



Role of insulin-like growth factor-I in allergen-induced airway inflammation and remodeling

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Abstract

Insulin-like growth factor (IGF)-I is known to act on fibroblasts as a progression factor to push cells toward proliferation and activation to synthesize collagen. Subepithelial fibrosis, collagen deposition at the lamina reticularis, is part of the process of so-called remodeling and is a characteristic finding in the asthmatic airway. To study the role of IGF in the evolution of asthma, we used a model that involved immunization of mice with ovalbumin and alum, followed by an inhaled challenge of ovalbumin. IGF-I neutralizing antibody was continuously infused with an osmotic pump. Pulmonary function was analyzed using whole-body plethysmography before and after acetylcholine administration. It was found that OVA inhalation induced IGF-I expression at the site of the airway. IGF-I neutralizing Ab inhibited the elevation of airway resistance, airway inflammation, and an increase in airway wall thickening. The depression of ICAM-1 expression was accompanied by a diminution in airway inflammation. In conclusion, these results suggest that IGF-I is likely to be an important mediator of inflammation and remodeling in the asthmatic airway.
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1. Introduction

Airway inflammation and airway remodeling are major characteristics of asthma [1,2]. Remodeling was originally considered to be repair of damage associated with asthma. However, in asthmatic patients, airway remodeling is the predecessor of various structural changes, such as airway wall thickening (AWT), subepithelial fibrosis, and mucus metaplasia, which are manifestations of the irreversible pathology of severe asthma [3–5].

During subepithelial fibrosis, the fibroblast is activated and collagen synthesis increases. Several cytokines

are involved in fibroblastic proliferation. Among those, platelet-derived growth factor (PDGF) and fibronectin function as competence factors, while insulin-like growth factor (IGF)-I, which was originally called macrophage-derived growth factor of fibroblasts (MDGF), is a progression factor [3,4]. We previously reported that PDGF plays a critical role in remodeling in asthma [5]. Neutralizing antibody to PDGF-B inhibited both diesel exhaust particulate (DEP)-induced AWT and hyperresponsiveness to acetylcholine (ACh), suggesting that airway wall thickness is directly related to airway hyperresponsiveness. PDGF plays the role of stimulating deposition of the extracellular matrix (ECM) beneath the basement membrane. Although the involvement of IGF-I in remodeling in asthma is also suggested [6,7], the exact relation between IGF-I and the pathogenesis of asthma must be elucidated.

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Among IGFs, IGF-I, and IGF-II are single chain polypeptides that have a high degree of homology to insulin and that act in the manner of endocrines [8]. IGF-I has been shown to be the same molecule designated as MDGF [3]. In addition, IGF-I is known to produce effects on immune cells, such as inducing T-cell proliferation and preventing T-cell apoptosis [9,10]. In this study, we investigated the role of IGF-I in airway inflammation and remodeling in asthma. Our data suggested that IGF-I neutralizing antibody inhibited both airway inflammation and AWT, resulting in improvement of airway hyperresponsiveness.

2. Materials and methods

2.1. Reagents

Neutralizing antibody for murine IGF-I was kindly provided by Dr. Jui-Lan Su [11]. This antibody was originally made against human IGF-I but is fully cross-reactive with murine IGF-I [11]. Goat neutralizing antibodies to IGF-I were purchased from Becton–Dickinson (Mountain View, CA). Both mouse IgG and goat IgG were obtained from Chemicon International (Temecula, CA). Rabbit anti-IGF-I and anti-ICAM-1 for immunohistochemical staining were also purchased from Chemicon International. Pentobarbital sodium was from Pitman Moore (Mundelein, IL), pancuronium bromide was from Organ-Teknika (the Netherlands), and ACh was from Wako Chemicals (Tokyo, Japan).

2.2. Sensitization of mice

Specific pathogen-free male A/J mice (10–12 weeks old), which have native airway hyperresponsiveness to ACh [23,24], were purchased from SLC (Shizuoka, Japan). The mice were initially immunized four times with 10 µg OVA + 2 mg alum on days 0, 28, 35, and 49. After the four injections were finished, ELISA titers of OVA-specific IgE were significantly elevated following inhalation of 20 mg/ml OVA for 10 min every other day from day 49 to day 63, as previously reported [12]. The mice were divided into four groups for administration of inhaled challenge: (1) 0.9 M NaCl, (2) OVA + sham control goat IgG (4 mg/mouse in 0.9 M NaCl), (3) OVA + anti-IGF-I (4 mg/mouse in 0.9 M NaCl), and (4) OVA + anti-IGF-I (0.4 mg/mouse in 0.9 M NaCl). The antibodies were administered using osmotic pumps. An Alzet Minipump, which continuously released its contents for two weeks, was implanted subcutaneously in the anterior back region.

2.3. Assessment of airway responsiveness

The mice were anesthetized with pentobarbital and were tracheotomized. The animals were connected to a

Harvard ventilator with 0.25 ml tidal volume and a respiratory frequency of 120/min, as previously reported [5], after which they were given an injection of pancuronium bromide. Airway resistance (Raw) was measured using whole-body plethysmographs (Buxo Electronics, Troy, NY) after 24 h of the last OVA inhalation. Increasing doses of ACh (1250–5000 µg/ml) were administered by ultra-nebulization for 3 min. Data are expressed as [(Raw after inhalation of ACh/raw before inhalation) × 100 (%)].

2.4. BALF cell analysis and histological examination

After measurement of pulmonary function, each mouse was exsanguinated. BALF was obtained from selected mice by intubating and washing the lungs with 1 ml saline until the recovered fluid reached 5 ml. The lungs were fully inflated using 10 cm H₂O pressure and fixed with 20% formaldehyde for hematoxylin–eosin (HE) and elastica van Gieson (EVG) staining.

Thickening beneath the basement membrane was analyzed using an image analyzer and standardized. The AWT index was calculated as the (external perimeter – inner perimeter)/inner perimeter, as described previously [5].

Collagen deposition was assessed indirectly from the production of hydroxyproline. To measure hydroxyproline, the excised lung was washed in saline and freeze dried at –70 °C. Hydroxyproline content was then determined in triplicate using a previously described method [5].

2.5. Analysis of mRNA expression

Lungs of mice were frozen in liquid nitrogen immediately after harvest and were used for RNA extraction. Each lung tissue was moved quickly into 1 ml Isogen (Nippon Gene, Tokyo, Japan). Lung tissue was homogenized and total RNA was extracted, using a modified acid guanidium–phenol–chloroform method. To synthesize cDNA, 5 µg of total RNA was incubated with 5 mM MgCl₂, 1 mM dNTP mixture, 0.25 U reverse transcriptase, 1 U RNase inhibitor, and 0.125 µM oligo(dT) (Takara Biochemicals, Tokyo, Japan). Amplification cycles were 42 °C for 15 min, 99 °C for 5 min, and then 5 °C for 5 min using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

The mRNA levels of cytokines were quantified by real-time polymerase chain reaction (PCR) using the Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) for amplification of cDNA. Quantification of cytokines and β-actin was performed on the basis of standard curves obtained using 5-, 25-, and 125-fold dilutions of standard cDNA. Results are shown as ratios of quantities of cytokine to β-actin mRNA. Primers were

synthesized by Nihon Gene Research Lab. (Sendai, Japan) and are shown in Table 1. Amplified products were also electrophoresed on 2% agarose gels.

2.6. Western blot analysis

Mouse sera and BALF were first precleared using protein A columns (Pharmacia Biotech, Wilstrom, Sweden) followed by incubation with goat anti-IGF-I (Becton–Dickinson) and protein G Microbeads (Miltenyi Biotec, Gladbach, Germany) for 30 min. Subsequently, immunoprecipitates were separated in the magnetic field of an μ MACS separator. The immunoprecipitates were eluted with SDS sample buffer, after which they were separated by SDS–PAGE and electrotransferred onto Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then blocked in buffer containing 4% BSA, and incubated first with rabbit anti-IGF-I (Chemicon), and then with horseradish peroxidase-conjugated F(ab')₂ donkey anti-rabbit antibody (1:2000) (Amersham Pharmacia Biotech). The immunoblotted proteins were visualized using an enhanced chemiluminescence (ECL) kit from Amersham Pharmacia Biotech.

2.7. Immunohistological analysis

Tissue sections were deparaffinated and rehydrated. Samples were treated with Target Retrieval (Dako, Glodstrup, Denmark) at 95 °C, blocked at room temperature using Protein Block Serum-Free (Dako), and incubated with either anti-ICAM-1, or control rabbit IgG. Antigen–antibody complexes were detected using a streptavidin–biotin–peroxidase technique (Immunotech, Cedex, France), and visualized colorimetrically after conversion of AEC peroxidase substrate.

2.8. Statistics

Multiple parameters were compared by analysis of variance (ANOVA) with the Bonferroni post test. Data are expressed as means $\pm$ SEM. Significance levels are at $P < 0.05$.

3. Results

3.1. Synthesis of IGF-I is observed in OVA-challenged mice

First we monitored whether IGF-I synthesis became elevated after OVA inhalation in our murine model. IGF-I protein was detected in both serum and BALF samples obtained from OVA-sensitized mice (Fig. 1A). The majority of IGF-I positive cells were macrophages and airway epithelial cells (Fig. 1B). Inhalation of OVA resulted in detection of mRNA expression for IGF-I in lung tissue at 3–24 h. (Fig. 1C). IGF-I mRNA and TGF- β were detected from total lung lysate 24 h from the last OVA inhalation (Fig. 1D).

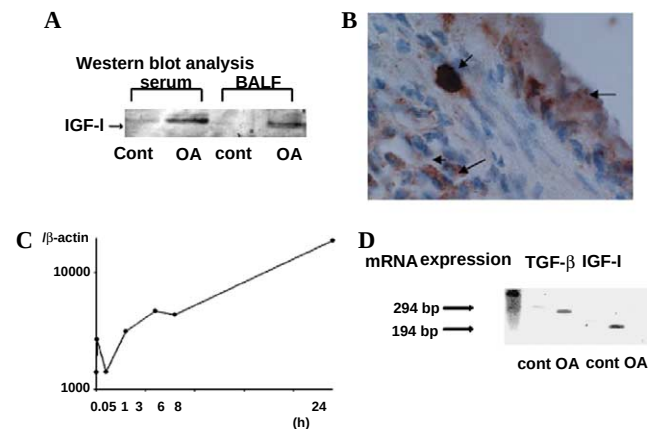


Fig. 1. IGF-I mRNA and protein expressions. (A) Western blot analysis of IGF-I. IGF-I protein was detected in serum and BALF. (B) Histological analysis of IGF-I protein expression. Positive cells are indicated by arrows. (Original magnification: 400 $\times$) (C) mRNA expression of IGF-I in BALF cells. BALF cells were examined for mRNA of IGF-I expression by real-time PCR. At each time point, BALF cells from five mice were pooled and used for mRNA extraction. (D) IGF-I mRNA expression in the lung. Total lung mRNA was extracted as described in Section 2. Left line is DNA size marker. Expected size of IGF-I PCR is 194 bp indicated by an arrow.

Table 1
Primer sequences

Gene	Primer	Size (bp)
β -Actin	Forward	5'-CCTGTATGCCTCTGGTCGTA-3'
	Reverse	5'-CCATCTCCTGCTCGAAGTCT-3'
IGF-I	Forward	5'-TACTTCAACAAGCCCACAGG-3'
	Reverse	5'-ACTTCCTTCTGAGTCTTGGG-3'
TGF- β 1	Forward	5'-AACAACGCCATCTATGAG-3'
	Reverse	5'-TATTCCGTCTCCTTGGTT-3'
ICAM-1	Forward	5'-GAACCCATCTCCTAAAATGA-3'
	Reverse	5'-TGGCACCAGAATGATTATAG-3'
IGFBP3	Forward	5'-AGCGGGAGACAGAATACGGT-3'
	Reverse	5'-GCTGCCCATACTTGTCCACA-3'

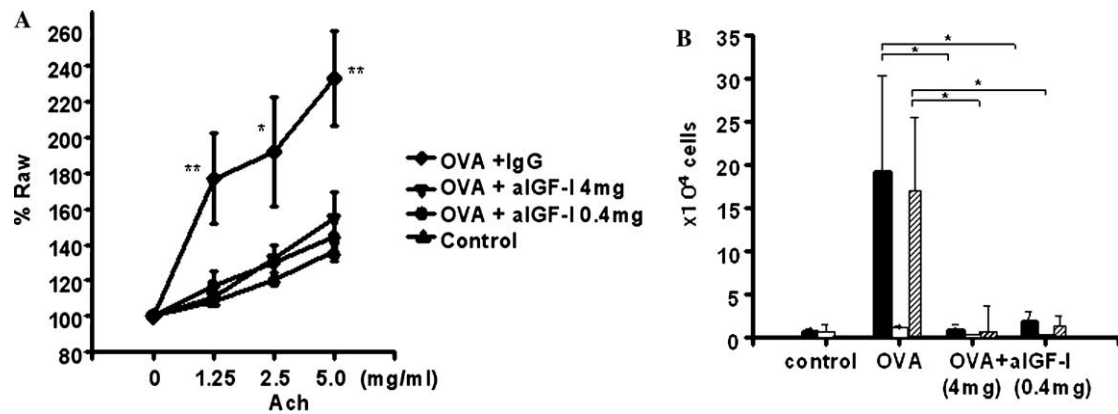


Fig. 2. Effects of neutralization of IGF-I on bronchoconstriction and inflammation. (A) Bronchoconstriction induced with ACh inhalation. IGF-I neutralizing Abs were administered by osmotic pump for 2 weeks during OVA inhalation. The indicated concentrations of ACh were inhaled for 3 min. Raw was measured using whole-body plethysmographs as described in Section 2. ACh-evoked changes in Raw are expressed as a percentage of Raw observed before ACh inhalation (100%). Data shown are means $\pm$ SEM for six mice. Similar results were obtained from three same kinds of experiments. (B) BALF cell analysis. Lungs were intubated for lavage with a total of 5 mL saline (0.9 M NaCl). Cells were pelleted from the lavage fluid, resuspended in 1 mL saline and placed on glass slides for counting and fixation using Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. Data show means $\pm$ SEM of six mice. The same kinds of experiments were repeated three times. Similar results were obtained from three same kinds of experiments. * $P < 0.05$, ** $P < 0.01$. (■) Total cells, (□) macrophages, and (▨) eosinophils.

3.2. IGF-I neutralization inhibits airway hyperresponsiveness and inflammation

The effect of murine IGF-I neutralizing antibody on airway response to ACh was tested by administering the antibody continuously for two weeks using an osmotic pump. Baseline airway resistance was the same in both OVA- and non-treated mice. After the inhalation of 1.25–5.0 mg/ml ACh, OVA-treated mice exhibited higher Raw than control mice (Fig. 2A). Airway response following doses of 5, 2.5, and 1.25 mg/ml ACh was significantly inhibited in the anti-IGF-I antibody-infused mice (Fig. 2A). Inhibitory effects on airway response were also observed after the administration of goat anti-IGF-I (0.1 mg/mouse) (OVA vs OVA + anti-IGF-I, $191 \pm 6.7\%$ ($n = 6$) vs $129 \pm 6.1\%$ ($n = 5$), $P < 0.01$).

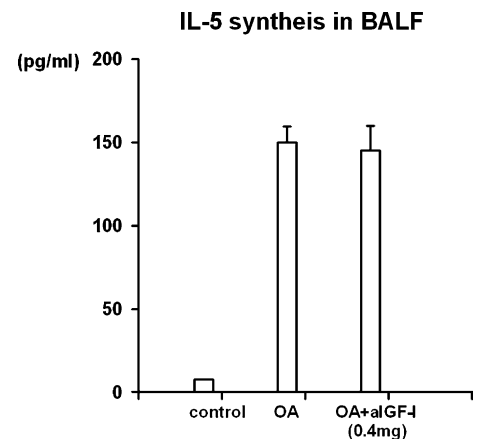


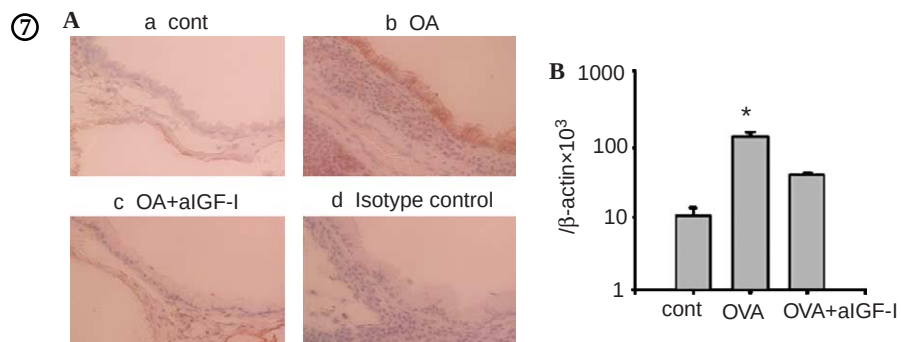
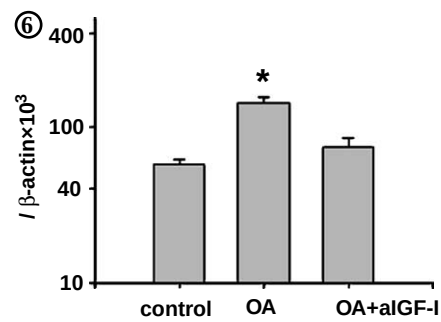
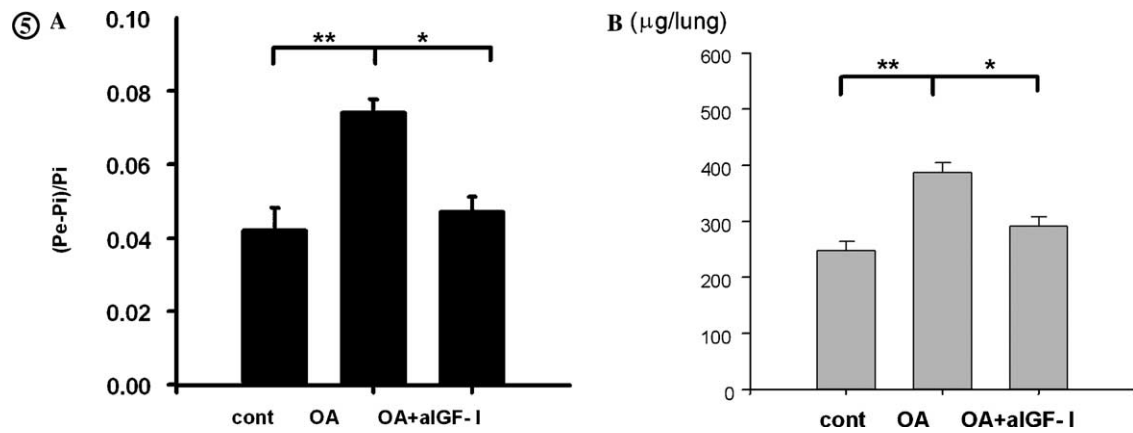
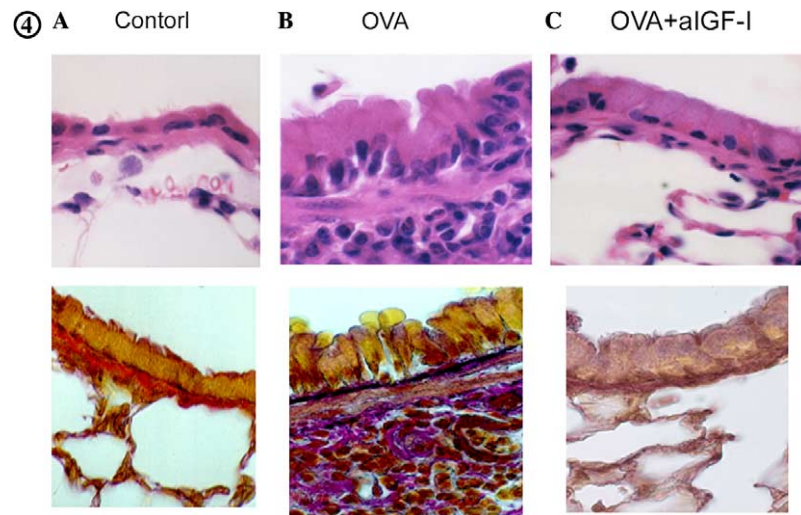
Fig. 3. Effect of IGF-I neutralizing antibody on IL-5 production. The IL-5 level in the BALF supernatant was examined using ELISA Quantikine kit (R&D System, Minneapolis, MN). Each bar consist of mean $\pm$ SEM from five samples.

Fig. 4. Effect of IGF-I neutralizing antibody on the histological characteristics of lung tissue. Sections of lung were stained with HE (upper panel) and EVG (lower panel) stain. (A) Control untreated mouse; (B) OVA inhalation and sham IgG-treated mouse; (C) OVA inhalation and anti-IGF-I-treated mouse. Original magnification: 400 $\times$.

Fig. 5. Analysis of airway wall thickness. (A) Analysis of airway wall thickening index. The sample stained with elastica van Gieson was analyzed using an image analyzer. The airway wall thickening index was calculated as the external perimeter-internal perimeter/internal perimeter of bronchioles. Data are means $\pm$ SEM of six bronchioles from at least three different sites in eight mice. * $P < 0.05$, ** $P < 0.01$. (B) Hydroxyproline content. Total lung was freeze dried and measured for weight. The hydroxyproline content was measured as described in Section 2. Control, mice with intranasal administration of saline only; OVA, mice treated with OVA + sham IgG; OVA + aIGF-I, mice treated with both OVA and anti-IGF-I. Data are means $\pm$ SEM of six mice. * $P < 0.05$, ** $P < 0.01$.

Fig. 6. mRNA expression of IGFBP-3 examined by real-time PCR. Total RNA from the lung was examined for IGFBP-3 mRNA expression. Results are shown as ratios of quantities of cytokine to β -actin mRNA. Data show means $\pm$ SEM of four lungs. * $P < 0.05$.

Fig. 7. Analysis of ICAM-1 expression. (A) Immunohistochemical analysis of ICAM-1 expression at the airway. Original magnification: 400 $\times$. a, control mice; b, OA-treated mice; c, OA + aIGF-I treated mice; d, specimen from OA-treated mice were stained by isotype control antibody. (B) ICAM-1 mRNA expression was quantified using real-time PCR. Results are shown as ratios of quantities of cytokine to β -actin mRNA. Data show means $\pm$ SEM of four lungs. * $P < 0.05$.



Airway inflammation was analyzed by BALF cell analysis. Following OVA inhalation the number of cells in BALF increased, due largely to an increase in eosinophils (Fig. 2B). In mice treated with both OVA and anti-IGF-I, the increase in BALF cell numbers was significantly blunted (Fig. 2B).

To identify the effects of anti-IGF-I on eosinophil related cytokine, we examined the level of IL-5 by ELISA. As shown in Fig. 3, IL-5 level is not influenced by anti-IGF-I antibody treatments.

3.3. Histopathological improvement is observed by IGF-I neutralization

The inflammation and airway remodeling that occurred after OVA inhalation were evaluated histologically. HE staining revealed that airway inflammation was attenuated by the administration of anti-IGF-I (Fig. 4A). Fig. 4B shows EVG-stained lung sections. Mice treated with OVA had both thickening of the airway wall and deposition of pink-stained elastic fibers. However, stained lung sections from the antibody-treated mice had fewer pink areas beneath the epithelial cells compared with sections from OVA-treated mice. When the AWT index was calculated, anti-IGF treatment induced a significant depression of the AWT index compared to treatment with sham IgG (Fig. 5A). To confirm this result, we examined the hydroxyproline content of the lung (Fig. 5B). Like wall thickness, hydroxyproline content was inhibited by IGF-I neutralization compared to OA inhalation with control IgG.

3.4. Analysis of IGF-binding protein

IGF-I activity is also controlled by IGF-binding protein (IGFBP). Reversely, it has been reported that IGF-I influences IGFBP expression [11,13,14]. Next, we examined mRNA expression of IGFBP3 by real-time PCR. An increase of IGFBP 3 following OVA inhalation was observed (Fig. 6). The IGFBP3 mRNA expression was significantly decreased by neutralization of IGF-I (Fig. 6).

3.5. Expression of ICAM-1 mRNA is reduced by IGF-I neutralization

Recruitment of inflammatory cells at the airway site was attenuated by IGF-I neutralization in addition to reduction in deposition of the ECM beneath the basement membrane. Since IGF-I has been reported to regulate expression of adhesion molecules [15,16], we explored the expression of ICAM-1 by immunohistochemically (Fig. 7A). The expression of ICAM-1 protein was observed in the endothelium and epithelium after OVA inhalation and was reduced in the IGF-I-treated

group. To quantify the expression, ICAM-1 mRNA in the lung was studied. ICAM-1 mRNA expression of ICAM-1 was significantly reduced by neutralization of IGF-I (Fig. 7B).

4. Discussion

Data from our study show that neutralization of IGF-I with antibody inhibits the airway inflammation and AWT that are involved in the remodeling process, resulting in abolishment of airway responsiveness to ACh. These anti-inflammatory effects were partly attributed to suppression of expression of adhesion molecules.

Our data show that neutralization of IGF-I, a progression factor acting on fibroblasts, diminishes subepithelial fibrosis. Components of airway remodeling, such as AWT, subepithelial fibrosis, and mucus metaplasia are related to the irreversibility of effects of asthma [2,17,18]. Subepithelial fibrosis follows the deposition of collagen and laminin that are secreted by myofibroblasts. The layer beneath the basement membrane composed of deposits of collagen and laminin [19,20] was positive for ECM deposition by EVG staining. We previously reported that subepithelial fibrosis was directly related to airway responsiveness [5], which is consistent with previous reports [21–23]. Airway smooth muscle thickening was also reported to be involved in airway remodeling in asthma and to be related to airway hyperresponsiveness [21]. Although IGF-I also has been associated with smooth muscle hyperplasia [24,25], an increase in smooth muscle mass was not obvious in our model. We concluded that the attenuation of airway responsiveness by IGF-I neutralization can be ascribed to diminution of subepithelial fibrosis. In addition, IGF-I neutralization also attenuates subepithelial fibrosis in the DEP-induced model as does PDGF neutralization (data not shown). It also has been reported anti-fibrotic treatment by anti-TGF- β can suppress airway remodeling without affecting airway inflammation [26]. These data suggested that there are direct effects on airway remodeling, although anti-IGF-I possess anti-inflammatory effects and some part of suppression of airway remodeling may be through the suppression of inflammation.

We found marked decrease in airway inflammation. For the mechanism to suppress airway inflammation, decrease in (1) Th2 cytokine and chemokine or (2) adhesion molecules, and (3) induction apoptosis can be proposed. Lymphocytes, monocytes, neutrophils, endothelial, and smooth muscles cells express IGF-1 receptors [8]. IGF-I possesses various functions on immune cells as well as acting as hormone. As shown in the Fig. 3, IL-5 level is not changed by anti-IGF-I treatment. Furthermore, when we examined cytokine profile by cDNA array, we did not observe suppression in the levels of IL-4, 5, and 13, which are primarily derived from Th2 cells, by anti-IGF-I

treatment (data not shown), suggesting that the first possibility is very low. We found the decrease in ICAM-1 expression after neutralization of IGF-I, suggesting that the second possibility is likely. It has been reported that IGF-I is associated with ICAM-1 gene expression [15,16]. The dose-dependent administration of IGF-I directly increased ICAM-1 expression on endothelial cells [15]. In addition, the neutralization of ICAM-1 suppressed eosinophil migration to the airway in a monkey model for asthma [27]. Thus, the anti-inflammatory effects of IGF-I neutralization can be ascribed partly to the attenuation of ICAM-1 expression. It has also been known that IGF-I prevents apoptosis, and that interfering with IGF-I results in induction of apoptosis [10,28]. Thus, induction of apoptosis is another possibility of anti-inflammatory effects of IGF-I. This possibility is now under investigation.

We also found that IGFBP3 mRNA increased in mice that inhaled OVA and decreased after IGF-I neutralization. It has been reported that IGF-I-induced IGFBP expression. Among many IGFBPs we studied IGFBP3, because it has been reported to be related to pulmonary diseases and airway smooth muscles [13,25]. Idiopathic pulmonary fibrosis, which is caused by a deposition of interstitial matrix, is accompanied by a spontaneous increase of both IGF-I and IGFBP3 synthesis [29–32]. Thus, the increase in IGFBP3 also may be related to the increase in the extracellular matrix. IGFBP also controls the action of IGF-I; for example, both IGF-I or IGF-I/IGFBP3 complexes will delay the onset of experimental autoimmune encephalomyelitis [16]. Thus, augmentation of IGFBP3 mRNA results in an increase of IGF-I/IGFBP3 complexes and amplifies the effects of IGF-I on OVA-induced airway inflammation and remodeling.

To modulate the IGF-I axis, not only administration of IGF-I neutralizing antibody but also neutralization of IGF-IR and IGFBP have been explored and successful modulation of the IGF-I axis has been reported in cancer and neurogenic diseases [33,34]. In addition, intracellular signal modulation has been reported [35,36]. Our data support the possibility that modulation of the IGF-I axis may control allergic inflammation and remodeling in asthma.

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