

Original article

Effect of inhaled corticosteroid on an immunoreactive thymus and activation-regulated chemokine expression in the bronchial biopsies from asthmatics

Background: Bronchial asthma is characterized by airway inflammation, notably because of eosinophils and T cells. Thymus and activation-regulated chemokine (TARC) is known to selectively attract Th2 cells, and is increased in response to interleukin (IL)-4 and IL-13, which share a common receptor, IL-4 receptor alpha (IL-4R α). While corticosteroids have proven, very effective in modifying airway inflammation, the effect of corticosteroids on TARC in asthmatics has been little studied.

Objective: We examined the effects of inhaled budesonide (BUD) on the expression of TARC and the number of inflammatory cells in bronchial biopsy specimens taken from asthma patients.

Methods: Inhaled BUD 800 μ g daily, or placebo was administered for 3 months in a double-blind, parallel-group study, and bronchial biopsies were performed before and after treatment. Biopsy specimens were examined by immunocytochemistry.

Results: We observed a significant decrease in the epithelial expression of TARC ($P < 0.01$) in the BUD group compared with the placebo group. This was accompanied by decreases in the number of eosinophils ($P < 0.01$), CD3⁺ T cells ($P < 0.05$), and CD4⁺ T cells ($P < 0.01$). A significant correlation was found between changes in epithelial TARC and in IL-4R α immunoreactivity ($r_s = 0.66$, $P < 0.01$).

Conclusions: These findings suggest that corticosteroid asthma treatment can reduce infiltration of the airway by inflammatory cells, an effect modulated by down-regulation of bronchial epithelial TARC expression.

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Key words: airway inflammation; bronchial asthma; budesonide; IL-4 receptor alpha; thymus and activation-regulated chemokine; Th2 cytokine.

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Bronchial asthma is a chronic inflammation of the airways, particularly because of infiltration of eosinophils and T cells (1). CD4⁺ Th2 cells produce cytokines such as interleukin (IL)-4, IL-5 and IL-13, which induce immunoglobulin (Ig)E production and eosinophil activation (2). However, the mechanism controlling recruitment of T cells into sites of allergic inflammation and subsequent events including eosinophil infiltration remains largely unknown.

Thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) have been identified as specific ligands for CCR4 (3, 4) and were shown to induce chemotaxis of CD4⁺ T cells (5, 6). We have previously demonstrated that TARC expression is increased in the bronchial epithelium, but not MDC expression, asthmatic patients (7). *In vitro* studies of bronchial TARC production have shown that overlapping, but different, effector profiles are induced by combined stimulation with IL-4 and tumor necrosis

factor (TNF)- α , as well as IL-13 and TNF- α (7, 8). The IL-4 and IL-13 share a common receptor chain, IL-4 receptor alpha (IL-4R α), and activate the signal transduction mechanism (9). Furthermore, overexpression of IL-4R α has been reported in asthmatic bronchial epithelial cells (10). The fact that TARC production is induced by stimulation with these cytokines suggests IL-4R α plays a major role in the amplification of recruitment of CD4⁺ Th2 cell recruitment.

Inhaled corticosteroids (ICS) are currently the recommended first line of treatment for asthma. However, clinical studies investigating the effects of corticosteroids on local TARC production in patients with asthma have not yet been performed. We have hypothesized that the beneficial effect of ICS may be due to inhibition of TARC generation by epithelial cells. The purpose of this study was to examine the effect of treatment with ICS on TARC expression in asthmatic patients. As regulation of TARC generation has therapeutic potential in the treatment of

asthma, we evaluated the infiltration of inflammatory cells into airway mucosa and also the immunoreactivity of IL-4R α .

Material and methods

Subjects

Twenty-seven nonsmoking subjects with asthma were recruited for this study. Asthma was defined on the basis of (1) a clear history with current clinical symptoms and (2) a >20% reversibility of forced expiratory volume in 1 s (FEV₁) spontaneously or after β_2 -agonist inhalation and airway hyperresponsiveness to methacholine. The severity of asthma was classified according to a combination of symptoms, the amount of β_2 -agonist use to treat symptoms, and lung function (11). Four subjects had mild asthma, 23 had moderate asthma, and none had severe asthma. Atopy was defined as a positive skin prick test (wheal > 3 mm in diameter in the presence of positive histamine and negative diluent controls) to one or more common aeroallergen extracts. None of the subjects received inhaled or oral corticosteroids or any other anti-inflammatory drugs in the previous 3 months and an inhaled β_2 -agonist was the only bronchodilator used. No subjects had any upper or lower respiratory infections for at least 2 weeks before entering the study. This study was approved by the ethics committee of our hospital, and written informed consent was obtained from each subject.

Study design

We conducted a double-blind, placebo-controlled, parallel-group study. Each patient received a 3-month course of either 400 μ g budesonide (BUD) (AstraZeneca, Osaka, Japan) administered twice daily or a matched placebo. Spirometry was performed and airway methacholine responsiveness measured using an Astograph device (TCK-6000CV, Chest MI. Co, Tokyo, Japan), according to the method of Takishima et al. (12). Subjects returned 7 days later and underwent fiberoptic bronchoscopy. At the end of the course, patients' spirometric values, airway responsiveness were measured and fiberoptic bronchoscopy was repeated.

Bronchial biopsy and immunocytochemistry

A bronchoscope (BF type 1T-30; Olympus Co., Tokyo, Japan) and biopsy forceps (FB-15C, Olympus Co.) were used to take biopsy specimens from the subcarina. Biopsy specimens were mounted in optimal cutting temperature (OCT) compound, snap frozen in liquid nitrogen-cooled isopentane, and stored at -70°C until use. Cryostat serial sections (4 μ m) were cut from the frozen biopsy specimens, air-dried for 1 h, fixed in acetone/methanol solution, and stained using the alkaline phosphatase antialkaline phosphatase (APAAP) (Dako, Glostrup, Denmark) method as previously described (13), and slides were developed in fast red. For negative controls, the primary antibody was replaced by nonspecific mouse IgG or by a tris-buffered saline solution.

Antibodies

Sections were incubated with the following primary mouse monoclonal antibody (mAb), anti-major basic protein (MBP) (dilution 1 : 20; MAS529; Harlan Sera-Lab, Crawly Down, UK) to detect eosinophils, anti-CD3 (No.7340; Becton Dickinson, San Jose, CA,

USA) to detect total T cells, anti-CD4 (No. 6320; Becton Dickinson) and anti-CD8 (No. 6310; Becton Dickinson) to detect helper T cells and cytotoxic T cells, respectively (dilution 1 : 30). Immunolocalization of TARC and IL-4R α were examined with an anti-TARC mAb (dilution 1 : 100; a gift from A. Morita, Shionogi Institute Medical Science, Osaka, Japan) (14) and an anti-IL-4R α mAb (dilution 1 : 200; huIL-4R-M57; Immunex, Seattle, WA, USA).

Quantitation

All slides were coded before analysis by one observer and read blindly. Airway epithelial expression of immunoreactive TARC and IL-4R α in cryosections was quantitated using computerized colour image analysis (Win ROOF; Mitani Corp., Fukui, Japan) (15). The epithelium staining in each section was assessed by viewing several high-magnification fields covering the entire section, and the results are expressed as the percentage of the total epithelial area staining for either TARC or IL-4R α . As the bronchial epithelium, either intact or focally stripped, was present in all biopsy specimens, epithelial integrity was also assessed. The length of damaged epithelium was expressed as a percentage of the total length of epithelium measured along the basement membrane in each section. The number of inflammatory cells was counted for the entire lamina propria, excluding mucosal glands and vessels. Results are expressed as the number of positive cells per square millimetre of lamina propria.

Statistical analysis

Clinical values are expressed as mean values (ranges). Results for clinical data were analysed using the Student's *t*-test for within-group comparisons and the two-sample *t*-test for between-group comparisons. The percentages of TARC and IL-4R α expression and the number of positive cells are expressed as median values (ranges). Paired biopsy data were analysed using the Wilcoxon matched pairs signed rank test. For between-group comparisons, the Mann-Whitney *U*-test was performed on the delta values (before – after) for the two treatment groups. Intraobserver reproducibility was assessed by the coefficient of variation, and the same observer performed at least three replicate measurements of morphometric parameters. The mean coefficient of variation for repeated measurements ranged from 4 to 9% for percentage expression, and 5–14% for number of each cell type. There was no statistically significant difference in the proportion of damaged epithelium between the subjects treated with BUD and with a placebo. Correlation coefficients were obtained using Spearman's rank order method. A *P* < 0.05 was considered statistically significant.

Results

There were no significant differences between the two groups in terms of age, sex, asthma severity, FEV₁%, airway responsiveness, or IgE value. In addition, all asthmatic patients were atopic.

Clinical data

There were significant improvements in FEV₁% (*P* < 0.01) and Dmin (*P* < 0.01) in BUD-treated, but not placebo-treated, patients. Compared with the

Table 1. Clinical data before and after treatment with budesonide (BUD) or placebo

	BUD		Placebo	
	Before	After	Before	After
n (male : female)		14 (9 : 5)		13 (9 : 4)
Age (years)		34 (18–46)		32 (21–44)
Severity (mild/moderate/severe)		2/12/0		2/11/0
FEV ₁ (% predicted)	70.7 (62.8–82.0)	80.8 (71.2–91.6)*, **	71.7 (63.2–82.4)	70.9 (60.4–82.6)
Dmin (unit)	1.01 (0.24–1.89)	1.98 (0.78–2.73)*, **	1.00 (0.25–1.73)	0.90 (0.19–1.62)
Positive skin test		14/14		13/13
Serum immunoglobulin (Ig)E (IU/ml)		743 (172–2289)		611 (204–2370)

Values are mean (range).

Dmin = minimum dose of methacholine required to reduce airway responsiveness unit equal to 1 min of 1.0 mg/ml aerosol inhalation of methacholine.

* $P < 0.01$: for comparison between values before and after treatment (within-group).

** $P < 0.01$: for comparison treatment effects on values (before – after treatment) for BUD vs placebo.

placebo-treated group, there were significant improvements in FEV₁% and Dmin ($P < 0.01$, for each) in the BUD-treated group (Table 1).

Bronchial biopsy

At baseline, there were no significant differences in TARC or IL-4R α expression, or number of inflammatory phenotypic cells, between subjects who were assigned to the BUD- or placebo-treatment group. The percentage of epithelial cells expressing TARC significantly decreased from 16.6 (9.7–25.4) to 9.1% (4.7–15.4) in the BUD-treated group ($P < 0.01$). However, TARC expression remained unchanged after placebo treatment between 15.0 (9.8–26.0) and 17.0% (10.4–26.2). The difference between BUD and placebo treatment in TARC expression was statistically significant ($P < 0.01$, Fig. 1).

Within the BUD-treated group, there were significant reductions in the number of MBP⁺ eosinophils, CD3⁺ T cells, and CD4⁺ T cells before vs after treatment ($P < 0.01$, each). Within the placebo-treated group, there were no significant changes in any of the cell counts before vs after treatment. A comparison between the two groups showed that the differences in MBP⁺ eosinophils ($P < 0.01$), CD3⁺ T cells ($P < 0.05$), and CD4⁺ T cells ($P < 0.01$) were significant (Table 2).

The percentage of epithelial cells exhibiting IL-4R α was significantly decreased from 20.6 (11.7–28.8) to 9.5% (5.2–16.4) in the BUD-treated group ($P < 0.01$). No significant change was observed in expression of IL-4R α , from 20.5 (11.7–29.4) to 20.6% (10.8–31.1), in the placebo-treated group. The difference in IL-4R α expression between the two groups was statistically significant ($P < 0.05$, Fig. 2). An example of positive staining for TARC and IL-4R α in biopsy specimens before and after treatment with BUD is shown in Fig. 3.

There were significant correlations between changes in TARC expression and changes in the number of MBP⁺ eosinophils ($r_s = 0.62$, $P < 0.05$), CD3⁺ T cells ($r_s = 0.55$, $P < 0.05$), and CD4⁺ T cells ($r_s = 0.97$,

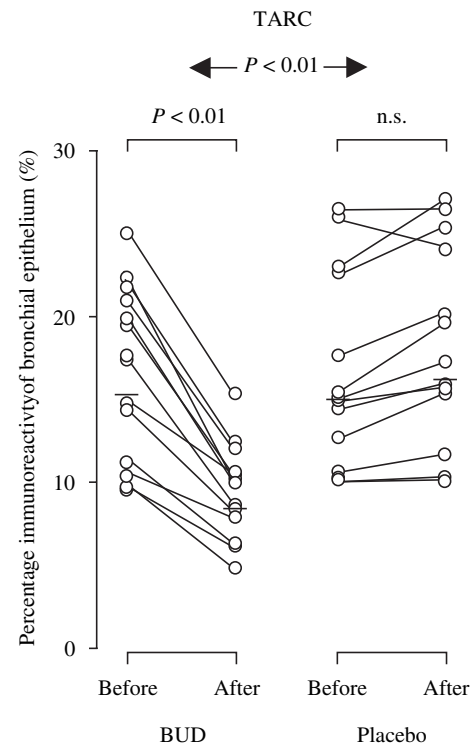


Figure 1. Effect of treatment for 3 months with 400 μ g budesonide (BUD) twice a day or placebo on the percentage expression of thymus and activation-regulated chemokine (TARC) in the bronchial epithelium. Bars represent median values.

$P < 0.001$). Moreover, a significant correlation was found between changes in expression of TARC and in IL-4R α ($r_s = 0.66$, $P < 0.01$).

Discussion

In this study, we demonstrated that (i) BUD decreases bronchial epithelial TARC expression and this change is associated with a reduction in eosinophils and CD3⁺

Table 2. Number of inflammatory cells in bronchial biopsy specimens from BUD or placebo group

	MBP	CD3	CD4	CD8
BUD				
Before treatment	77 (25–120)	165.5 (90–252)	136 (73–210)	48 (23–80)
After treatment	41 (7–83)*,***	126.5 (75–204)*, **	84.5 (52–140)*, ***	44 (20–74)
Placebo				
Before treatment	66 (20–108)	160 (102–243)	120 (74–213)	41 (24–75)
After treatment	65 (28–114)	170 (90–255)	132 (68–215)	45 (20–80)

Data were expressed as medians (range) and represented number of cells per square millimetre of lamina propria.

BUD, budesonide; MBP, eosinophilis; CD3, total T cells; CD4, helper/inducer T cells; CD8, suppressor/cytotoxic T cells.

* $P < 0.01$: for comparison between values before and after treatment (within-group).

** $P < 0.05$, *** $P < 0.01$: for comparison treatment effects on values (before – after treatment) for BUD vs placebo.

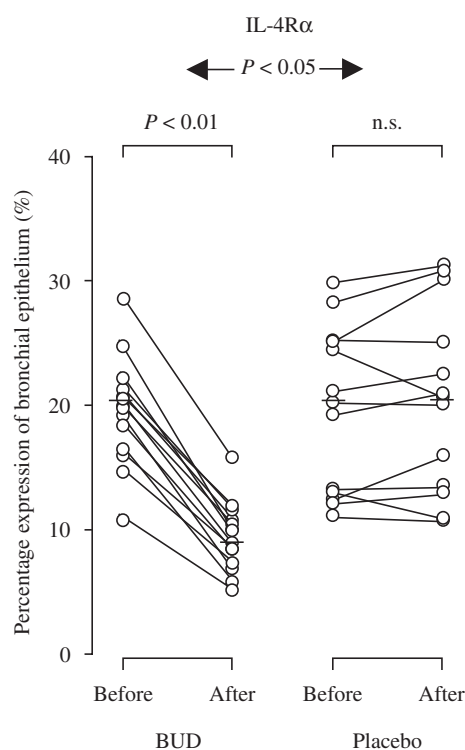


Figure 2. Effect of treatment for 3 months with 400 µg budesonide (BUD) twice a day or placebo on the percentage expression of IL-4 receptor alpha (IL-4Rα) in the bronchial epithelium. Bars represent median values.

T cells, and CD4⁺ T cells infiltration into the airway; (ii) there is a significant correlation between changes in TARC and in IL-4Rα immunoreactivity.

The TARC and MDC are key chemokines in the migration of Th2 cells to sites of inflammation (5, 6).

Using immunocytochemistry we showed that the bronchial epithelium of asthmatics expresses higher levels of TARC than controls. In contrast, expression of MDC was only weakly detected in asthmatics (7). The TARC levels in both serum and sputum of asthmatics were increased compared with control subjects (16, 17), and plasma TARC concentration is a useful marker for the monitoring of asthmatics exacerbation (18). These findings suggest that TARC is a pivotal chemokine in the development of allergic airway inflammation. Higher TARC expression was observed in the nasal epithelium of allergic rhinitis patients (8). Furthermore, skin biopsies also implicated TARC in atopic dermatitis (19).

Our findings regarding the effect of BUD show that inhibition of TARC expression in the bronchial epithelium is associated with decreased airway inflammation. Significant correlations were found among the changes in TARC expression and in eosinophils, CD3⁺ T cells, and CD4⁺ T cells. Anti-inflammatory effects of ICS were established in bronchial asthma. Recently, Kawasaki et al. (20) reported that pretreatment of ovalbumin-sensitized mice with an anti-TARC antibody prevented airway infiltration by eosinophils and CD4⁺ T cells, while production of Th2 cytokines (IL-4 and IL-13), further reduced eosinophilic chemokine expression of eotaxin and RANTES mRNA. As BUD reduces the expression of TARC, we assume that there were not only positive relationships between TARC and CD4⁺ T cells, but also eosinophils and CD3⁺ T cells. This is supported by a previous report showing that plasma TARC concentration was lower in corticosteroid-treated than in steroid-naïve asthmatic patients (21). Moreover, many reports have indicated that treatment with topical corticosteroids and oral antihistamines decreased levels of serum TARC and blood eosinophils in patients with allergic dermatitis (19, 22).

Recent evidence indicates that IL-4 and IL-13, in combination with TNF-α, can up-regulate TARC production by epithelial cells (7, 8), suggesting the presence of an amplification circuit of Th2 responses, based on induction of TARC by IL-4/IL-13 and selective responses to this chemokine by Th2 cells. The actions of IL-4 are elicited through a cell surface receptor composed of an α-chain – also a component of the IL-13 receptor – and the common γc chain used by a number of other cytokines (23). It was reported that expression of IL-4Rα was significantly increased in biopsy specimens from asthmatics compared with controls (10). As a significant correlation was also found between the change in TARC and in IL-4Rα immunoreactivity, one mechanism that has been proposed is that TARC expression of the bronchial epithelium, which is recognized as a major cellular source of this chemokine, was inhibited by BUD. Many reports suggested that steroid treatment reduced the Th2 type cytokine gene expression in allergic inflammation (24–26). The TARC expressing areas coincided with the surface epithelial areas positively stained by IL-4Rα

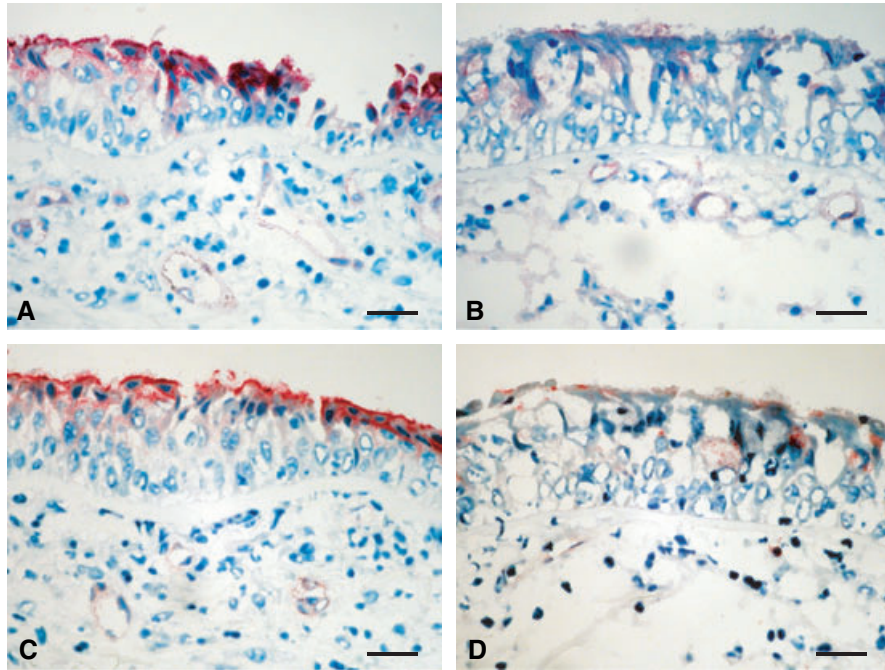


Figure 3. Microphotograph showing immunostaining for thymus and activation-regulated chemokine (TARC) (upper) and IL-4 receptor alpha (IL-4R α) (lower) expression in bronchial epithelium of a subject with asthma before (A, C) and after (B, D) treatment with 400 μ g budesonide (BUD) twice a day. Scale bar = 50 μ m.

immunopositive cells. As IL-4 and IL-13 share one common receptor subunit, IL-4R α , it seemed to be promising to inhibit both cytokines with one molecule by blocking this receptor subunit. Therefore, our result shows that a significant correlation was observed between changes in epithelial TARC and IL-4R α expression after BUD. Transcription factors such as nuclear factor (NF)- κ B and signal transducer and activator of transcription 6 (STAT6) are important in allergic inflammation. Although the signal transduction involved in TARC regulation has yet to be clarified, Matsukura et al. reported that the combination of TNF- α and IL-4 led to activation of the eotaxin promoter, which contains overlapping consensus-binding sites of NF- κ B and STAT6 (27). Considering that both NF- κ B and STAT6 are expressed in airway epithelial cells (28, 29), it is

possible that BUD causes these transcription factors to act negatively at a site on the TARC promoter containing overlapping-binding sites.

The specific antibody against TARC attenuated airway inflammation and decreased airway hyperresponsiveness after allergen challenge in mice (20). Because regulation of TARC generation has therapeutic potential for asthma treatment, studies involving the anti-TARC antibodies in human bronchial asthma are needed. Our findings support the hypothesis that the anti-inflammatory effects of BUD in asthma result in part from modulation of TARC.

In conclusion, we have provided the first evidence that inhaled BUD can significantly reduce not only inflammatory cells, but also epithelial TARC expression in asthma patients.

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