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ORIGINAL ARTICLE

Effect of Procaterol, a β_2 Selective Adrenergic Receptor Agonist, on Airway Inflammation and Hyperresponsiveness

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ABSTRACT

Background: β-agonists are frequently used as bronchodilators for asthma as not only a reliever but also a controller, and their utility has increased with the development of long-acting β2 selective drugs. Although anti-inflammatory effects of β2 selective-agonists have been reported *in vitro*, side effects on augmentation of airway hyperresponsiveness by chronic use of β2 selective-agonists have been described in several reports. In this study, we investigated the effects of procaterol, a second-generation β2-agonist, on airway inflammation *in vivo* using an antigen-specific murine model of asthma.

Methods: Mice immunized with ovalbumin (OVA) + alum and challenged with inhaled ovalbumin were orally administered procaterol during the challenge. After inhalation, the mice were tracheostomized and placed in a body box under controlled ventilation to measure airway resistance before and after acetylcholine inhalation.

Results: Administration of procaterol at a clinical dose equivalent did not augment airway hyperresponsiveness, inflammation of the airway wall, or subsequent airway wall thickening induced by OVA inhalation. BALF cell analysis revealed that the eosinophil number in the BALF was significantly reduced in procaterol-treated mice compared to untreated mice.

Conclusions: Oral administration of procaterol at a clinical dose did not augment airway responsiveness, but did reduce eosinophil inflammation.

KEY WORDS

airway hyperresponsiveness, allergic inflammation, eosinophil, murine model, β2 adrenergic receptor agonist

INTRODUCTION

Currently, the main target of asthma therapies is chronic airway inflammation.[1-3] The steroid inhaler has become a basic long-term therapy for management of chronic airway inflammation.[4] For combination therapy, steroid inhalers have been supplemented with long-acting β2 agonists, theophylline, or leukotriene receptor agonists.[5-7] Clinically, it has been observed that addition of β2 selective-agonists is more effective than doubling the dose of steroid inhaler.[4,8-10]

Studies *in vitro* have demonstrated that β2 selective-agonists possess anti-inflammatory effects. β2 selective-agonists increase cyclic AMP levels, which in turn inhibit mast cell and eosinophil degranulation, induction of apoptosis, and cytokine production.[11-16] In contrast, human studies as well as *in vivo* studies have shown that chronic use of β2 agonists worsen airway hyperresponsiveness.[17-19] The anti-inflamma-tory effects of salmeterol, a new long-acting β2 agonist, have been intensively studied.[11,20-22] Salmeterol shows superior anti-inflammatory activities over salbutamol,[23] and in addition it possesses synergistic effects with steroids.[24-26] However, there are contradictory data regarding the anti-

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inflammatory effects and the synergistic effects of salmeterol.[27-29] In this study, we investigated the *in vivo* effects of a clinical dose of procaterol on airway inflammation as well as on airway hyperresponsiveness. Procaterol is β2-selective full agonist that is used as a rescue from asthmatic attack when inhaled and as a controller when taken orally. We found that a clinical oral dose of procaterol did not augment airway responsiveness. Rather, procaterol exhibited a tendency to reduce eosinophil infiltration.

METHODS

MEASUREMENT OF SERUM PROCATEROL CONCENTRATIONS

All mice were orally administered procaterol (Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan) dissolved in distilled water at doses of 0.1, 1, or 10 mg/kg in a volume of 10 mL/kg. At 1 hour and 6 hours after administration, a venous blood sample was collected from the large abdominal vein of mice anesthetized with ether. The blood was transferred to a sample tube and centrifuged at 3000 rpm for 30 minutes, and the serum was frozen until analysis by liquid chromatography-tandem mass spectrometry. Each sample comprised sera obtained from 5 mice.

TREATMENT OF MICE

Specific pathogen-free male A/J mice (10–12 weeks old) with native airway hyperresponsiveness to acetylcholine (ACh)[30,31] were purchased from SLC (Shizuoka, Japan). Mice were bred in the animal facilities of Teikyo University School of Medicine under Specific Pathogen-Free (SPF) conditions. Care and use of the animals followed the guidelines of the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research.

The mice were initially immunized four times with 10 μg OVA + 2 mg alum on days 0, 28, 35, and 49. ELISA titers of OVA-specific IgE were significantly elevated after the immunizations as previously reported.[32] After immunization, the mice were divided into four groups for administration of inhaled challenge from day 49 to day 63 (inhalation of 20 mg/ml OVA for 10 minutes every other day, total 7 times): (1) 0.9 M NaCl, (2) OVA, (3) OVA + procaterol (orally) (4) OVA + dexamethasone (1 mg/kg, intraperitoneally). Procaterol in distilled water and dexamethasone was dissolved in saline and saline only was administered as a control. Procaterol and dexamethasone were administered once a day, at 1 hour before each OVA inhalation. Four to six mice were used in each group for one experiment.

ASSESSMENT OF AIRWAY RESPONSIVENESS

Twenty-four hours after the final OVA inhalation, airway responsiveness was analyzed. The mice were anesthetized with pentobarbital and were tracheostomized. The animals were connected to a Harvard ventilator with 0.25 ml tidal volume and a respiratory frequency of 120/minute, as previously reported,[33] after which they were given an injection of pancuronium bromide. Airway resistance (Raw) was measured using a whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY). ACh was administered by ultra-nebulization for 3 minutes. Data were expressed as [(Raw after inhalation of ACh/Raw before inhalation) ×100 (%)].

BALF CELL ANALYSIS AND HISTOLOGICAL EXAMINATION

BALF was obtained from selected mice by intubating and washing the lungs with 1 ml of saline until the recovered fluid reached 5 ml. BALF was centrifuged at 1500 rpm for 10 minutes at 4℃. Pellets were dissolved in 1 ml PBS and the number of the cells was counted. Cytospin specimen was obtained by rotating at 640 rpm for 2 minutes. Then, the cells were stained with Diff Quik (International Reagents Corporation) and the cell differentiation counts were examined by microscope.

The lungs were fully inflated using 10 cm H_2O pressure and fixed with 20% formaldehyde for hematoxylin-eosin (HE) and elastica van Gieson (EVG) staining.

ANALYSIS OF MRNA EXPRESSION

Lungs of mice were frozen in liquid nitrogen immediately after harvest and were used for RNA extraction. Each lung tissue was moved quickly into 1 ml ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan). Lung tissue was homogenized and total RNA was extracted, using a modified acid guanidium-phenol-chloroform method. To synthesize cDNA, 5 μg of total RNA was incubated with 5 mM $MgCl_2$, 1 mM dNTP mixture, 0.25 U reverse transcriptase, 1 U RNase inhibitor, and 0.125 μM oligo (dT) (Takara Biochemicals, Tokyo, Japan). Amplification cycles were 42℃ for 15 minutes, 99℃ for 5 minutes, and then 5℃ for 5 minutes using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

The mRNA levels of cytokines were quantified by real-time polymerase chain reaction (PCR) using the Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) for amplification of cDNA. The reaction was undertaken in 20 μl, containing 3 mM MgCl2, 1 μM primers, FastStart Taq DNA polymerase, dNTP mix and SYBR Green I (Light Cycler-Fast Start DNA Master SYBR GreenI kit, Roche Diagnostics). Quantification was performed with a standard curve obtained using 5 dilutions of cDNA. Results are shown as ratios of the level of mRNAs standardized to the level of β-actin mRNA. The primers used were as follows: β-actin 5'-CCTGTATGCCTCTGGTCGTA-3' 5'-CCATCTCCTG-CTCGAAGTCT-3' 260bp, IL-13 5'-GAGGAGCTGAG-CAACATCAC-3' 5'-GCAATATCCTCTGGGTCCTG-3'

Table 1 Serum Procaterol Concentrations in A/J Strain Mice

Dose of Procaterol	Serum Concentrations of Procaterol (ng/mL)	
	1 hour after administration	6 hours after administration
0.1 mg/kg	0.212	0.032
1 mg/kg	3.272	0.557
10 mg/kg	23.861	4.098

Each value is the mean of 2 samples. Each sample comprised serum obtained from 5 animals.

157bp, eotaxin 5'-TCCCCAACACACTACTGAAG-3' 5'-AGGCTCTGGGTTAGTGTCAA-3' 217bp, TGF-β1 5'-AACAACGCCATCTATGAG-3', 5'-ATTCCGTCTCCT-TGGTT-3' 294bp.

STATISTICAL ANALYSIS

Data were statistically analyzed by Student's t-test and ANOVA. Statistical significance was accepted at $p < 0.05$.

RESULTS

INFLUENCE OF PROCATEROL ON AIRWAY HYPERRESPONSIVENESS

First, we tried to set the concentration of procaterol at a clinical dose. A single clinical dose of procaterol in humans reaches 0.2 ng/mL of serum level.[34,35] We found that oral administration of 0.1 mg/kg procaterol reached the human effective serum concentration (Table 1). Next, we examined whether continuous treatment with procaterol (0.1 mg/kg) augments airway responsiveness. The dose response curve was examined using three different mice in each group as shown in Figure 1A. OVA inhalation significantly increased airway responsiveness after 2.5 mg and 5 mg ACh inhalation ($p < 0.05$). Treatment with procaterol before OVA inhalation did not augment airway responsiveness under these conditions (Figs. 1A, B). Because we examined airway hyperresponsiveness 25 hours after the final procaterol administration, the direct effect of bronchodilation by procaterol was negligible. We hypothesize that the slight decrease in airway response by procaterol is attributable to its influence on airway inflammation.

EFFECT OF PROCATEROL ON AIRWAY INFLAMMATION

Next, we examined the influence of procaterol on airway inflammation. BALF cell analysis was performed 24 hours after the final inhalation of OVA. The total number of cells in BALF was significantly increased in OVA-treated groups compared with the non-treated groups ($p < 0.01$). Macrophages were dominant in non-treated groups. In contrast, an increase in eosinophils was prominent in OVA-treated groups ($p < 0.01$) (Fig. 2). A significant decrease in eosinophil number was observed in OVA inhalation group ($p < 0.05$) (Fig. 2). We also performed histological examination with HE staining and EVG staining. Histological analysis confirmed deceased infiltration of eosinophils in the submucosal area in procaterol-treated mice (Fig. 3A). EVG staining showed that subepithelial fibrosis, which represents airway remodelling, did not worsen after procaterol treatment (Fig. 3B).

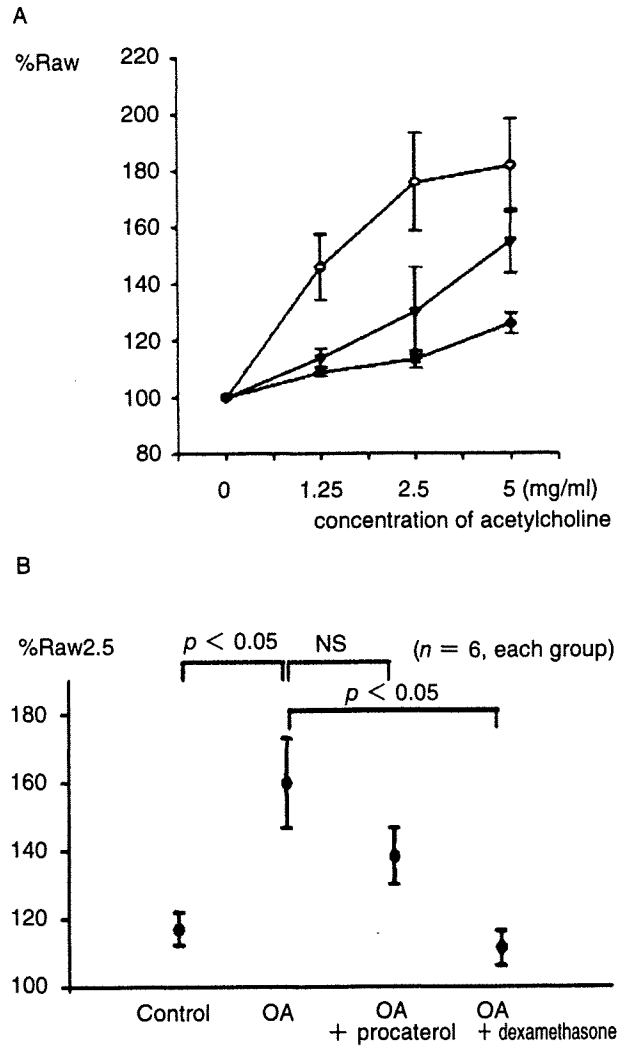

Fig. 1 Airway Responsiveness. Bronchoconstriction induced with ACh inhalation. ACh 1.25, 2.5 5 mg/ml were inhaled for 3 minutes. Raw was measured using whole body plethysmographs as described in the Methods. ACh-evoked changes in Raw are expressed as a percentage of Raw observed before ACh inhalation (100%). Data shown are the mean ± SEM. (**A**) Dose response curve. ○ OVA ▼ OVA + procaterol ● control mice (**B**) Airway responsiveness at 2.5 mg/ml Ach.

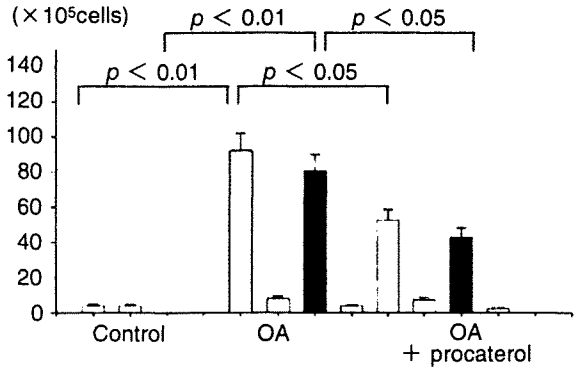

Fig. 2 BALF Cell Analysis. Lungs were subjected to lavage through intubations until 5 ml of BALF was obtained. Cells present in the BALF were pelleted, resuspended in 1 mL of saline and placed on glass slides, where they were counted and fixed by Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. Each bar indicates means ± SEM of seven mice. □ Total cells □ macrophage ■ eosinophils □ lymphocytes. Similar experiments were undertaken at least three times.

EFFECT OF PROCATEROL ON CYTOKINE mRNA AND PROTEIN SYNTHESIS

β2 agonists have been reported to suppress cytokine production *in vitro*. Next, we examined the effect of procaterol on cytokine mRNA synthesis *in vivo*. Figure 4 shows that procaterol itself did not significantly reduce IL-13 and eotaxin mRNA, the products of which mediate eosinophil-associated inflammation. TGF-β, which is involved in airway remodelling, also exhibited no change after procaterol administration.

DISCUSSION

β2 selective agonists function as bronchiodilators and are used as relievers and controllers.[34,35] Recently, inhaled steroids have been recognized as the most effective anti-inflammatory drugs and are the most common choice for controlling asthma.[36] A combined therapy comprising inhaled steroids and a long acting β2 selective agonist is recommended for controlling asthma.[25] Synergistic effects have been reported, and in fact, the combined therapy is more effective than doubling the dose of inhaled steroids.[4,8-10] Thus new aspects of the usefulness in β2 selective agonists are considered. However, the adverse effects of chronic

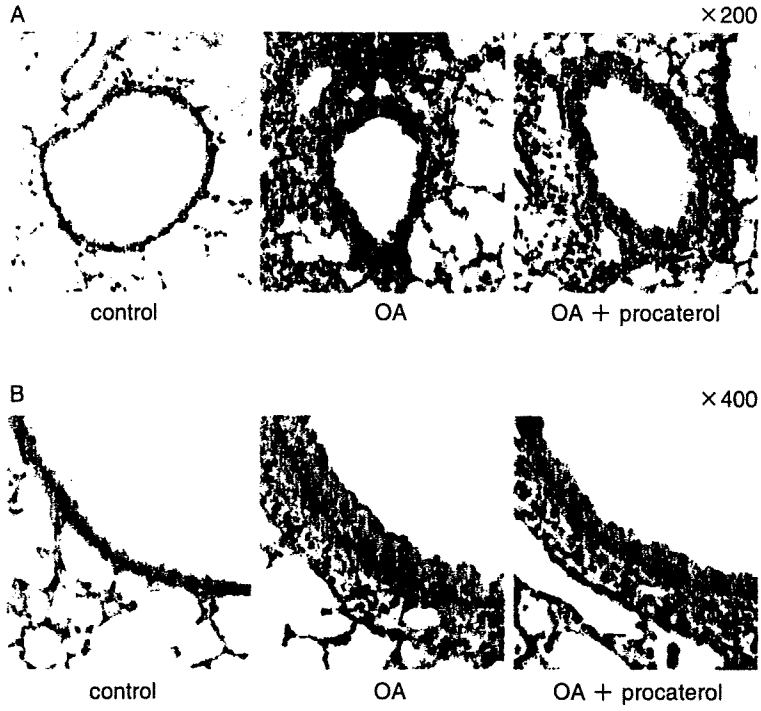

Fig. 3 Histological Analysis. Hematoxylin eosin (**A**) and Elastica van Gieson staining (**B**) were performed on paraffin-embedded sections. Lungs were extracted and fixed overnight with intra-tracheal infusion of 10% formalin maintaining the airway pressure at 10 cm H_2O lung.

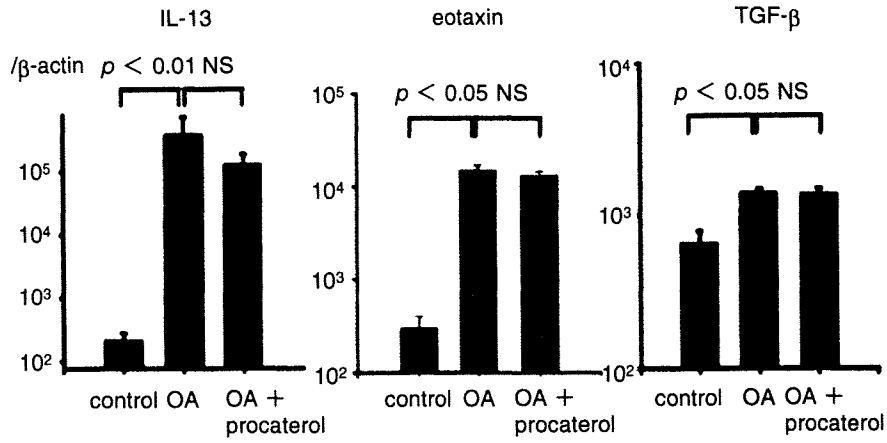

Fig. 4 Effect of Procaterol on IL-13, Eotaxin and TGF-β Gene Expression. RNA was extracted from whole lung of OVA treated mice and mRNA expression for various genes was determined by real-time PCR. Data are mean ± SEM of three different mice.

use by inhalation, including increases in airway hyperresponsiveness, have been reported.[17,37,38] The short acting β2 selective agonist, salubutamol, has been shown to worsen the airway hyperresponsiveness in animal model and in human studies.[17-19,37-39] While the anti-inflammatory activities of salmeterol, a long acting β2 selective agonist have been reported,[11,13,23] some reports deny the effects.[27,28] In this study, we investigated the effect of procaterol, a β2 selective full agonist, which is commonly used as controller by oral tablet administration. We found that clinical dose of oral procaterol did not augment airway responsiveness and airway remodelling. Rather, procaterol significantly reduced eosinophil infiltration.

Mast cells, eosinophils, and smooth muscle cells at the site of asthmatic inflammation possess β2 receptors,[40] and β2 agonists have been reported to block mast cell and eosinophil degranulation.[13,14] β2 agonists function by increasing the concentration of intracellular cAMP,[41] which result in inhibition of cytokine synthesis and induction of apoptosis on eosinophils *in vitro*.[42] However, a report which studied spontaneous apoptosis showed that β2 agonists and cAMP increasing reagents decrease apoptosis of eosinophils.[43] In contrast to spontaneous apoptosis, it has been shown that cytokine mediated survival of eosinophils is inhibited by the increase of cAMP, through accelerated induction of apoptosis.[16,44] Theophylline, another cAMP increasing drug, has been shown to reduce cytokine mediated eosinophil survival, which is relevant to the *in vivo* condition.[45-47] We can hypothesize that the *in vivo* effects of a decrease in eosinophils occurred via induction of apoptosis. Another possible mechanism of a decrease in airway inflammation by procaterol is down-regulation of adhesion. Procaterol has been proven to reduce adhesion molecules in vitro studies.[16,48] It was also reported that systemic administration of tulobuterol, a β2 selective agonist, attenuates eosinophil adhesion to endothelial cells, which results in reduction of eosinophil inflammation.[49] Systemic but not inhalational administration can modulate endothelial cells.[49]

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Original Paper

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Effect of Interleukin-13 or Tumor Necrosis Factor-Alpha on Eosinophil Adhesion to Endothelial Cells under Physiological Flow Conditions

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Key Words
Eosinophils · Endothelial cells · Adhesion molecules · Physiological flow conditions

Abstract
Rationale: We examined the mechanisms used by eosinophils to accumulate on IL-13- or TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) under flow conditions. ***Methods:*** HUVECs were treated for 1, 3, 6, 18 and 24 h with IL-13 or TNF-α (1–100 ng/ml). Human eosinophils were infused at physiologic flow rates (0.5 dyn/cm^2) for 10 min, and attached eosinophils were counted. ***Results:*** Under these flow conditions, eosinophils accumulated efficiently on IL-13-stimulated (109 ± 18 cells/field) or TNF-α-stimulated (96 ± 27 cells/field) HUVECs in a concentration-dependent manner. Eosinophil accumulation on IL-13-activated HUVECs was first observed at 3 h and reached a maximum at 24 h. On the other hand, the levels of eosinophils accumulating on TNF-α-activated HUVECs were the same at all time points (1, 3, 6, 18 and 24 h). Anti-α4 integrin mAb inhibited eosinophil accumulation on both IL-13- and TNF-α-activated HUVECs. ***Conclusions:*** Eosinophil accumulation on HUVECs under physiologic flow conditions is differentially regulated by IL-13 and TNF-α.

Introduction

Eosinophil recruitment during allergic inflammation is a complex process initiated by the interaction of leukocyte adhesion molecules with counterligands on vascular endothelial cells [1]. A variety of in vitro assays have been developed to study the key steps involved in leukocyte recruitment. These include parallel-plate adhesion assays performed under conditions that mimic blood flow conditions, as well as those that examine leukocyte-endothelial adhesion, transendothelial migration and chemotaxis [2–4]. We examined the mechanisms used by eosinophils to accumulate on IL-13- or TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) under flow conditions.

Materials and Methods

Human eosinophils were isolated from EDTA-anticoagulated venous blood of donors with mild allergic rhinitis or asthma by Percoll density gradient centrifugation at room temperature and removal of CD16-positive cells using immunomagnetic microbeads (Militenyi Biotec, Inc.) as previously described [4]. Eosinophil purity (based on examination of Diff-Quik-stained cytocentrifugation preparations) was >98% and viability (trypan blue stain) was >99%.

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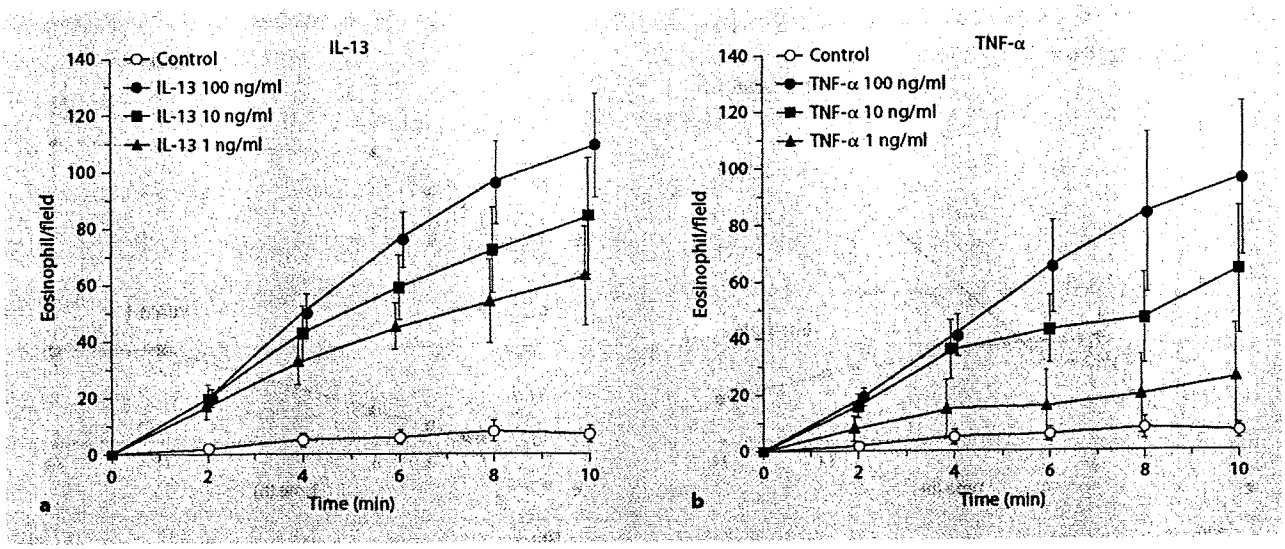

Fig. 1. Eosinophil accumulation on IL-13- or TNF-α-activated HUVECs. HUVECs were treated for 24 h with IL-13 (**a**) or TNF-α (**b**). Human eosinophils were infused at physiologic flow rates (0.5 dyn/cm^2) for 10 min and attached eosinophils were counted (n = 4, mean ± SEM).

First passage HUVECs were isolated as described [5] and maintained in M199 with 20% fetal calf serum. For all experiments, HUVECs were used 2 days after confluence. HUVECs were incubated with IL-13 (1–100 ng/ml, TECHNE corporation, USA) or TNF-α (1–100 ng/ml, Incitrogen TECH-Line, USA) for 1–24 h.

The assembled parallel plate flow assay system consisted of (1) a Plexiglas flow chamber (Glylotech, Rockville, Md., USA) with inlet/outlet ports, a vacuum line, and silicone gasket; (2) an Olympus inverted phase contrast microscope with video capacity (Olympus, Tokyo, Japan); (3) a high-resolution CCD camera (Olympus); (4) a black-and-white high-resolution monitor and videocassette recorder (Sony Corp., Tokyo, Japan); (5) an infuse/withdrawal syringe pump (Nihon Kohden, Tokyo, Japan). Before assemblage, the flow chamber was filled with media and all air removed from the system. The flow chamber was inserted with the gasket in place and media placed on the flow path. A 35-mm tissue culture plate on to which HUVECs were grown to confluence was then placed on top of the chamber and a vacuum created. Once assembled, the chamber and plate were placed on the microscope stage and flow of cells was initiated by the syringe pump attached to the outlet port, so that cells were drawn rather than pushed through the chamber. Eosinophils (2 × 10^5 cells/ml in RPMI1640 containing 1% BSA) were drawn at a constant flow of 0.5 dyn/cm^2 for 10 min, conditions previously shown to be optimal for integrin-mediated rolling [6]. Throughout the infusion, cells were kept at 37°C. In some experiments, eosinophils were pre-incubated with saturating concentrations of mAb against CD162 (PL-1, Immunotech, France), CD49d (HP2/1, Immunotech), CD62L (Dreg 56, Immunotech), CD18 (7E4, Immunotech) or an isotype control mAb for 15 min before perfusion. Interac-

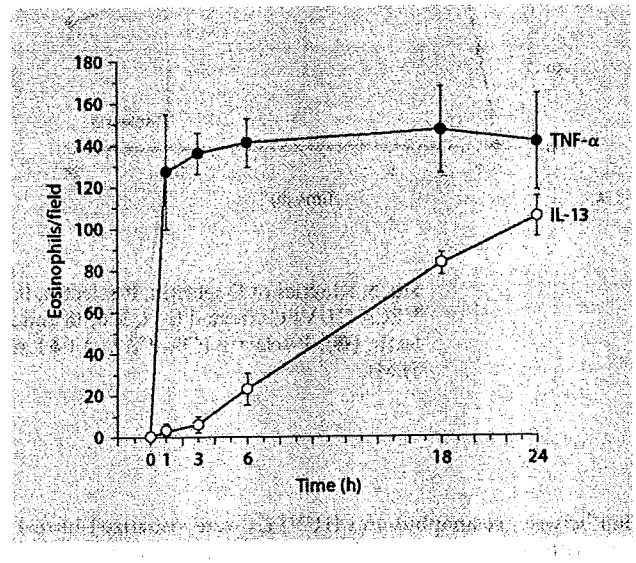

Fig. 2. The kinetics of eosinophil accumulation on IL-13- or TNF-α-activated HUVECs. HUVECs were treated for 1, 3, 6, 18 and 24 h with IL-13 (100 ng/ml) or TNF-α (100 ng/ml). Human eosinophils were infused and counted as in figure 1 (n = 4, mean ± SEM).

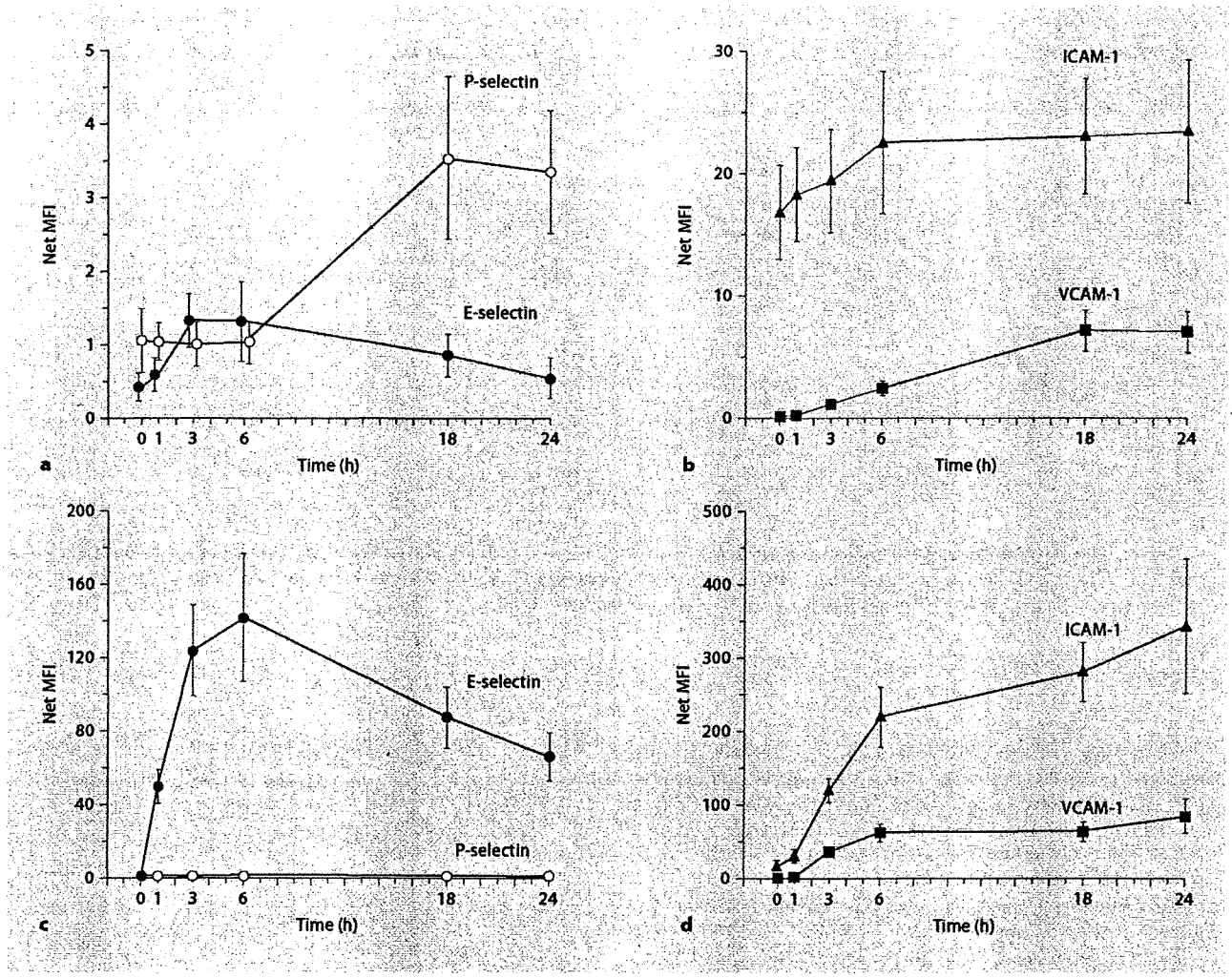

Fig. 3. Kinetics of E-selectin, P-selectin, ICAM-1 and VCAM-1 expression on IL-13- or TNF-α-activated HUVECs. HUVECs treated for 1, 3, 6, 18 and 24 h with 100 ng/ml IL-13 (**a**, **b**) or TNF-T (**c**, **d**). Expression of E-selectin (●), P-selectin (○), ICAM-1 (▲) and VCAM-1 (■) was examined by flow cytometry (n = 7, mean ± SEM).

tion between eosinophils and HUVECs were visualized in real time with video microscopy. Images were digitized from the videotape recorder and the number of adherent cells that accumulated for 20 s at 2-min intervals was determined by visual counts of the videotapes.

Expression of adhesion molecules on HUVECs was tested by indirect immunofluoresence and flow cytometry as previously reported [7]. The first passages of HUVECs were incubated with IL-13 (100 ng/ml) or TNF-α (100 ng/ml) for 1, 3, 6, 18 and 24 h at 37°C. HUVECs were washed with PBS and incubated 0.05% trypsin-EDTA-PBS for 5 min and loosened cells dislodged using cell scraper (BD Falcon, USA). Then HUVECs were incubated for 30 min at 4°C in PBS solution containing 0.2% BSA (Sigma) and 4 mg/ml human IgG1 (Sigma) with saturating concentrations of mAb against P-selectin, E-selectin, ICAM-1, VCAM-1 or equivalent concentration of irrelevant IgG control mAb. Cells were washed and incubated with PE-conjugated F(ab')2 goat anti-mouse IgG Ab (Biosource, Camarillo, Calif., USA) for another 30 min at 4°C. After fixation in 1% paraformaldehyde in PBS, 5,000 cells were evaluated using a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif., USA).

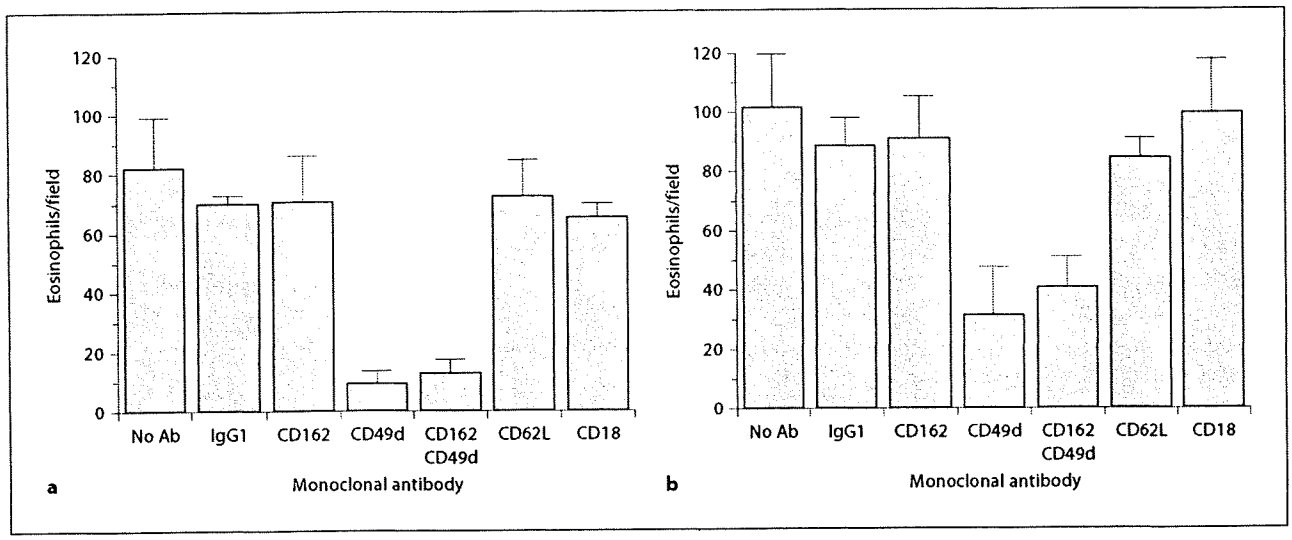

Fig. 4. Eosinophil accumulation on IL-13- or TNF-α-activated HUVECs was inhibited by anti-α4 integrin mAb. HUVECs stimulated with 100 ng/ml of IL-13 (**a**) or TNF-α (**b**) for 24 h. Human eosinophils were pretreated with indicated monoclonal antibodies. Eosinophils were infused and counted as in figure 1 (n = 5, mean ± SEM).

Results and Discussions

HUVECs were incubated with 1–100 ng/ml of IL-13 or TNF-α or for 24 h and then placed into the parallel plate flow chamber system. Eosinophils were infused at 0.5 dyn/cm^2 for 10 min. Under these physiological flow conditions, eosinophils accumulated efficiently on IL-13-stimulated (100 ng/ml: 109 ± 18 cells/field) or TNF-α-stimulated (100 ng/ml: 96 ± 27 cells/field) HUVECs in a concentration-dependent manner (fig. 1). Although both cytokines have similar efficacy at 100 ng/ml stimulation, IL-13 seems more potent than TNF-α at 1 ng/ml stimulation. In the next experiments, we examined the kinetics of the effect of IL-13 or TNF-α on eosinophil accumulation on activated HUVECs. Eosinophil accumulation on IL-13-activated HUVECs was observed at 3 h and reached a maximum at 24 h. On the other hand, 1-hour activation of HUVECs by TNF-α caused maximal eosinophil accumulation (fig. 2), as the levels of eosinophil accumulating on TNF-α-stimulated HUVECs were the same at all time points (1, 3, 6, 18 and 24 h). Thus, the kinetics of eosinophils accumulation to HUVECs was differently regulated by TNF-α and IL-13 under physiological flow conditions.

To clarify the difference between IL-13 and TNF-α in eosinophil accumulation under flow condition, we examined the effect of IL-13 or TNF-α on the expression of adhesion molecules on HUVECs. HUVECs were incubated with 100 ng/ml of IL-13 or TNF-α for 1, 3, 6, 18 and 24 h. Expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 on HUVECs was analyzed by flow cytometry. IL-13 induced both P- and E-selectin on HUVECs. Although induction of E-selectin expression was observed after 3–6 h, levels of E-selectin were weak (fig. 3a). Inductions of P-selectin expressions were observed from 18 h after IL-13 stimulation (fig. 3a). TNF-α induced E-selectin but not P-selectin on HUVECs (fig. 3c). Expression of E-selectin on HUVECs was observed at 1 h after the stimulation, reached maximum expression at 6 h and declined by 24 h of stimulation (fig. 3c). These data were consistent with previous reports in mouse and human models [8]. ICAM-1 but not VCAM-1 was expressed on resting HUVECs [7]. IL-13 induced both ICAM-1 and VCAM-1 on HUVEC (fig. 3b). TNF-α induced both ICAM-1 and VCAM-1 on HUVECs (fig. 3d). The levels of induction of ICAM-1 and VCAM -1 by IL-13 were less than the levels of induction of ICAM-1 and VCAM-1 by TNF-α.

To clarify which adhesion molecules on eosinophil were important in this attachment assay, we examined the effect of monoclonal antibodies (mAb) on eosinophil accumulation on activated HUVECs. HUVECs were in-

cubated with IL-13 or TNF-α (100 ng/ml) for 24 h. Eosinophils were pretreated with mAb against PSGL-1 (CD162), L-selectin (CD62L), β2 integrin (CD18), α4 integrin (CD49d) or control. Eosinophils were infused at 0.5 dyn/cm^2 for 10 min and attached eosinophils were counted. As shown in figure 4, anti-CD49d mAb effectively inhibited eosinophil accumulation on both IL-13 and TNF-α-activated HUVECs. These data indicate that interaction between VCAM-1 on endothelial cells and VLA-4 on eosinophils play an important role in eosinophil recruitment on HUVECs stimulated with IL-13 or TNF-α for 24 h. On the other hand, much eosinophil accumulation was observed at 1 h stimulation with TNF-α. HUVECs expressed E-selectin and ICAM-1 but not P-selectin and VCAM-1 at 1 h after TNF-α stimulation. Recently it has been reported that VCAM-1 but not ICAM-1 supported eosinophil rolling under conditions of shear flow [9, 10]. Taken together, eosinophil accumulation at 1 h after TNF-α stimulation may mediated by E-selectin.

These results indicate that eosinophil accumulation on HUVECs under these physiological flow conditions is differently regulated by TNF-α and IL-13.

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REVIEW

CCR3-active chemokines influence eosinophil adhesion to endothelial cells under static and flow conditions

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Clinical and Experimental Allergy Reviews

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Conflicts of interest:
The authors have declared no conflicts of interest.

Summary

We recently demonstrated that CCR3-active chemokines promote rapid detachment of eosinophils bound to vascular cell adhesion molecule-1 (VCAM-1) *in vitro*. Eosinophils adhered well to immobilized human recombinant VCAM-1 primarily via $\alpha_4\beta_1$ integrin. Eotaxin-2, a CCR3-specific chemokine, induced eosinophil de-adhesion from VCAM-1. In contrast, very few eosinophils spontaneously adhered to bovine serum albumin (BSA), and eosinophil adhesion to BSA was enhanced by eotaxin-2 over a similar nM range of concentrations. This enhancement of BSA adhesion was dependent on β_2 integrins. Eosinophil $\alpha_4\beta_1$ integrins can mediate rolling on VCAM-1 under physiological flow conditions. Although we observed a reduction of eosinophil accumulation on immobilized VCAM-1 in response to eotaxin-2 under physiological flow conditions, this reduction of adhesion was not observed when VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) were co-immobilized. Based on antibody-blocking studies, this appears to be caused by a chemokine-induced shift in integrin usage away from β_1 integrin-dominated interactions with VCAM-1 towards β_2 integrin-dominated interactions with ICAM-1. Our results confirm the important role of integrins and chemokines in selective eosinophil migration processes. CCR3-active chemokines may be necessary to facilitate de-adhesion from luminal VCAM-1 and to facilitate the process of diapedesis by shifting integrin usage in eosinophils away from β_1 integrin-dominated interactions with VCAM-1 towards β_2 integrin-dominated interactions with ICAM-1. The critical importance of integrins and chemokines in eosinophilic inflammation lends support for targeting these molecules with novel therapeutic agents.

Keywords human, eosinophils, cell trafficking, chemokines, adhesion molecules

Introduction

Eosinophils selectively accumulate at allergic inflammation sites. Numerous studies have been performed to clarify the mechanisms of selective eosinophil accumulation during allergic inflammation. Adhesion molecules and chemokines play important roles in selective eosinophil accumulation [1]. For example, eosinophils but not neutrophils express $\alpha_4\beta_1$ integrins, one of the ligands for vascular cell adhesion molecule-1 (VCAM-1). Among chemokine receptors, eosinophils express CCR3. Like $\alpha_4\beta_1$ integrins, CCR3 is not expressed on neutrophils and so its activation effectively and selectively induces eosinophil chemotaxis. Eosinophil infiltration into inflammatory sites is regulated by the expression of adhesion molecules and chemokines; yet, the role of these proteins in shear-dependent adhesion and transmigration is poorly understood. In this review, we discuss the regulation of eosinophil integrin function by CCR3-active chemokines.

Expression of integrins on eosinophils

Eosinophil recruitment to sites of allergic inflammation is probably the result of multiple interactions between endothelial cells and eosinophils involving selective adhesion and migration. Eosinophil migration from within blood vessels into tissue requires several steps such as rolling, tethering, firm adhesion, and transendothelial migration. Rolling of eosinophils is believed to be mediated primarily by selectins and their ligands. Rolling of eosinophils on endothelial cells may be followed by the induction of firm adhesion. Firm adhesion and transmigration are mediated by integrins. Integrins have two

Table 1. Integrins on eosinophils and their ligands

Integrin	Ligand
$\alpha_4\beta_1$	VCAM-1, fibronectin
$\alpha_4\beta_7$	VCAM-1, MAdCAM-1, fibronectin
$\alpha_6\beta_1$	Laminin
$\alpha_L\beta_2$	ICAM-1,2,3
$\alpha_M\beta_2$	ICAM-1, iC3b
$\alpha_X\beta_2$	Fibrinogen, iC3b
$\alpha_D\beta_2$	ICAM-3, VCAM-1

ICAM, intercelluar adhesion molecule-1; VCAM, vascular cell adhesion molecule-1; MAdCAM, mucosal addressin cell adhesion molecule-1.

components – α and β subunits. Eosinophils express β_1, β_2, and β_7 integrins [2] as shown in Table 1. For example, eosinophil $\alpha_4\beta_1$ integrin binds to VCAM-1 on endothelial cells, which plays an important role in selective eosinophil accumulation to tissue. Interestingly, $\alpha_4\beta_1$ integrin has the ability not only to mediate firm adhesion but also to participate in tethering on endothelial VCAM-1 under shear conditions *in vitro* [3], a property shared by $\alpha_D\beta_2$ integrin [4, 5]. Another ligand of $\alpha_4\beta_1$ integrin, albeit of lower affinity, is CS-1 peptide, a fragment of fibronectin [6]. The only other β_1 on eosinophils is $\alpha_6\beta_1$ integrin, a ligand of laminin [7]. Eosinophils also express $\alpha_4\beta_7$ integrins, which bind to the gut-expressed mucosal addressin cell adhesion molecule-1 (MAdCAM-1), fibronectin, and VCAM-1 [8]. Eosinophils express all four β_2 integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, and $\alpha_D\beta_2$). Their ligands include intercelluar adhesion molecule-1 (ICAM-1) ($\alpha_L\beta_2$, $\alpha_M\beta_2$), ICAM-2 ($\alpha_L\beta_2$), ICAM-3 ($\alpha_L\beta_2$, $\alpha_D\beta_2$), fibrinogen ($\alpha_M\beta_2$), the complement fragment C3bi ($\alpha_M\beta_2$, $\alpha_X\beta_2$), and VCAM-1 ($\alpha_D\beta_2$) [9].

Expression of chemokine receptors on eosinophils

Eosinophil migration to sites of allergic inflammation is influenced by the presence of chemoattractants. Much effort has been devoted to the identification of selective and potent chemoattractants for eosinophils. The growing list of eosinophil-active chemoattractants includes LTB_4, LTD_4, LTE_4, PAF, C3a, and C5a. Although LTD_4 and LTE_4 induce eosinophil but not neutrophil chemotaxis, many of the other chemoattractants are not selective for eosinophils and several have at best only modest activity [10]. As a result, numerous studies have focused on the chemotactic cytokines termed chemokines and their receptors. Among the numerous known chemokine receptors, eosinophils express CCR1, CCR3, CCR6, and CXCR4 Table 2 [11, 12]. Perhaps most notable is the expression of CCR3, which binds eotaxin, eotaxin-2, eotaxin-3, monocyte chemotactic protein (MCP)-2, MCP-3, MCP-4, and regulated on activation normal T cell expressed and secreted (RANTES). Macrophage-derived chemokine (MDC), a CCR4-active chemokine, also induces modest eosinophil chemotaxis [13]. Eosinophils do not express CCR4, and so the receptor for MDC on eosinophils remains unknown.

Table 2. Chemokine receptors on eosinophils and their ligands

Receptor	Ligand
CCR1	RANTES, MCP-3, MIP-1α
CCR3	Eotaxin-1,2,3, MCP-2,3.4, RANTES
CCR6	MIP-3α, LARC, exodus
CXCR4	SDF-1

RANTES, regulated on activation normal T cell expressed and secreted; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein.

Regulation of eosinophil integrin function by CCR3-active chemokines and other chemoattractants

Preferential eosinophil accumulation at sites of allergic inflammation is the result of interactions involving adhesion molecules, chemokines, and other molecules during which selective adhesion and detachment events allow for directed cellular migration. We recently demonstrated that CCR3-active chemokines promote rapid detachment of eosinophils bound to VCAM-1 *in vitro* [14–19]. Eosinophils adhered well to immobilized human recombinant VCAM-1 primarily via $\alpha_4\beta_1$ integrin. Eotaxin-2, a CCR3-specific chemokine, induced eosinophil de-adhesion from VCAM-1. In contrast, very few eosinophils spontaneously adhered to bovine serum albumin (BSA), and eosinophil adhesion to BSA was enhanced by eotaxin-2 over a similar nM range of concentrations. This enhancement of BSA adhesion was dependent on β_2 integrins. The effect of eotaxin-2 on eosinophil adhesion to VCAM-1 and BSA occurred within 3 min and was sustained for $\geqslant$60 min. Similar effects were seen with other chemoattractants such as RANTES and PAF. The reduction of eosinophil adhesion to VCAM-1 and enhancement of eosinophil adhesion to BSA by eotaxin-2 were prevented by a CCR3-blocking mAb.

Integrins on eosinophils exist in high- and low-affinity states [6, 17]. To explore the mechanism by which chemokines affect adhesion, their effect on integrin expression was determined [15]. Levels of α_4 and β_1 integrin expression on eosinophils were unchanged or slightly decreased by eotaxin-2 treatment. More impressive was the finding that levels of the activated β_1 integrin epitope were remarkably decreased. It appears likely, therefore, that the reduction of eosinophil adhesion to VCAM-1 in response to eotaxin-2 is primarily caused by changes of β_1 integrin function rather than expression.

Eosinophil $\alpha_4\beta_1$ integrins can mediate rolling on VCAM-1 under physiological flow conditions [3, 5]. Although we observed a reduction of eosinophil accumulation on immobilized VCAM-1 in response to eotaxin-2 under physiological flow conditions, this reduction of

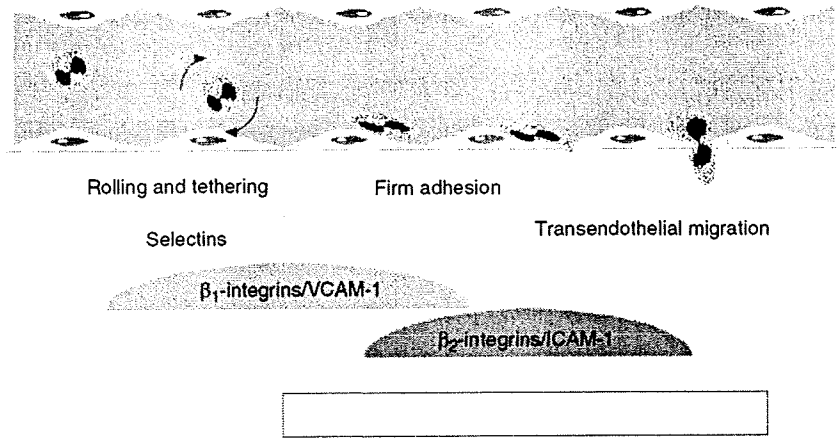

Fig. 1. CCR3-active chemokines regulate integrin function on eosinophils. VCAM, vascular cell adhesion molecule-1; ICAM, intercellular adhesion molecule-1

adhesion was not observed when both VCAM-1 and ICAM-1 were co-immobilized [18]. Based on antibody-blocking studies, this appears to be caused by a chemokine-induced shift in integrin usage away from β_1 integrin-dominated interactions with VCAM-1 towards β_2 integrin-dominated interactions with ICAM-1. In some ways, our results seem to differ from those of previous reports. Our data showed that a CCR3-active chemokine activated eosinophil β_2 integrin function, consistent with the work of others [14, 16, 19]. However, it has been reported that chemokines and C5a transiently activated $\alpha_4\beta_1$ integrin, followed by subsequent inhibition [16, 20]. Other researchers have reported that maximally activated adhesion, induced through β_1 integrins with a unique integrin-activating mAb, actually inhibited eosinophil transmigration by preventing cell movement [21]. Furthermore, eosinophil migration across human pulmonary microvascular endothelial cells inversely correlated with eosinophil adhesion and VCAM-1 expression [22]. These data suggest that activation of β_1 integrins can inhibit migration and that chemokines down-regulate β_1 integrin function during eosinophil migration.

Recently, Culvelier and Patel [23] reported that eotaxin-3, a CCR3-active chemokine, is released by IL-4-activated human umbilical vein endothelial cells and is displayed on the surface of endothelial cells. They also demonstrated that shear-dependent eosinophil transmigration is regulated in association with chemokines on the surface of activated endothelial cells. These data support our results, suggesting that CCR3-active chemokines regulate eosinophil–integrin function under flow conditions.

Conclusion

Our results confirm the important role of integrins and chemokines in selective eosinophil migration processes. CCR3-active chemokines may be necessary to facilitate de-adhesion from luminal VCAM-1 and facilitate the process of diapedesis by shifting integrin usage in eosinophils away from β_1 integrin-dominated interactions with VCAM-1 towards β_2 integrin-dominated interactions with ICAM-1 (Fig. 1). The critical importance of integrins and chemokines in eosinophilic inflammation lends support for targeting these molecules with novel therapeutic agents.

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ORIGINAL PAPER

Transforming growth factor-β_2 polymorphisms are associated with childhood atopic asthma

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Summary

Background Transforming growth factor (TGF)-β plays an important role in the regulation of airway inflammation and remodelling in asthma. Recent studies suggest that TGF-β_2 is a predominant isoform expressed in severe asthma and it is also associated with airway remodelling.

Objective To determine whether the polymorphisms in *TGF-β_2* are associated with childhood atopic bronchial asthma in a Japanese population.

Methods We identified a total of eight polymorphisms and characterized the linkage disequilibrium (LD) mapping of the gene. Three variants in the promoter and 3′UTR were genotyped, and we conducted an association study of *TGF-β_2* (childhood atopic asthma $n = 297$, normal controls $n = 555$). An association analysis of these variants and an expression and functional analysis were performed.

Results 3′UTR 94862T > A was found to be significantly associated with the risk of childhood atopic asthma ($P = 0.00041$). The − 109 → ACAA ins promoter variant was also associated with the risk of childhood atopic asthma ($P = 0.0037$). TGF-β_2 expression was observed in both the normal and asthmatic bronchial epithelium, and both real-time PCR and an ELISA showed a significant basal and TGF-β_1-induced TGF-β_2 expression in the bronchial epithelial cell line BEAS2B. Furthermore, the promoter variant − 109 → ACAA ins increased the TGF-β_2 promoter-reporter activity in BEAS2B cells.

Conclusions Our data suggest that *TGF-β_2* may therefore be involved in the development of childhood atopic asthma by means of functional genetic polymorphism. The polymorphisms in *TGF-β_2* may become important information for asthma susceptibility in children.

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Introduction

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that is involved in a variety of cellular programs (proliferation, differentiation, extracellular matrix regulation, and survival), affecting multiple biological processes including immune responses and tissue repair [1–3]. There are three isoforms of TGF-β: TGF-β_1, TGF-β_2, and TGF-β_3 in mammals. Each isoform is encoded by a distinct gene and it is expressed in both a tissue-specific and a developmentally regulated fashion. The deletion of individual isoforms in mice results in a different phenotype, thus suggesting their distinct roles *in vivo* [4].

[1]Both the authors contributed equally to this work.

The roles of TGF-β_1 in asthma have been intensively studied. TGF-β_1 expression was significantly increased in the bronchoalveolar (BAL) lavage fluids and lung tissue obtained from asthmatic patients [5–9]. The expression of TGF-β_1 in the asthmatic airways was predominantly detected in eosinophils and fibroblasts [6, 7] and it was significantly correlated with the severity of the disease, basement membrane thickness, and submucosal fibroblast number [5–8], thus suggesting that TGF-β_1 is involved in airway remodelling in adult asthma. In addition, a C to T single-nucleotide polymorphism (SNP) (− 509C/T) in the *TGF-β_1* gene promoter was associated with the diagnosis of adult asthma in Caucasian, Indian, and Chinese populations [10–15].

Recent studies have highlighted the roles of TGF-β_2 in asthma. High levels of TGF-β_2 have also been reported in the BAL fluid of asthmatics and the levels were observed to increase after an allergen challenge [9]. Chu et al. [16] reported the epithelial TGF-β_2 expression levels to be higher than TGF-β_1 in both asthmatics and normal subjects, and TGF-β_2 also increased in asthmatics in comparison with normal subjects. Balzar et al. [17] recently showed that TGF-β_2 is significantly up-regulated in severe asthma, with eosinophils identified as a major cell source of the protein in patients with persistent eosinophilic inflammation. The TGF-β_2 production and release from primary human bronchial epithelial cells (HBECs) were induced by IL-4 and IL-13 [18, 19], which are key factors contributing to the chronic inflammatory state characterizing asthma [20]. These findings suggest that not only TGF-β_1 but also TGF-β_2 plays an important role in the pathophysiology of asthma.

Although genetic studies in asthma have been conducted to clarify the polymorphisms in *TGF-β_1* genes, the genetic influences of the polymorphisms of *TGF-β_2* still remain unclear. Hobbs et al. screened the promoter region of *TGF-β_2* using the single-strand conformational polymorphism (SSCP) method, and thus found a variant in the region. However, no association with either an allergy or asthma-related phenotype was identified [10]. To elucidate whether genetic variations of *TGF-β_2* contribute to asthma susceptibility or asthma-related phenotypes such as a high eosinophil count, a high serum IgE level, and disease severity, we first carried out linkage disequilibrium (LD) mapping of the gene, and then conducted an association study. We next investigated whether the expression and secretion of TGF-β_2 are influenced by other cytokines in bronchial epithelial cells. Furthermore, we also performed functional analyses of the associated polymorphisms.

Methods

Study subjects

All subjects with asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute) [21] and demonstrated at least a 12% improvement in their forced expiratory volume in 1 s measurement after β_2-agonist inhalation. Peripheral blood samples were obtained from each of 297 paediatric atopic asthma outpatients at the Osaka Prefectural Habikino Hospital and National Sagamihara Hospital [mean age 9.6, 3–15 years; male : female ratio = 1.63 : 1.0; mean serum IgE level, 490 U/mL; *Dermatophagiodes pteronyssinus* (Derp) or *Dermatophagiodes farinae* (Derf) RAST positive 80.0%], and the asthmatic profiles of these patients have been provided in our recent report [22]. The mean of $\log_{10}$[total IgE (tIgE) (IU/mL)] of patients with childhood atopic BA was 2.69 [$= \log_{10}$(490 IU/mL)]. In this study, 'high IgE' levels were defined as those values in the 75th percentile or higher for total IgE. The 75th percentile value of $\log_{10}$ (tIgE) in patients with childhood atopic BA was 3.04 [$= \log_{10}$(1106 IU/mL)]. The severity of asthma was defined according to the degree of therapy required to control symptoms at the time of entry into the study. The grades were as follows: grade 1, β stimulants only; grade 2, sodium cromoglycate and/or theophylline; grade 3, inhaled beclomethasone, 400 μg/day or less; and grade 4, inhaled beclomethasone of more than 400 μg/day. Patients classified as having grades 3 and 4 were treated with inhaled steroids and they accounted for 46% of all patients. A total of 555 healthy individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age 45, 18–75 years; male : female ratio = 2.44 : 1.0) were recruited after being interviewed by physicians regarding whether or not they had been diagnosed with asthma, atopic dermatitis, or nasal allergies. As there was a large age difference between the cases and controls, correlation between age and genotype frequencies of genotyped SNPs were analysed using Spearman's correlation coefficient by a rank test. *Rs* (rank correlation coefficient) of all SNPs were < 0.1 ($P > 0.05$), and so there was no evidence of a correlation between age and allele frequencies. No smoking data are available for 284 control subjects. Other control subjects were 110 smokers, 52 ex-smokers, and 109 non-smokers. No lung function or serum IgE data were available for this population. All individuals were Japanese and gave their written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the rules of the process committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping

To identify polymorphisms in the human *TGF-β_2* gene, we sequenced all seven exons, including a minimum of 100 bases of the flanking intronic sequence and 3 kb of the 5′ flanking region from 24 asthmatic subjects. Eleven primer sets were designed on the basis of the *TGF-β_2* genomic

sequence from the GenBank database (accession number AC096638.2; see Table 1). The sequences were analysed and polymorphisms were identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA). The selected three polymorphisms were genotyped by the TaqMan™ allele-specific amplification (TaqManASA) method (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time polymer chain reaction of multi-tissues panel

The tissue expression of mRNAs of the three *TGF-β* isoforms was quantitatively evaluated using quantitative real-time PCR. Total RNAs of human multi-tissues were purchased from Clontech Laboratories Inc. (Mountain View, CA, USA). cDNAs were prepared from 5 μg of total RNA and synthesized using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). We quantified mRNA using SYBR® Premix Ex Taq™ (TAKARA Bio Inc., Chuo-ku, Tokyo, Japan) and an ABI Prism 7900 sequence detector (Applied Biosystems) according to the manufacturers' instructions. The primers were: for *TGF-β_1*, 5′-AGC GACTCGCCAGAGTGGTTA-3′ and 5′-GCAGTGTGTTATCC CCTGCTGTCA-3′, for *TGF-β_2*, 5′-GCTTTGGATGCGGCCTA TTG-3′ and 5′-TTCGTGTATCCATTTCCACCCTAGA-3′, and for *TGF-β_3*, 5′-GGGTCCATGAACCTAAGGGCTACTA-3′ and 5′-GATGCTTCAGGGTTCAGAGTGTTG-3′. The data were analysed using relative real-time PCR quantification based on the delta delta Ct method [23]. The endogenous reference gene was GAPDH, and the control organ was the small intestine.

Immunostaining of transforming growth factor-β_2

Lung biopsy specimens were obtained from two normal adult subjects and three adult patients with moderate asthma as defined by the criteria of the Japanese Society of Allergology [24] as described previously [25]. In addition, these patients were not treated with systemic steroids within 4 weeks before the lung biopsy. The investigation was approved by the Ethics Committee of Dokkyo University School of Medicine, and all subjects gave their written informed consent. Each biopsy specimen was immediately placed in OCT compound, snap-frozen in liquid nitrogen, and stored at −80 °C until cryosectioned. The sections were stained with an anti-TGF-β_2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) using peroxidase-based VECTASTAIN ABC kits with a DAB substrate (Vector Laboratories, Burlingame, CA, USA).

Cell culture

The simian virus 40 (SV-40)-transformed human bronchial epithelial cell line, BEAS-2B [26], was obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were cultured in bronchial epithelial basal medium (BEBM™) (Cambrex, East Rutherford, NJ, USA) supplemented with 50 μg/mL bovine pituitary extract, 0.5 μg/mL hydrocortisone, 0.5 ng/mL human epidermal growth factor, 0.5 μg/mL epinephrine, 10 μg/mL transferrin, 5 μg/mL insulin, 0.1 ng/mL retinoic acid, 6.5 ng/mL triiodothyronine, 50 μg/mL gentamicin, and 50 ng/mL amphotericin-B (all materials from Cambrex).

Transforming growth factor-β_1 and -β_2 mRNA expression in BEAS2B cells

For conventional RT-PCR, total RNA was extracted from BEAS2B cells ($1-2 \times 10^6$) using Isogen solution (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. cDNA was then synthesized from 2 μg of total RNA using a Reverse Transcriptase System (Applied Biosystems). PCR amplification (TGF-β_1, TGF-β_2, and Histone 3.3; 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; 35 cycles) was then performed in a DNA engine cycler (MJ Research Inc., Waltham, MA, USA). The PCR products were separated by 2.0% agarose gel electrophoresis and stained with 0.5 μg/mL ethidium bromide. The specific primers were purchased from R&D Inc.

Table 1. Primers used in screening for polymorphisms in *TGF-β_2*

	Forward primer		Reverse primer
F1	CTGAACCTGTATCCTCAGCA	R1	TCAGATGTTCCTGAGTTCAGA
F2	CTGGGACCATTGTTTCTCAGA	R2	GATACCTTAGCAGGTTGCCAT
F3	AAGAAGTGGCTTAGGCAGCA	R3	TGTCAGGAGCTTCTGGAGCT
F4	CTTCTGACTGTAATCCTAGCA	R4	CTTGGTTACTCCACGTTGCT
F5	GCAAGGGCTGCCGTTGTGAT	R5	GAACCCTGACTTTGGCGAGT
F6	GGAGGAGCCGAGTTCAGAT	R6	GGCCTCAAGCATACAACTGA
F7	GCTACTCCTGTAACTAAATGCA	R7	CTCCTGCAGTCCCATTGACT
F8	TGAGATGACAATGCATGGCTA	R8	ATCCAACCATATCACTGTCCA
F9	TCACGTGGGTTAGTCGTAGT	R9	TCACTGCTAAAGACCTGATGA
F10	TAAGGGCAAGTAGTCCAGCA	R10	GTTTGTAACCGATCAGTGTCA
F11	GGTCACATATCAGTTTGACTG	R11	AGGGTGCCTATTGCATAGCA

(Minneapolis, MN, USA) and used according to the manufacturer's instructions.

For real-time PCR, BEAS2B cells ($1-2 \times 10^6$) were stimulated with recombinant human TGF-β_1 (10 ng/mL), IL-6 (10 ng/mL), granulocyte–monocyte colony stimulating factor (GM-CSF) (10 ng/mL), TNF-α (10 ng/mL), IL-13 (10 ng/mL), or TNF-α (10 ng/mL) plus IL-13 (10 ng/mL) (R&D Inc) for 1 or 3 h. Total RNAs were then extracted from the cells and cDNAs were synthesized as described above. Real-time PCR was then performed to quantify *TGF-β_1*, *TGF-β_2*, and *GAPDH* mRNA as described above.

Enzyme-linked immunosorbent assay

BEAS2B cells (1×10^6) were challenged with recombinant human TGF-β_1 (10 ng/mL), TNF-α (10 ng/mL), IL-13 (10 ng/mL), TNF-α (10 ng/mL) plus IL-13 (10 ng/mL), IL-6 (10 ng/mL), or GM-CSF (10 ng/mL), (all purchased from R&D Inc.). After stimulation for 48 h or 72 h, the culture supernatants were harvested and kept at −80 °C until analysed. The amounts of TGF-β_2 in the culture supernatants were determined using the human TGF-β_2 ELISA kit (R&D) according to the manufacturer's instructions. The samples were activated by an acidification procedure before the ELISA assay in order to determine the total TGF-β_2 concentration.

Luciferase assay

DNA fragments were cloned into pGL3-basic vector (Promega, Madison, WI, USA) in the 5′–3′ orientation (Fig. 5). We then transfected subconfluent BEAS-2B cells (5×10^4) cultured in 24-well plates with 0.125 μg of each construct and 0.0025 μg of pRL-TK Renilla luciferase vector (Promega), an internal control for transfection efficiency, using 0.75 μL of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we lysed the cells and measured the firefly and Renilla luciferase activities in a luminometer using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity of the mock and *TGF-β_2* reporter construct is represented as the ratio of the firefly luciferase activity to that of Renilla. Each experiment was repeated three times, and each sample was studied in triplicate. The average luciferase activity of the mock-transfected cells was designated as 1.

Statistical analysis

Pairwise LD was calculated as $|D'|$ and r^2 using the Haploview 3.2 software program (http://www.broad.mit.edu/mpg/haploview/). We calculated the allele frequencies and tested the agreement with Hardy–Weinberg equilibrium using the χ^2 goodness-of-fit test at each locus. We then compared the differences in allele frequencies and genotype distribution of each polymorphism between the case and control subjects using the χ^2 test, and calculated the odds ratios (ORs) with 95% confidence intervals (95% CI). We applied Bonferroni corrections, the multiplication of *P* values by the number of variants. Corrected *P* values of < 0.05 were considered to be significant. Haplotype frequencies for two loci were estimated, and haplotype association tests were performed using Haploview 3.2. Serum total IgE and eosinophil counts were analysed as quantitative levels by the Mann–Whitney *U*-test. Comparisons in real-time PCR, ELISA, and reporter assays were performed using an unpaired Student's *t*-test. A *P* value of < 0.05 was considered to be statistically significant.

Results

Transforming growth factor-β_2 polymorphism

We identified eight biallelic polymorphisms in *TGF-β_2*: three in the 3′ untranslated region and five in the promoter region (Table 2 and Fig 1). Pairwise LD among eight SNPs with a frequency > 0.05 was measured by D′ and r^2 using the Haploview software program (Table 3). The promoter variant, −677T > C, was in complete LD with another promoter variant, −109 → ACAA ins ($D' = 1.00$ and $r^2 = 1.00$). In this study, we finally genotyped three polymorphisms, namely promoter variant −1615 → CTTC ins, −109 → ACAA ins, and 3′UTR variant 94862T > A.

Association of transforming growth factor-β_2 polymorphisms with childhood atopic asthma

All these loci were in Hardy–Weinberg equilibrium in the control group. The allele frequency of each selected polymorphism was compared between the patients and the normal controls by the χ^2 test using genetic, allelic,

Table 2. Locations and allele frequencies of polymorphisms in *TGF-β_2* screened with 24 subjects

SNP*	Location	Allele frequency	NCBI†
−3076 G/A	5′ flanking region	0.042	rs12058014
−1615 -/CTTC ins‡	5′ flanking region	0.250	rs9331507
−1529 A/C	5′ flanking region	0.042	rs7550232
−677 T/C	5′ flanking region	0.292	-
−109 -/ACAA ins‡	5′ flanking region	0.292	rs10482719
94700 G/A	3′UTR	0.021	rs11466412
94796 G/A	3′UTR	0.021	-
94862 T/A‡	3′UTR	0.229	rs900

*Numbering according to the genomic sequence of *TGF-β_2* (AC096638.2), and position 1 is the A of the initiation codon.
†Number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
‡SNPs were genotyped in this study.
SNP, single-nucleotide polymorphism.

