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Recombinant IGFBP-3 inhibits allergic lung inflammation, VEGF production, and vascular leak in a mouse model of asthma

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Keywords

allergic airway disease; HIF- α ; IGFBP-3; vascular endothelial growth factor; vascular permeability.

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Abstract

Background: Vascular endothelial growth factor (VEGF) plays a pro-inflammatory mediator as well as a vascular permeability factor in bronchial asthma. Insulin-like growth factor (IGF)-I is also involved in the inflammatory process associated with bronchial asthma and stimulates VEGF expression. The IGF-binding proteins (IGFBPs), especially IGFBP-3, display distinctive properties and can interfere with various biological processes.

Methods: In this study, an ovalbumin (OVA)-induced murine model of allergic airway disease was used to investigate which mechanism is implicated in the preventive and therapeutic actions of IGFBP-3 administered exogenously on allergen-induced bronchial inflammation and airway hyper-responsiveness, in particular focusing on the regulation of VEGF expression.

Results: Administration of recombinant human IGFBP-3 to OVA-inhaled mice substantially attenuated the increases in hypoxia-inducible factor (HIF)- α activity, IGF-I production, and VEGF protein levels in the lung. In addition, the blockade of IGF-I action decreased the OVA-induced VEGF expression, airway inflammation, and bronchial hyper-responsiveness. The administration of recombinant human IGFBP-3 or CBO-P11 also reduced significantly increases in inflammatory cells, airway hyper-responsiveness, levels of IL-4, IL-5, IL-13, and vascular permeability in the lung of OVA-inhaled mice. Moreover, when recombinant human IGFBP-3 was administered after the completion of OVA inhalation, these therapeutic effects of IGFBP-3 were also observed.

Conclusions: These results indicate that IGFBP-3 administered exogenously may attenuate antigen-induced airway inflammation and hyper-responsiveness through the modulation of vascular leakage and VEGF expression mediated by HIF-1 α /HIF-2 α signaling as well as IGF-I action in allergic airway disease of mice.

Inflammation of the asthmatic airway is usually accompanied by increased vascular permeability and plasma exudation (1). Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability-enhancing peptide linked to edema and profound alterations in the extracellular matrix (2). Furthermore, we have recently demonstrated that VEGF is implicated in the pathogenesis of asthma, and thus, the inhibition of VEGF receptor and/or its signaling pathway may be a good therapeutic strategy (3).

Hypoxia-inducible factors (HIFs) are known to stimulate the expression of genes that promote angiogenesis, vasodila-

tion, vascular permeability, and glucose uptake (4). To date, three HIF isoforms have been identified with an obvious tissue-restricted expression pattern. Among them, HIF-1 is the most prevalent isoform (5). Binding of HIF-1 to the hypoxia response element of the VEGF promoter results in the enhancement of VEGF expression (5). HIF-2 α is also referred to as endothelial PAS domain protein-1 or HIF-1 α -like factor, and bears functional resemblance to HIF-1 α regarding hypoxic stabilization and binding to HIF-1 β , although it has also different roles in tumorigenesis (6). In fact, HIF-2 α can directly activate the expression of genes

encoding a number of pro-angiogenic factors including VEGF, erythropoietin, angiopoietin, and Tie-2 receptors (7). Furthermore, our recent study has demonstrated that HIF-1 α /HIF-2 α inhibition dramatically reduces the VEGF expression in allergic airway diseases of mice (8).

The insulin-like growth factor (IGF) axis is a multicomponent network of molecules, which is ubiquitously involved in the regulation of growth, proliferation, and differentiation of a variety of cell types (9). The IGF system is composed of growth hormone, IGF-I/II peptides, type I and II IGF receptors (IGF-IR), IGF-binding proteins (IGFBPs), and IGFBP proteases (10). Because IGFs bind with higher affinity to IGFBPs than to the IGF-IR, IGFBPs are capable of acting as antagonists by reducing IGF bioavailability through sequestration (11). It is well established that IGFBPs possess intrinsic biological activity (i.e., IGF-/IGF-IR-independent actions) in addition to their actions to bind IGFs and sequester the active hormone, thereby reducing IGF biological activity (i.e., IGF-/IGF-IR-dependent actions) (12). A total of six high-affinity binding proteins (IGFBP-1 through IGFBP-6) have been identified (10). Among them, IGFBP-3 is a major species in circulation, binds 75–90% of circulating IGF-I in a large ternary complex consisting of IGFBP-3, acid-labile subunit, and IGF (10). Although the role of IGF-I and the IGFBP-3 in allergic airway disease remains elusive, IGF-I is up-regulated in the airway of mice after allergen challenge with a concomitant increase in IGFBP-3 mRNA (13), and IGF-I levels in patients with asthma correlate with subepithelial fibrosis (14). Very recently, we have described that IGFBP-3 protein levels are significantly suppressed in the lung isolated from ovalbumin (OVA)-inhaled asthmatic mice and that IGFBP-3 inhibits airway inflammation and hyper-responsiveness via an IGF-independent mechanism that involves the activation of IGFBP-3R signaling and cross-talk with nuclear factor- κ B (NF- κ B) signaling (15).

While a growing body of evidence has demonstrated that IGF-I can stimulate HIF-1 activity and the secretion of VEGF *in vivo* and *in vitro* (16, 17), information on the relationship between IGF-I/IGFBP-3 and VEGF in the pathogenesis of bronchial asthma is limited. In this study, we used OVA-inhaled mice to investigate the effects of recombinant human IGFBP-3 on asthmatic features including vascular leakage and VEGF expression and the molecular basis of IGFBP-3 signaling. Our results showed that IGFBP-3 administered exogenously exerts a therapeutic role against allergic airway inflammation through blockade of VEGF expression mediated by HIF- α activation and IGF-I action.

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. (Seongnam, Korea). Mice were sensitized and challenged with OVA (Sigma-Aldrich, St Louis, MO, USA) as previously described with some modifications (18, 19). Bronchoalveolar lavage (BAL) was performed at 48 h after the last

challenge, and smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA, USA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc. Aguada, Puerto Rico) in order to examine the cell differentials.

Administration of recombinant human IGFBP-3, CBO-P11, LY 294002, wortmannin, or anti-IGF-I antibody

Recombinant human IGFBP-3 (10 μ g/kg or 50 μ g/kg body weight per day; Upstate Biotechnology, Lake Placid, NY, USA) was suspended in phosphate-buffered saline (PBS) and administered intratracheally two times to each animal, once at 1 h before the first airway challenge with OVA and the second time at 3 h after the last airway challenge with OVA. Cyclopeptidic VEGF receptor inhibitor, CBO-P11 (2 mg/kg body weight/day; Calbiochem, San Diego, CA, USA), or vehicle control (0.05% DMSO) was administered intraperitoneally four times at 24-h intervals, beginning at 1 h before the first inhalation of OVA (20). LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (1.5 mg/kg body weight/day; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) or wortmannin (100 μ g/kg body weight/day; Calbiochem) dissolved in DMSO and diluted with 0.9% NaCl was administered in a volume of 50 μ l. LY-294002 or wortmannin was administered intratracheally two times to each treated animal, once at 1 h before the first challenge with OVA and the second time at 3 h after the last challenge with OVA. In addition, anti-IGF-I antibody or control polyclonal antibody (2.5 mg/kg body weight/day; R&D Systems, Inc., Minneapolis, MN, USA) was administered to each animal intratracheally at 3 h after the last challenge with OVA.

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis as described previously (19).

Measurement of T-helper type 2 (Th2) cytokines and VEGF

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (IL-4, IL-5, and IL-13; Invitrogen Corp., Carlsbad, CA, USA; VEGF; R&D Systems, Inc.).

Measurement of serum OVA-specific IgE

OVA-specific IgE levels were measured by ELISA according to the manufacturer's protocol, using a mouse OVA-IgE ELISA kit (MD Biosciences, Inc., St Paul, MN, USA).

Nuclear protein extractions for the analysis of HIF-1 α , HIF-2 α , and HIF-1 β

Nuclear extraction was performed as described previously (8).

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was used as described previously (18).

Histology

For histologic examination, 4- μ m 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 hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere (19, 21).

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airway, as described elsewhere (22).

Results

Recombinant human IGFBP-3 or CBO-P11 reduces VEGF protein levels and plasma exudation in the lung of OVA-sensitized and OVA-challenged mice

To examine whether exogenous IGFBP-3 regulates VEGF expression, effect of IGFBP-3 on VEGF expression and vascular permeability was examined. Western blot analysis revealed that levels of VEGF protein in the lung tissues were significantly increased at 48 h after OVA inhalation compared with the levels after saline inhalation (Fig. 1A,B). The increased levels of VEGF protein in lung tissues after OVA inhalation were dramatically reduced by the administration of IGFBP-3 (50 μ g/kg) or CBO-P11. Consistent with these results, enzyme immunoassay also showed that the administration of IGFBP-3 (10 and 50 μ g/kg) or CBO-P11 decreased significantly the increased levels of VEGF in BAL fluids after OVA inhalation (Fig. 1C).

Evans blue dye assay revealed that plasma exudation was significantly increased at 48 h after OVA inhalation compared with the levels after saline inhalation. The increase in plasma exudation in OVA-inhaled mice was significantly reduced by the administration of IGFBP-3 (10 and 50 μ g/kg) or CBO-P11 (Fig. 1D). In addition, the levels of VEGF protein in BAL fluids correlated significantly with the levels of plasma exudation in OVA-sensitized and OVA-challenged mice ($r = 0.799$; $P < 0.05$) (Fig. 1E).

Recombinant human IGFBP-3 inhibits Akt phosphorylation in lung tissues of OVA-sensitized and OVA-challenged mice

The activation of Akt which is known to activate the HIF pathway was determined by Western blotting. Levels of phosphorylated Akt (p-Akt) protein in lung tissues were significantly increased at 48 h after OVA inhalation compared

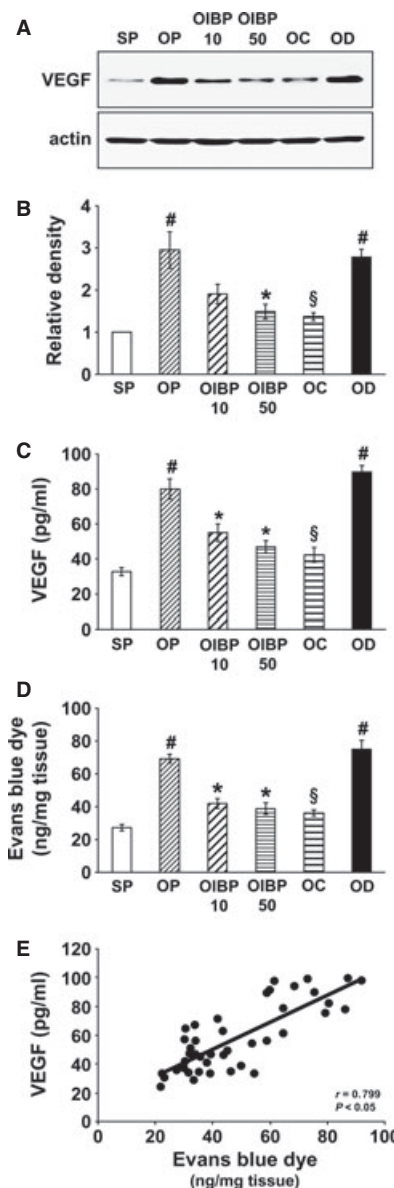


Figure 1 Effect of IGFBP-3 or CBO-P11 on VEGF protein expression in lung tissues and BAL fluids and plasma exudation of OVA-sensitized and OVA-challenged mice. (A) Western blotting of VEGF. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), OVA-inhaled mice administered IGFBP-3 10 μ g/kg (OIBP 10), OVA-inhaled mice administered IGFBP-3 50 μ g/kg (OIBP 50), OVA-inhaled mice administered CBO-P11 (OC), and OVA-inhaled mice administered 0.05% DMSO (OD). (B) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of SP is arbitrarily presented as 1. (C) Enzyme immunoassay of VEGF in BAL fluids. (D) Evans blue dye assay in lung tissues. Data represent mean $\pm$ SEM from seven mice per group. # $P < 0.05$ vs SP; * $P < 0.05$ vs OP; § $P < 0.05$ vs OD. (E) Correlation between VEGF levels in BAL fluids and plasma exudation.

with the levels after saline inhalation (Fig. 2A,B). However, no significant changes in Akt protein levels were observed in any of the groups tested. The increased levels of p-Akt protein in lung tissues after OVA inhalation were significantly reduced by the administration of IGFBP-3 (10 and 50 $\mu\text{g/kg}$).

PI3K inhibitor or recombinant human IGFBP-3 decreases HIF-1 α and HIF-2 α activation in lung tissues of OVA-sensitized and OVA-challenged mice

The HIF- α activation that leads to VEGF expression was determined by Western blot analysis. Levels of HIF-1 α and HIF-2 α in nuclear protein extracts from lung tissues were increased at 48 h after OVA inhalation compared with the

levels after saline inhalation (Fig. 2C–J). The increased HIF-1 α and HIF-2 α levels in nuclear protein extracts were decreased by the administration of IGFBP-3 (10 and 50 $\mu\text{g/kg}$), LY 294002, or wortmannin in OVA-sensitized and OVA-challenged mice.

Recombinant human IGFBP-3 inhibits VEGF expression through the reduction in IGF-I levels in lung tissues of OVA-sensitized and OVA-challenged mice

As expected, the levels of IGF-I, one of VEGF expression inducers, in lung tissues were substantially increased at 48 h after OVA inhalation compared with the levels after saline inhalation, which was revealed by Western blot analysis

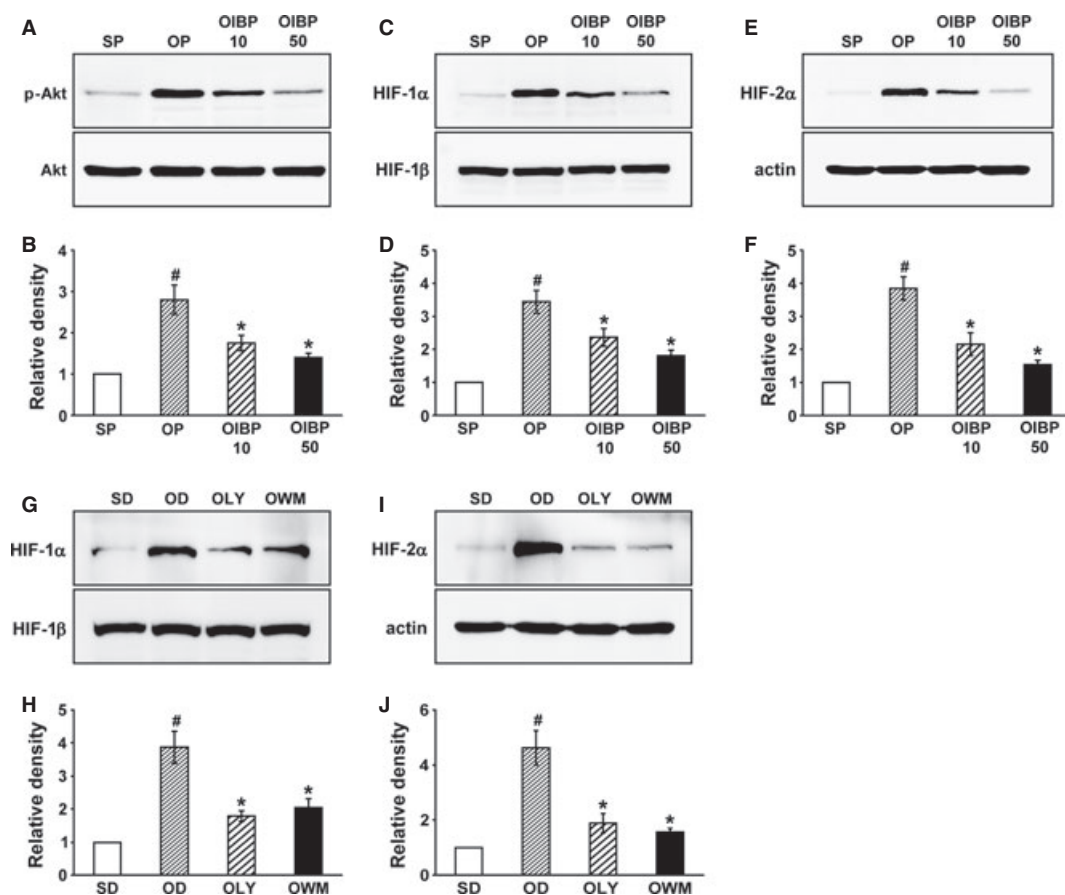


Figure 2 Effect of IGFBP-3 on p-Akt, Akt, HIF-1 α , HIF-2 α , and HIF-1 β levels in nuclear protein extracts of lung tissues of OVA-sensitized and OVA-challenged mice. Levels of HIF-1 α and HIF-1 β were measured at 48 h after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), saline-inhaled mice administered DMSO (SD), OVA-inhaled mice administered DMSO (OD), OVA-inhaled mice administered IGFBP-3 10 $\mu\text{g/kg}$ (OIBP 10), OVA-inhaled mice administered IGFBP-3 50 $\mu\text{g/kg}$ (OIBP 50), OVA-inhaled mice administered LY294002 (OLY), or OVA-inhaled mice administered wortmannin (OWM). (A) Western blot analysis of p-Akt and Akt in lung tissues. (B) Densito-

metric analyses are presented as the relative ratio of p-Akt to Akt. (C and G) Western blot analyses of HIF-1 α and HIF-1 β levels in nuclear protein extracts from lung tissues. (D and H) Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . (E and I) Western blot analyses of HIF-2 α levels in nuclear protein extracts from lung tissues. (F and J) Densitometric analyses are presented as the relative ratio of HIF-2 α to actin. The relative ratio of p-Akt, HIF-1 α or HIF-2 α in nuclear protein extracts from the lung tissues of SP or SD is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group (A–F) and six mice per group (G–J). # P < 0.05 vs SP or SD; * P < 0.05 vs OP or OD.

(Fig. 3A,B). The increased IGF-I levels in lung tissues were significantly decreased by the administration of IGFBP-3 (50 μ g/kg) in OVA-sensitized and OVA-challenged mice.

In addition, we found that levels of VEGF protein in lung tissues were significantly increased at 48 h after OVA inhalation compared with the levels after saline inhalation (Fig. 3C, D). The increased levels of VEGF protein in lung tissues after OVA inhalation were dramatically reduced by the administration of anti-IGF-I antibody.

Anti-IGF-I antibody reduces inflammatory cells in BAL fluids and airway hyper-responsiveness of OVA-sensitized and OVA-challenged mice

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 48 h after OVA inhalation compared with the numbers after saline inhalation (Fig. 3E). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of anti-IGF-I antibody.

In addition, we measured respiratory system resistance (R_{rs}) after the administration of anti-IGF-I antibody to OVA-inhaled mice. Airway responsiveness was assessed as a percent increase in R_{rs} in response to increasing doses of

methacholine. In OVA-inhaled mice, the dose-response curve of R_{rs} shifted to the left compared with that of saline-inhaled mice (Fig. 3F). In addition, the percent R_{rs} produced by methacholine administration (at dose of 2.5–50 mg/ml) increased significantly in the OVA-inhaled mice compared with that of the saline-inhaled mice. Anti-IGF-I antibody administered into the OVA-inhaled mice significantly reduced R_{rs} at 50 mg/ml of methacholine compared with that of OVA-inhaled mice administered control polyclonal antibody. These results indicate that blockade of IGF-I reduces OVA-induced airway hyper-responsiveness.

Recombinant human IGFBP-3 or CBO-P11 reduces Th2 cytokine levels in the lung of OVA-sensitized and OVA-challenged mice

Western blot analysis revealed that IL-4, IL-5, and IL-13 protein levels in lung tissues were increased significantly at 48 h after OVA inhalation compared with the levels after saline inhalation (Fig. 4A,B). The increased levels of IL-4, IL-5, and IL-13 protein in lung tissues after OVA inhalation were significantly reduced by the administration of IGFBP-3 (50 μ g/kg) or CBO-P11. In case of IL-13, the treatment with 10 μ g/kg of IGFBP-3 also reduced significantly OVA-induced

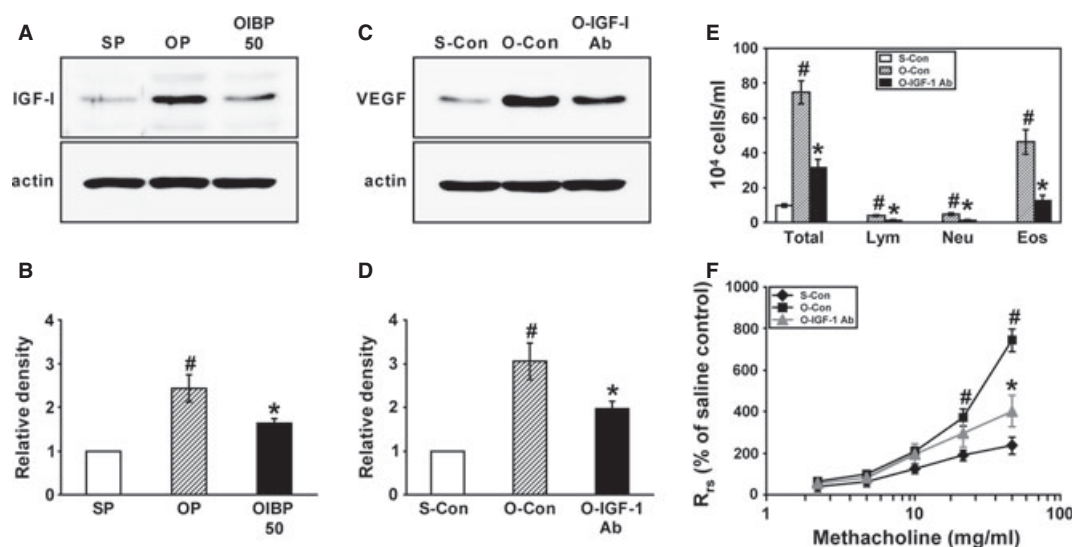


Figure 3 The relationships between IGF-I, IGFBP-3, and VEGF in the lung of OVA-inhaled mice. (A–B) Effect of IGFBP-3 on IGF-I levels in lungs of OVA-inhaled mice. Levels of IGF-I were measured at 48 h after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), and OVA-inhaled mice administered IGFBP-3 50 μ g/kg (OIBP 50). (A) Western blot analyses of IGF-I in lung tissues. (B) Densitometric analyses are presented as the relative ratio of IGF-I to actin. The relative ratio of each molecule in the lung tissues of SP is arbitrarily presented as 1. Data represent mean $\pm$ SEM from six mice per group. # P < 0.05 vs SD; * P < 0.05 vs OD. (C–F) Effect of anti-IGF-I antibody on VEGF expression, inflammatory cells, and airway responsiveness in the lung of OVA-inhaled mice. Sampling was

performed at 48 h after the last challenge in saline-inhaled mice administered control polyclonal antibody (S-Con), OVA-inhaled mice administered control polyclonal antibody (O-Con), and OVA-inhaled mice administered anti-IGF-I antibody (O-IGF-I Ab). (C) Western blot analyses of VEGF in lung tissues. (D) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of each molecule in lung tissues of S-Con is arbitrarily presented as 1. (E) Total cells and differential cellular components in BAL fluids of OVA-inhaled mice. (F) R_{rs} values were obtained in response to increasing doses (2.5–50 mg/ml) of methacholine. Total, total cell; Lym, lymphocyte; Neu, neutrophil; Eos, eosinophil. Data represent mean $\pm$ SEM from six mice per group. # P < 0.05 vs S-Con; * P < 0.05 vs O-Con.

increase in IL-13 protein levels from lung tissues. Consistent with these results, enzyme immunoassays also showed that the increase in IL-4, IL-5, and IL-13 protein levels in BAL fluids after OVA inhalation was substantially decreased by the administration of IGFBP-3 (10 and 50 $\mu\text{g/kg}$) or CBO-P11 (Fig. 4C).

Recombinant human IGFBP-3, CBO-P11, or anti-IGF-I antibody decreases the levels of serum OVA-specific IgE in OVA-inhaled mice

Significant decreases in OVA-specific IgE level in the serum of OVA-inhaled mice were observed with the administration of IGFBP-3, CBO-P11, or anti-IGF-I antibody compared with the levels in OVA-inhaled mice administered PBS, DMSO, and control polyclonal antibody, respectively (Fig. 4D).

Recombinant human IGFBP-3 or CBO-P11 reduces inflammatory cells in BAL fluids of OVA-sensitized and OVA-challenged mice

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 48 h after OVA inhalation compared with the numbers after saline inhalation (Fig. 5A). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of IGFBP-3 (10 and 50 $\mu\text{g/kg}$) or CBO-P11.

Recombinant human IGFBP-3 or CBO-P11 decreases OVA-induced airway hyper-responsiveness

To investigate the changes in airway hyper-responsiveness by IGFBP-3 or CBO-P11, we measured R_{rs} after the administration of IGFBP-3 or CBO-P11 to OVA-inhaled mice. Airway responsiveness was assessed as a percent increase in R_{rs} in response to increasing doses of methacholine. In OVA-inhaled mice, the dose-response curve of R_{rs} shifted to the left compared with that of saline-inhaled mice (Fig. 5B). In addition, the percent R_{rs} produced by methacholine administration (at dose of 25–50 mg/ml) increased significantly in the OVA-inhaled mice compared with that of the saline-inhaled mice. The levels of R_{rs} of OVA-inhaled mice administered IGFBP-3 or CBO-P11 were significantly reduced compared with those of OVA-inhaled mice administered drug vehicle. These results indicate that IGFBP-3 or CBO-P11 reduces OVA-induced airway hyper-responsiveness.

Recombinant human IGFBP-3 attenuates pathological changes in OVA-induced airway disease

To assess the pathologic changes in the lung of OVA-inhaled mice by the administration of IGFBP-3, histologic analyses were performed at 48 h after OVA inhalation. The results revealed typical pathologic features of allergic airway disease in the OVA-exposed mice (Fig. 6B). Numerous inflammatory cells infiltrated around the bronchioles and mucus and debris had accumulated in the lumen of bronchioles of OVA-

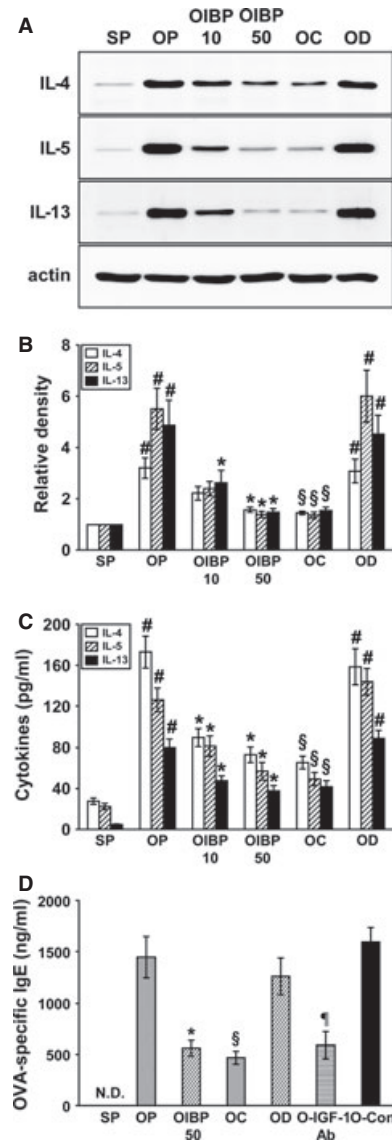


Figure 4 Effect of IGFBP-3, CBO-P11, or anti-IGF-I antibody on levels of Th2 cytokines in the lung and OVA-specific IgE in serum of OVA-sensitized and OVA-challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), OVA-inhaled mice administered IGFBP-3 10 $\mu\text{g/kg}$ (OIBP 10), OVA-inhaled mice administered IGFBP-3 50 $\mu\text{g/kg}$ (OIBP 50), OVA-inhaled mice administered CBO-P11 (OC), OVA-inhaled mice administered 0.05% DMSO (OD), OVA-inhaled mice administered anti-IGF-I antibody (O-IGF-I Ab), or OVA-inhaled mice administered control polyclonal antibody (O-Con). (A) Western blot analyses of IL-4, IL-5, or IL-13 levels in lung tissues. (B) Densitometric analyses are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissues of SP is arbitrarily presented as 1. (C) Enzyme immunoassay of IL-4, IL-5, or IL-13 in BAL fluids. (D) Changes in OVA-specific IgE levels in serum. Data represent mean $\pm$ SEM from seven mice per group (A–C) and six mice per group (D). # P < 0.05 vs SP; * P < 0.05 vs OP; § P < 0.05 vs OD; ¶ P < 0.05 vs O-Con.

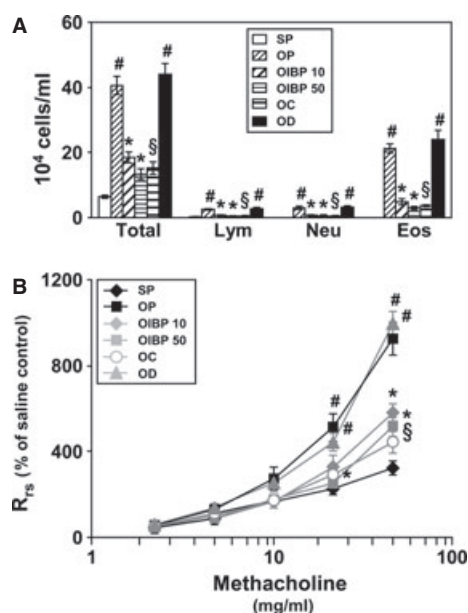


Figure 5 Effect of IGFBP-3 or CBO-P11 on total cells and differential cellular components in BAL fluids (A) and airway responsiveness (B) of OVA-sensitized and OVA-challenged mice. The numbers of each component of BAL cells and airway responsiveness were measured at 48 h after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), OVA-inhaled mice administered IGFBP-3 10 µg/kg (OIBP 10), OVA-inhaled mice administered IGFBP-3 50 µg/kg (OIBP 50), OVA-inhaled mice administered CBO-P11 (OC), and OVA-inhaled mice administered 0.05% DMSO (OD). R_{rs} values were obtained in response to increasing doses (2.5–50 mg/ml) of methacholine. Data represent mean $\pm$ SEM from seven mice per group. Total, total cell; Lym, lymphocyte; Neu, neutrophil; Eos, eosinophil. # P < 0.05 vs SP; * P < 0.05 vs OP; § P < 0.05 vs OD.

sensitized and OVA-challenged mice (Fig. 6B) as compared with the control (Fig. 6A). Mice treated with IGFBP-3 of 10 µg/kg (Fig. 6C) and IGFBP-3 of 50 µg/kg (Fig. 6D) showed a marked reduction in the thickening of airway epithelium, the infiltration of inflammatory cells in the peribronchiolar region, the number of inflammatory cells, and the amount of debris in the airway lumen. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 48 h after OVA inhalation compared with the scores after saline inhalation (Fig. 6E). The increased inflammation scores after OVA inhalation were significantly reduced by the administration of IGFBP-3.

Discussion

In this study, we have found that recombinant human IGFBP-3 exerts a preventive and therapeutic role against allergen-induced airway inflammation and airway hyper-responsiveness via modulation of VEGF expression mediated by HIF- α activation and IGF-I action. Actually, restoration of IGFBP-3 by the intratracheal administration of recombi-

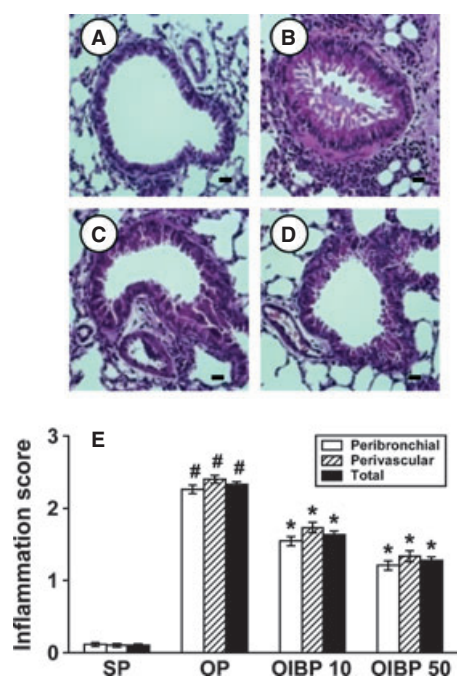


Figure 6 Effect of IGFBP-3 on pathologic changes in lung tissues of OVA-sensitized and OVA-challenged mice. Representative hematoxylin- and eosin-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered PBS (A), OVA-inhaled mice administered PBS (B), OVA-inhaled mice administered IGFBP-3 10 µg/kg (C), and OVA-inhaled mice administered IGFBP-3 50 µg/kg (D). Bars indicate scale of 20 µm. (E) Peribronchial and perivascular lung inflammation was measured, and total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Data represent mean $\pm$ SEM from seven mice per group. # P < 0.05 vs SP; * P < 0.05 vs OP.

nant human IGFBP-3 attenuated the increases in inflammatory cells in the airway, Th2 cytokines in the lung, OVA-specific IgE in serum, plasma exudation, and airway hyper-responsiveness in OVA-inhaled mice. In addition, the administration of IGFBP-3 reduced the HIF-1 α /HIF-2 α activation as well as IGF-I production increased after OVA inhalation in lung tissues, and subsequently decreased the VEGF expression in BAL cells and lung tissues.

IGFBP-3 is a well-documented inhibitor of cell growth and/or promoter of apoptosis. Although its antiproliferative action is achieved through the attenuation of IGF/IGF-IR interaction (23), to date much light has been shed on the ability of these bioactivities to occur via an IGF/IGF-IR axis-independent means (9, 24). This growth-inhibitory character of IGFBP-3 has also been reported in cancer cells, and its antiproliferative action is exerted through the suppression of VEGF expression (25). As for VEGF expression, a number of factors regulate VEGF production; among them, IGF-I has been demonstrated to stimulate VEGF expression (26). In addition, there is ample evidence that IGF-I can activate HIF-1, which is a very powerful transcription factor for

VEGF (16, 17). Because IGFs bind with stronger affinity to IGFBPs than to the IGF-IR, it can be expected that IGFBP-3 acts as an antagonist for IGF-I-stimulating VEGF production as well as functions via its intrinsic biological property. Supporting this contention, our current data have shown that IGFBP-3 administered exogenously suppressed VEGF expression and inhibited HIF-1 α /HIF-2 α activation in lung tissues of OVA-inhaled mice. In addition, recombinant human IGFBP-3 reduced the levels of IGF-I in lung tissues of OVA-inhaled mice, and blockade of IGF-I action using anti-IGF-I antibody inhibited OVA-induced VEGF expression, airway inflammation, and bronchial hyper-responsiveness. Taken together, IGFBP-3 seems to regulate VEGF production via suppression of HIF-1 α /HIF-2 α activity through IGF-I-dependent and/or IGF-I-independent manner in allergic airway disease.

Meanwhile, VEGF is not only implicated in angiogenesis but also contributes to asthmatic tissue edema through its effect on vascular permeability (27). Moreover, VEGF is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and Th2 cell-mediated inflammation (28). The major role of VEGF in asthma appears to be the enhancement of vascular permeability (2, 3). Consistent with these observations, our results have shown that VEGF expression was up-regulated and that vascular permeability was increased, accompanying Th2 cell-mediated airway inflammation and airway hyper-responsiveness in OVA-induced allergic airway disease. Interestingly, these typical asthmatic features were ameliorated by the administration of recombinant human IGFBP-3 or a VEGF receptor inhibitor, CBO-P11. These findings suggest that IGFBP-3 attenuates the allergic airway inflammatory responses through blockade of VEGF actions.

VEGF expression is regulated through HIF-1 α (29), which plays an important role in immune and inflammatory responses (18). In fact, HIF-1 α is activated oxygen dependently or independently by various mediators including hypoxia, nitric oxide, reactive oxygen species, cytokines, apoptotic cell debris, and infectious pathogens in inflammatory tissues (30). Studies have shown HIF-1 α activation during inflammation, leading to the enhancement of its target gene expression such as VEGF, glucose transporter 1, and metalloproteinase (30, 31). In addition, HIF-2 α sharing the pronounced sequence homology with HIF-1 α is linked to up-regulation of VEGF expression and tumor angiogenesis (32). Actually, we have revealed that HIF- α inhibition decreases the VEGF expression in allergic airway diseases using both *in vivo* and *in vitro* system (8). Furthermore, we have demonstrated that PI3K/Akt signaling is required for HIF-1 α activation in airway inflammatory diseases such as asthma (8). Recent works have revealed that HIF-2 α -overexpressed tumor cells exhibit enhanced activation of PI3K/Akt/mTOR (33). In our present study, recombinant human IGFBP-3

inhibited Akt phosphorylation and reduced HIF-1 α /HIF-2 α activity in the lung of OVA-inhaled mice. In addition, pharmacological PI3K inhibition significantly reduced HIF-1 α /HIF-2 α activity in the current OVA-inhaled mice. Supporting our findings, studies have reported that IGFBP-3 blocks the phosphorylation of Akt in various cells (34, 35). Taken together, exogenous IGFBP-3 suppresses allergen-induced VEGF expression as well as PI3K/Akt-HIF- α activation in mice.

In summary, our study has shown that IGF-I production, HIF- α activity, VEGF expression, and vascular permeability are increased in the lung of a murine model of allergic airway disease and that administration of recombinant human IGFBP-3 to allergen-inhaled mice attenuates the increased IGF-I production, HIF- α activity, VEGF expression, and vascular permeability as well as airway hyper-responsiveness. These findings suggest that IGFBP-3 signaling involves HIF-1 α /HIF-2 α -VEGF axis through IGF-I-dependent and/or IGF-I-independent manner, thereby altering vascular permeability.

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Authors' contribution

So Ri Kim designed the research, performed the experiments, interpreted the experimental data, and wrote the manuscript; Kyung Sun Lee designed and performed the experiments; Kyung Bae Lee performed the experiments and reviewed the manuscript; Yong Chul Lee designed the research, performed the experiments, interpreted the data, wrote and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods and results.

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[Supporting Information]

**Recombinant IGFBP-3 inhibits allergic lung inflammation,
VEGF production and vascular leak in a mouse model of
asthma**

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Short title: IGFBP-3 and VEGF in allergic airway disease

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, Korea), were housed throughout the experiments in a laminar flow cabinet and 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. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 µg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 µl, as previously described with some modifications (1, 2). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes 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 48 hours after the last challenge. At the time of lavage, the mice were sacrificed by ether inhalation (Junsei Chemical Co., Ltd, Tokyo, Japan). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lung and withdrawn. The aliquots were pooled and then kept at 4°C. Part of each pool was then centrifuged and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc. Aguada, Puerto Rico) in order to examine the cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Inter-investigator variation was < 5%. The mean number from the two investigators was used to estimate the cell differentials.

Administration of recombinant human IGFBP-3, IGFBP-1, CBO-P11, LY 294002, wortmannin, or anti-IGF-I antibody

Recombinant human IGFBP-3 (10 µg/kg or 50 µg/kg body weight per day; Upstate Biotechnology, Lake Placid, NY) was suspended in phosphate buffered saline (PBS) and administered intratracheally two times to each animal, once at 1 hour before the first airway challenge with OVA and the second time at 3 hours after the last airway challenge with OVA. In addition, recombinant human IGFBP-1 (50 µg/kg body weight per day; R&D Systems, Inc., Minneapolis, MN) suspended in PBS was administered intratracheally two times to each animal according to same schedule for administration of IGFBP-3. For evaluating of the effect of IGFBP-3 on OVA-induced ongoing allergic airway inflammation, recombinant human IGFBP-3 (50 µg/kg body weight/day) was administered intratracheally to each animal at 3 hours after the last OVA challenge. Cyclopeptidic VEGF receptor inhibitor, CBO-P11 (Calbiochem, San Diego, CA) was used to inhibit VEGF activity. CBO-P11 (2 mg/kg body weight/day) or vehicle control (0.05% DMSO) was administered intraperitoneally four times at 24 hour-intervals, beginning at 1 hour before the first inhalation of OVA (3). LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (1.5 mg/kg body weight/day; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) or wortmannin (100 µg/kg body weight/day; Calbiochem) dissolved in DMSO and diluted with 0.9% NaCl was administered in a volume of 50 µl. LY-294002 or wortmannin was administered intratracheally two times to each treated animal, once at 1 hour before the first airway challenge with OVA and the second time at 3 hours after the last airway challenge with OVA. In addition, anti-IGF-I antibody or control polyclonal antibody (2.5 mg/kg body weight/day; R&D Systems, Inc.) was administered to each animal intratracheally at 3 hours after the last challenge with OVA.

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors, and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc.,

Hercules, CA), as described previously (2). Samples were loaded on SDS-polyacrylamide gel electrophoresis gel. After electrophoresis at 120 V for 90 minutes, separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by the wet transfer method (250 mA, 90 minutes). Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween 20 (TBST; 25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20) for 1 hour, and the blots were then incubated overnight at 4°C with an anti-IGF-I antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-4 antibody (Serotec Ltd., Oxford, United Kingdom), anti-IL-5 antibody (Santa Cruz Biotechnology), anti-IL-13 antibody (R&D Systems, Inc.), anti-p-Akt antibody (R&D Systems, Inc.), anti-Akt antibody (Cell Signaling Technology Inc., Beverly, MA), or anti-VEGF antibody (Santa Cruz Biotechnology). Anti-rabbit or -mouse horseradish peroxidase conjugated-IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with an anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibodies was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare). The film was scanned and quantified using the quantification software (Gel Doc XR, Bio-Rad Laboratories Inc.). For the quantification of specific bands, the same size square was drawn around each band to measure the density and then the value was adjusted by the density of the background near that band. The results of densitometric analyses were expressed as the relative ratio of the target protein to reference protein. The relative ratio of the target protein of control group is arbitrarily presented as 1.

Measurement of T-helper type 2 (Th2) cytokines and VEGF

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by

enzyme immunoassays according to the manufacturer's protocol (IL-4, IL-5, and IL-13; Invitrogen Corp., Carlsbad, CA, USA; VEGF; R&D Systems, Inc.). Sensitivities for IL-4, IL-5, IL-13, and VEGF assays were 5, 3, 2, and 3.0 pg/ml, respectively.

Measurement of Serum OVA-Specific IgE

OVA-specific IgE levels were measured by ELISA according to the manufacturer's protocol, using a mouse OVA-IgE ELISA kit (MD Biosciences, Inc., St. Paul, MN). Briefly, microtiter plates were coated with purified monoclonal anti-mouse OVA-IgE. Appropriately diluted serum samples were added to the plates and incubated for 1 hour at room temperature. Biotinylated OVA was added to each well, and the samples were incubated for 30 minutes at room temperature with gentle shaking. After adding the diluted streptavidin-horseradish peroxidase, the mixture was incubated in the dark for 30 minutes without shaking. The levels were quantified by reading the plates at 450 nm. The sensitivity for the OVA-IgE ELISA is typically less than 3.8 ng/ml.

Nuclear protein extractions for analysis of HIF-1 $\sim$, HIF-2 $\sim$, and HIF-1

Lung tissues were homogenized in 8 volumes of a lysis buffer containing 1.3 mol/L sucrose, 1.0 mmol/L MgCl₂, and 10 mmol/L potassium phosphate buffer (pH 7.2). The homogenate was filtered through four layers of gauze and centrifuged at $1,000 \times g$ for 15 minutes. The resulting pellets were harvested and resuspended in 10 mmol/L potassium phosphate buffer (pH 7.2) containing 2.4 mol/L sucrose and 1.0 mmol/L MgCl₂ to maintain a final 2.2 mol/L sucrose concentration and centrifuged at $100,000 \times g$ for 1 hour. The resulting nuclear pellets were washed once with a buffer (pH 7.2) containing 0.25 mol/L sucrose, 0.5 mmol/L MgCl₂, and 20 mmol/L Tris-HCl and centrifuged at $1,000 \times g$ for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150

mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid (EDTA), 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor cocktails. The mixture was kept on ice for 2 hours with gentle stirring and centrifuged at $12,000 \times g$ for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for detection of HIF-1 α or HIF-1 β . Western blotting was performed using an antibody against HIF-1 α (R&D Systems, Inc.), HIF-1 β (Cell Signaling Technology Inc.), or HIF-2 α (Novus Biologicals Inc., Littleton, CO). Anti-rabbit horseradish peroxidase conjugated-IgG was used to detect binding of antibody.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/ml. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 minutes, the animals were killed and their chests were opened. Normal saline containing 5 mmol/L EDTA was perfused through the aorta until all venous fluid returned to the opened right atrium was clear. Lungs were removed and weighed wet. EBD was extracted in 2 ml formamide kept in a water bath at 60°C for 3 hours and the absorption of light at 620 nm was measured using a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01-10 μ g/ml and is expressed as ng of dye/mg of wet lung.

Histology

At 48 hours after the last challenge, mice were euthanized for histological assessment. Lungs and trachea of mice were filled with 10% (v/v) neutral buffered formalin intratracheally and

then were removed from the mice. For fixation, the neutral buffered formalin was also used. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μ m 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 hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (2, 4). A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airway, as described elsewhere (5). Anesthesia was achieved with 45 mg/kg 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 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O 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. 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 in saline). After each methacholine challenge, the data of respiratory system resistance (R_{rs}) was continuously collected. Maximum values of R_{rs} were selected to express changes in airway function which was represented as a percentage change from baseline after saline aerosol.

Densitometric analysis and statistics

All immunoreactive and phosphorylative signals were analyzed by densitometric scanning (Gel Doc XR, Bio-Rad Laboratories Inc.). We used SPSS statistical software (version 16.0, SPSS, Chicago, IL). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Pearson's correlation was calculated to assess the correlation between data. Statistical significance was set at $P < 0.05$.

Results

Recombinant human IGFBP-3 administered after completion of OVA inhalation reduces cellular changes in BAL fluids and airway hyperresponsiveness of OVA-inhaled mice

To investigate the effect of IGFBP-3 on the ongoing or established asthmatic symptoms, 50 µg/kg of IGFBP-3 was administered after OVA challenge was completed, and then the changes in BAL cells and airway hyperresponsiveness were determined. Numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly increased in the BAL fluid at 48 hours after the last OVA inhalation compared with the numbers after saline inhalation (Fig. S1A). The increased numbers of these cells after OVA inhalation were substantially reduced by the administration of IGFBP-3.

In OVA-sensitized and -challenged mice, the dose-response curves of percent R_{rs} shifted to the left compared with that of saline-inhaled mice (Fig. S1B). In addition, the percent R_{rs} produced by administration of 25 to 50 mg/ml methacholine increased significantly in OVA-inhaled mice compared with the saline-inhaled mice. OVA-sensitized and -challenged mice treated with IGFBP-3 showed a substantial reduction of R_{rs} at 50 mg/ml of methacholine compared with that of mice treated with drug vehicle only. These results indicate that IGFBP-3 attenuates OVA-induced airway hyperresponsiveness that is a typical asthmatic symptom.

Effects of recombinant IGFBP-1 on inflammatory cells in BAL fluids and airway hyperresponsiveness of OVA-sensitized and -challenged mice

To specify effects of IGFBP-1 as a control protein on the asthmatic features in the current OVA-inhaled mice, 50 µg/kg of IGFBP-1 was administered intratracheally according to the same schedule for IGFBP-3. Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 48 hours after OVA inhalation

compared with the numbers after saline inhalation (Fig. S2A). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were not affected by the administration of IGFBP-1.

In addition, we measured R_{rs} after the administration of IGFBP-1 to OVA-inhaled mice. Airway responsiveness was assessed as a percent increase of R_{rs} in response to increasing doses of methacholine. In OVA-inhaled mice, the dose-response curve of R_{rs} shifted to the left compared with that of saline-inhaled mice (Fig. S2B). In addition, the percent R_{rs} produced by methacholine administration (at dose of 25 to 50 mg/ml) increased significantly in the OVA-inhaled mice compared with the saline-inhaled mice. The levels of R_{rs} of OVA-inhaled mice administered IGFBP-1 were not changed significantly compared with that of OVA-inhaled mice administered PBS.

These results indicate that IGFBP-1 has no effect on the OVA-induced airway inflammation and hyperresponsiveness.

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Figure Legends

Figure S1. Effect of IGFBP-3 administered after OVA inhalation on total cells and differential cellular components in BAL fluids and on airway responsiveness of OVA-sensitized and -challenged mice. (A) The numbers of total and differential cellular component of BAL fluids. (B) Airway responsiveness. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), and OVA-inhaled mice administered 50 $\mu\text{g/kg}$ of IGFBP-3 (OIBP 50). R_{rs} values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Bars represent mean $\pm$ SEM from 7 mice per group. Total, total cell; Lym, lymphocyte; Neu, neutrophil; Eos, eosinophil. $\#P < 0.05$ versus SP; $*P < 0.05$ versus OP.

Figure S2. Effect of IGFBP-1 on total cells and differential cellular components in BAL fluids (A) and airway responsiveness (B) of OVA-sensitized and -challenged mice. The numbers of each component of BAL cells and airway responsiveness were measured at 48 hours after the last challenge in saline-inhaled mice administered PBS (SP), OVA-inhaled mice administered PBS (OP), and OVA-inhaled mice administered IGFBP-1 (O-IGFBP-1). R_{rs} values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Data represent mean $\pm$ SEM from 6 mice per group. Total, total cell; Lym, lymphocyte; Neu, neutrophil; Eos, eosinophil. $\#P < 0.05$ versus SP.