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ORIGINAL ARTICLE

Increased Levels of CTGF mRNA Expression in a Murine Model of Allergic Airway Inflammation

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ABSTRACT

Background: Connective tissue growth factor (CTGF) is known to play a direct role in fibrosis in various organs as a downstream mediator of TGF-β.

Objective: To evaluate a role in subepithelial fibrosis in the asthmatic airway, we investigated CTGF mRNA expression and CTGF producing cells in the airways of a murine asthma model with allergic inflammation.

Methods: After repetitive inhalation challenges with ovalbumin (OVA), cell numbers and TGF-β1 concentrations in bronchoalveolar lavage fluid from immunized mice were measured. Collagen deposition in lung tissue was estimated by measuring hydroxyproline content. CTGF mRNA and GAPDH mRNA levels were determined by quantitative RT-PCR method. Immunohistochemistry for CTGF with anti-CTGF antibody was performed.

Results: Numbers of eosinophils and TGF-β1 concentration increased markedly in BALF on the 7th day and 14th day after inhalation challenge with OVA. Hydroxyproline content in lung tissue increased significantly on the 14th day after inhalation challenge of OVA compared to control. The ratio of CTGF mRNA /GAPDH mRNA in lung tissue in mice exposed to OVA increased 10-fold compared to those exposed to saline. Immunohistochemistry revealed that the number of CTGF-positive cells increased in bronchial submucosa after inhalation challenge of OVA.

Conclusions: Our results suggested that CTGF might be one of the potential molecules involved in subepithelial fibrosis in murine airways with allergic inflammation.

KEY WORDS

asthma, collagen, remodeling, subepithelial fibrosis, TGF-β

INTRODUCTION

Airway remodeling in asthma has been characterized by goblet cell hyperplasia, subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia and submucosal gland hypertrophy/hyperplasia. These structural changes result in thickening of the airway wall which induces the maximal degree of airway narrowing when the smooth muscle constricts. In this regard, the airway remodeling has been thought to contribute to the development of airway hyperresponsiveness and the progressive impairment of lung function over time.[1,2]

To elucidate the mechanism of airway remodeling in asthma, roles of several growth factors in the structural changes have been evaluated in asthmatic airways and in experimental animal models with bronchial asthma. Several reports have suggested that thickening of the reticular lamina results from interstitial collagen and fibronectin deposition.[3,4]

Transforming growth factor-β (TGF-β) is a potent profibrotic cytokine which stimulates fibroblasts to promote the synthesis and secretion of collagens and other extracellular matrix proteins. Ohno *et al.* dem-

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onstrated that eosinophils express TGF-β mRNA in the airway of patients with severe asthma.[5] Minshall *et al.* reported TGF-β mRNA expression correlated with the thickness of the airway basement membrane.[6] These results suggested TGF-β may play a role in subepithelial fibrosis.

Connective tissue growth factor (CTGF), which is a cysteine-rich 38-kDa mitogenic peptide, functions as a downstream mediator of TGF-β action in connective tissue cells.[7,8] Previous studies demonstrated that there were CTGF-dependent and CTGF-independent signaling pathways activated by TGF-β in fibroblasts.[9] It is known that CTGF expression was upregulated in bleomycin-induced lung fibrosis, and idiopathic pulmonary fibrosis, suggesting that CTGF may play a role in pulmonary fibrosis.[10-12]

However, to date, little is understood of the roles of CTGF in airway remodeling in asthma. To extend the findings and investigate whether CTGF is involved in subepithelial fibrosis in asthma, we studied the immunohistochemical localization of CTGF protein, and analyzed the quantitative CTGF mRNA expression with experimental murine asthma model sensitized with ovalbumin (OVA).

METHODS

ANIMALS

Female BALB/c mice (6–8 wk old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under specific pathogen-free conditions following a 12-hour light-dark cycle and fed a standard laboratory diet and given water *ad libitum*. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animal Science in 1987.

IMMUNIZATION AND AEROSOLIZATION PROTOCOL

The mice were sensitized according to the methods described in a previous paper.[13] In brief, mice were sensitized at days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 μg OVA (Sigma Chemical Co., St. Louis, MO) adsorbed overnight at 4°C to 4 mg of aluminium hydroxide (Wako Chemical Co., Tokyo, Japan) in phosphate-buffered saline (PBS). 12 days later, mice were divided into 5 groups which consisted of 6–8 animals. One group of mice was killed for analysis as a control without inhalation. Two groups of the mice were placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 h. In the case of controls, the other 2 groups of the mice were exposed to 0.9% saline only. The aerosolized OVA was produced by Pulmo-Aide Compressor/Nebulizer (Devilbiss) (Sunrise Medical HHG, Inc, Somerset, PA) at a flow rate of 5–7 liter/min.

COLLECTION AND MEASUREMENT OF SPECIMENS

After being exposed to aerosolized saline or OVA every day over periods of 6 and 13 days, each group of mice was killed on the 7th and the 14th day, 24 hours after final inhalation, and bronchoalveolar lavage fluid (BALF) and lung tissue were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The right lungs were lavaged twice with 0.5 ml PBS, and–0.8 ml of the instilled fluid was consistently recovered. The recovered fluid was centrifuged (300 × *g* for 6 min) and the cells were resuspended in 0.5 ml PBS. The total number of cells was counted using an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made of each sample containing 10,000 cells by cytospin (Cytocentrifuge, Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard error of the mean (SEM) for each treatment group.

After centrifugation, supernatants were stored at – 80°C for measurement of TGF-β1 by ELISA. After harvesting BALF, the right lungs were frozen at – 80°C for measurement of hydroxyproline. The sections of left lungs were fixed with periodate-lysin-paraformaldehyde solution at 4°C, and were embedded in OTC-compound in liquid nitrogen for immunohistochemistry for CTGF. The other lung tissues were fixed with formaldehyde and were embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin eosin (HE), and Elastica Masson's trichrome (EM). In addition, the other parts of left lungs were stored at – 80°C for RNA extraction.

ISOLATION OF TOTAL RNA AND REAL-TIME QUANTITATIVE PCR

Total RNA from tissues was obtained using ISOGEN (Wako Pure Chemicals, Osaka, Japan), quantitated by spectrophotometry. The quality of the obtained RNA was examined by agarose gel electrophresis.

To quantify the mRNA of CTGF and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) expression in murine lungs, quantitative PCR was carried out using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) as previously described.[14]

Oligonucleotide PCR primer pairs and fluorogenic probes for murine CTGF were designed from the published sequences using Primer Express software (Perkin-Elmer) (sense primer ; 5'-GTCAACCTCAGACACTGGTTTCG-3', antisense primer ; 5'-CCACTGTTCCAGGAGACTCACC-3', Taqman probe ; 5'-[FAM] TCATTAGCGCACAGTGCCAGAACGC [TAMRA]-3').[15] Primers and labeled probe (VIC) for

Table 1 Total cell number and cell differential in BALF of mice after saline and OVA inhalation

	saline, 7th day	saline, 14th day	OVA, 7th day	OVA, 14th day
total cells ($\times 10^4$/ml)	12.1 ± 2.3 (n = 6)	22.1 ± 5.5 (n = 6)	297.3 ± 68.8* (n = 6)	302.3 ± 47.0 * (n = 6)
alveolar macrophages ($\times 10^4$/ml)	10.4 ± 0.75 (85.6 ± 6.2%)	20.2 ± 1.66 (91.4 ± 7.5%)	69.9 ± 20.8* (23.5 ± 7.0%) *	73.2 ± 21.5* (24.2 ± 7.1%)*
lymphocytes ($\times 10^4$/ml)	1.74 ± 0.75 (14.4 ± 6.2%)	1.9 ± 1.7 (8.6 ± 7.5%)	24.7 ± 7.4* (8.3 ± 2.5%)	22.1 ± 10.6* (7.3 ± 3.5%)
eosinophils ($\times 10^4$/ml)	< 0.1%	< 0.1%	203.1 ± 21.7* (68.3 ± 7.3%)*	207.1 ± 21.2* (68.5 ± 7.0%)*
neutrophils	< 0.1%	< 0.1%	< 0.1%	< 0.1%
epithelial cells	< 0.1%	< 0.1%	< 0.1%	< 0.1%

The number of cells in BALF on 7th day and 14th day after saline and OVA inhalation in mice sensitized with OVA. BALF was collected as described in "Methods". Values were expressed as the means ± SEM of 6 mice. *p < 0.01 relative to the respective value of saline inhalation

rodent GAPDH were purchased from Perkin-Elmer Applied Biosystems. Briefly, 100 ng of RNA dissolved in 10 µl of water from each aliquot of murine lung tissue were denatured at 90°C for 90 s. Each RNA sample (100 ng/10 µl of water) was mixed in 40 µl of buffer containing the following reagents for the one-step RT-PCR reaction : 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 5 mM MgCl2, 100 nM sense primer, 100 nM antisense primer, 0.3 mM deoxynucleoside triphosphate (Boehringer), 0.4 U/µl RNase inhibitor (Promega), 0.4 U/µl Moloney murine leukemia virus RT (Perkin Elmer), 0.0025 U/µl *Taq* Gold Polymerase (Perkin Elmer), and 100 nM Taqman probe as described above. The fragment of mRNA for CTGF and GAPDH was reverse transcribed into cDNA (30 min at 48°C) and amplified by PCR for 40 cycles (15 s at 95°C and 1 min at 60°C). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (C), which is predictive of the quantity of an input target fragment.[16] The standard curve was obtained between the fluorescence emission signals and C by means of duplicated serial dilutions of the total RNA from murine lung in medium alone. Expression of CTGF mRNA was normalized to a constitutive expression of GAPDH mRNA.

ANTIBODIES AND IMMUNOHISTOCHEMICAL STAINING

We adopted the biotin-streptavidin system using a Histofine Kit (Nichirei, Tokyo, Japan) for immunohistochemical staining. The sections were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. The sections were incubated with 10% normal rabbit serum for 30 minutes at room temperature to block the non-specific antibody reaction. We used anti-human CTGF rabbit polyclonal antibody which also reacted with murine CTGF.[12] This cross reactivity was confirmed on western blotting (data not shown). The sections were incubated overnight at 4°C with the primary antibodies. 3'3-diaminobenzidine (DAB) was used as the chromogenic substrate.

TGF-β1 ASSAY

Murine TGF-β1 concentration in BALF was measured according to the steps described in a previous paper.[17] BALF was applied to 96-well microtiter plates coated with chicken anti-human TGF-β1antibody (5 µg/ml) (R & D Systems, Minneapolis, MN). Bound TGF-β1 was detected by monoclonal anti-TGF-β1 (1 µg/ml) (Genzyme), followed by peroxidase-labeled goat anti-mouse IgG (1 µg/ml) (Kierkegaard & Perry Laboratories, Gaithersburg, MD) and tetramethylbenzidine reagent (Kierkegaard & Perry). In this assay, concentration of total TGF-β1 including both active and inactive molecules was measured. Optical densities were determined at 450 nm and converted to concentrations (ng/ml) according to the standard curve obtained with titrated concentrations of human recombinant TGF-β1 (R & D Systems). The lower limit of this method was 7.0 pg/ml.

HYDROXYPROLINE ANALYSIS

Total collagen content of the right lung was estimated by an assay of hydroxyproline.[18] Briefly, after acid hydrolysis of the lung was completed with 6 N HCl at 110°C for 16 h in a sealed glass tube (Iwaki, Tokyo, Japan), 50-µl aliquots were added to 1 ml of 1.4% chloramine T (Sigma Chemical Co., St. Louis, MO), 10% *n*-propanol, and 0.5 M sodium acetate, pH 6.0. After 20 min of incubation at room temperature, 1 ml of Erlich's solution (1 M *p*-dimethylaminobenzaldehyde in 70% *n*-propanol, 20% perchloric acid) was added and allowed to incubate at 65°C for 15 min. Absorbance was measured at 550 nm and the amount of hy-

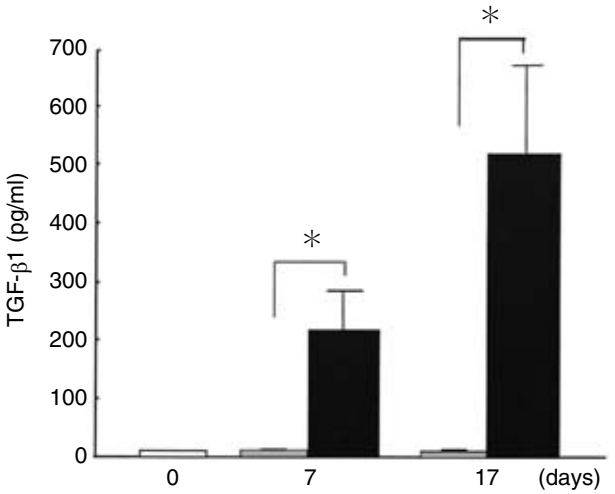

Fig. 1 TGF-β1 concentration in BALF after antigen inhalation in BALB/c mice sensitized with OVA. As described in "Methods", after the exposure to aerosolized saline or OVA every day over periods of 6 or 13 days, mice were killed on the 7th and the 14th day, and BALF was collected. Values are expressed as the means ± SEM of 6–8 mice. A value less than 7.0 pg/ml could not be detected. Open column: sensitized mice with OVA before inhalation of OVA (0 day); gray column: sensitized mice with OVA after saline; black column: sensitized mice with OVA after inhalation of OVA. * $p < 0.001$

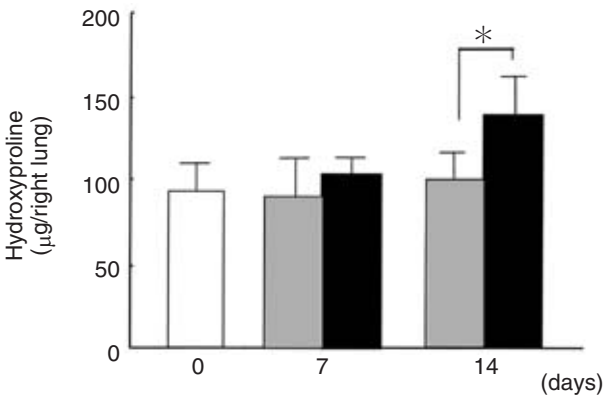

Fig. 2 The content of hydroxyproline in the lungs after antigen inhalation in mice sensitized with OVA. As described in "Methods", mice were exposed to aerosolized saline or OVA successively for 6 or 13 days, and the lungs were excised from these mice 24 hour after final inhalation of saline or OVA. Values are expressed as the means ± SEM of 6 mice. Open column: sensitized mice with OVA before inhalation of OVA; gray column: sensitized mice with OVA after saline; black column: sensitized mice with OVA after inhalation of OVA. * $p < 0.001$

droxyproline was determined against a standard curve generated using known concentrations of reagent hydroxyproline (Sigma Chemical Co.).

STATISTICAL ANALYSIS

Mann-Whitney *U* Test was used in the analysis of results. All values are expressed as means ± SEM. Values of $p < 0.05$ were considered statistically significant.

RESULTS

NUMBERS OF TOTAL CELLS AND EOSINOPHILS IN BALF OF SENSITIZED MICE AFTER INHALATION CHALLENGE OF OVA

While total numbers of cells and eosiophils in BALF of OVA-sensitized mice showed no change on the 7th and 14th day after saline inhalation, in contrast, these increased markedly on the 7th and 14th day after inhalation challenge with OVA (Table 1).

TGFβ1 IN BALF OF SENSITIZED MICE AFTER INHALATION CHALLENGE OF OVA

TGFβ1 concentration was very low without inhalation on the 7th and 14th day in BALF from OVA-sensitized mice after inhalation of saline. In contrast, TGFβ1 concentration increased markedly on the 7th and 14th day in BALF from OVA-sensitized mice after inhalation of OVA (Fig. 1).

COLLAGEN CONTENT IN LUNG TISSUES OF SENSITIZED MICE AFTER INHALATION CHALLENGE OF OVA

Determination of collagen content in lung tissues was expressed as hydroxyproline content in the tissue. Hydroxyproline content increased significantly on the 14th day in the lung tissues from OVA-sensitized mice after inhalation of OVA, compared to those from OVA-sensitized mice after inhalation of saline (Fig. 2).

CTGF mRNA LEVEL IN LUNG TISSUES FROM OVA-SENSITIZED MICE AFTER INHALATION OF OVA

CTGF mRNA levels were determined by quantitative RT-PCR as described in "Methods". The ratios of CTGF mRNA/GAPDH mRNA were calculated to examine the change of CTGF mRNA level in the lung from OVA-sensitized mice after inhalation of OVA. The ratios of CTGF mRNA/GAPDH mRNA did not change in the lung of OVA-sensitized mice after inhalation of saline. In contrast, these greatly increased on the 7th and 14th day in the lung of OVA-sensitized mice after inhalation of OVA (Fig. 3).

HISTOLOGICAL CHANGES IN AIRWAYS OF SENSITIZED MICE AFTER INHALATION OF OVA

After inhalation of OVA, dramatic histological changes were observed in the airways of sensitized

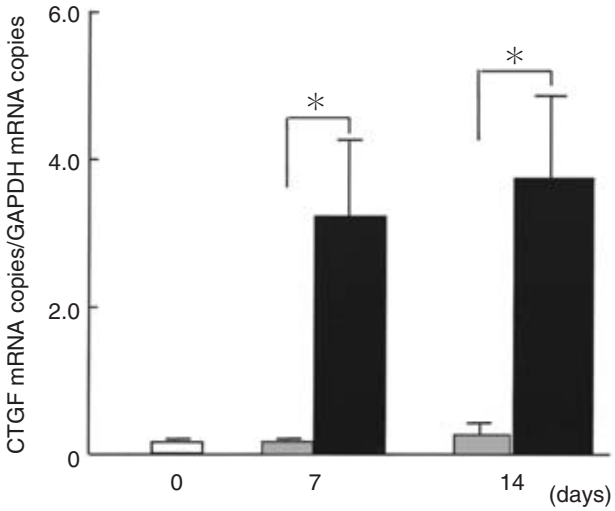

Fig. 3 Expression of CTGF gene in lung tissues after allergen inhalation. The amount of CTGF mRNA in lung tissues was quantified by real-time RT-PCR as described in "Methods", and the expression of CTGF genes is represented as the ratio of CTGF-specific mRNA copy numbers to those of the GAPDH (housekeeping gene). Values are expressed as the means ± SEM of 6 mice. Open column: sensitized mice with OVA before inhalation of OVA; gray column: sensitized mice with OVA after saline; black column: sensitized mice with OVA after inhalation of OVA. * $p < 0.001$

mice. Submucosal cellular infiltration including eosinophils and mononuclear cells was observed in submucosa of bronchi and bronchioles (Fig. 4B,4C). Goblet cell metaplasia occurred in bronchi and bronchioles within 7 days after inhalation of OVA, and almost all epithelial cells changed into goblet cells on the 14th day after the inhalation. Fibrous materials in submucosa of bronchi also became marked on the 14th day after the inhalation as expressed as blue color in Elastica Masson's trichrome staining (Fig. 4 F,4G). On the other hand, fibrotic changes were not observed in alveolar septum. Increase of bronchial muscle layer became prominent in submucosa.

After 14-day inhalation of saline to sensitized mice, there were few goblet cells in epithelium and a small number of infiltrated cells in airway submucosa (Fig. 4D,4H).

IMMUNOHISTOCHEMISTRY FOR CTGF

Some bronchial epithelial cells were weakly stained in both groups of mice after inhalation of saline (Fig. 5A). However, CTGF positive epithelial cells were much more prominent in mice after inhalation of OVA (Fig. 5B) compared to those after inhalation of saline. Many positive cells were seen in the submucosa of bronchi after OVA inhalation. Smooth muscle cells and infiltrated round cells were positively stained in submucosa of bronchi on the 14th day in the lungs of OVA-sensitized mice after inhalation of OVA (Fig. 5C). Spindle-like cells were also positively stained in fibrotic tissue in the submucosa of bronchi on the 14th day after inhalation of OVA (Fig. 5D).

In Panel E treated with normal rabbit serum instead of anti-CTGF antibody, no positive cells were detected in airway tissue.

DISCUSSION

The present study demonstrated the increase of CTGF mRNA level in the lung of OVA-sensitized mice after repetitive inhalation of OVA. In this murine asthma model, structural changes occurred, such as goblet cell metaplasia and collagen deposition in bronchial submucosa after 13 days of repetitive OVA inhalation. These results suggest CTGF may play a role in collagen deposition in the bronchial wall in a murine model of allergic airway inflammation.

Thickening of basement membrane in the central airway, which is widely recognized as one of the structural changes characteristic in asthma, has been attributed to deposition of extracellular matrix molecules including collagen induced by fibroblast activation.[3] In our murine model, histological study with EM staining demonstrated an increased level of fibrous proteins in bronchial submucosa on 7th and 14th day after antigen inhalation. In addition, collagen content also increased on the 14th day after antigen inhalation. We speculated that the delay of the increase of hydroxyproline content in the lung tissue compared to that of TGF-β1 in BALF was partially due to the time required for the process including activation of TGF-β signaling, collagen gene expression, collagen synthesis, collagen secretion and collagen deposition in interstitium. In this time course, no fibrotic change was detected in alveolar tissue. We thought that allergic reactions mainly occurred in bronchi and bronchioles due to the deposition of OVA, and this was the reason why fibrotic response possibly induced by TGF-β1 produced from inflammatory cells was observed in the airway not in alveolar tissue. These results suggested that subepithelial fibrosis in the airway proceeds after14 days with repetitive daily antigen inhalation in the airway of OVA-sensitized mice.

TGF-β, a potent fibrogenic cytokine, has been involved in tissue fibrosis, including pulmonary fibrosis.[19,20] TGF-β has also been suggested to play an important role in subepithelial fibrosis by the following evidence : (a) TGF-β is detected in exaggerated quantities in asthmatic BAL fluid before and after antigen challenge ; (b) TGF-β is produced by eosinophils and fibroblasts from patients with severe and mild asthma and (c) TGF-β1 expression correlates with basement membrane thickness and fibroblast number and/or disease severity.[5,6,21,22] The present study confirmed the increase of TGF-β1 in BALF during antigen inhalation to sensitized mice with OVA, suggesting that

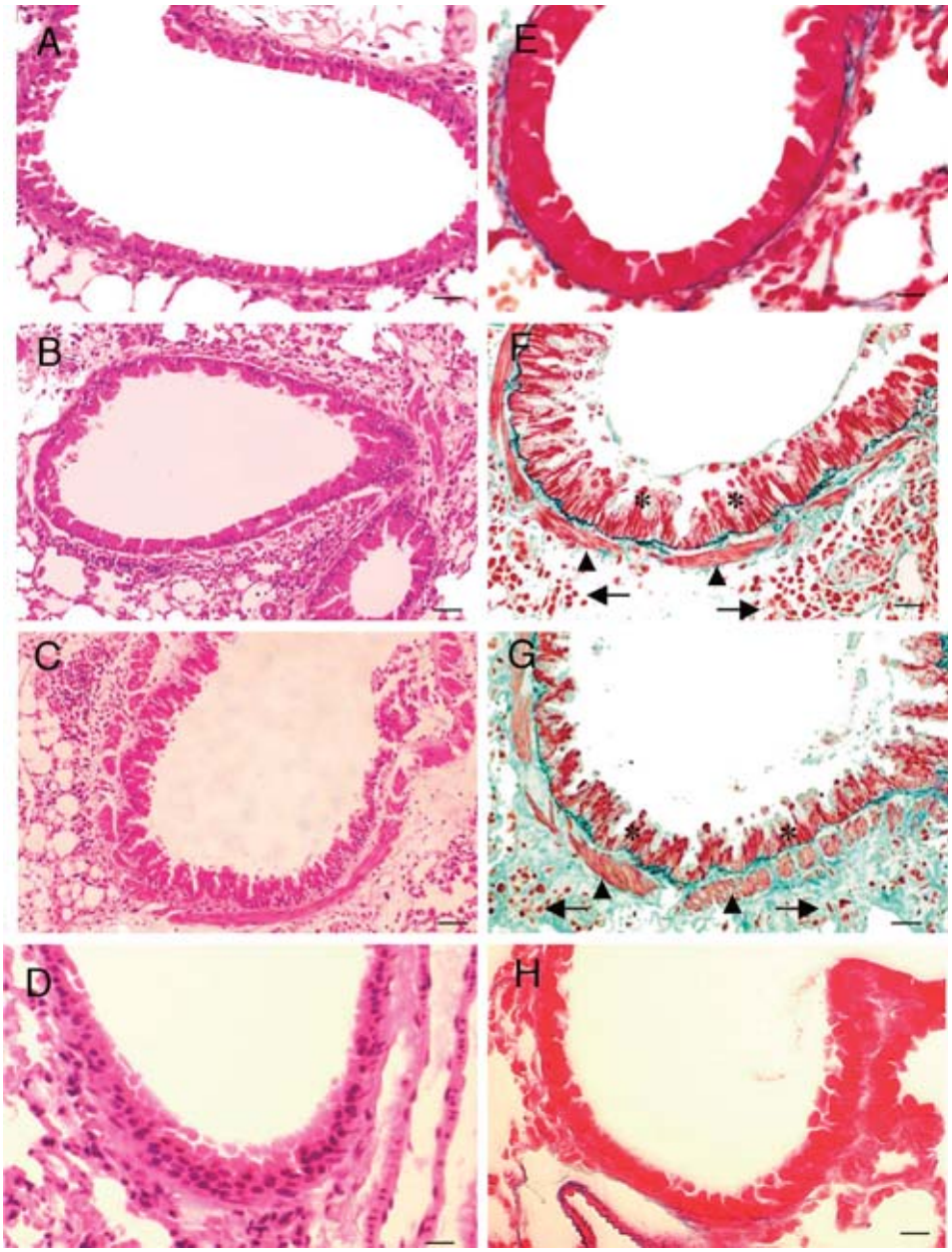

Fig. 4 Histological changes in sensitized murine lungs after inhalation of OVA. Panels **A**, **B**, **C** and **D** are sections with HE staining (× 20 magnification), and **E**, **F**, **G** and **H** are sections stained with EM (× 25). Panels **A** and **E**: Bronchial tissue from the sensitized mouse before OVA inhalation; Panels **B** and **F**: Bronchial tissue from the sensitized mouse on the 7th day after OVA inhalation; Panels **C** and **G**: Bronchial tissue from the sensitized mice on the 14th day after OVA inhalation. Panels **D** and **H**: Bronchial tissue from the sensitized mice on the 14th day after saline inhalation. In Panels **B** and **C**, submucosal cellular infiltration was prominent (internal scale bar = 50 μm). In Panel **E**, no goblet cell was observed in bronchial epithelium. In contrast, almost all bronchial epithelial cells were replaced by goblet cells (*) in Panels **F** and **G**. In Panels **F** and **G**, bronchial epithelial basement membrane, colored blue, was prominent and submucosal collagen deposition expressed by a blue color appeared to be increased in Panel **F** (internal scale bar = 40 μm). In Panels **D** and **H**, there were few goblet cells in epithelium and a small number of infiltrated cells in airway submucosa. In addition, submucosal collagen deposition was not detected. Arrows indicate infiltrated cells and arrow-heads indicate smooth muscle.

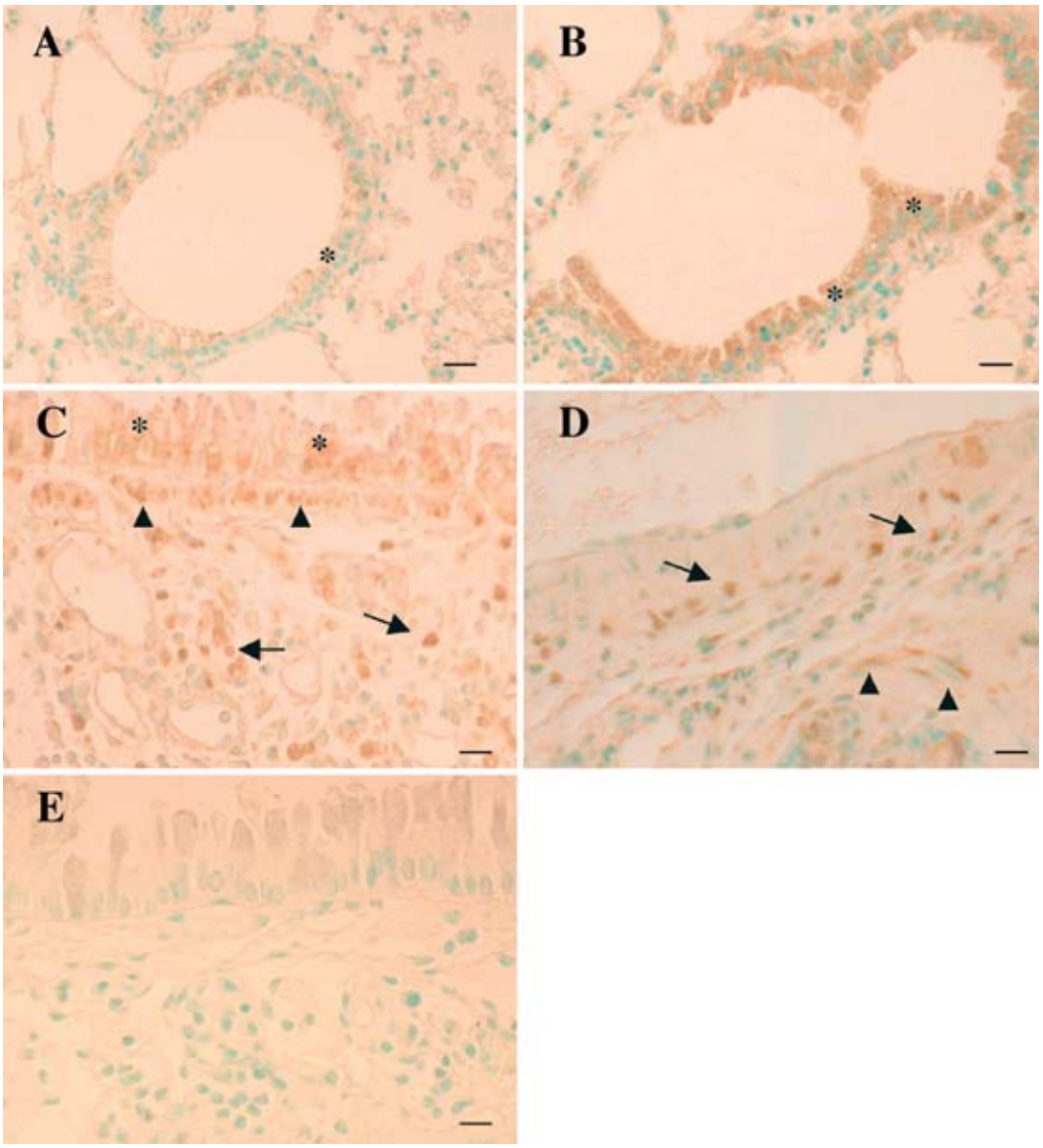

Fig. 5 Immunohistochemistry for CTGF. Bronchial tissues excised from the sensitized mice were stained with anti CTGF antibody as described in "Methods". Positive signals were expressed by a brown color. Panel **A**: on the 7th day after daily saline inhalation (× 20); Panel **B**: on the 7th day after daily OVA inhalation (× 20); Panel **C** and Panel **D**: on the 14th day after daily OVA inhalation (× 50). Panel **E**: bronchial tissue on the 14th day after daily OVA inhalation (×50) as a control with use of normal rabbit serum for blocking and without anti CTGF antibody. In Panels **A**, a small number of cells were weakly stained and sparse in bronchial epithelium (*). In Panel **B**, almost all bronchial epithelial cells (*) were stained strongly (internal scale bar = 50 μm). In Panel **C**, epithelial cells (*), submucosal smooth muscle (arrow-heads) and infiltrated cells (arrows) were stained. In Panel **D**, round cells (arrows) and spindle-like cells (arrow-heads) were stained in the airway submucosa (internal scale bar = 20 μm). In Panel **E**, no positive cells were detected in airway submucosa, suggesting that normal rabbit serum did not stain falsely positive.

TGF-β may play an important role in collagen deposition in bronchial submucosa of mice with allergic inflammation.

CTGF is a cysteine-rich secretory protein of 36–38 kDa, which is composed of 349 amino acid residues, and its gene belongs to the CCN family.[23,24] Because of its ability to induce the expression of the extracellular matrix (ECM) molecules, CTGF has been pro-

posed to play an important role in connective tissue cell proliferation and ECM deposition as one of the mediators of TGF-β.[25] Increased CTGF expression has been shown in a variety of human and experimental diseases characterized by fibrosis, including studies in the kidney, skin, blood vessels, lung, and liver.[10,12,26,27] This present study demonstrated the increase of CTGF mRNA levels with real time quantitative PCR and the increased number of CTGF containing cells in airways of our murine model. Combined with previous results, our findings indicated that CTGF may play a role in collagen deposition in bronchial submucosa of experimental murine model with allergic airway inflammation.

Recently, Abreu *et al.* reported that CTGF directly binds TGF-β1 through its cysteine-rich domain and potentiates TGF-β1 receptor binding and signaling.[28] This new type of interaction between CTGF and TGF-β1 may work widely in tissue fibrosis of multiple organs including pulmonary fibrosis in which simultaneous induction of CTGF and TGF-β1 were evaluated. In our model, induction of TGF-β1 protein was evaluated and induction of CTGF protein was strongly suggested because of up-regulation of CTGF mRNA level. In the present study, we could not measure CTGF protein concentration because an appropriate anti-mouse CTGF antibody was not available. However, to evaluate the interactions between CTGF and TGF-β1, both CTGF and TGF-β1 protein levels need to be studied.

Immunohistochemical staining demonstrated localization of CTGF protein in murine airways when exposed to saline or OVA. We used anti-human CTGF rabbit polyclonal antibody which also reacted with murine CTGF. Bronchial epithelial cells in mice exposed to saline for 6 or 13 days were weakly stained with anti-CTGF antibody. We also could detect CTGF mRNA expression in lung tissues of the mice exposed to saline for 6 or 13 days. These results suggested that bronchial epithelial cells constitutively express CTGF mRNA and produce CTGF. In contrast to the mice exposed to saline, bronchial epithelial cells were strongly stained in those exposed to OVA. In addition, round cells and spindle-like cells in submucosa were positively stained. Smooth muscle cells were also found to be positively stained in bronchial submucosa. Fan *et al.* reported that CTGF was expressed in vascular smooth muscle cells and might play a role in migration and proliferation of these cells.[30] Recently Burgess *et al.* reported that TGF-β specifically induces mRNA and protein for connective tissue growth factor in airway smooth muscle, and the connective tissue growth factor response is greater in airway smooth muscle cultured from patients with asthma compared with patients without asthma.[31] This result suggested that CTGF may also play a role in migration and proliferation of airway smooth muscle cells in asthma.

Ricupero *et al.* reported that PGE_2 reduced mRNA and protein level of CTGF in fibroblasts stimulated by TGF-β.[32] This down-regulation of CTGF mRNA level was thought to be induced by elevated intracellular cAMP levels.[30] On the other hand, Rishikof *et al.* reported that IL-4 attenuated the TGF-β-stimulated induction of CTGF mRNA expression in human lung fibroblasts.[33] Having taken these results into consideration, CTGF mRNA level was thought to be influenced by PGE2 and IL-4 produced in murine allergic airway inflammation. In addition, we reported recently that myofibroblasts contained CTGF in fibrotic pulmonary tissue of patients with idiopathic pulmonary fibrosis.[12] This result suggested spindle-like cells in submucosa to be myofibroblasts. On the other hand, round cells were not evaluated. To date, TGF-β is known to be produced by bronchial epithelial cells, eosinophils, macrophages and fibroblasts, especially myofibroblasts. These consequences suggested TGF-β produced by these cells may be involved in the induction of the CTGF gene in the airway in an autocrine or paracrine manner. Furthermore, it is feasible to assume that CTGF overproduced in the airway may be involved in proliferation and activation of fibroblasts which cause extracellular matrix deposition in the submucosa of the airway.

In conclusion, CTGF may be one of potential molecules involved in subepithelial fibrosis in murine airways with allergic inflammation.

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