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H. 知的財産権の出願・登録状況

なし

III. 研究成果の刊行に関する一覧表

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Leukotriene D_4 stimulates collagen production from myofibroblasts transformed by TGF-β

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Mechanisms of asthma and allergic inflammation

Background: Airway remodeling has an important role in the pathogenesis of bronchial asthma. Many mediators that influence the pathophysiology of bronchial asthma, especially cysteinyl leukotrienes (CysLTs) and TGF-β_1, are involved in airway remodeling.

Objective: To know whether TGF-β_1 alters fibroblast responsiveness to CysLTs, we examined the effects of leukotriene (LT) D_4 on collagen production from fibroblasts and from myofibroblasts transformed by TGF-β_1. We also examined whether TGF-β_1 upregulates CysLT1 receptor (CysLT1R) expression in fibroblasts.

Methods: Concentrations of procollagen in the human fetal lung fibroblast (HFL) 1 cell supernatant were measured by using an enzyme immunoassay kit in the presence or absence of various concentrations of LTD_4, TGF-β_1, CysLT1R antagonist, or some combination of these. The mRNA expression of CysLT1R and α-smooth muscle actin as a marker of myofibroblasts was measured by means of real-time PCR. Furthermore, protein expression of CysLT1R on fibroblasts was measured by means of flow cytometric analysis.

Results: TGF-β_1 stimulated collagen production from HFL-1 cells, but LTD_4 alone did not. LTD_4 in combination with TGF-β_1 increased collagen production compared with TGF-β_1 alone. Real-time PCR showed that stimulation with TGF-β_1 significantly upregulated CysLT1R and α-smooth muscle actin mRNA expression in HFL-1 cells.

Conclusions: LTD_4 increased collagen production by upregulating CysLT1R induced by TGF-β_1. In the TGF-β–rich milieu, activated myofibroblasts expressing CysLT1R can respond to CysLTs and produce large amounts of extracellular matrix, thereby contributing to airway remodeling. These data suggest that treatment with leukotriene receptor antagonists might prevent airway remodeling in patients with asthma.

(J Allergy Clin Immunol 2004;114:310-5.)

Key words: Cysteinyl leukotrienes, CysLT1 receptor, growth factor, transforming growth factor β, fibroblast, myofibroblast, airway remodeling, bronchial asthma, montelukast, α-smooth muscle actin

Abbreviations used
BrdU: 5-Bromo-2′-deoxyuridine
CT: Threshold cycle
CysLT: Cysteinyl leukotriene
CysLT1R: CysLT receptor type 1
ECM: Extracellular matrix
HFL-1: Human fetal lung fibroblast 1
IGF: Insulin-like growth factor
LT: Leukotriene
LTRA: Leukotriene receptor antagonist
PIP: Procollagen type I carboxy-terminal peptide
α-SMA: α-Smooth muscle actin

Airway remodeling is an established pathologic feature of bronchial asthma. It is characterized by deposition of subepithelial collagen, hypertrophy of bronchial smooth muscle, and transformation of fibroblasts to myofibroblasts.[1] Transformed myofibroblasts produce large quantities of extracellular matrix (ECM) proteins, including collagen and fibronectin, and participate in the development of subepithelial hypertrophy and fibrosis.[2,3] Even slight contractions of airway smooth muscle can substantially increase airway resistance in patients with remodeled airways and increased airway wall thickness.[4] Thus it is thought that airway remodeling might be associated with severe and intractable disease in patients with asthma.

Many mediators that influence the pathophysiology of bronchial asthma participate in airway remodeling. Cysteinyl leukotrienes (CysLTs; leukotriene [LT] C_4, LTD_4, and LTE_4) produced by eosinophils and mast cells play an important role in the pathogenesis of bronchial asthma. Levels of CysLTs are increased in asthmatic patients. Eosinophils from patients with bronchial asthma synthesize significantly (5- to 10-fold) more LTC_4 than do eosinophils from healthy individuals.[5] Many studies have shown increased levels of CysLTs in bronchoalveolar lavage fluid and urine samples from patients with asthma.[6-8] In addition to acting mainly as smooth muscle contractants,[9,10] CysLTs are associated with mucus hypersecretion,[11-13] increased microvascular permeability,[12,14,15] decreased ciliary movement,[16,17] and eosinophil recruitment.[17-20]

Recent studies have shown that CysLTs participate in airway remodeling in animal models of asthma.[21,22] When combined with an appropriate growth factor, such as epidermal growth factor, CysLTs induce proliferation of airway smooth muscle.[23] However, few studies have examined the role of CysLTs in airway wall fibrosis.

TGF-β plays a central role in airway remodeling in patients with asthma. TGF-β stimulates proliferation and chemotaxis of fibroblasts and smooth muscle cells.[24] It also simulates production of ECM molecules, such as collagen. Moreover, TGF-β can induce differentiation of

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myofibroblasts from fibroblasts.[2,3] Myofibroblasts contribute to subepithelial fibrosis of the airway in asthma[25-28] by producing large quantities of ECM proteins. Asthmatic individuals express higher levels of TGF-β mRNA in the airway submucosa and eosinophils than do healthy control subjects.[29-31]

In the TGF-β–rich and CysLT-rich milieu associated with bronchial asthma, both mediators might act synergistically to promote airway remodeling. We hypothesized that TGF-β alters fibroblast responsiveness to CysLTs, whereas TGF-β simultaneously transforms fibroblasts to myofibroblasts. To examine this hypothesis, we investigated whether LTD_4, alone or in combination with TGF-β, can stimulate collagen production by fibroblasts. Furthermore, we examined whether TGF-β upregulates CysLT1 receptor (CysLT1R) expression in fibroblasts.

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 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°C in a 5% CO_2-humidified incubator in Ham's F12K medium (Sigma, St Louis, Mo) containing 10% heat-inactivated 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 hours, the cells were washed twice and cultured for another 24 hours in the presence or absence of various concentrations of LTD_4 (Cayman Chemical, Ann Arbor, Mich), recombinant human TGF-β_1 (R&D Systems Inc, Minneapolis, Minn), or both. Cell proliferation was evaluated on the basis of DNA synthesis assessed by measuring BrdU incorporation with an ELISA kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). This assay was performed according to the manufacturer's instructions. Briefly, after stimulus treatment, cells were allowed to grow, and then BrdU-labeling solution was added overnight at a concentration found to be nontoxic to all cell lines (10 μmol/L). The cells were then fixed to the plate with methanol and probed with an anti-BrdU antibody peroxidase conjugete, tetramethylbenzidine was added, and the color change corresponding to cell proliferation was measured by using a colorimetric plate reader at 450 nm.

Measurement of PIP 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 hours, the cells were washed once. The cells were then cultured with LTD_4 in the presence or absence of a leukotriene receptor antagonist (LTRA), montelukast, for 24 hours. 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 enzyme immunoassay kit (Takara Bio Inc, Shiga, Japan). The minimum PIP concentration detected by using this method was 10 ng/mL.

RT-PCR analysis

Total RNA was extracted with the use of TRIZOL Reagent (Life Technologies, Frederick, Md) from HFL-1 cells cultured in 6-well tissue-culture plates. Total RNA was reverse transcribed with a Thermoscript RT-PCR System (Life Technologies) according to the manufacturer's protocol. The sequences of the 5′ sense primers and the 3′ antisense primers synthesized on the basis of published sequence data[32,33] and used in this study were as follows: CysLT1R, 5′-GACAGCCATGAGCTTTTTCC-3′ (sense) and 5′-ATGCAC-CCAGAGACAAGGTT-3′ (antisense; product size, 514 bp); β-actin, 5′-AAGAGAGGCATCCTCACCCT-3′ (sense) and 5′-TACATGG-CTGGGGTGTTGAA-3′ (antisense; product size, 234 bp). PCR reactions were performed with a thermal cycler (Takara Bio Inc) under previously described conditions.[34] Products were analyzed by means of 2% (wt/vol) agarose gel electrophoresis.

Real-time PCR analysis

Reverse transcription was performed with a TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, Calif). The following are sequences for CysLT1R and β-actin, respectively, on the basis of published data[33,34]: forward primer, 5′-GCACCT-ATGCTTTGTATGTCAACC-3′; reverse primer, 5′-ATACCTAC-ACACACAAACCTGGC-3′; forward primer, 5′-AAGAGAGGCA-TCCTCACCCT-3′; reverse primer, 5′-TACATGGCTGGGGTG-TTGAA-3′. Real-time PCR was performed on an ABI Prism 7700 sequence detection system (PE Applied Biosystems), as described previously,[34] by using SYBR green (Roche Diagnostics, Somerville, NJ) as a double-stranded DNA–specific binding dye. The PCR was cycled 40 times after initial denaturation (at 95°C for 2 minutes) with the following parameters: denaturation, 95°C for 15 seconds; and annealing and extension, 60°C for 1 minute. 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 concentrations 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 compared with that seen in unstimulated cells, ΔΔCT was calculated as follows: ΔΔCT = ΔCT stimulus − ΔCT nonstimulated cells. CysLT1R mRNA was indexed to the β-actin by 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 compared with that seen in unstimulated cells.

Flow cytometric analysis

The expression of CysLT1R on HFL-1 cells was analyzed by means of flow cytometry, as described by Thivierge et al.[35] The cells were washed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature, followed by permeabilization with DAKO IntraStain reagent B (DAKO, Copenhagen, Denmark) for 15 minutes at room temperature. The cells were resuspended with PBS and labeled with anti-CysLT1R antibody (1:1000; Cayman Chemical) or isotype control antibody for 60 minutes at 4°C. After washing with cold PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG F(ab′)2 (Rockland, Gilbertsville, Pa) for 60 minutes at 4°C. 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, Calif).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by means of 1-way ANOVA or paired *t* test. A *P* value of less than .05 was considered to indicate statistical significance.

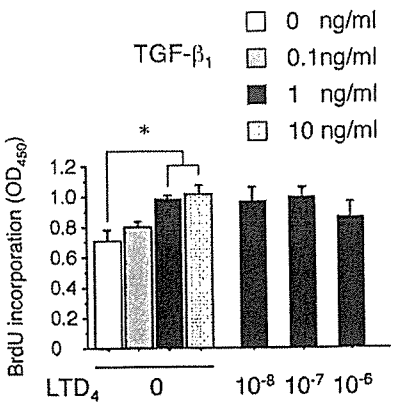

FIG 1. Proliferation of HFL-1 cells. HFL-1 cells were stimulated by various concentration of TGF-β_1 on LTD_4 in combination with TGF-β_1 (1 ng/mL). TGF-β_1 (1-10 ng/mL) alone significantly induced cell proliferation. In contrast, LTD_4 did not augment TGF-β_1–induced HFL-1 cell proliferation. Data are expressed as means±SEM (n = 4). *$P < .01$.

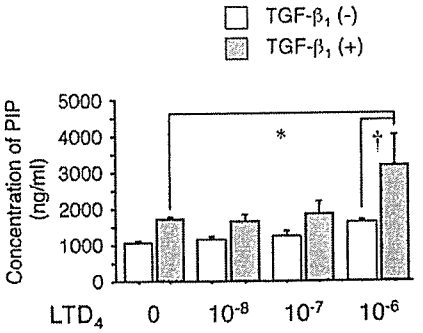

FIG 2. Production of PIP in the culture supernatant stimulated by LTD_4 in combination with TGF-β_1. LTD_4 (10^{-6} mol/L) in combination with TGF-β_1 (1 ng/mL) induced PIP production from HFL-1 cells. Data are expressed as means±SEM (n = 4). *$P < .005$ versus TGF-β_1 alone; †$P < .001$ versus LTD_4 (10^{-6} mol/L) alone.

RESULTS

Effect of LTD_4 and TGF-β on cell proliferation

LTD_4 alone (10^{-10} to 10^{-6} mol/L) did not induce proliferation of HFL-1 cells (data not shown). Meanwhile, TGF-β_1 (0.01-10 ng/mL) induced proliferation of HFL-1 cells in a concentration-dependent manner. LTD_4 (10^{-8} to 10^{-6} mol/L) did not augment TGF-β_1 (1 ng/mL)–induced HFL-1 cell proliferation (Fig 1).

Production of PIP in culture supernatant of HFL-1 cells stimulated with LTD_4 and TGF-β

LTD_4 alone (10^{-10} to 10^{-6} mol/L) did not induce PIP production from HFL-1 cells. Meanwhile, TGF-β_1 (1-10 ng/mL) induced PIP production from HFL-1 cells in a concentration-dependent manner (data not shown). LTD_4 in combination with TGF-β_1 (1 ng/mL) enhanced the PIP production from HFL-1 cells compared with TGF-β_1 (1 ng/mL) alone (3194 ± 810 vs 1724 ± 66 ng/mL; $P < .001$; Fig 2).

Production of PIP in culture supernatant previously treated with TGF-β

To determine whether the observed synergy between LTD_4 and TGF-β depends on priming of HFL-1 cells by TGF-β, we examined the effect of LTD_4 on PIP production by HFL-1 cells previously stimulated with TGF-β. After preincubation of HFL-1 cells with 1 ng/mL TGF-β_1 for 24 hours, the cells were washed twice and cultured for another 24 hours in the presence or absence of various concentrations of LTD_4. At a concentration of 10^{-6} mol/L, LTD_4 augmented PIP production from HFL-1 cells prestimulated with 1 ng/mL TGF-β_1 (from 1437 ± 99 ng/mL to 1795 ± 136 ng/mL; $P < .05$; Fig 3). These results suggest that TGF-β can alter the response of HFL-1 cells to LTD_4. Furthermore, a CysLT1 receptor antagonist, montelukast, at a concentration of 10^{-6} mol/L significantly blocked the augmentation of PIP production by LTD_4 (from 1795 ± 136 ng/mL to 1321 ± 124 ng/mL; $P < .05$; Fig 3). This finding strongly suggests that the synergism between LTD_4 and TGF-β depends on CysLT1R.

Expression of CysLT1R mRNA and protein in HFL-1 cells

To investigate the mechanism underlying increased responsiveness of HFL-1 cells to LTD_4, the expression of CysLT1R mRNA in HFL-1 cells was examined by means of RT-PCR and real-time PCR. Although baseline expression of CysLT1R mRNA on HFL-1 was very weak, stimulation with TGF-β_1 (0.1-10 ng/mL) for 24 hours significantly (3- to 4-fold) upregulated the expression (Fig 4, *A*). Increased CysLT1R mRNA expression was confirmed quantitatively by means of real-time PCR (Fig 4, *B*). Flow cytometric analysis similarly showed that treatment with TGF-β_1 (1 ng/mL) for 24 hours significantly upregulated CysLT1R protein expression in HFL-1 cells (Fig 5).

Expression of α-SMA mRNA on HFL-1 cells

To know whether CysLT1R-expressed fibroblasts induced by TGF-β are transformed myofibroblasts, the expression of α-SMA was examined. After incubation with TGF-β_1 (1 and 10 ng/mL) for 24 hours, expression of α-SMA mRNA in HFL-1 cells increased up to 3-fold in a concentration-dependent manner (Fig 6).

DISCUSSION

Our study showed that LTD_4 alone had no effect on fibroblast proliferation or on collagen production from normal fibroblasts. LTD_4 augmented TGF-β–induced collagen production from fibroblasts but did not enhance TGF-β–induced proliferation of fibroblasts. LTD_4-induced enhancement of collagen production from fibroblasts was also seen when fibroblasts were pretreated with TGF-β for 24 hours. These findings indicate that TGF-β altered fibroblast responsiveness to LTD_4. We next focused on the expression of the receptor for LTD_4, CysLT1R, and examined whether TGF-β upregulates expression of CysLT1R in fibroblasts. CysLT1R expression in fibroblasts was augmented by incubation with TGF-β, thereby producing more collagen in response to LTD_4.

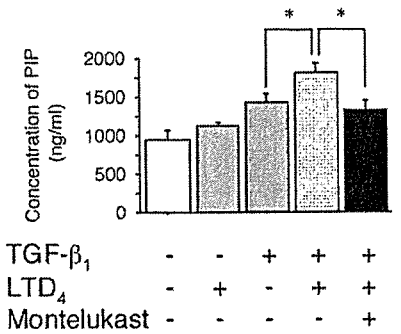

FIG 3. Effect of LTD_4 on PIP production from HFL-1 cells previously stimulated with TGF-β_1. HFL-1 cells prestimulated with 1 ng/mL TGF-β_1 responded to LTD_4 (10^{-6} mol/L) and produced more PIP than did cells treated with TGF-β_1 alone. Montelukast significantly blocked augmentation of PIP production by LTD_4. Data are expressed as means±SEM (n = 4). *P < .05.

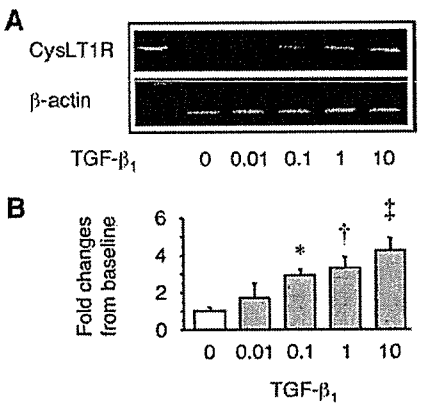

FIG 4. Expression of CysLT1 receptor mRNA in HFL-1 cells stimulated by TGF-β_1. **A,** Representative RT-PCR showing CysLT1R and β-actin. **B,** Quantitative analysis of CysLT1R mRNA expression by means of real-time PCR. Data are expressed as means±SEM (n = 3). *P < .05, †P < .01, and ‡P < .001 versus 0 ng/mL TGF-β_1.

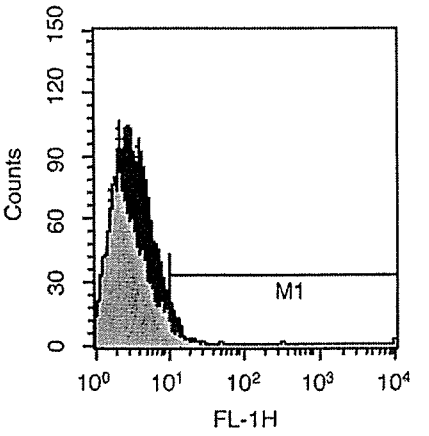

FIG 5. Flow cytometric analysis of CysLT1R expression in HFL-1 cells stimulated by TGF-β_1 for 24 hours. The *gray area* represents TGF-β_1 (1 ng/mL)–treated cells labeled with isotype control antibody. The *black area* represents TGF-β_1 (1 ng/mL)–treated cells labeled with anti-CysLT1R antibody. The *vertical line* expresses number of cells, and the *horizontal line* expresses intensity of immunofluorescence. Untreated cells showed no detectable expression.

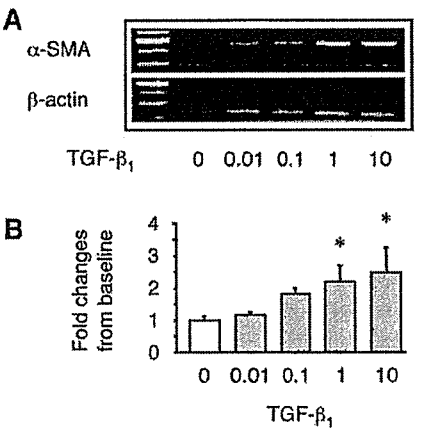

FIG 6. Expression of α-SMA mRNA in HFL-1 cells stimulated by TGF-β_1. **A,** Representative RT-PCR showing α-SMA and β-actin. **B,** Quantitative analysis of α-SMA mRNA expression by means of real-time PCR. α-SMA mRNA expression is presented as the fold changes from baseline mRNA expression in the untreated cells. Data are expressed as means±SEM (n = 3). *P < .05 versus 0 ng/mL TGF-β_1.

CysLTs play an important role in airway remodeling in asthma.[21-23,36] Henderson et al[21] reported that LTRA inhibited airway remodeling, including subepithelial matrix deposition and fibrosis, in a mouse model of OVA-induced asthma. Wang et al[22] demonstrated that the increase in rat airway smooth muscle cells after repeated ovalbumin exposure was abrogated by a CysLT antagonist. 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 from rat lung fibroblasts at relatively low concentrations (10^{-11} to 10^{-8} mol/L). In our study, however, LTD_4 in concentrations up to 10^{-6} mol/L did not stimulate collagen production from unstimulated human fibroblasts (HFL-1). This discrepancy might have arisen from differences in species or cell culture conditions. Baud et al[38] reported that CysLTs stimulate growth of human skin fibroblasts in a dose-dependent manner. However, the effects of CysLTs on fibroblast proliferation were apparent only after addition of indomethacin to the incubation medium. Indomethacin is well known to block cyclooxygenase activity 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 LTD_4 alone apparently did not stimulate fibroblast proliferation.

Several recent studies have shown that CysLTs act in conjunction with other mediators, such as cell growth factors and cytokines. Cohen et al[39] reported that LTD_4 combined with insulin-like growth factor (IGF) 1 synergistically promotes the growth of airway smooth muscle cells. LTD_4-induced matrix metalloproteinase 1 was proposed to act as an IGF binding protein protease and modulate the action of IGF, thereby promoting smooth muscle proliferation.[40] Panettieri et al[23] reported that LTD_4 augments proliferation of human airway smooth muscle induced by epidermal growth factor. The precise mechanism remains unknown but can be explained by convergence between LTD_4 signals through a G protein–coupled

receptor and epidermal growth factor signals through epidermal growth factor receptor tyrosine kinase. Among potential mechanisms for the synergism between CysLTs and growth factors, we focused on receptor-dependent pathways. We recently reported that unstimulated HFL-1 cells do not produce eotaxin in response to CysLTs but become responsive and produce eotaxin after stimulation with IL-13.[34] Expression of CysLT1R in fibroblasts is upregulated by IL-13; the fibroblasts can thereby respond to LTC_4 and produce eotaxin. Espinosa et al[41] also have reported a similar mechanism to that seen in our previous report[34] and the present study. They demonstrated that CysLT1 receptor upregulation by TGF-β enabled bronchial smooth muscle cells to respond to LTD_4, resulting in smooth muscle cell proliferation. TGF-β increased CysLT1R protein expression without significantly affecting CysLT1R mRNA expression. In contrast, TGF-β increased both CysLT1R protein and mRNA expression in fibroblasts in the present study. Although the reason for this discrepancy is not known, it might possibly be attributed to the difference in cell sources.

Little is known about the expression of CysLT1R in fibroblasts. CysLT1R is most highly expressed in spleen and peripheral blood leukocytes, including eosinophils and monocytes. CysLT1R mRNA expression in unstimulated HFL-1 cells is very weak compared with that seen in smooth muscle cells and alveolar macrophages.[34] In normal human lung the *in situ* hybridization pattern of CysLT1R is characterized by strict localization to smooth muscle cells and some macrophages.[5] To our knowledge, however, the localization of CysLT1R has not been studied in asthmatic patients. Thivierge et al[42] showed that T_H2 cytokines, such as IL-4, IL-5, and IL-13, regulate CysLT1R expression in monocytes-macrophages[35] or HL-60 cells.[42] Our previous study showed that IL-13 induces upregulation of CysLT1R expression in HFL-1 cells.[34] In the present study TGF-β similarly increased CysLT1R expression in HFL-1 cells. These findings suggest that CysLT1R expression is increased in lung fibroblasts or myofibroblasts in TGF-β–rich milieu, such as that found in asthma. It is necessary to confirm whether similar findings obtained in this *in vitro* study using a cultured fibroblast cell line are observed in the patients with bronchial asthma.

TGF-β has been established to play a central role in the pathogenesis of airway remodeling in asthmatic patients. TGF-β is produced by various cells, including bronchial epithelial cells, lung fibroblasts, smooth muscle cells, and eosinophils.[31,43] Airway tissue levels of TGF-β are higher in asthmatic patients than in healthy subjects.[31,44,45] TGF-β has a wide range of functions, depending on the specific target cells, cell culture conditions, and mediator concentrations. Although TGF-β acts as a growth inhibitor for many types of cells, it can stimulate proliferation and chemotaxis of fibroblasts and smooth muscle cells.[24] TGF-β stimulates production of ECM molecules, such as collagen and fibronectin. It also inhibits proteolysis by suppressing production of proteases and by producing inhibitors of proteinases, such as tissue inhibitor of metalloproteinase and plasminogen activator inhibitor. These mechanisms promote accumulation of ECM.[46-48]

TGF-β also plays an important role in the differentiation of myofibroblasts from fibroblasts. Myofibroblasts are 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 airways in asthma.[25-28] Zhang et al[49] reported that newly reactive α-SMA–positive cells strongly express procollagen mRNA in pulmonary fibrosis, suggesting that myofibroblasts synthesize considerable amounts of collagens. In the 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 unclear, but TGF-β can obviously induce myofibroblasts characterized by α-SMA expression and alter the responsiveness of fibroblasts to LTD_4.

Consequent upregulation of CysLT1R promotes collagen production. This seems to be consistent with the previously reported findings that myofibroblasts can produce more collagen than fibroblasts.

LTRAs have been clinically established to be effective for bronchial asthma because of their anti-inflammatory effects, as well as their bronchodilator activity. We found that the LTRA montelukast inhibited LTD_4-induced augmentation of collagen production from TGF-β–induced myofibroblasts. This finding supports the potential therapeutic usefulness of LTRA for airway remodeling in asthmatic patients.

In contrast to its effect on PIP production, LTD_4 did not augment TGF-β–induced cell proliferation. No satisfactory explanation can be given for this difference, but distinct signaling pathways might be involved.

In conclusion, LTD_4 increased production of type I collagen by upregulating CysLT1R induced by TGF-β. In TGF-β–rich milieu, such as that in the bronchial mucosa of asthmatic patients, activated myofibroblasts expressing CysLT1R can respond to CysLTs and produce abundant quantities of ECM, thereby promoting airway remodeling. LTRA can inhibit matrix production and slow the progression of airway remodeling.

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Clinical features of asthmatic patients with increased urinary leukotriene E4 excretion (hyperleukotrienuria): Involvement of chronic hyperplastic rhinosinusitis with nasal polyposis

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Background: The urinary leukotriene E4 (U-LTE4) concentration is significantly increased in patients with aspirin-intolerant asthma (AIA). However, the relationship between the clinicopathogenetic factors of asthma and the U-LTE4 concentration remains undetermined.
Objective: We sought to examine the clinical features of asthmatic patients with increased excretion levels of U-LTE4 (hyperleukotrienuria).
Methods: We measured the U-LTE4 concentrations in 137 asthmatic patients (including 64 patients with AIA) who were in clinically stable condition. A U-LTE4 concentration of 150 pg/mg creatinine or greater (mean U-LTE4 + 3 SDs of normal healthy control subjects) was indicative of hyperleukotrienuria.
Results: The basal concentration of U-LTE4 was significantly higher in the patients with AIA than in those with aspirin-tolerant asthma (ATA; median, 227.2 vs 90.3 pg/mg creatinine; $P < .01$). Compared with normal leukotrienuria in the patients with AIA, hyperleukotrienuria in the patients with AIA was associated with older age and decrease in pulmonary function. On the other hand, compared with normal leukotrienuria in the patients with ATA, hyperleukotrienuria in the patients with ATA was associated with severe asthma and chronic hyperplastic rhinosinusitis with nasal polyposis (CHRS/NP), which are well-known symptoms of the aspirin triad, as well as hypereosinophilia and anosmia. The patients with ATA with CHRS/NP excreted U-LTE4 at significantly high concentrations. There were significant decreases in the U-LTE4 concentrations before and after the sinus surgery in both the AIA and ATA groups ($P < .05$).
Conclusion: Cysteinyl leukotrienes are not strictly associated with aspirin intolerance itself but rather with clinical features, such as CHRS/NP, that are similar to those seen in AIA. CHRS/NP might be involved in cysteinyl leukotriene overproduction in asthmatic patients. (J Allergy Clin Immunol 2004;113:277-83.)

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Cysteinyl leukotrienes (cys-LTs), namely leukotriene C4 (LTC4), LTD4, and LTE4, play an extremely important role in the pathophysiology of asthma.[1] Cys-LTs cause potent bronchoconstriction, mucosal edema, and increased mucus secretion within the airways of asthmatic patients.[2,3] LTE4 has been identified as a major metabolite of LTC4,[4,5] and urinary LTE4 (U-LTE4) is now considered as the most reliable analytic parameter for monitoring the endogenous synthesis of cysLTs.[6,7] Previous studies have shown that the U-LTE4 concentration is useful in demonstrating cys-LT release in vivo during allergen challenge[8,9] and acute exacerbation of asthma.[10,11] Moreover, even in clinically stable conditions, basal urinary excretion levels of LTE4 in patients with aspirin-intolerant asthma (AIA) are significantly higher than those in patients with aspirin-tolerant asthma (ATA).[12,13] The cellular source of cys-LTs is as yet unknown, but it possibly includes eosinophils and mast cells.[14,15] It also remains unclear which step in the pathway is responsible for cys-LT overproduction in patients with AIA. Cowburn et al[16] reported that the number of cells expressing LTC4 synthase (LTC4S) is higher in the bronchial mucosa of patients with AIA than in that of patients with ATA and that the amount of cys-LTs in bronchoalveolar lavage fluid is strongly correlated with the number of LTC4S-positive cells. In addition, Sanak et al[17,18] found that the polymorphism (ie, A to C transversion at 444 nucleotides upstream of the ATG translation start site) in the promoter region of the *LTC4S* gene is associated with the development of AIA in a Polish population. However, this polymorphism of the *LTC4S* gene might not be the probable mechanism underlying cys-LT overproduction in patients with AIA. Van Sambeek et al[19] reported that the C allele in the *LTC4S* gene is not correlated with the phenotype of AIA in the United States and that this polymorphism does not affect the transcriptional activity of the *LTC4S* gene. More recently, Kawagishi et al[20] also reported similar findings that there is no clear association between this polymorphism of the *LTC4S* gene and the aspirin intolerance or the enzymatic activity of the *LTC4S* gene in eosinophils. There has been no

Abbreviations used
AIA: Aspirin-intolerant asthma
ATA: Aspirin-tolerant asthma
CHRS/NP: Chronic hyperplastic rhinosinusitis with nasal polyposis
cys-LT: Cysteinyl leukotriene
LTC4S: Leukotriene C4 synthase
LTE4: Leukotriene E4
U-LTE4: Urinary leukotriene E4

report demonstrating that the basal concentration of U-LTE4 differs between wild-type A homozygotes and variant C allelic carriers in patients with AIA[18,20] or in patients with ATA.[20] The clinicopathogenetic factors associated with the increased excretion levels of U-LTE4 in asthmatic patients have not been determined. In our study we evaluated the clinicopathogenetic factors that might be associated with the increased excretion level of U-LTE4 in asthmatic patients and demonstrated that the cys-LT overproduction is associated with some clinical features also observed in AIA, such as chronic hyperplastic rhinosinusitis with nasal polyposis (CHRS/NP).

METHODS

Subjects

The hospital-based case-control study was conducted from June 1998 through March 2002. The subjects of this study were 64 nonsmoking asthmatic outpatients with AIA (age range, 21-79 years; mean age, 53.3 years; 23 male and 41 female patients) and 73 control asthmatic outpatients who tolerated aspirin well (ATA; age range, 21-80 years; mean age, 51.2 years; 38 male and 35 female patients; Table I). The diagnosis of asthma was based on the American Thoracic Society criteria.[21] Asthma severity was classified on the basis of daily medication regimen and response to treatment according to the Global Initiative for Asthma guidelines.[22] All the patients with AIA have been proved to have histories of severe bronchoconstriction and nasal symptoms after ingestion of at least 2 different nonsteroidal anti-inflammatory drugs or have had a positive reaction to aspirin systemic challenge.[20] All the patients with ATA have had a negative reaction to the aspirin challenge. All the patients were in clinically stable condition. None of the patients had complications of cystic fibrosis, immotile cilia syndrome, or autoimmune diseases, and none of them had an upper respiratory tract infection in the 6 weeks preceeding the study. Thirty-five healthy volunteers without subjective symptoms or objective findings of diseases, including asthma and allergic rhinitis, were also enrolled in this study as healthy control subjects (age range, 25-81 years; mean age, 50.1 years; 23 male and 12 female subjects). Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital, and all the patients who participated in the study provided informed consent.

Study design

On entry into this study, the patients were documented according to a detailed structured questionnaire. The age at onset of asthma was determined as accurately as possible. In case of uncertainty, the earliest respiratory symptoms, including cough, wheezing, and episodic dyspnea, were taken into account. Nasal symptoms were also assessed. Self-assessment of olfactory disturbance was performed by the patients using an analog scale for rating symptoms from 0 (none) to 4 points (anosmia) by evaluating the degree at which the disturbance interfered with their everyday life. When a 4-point (anosmia) score was obtained, we reconfirmed the presence of anosmia by using cards or smell bottles containing cigarettes, coffee, deodorants, and cloves, as previously described but with some modifications.[23] Subjective assessment of olfactory disturbance in asthmatic patients with seasonal allergic rhinitis was performed during the off season of pollen allergens. The diagnosis of CHRS/NP[24-26] was assessed by an independent observer on the basis of clinical histories, rhinoscopic findings, and sinus radiographic or computed tomographic scans. In this article CHRS/NP is determined by evaluating the clinical symptoms of chronic rhinosinusitis with evidence of bilateral sinus mucosal thickening on radiographic studies in conjunction with nasal polyposis.[27-30] In contrast, infectious chronic rhinosinusitis,[31] as determined by means of clinical definition, such as the presence of facial pain, purulent drainage, or an extensive air fluid level on sinus radiographs, was an exclusion criterion.

We collected blood and urine samples from the patients, and then pulmonary function tests were performed between 9 and 11 AM. The number of eosinophils in peripheral blood was determined by using a standard automated cell counter. Because treatment with β_2-agonists or with anti-inflammatory drugs, including oral or inhaled corticosteroids, sodium cromoglycate, or oral leukotriene receptor antagonists themselves, does not affect U-LTE4 levels,[32] these medications were not withheld at the time of urine sample collection in this study.

To reconfirm the involvement of CHRS/NP in the increase in the excretion level of U-LTE4, we compared the LTE4 concentration in urine between before and after the elective endoscopic surgery for CHRS/NP (3-4 weeks after the surgical treatment), which is not part of the goal of this study. The subjects in the reconfirmatory study were 7 patients with AIA (age range, 37-68 years; mean age, 52.9 years; 2 male and 5 female patients) and 8 patients with ATA (age range, 22-71 years; mean age, 48.4 years 5 male and 3 female patients). Before and after the endoscopic surgery for CHRS/NP (sinus surgery), all these patients were in clinically stable condition, with no changes in asthma symptoms or the kind and dose of medication. Treatments included only inhaled steroids, leukotriene receptor antagonists, and single use of short-acting β_2-agonists but not systemic corticosteroid administration.

Measurements

We measured the LTE4 concentration in spot urine by using a method previously described.[20,33-35] The U-LTE4 concentration is expressed as picograms per milligram of creatinine.

Analysis of data

Demographic characteristics are expressed as means ± SD. The U-LTE4 concentration is shown as the median and the range. U-LTE4 concentrations in the AIA, ATA, and healthy control groups were first compared by using the Kruskal-Wallis test. When a significant difference was found, the Mann-Whitney *U* test with the Bonferroni correction for comparison between groups was performed. U-LTE4 concentrations in the patients with different clinical asthma severity levels were compared by using the same statistical analyses described above. U-LTE4 concentrations in patients with CHRS/NP and those with normal sinuses were compared with the Mann-Whitney *U* test. Similarly, U-LTE4 concentrations in patients with AIA and ATA were compared with the Mann-Whitney *U* test. U-LTE4 concentrations before and after the sinus surgery were compared by using the Wilcoxon *t* test.

In this study the U-LTE4 concentration was the basis for classifying the patients into those showing either increased excretion levels of U-LTE4 (hyperleukotrienuria) or nonincreased excretion levels of U-LTE4 (normal leukotrienuria). A U-LTE4 concentration of

TABLE I. Demographic characteristics of the patients with AIA and those with ATA

	AIA	ATA	*P* value
No.	64	73	
Male/female sex	23/41	38/35	NS
Age (y), mean (SD)	53.3 (13.5)	51.2 (15.2)	NS
Onset age (y), mean (SD)	38.1 (14.0)	37.3 (17.7)	NS
Atopic status, n (%)	32 (50.0)	51 (69.9)	<.05
Blood eosinophil (×10^6/mL), median (range)	585 (5-3520)	430 (20-4160)	NS
Serum IgE-RIST (IU/mL), median (range)	114 (14-1390)	233 (15-2290)	<.05
U-LTE4 (pg/mg creatinine), median (range)	227.2 (30.9-1465.7)	90.3 (16.5-915.8)	<.01
Pulmonary function tests			
FEV_1 (mL), mean (SD)	2043 (757)	2100 (796)	NS
FEV_1 (% predicted), mean (SD)	77.3 (19.8)	80.7 (21.5)	NS
Clinical features			
Severity			NS
Intermittent, n (%)	5 (7.8)	10 (13.7)	
Mild persistent, n (%)	12 (18.8)	23 (31.5)	
Moderate persistent, n (%)	17 (26.6)	17 (23.3)	
Severe persistent, n (%)	30 (46.9)	23 (31.5)	
CHRS/NP, n (%)	43* (71.7)	27† (40.3)	<.001
Anosmia, n (%)	41‡ (67.2)	16§ (24.2)	<.001
Treatment			
Oral systemic CS, n (%)	22 (34.4)	11 (15.1)	<.05
LTRAs, n (%)	11 (17.2)	18 (24.7)	NS
Dose of inhaled CS (mg/d), median‖	800	800	NS

NS, Not significant; *RIST*, radioimmunosorbent test; *CS*, corticosteroids.
*n = 60 and ‡n = 61, patients with AIA.
†n = 67 and §n = 66, patients with ATA.
‖Dose of inhaled corticosteroids is the median daily dose in beclomethasone equivalent units. Beclomethasone is assumed to be equipotent with budesonide and has a potency half that of fluticasone.

greater than 150 pg/mg creatinine, which corresponds to the mean U-LTE4 plus 3 SDs of the healthy control subjects, indicated hyperleukotrienuria, and a U-LTE4 concentration of less than 100 pg/mg creatinine (mean U-LTE4 + 1 SD of the healthy control subjects) indicated normal leukotrienuria. We compared the clinical features between asthmatic patients with hyperleukotrienuria and those with normal leukotrienuria. The differences in demographic characteristics between these 2 groups were analyzed by using the unpaired Student *t* test and the χ^2 test. Relationships were analyzed by using the Spearman rank correlation test. *P* values of less than .05 were regarded as statistically significant.

RESULTS

Table I shows the clinical characteristics of the patients with AIA and those with ATA. These 2 groups were well matched for age, sex, severity of asthma, respiratory function, and doses of both inhaled steroids and leukotriene receptor antagonists. In the ATA group, however, both the frequency of atopic asthmatic patients (69.9% vs 50.0%, $P < .05$) and the level of serum IgE on radioimmunosorbent testing (median, 233 vs 114 IU/mL; $P < .05$) were significantly higher than those in the AIA group. The frequency of the complication of CHRS/NP was higher in the patients with AIA than in the patients

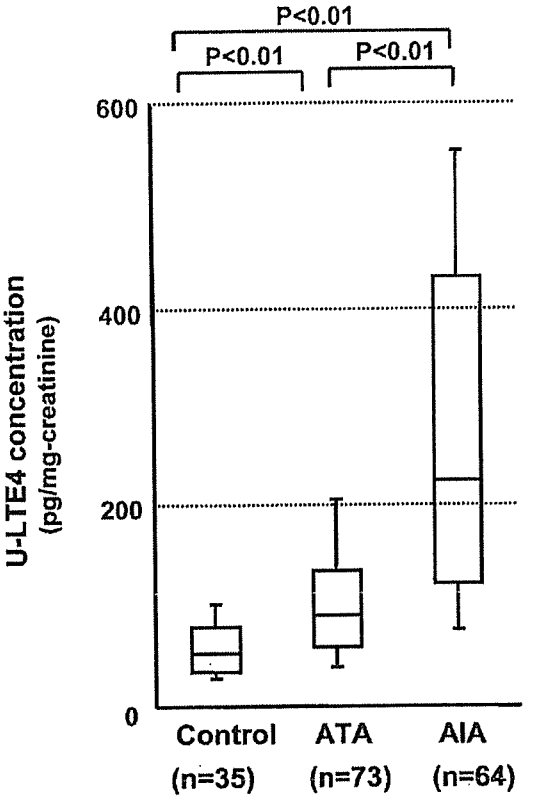

FIG 1. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with AIA, patients with ATA, and healthy control subjects. U-LTE4 concentration in each group is expressed as median plus percentile (10%, 25%, 75%, and 90%).

with ATA (71.7% vs 40.3%, $P < .001$). In addition, the frequency of anosmia was higher in the patients with AIA (frequency, 67.2% vs 24.2%; $P < .001$).

The basal excretion level of U-LTE4 was significantly higher in patients with AIA than in those with ATA (median, 227.2 vs 90.3 pg/mg creatinine; $P < .01$; Fig 1 and Table I). There was no significant difference in the U-LTE4 concentration between the sexes for these 2 groups (data not shown). When U-LTE4 concentrations between these 2 groups with different clinical asthma severity levels were compared, the U-LTE4 concentrations in patients with AIA with moderate or severe persistent asthma were significantly higher than those in patients with ATA with the same severity of asthma ($P < .01$ and $P < .05$, respectively; Fig 2). Table II shows the clinical characteristics of patients with hyperleukotrienuria and those with normal leukotrienuria in each group. The frequency of the patients with AIA with hyperleukotrienuria was 67.2%. Compared with the patients with AIA with normal leukotrienuria, the patients with AIA with hyperleukotrienuria were significantly older (mean, 56.1 vs 44.8 years, $P < .05$) and had a lower pulmonary function (mean FEV_1, 1907 vs 2401 mL; $P < .05$). In contrast, the frequency of patients with ATA with hyperleukotrienuria was 21.9%, and patients with ATA with hyperleukotrienuria had more severe asthma ($P < .05$) and a higher frequency of anosmia (frequency, 50.0% vs 13.9%; $P < .05$). In addition, 81.3% of the

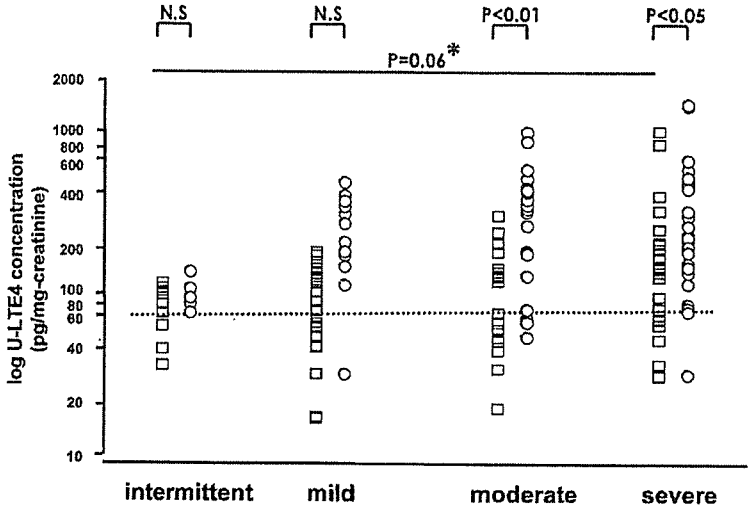

FIG 2. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with AIA and patients with ATA classified according to clinical severity of asthma. U-LTE4 concentration is expressed by using the log scale. Patients with ATA and patients with AIA are denoted by an *open square* and an *open circle*, respectively. The *dotted line* indicates the mean level of U-LTE4 in healthy control subjects. *U-LTE4 concentrations in the patients with ATA with different clinical asthma severity levels were compared by using the Kruskal-Wallis test.

patients with ATA with hyperleukotrienuria appeared to have CHRS/NP compared with 22.9% of the patients with ATA with normal leukotrienuria ($P < .001$). The number of eosinophils in peripheral blood was greater in the patients with ATA with hyperleukotrienuria (median, 860/μL vs 330/μL, $P < .01$). Female patients with ATA with hyperleukotrienuria showed a higher U-LTE4 concentration than male patients with ATA (median, 258.7 vs 184.3 pg/mg creatinine; $P < .05$).

Between the 2 groups with hyperleukotrienuria, the frequency of atopic status was significantly less in the patients with AIA (frequency, 48.8% vs 87.5%; $P < .05$). In contrast, between the 2 groups with normal leukotrienuria, anosmia had a greater frequency in the patients with AIA (frequency, 50.0% vs 13.9%; $P < .05$).

When we examined the correlations between the U-LTE4 concentration and other clinical parameters, a statistically nonsignificant but noteworthy correlation between the U-LTE4 concentration and clinical asthma severity level in the patients with ATA was found ($P = .06$, Fig 2). In addition, U-LTE4 concentrations in the patients with ATA with CHRS/NP (n = 27) were higher than those in the patients with ATA with normal sinuses (n = 30; median, 148.0 vs 81.2 pg/mg creatinine; $P < .05$; Fig 3). In contrast, there were 43 patients with CHRS/NP and only 8 with normal sinuses in the AIA group. No significant correlations between the U-LTE4 concentration and clinical parameters, such as asthma severity or the complication of CHRS/NP, were observed in the AIA group. There was a significant decrease in the U-LTE4 concentration after the sinus surgery in 15 asthmatic patients (median, 221.3 vs 72.3 pg/mg creatinine; $P < .01$). Six of 9 asthmatic patients with hyperleukotrienuria before the sinus surgery were not classified into the hyperleukotrienuria group after the sinus surgery. Fig 4 shows significant decreases in the U-LTE4 concentrations before and after the sinus surgery in both the AIA and ATA groups (median, 322.7 pg/mg creatinine [before] vs 98.2 pg/mg creatinine [after], $P < 0.05$, for the AIA group; 93.4 pg/mg creatinine [before] vs 80.9 pg/mg creatinine [after], $P < .05$, for the ATA group). Before the sinus surgery, the U-LTE4 concentrations of both groups were not significantly different. Similarly, the U-LTE4 concentrations of both groups after the sinus surgery showed no significant difference. All the nasal polyp and sinus sections revealed a diffused and marked infiltration of eosinophils, leading to the diagnosis of CHRS/NP.[36-38]

DISCUSSION

There is accumulating evidence that supports the central role of cys-LTs as mediators of inflammatory diseases.[1] An increase in the U-LTE4 concentration has been observed not only in asthma but also in autoimmune diseases, including rheumatoid arthritis[39] and systemic lupus erythematosus.[40] However, the relationship between the clinicopathogenetic factors of asthma and U-LTE4 concentration remains undetermined. We have demonstrated for the first time that there are some important clinicopathogenetic factors associated with increased excretion levels of U-LTE4 (ie, hyperleukotrienuria) in asthmatic patients. Hyperleukotrienuria in patients with AIA (67.2% of patients with AIA in this study) was associated with older age and decrease in pulmonary function. On the other hand, hyperleukotrienuria in patients with ATA (21.9% of the patients with ATA in this study) was associated with severe asthma and CHRS/NP, which are well-known symptoms of the aspirin triad,[41] as well as hypereosinophilia and anosmia. Conversely, the U-LTE4 concentration in the patients with ATA with CHRS/NP was

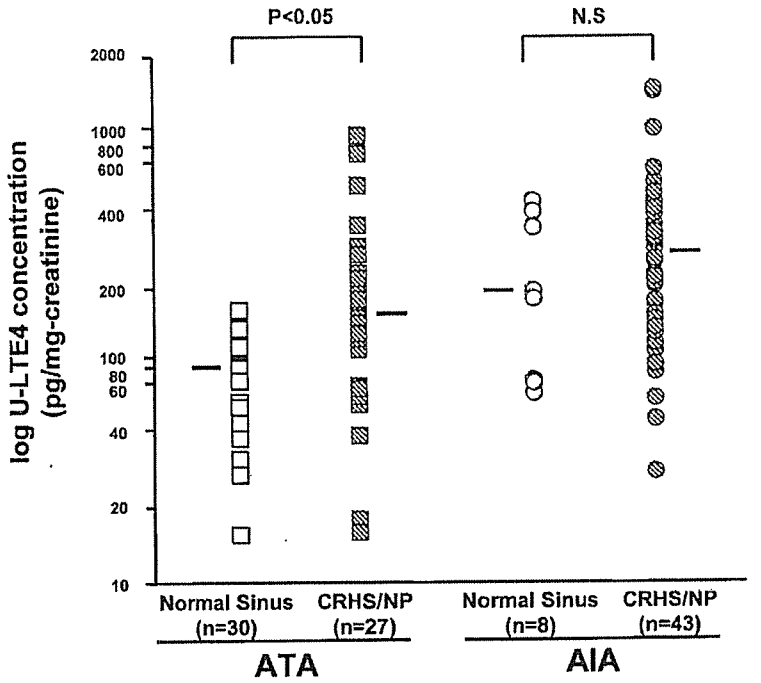

FIG 3. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with CHRS/NP and those with normal sinuses. U-LTE4 concentration is expressed by using the log scale. The *horizontal bars* indicate medians. Patients with ATA and patients with AIA are denoted by an *open square* and an *open circle*, respectively.

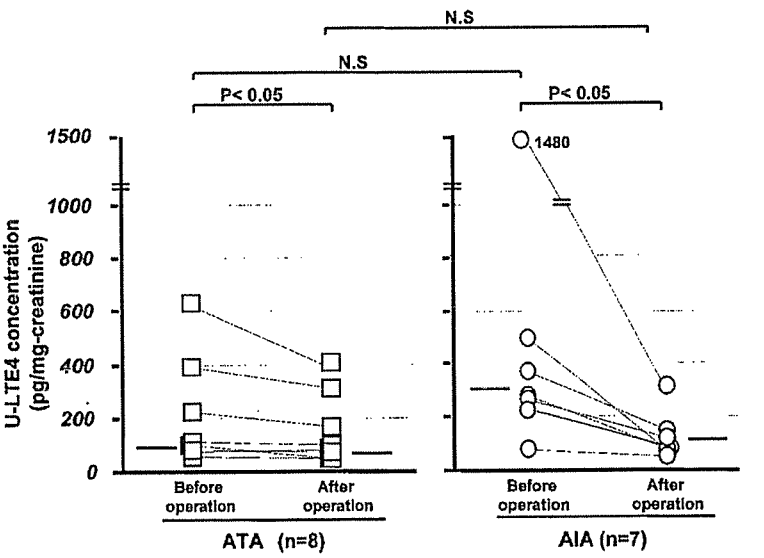

FIG 4. A significant decrease in the U-LTE4 concentration between before and after the endoscopic surgery of rhinosinusitis with nasal polyposis. *Horizontal bars* indicate medians. Patients with ATA and patients with AIA are denoted by *squares* and *circles*, respectively.

higher than that in the patients with ATA with normal sinuses. In addition, a statistically nonsignificant but noteworthy correlation between the U-LTE4 concentration and clinical asthma severity in patients with ATA was found. In support of our findings, ten Brinke et al[42] have suggested a direct relationship between hyperplastic rhinosinusitis and lower airway inflammation in severe asthma, and they have suggested that extensive rhinosinusitis is associated with adult-onset asthma, aspirin intolerance, and nasal polyposis.

Taken together, these findings led us to the following generalizations. First, cys-LT overproduction is not strictly associated with aspirin intolerance itself but is associated with clinical features, such as CHRS/NP, that are similar to those of AIA. Second, CHRS/NP might play an important role in cys-LT overproduction in asthmatic

TABLE II. Demographic characteristics of each group

	AIA			ATA				
	Hyperleukotrienuria	Normal leukotrienuria	P_{AIA} (h-n)	Hyperleukotrienuria	Normal leukotrienuria	P_{ATA} (h-n)	PG(h-h)	PG(n-n)
No.	43	12		16	40			
Male/female sex	16/27	3/9	NS	6/10	21/19	NS	NS	NS
Age (y), mean (SD)	56.1 (13.0)	44.8 (11.4)	<.05	52.1 (12.2)	49.2 (16.1)	NS	NS	NS
Onset age (y), mean (SD)	39.2 (14.1)	32.3 (15.9)	NS	39.4 (16.9)	33.5 (17.1)	NS	NS	NS
Atopic status (n)	21 (48.8)	7 (58.3)	NS	14 (87.5)	23 (57.5)	NS (.07)	<.05	NS
Blood eosinophil ($\times 10^6$/mL), median (range)	535 (12-3520)	560 (5-2460)	NS	860 (200-4163)	330 (20-930)	<.01	NS	NS
Serum IgE-RIST (IU/mL), median (range)	114 (14-1390)	119 (15-600)	NS	314 (44-2290)	162 (15-2050)	NS	NS	NS
Pulmonary function tests								
FEV_1 (mL), mean (SD)	1907 (746)	2401 (622)	<.05	2117 (923)	2308 (788)	NS	NS	NS
FEV_1 (% predicted), mean (SD)	73.5 (19.5)	91.1 (18.8)	<.05	81.1 (26.3)	84.1 (20.4)	NS	NS	NS
Clinical features								
Severity			NS			<.05	NS	NS
Intermittent, n (%)	0 (0)	3 (25.0)		0 (0)	8 (20.0)			
Mild persistent, n (%)	9 (20.9)	1 (8.3)		2 (12.5)	15 (37.5)			
Moderate persistent, n (%)	12 (27.9)	4 (33.3)		4 (25.0)	8 (20.0)			
Severe persistent, n (%)	22 (51.2)	4 (33.3)		10 (62.5)	9 (22.5)			
CHRS/NP, n (%)	30* (75.0)	6 (50.0)	NS	13 (81.3)	8† (22.9)	<.001	NS	NS
Anosmia, n (%)	28* (70.0)	6 (50.0)	NS	8 (50.0)	5‡ (13.9)	<.05	NS	<.05
Treatment								
Oral systemic CS, n (%)	12 (27.9)	4 (33.3)	NS	1 (6.3)	6 (15.0)	NS	NS	NS
LTRAs, n (%)	12 (27.9)	4 (33.3)	NS	3 (18.8)	9 (22.5)	NS	NS	NS
Dose of inhaled CS (mg/d), median§	800	950	NS	800	800	NS	NS	NS

*n = 40, patients with AIA with hyperleukotrienuria.

†n = 35 and ‡n = 36, patients with ATA with normal leukotrienuria.

§Dose of inhaled corticosteroids is the median daily dose in beclomethasone equivalent units. Beclomethasone is assumed to be equipotent with budesonide and has a potency half of that of fluticasone.

P(h-n), Significant differences between asthmatic patients with hyperleukotrienuria and those with normal leukotrienuria; *PG*, significant differences between the 2 groups; *PG(h-h)*, significant differences between the 2 groups with hyperleukotrienuria; *PG(n-n)*, significant differences between the 2 groups with normal leukotrienuria; *NS*, not significant; *RIST*, radioimmunosorbent test; *CS*, corticosteroids.

patients. The precise pathophysiologic mechanism underlying hyperleukotrienuria remains obscure. We attempted to reconfirm the involvement of CHRS/NP in the pathogenesis of hyperleukotrienuria, and thus we compared the U-LTE4 concentrations between before and after the sinus surgery. We demonstrated for the first time that there is a significant decrease in the U-LTE4 concentrations after the sinus surgery, suggesting that sinonasal tissue might be one of the main tissues that produce cys-LTs in asthmatic patients. Surgical resection of both large nasal polyps and extensive hyperplastic sinonasal mucosa contributes to the termination of the vicious cycle in which cys-LTs and chemotactic factors produced in the sinonasal tissue[43] promote the chemotaxis of eosinophils.[44] More recently, it has been reported that cys-LT concentrations were significantly higher in sinus tissues obtained from patients with CHRS/NP than in healthy sinus tissues and noneosinophilic sinus tissues.[45] Thus the complication of severe CHRS/NP might affect the U-LTE4 concentration, and sinonasal tissues might be one of the main tissues that produce cys-LTs in asthmatic patients. Interestingly, we reconfirmed the decrease in U-LTE4 concentration after surgery for CHRS/NP (72.4 pg/mg creatinine to 32.8 pg/mg creatinine) in only one nonasthmatic patient. Because U-LTE4 has a detection limit to serve as a biomarker of airway inflammation,[32,35] it is necessary to carry out further investigations with a large number of patients. Considering the high recurrence rate of nasal polyposis in patients with AIA,[46] we should examine whether those with CHRS/NP recurrence have significant increases in U-LTE4 levels over time and whether aspirin intolerance will develop in patients with ATA with hyperleukotrienuria exhibiting clinical characteristics similar to those of patients with AIA.

In conclusion, our study showed, for the first time, a close relationship between the U-LTE4 concentration and clinical features of asthmatic patients. We suggest the possibility that cys-LTs are not strictly associated with aspirin intolerance itself but with clinical features such as CHRS/NP that are similar to those of AIA. In particular, our study suggests the involvement of CHRS/NP in cys-LT overproduction in asthmatic patients.

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Urinary eicosanoid and tyrosine derivative concentrations in patients with vasculitides

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Background: Vasculitides are classified on the basis of the type of cell involved, namely, eosinophilic vasculitides such as Churg-Strauss syndrome (CSS) and noneosinophilic vasculitides. However, knowledge on inflammatory mediators and oxidative tissue damage associated with vasculitides is insufficient.

Objective: We measured the urinary concentrations of inflammatory mediators and tyrosine derivatives to assess biomarkers associated with the pathophysiology of vasculitides.

Methods: Urine was collected from 9 patients with CSS during acute exacerbation and during clinical remission, 24 patients with rheumatoid arthritis in stable condition, and 8 patients with vasculitis diseases (VDs) during acute exacerbation. Leukotriene E_4 (LTE_4), 9α,11β prostaglandin F_2, and eosinophil-derived neurotoxin (EDN) concentrations were determined by enzyme immunoassay. 3-Bromotyrosine (BrY) and 3-chlorotyrosine (ClY) concentrations were determined by gas chromatography-mass spectrometry.

Results: The urinary LTE_4, EDN, BrY, and ClY concentrations were significantly higher in the patients with CSS during acute exacerbation than in healthy control subjects and, except for urinary ClY concentration, significantly decreased during clinical remission. The urinary EDN and BrY concentrations were significantly higher in patients with CSS during acute exacerbation than in patients with VD during acute exacerbation. Only urinary LTE_4 concentration was significantly different between the patients with rheumatoid arthritis in stable condition and the patients with VD during acute exacerbation.

Conclusion: Oxidative tissue damage caused by eosinophil peroxidase is a pathophysiological characteristic of eosinophil-associated diseases such as CSS. Urinary LTE_4 concentration may reflect a pathophysiological event involved in eosinophilic and noneosinophilic vasculitides. Cysteinyl-leukotriene pathways are potential therapeutic targets for small-vessel vasculitides. (J Allergy Clin Immunol 2004;114:1353-8.)

Key words: Churg-Strauss syndrome, vasculitides, 3-bromotyrosine, 3-chlorotyrosine, leukotriene E_4

Abbreviations used
ANCA: Antineutrophil cytoplasmic autoantibody
BrY: 3-Bromotyrosine
ClY: 3-Chlorotyrosine
cr: Creatinine
CSS: Churg-Strauss syndrome
cysLT: Cysteinyl-leukotriene
EDN: Eosinophil-derived neurotoxin
EPO: Eosinophil peroxidase
HC: Healthy control
HOBr: Hypobromous acid
LT: Leukotriene
MPA: Microscopic polyangiitis
PG: Prostaglandin
RA: Rheumatoid arthritis
TA: Temporal arteritis
VD: Vasculitis disease
WG: Wegener granulomatosis

Eosinophils possess a wide range of biological properties. Namely, eosinophils release proteins, inflammatory cytokines, and mediators, such as eicosanoids and platelet-activating factors, and can cause tissue injury by releasing a spectrum of toxic products. Eosinophil peroxidase (EPO)[1-3] also resides in a matrix of cytoplasmic granules and is one of the most abundant proteins in eosinophils.[4] EPO plays a role in mediating the host-defense mechanism, such as the destruction of invading parasites and the pathological damage of host tissue by oxidizing intermediates. Briefly, activated eosinophils generate superoxide (O_2^-) by using a membrane-associated nicotinamide adenine dinucleotide phosphate oxidase,[5] and its dismutation product, H_2O_2.[1] By using H_2O_2 as a cosubstrate, EPO in eosinophils generates a halogenating oxidant, which is a potent reactive, cytotoxic, and diffusible species. Despite the fact that the plasma chloride (Cl^-) concentration is 1000-fold higher than that of bromide (Br^-), interestingly, the major product of the EPO-H_2O_2 system is hypobromous acid (HOBr): $Br^- + H_2O_2 + H^+ \rightarrow HOBr + H_2O$.[6] *In vitro*, HOBr reacts with primary amines to form N-mono-bromamines, and it converts tyrosine to 3-bromotyrosine (BrY).[7,8] Similarly, myeloperoxidase, a structurally and functionally distinct enzyme produced by neutrophils, monocytes, and certain tissue macrophages,[9] also contributes to inflammatory tissue injury. Neutrophils selectively use Cl^- in plasma to

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TABLE I. Demographic characteristics of patients with CSS

Male/female sex	2/7
Age, y, mean (SD)	51.8 (15.2)
Age at onset, y, mean (SD)	
Asthma onset	42.3 (14.3)
CSS onset	49.8 (14.5)
Blood eosinophil $\times 10^6$/L, median (range)	
During acute exacerbation	6590 (2510-17,880)
During remission	70 (10-700)
IgE-radioimmunosorbent test, IU/mL, median (range)	467 (18-3360)
Cumulative organ involvement, n	
Mononeuritis multiplex	9
Lung	9
Sinus	7
Kidney/urinary tract	7
Heart	4
Skin	2
Mediastinum	1

generate chlorinating oxidants.[10-12] Thus, 3-chlorotyrosine (ClY) is considered to be a selective marker of myeloperoxidase-catalyzed oxidation, whereas BrY is that of EPO-catalyzed oxidation.[13]

The characteristic feature of Churg-Strauss syndrome (CSS),[14,15] an eosinophilic necrotizing vasculitis,[16] is hypereosinophilia in blood and tissues, such as those of the lungs, gastrointestine, nerves, and kidneys. The extent of eosinophilia commonly reflects clinical disease activity.[14,15] Previous studies demonstrated that the seromarkers of the activation of eosinophils, such as eosinophil cationic protein and eosinophil-derived neurotoxin (EDN), can predict a relapse.[17] However, measurement of these seromarkers is not applicable to patients with noneosinophilic small-vessel vasculitides, such as Wegener granulomatosis (WG) and microscopic polyangiitis (MPA). Considering the oxidative reaction-associated myeloperoxidase in the human artery,[12,18] we hypothesized that measuring both BrY and ClY may serve as a powerful method for estimating oxidative tissue damage and the relative contributions of eosinophils versus neutrophils *in vivo*. Furthermore, the high levels of antibody against myeloperoxidase-specific antineutrophil cytoplasmic autoantibody (ANCA) are observed in patients with myeloperoxidase-ANCA–related vasculitides such as WG and MPA.[19,20] Because ANCA-activated neutrophils can adhere to and destroy endothelial cells *in vitro*, ANCA is considered to inhibit the inactivation of myeloperoxidase, resulting in tissue damage.[21,22] In addition, Mayatepek and Lehmann[23] demonstrated a high urinary leukotriene (LT) E_4 concentration in patients with Kawasaki disease, which is the most common childhood vasculitis. However, there has been little experimental evidence to substantiate the close relationship between cysteinyl-leukotriene (cysLT) and vasculitides. According to the National Institutes of Health workshop report,[24] there is no objective evidence that CSS is actually caused by LT receptor antagonists. Because there have been no comparative studies of urinary eicosanoid concentrations and clinical characteristics, we aimed to characterize the profiles of eicosanoid, BrY, and ClY concentrations in patients with systemic small-vessel vasculitides, including CSS.

METHODS

Subjects

The subjects of this study were 9 patients with CSS (age range, 27-75 years; mean age, 51.8 years; 2 male and 7 female), 8 patients with vasculitis diseases (VDs) during acute exacerbation (age range, 50-77 years; mean age, 66.0 years; 2 male and 6 female), 24 patients with rheumatoid arthritis (RA) in a clinically stable condition (age range, 39-77 years; mean age, 60.1 years; 6 male and 18 female), and 8 healthy control (HC) subjects (age range, 27-55 years; mean age, 37.3 years; 4 male and 4 female). CSS was diagnosed according to the 1990 American College of Rheumatology criteria and the 1992 Chapel Hill definition.[25,26] Three patients had recurrent CSS. The mean age at onset of CSS was 49.8 (Table I). Histopathological confirmations, such as necrotizing vasculitis, extravascular necrotizing granulomas, and/or hypereosinophilia in extravascular tissues, were present in all 9 patients with CSS. Clinical examinations and staging included the lung function test, chest radiography, bronchoalveolar lavage test, echocardiography, radioisotope scintigraphy, otorhinolaryngologic and neurologic examinations, and laboratory screening for ANCA.[27] During the acute exacerbation of CSS, all patients with CSS showed vasculitis symptoms involving multiple organs, such as eosinophilic pneumonia and cardiopathy, in addition to peripheral hypereosinophilia (mean %, 58.1%) and mononeuritis multiplex, as shown in Table I. After intensive immunosuppressive therapies with drugs including systemic corticosteroids (n = 5 with intravenous administration of 1000 mg/d methylprednisolone for 3 days; n = 9 with 30-40 mg/d prednisolone), cyclosporine (n = 5, 50-100 mg/d), and/or intravenous immunoglobulin[27] (n = 7), all 9 patients with CSS were clinically in a disease remission phase, maintained at a dose of 5 to 25 mg/d prednisolone (n = 7) in addition to cyclosporine (n = 4, 50-100 mg/d) at the time of follow-up examination. The mean percentage of peripheral eosinophils was 2.3%. The duration between acute exacerbation and remission was 6.1 ± 2.5 months.

The patients with VD had an acute exacerbation of vasculitis accompanied by autoimmune diseases, such as MPA,[26] WG,[26,28] temporal arteritis (TA, giant-cell arteritis),[29] and RA[30] (Table II). The patients with VD were diagnosed on the basis of clinical and laboratory examination findings, such as the presence of ANCA (n = 4) and immunocomplex C1q. Pathological vasculitis was confirmed in 5 of 8 patients. In contrast, the stable RA group, composed of 24 patients with RA in a stable condition, was a comparative control for patients with an acute exacerbation of VD. Nine patients received systemic corticosteroid (mean prednisolone dose, 4.2 mg/d), whereas 12 patients received methotrexate therapy. None of the patients had an upper respiratory tract infection in the 4 weeks preceding the study. Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital, and all of the patients who participated gave their informed consent.

Measurements

Spot urine was collected between 9:00 and 11:00 AM from patients with CSS during acute exacerbation and during clinical remission, patients with VD during acute exacerbation, patients with stable RA,

TABLE II. Demographic characteristics of patients with VD

Male/female sex	2/6
Age, y, mean (SD)	66.0 (8.4)
White blood cells $\times 10^6$/L	9453 (3024)
Neutrophil %, mean (SD)	81.0 (8.7)
Eosinophil %, mean (SD)	2.1 (1.9)
C-reactive protein, mg/dL, mean (SD)	9.7 (6.6)
C1q, mg/mL, median (range)*	5.0 (1.6-29.6)
Underlying diseases, n	
RA	3
MPA	1
MPA, PSS, and Sjogren syndrome	1
WG	1
Mixed-connective tissue disease and RA	1
TA and Behcet disease	1
Cumulative vasculitis-associated symptoms, n	
Mononeuritis multiplex	5
Skin ulcer	2
Scleritis	2
RA nodule/TA nodule	3/1
Pathological findings, n	5
Obstructive vasculitis/necrotizing vasculitis	3/2

*n = 7.

and HC subjects. In particular, in the cases of acute exacerbations of CSS and VD, urine was collected before intensive immunosuppressive therapy. We determined the urinary concentrations of LTE_4 (Cayman, Ann Arbor, Mich), 9α,11β prostaglandin (PG) F_2 (Cayman), which corresponds to the PGD_2 metabolite, and EDN (MBL, Nagoya, Japan) by enzyme immunoassay as previously reported.[31] The urinary concentrations of BrY and ClY, the selective markers of EPO-catalyzed and myeloperoxidase-catalyzed oxidations, respectively, were determined by gas chromatography-mass spectrometry by using ^{13}C-labeled compounds as internal standards, as reported elsewhere.[32] Briefly, after the addition of $^{13}C_6$-BrY (50 ng) and $^{13}C_6$-ClY (30 ng) to 2 mL urine, BrY and ClY were extracted with 25% methanol by using a reverse-phase column and then converted to the corresponding heptafluorobutyryl *tert*-butyldimethylsilyl derivatives.[33,34] BrY and ClY concentrations were determined by using Shimadzu gas chromatography-mass spectrometry QP2010 (Kyoto, Japan) equipped with a SPD-5 capillary column (15 m; 0.25-mm internal diameter; 0.25-μm film thickness; Supelco, Bellefonte, Pa) in the negative ion chemical ionization mode with methane as the reagent gas. BrY and ClY concentrations were determined by measuring the fragment ions at mass-to-charge ratio (m/z) 489.10 for endogenous compounds and m/z 495.15 for the internal standards. Urinary LTE_4, 9α,11βPGF_2, EDN, BrY, and ClY concentrations were normalized to urinary creatinine (cr) concentration.

Analysis of data

Demographic characteristics are expressed as means ± SDs. The urinary eicosanoid, EDN, BrY, and ClY concentrations are expressed on a log scale in the figures. These urinary concentrations in the 4 groups (CSS during acute exacerbation, VD during acute exacerbation, stable RA, and HC groups) were first compared by using the Kruskal-Wallis test. When a significant difference was found, the Mann-Whitney *U* test with the Bonferroni correction for comparison between groups was performed. The urinary concentrations of the 5 biomarkers in CSS patients during acute exacerbation and clinical remission were compared by using the Wilcoxon *t* test. Relationships were analyzed by using the Spearman rank correlation test. *P* values of less than .05 were regarded as statistically significant.

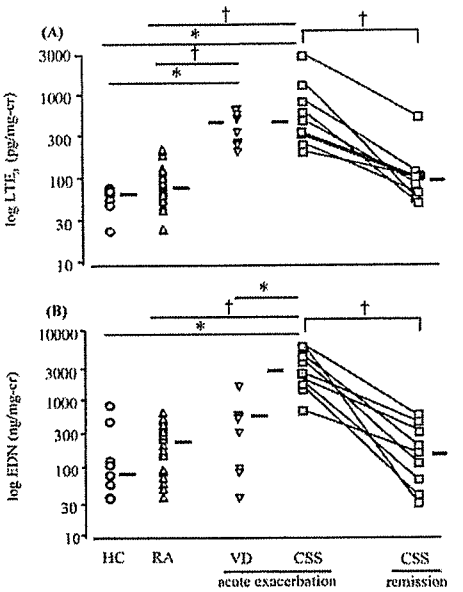

FIG 1. Urinary LTE_4 **(A)** and EDN **(B)** concentrations in each group. Urinary concentrations are expressed by using the log scale. Patients with CSS, VD, and RA and HC subjects are denoted by *closed squares, closed triangles, open triangles,* and *open circles,* respectively. *Horizontal bars* indicate medians. *$P < .05$; †$P < .01$.

RESULTS

As shown in Fig 1, the urinary LTE_4 concentration was significantly higher in the patients with CSS during acute exacerbation (median, 449.6 pg/mg-cr) than in the patients with stable RA (median, 79.3 pg/mg-cr; $P < .01$) and the HC subjects (67.5 pg/mg-cr; $P < .05$). A significantly higher urinary EDN concentration was observed in the patients with CSS during acute exacerbation (2404.0 ng/mg-cr) than in the patients with VD (432.3 ng/mg-cr; $P < .05$), the patients with stable RA (296.7 ng/mg-cr; $P < .01$), and the HC subjects (94.9 ng/mg-cr; $P < .05$), respectively. Fig 2 shows the urinary BrY and ClY concentrations in each group. The urinary BrY concentration was significantly higher in the patients with CSS during acute exacerbation (182.6 ng/mg-cr) than in the patients with stable RA (36.8 ng/mg-cr; $P < .01$) and the HC subjects (25.2 ng/mg-cr; $P < .05$). The urinary ClY concentration was significantly higher in the patients with CSS during acute exacerbation (6.1 ng/mg-cr; $P < .05$), the patients with VD during acute exacerbation (9.2 ng/mg-cr; $P < .05$) and the patients with stable RA (4.7 ng/mg-cr; $P < .05$) than in the HC subjects (1.2 ng/mg-cr). No significant difference in urinary 9α,11βPGF_2 concentration was observed among the 4 groups (data was not shown). Next, we examined the correlation between these urinary parameters and the involvement of vasculitis. As shown in Figs 1 and 2, there were significant differences in urinary LTE_4, EDN, and BrY concentrations in the patients with CSS during acute exacerbation and clinical remission (median, for LTE_4, 449.6 pg/mg-cr vs 91.2 pg/mg-cr;

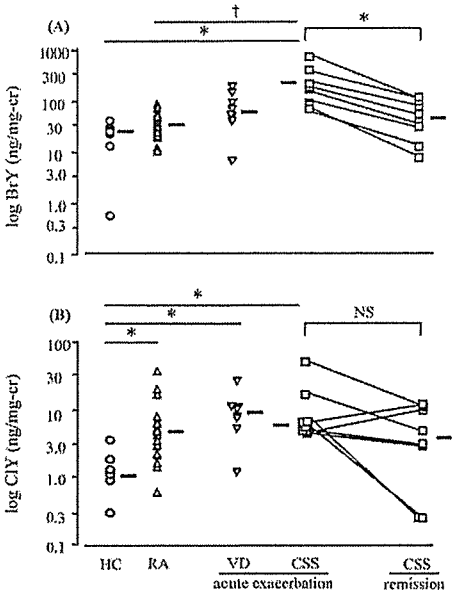

FIG 2. Urinary BrY (A) and ClY (B) concentrations in each group. Results are expressed as in Fig 1. *$P < .05$; †$P < .01$.

$P < .01$; for EDN, 2404.0 ng/mg-cr vs 151.7 ng/mg-cr; $P < .01$; for BrY, 182.6 ng/mg-cr vs 44.4 ng/mg-cr; $P < .05$). Only urinary LTE_4 concentration was significantly different between the patients with stable RA and the patients with VD during acute exacerbation (median, 79.3 pg/mg-cr vs 434.0 pg/mg-cr; $P < .01$). No correlation was found among these 5 urinary markers in any of the 4 groups.

DISCUSSION

Leukotriene E_4 has been identified as a major metabolite of LTC_4, and urinary LTE_4 concentration is now considered the most appropriate analytical parameter for monitoring the endogenous synthesis of cysLTs.[35] In this study, we demonstrated for the first time that urinary LTE_4 concentration was significantly higher in patients with CSS during acute exacerbation than in HC subjects, and significantly decreased during clinical remission. It is most interesting to note that the increased urinary LTE_4 concentration in patients with VD during acute exacerbation was observed despite relatively low EDN concentrations. Recent studies have demonstrated a close relationship between cysLTs and vascular events. Sjöström et al[36] demonstrated that microsomal glutathione S-transferase 2, a distant homologue of LTC_4 synthase, is a critical enzyme present in vascular walls for LTC_4 biosynthesis, originating from the transfer of LTA_4 from granulocytes to endothelial cells. In addition, an increased urinary LTE_4 concentration was observed in patients with ischemic heart diseases.[37-39] Taking these findings together, transcellular biosynthesis among mononuclear cells and endothelial cells plays an important role in the cysLT overproduction in vasculitides. Aspirin intolerance is also characterized by a cysLT overproduction profile.[40-42] In particular, the clinical features of CSS are quite similar to those of the aspirin intolerance phenotype—namely, bronchial asthma, eosinophilic sinusitis, and hypereosinophilia. We previously demonstrated that basal urinary LTE_4 concentration in patients with asthma is higher than that in HC subjects, and that basal urinary LTE_4 concentration in asthmatic patients with eosinophilic sinusitis is higher than that in asthmatic patients without eosinophilic sinusitis.[40] In addition, we preliminarily confirmed in this study that markedly high urinary LTE_4 concentrations (167.6, 188.3, and 199.0 pg/mg-cr) were observed in 3 patients with nonvasculitic eosinophil diseases (acute eosinophilic pneumonia, episodic eosinophilic angioedema, and bronchial asthma with hypereosinophilia). However, despite markedly high percentages of blood eosinophils (mean, 34.6%), the extents of increase in urinary LTE_4 concentrations in these 3 patients with nonvasculitic eosinophil diseases were relatively smaller than in patients with CSS. Thus, particularly in CSS, eosinophilic vasculitides may be involved in cysLT overproduction in addition to eosinophilic pneumonia and sinusitis. Transcellular biosynthesis among endothelial cells and LTC_4 synthase–positive cells, including eosinophils, plays a key role in the mechanism underlying cysLT production in CSS. The vicious cycle, in which cysLTs promote the progenitor effect of LTC_4-producing cells,[43] possibly contributes to the further increased production of cysLTs in patients with an acute exacerbation of vasculitides. At least, this study demonstrated that urinary LTE_4 concentration as a new biomarker determined by a noninvasive methodology possibly contributes to the early diagnosis of small-vessel vasculitides.

In this study, we determined the urinary concentrations of 2 halogenated oxidation products—that is, BrY and ClY. BrY is considered a candidate marker of eosinophil activation,[13,32] ClY of neutrophil and monocyte activation. BrY and ClY concentrations in biological samples such as bronchoalveolar lavage fluid[13,44] and sputum[45] have been determined. Thus, we hypothesized that the adaptation of this methodology is expected to identify oxidative tissue damage and the involvement of specific inflammatory cells in vasculitides and hypereosinophilia.[46,47] In the patients with CSS, the urinary BrY concentration significantly increased during acute exacerbation and decreased during clinical remission. We previously demonstrated the significantly higher urinary BrY and ClY concentrations in patients with asthma than in the HC subjects.[32] Similarly, 3 patients with nonvasculitic eosinophil diseases described previously also showed high urinary BrY concentrations (97.4, 122.9, and 62.0 ng/mg-cr). Thus, these findings suggest that the oxidative tissue damage caused by activated eosinophils is a pathophysiological characteristic of eosinophil-associated diseases such as bronchial asthma and CSS. In contrast, we also analyzed the time course of the concentrations of urinary tyrosine derivatives in 2 patients with severe anaphylactic shock. This additional analysis showed normal urinary tyrosine derivative concentrations despite