

Suplatast tosilate inhibits thymus- and activation-regulated chemokine production by antigen-specific human Th2 cells

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Summary

Background Suplatast tosilate is an anti-allergic agent that suppresses cytokine production by human Th2 cells.

Objective We investigated the effects of suplatast tosilate on the production of thymus- and activation-regulated chemokine (TARC) by T cells from allergic patients with asthma.

Methods Purified protein derivative (PPD)-specific Th1 cell lines and *Dermatophagoides farinae* (Der f)-specific Th2 cell lines were established from nine patients with house dust mite-allergic asthma. The effects of suplatast tosilate on mRNA expression of TARC and protein production of TARC from antigen-specific Th1 or Th2 cell lines were investigated after stimulation with relevant antigens or phytohemagglutinin (PHA). In addition, the effects of IL-4, IL-10, and IFN- γ on TARC production by Der f-specific Th2 cell lines in the presence or absence of suplatast tosilate were studied.

Results Although PPD-specific Th1 cell lines did not produce TARC after stimulation with PPD antigen or PHA, stimulation of Der f-specific Th2 cell lines with Der f antigen or PHA increased production of TARC. Suplatast tosilate significantly and dose-dependently inhibited production of TARC by Der f-specific Th2 cell lines stimulated with either Der f antigen (76.5% inhibition at 100 μ g/mL, $P < 0.01$) or PHA (81.9% inhibition at 100 μ g/mL, $P < 0.01$). TARC production by Der f-specific Th2 cell lines was significantly increased only by activation with IL-4 but not with IL-10 or IFN- γ ; this increase in TARC production was significantly inhibited by suplatast tosilate (97.5% inhibition at 100 μ g/mL, $P < 0.01$).

Conclusion Suplatast tosilate inhibits TARC production by human Th2 cells. Therefore, this agent inhibits both Th2 cytokine and Th2 chemokine and may be a useful anti-allergic agent.

Keywords asthma, suplatast tosilate, T cell, thymus- and activation-regulated chemokine, interleukin-4, interferon- γ

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Introduction

Bronchial asthma is characterized by persistent airway inflammation induced by several types of inflammatory cells, including CD4⁺ T cells, mast cells, and eosinophils [1–3]. Because airway inflammation is closely associated with airway hyper-responsiveness, airflow limitation, and asthma symptoms, anti-inflammatory treatments are necessary for the management of chronic asthma [4].

CD4⁺ T cells can be divided into two subsets on the basis of cytokine production. Th1 cells produce mostly IFN- γ and play an important role in cell-mediated immunity. Th2 cells produce mostly IL-4, IL-5, and IL-13 and play important roles in humoral immunity and allergy [5]. Both messenger (m)RNA and protein production of Th2 cytokines are increased in CD4⁺ T cells from bronchoalveolar lavage fluid (BALF) and from

bronchial biopsy specimens from sensitized, allergic patients with asthma; these results suggest that Th2 cells are persistently activated and release Th2 cytokines in the airway [6, 7].

Thymus- and activation-regulated chemokine (TARC) is a member of the CC chemokine family which is constitutively expressed in the thymus and produced by monocyte-derived dendritic cells, endothelial cells, bronchial epithelial cells, and Th2 cells [8, 9]. Because TARC leads to the recruitment of Th2 cells, it is classified as a Th2-active chemokine. Levels of TARC in serum are elevated and are correlated with disease activity in patients with atopic dermatitis [10]. In patients with asthma, TARC expression is increased in the bronchial epithelium [11]. In addition, anti-TARC antibodies inhibit allergic reactions in ovalbumin-sensitized mice [12]. Because TARC plays an important role in allergic inflammation, the inhibition of TARC and Th2 cytokine production may be useful in the treatment of allergic diseases, including asthma.

Suplatast tosilate is one of a class of sulphur-containing agents with immunomodulatory actions developed for use against allergic diseases. Although precise mechanisms of the action of suplatast tosilate have not been completely elucidated,

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suplatast tosilate inhibited passive cutaneous anaphylaxis and allergic rhinitis [13, 14]. Furthermore, suplatast tosilate class-specifically inhibited production of IgE antibody but not IgM or IgG antibody, and suppressed Th2 cytokine production and goblet-cell metaplasia [15–17]. It has been reported that suplatast tosilate reduces the number of airway eosinophils in patients with asthma [18]. Moreover, treatment with suplatast tosilate allows doses of inhaled corticosteroids to be reduced in patients with severe asthma [19].

In the present study, we examined the effect of suplatast tosilate on TARC production by Th2 cells of allergic patients with asthma after antigen-specific and non-specific stimulation. We also analysed the effects of IL-4, IL-10, and IFN- γ on TARC production by Th2 cells in the presence or absence of suplatast tosilate.

Materials and methods

Reagents

The following reagents were used: phytohemagglutinin (PHA; Sigma Chemical Co., St Louis, MO, USA); recombinant human IL-2 (a gift from Shionogi Pharmaceuticals, Co., Osaka, Japan); recombinant human IL-4, IL-10, and IFN- γ (R & D Systems, Minneapolis, MN, USA); *Dermatophagoides farinae* (Der f) antigen (a gift from Torii Pharmaceuticals Co., Ltd, Tokyo, Japan); and purified protein derivative (PPD) extract (Japan BCG LA, Tokyo, Japan).

Subjects

Nine non-smoking patients with allergic asthma (five women and four men), aged 21–36 years were selected on the basis of (a) positive cutaneous reactions to house dust mite extract; (b) high levels of house dust- and Der f-specific IgE in sera; (c) baseline FEV₁ of more than 70% of the predicted value; (d) increased bronchial responsiveness to histamine; and (e) no treatment other than rescue β_2 -agonists for at least 3 months. The titre of Der f-specific IgE was 54.2 ± 14.1 IU/mL. The mean duration of asthma was 6.2 ± 2.4 years. The percentage predicted FEV₁ was $83.8 \pm 2.48\%$. No subjects had had respiratory infections in the previous 4 weeks. Informed consent was obtained before the study.

Establishment of antigen-specific T cell lines

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood on Ficoll-Hypaque gradients (Nacalai Tesque Co., Kyoto, Japan). PBMCs were cultured with 5 μ g/mL of PPD or 10 μ g/mL of Der f antigens in RPMI-1640 supplemented with 10% v/v human AB serum, 100 U penicillin, and 100 μ g/mL streptomycin in flat-bottomed 24-well plates in an atmosphere containing 5% CO₂ at 37 °C. Cells were stimulated every week in the presence of autologous PBMCs treated with 50 μ g/mL of mitomycin C (MMC) as antigen-presenting cells (APCs) and 5 μ g/mL of PPD or 10 μ g/mL of Der f antigens and IL-2 at 4 ng/mL. On day 28, T cell lines (5×10^4) were tested for specificity with a 3-day proliferation assay performed in 96-well round-bottomed plates in tissue culture medium supplemented with 5 μ g/mL of PPD or 10 μ g/mL of Der f antigens and MMC-treated PBMCs (5×10^4) as APCs.

Cytokine and chemokine production

To assess cytokine and chemokine production, T cell lines (5×10^5 cells) were either activated with PHA for 48 h, activated with 10 μ g/mL of Der f or 5 μ g/mL of PPD antigens for 48 h in the presence of MMC-treated PBMCs (5×10^5 cells), or were not activated. To investigate the effects of suplatast tosilate (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan), both types of T cell line were incubated with 1, 10, or 100 μ g/mL of suplatast tosilate. In some experiments, Der f-specific Th2 cell lines were stimulated with IL-4 (10 ng/mL), IL-10 (1 ng/mL), or IFN- γ (500 ng/mL) for 48 h. Concentrations of TARC, IL-4, and IFN- γ in the supernatant were measured with ELISA kits obtained from Biosource International (Camarillo, CA, USA) or R & D Systems. The ELISA kits could detect IL-4 at concentrations as low as 10 pg/mL, IFN- γ at 2 pg/mL, and TARC at 7 pg/mL.

RT-PCR analysis

The T cell lines were stimulated with PHA for 12 h or Der f or PPD antigens in the presence of MMC-treated PBMCs for 24 h. Total cellular RNA was separated from 1×10^6 cells with the guanidinium thiocyanate-phenol-chloroform extraction method. After precipitation with ethanol, RT with oligo dT primers was performed for 30 min at 55 °C with 1 μ g of total RNA using cDNA synthesis kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Amplification of each cDNA was performed with a SuperTaq Premix kit (Sawady Technology, Tokyo, Japan) using specific primers for TARC (sense, CCACTGAAGATGCTGGCCCTG; antisense, GAGGCTTCAAGACCTCTCAAG) and β -actin. The PCR conditions included 5 min of preheating at 95 °C followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, elongation at 72 °C for 120 s, and final elongation at 72 °C for 10 min. The PCR products were analysed with electrophoresis through 2% agarose gels and visualized under ultraviolet light after ethidium bromide staining. Ethidium bromide intensity was measured and standardized with the β -actin mRNA level.

Statistical analysis

The significance of differences was assessed using a Bonferroni correction for multiple comparisons. The significance of differences between groups was calculated with Wilcoxon rank test. Data are expressed as mean $\pm$ standard error of mean (SEM), and a probability less than 0.05 was considered to indicate significance.

Results

As we have reported previously, suplatast tosilate did not inhibit IFN- γ production by PPD-specific Th1 cell lines, but 100 μ g/mL of suplatast tosilate significantly inhibited IL-4 production by Der f-specific Th2 cell lines (426.0 ± 40.4 pg/mL to 182.4 ± 29.1 pg/mL; $P < 0.01$). The total number of cells was counted and cell viability was analysed by staining with trypan blue under the concentration of 100 μ g/mL of suplatast tosilate for up to 7 days. 100 μ g/mL of suplatast tosilate did not affect either total cell number or cell viability (data not shown). These results reconfirm the previous results that inhibitory effect of suplatast tosilate was not due to the toxic effect of this agent.

Effect of suplatast tosilate on TARC production

The effects of suplatast tosilate on TARC production by antigen-specific Th1 and Th2 cell lines were investigated. PPD-specific Th1 cell lines produced IFN- γ after stimulation with antigen, but TARC was not detected in the culture supernatant (data not shown). In addition, TARC was not produced by Th1 cell lines after stimulation with PHA (data not shown). Stimulation with Der f antigen (71.9 ± 9.9 pg/mL to 413.1 ± 40.0 pg/mL) or PHA (64.0 ± 6.2 pg/mL to 394.2 ± 25.2 pg/mL) increased TARC production by Der f-specific Th2 cell lines (Fig. 1). The addition of suplatast tosilate significantly decreased TARC production in a dose-dependent manner by Der f-specific Th2 cell lines stimulated with Der f antigen (413.1 ± 40.0 pg/mL to 151.9 ± 21.0 pg/mL at $100 \mu\text{g/mL}$ [76.5% inhibition]; $P < 0.01$; Fig. 1). Suplatast tosilate similarly inhibited TARC production by Der f-specific Th2 cell lines stimulated with PHA (394.2 ± 25.2 pg/mL to 123.8 ± 26.1 pg/mL at $100 \mu\text{g/mL}$ [81.9% inhibition]; $P < 0.01$; Fig. 1).

We next studied the effects of suplatast tosilate on mRNA expression of TARC in Der f-specific Th2 cell lines. The addition of suplatast tosilate significantly inhibited TARC mRNA expression in a dose-dependent manner after stimulation with

PHA ($P < 0.01$; Fig. 2) and after stimulation with Der f antigen (data not shown).

Effect of IL-4 on TARC production by Th2 cells

To investigate the effects of activation with Th1 and/or Th2 cytokines on TARC production, we next stimulated Der f-specific Th2 cell lines with IL-4, IL-10, or IFN- γ . Stimulation of Der f-specific Th2 cell lines with IL-4 significantly increased TARC production from the concentration at 10 ng/mL (80.7 ± 7.6 pg/mL to 199.2 ± 37.2 pg/mL; $P < 0.05$; Fig. 3a). In addition, TARC production from Der f-specific Th2 cell lines was not inhibited or increased by the addition of IL-10 or IFN- γ (Fig. 3b).

Effect of suplatast tosilate on IL-4-induced TARC production

Because suplatast tosilate significantly inhibited the production of TARC after stimulation with Der f antigen or PHA, we next investigated the effect of suplatast tosilate on TARC production by the stimulation with 10 ng/mL of IL-4. The increase in IL-4-induced TARC production was significantly inhibited by treatment with $100 \mu\text{g/mL}$ of suplatast tosilate (199.2 ± 37.2 pg/mL to 84.8 ± 7.5 pg/mL [97.5% inhibition]; $P < 0.01$, Fig. 3b).

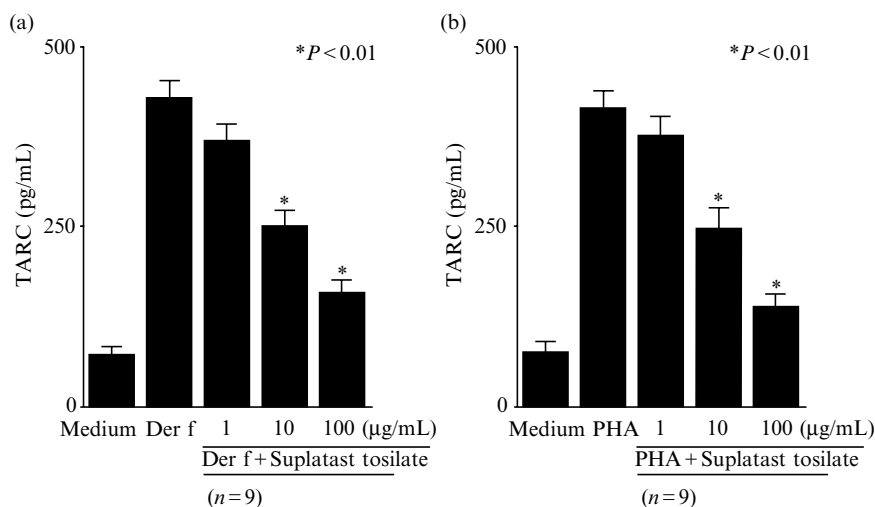


Fig. 1. Inhibitory effects of suplatast tosilate on TARC production by Der f-specific Th2 cell lines. Der f-specific Th2 cell lines were stimulated with Der f antigen (a), or PHA (b) for 48 h in the presence or absence of suplatast tosilate (1, 10, $100 \mu\text{g/mL}$). Culture supernatants were harvested, and TARC concentrations were measured with ELISA. Results are mean $\pm$ SEM of nine different T cell lines established from nine different patients.

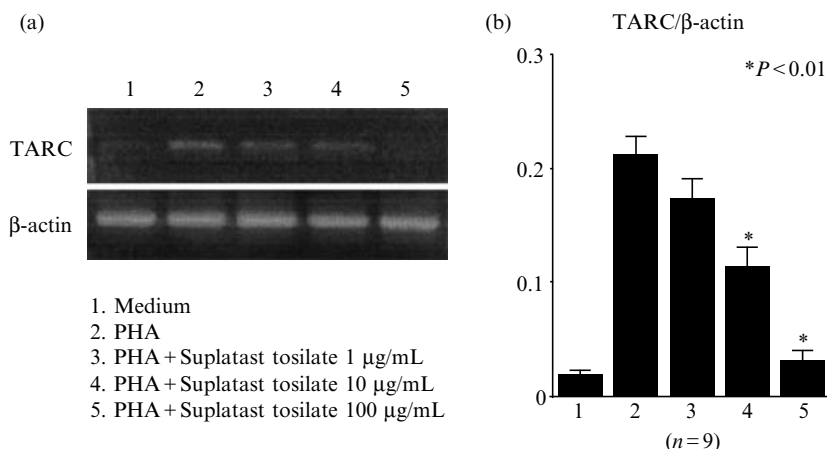
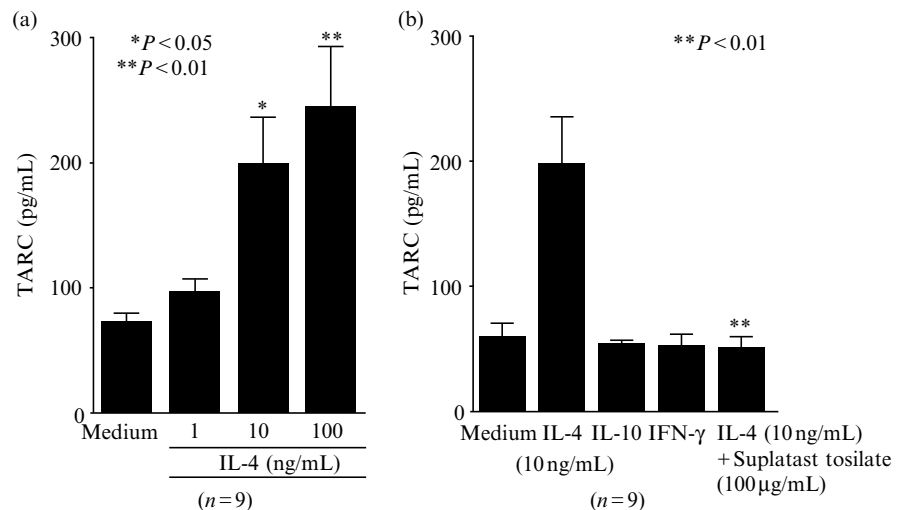


Fig. 2. Inhibitory effects of suplatast tosilate on TARC mRNA expression in Der f-specific Th2 cell lines. Der f-specific Th2 cell lines were stimulated with PHA for 12 h in the presence or absence of suplatast tosilate. (a) The PCR products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. One representative of nine is shown. (b) Densitometric analysis of results of RT-PCR is shown with the intensity of the TARC signal normalized against the β -actin signal. Lane 1: medium alone; lane 2: PHA; lane 3: PHA + suplatast tosilate $1 \mu\text{g/mL}$; lane 4: PHA + suplatast tosilate $10 \mu\text{g/mL}$; lane 5: PHA + suplatast tosilate $100 \mu\text{g/mL}$. Results are mean $\pm$ SEM of nine different T cell lines established from nine different patients.

Fig. 3. Inhibitory effects of suplatast tosilate on IL-4-induced TARC production by Der f-specific Th2 cell lines. (a) Der f-specific Th2 cell lines were stimulated with various concentration of IL-4 (1, 10, 100 ng/mL) for 48 h. (b) Der f-specific Th2 cell lines were stimulated with IL-4 (10 ng/mL), IL-10 (1 ng/mL), or IFN- γ (500 ng/mL) for 48 h. In some experiments, cells were pretreated for 24 h with suplatast tosilate (100 μ g/mL) and then stimulated with IL-4 for 48 h in the presence of suplatast tosilate (100 μ g/mL). Culture supernatants were harvested, and TARC concentrations were measured with ELISA. Results are mean $\pm$ SEM of nine different T cell lines established from nine different patients.



Discussion

Although suplatast tosilate does not inhibit proliferative responses, it significantly inhibits Th2 cytokine production by antigen-specific Th2 cell lines [16]. In this study, we have demonstrated that suplatast tosilate inhibits production of TARC, a Th2 chemokine, by human Th2 cells in a dose-dependent manner after stimulation with antigen or PHA. Furthermore, TARC production by antigen-specific Th2 cells was increased by the addition of IL-4 but was not suppressed by the addition of IFN- γ or IL-10; this increase in TARC production was significantly inhibited by suplatast tosilate. Therefore, these results suggest that suplatast tosilate inhibits both Th2 cytokine and Th2 chemokine production by human antigen-specific Th2 cells.

Th2-type CD4⁺ T cells play an important role in the development of airway inflammation in asthma. Expression of the Th2 cytokines IL-4, IL-5 and IL-13 is increased in bronchial biopsy specimens, BALF cells, and peripheral blood T cells from allergic patients with asthma [6, 7, 20]. Moreover, treatment with antigen-specific immunotherapy converts cytokine production from Th2-type to Th0 or Th1-type and thus may decrease airway inflammation and airway hyper-responsiveness [20, 21]. We have previously reported that suplatast tosilate inhibits production by human antigen-specific T cell lines of the Th2 cytokines IL-4 and IL-5 but not of the Th1 cytokine IFN- γ [16]. Therefore, suplatast tosilate is a useful inhibitor of Th2 cytokines in the treatment of allergic diseases. In fact, treatment with suplatast tosilate decreases airway hyper-responsiveness and eosinophil number in bronchial biopsy specimens in patients with asthma [18].

Recent studies suggest that several chemokines are involved in the recruitment of inflammatory cells to the airway. TARC and macrophage-derived chemokine (MDC) are CC chemokines that selectively attract Th2 cells. Kawasaki et al. have recently described the role of TARC in a murine asthma model [12]. They found that treatment with anti-TARC antibodies reduces the number of CD4⁺ T cells and eosinophils in BALF, inhibits production of the Th2 cytokines IL-4 and IL-13, and decreases airway hyper-responsiveness after allergen challenge. Moreover, specimens of bronchial mucosa from

patients with asthma show increased TARC expression [11]. In the present study, suplatast tosilate significantly inhibited TARC production by human Th2 cells. In addition, we have observed that suplatast tosilate significantly inhibits TARC production by monocyte-derived dendritic cells (unpublished observation). These findings suggest that suplatast tosilate inhibits Th2 chemokine TARC production and may decrease the recruitment of Th2 cells into the airway and reduce airway inflammation.

Studies of the mechanisms of TARC production have shown that Th2 cytokines, including IL-4 and IL-13, induce TARC production by macrophages and respiratory epithelial cells [11]. Although TARC production by PBMCs is enhanced by granulocyte macrophage colony-stimulating factor, IL-3 and IL-4, and is suppressed by IFN- γ and IL-10, TARC production by bronchial epithelial cells is enhanced by IFN- γ [9, 11]. In the present study, we found that TARC production by Th2 cells was induced by IL-4 but was not affected by IL-10 or IFN- γ . Studies in a mouse model of asthma have shown that eotaxin-1, eotaxin-2, MDC, and TARC expression in the allergic lung is dependent on Stat6 [22]. The fact that TARC production is induced by stimulation with IL-4 suggests that the functional Stat6 binding site is in the promoter region of TARC in human Th2 cells. Because suplatast tosilate significantly inhibits TARC production by Th2 cells after stimulation with IL-4, this agent may inhibit the function of Stat6. Further studies are necessary to elucidate the inhibitory mechanisms of suplatast tosilate.

The receptor for both TARC and MDC is CCR4 [9]. CCR4 is preferentially expressed on Th2 cells, whereas CCR5 is expressed on Th1 cells [23]. Although the precise mechanisms of CCR4 expression on Th2 cells are unknown, a recent study has demonstrated that high circulating levels of TARC in the serum of patients with chronic hypereosinophilia down-regulate CCR4 expression on Th2 cells [24]. We have observed that mRNA expression of CCR4 in PBMCs was increased after the stimulation with PHA and suplatast tosilate inhibited the increase in mRNA expression of CCR4 (unpublished observation). Further study is necessary to elucidate the inhibitory effect of suplatast tosilate on protein expression of CCR4 on CD4⁺ T cells.

In conclusion, our findings indicate that suplatast tosilate selectively inhibits production of both Th2 cytokine and Th2 chemokine by antigen-specific human Th2 cells. Therefore, suplatast tosilate may be a useful agent for the treatment of allergic diseases.

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