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Originals

Leukotriene C_4 in Combination with Transforming Growth Factor - β Augments Extracellular Matrix Production from Human Lung Fibroblasts

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SUMMARY

Airway remodeling has an important role in the pathogenesis of bronchial asthma and is associated with severe, intractable disease. A lot of mediators, especially cysteinyl leukotrienes (CysLTs) and transforming growth factor (TGF) - β that influence pathophysiology of bronchial asthma are involved in the formation of airway remodeling. We hypothesized that responsiveness of fibroblasts to CysLTs is not the same as that of activated myofibroblasts induced by TGF - β. We examined the effects of leukotriene C_4 (LTC_4) on collagen production from fibroblasts and myofibroblasts transformed by TGF - β. Furthermore, we investigated mRNA expression of CysLT1 receptor and α - smooth muscle actin (α - SMA) as a marker of myofibroblast. TGF - β_1 stimulated collagen production from human fetal lung fibroblasts (HFL - 1), but LTC_4 alone did not. Meanwhile, LTC_4 in combination with TGF - β_1 enhanced collagen production as compared with TGF - β alone. Real time PCR revealed that stimulation with TGF - β significantly upregulated CysLT1R and α - SMA mRNA expression in HFL - 1. LTC_4 increased the collagen production through upregulation of CysLT1R induced by TGF - β. In the TGF - β - rich milieu such as asthma, activated myofibroblasts expressing CysLT1R can respond to CysLTs and produce abundant extracellular matrix thereby forming airway remodeling. These data suggest that leukotriene receptor antagonists may be an effective therapeutics for preventing airway remodeling in asthmatics.

Key Words : cysteinyl leukotrienes, CysLT1 receptor, growth factor, fibroblast, airway remodeling

INTRODUCTION

Airway remodeling is an established pathological feature in bronchial asthma. It includes deposition of subepithelial collagen, hypertrophy of bronchial smooth muscle and transformation of fibroblasts to myofibroblasts [1]. The transformed myofibroblasts produce extracellular matrix proteins including collagen and fibronectin in large quantities, and participate in formation of subepithelial hypertrophy and fibrosis [2, 3]. Even slight contraction of airway smooth muscle can substantially increase airway resistance in patients with remodeled airways and increased airway wall thickness [4]. Therefore, airway remodeling is thus associated with severe, intractable disease in patients with asthma.

Many mediators that influence pathophysiology of bronchial asthma are involved in the formation of airway remodeling. Cysteinyl leukotrienes (CysLTs) including leukotniene C_4 (LTC_4), LTD_4, and LTE_4 produced by eosinophils and mast cells play an important role in the pathophysiology of bronchial asthma. The levels of CysLTs are elevated in the asthmatic patient. The eosinophils of bronchial asthmatics synthesize significant-

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ly more LTC_4 than the eosinophils from healthy individuals (five to ten-fold)[5]. A large number of studies have shown increased level of CysLTs in bronchoalveolar lavage fluid (BALF) and urine samples of patients with asthma[6~8]. Besides CysLTs mainly act as smooth muscle contractants[9, 10], they have multifunction such as mucus hypersecretion[11~13], increased microvascular permeability[12, 14, 15], decreased ciliary movement[16, 17] and eosinophil recruitment[17~20].

Recently, CysLTs have been shown to be involved in the pathogenesis of airway remodeling in an animal model of asthma[21]. CysLTs have been reported to induce airway smooth muscle proliferation when combined with an appropriate growth factor such as epidermal growth factor[22]. However, few studies have examined the role of CysLTs on airway wall fibrosis.

Transforming growth factor (TGF)-β plays a central role in the pathogenesis of airway remodeling in asthmatics. TGF-β stimulates proliferation and chemotaxis of fibroblasts and smooth muscle cells[23]. It also stimulates production of extracellular matrix (ECM) molecules such as collagen. Furthermore, TGF-β can induce myofibroblast differentiation from the fibroblasts[2, 3]. Myofibroblasts are involved in subepithelial fibrosis of the airway in asthma[24~27] because they can produce a large amount of ECM proteins. Asthmatic individuals exhibit a greater expression of TGF-β mRNA in the airway submucosa and eosinophil than healthy control subjects[28~30].

In the real world, a large number of fibroblasts are present in the airway of asthmatic patients, and the production of CysLTs and TGF-β are increased. Under these conditions, both mediators may act synergistically to form airway remodeling. We hypothesized that responsiveness of fibroblasts to CysLTs is altered from that of activated myofibroblasts induced by TGF-β. Therefore, we examined the effects of CysLTs on cell proliferation of fibroblasts and myofibroblasts transformed by TGF-β and collagen production by both cells. We report here that LTC_4 enhances collagen production by TGF-β-induced myofibroblasts but not by nonstimulated fibroblasts. It was founded that CysLT1 receptor-dependent mechanism was involved in this response.

MATERIALS AND METHODS

Cell culture

Human fetal lung fibroblasts (HFL-1, passage 16 – 20) were obtained from the American Type Tissue Culture Collection (Rockville, MD). HFL-1 cells were seeded in 12-well tissue culture plates at a density of 1×10^5 cells/ml for procollagen type I carboxy-terminal peptide (PIP) enzyme immunoassay (EIA) and in 96-well tissue culture plates at a density of 2×10^4 cells/cm^2 for proliferation assay. HFL-1 cells were cultured at 37℃ in a 5% CO_2-humidified incubator in Ham's F12K medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS).

Cell proliferation assay

We evaluated 5-bromo-2′-deoxyuridine (BrdU) incorporation by HFL-1 cells to assess cell proliferation. HFL-1 cells were cultured until subconfluence. The medium was then replaced with FBS-free F12K. After culture for 48 h, the cells were washed twice and cultured for another 24 h in the presence or absence of various concentrations of LTC_4 (Cayman Chemical, Ann Arbor, MI) and/or recombinant human TGF-β (R & D Systems Inc., Minneapolis, MN). Cell proliferation was evaluated by measuring BrdU incorporation with an ELISA kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). This assay was performed according to the manufacture's instructions. Briefly, after stimulus treatment, cells were allowed to grow then BrdU labeling solution was added overnight at a concentration found non-toxic to all cell line (10 μM). The cells were then fixed to the plate with methanol and probed with an anti-BrdU antibody peroxidase conjugate. After tetramethylbenzidine was added, the color change corresponding to cell proliferation was measured using a colorimetric plate reader at 450nm.

Measurement of procollagen type I peptide in culture supernatant

HFL-1 cells were cultured to subconfluence. The medium was then replaced with FBS-free F12K, and the cells were stimulated with TGF-β_1. After culture for 24 h, the cells were washed once. The cells were then cultured with LTC_4 in the presence or absence of montelukast, a leukotriene receptor antagonist (LTRA), for 24 h. The montelukast was a gift from Merck & Co., Inc. (Whitehouse Station, NJ). Concentrations of PIP in the HFL-1 cell supernatant were measured with the use of an EIA kit (Takara Bio Inc., Shiga, Japan). The minimum

PIP concentration detected by this method was 10 ng/ml.

RT-PCR analysis

Total RNA was extracted with the use of TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA) from HFL-1 cells cultured in 6-well tissue culture plates. Total RNA was reverse transcribed with a Thermoscript RT-PCR System (Life Technologies, Rockville, MD) according to the manufacturer's protocol. The sequences of the 5′ sense primers and the 3′ antisense primers synthesized based on published sequence data[31,32] and used in this study were as follows : CysLT1R, 5′-GACAGCCATGAGCTTTTTCC-3′ (sense), 5′-ATGCAC – CCAGAGACAAGGTT-3′ (antisense) (product size, 514 bp) ; β-actin, 5′-AAGAGAGGCATCCTCACCCT-3′ (sense), 5′-TACATGGCTGGGG-TGTTGAA-3′ (antisense) (product size, 234 bp). PCR reactions were performed with a thermal cycler (Takara Bio Inc.) under previously described conditions[33]. Products were analyzed by 2% (w/v) agarose gel electrophoresis.

Real-time PCR analysis

Reverse transcription was performed with a TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA). The following are sequences for CysLT1R and β-actin on the basis of published data[32,33] : forward primer, 5′-GCACCTATGCTTTGTATGTCAACC-3′ ; reverse primer, 5′-ATACCTACACACACAAACCTGGC-3′ ; forward primer, 5′-AAGAGAGGCATCCTCACCCT-3′ ; reverse primer, 5′-TACATGGCTGGGG-TGTTGAA-3′. Real-time PCR was performed on an ABI Prism 7700 sequence detection system (PE Applied Biosystems) as described previously[33], using SYBR green (Roche Diagnostics, Somerville, NJ) as a double-strand DNA specific binding dye. The PCR were cycled 40 times after initial denaturation (95℃, 2 minutes) with the following parameters : denaturation, 95℃, 15 sec ; and annealing and extension, 60℃, 1 min. The threshold cycle (CT) was recorded for each sample to reflect the mRNA expression level. A validation experiment proved the linear dependence of the CT value for both CysLT1R and β-actin concentration and consistency of ΔCT (CysLT1R average CT-β-actin average CT) in a given sample at different RNA concentration. Therefore, ΔCT was used to reflect the relative CysLT1 expression levels. To determine the effects of different stimuli on CysLT1R gene expression as compared with unstimulated cells, ΔΔCT was calculated (ΔΔCT = ΔCT stimulus - ΔCT nonstimulated cells). CysLT1R mRNA was indexed to the β-actin using the following formula : $1/(2^{\Delta CT}) \times 100\%$. The value of $2^{\Delta\Delta CT}$ was calculated to demonstrate the fold changes of CysLT1R gene expression in stimulated cells as compared with unstimulated cells.

Flow cytometric analysis

The expression of CysLT1R on HFL-1 cells was analyzed by flow cytometry as described by Thivierge et al.[34]. The cells were washed with PBS and fixed in 2% paraformaldehyde for 15 min at room temperature, followed by permeabilization with DAKO IntraStain reagent B (DAKO, Copenhagen, Denmark) for 15 min at room temperature. The cells were resuspended with PBS and labeled with anti-CysLT1R antibody (Cayman Chemicals, Ann Arbor, MI) (1 : 1000) or isotype control antibody for 60 min at 4℃. After washing with cold PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG F (ab') 2 (Rockland, Gilbertsville, PA) for 60 min at 4℃. Finally, the cells were washed again and resuspended in PBS. Single color immunofluorescence analysis of 5000 cells was performed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by one-way analysis of variance or by paired *t*-test. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

HFL-1 Cell proliferation stimulated by LTC_4 and TGF-β_1

LTC_4 alone (10^{-10} to 10^{-6} M) did not induce cell proliferation of HFL-1 (data not shown). On the other hand, TGF-β_1 (0.01 to 10 ng/ml) induced cell proliferation of HFL-1 in a concentration-dependent manner (data not shown). LTC_4 (10^{-6} M) did not augment TGF-β_1 (1 ng/ml) -induced HFL-1 cell proliferation (Fig. 1).

Production of PIP in the culture supernatant of HFL-1 stimulated with LTC_4 and TGF-β_1

LTC_4 alone (10^{-8} to 10^{-6} M) did not induce PIP production from HFL-1 cells (Fig. 2A). In contrast, TGF-β_1 (1 and 10 ng/ml) induced PIP production from HFL-

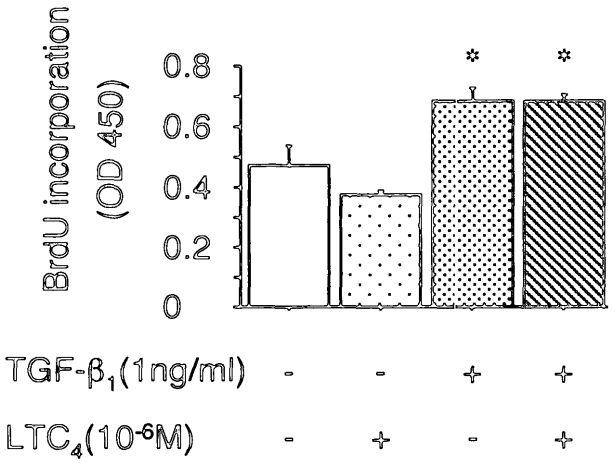

Fig. 1 Human fetal lung fibroblasts (HFL-1) cell proliferation stimulated by leukotriene C_4 (LTC_4) in combination with transforming growth factor (TGF)-β_1. TGF-β_1 (1 ng/ml) alone significantly induced cell proliferation. However, LTC_4 did not augment TGF-β_1-induced HFL-1 cell proliferation. Data are expressed as means ± SEM (n = 3). *$p < 0.005$ vs. 0 ng/ml of TGF-β_1 and LTC_4

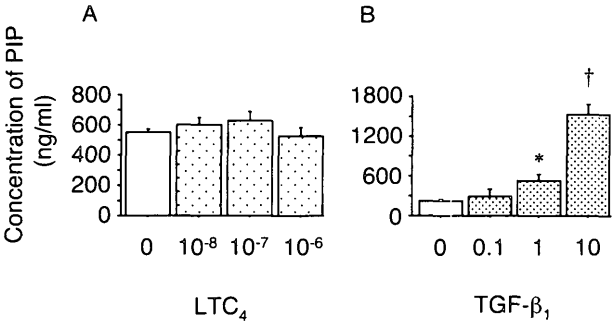

Fig. 2 A) Production of procollagen type I peptide (PIP) in the culture supernatant stimulated with LTC_4. 10^{-8} – 10^{-6} M concentration of LTC_4 did not induce PIP production from HFL-1 cells. Data are expressed as means ± SEM (n = 3).
B) Production of PIP in the culture supernatant stimulated with TGF-β_1. TGF-β_1 at the concentration of 1 and 10 ng/ml induced PIP production by HFL-1 cells in a concentration-dependent manner. Data are expressed as means ± SEM (n = 3). * $p < 0.005$ and † $p < 0.0001$ vs. the control

1 cells in a concentration – dependent manner (Fig. 2B).

Production of PIP in the culture supernatant previously treated with TGF-β_1

To determine whether the observed synergy between LTC_4 and TGF-β_1 depends on the priming of HFL-1 by

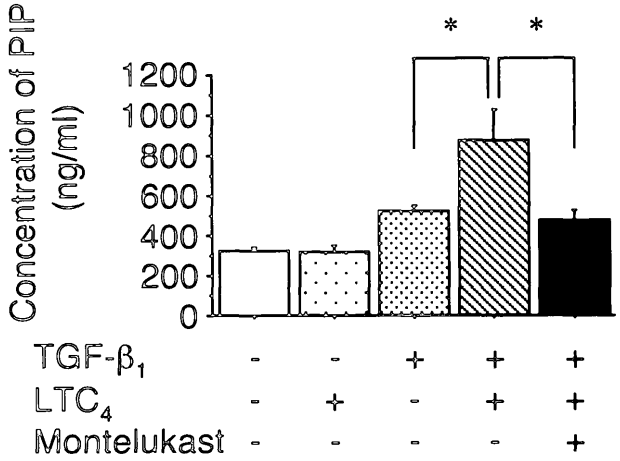

Fig. 3 Effect of LTC_4 on PIP production from HFL-1 previously stimulated with TGF-β_1. After preincubation of HFL-1 with 1 ng/ml of TGF-β_1 for 24 h, cells were washed twice and further cultured for 24 h in the presence or absence of various concentrations of LTC_4. LTC_4 alone showed no stimulation, but HFL-1 prestimulated with 1 ng/ml of TGF-β_1 could respond to LTC_4 (10^{-6} M) and produced more PIP than TGF-β_1 did. Cysteinyl leukotriene (CysLT) 1 receptor antagonist, montelukast, at the concentration of 10^{-6} M significantly prevented the augmentation of PIP production by LTC_4. Data are expressed as means ± SEM (n = 3). *$p < 0.05$

TGF-β_1, we examined the effect of LTC_4 on PIP production by HFL-1 previously stimulated with TGF-β_1. After preincubation of HFL-1 with 1 ng/ml of TGF-β_1 for 24 h, cells were washed twice and further cultured for 24 h in the presence or absence of various concentrations of LTC_4. HFL-1 prestimulated with 1 ng/ml of TGF-β_1 could respond to LTC_4. At a concentration of 10^{-6} M, LTC_4 augmented PIP production from HFL-1 cells. These results suggest that TGF-β_1 can alter the response of HFL-1 to LTC_4. Furthermore, a CysLT1 receptor antagonist, montelukast, at a concentration of 10^{-6} M significantly prevented the augmentation of PIP production induced by LTC_4. This strongly suggests that the synergy effect of LTC_4 and TGF-β_1 is dependent to CysLT1 receptor (Fig. 3).

Expression of CysLT1R mRNA and protein and α-SMA mRNA on HFL-1

To investigate the mechanism of increased responsiveness of HFL-1 to LTC_4, the expression of CysLT1R mRNA on HFL-1 was examined by RT-PCR and real-

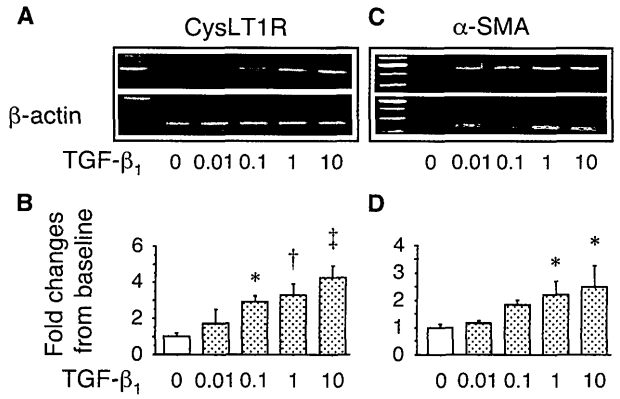

Fig. 4 Expression of CysLT1 receptor mRNA on HFL-1 cells stimulated by TGF-β_1. TGF-β_1 at the concentration of 0.1 – 10 ng/ml significantly upregulated CysLT1R mRNA expression in a concentration-dependent manner. A) Representative RT-PCR patterns showing CysLT1R and β-actin. B) Quantitative analysis of CysLT1R mRNA expression by real-time PCR. Expression of α-smooth muscle actin (α-SMA) mRNA on HFL-1 cells stimulated by TGF-β_1. TGF-β_1 at a concentration of 0.1 – 10 ng/ml significantly upregulated α-SMA mRNA expression in a concentration-dependent manner. C) Representative RT-PCR patterns showing α-SMA and β-actin. D) Quantitative analysis of α-SMA mRNA expression by real-time PCR. CysLT1R and α-SMA mRNA expression were normalized by the corresponding β-actin expression and presented as the fold changes from baseline mRNA expression in the untreated cells. Data are expressed as means ± SEM (n = 3). *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$ vs. 0 ng/ml of TGF-β_1

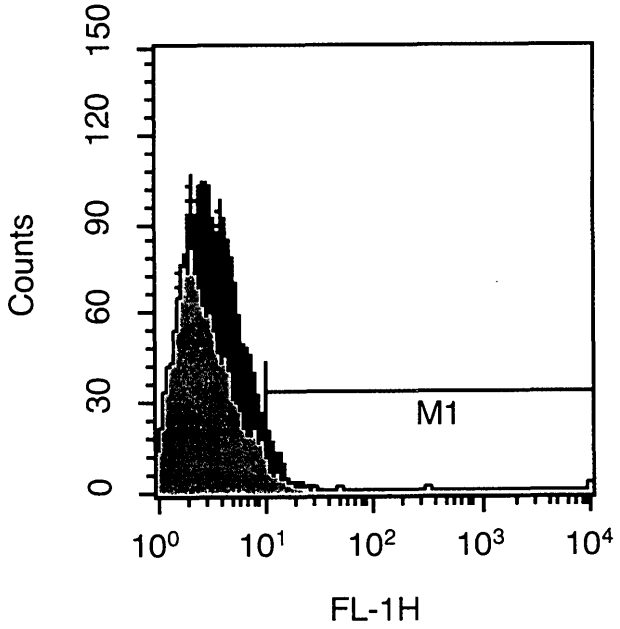

Fig. 5 Flow cytometric analysis of CysLT1R expression on HFL-1 cells stimulated by TGF-β_1. Gray area represents TGF-β_1 (10 ng/ml)-treated cells labeled with isotype control antibody. Black area represents TGF-β_1 (10 ng/ml)-treated cells labeled with anti-CysLT1R antibody. Untreated cells showed no detectable expression (data not shown).

time PCR. RT-PCR revealed that stimulation with TGF-β_1 (0.1 to 10 ng/ml) for 24 h significantly upregulated CysLT1R mRNA expression 3 to 4-fold in HFL-1 (Fig. 4A). Using real-time PCR, this increased expression was confirmed quantitatively (Fig. 4B). After incubation with TGF-β_1 (1 and 10 ng/ml) for 24 h, expression of α-SMA mRNA in HFL-1 cells increased up to 3-fold in a dose-dependent manner (Fig. 4C, 4D). In addition, flow cytometric analysis revealed that TGF-β_1 (1 ng/ml) significantly upregulated CysLT1R protein expression in HFL-1 (Fig. 5).

DISCUSSION

In our present study, LTC$_4$ alone had no effect on fibroblast proliferation and collagen production from normal fibroblasts. In contrast, LTC$_4$ augmented TGF-β-induced PIP production from fibroblasts. Proliferation of fibroblasts induced by TGF-β was not further enhanced by LTC$_4$. The enhancing-effect of LTC$_4$ on collagen production from fibroblasts was also shown when fibroblasts were pre-treated with TGF-β for 24 h. These findings indicated that TGF-β altered fibroblast responsiveness to LTC$_4$. CysLT1R expression on fibroblasts was augmented by incubation with TGF-β thereby producing more collagen in response to LTC$_4$.

Several previous reports have indicated that CysLTs play an important role in the pathogenesis of airway remodeling in asthma[21, 22, 35, 36]. Henderson et al.[21] reported that LTRA inhibited airway remodeling including subepithelial matrix deposition and fibrosis in a mouse ovalbumin-induced asthma model. However, little is known about the direct effect of CysLTs on fibroblasts. Phan et al.[37] reported that LTC$_4$ and to a lesser extent LTD$_4$ stimulated collagen synthesis by rat lung fibroblasts at quite low concentrations (10^{-11} – 10^{-8} M). On the contrary, LTC$_4$ up to 10^{-6} M did not stimulate collagen production from unstimulated human fibroblasts (HFL-1) in the present study. This discrepancy may be attrib-

uted to the difference of species or cell culture conditions. Baud et al.[38] described CysLTs stimulated human skin fibroblasts growth in a dose-dependent manner. However, the effects of CysLTs on fibroblast proliferation were observed only after addition of indomethacin to the incubation medium. Indomethacin is a well-known inhibitor of cyclooxygenase and synthesis of prostaglandin E_2, a suppressor of fibroblast cell growth. In the present study, we did not examine the effect of indomethacin and LTC_4 alone showed no stimulatory effect on fibroblast proliferation.

Recently, several reports have indicated that CysLTs exert their effects in combination with other mediators such as cell growth factors and cytokines. Cohen et al.[39] reported that LTD_4 in combination with insulin-like growth factor (IGF)-1 synergistically increased airway smooth muscle cell growth. This mechanism can be explained by the fact that LTD_4-induced matrix metalloproteinase-1 acts as an IGF binding protein protease, which modulates IGF action thereby promoting smooth muscle proliferation[40]. Panettieri et al.[22] have reported that LTD_4 augment proliferation of human airway smooth muscle induced by epidermal growth factor (EGF). The precise mechanism is unknown, but it could be explained by convergence between LTD_4 signals via a G protein-coupled receptor and EGF signals via EGFR tyrosine kinase. Among various mechanisms considered to be involved in the synergism of CysLTs and growth factors, we paid our attention to the receptor-dependent pathway. Recently, we have reported that unstimulated HFL-1 cell did not produce eotaxin in response to CysLTs but they acquired responsiveness and produced eotaxin when they were stimulated by IL-13[33]. Expression of CysLT1R on fibroblasts was upregulated by IL-13 thereby fibroblasts could respond to LTC_4 and produce eotaxin.

Little is known about the expression of CysLT1R on fibroblasts. The CysLT1R is most highly expressed in the spleen, peripheral blood leukocytes including eosinophils and monocytes. CysLT1R mRNA expression in unstimulated HFL-1 cells were very weak as compared with smooth muscle cells and alveolar macrophages[33]. In normal human lung, the *in situ* hybridization pattern of CysLT1R is characterized by strict localization to smooth muscle cells and some macrophages[5]. However, there is no report on localization of CysLT1R in asthmatic patients. Thivierge et al. showed that Th2 cytokines such as IL-4, IL-5, and IL-13 have a regulatory role in CysLT1R expression in monocytes/macrophages[34] or HL-60 cells[41]. Our previous report indicated that IL-13 induced upregulation of CysLT1R expression in HFL-1[33]. In the present study, TGF-β also increased CysLT1R expression in HFL-1. These findings suggest that CysLT1R expression is possibly increased in lung fibroblasts or myofibroblasts in TGF-β rich milieu like asthmatics.

It has been well established that TGF-β plays a central role in the pathogenesis of airway remodeling in asthmatics. TGF-β is produced by various cells including bronchial epithelial cells, lung fibroblasts, smooth muscle cells and eosinophils[30, 42]. Higher levels of TGF-β are detectable in the airways of asthmatic patients in comparison with normal subjects[30, 43, 44]. TGF-β has multifunction depending on the specific target cells, conditions of cell culture, and concentrations. Although TGF-β acts as a growth inhibitor for many cells, it can stimulate proliferation and chemotaxis of fibroblasts and smooth muscle cells[23]. Furthermore, TGF-β simulates production of ECM molecules such as collagen and fibronectin. It also inhibits a proteolysis by suppressing production of protease and produces inhibitors of proteinases such as tissue inhibitor of metalloproteinase (TIMP) and plasminogen activator inhibitor (PAI), and it promotes accumulation of ECM[45~47].

TGF-β also plays an important role in myofibroblast differentiation from the fibroblast. The myofibroblast is characterized by α-SMA expression which is induced by TGF-β in growing and quiescent cultured fibroblasts[2, 3]. Myofibroblasts are involved in subepithelial fibrosis of the airway in asthma[24~27]. Zhang et al. reported that the newly reactive α-SMA-positive cells strongly expressed procollagen mRNA in pulmonary fibrosis. These findings suggested that myofibroblasts markedly synthesized collagens[48]. In our present study, TGF-β induced upregulation of CysLT1R expression simultaneously with expression of α-SMA in fibroblasts. The relationship between CysLT1R and α-SMA expression in fibroblasts is not clear but it is obvious that TGF-β can induce myofibroblasts characterized by α-SMA expression. TGF-β also alters responsiveness of fibroblasts to LTC_4, which results in enhanced collagen synthesis through the mechanism of CysLT1R upregulation.

LTRAs are clinically established therapeutics for

bronchial asthma because of their anti − inflammatory effects as well as bronchodilater effects. In the present study, an LTRA, montelukast, inhibited LTC_4-induced augmentation of collagen production by TGF-β-induced myofibroblasts. This finding supports the therapeutic usefulness of LTRAs for airway remodeling in asthmatics.

In comparison with production of PIP, LTC_4 did not augment TGF-β-induced cell proliferation. No satisfactory explanation can so far be given of this difference, but the different signaling pathways may be involved in these two systems.

In conclusion, LTC_4 increases the production of type I collagen through upregulation of CysLT1R induced by TGF-β. In the TGF-β-rich milieu such as bronchial mucosa of asthmatic patients, activated myofibroblasts expressing CysLT1R can respond to CysLTs and produce abundant ECM thereby forming airway remodeling. Therefore, LTRA can inhibit the matrix production and suppress the progression of airway remodeling formation.

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