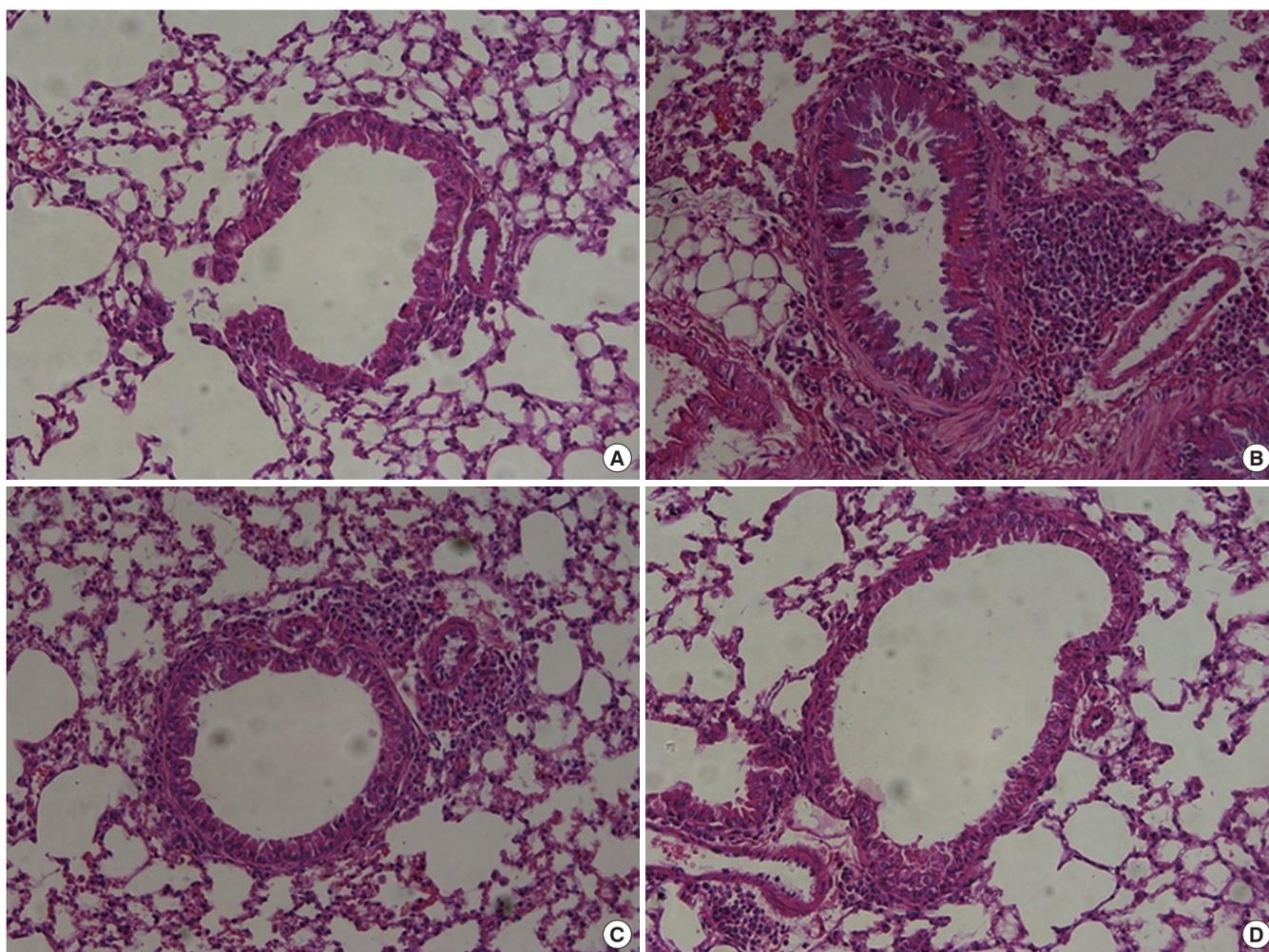


Fig. 2. PEDF attenuates airway inflammation in a murine model of asthma. Hematoxylin and eosin staining (original magnification $\times 100$): (A) control group, (B) OVA group, (C) PEDF low group, and (D) PEDF high group.

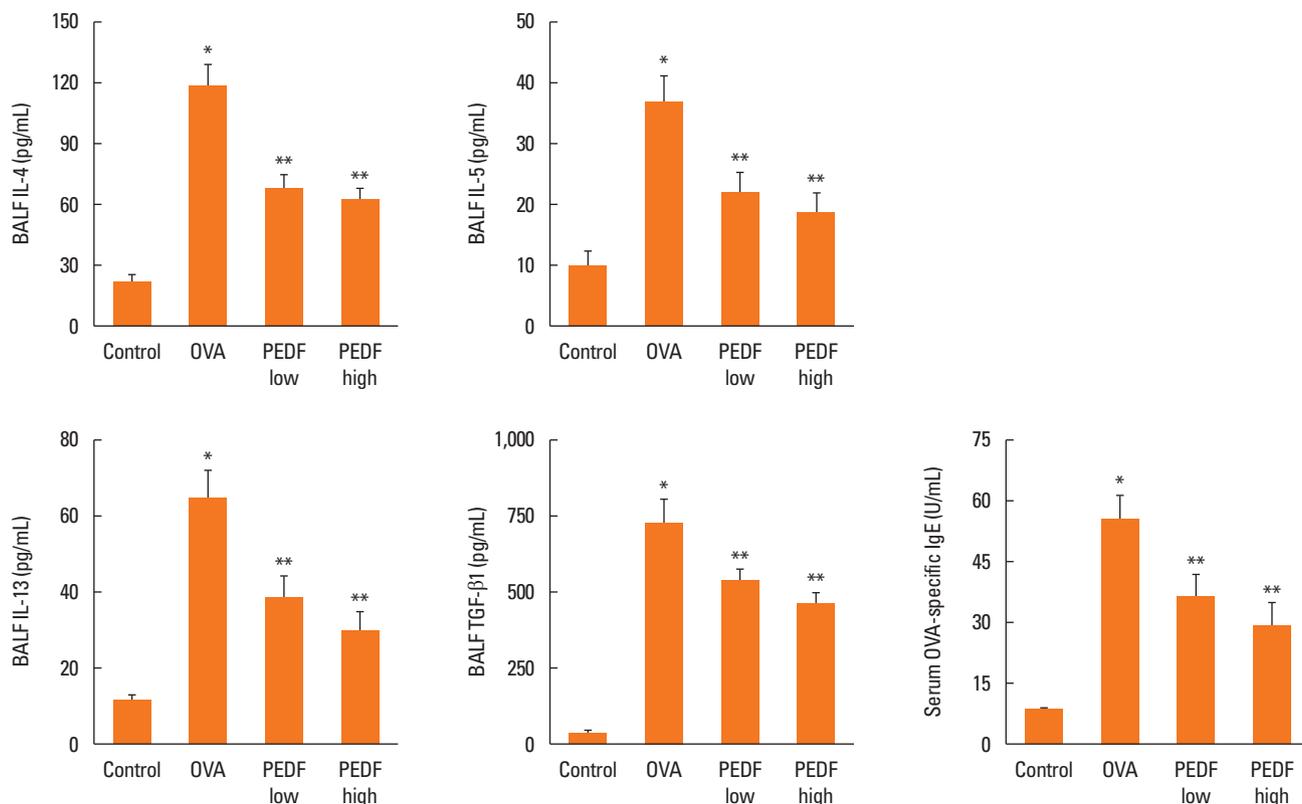


Fig. 3. PEDF inhibits IL-4, IL-5, IL-13, and TGF-β1 in BALF and OVA-specific IgE in serum in a murine model of asthma. Data are presented as means ± SEM (n=8 per group). *P<0.05 compared to the control group; **P<0.05 compared to the OVA group.

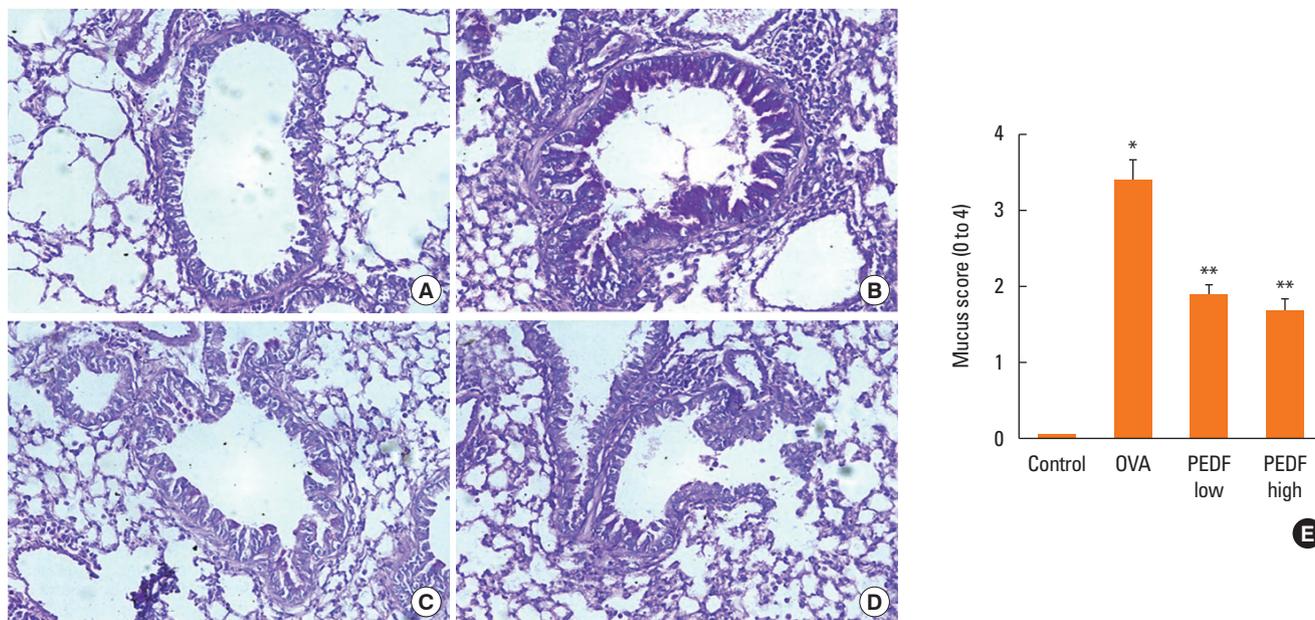


Fig. 4. PEDF attenuates airway goblet cell hyperplasia and mucus production in a murine model of asthma. Periodic acid-Schiff (PAS) staining (original magnification ×200): (A) control group, (B) OVA group, (C) PEDF low group, and (D) PEDF high group. (E) Quantitative analyses of mucus production in lung sections were performed as described in the Methods. Data are presented as means ± SEM (n=8 per group). *P<0.05 compared to the control group; **P<0.05 compared to the OVA group.

PEDF inhibits airway remodeling in a chronic asthma model

To determine the effects of PEDF on airway remodeling

changes, we determined the effects of PEDF on goblet cell metaplasia, smooth muscle proliferation, and subepithelial fi-

brosis. PAS staining was used to identify mucus-containing cells in the airway epithelium (Fig. 4A-D). Significant goblet cell

hyperplasia and mucus hypersecretion were found in the OVA mice compared to the control group ($P < 0.05$, Fig. 4E). This in-

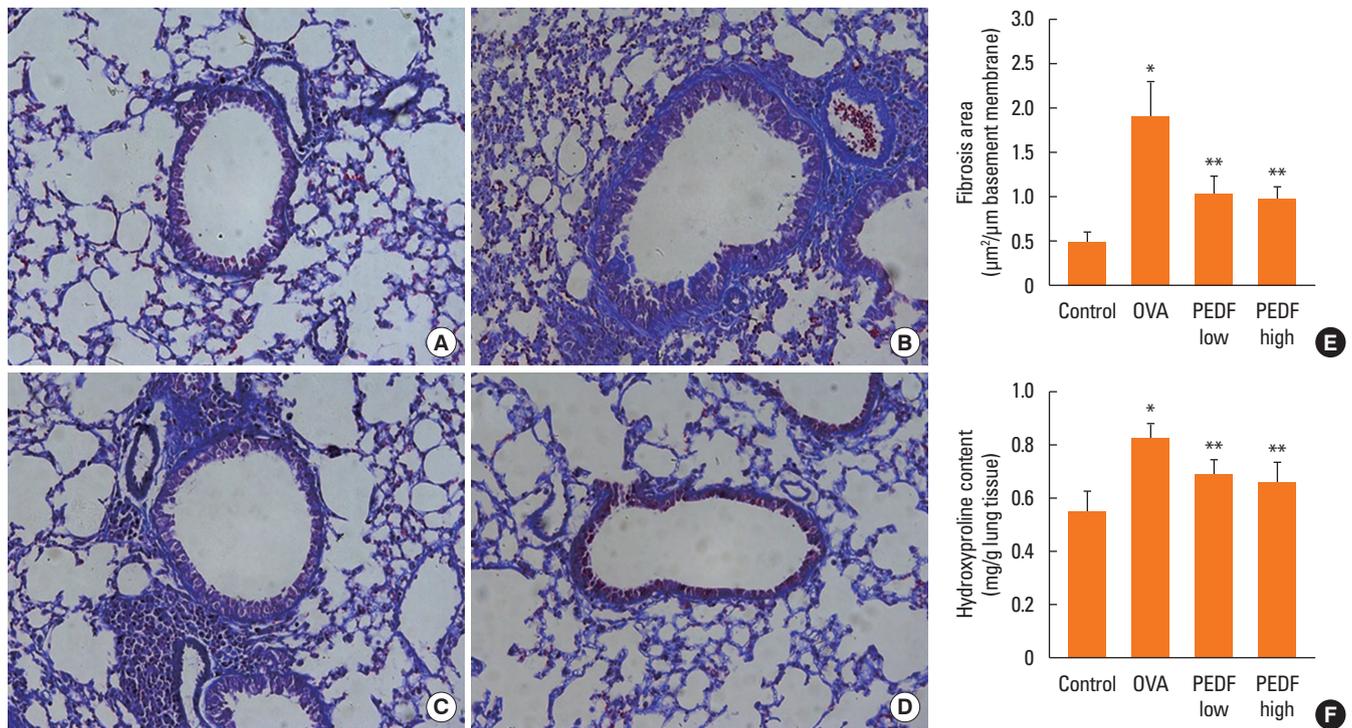


Fig. 5. PEDF inhibits peribronchiolar fibrosis in chronic experimental asthma. Masson's trichrome-positive peribronchiolar collagen layer (original magnification $\times 200$) of lung tissue from (A) control group, (B) OVA group, (C) PEDFlow group, and (D) PEDFhigh group. (E) Quantitative analyses of the mean area of airway fibrosis and (F) total lung hydroxyproline content were determined as described in the Methods. Data are presented as means \pm SEM ($n = 8$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the OVA group.

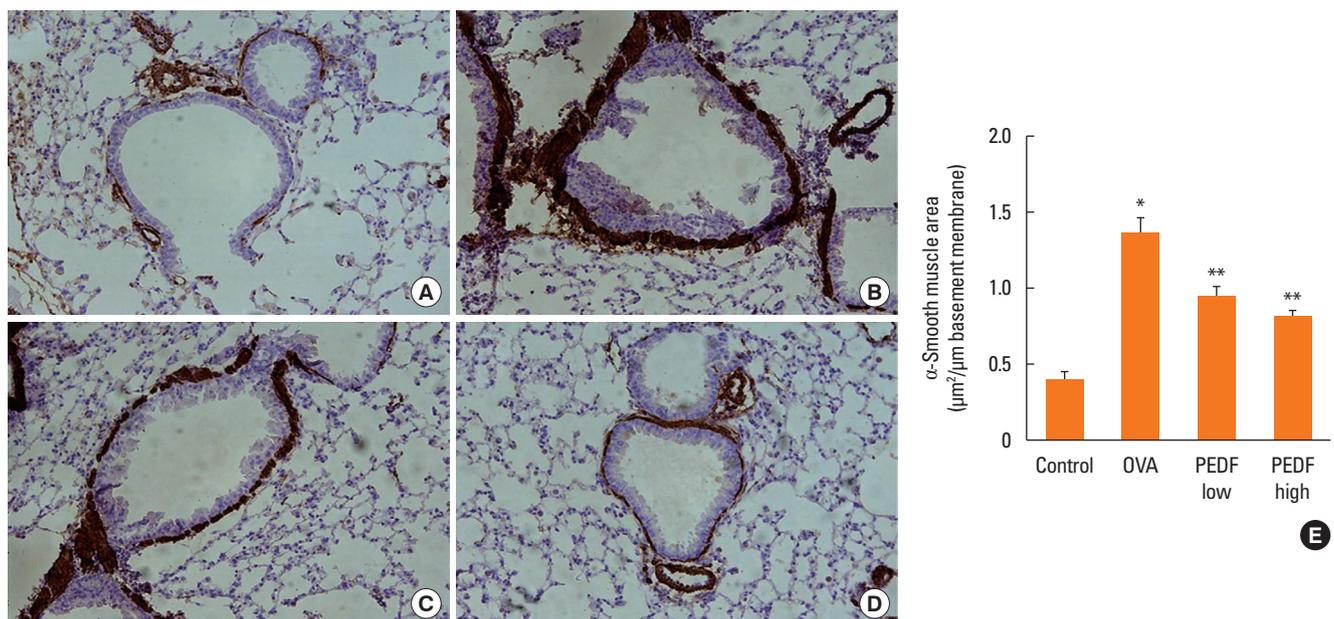


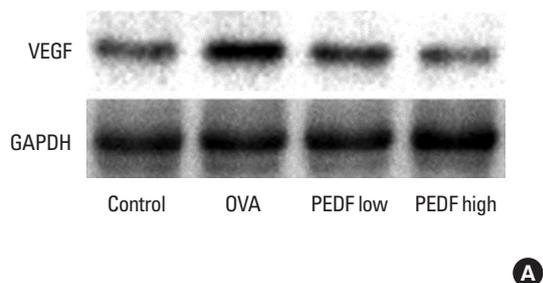
Fig. 6. PEDF inhibits the areas of α -smooth muscle actin (SMA) in a murine model of asthma. Peribronchial α -SMA immunostaining (immunohistochemistry, original magnification $\times 200$) of lung tissue from (A) control group, (B) OVA group, (C) PEDF low group, and (D) PEDF high group. (E) Quantitative analyses of immunostaining area for peribronchial α -SMA. Data are presented as means \pm SEM ($n = 8$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the OVA group.

crease was evidently inhibited in mice treated with PEDF. Furthermore, PEDF reduced the number of goblet cells in the lumen of the bronchioles in a dose-dependent manner.

Masson's trichrome staining revealed dense collagen deposition/sub-epithelial fibrosis throughout the interstitium surrounding the airways and vessels of lung tissues in the OVA group compared to the control group (Fig. 5A-D). PEDF treatment obviously reduced collagen deposition compared to the OVA group ($P < 0.05$, Fig. 5E).

Total lung hydroxyproline content was significantly increased in the OVA group compared to the control mice ($P < 0.05$, Fig. 5F). In contrast, treatment with PEDF resulted in a significant reduction in total lung hydroxyproline content compared to the OVA group.

Additionally, α -SMA immunostaining was used to evaluate the thickness of airway smooth muscle. A marked increase in the α -SMA-staining areas in the peribronchiolar region was found in the OVA mice compared with the control mice (Fig. 6A-D). Administration of PEDF significantly reduced the area of the α -SMA-stained smooth muscle layer in a dose-dependent manner compared to the OVA group ($P < 0.05$, Fig. 6E).



PEDF ameliorates the expression of VEGF protein in lung tissue and BALF in chronic experimental asthma

To determine whether VEGF is inhibited by PEDF in this model, we evaluated VEGF protein expression in mouse pulmonary tissue and VEGF levels in BALF. Compared to the control group, the OVA group had higher VEGF protein levels in the pulmonary tissue, and PEDF treatment prevented this increase ($P < 0.05$, Fig. 7). ELISA also showed a marked increase in BALF VEGF levels in OVA-challenged mice. The mice treated with PEDF demonstrated lower levels of VEGF in BALF compared to the OVA group (Fig. 8). We also observed that PEDF administration reduced VEGF in a dose-dependent manner in the BALF and lung tissue of OVA-challenged mice.

PEDF suppresses IL-1 β -induced VEGF release in BEAS-2B cells

VEGF released from airway epithelial cells can aggravate airway inflammation and airway remodeling. To further ascertain the anti-inflammatory and antiremodeling mechanisms of PEDF, we studied the effects of PEDF on IL-1 β -induced VEGF expression in BEAS-2B cells. The levels of VEGF in culture supernatants from BEAS-2B cells induced by IL-1 β were signifi-

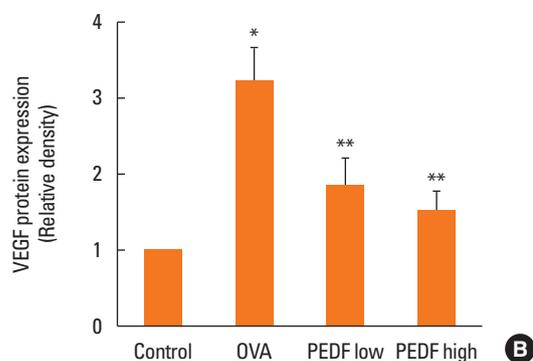


Fig. 7. PEDF ameliorates the expression of VEGF protein in lung tissue in chronic experimental asthma. (A) Total protein from lung tissue was extracted 24 hours after the final OVA challenge and subjected to Western blot analysis of VEGF. GAPDH was utilized as the standard control. (B) The band signal strength of VEGF expressed as a ratio to GAPDH. Data are presented as means \pm SEM ($n = 8$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the OVA group.

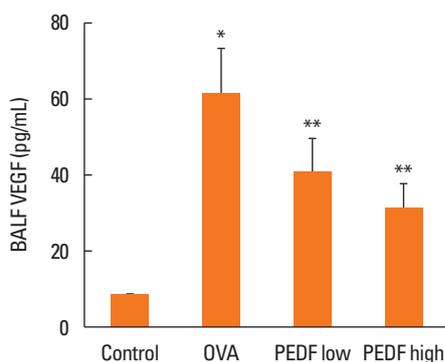


Fig. 8. PEDF reduces BALF VEGF levels in chronic experimental asthma. Data are presented as means \pm SEM ($n = 8$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the OVA group.

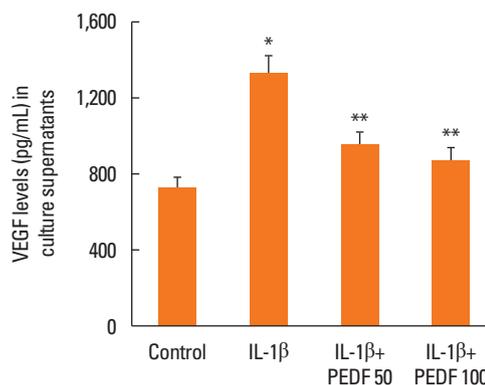


Fig. 9. Recombinant PEDF suppresses IL-1 β -induced VEGF release in BEAS-2B cells. Data are presented as means \pm SEM ($n = 3$ per group). * $P < 0.05$ compared to the control group; ** $P < 0.05$ compared to the IL-1 β group.

cantly increased compared to unstimulated cells. PEDF evidently inhibited the release of VEGF from BEAS-2B cells stimulated with IL-1 β in a dose-dependent manner ($P < 0.05$, Fig. 9).

DISCUSSION

Airway remodeling is an important pathophysiological characteristic of chronic asthma and correlates with the duration and severity of asthma.⁸ In this study, we evaluated the effects of recombinant PEDF on airway remodeling in chronic allergic asthma. Our results showed that recombinant PEDF had significant inhibitory effects on eosinophilic airway inflammation, AHR, and airway remodeling in this asthma model. In addition, treatment with recombinant PEDF resulted in marked inhibitory effects on the high expression of VEGF in lung tissues of OVA-challenged mice. Furthermore, recombinant PEDF administration suppressed VEGF release from BEAS-2B cells stimulated with IL-1 β .

Th2-mediated airway inflammation present within the lung is fundamental in asthma. The Th2 cytokines IL-4, IL-5, and IL-13 are considered the key molecular mechanisms of asthma and have been shown to be associated with airway inflammation.²⁰⁻²⁴ In the present study, the production of Th2 cytokines was significantly increased in OVA-challenged mice. Treatment with PEDF inhibited the levels of Th2 cytokines, such as IL-4, IL-5, and IL-13 in BALF, compared to OVA-challenged mice. In addition, recombinant PEDF prevented an increase in total inflammatory cells, especially eosinophils. Lung histological analysis also demonstrated that recombinant PEDF suppressed inflammatory cell infiltration, mucus hypersecretion, and goblet cell metaplasia in the airway. The results indicated that recombinant PEDF exerts significant anti-inflammation effects in chronic OVA-induced allergic mice.

Collagen deposition/subepithelial fibrosis in the airways, an important aspect of airway remodeling, is related to clinically severe asthma and a decline in pulmonary function.²⁵ Previous studies showed that PEDF possesses antifibrosis properties exerted via inhibition of the TGF- β 1 expression.^{26,27} TGF- β 1, a profibrotic cytokine, leads to subepithelial collagen and extracellular matrix protein deposition in asthma.²⁸ Thus, a marked increase in collagen deposition was observed around the airway in chronic OVA-induced mice. We show for the first time that the administration of recombinant PEDF not only significantly inhibits the TGF- β 1 level in BALF in ova-induced mice, but also prevents subepithelial collagen deposition, as determined by Masson's trichrome staining and hydroxyproline analyses in chronic allergic asthma.

Airway smooth muscle hyperplasia is another key characteristic of airway remodeling and appears to be attributable to exaggerated AHR in asthma.²⁹ In this study, the α -SMA stained area was significantly increased in chronic OVA-exposed mice. The chronic OVA-induced increase in airway smooth muscle was

significantly inhibited by recombinant PEDF administration. The ability of recombinant PEDF to suppress airway smooth muscle thickness may be an important reason for its suppression of AHR.

VEGF is a potent stimulator of angiogenesis and structural changes in asthma.³⁰ VEGF can aggravate Th2-type airway inflammation via IL-13-dependent and -independent mechanisms.³¹ Epithelial cell-derived VEGF promotes airway remodeling in asthma.³² VEGF levels are increased in sputum and lung tissues from asthmatic patients and correlate with the disease severity of asthma.³³⁻³⁵ The inhibition of VEGF can lead to a significant reduction in basement membrane thickness and goblet cell hyperplasia.³⁶ Previous studies showed that PEDF counteracts the biological actions of VEGF via down-regulating VEGF expression or binding VEGF receptors.^{37,38} The results of our study showed that expression of the VEGF protein was enhanced after chronic OVA challenge, and treatment with recombinant PEDF decreased VEGF protein expression in the lung tissues and VEGF levels in the BALF of chronic OVA-induced allergic mice. In addition, recombinant PEDF significantly suppressed VEGF release from BEAS-2B cells stimulated by IL-1 β . Therefore, we hypothesized that recombinant PEDF may inhibit airway inflammation and airway remodeling by reducing the expression of VEGF in a chronic asthma model.

In conclusion, our data demonstrated that PEDF exerts significant anti-inflammatory and antiremodeling effects in a chronic allergic asthma model. In addition, recombinant PEDF obviously inhibited VEGF expression *in vivo* and *in vitro*. Our findings indicate that PEDF might be a therapeutic target for chronic allergic asthma.

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