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Age-Related Difference in the Persistency of Allergic Airway Inflammation and Bronchial Hyperresponsiveness in a Murine Model of Asthma

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Key Words
Cytokines · Development · Infant · Maturation · Mouse

Abstract
Aim: Asthmatic children are more likely to outgrow their symptoms than adult patients. Thus, we wanted to know whether there were any age-related differences in the time course of the allergic airway inflammation. ***Methods:*** BALB/C mice at different ages (young: 3 days after birth, and mature: 8 weeks of age) were sensitized with ovalbumin (OVA). Subsequently, animals were challenged with aerosolized OVA during 1, 2, 4 or 8 consecutive weeks. Bronchial hyperresponsiveness (BHR), serum IgE levels, the degrees of inflammatory cell infiltration (ICI) and goblet cell metaplasia (GCM) in the airways, and the number of eosinophils and cytokine levels in bronchoalveolar lavage fluid (BALF) were examined. ***Results:*** At 1 week, airway inflammation and BHR occurred similarly between young and mature mice. However, BHR disappeared at 4 weeks in young, whereas it persisted even at 8 weeks in mature mice. GCM, ICI and eosinophilia in BALF attenuated with time, with more remarkable reduction in young mice. The BALF IL-4 level was high during the first 2 weeks in both groups, while the IL-2 level was significantly increased at 2 weeks solely in young mice. ***Conclusion:*** Different time courses in airway inflammation and in BHR may relate to the different prognoses between childhood and adult asthma. The understanding of the mechanisms underlying this age-related differences may be helpful to induce remission in asthmatic patients.

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Introduction

Recently, asthma has been believed to be a chronic inflammatory and partly irreversible airway disease, mainly based on findings in adult patients [1]. In children, however, details on airway inflammation and remodeling are inconclusive compared with those in adult patients. A difference in the natural history of asthma has been shown in patients regarding the onset of illness in childhood and after adolescence. Longitudinal studies indicate that about 50% of all asthmatic children are virtually free of symptoms within 10–20 years [2], in contrast to only about 10% of asthmatic adults [3], showing that pediatric patients are more likely to outgrow their symptoms than adult patients, although the exact mechanism of this age-related difference remains to be elucidated. Most patients with childhood asthma are known

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to be atopic [4]. Therefore, an age-related difference in the development of allergic airway inflammation and remodeling may be responsible for the differences.

The pathophysiology of atopic asthma is characterized by airway eosinophilia, an elevated serum IgE level, bronchial hyperresponsiveness (BHR), inflammatory cell infiltration (ICI) and goblet cell metaplasia (GCM) in the airways. So-called T-helper (Th) 2 cytokines, e.g. interleukin (IL)-4, IL-5, and IL-13 produced by Th2 cell subsets, are thought to play a pivotal role in this process [5, 6]. The Th2 deviation has been reported to be induced by several factors, including the genetic background, the doses of antigen for sensitization [7] and the cytokines involved during early T-cell activation [6]. The details for these pathophysiological and immunological abnormalities have been based on data in humans and animal models of asthma.

It has been shown that immune responses in the perinatal and neonatal period were at variance with those seen in mature individuals [8, 9]. The different immune responses may induce altered airway inflammation and remodeling via immune modulators such as cytokines.

The purpose of the present study was to determine whether there are any differences in allergic airway inflammation and in BHR depending on the stages of maturation in animals. Thus, the time courses of airway inflammation and BHR were examined in sensitized young and mature mice. In addition, cytokine levels in the BALF were also assessed to know the role of immune responses in these changes.

Materials and Methods

Animals

BALB/C mice at different stages of maturation (young mice 3 days after birth and mature mice at 8 weeks of age) were studied. Eight-week-old adult mice and pregnant mice were obtained from Charles River Japan (Shizuoka, Japan). After delivery of newborn mice, each mother and the litter were housed separately. The protocol of the experimental study was approved by the institutional animal care and use committee.

Sensitization and Airway Challenge Protocol

Young and adult mice were randomly divided into two groups. One group of mice was immunized with 10 μg OVA i.p. (grade V, Sigma, St. Louis, Mo., USA) in 20 mg of alum ($AlOH_3$) on day 0 and boosted on days 7 and 14 as described previously [7, 10]. Thereafter, they were challenged with aerosolized 2.5% OVA solution using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) in a 4.5-liter inhalation box 3 times a week from 1 to 8 weeks. Another group of animals (non-immunized control group) received injections of alum alone (vehicle for OVA) for 3 times and were repeatedly challenged with vehicle (saline) under the same schedule. We chose 10 μg OVA as the dose of sensitization, because we have previously established that this dose, but not higher doses (e.g. 1,000 μg OVA), induced Th2-biased responses both in juvenile and mature mice [7]. Twenty-four hours after the last challenge of repetitive inhalation for either 1, 2, 4 or 8 weeks, BHR was examined in each animal. Thereafter, serum IgE levels, eosinophil and cytokine counts in BALF, and the degrees of ICI and GCM in the airways were examined.

Measurement of BALF Cytokines

The levels of eight BALF cytokines, including IL-2, IL-4, IL-5, IL-10 and IL-12, tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), were measured using a commercially available kit (Bio-Plex Suspension Array System; Bio-Rad, Richmond, Calif., USA). The detection range was 1.0–32,000 pg/ml for all cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α and IFN-γ).

Determination of OVA-Specific IgE

OVA-specific IgE levels were determined by ELISA as described previously [7]. Ninety-six-well microtiter plates were coated with 200 μg/ml of OVA (grade V, Sigma) diluted in 0.1 M $NaHCO_3$. After 2 h of incubation at 37°C, plates were washed with washing buffer (Sigma) and blocked with PBS-bovine serum albumin for 2 h at 37°C. After washing with the buffer for 5 times, serially diluted serum samples (100 μl) were added and incubated for 2 h at 37°C. Plates were washed for 5 times with 300 μl of the washing buffer. Subsequently, 100 μl of 1:800-diluted rat anti-mouse IgE monoclonal antibodies (Biosource International, Camarillo, Calif., USA) were added. After 2 h of incubation at 37°C, plates were washed for 5 times with 300 μl of the washing buffer. After 2 h of incubation at 37°C, the reaction chromogen was generated with FAST (Sigma). After the reaction was stopped with H_2SO_4, plates were read in a multiplate reader at 490 and 620 nm. The serum pooled from adult mice that was sensitized and challenged with OVA was used as a positive control. The OVA-specific IgE titer was determined as the reciprocal of the highest dilution giving a positive value. The results were expressed as indices (the ratio of test serum to positive control).

Determination of BHR

BHR to increasing concentrations of aerosolized methacholine (Mch) was studied on unstrained conscious mice as described previously [7]. Mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Sharon, Conn., USA), and continuous measurement of the pressure-time wave was made. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with airway resistance [7, 11], was calculated. Mice were challenged with Mch (3.13, 6.25, 12.50 and 25.00 mg/ml) aerosol generated by an ultrasonic nebulizer (NE-U12, Omron) for 2 min. Respiratory mechanics were measured for 3 min after each aerosol inhalation and averaged. BHR was evaluated utilizing two parameters, (1) the leftward shift of the dose-response curve and (2) the absolute value of Penh corresponding to the maximum Mch concentration (25.00 mg/ml) referred to as maximum reactivity.

The Sampling Procedure of Blood and BALF

After assessment of BHR, animals were killed with an overdose of pentobarbital (50 mg/animal i.p.) to obtain serum samples. After sampling, a 24-gauge cannula was introduced into the proximal portion of the trachea, and lungs were lavaged 3 times

with PBS. The amount of lavage fluid was 0.4 ml each time for mature mice and young animals exposed to OVA or vehicle for 4 and 8 weeks. From the young mice exposed to OVA or vehicle for less than 2 weeks, 0.3 ml of lavage fluid were sampled because of their smaller body size [7]. The BALF was centrifuged at 800 rpm for 5 min. For cytokine level measurements, the supernatant was stored at -70°C. The cell pellet was resuspended in 0.3 ml of RPMI-1640 medium (Sigma). Total cell counts were performed with a hemocytometer, and differential cell counts were performed on cytospin preparations stained with Diff-Quick (Kokusai-Siyaku, Tokyo, Japan). A blinded observer counted a minimum of 200 cells for each sample.

Tissue Preparation

After getting BALF, the lungs were inflated at a pressure of 25 cm H_2O. The trachea was clamped until fixation was completed. Tissue specimens were sectioned in the midsagittal plane to a thickness of 6 μm, embedded in paraffin and stained with hematoxylin-eosin and Alcian blue/periodic acid-Schiff (AB/PAS). The slides were coded and graded in a blinded fashion, and the degrees of ICI and GCM were examined.

Evaluation of ICI

ICI in the lung was evaluated using a modification of a reproducible scoring system described previously [7, 12]. A value from 0 to 3 per criterion was ascribed to each tissue section scored. Three criteria were scored to document the pulmonary inflammation: peribronchial inflammation, perivascular inflammation and alveolar inflammation. For peribronchial and perivascular lesions, a value of 0 was assigned when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value 2 when most bronchi or vessels were surrounded by a thin layer (1–5 cells thick) of inflammatory cells and a value of 3 when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells. To assess alveolar wall inflammation, a value of 1 was defined as increased numbers of inflammatory cells in alveolar walls, a value of 2 as 1–3 foci per section showing cellular alveolar exudate and atelectasis, and a value of 3 as >3 foci per section showing cellular alveolar exudate and atelectasis additionally. The total score (the cellular infiltration score) was the sum of these three subscores (ranging from 0 to 9).

Evaluation of GCM

The degree of GCM was analyzed using a semiquantitative 5-point-scale, as described previously [7, 13]. The AB/PAS-stained slides were examined with a light microscope (IX 70, Olympus, Tokyo, Japan) and graded on a 5-point scale: grade 0 = 0%, grade 1 = 0–25%, grade 2 = 25–50%, grade 3 = 50–75% and grade 4 = 75–100% of epithelial cells staining positive for AB/PAS, respectively. The mean of the grade in the main bronchus and the large membranous airways was scored separately in each animal. The average of both points was referred to as the mucus cell score.

Statistical Analysis

All data were expressed as means ± SEM unless otherwise mentioned. Non-parametric analysis of variance (Kruskal-Wallis test for unmatched pairs) was used to determine the significance of variance between groups. If a significant difference was found, the Mann-Whitney U test was performed to assess differences between groups. A p value of less than 0.05 was considered to indicate statistical significance. The statistical analysis was performed utilizing Statview version 4.5 (Abacus Concepts, Berkeley, Calif., USA).

Results

Cytokine Levels in BALF

Of the eight cytokines examined in this study, only IL-4, a Th2 cytokine, and IL-2, a Th1 cytokine, were significantly increased compared to control animals of corresponding age. Other cytokines, including IL-5 and IL-12, did not show any significant change. In both young and mature animals, the IL-4 level was increased during the first 2 weeks and then declined. In contrast, the IL-2 level was significantly increased at 2 weeks in young but not in mature animals (fig. 1). Around 2 weeks after the challenge, Th1 and anti-inflammatory cytokines, including TNF-α, IFN-γ and IL-10, were detected in some of the young animals sensitized and challenged with OVA. However, these changes did not reach statistical significance (fig. 1).

OVA-Specific IgE Antibodies

In both mature and young animals, the OVA-specific IgE antibody level was significantly higher in OVA-sensitized groups compared to the corresponding control, and did not fluctuate throughout the study period. Comparing sensitized animals of different age groups, there was a tendency to a higher level in mature mice compared to the young ones, which was significant 1 and 4 weeks after challenge ($p < 0.05$, fig. 2).

BHR to Mch Challenge

Figure 3 shows the results of BHR to Mch in mature and young mice. One and 2 weeks after exposure, BHR showed a marked leftward shift of the dose-response curve, and a significant increase in maximum reactivity in both age groups of animals sensitized and challenged with OVA compared to controls. In mature animals, this was also the case 4 and 8 weeks after repeated exposures. By contrast, in young mice, BHR disappeared 4 and 8 weeks after exposure compared to control animals.

The Number of Eosinophils in BALF

In both age groups of animals sensitized and challenged with OVA, the number of eosinophils was significantly elevated compared to controls 1 week after exposure. In both age groups, the number of eosinophils significantly declined with time, although it was significantly higher compared to control animals without sen-

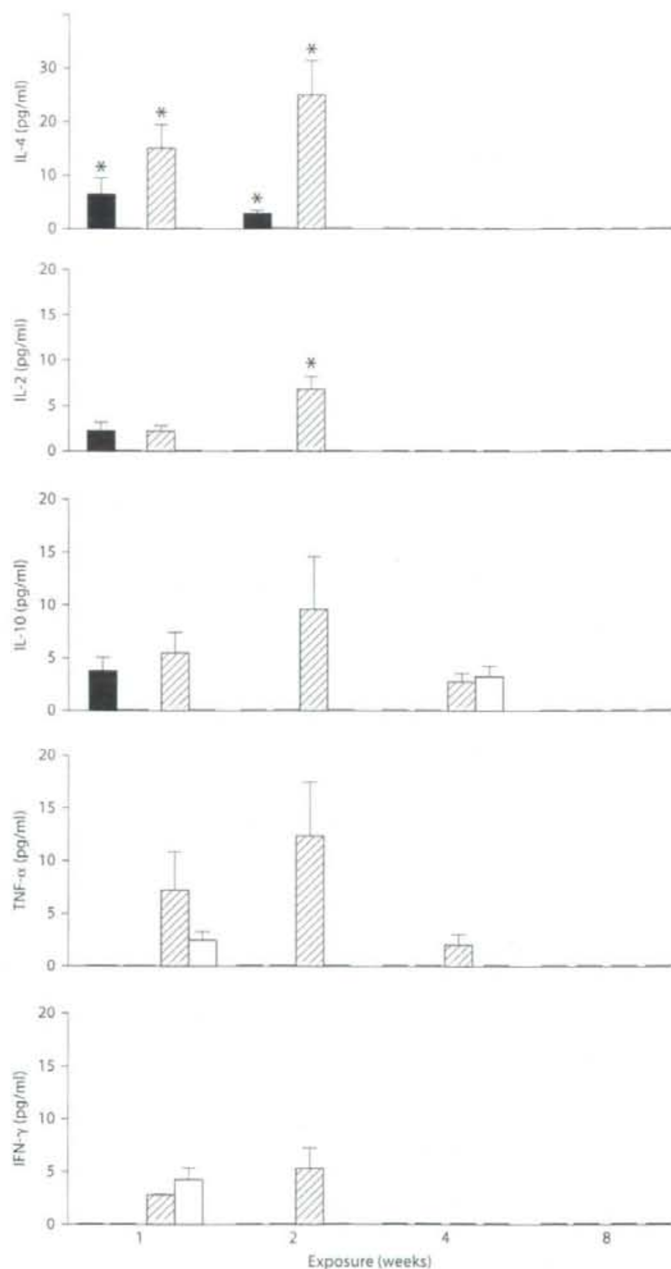
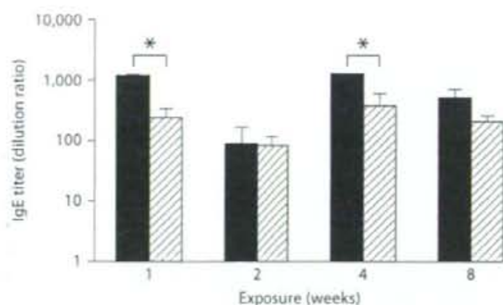


Fig. 1. Cytokine levels in BALF. The values of mature animals with (■) and without sensitization (-) and those of young ones with (▨) and without sensitization (□) were shown. Values are means ± SEM. * $p < 0.05$ vs. control animals at corresponding age (n = 5 for each group).

Fig. 2. OVA-specific IgE antibody titers determined as the reciprocal of the highest dilution giving a positive value. Mature (■) and young (▨) animals sensitized and challenged with OVA. Values are means ± SEM. * $p < 0.05$ between groups (n = 5 for each group).

Fig. 3. BHR to aerosolized Mch in adult (**a–d**) and juvenile (**e–h**) mice after exposure to OVA or vehicle (control) for 1 (**a**, **e**), 2 (**b**, **f**), 4 (**c**, **g**) and 8 weeks (**d**, **h**). Values are means ± SEM. * $p < 0.05$ between groups (OVA: n = 7–17, control: n = 4–10 for each group). BC = Before challenge.



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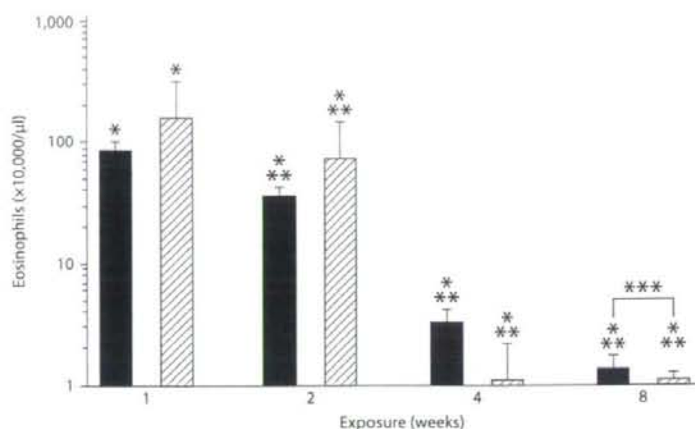

Fig. 4. Eosinophil counts in BALF of mature animals (■) and young ones (▨) with and without sensitization are shown. Values are means ± SEM. * $p < 0.05$ vs. control animals at corresponding age, ** $p < 0.01$ vs. animals exposed to OVA for 1 week, *** $p < 0.05$ between groups (n = 4–9 for each group).

sitization and challenge even at 8 weeks (fig. 4). Comparing sensitized animals at different age groups, the number at 8 weeks was significantly higher in mature mice than in young animals ($p < 0.05$).

ICI and GCM

In both age groups of animals sensitized and challenged with OVA, the cellular infiltration score was significantly higher than that in control animals 1 week after exposure (3.8 ± 0.7 vs. 0.5 ± 0.3 in mature mice, and 4.6 ± 1.2 vs. 0.4 ± 0.3 in young animals, respectively). However, a statistical difference was not seen after the 2-week exposure in animals of both age groups compared to control animals.

The mucus cell score, a parameter of GCM, was almost zero in both age groups of the controls, and that in sensitized and challenged mice of both age groups was significantly higher than in control animals even at 8 weeks. In young animals, however, the degree attenuated significantly at 4 and 8 weeks compared to that 1 week after exposure, although it did not change in mature animals (table 1).

Discussion

In the present study, we have demonstrated that the persistency of both airway inflammation and BHR were different between young and mature mice. One week after the aerosol challenge with OVA, airway inflammation and BHR occurred in both young and mature mice to a similar degree. However, at 4 weeks, BHR disappeared in young mice, whereas it persisted even at 8 weeks in mature animals. GCM, ICI and eosinophilia in BALF attenuated with time in both groups, being more remarkable in young than in mature animals.

In animal models of asthma, the time course of allergic airway inflammation after chronic exposure to antigen differs depending on the animal species or the strain even in the same species. In mice, Temelkovski et al. [14] sensitized BALB/c mice systemically with OVA and chronically challenged these animals with low particle mass concentrations of aerosolized OVA for up to 8 weeks. As a result, they showed that airway inflammation and BHR persisted during the study period. On the other hand, Shinagawa and Kojima [15] demonstrated that only A/J, but neither BALB/C, C57BL/6 nor C3H/Hej mice, developed airway inflammation and remodeling as well as BHR up to 12 weeks after exposure to antigen, when sensitizing animals by instillation via the nose. Yiamouyiannis et al. [16] recently reported that neither allergic airway inflammation nor BHR persisted in the airways of C57BL/6 after the exposure to antigen for 6 weeks, although these responses were seen in the period of acute challenge (10 days). Our present study confirmed the findings of Temelkovski et al. [14] that BHR persisted for 8 weeks after the aerosol challenge with antigen in mature BALB/C mice. However, airway inflammation gradually attenuated with time, although it persisted even at 8 weeks, but the reason for this difference remains to be elucidated.

Table 1. Mucus cell score at the different periods of exposure

Animals	Mucus cell score			
	1 week	2 weeks	4 weeks	8 weeks
Mature				
Control	0.00 ± 0.00	0.20 ± 0.20	0.25 ± 0.25	0.00 ± 0.00
OVA	3.00 ± 0.45*	2.17 ± 0.48*	2.10 ± 0.19*	2.00 ± 0.41*
Young				
Control	0.10 ± 0.10	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
OVA	3.20 ± 0.26*	2.92 ± 0.33*	2.14 ± 0.32*, **	1.00 ± 0.00*, **

OVA and control indicate animals given OVA and vehicle for sensitization and challenge, respectively. Values are means ± SEM. * $p < 0.05$ vs. control animals at corresponding age, ** $p < 0.01$ vs. animals exposed to OVA for 1 week (n = 4–9 for each group).

Previously, we have shown that the major area of airway remodeling after repeated exposure to antigen differed depending on the stage of maturation of guinea pigs in vivo [17]. In brief, the prominent areas of thickening caused by chronic exposure to antigen were different among juvenile, adult and old animals. Inner wall thickening was more remarkable in juvenile and adult animals than in old ones. By contrast, a thickened smooth muscle was only observed in old animals. In addition, it was suggested that increased renewal of epithelial cells contributed to the thickening of the inner wall in juvenile and adult animals, and that thickening of smooth muscle areas in old animals was due to factors such as hypertrophy. In the present study, BHR and airway inflammation subsided earlier in young than in mature mice, suggesting that the time course of allergic airway inflammation and BHR is also affected by the stage of maturation of animals.

The mechanism underlying the age-related differences in the persistency of airway inflammation and BHR observed in this study is particularly of concern. Recently, Schramm et al. [18] have reported that continuous inhalational exposure (6 weeks) to OVA resulted in attenuation of airway eosinophilia and BHR without reduction in OVA-specific IgE levels. The finding of our present study that elevated IgE levels persisted in the absence of allergic airway inflammation was similar to those of Schramm et al. [18]. Thus our blunted local airway responses were attributed to local mechanisms rather than systemic anergy.

Th2 cytokines, e.g. IL-4, IL-5 and IL-13, are responsible for the development of various features of allergic airway inflammation, including increased production of antigen-specific IgE, infiltration of eosinophils into the airways and GCM. In the present study, we have employed a relatively low dose of OVA in order to produce Th2-biased allergic airway inflammation [7, 19]. As a result, both allergic airway inflammation and BHR occurred in sensitized mice in both age groups. In BALF, IL-4 was increased until 2 weeks after sensitization and aerosol challenge, confirming that a low dose of antigen induced Th2-biased airway inflammation in both young and mature mice [7]. However, the time course of cytokine levels in BALF was slightly different between these groups, i.e. IL-2 was significantly increased at 2 weeks in young mice but not in mature animals. The cause and the role of the increased IL-2 level in the development of allergic inflammation and BHR in the young airways was not clear in the present study. IL-2 is necessary for any clonal expansion of T cells during immune responses irrespective of the Th type (Th1 or Th2) [20]. Thus, it might be possible that an increased level of local IL-2 modified the development of allergic inflammation and BHR by altering immune responses. In addition, Th1 cytokines, including TNF-α and IFN-γ, increased in young but not in mature mice around 2 weeks after challenge, although these changes did not reach statistical significance. The shift toward a Th1 response may partly contribute to the earlier disappearance of allergic airway inflammation in young animals. Further investigations assessing antibody production (IgG2a and IgG1) and subsets of T lymphocytes are required to elucidate the underlying mechanisms of these age-related differences.

In the present study, except for IL-2 and IL-4, cytokines did not significantly increase in the BALF. Of note, IL-5, a major survival and maturation factor for eosinophils, was not detected, although remarkable eosinophilia was observed in the BALF. In the present study, we collected BALF samples 24 h after the last challenge, as was report-

ed by Schramm et al. [18]. By contrast, Ohkawara et al. [21] examined a detailed time course of cytokine levels in BALF. They found that the inflammatory events were observed 3–24 h after antigen challenge. Thus, other cytokines might have been detected in earlier BALF samples.

The specific IgE antibody level in the peripheral blood was more prominent in mature than in young mice, whereas IL-4 responses in the BALF were similar or greater in juvenile animals, confirming our previous observation [7]. The results may indicate a functional immaturity of B lymphocytes or other cells that are responsible for the immune system in juvenile animals, although details remain to be elucidated.

In the present study, there was a tendency to an earlier attenuation in both BHR and airway inflammation in young compared to mature mice. The age-related differences in the persistency of airway responses may be partly responsible for the different outcome between patients with adult and childhood onset of asthma. Local cytokine levels may contribute to this difference.

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Differential Regulation of Eotaxin Expression by Dexamethasone in Normal Human Lung Fibroblasts

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Lung fibroblasts are a major source of several cytokines including CC chemokine eotaxin. We aimed to study the regulation of eotaxin-1/CCL11 production by dexamethasone and analyze its molecular mechanisms in human lung fibroblasts. Normal human lung fibroblast cells were exposed to IL-4 (40 ng/ml) and/or dexamethasone (10^{-6}–10^{-9} M), and eotaxin mRNA expression and production was evaluated. Mechanisms of transcriptional regulation were assessed by Western blotting and dual luciferase assay for eotaxin promoter. The effects of dexamethasone on suppressor of cytokine signaling (SOCS)-1 and eotaxin mRNA expression in the cells transfected with expression vector (pAcGFP1-C1) or short interfering RNA (siRNA) for SOCS-1 were also investigated. Within 24 hours, dexamethasone inhibited IL-4–induced eotaxin mRNA expression and protein production, while eotaxin production was markedly increased at 48 and 72 hours after coincubation with IL-4 and dexamethasone. IL-4–induced eotaxin promoter activity was inhibited by dexamethasone at 8 hours, but enhanced at 48 hours after coincubation. Dexamethasone suppressed SOCS-1 mRNA expression but enhanced IL-4–induced STAT6 phosphorylation at 36 to 48 hours after coincubation. Enhanced expression of eotaxin mRNA by dexamethasone 48 hours after coincubation was completely diminished in the cells transfected with either expression vector or siRNA for SOCS-1. These results indicated that dexamethasone, depending on the exposure duration, can either inhibit or enhance IL-4–induced expression and production of eotaxin in the lung fibroblasts. The mechanisms of later enhanced production may depend on the prolonged transcriptional activity of the eotaxin gene, in part due to inhibition of SOCS-1 expression.

Keywords: fibroblast; corticosteroid; eotaxin/CCL11; SOCS; airway remodeling

CLINICAL RELEVANCE

Our findings showing a lesser antiinflammatory effect of glucocorticoids in fibroblasts may be relevant to the relatively-insensitive-to-steroid therapy for difficult-to-treat asthma with increased progression of airway remodeling.

Asthma is a chronic inflammatory disorder of the airways in which many cells, especially eosinophils, may play important roles through the release of various mediators (1, 2). Chronic inflammation may be associated with bronchial hyperresponsiveness, variable airflow limitation, and respiratory symptoms. A prominent pathophysiologic feature of asthma is airway remodeling, along with airway inflammation. A link between airway inflammation and airway remodeling in asthma has recently been proposed (1–3).

In the airways of subjects with asthma, there is usually extensive infiltration of the airway lumen and wall with eosinophils and lymphocytes accompanied by vasodilatation, microvascular leakage, and epithelial disruption (1, 2). Eosinophil recruitment at the airway tissue is a complex mechanism. Chemokines involved in the migration and activation of blood eosinophils such as eotaxin may be produced by several types of cells, including airway fibroblasts, that have the potential to synthesize and release a variety of proinflammatory and profibrotic cytokines (4–7).

Eotaxin/CCL11, a CC chemokine with potent direct chemoattractant effects on eosinophils, is known to be regulated by Th2 cytokines, such as IL-4 and IL-13 (6, 8, 9). Eotaxin also regulates migration of mast cell progenitors into inflamed tissue and mast cell activation, and is likely to play an indirect role in airway remodeling through recruitment of eosinophils and mast cells, which have profibrogenic activity (2, 3, 7). It has been recently demonstrated that eotaxin has a direct and selective profibrogenic effect on lung and bronchial fibroblasts, providing a novel mechanism whereby eotaxin could participate in airway remodeling in asthma (7).

Glucocorticoids are a first-line therapy to control airway inflammation and to improve both bronchial hyperresponsiveness and hyperreactivity in patients with asthma (2). There are, however, conflicting results; showing that in regard to fibroblast function, glucocorticoids may either reduce or increase fibroblast proliferation that may be related to airway remodeling (10). It is unclear whether glucocorticoids either reduce or increase eotaxin production in lung fibroblasts, although they repressed the expression of eotaxin protein and mRNA induced by TNF-α and IL-4 in airway epithelial cells (11, 12). In the present study, we investigate the regulation of eotaxin expression by dexamethasone and analyze its molecular mechanisms in human lung fibroblasts.

MATERIALS AND METHODS

Cell Culture and Stimulation of the Cells

Normal Human Lung Fibroblasts (NHLF) (Clonetics, San Diego, CA) were cultured at 37°C with 5% CO_2 in fibroblast cell basal medium (Clonetics) supplemented with fibroblast growth medium-2 (FGM-2 Single Quots; Clonetics), 1.0 μg/L human Fibroblast Growth Factor-Basic (rhFGF-B), 5.0 mg/l insulin, 2% fetal bovine serum (FBS), 30 mg/ml gentamicin, and 15 μg/ml amphotericin. NHLF cells were seeded into 12-well plates for enzyme-linked immunosorbent assay (ELISA) and luciferase assay, and 6-cm dishes for Western blot and mRNA analysis.

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TABLE 1. PRIMERS USED FOR QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS OF GENE EXPRESSION

Target mRNA	Forward Primer (5′ to 3′)	Reverse Primer (3′ to 5′)
Eotaxin	CCA ACC ACC TGC TGC TTT AAC CTG	GCT TTG GAG TTG GAG ATT TTT GG
β-actin	GTG GGG CGC CCC AGG CAC CA	CTC CTT AAT GTC ACG CAC GAT TTC
IL-4Rα	ACA CCA ATG TCT CCG ACA CTC	GGA TGA CAA TGC AGG AAA CGC
SOCS-1	GGA ACT GCT TTT TCG CCC TTA	AGC AGC TCG AAG AGG CAG TC
SOCS-3	GTC CCC CCA GAA GAG CCT ATT A	TTG ACG GTC TTC CGA CAG AGA T

Definition of abbreviations: IL-4Rα, interleukin 4 receptor α chain; mRNA, messenger RNA; SOCS, suppressor of cytokine signaling.

Cells were allowed to grow to 70% confluence. In one type of experiment, cells were exposed to IL-4 (40 ng/ml) (R&D Systems, Minneapolis, MN) or dexamethasone (DEX, 10^{-6}–10^{-9} M) (Sigma-Aldrich Co., St. Louis, MO) alone, or a combination of both. In other experiments, cells were treated with tumor necrosis factor (TNF)-α (40 ng/ml) (R&D Systems) and DEX.

Assay of Eotaxin Protein Release into the Culture Medium

Concentrations of eotaxin in the collected culture medium were determined with a commercial system for ELISA (R&D Systems) in accord with the manufacturer's instructions. The limit of detection in the assay of eotaxin was 5 pg/ml.

Real-Time Quantitative PCR Analysis

Expressions of eotaxin, IL-4Rα, suppressor of cytokine signaling (SOCS)-1, and SOCS-3 mRNA in fibroblasts were determined by reverse transcription (RT), followed by real-time quantitative PCR. Total RNA was extracted from cells after incubation with or without indicated cytokines using Isogen reagent (Nippon Gene, Tokyo, Japan). Reverse transcription was performed using 1 μg of total

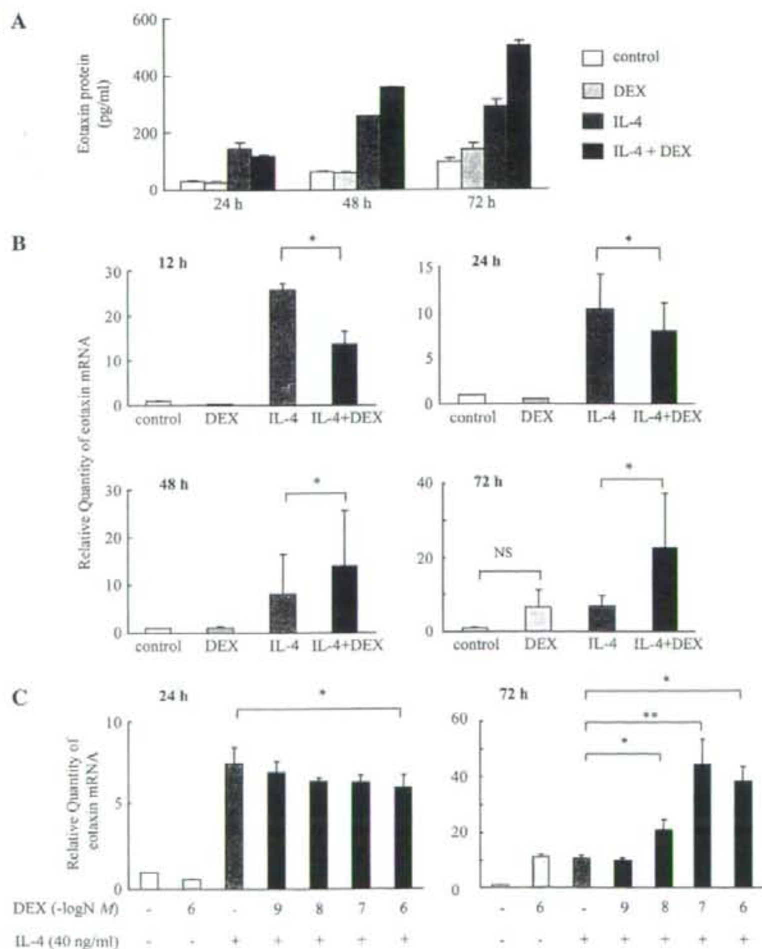

Figure 1. Effect of dexamethasone (DEX) and IL-4 on the production of eotaxin protein and mRNA in normal human lung fibroblast (NHLF) cells. (*A*) Cells were incubated with or without IL-4 (40 ng/ml) and/or DEX (10^{-6} M) for 24, 48, and 72 hours, and the concentration of eotaxin protein in the medium analyzed by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD of two independent experiments. (*B*) Quantitative real-time PCR assessment of the fold changes in eotaxin mRNA at 12, 24, 48, and 72 hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). Data are presented as the mean ± SD of four to six independent experiments (*$P < 0.05$). (*C*) Concentration-dependent effect of dexamethasone on expression of eotaxin mRNA. Cells were coincubated with IL-4 (40 ng/ml) and DEX (10^{-6}–10^{-9} M) for 24 and 72 hours. Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). Data are presented as the mean ± SD of four independent experiments (*$P < 0.05$, **$P < 0.01$).

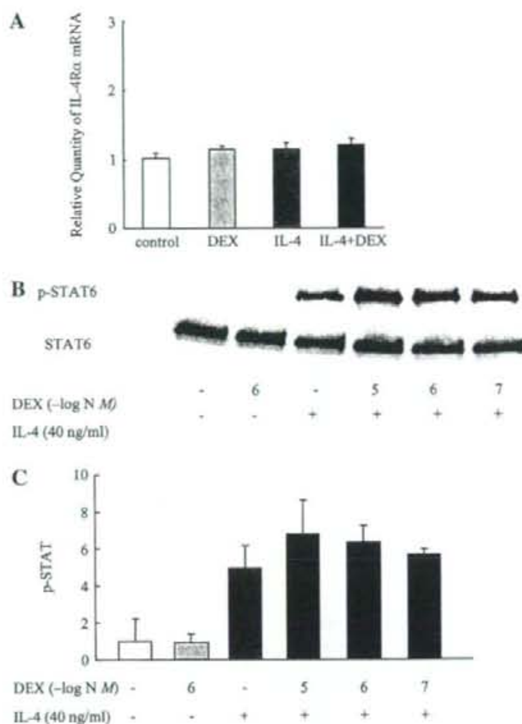

Figure 2. IL-4Rα mRNA analysis and representative Western blots. (*A*) Forty-eight hours after stimulation with or without IL-4 (40 ng/ml) and DEX (10^{-6} M), IL-4Rα mRNA expression was determined by quantitative real-time PCR. Data are presented as the mean ± SD of four independent experiments. Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). (*B*) Representative STAT6 and p-STAT6 Western blots from a single culture stimulated with medium alone, DEX (10^{-5}–10^{-7} M), IL-4 (40 ng/ml), or the combination for 36 hours. In the experiments, membranes were exposed to the film for 30 seconds. The results presented are from one of three experiments that produced similar results. (*C*) Phospho-STAT6 was expressed as a fold increase in relative intensity. Data are shown as the mean values ± SD of three independent experiments.

RNA and oligo (dT) primers in a 20-μl reaction in accord with the manufacturer's protocol (Applied Biosystems, Branchburg, NJ). The sequences of the specific primer sets that were used in the real-time PCR analysis are displayed in Table 1, as previously described (13–15).

Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA) using SYBR green (Applied Biosystems, Warrington, UK) as a dsDNA-specific binding dye. Reactions for eotaxin and β-actin were cycled 40 times after the initial denaturation of 95°C, 10 minutes followed by 95°C, 15 seconds. The annealing and extension temperature was 60°C, 1 minute, except for IL-4Rα, where it was 58°C, 1 minute. The threshold cycle (CT) was recorded for each sample to reflect mRNA expression levels. A validation experiment proved the linear dependence of the CT value for both eotaxin and β-actin concentrations and the consistency of ΔCT (eotaxin average CT minus β-actin average CT) in a given sample at different RNA concentrations. ΔCT was therefore used to reflect relative eotaxin mRNA expression levels. To determine the effects of different stimuli on eotaxin gene expression as compared with nonstimulated cells, ΔΔCT was calculated (ΔΔCT = ΔCT stimulus − ΔCT nonstimulated cells). Eotaxin mRNA was indexed to β-actin using the formula $1/(2^{\Delta CT}) \times 100\%$. $2^{\Delta\Delta CT}$ was calculated to demonstrate the fold change of eotaxin gene expression in stimulated cells as compared with nonstimulated ones.

Expressions of IL-4Rα, SOCS-1, and SOCS-3 mRNA in fibroblasts were determined in the same manner as eotaxin mRNA expression.

Western Blot Analysis

Cells stimulated with IL-4 were solubilized with NP-40 lysis buffer (0.5% NP-40, 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 3 mM *p*-amidinophenylmethanesulfonyl fluoride [Sigma, St. Louis, MO], 5 mg/ml aprotinin [Sigma], 2mM sodium orthovanadate [Sigma], 5 mM EDTA). Whole cell extracts were subjected to 7.5 to 12% Tris-glycine gel electrophoresis (XV Pantera Gel; DRC, Tokyo, Japan) and then transferred to Sequi-Blot polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked for 30 min with 5% skimmed milk in TBS-T (Tris-buffered saline with 0.05% Tween 20) before incubation with either rabbit anti-human STAT6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-human phospho-STAT6 (Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. Membranes were then washed by TBS-T and incubated with anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (Amersham, Buckinghamshire, UK) for 30 minutes. Enhanced chemiluminescence (ECL plus Western blot detection system; Amersham) substrate was added after further washing with TBS-T, and the membrane was then exposed to film.

Transient Transfection and Luciferase Assay

Eotaxin promoter-luciferase reporter plasmid, generously supplied by Prof. R. P. Schleimer (Division of Allergy-Immunology, Feinberg

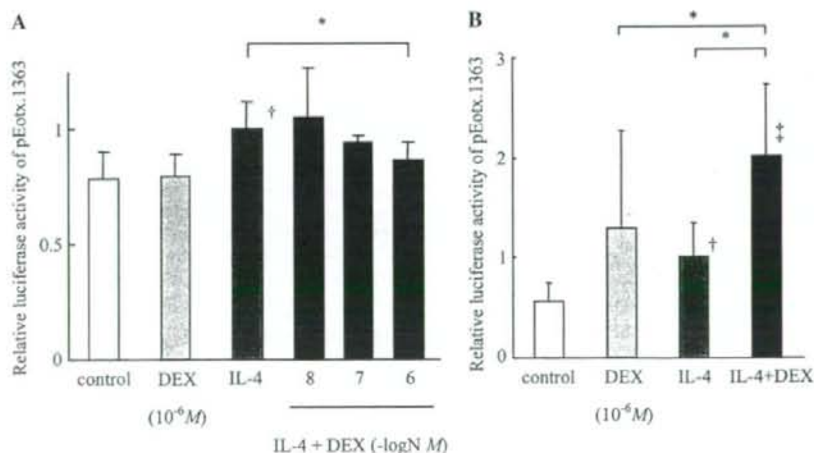

Figure 3. Activation of an eotaxin promoter-luciferase reporter plasmid (pEotax.1363) by IL-4 and/or DEX in NHLF cells. IL-4 (40 ng/ml) and/or DEX (10^{-6}–10^{-8} M) was added to the media, and after 8 hours (*A*) luciferase assay was performed. Forty-eight hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6} M), luciferase assay was done (*B*). Data are presented as the mean ± SD of a total of four (*A*) and six (*B*) independent experiments (*$P < 0.05$). †$P < 0.05$ and ‡$P < 0.01$ are compared with the value of control.

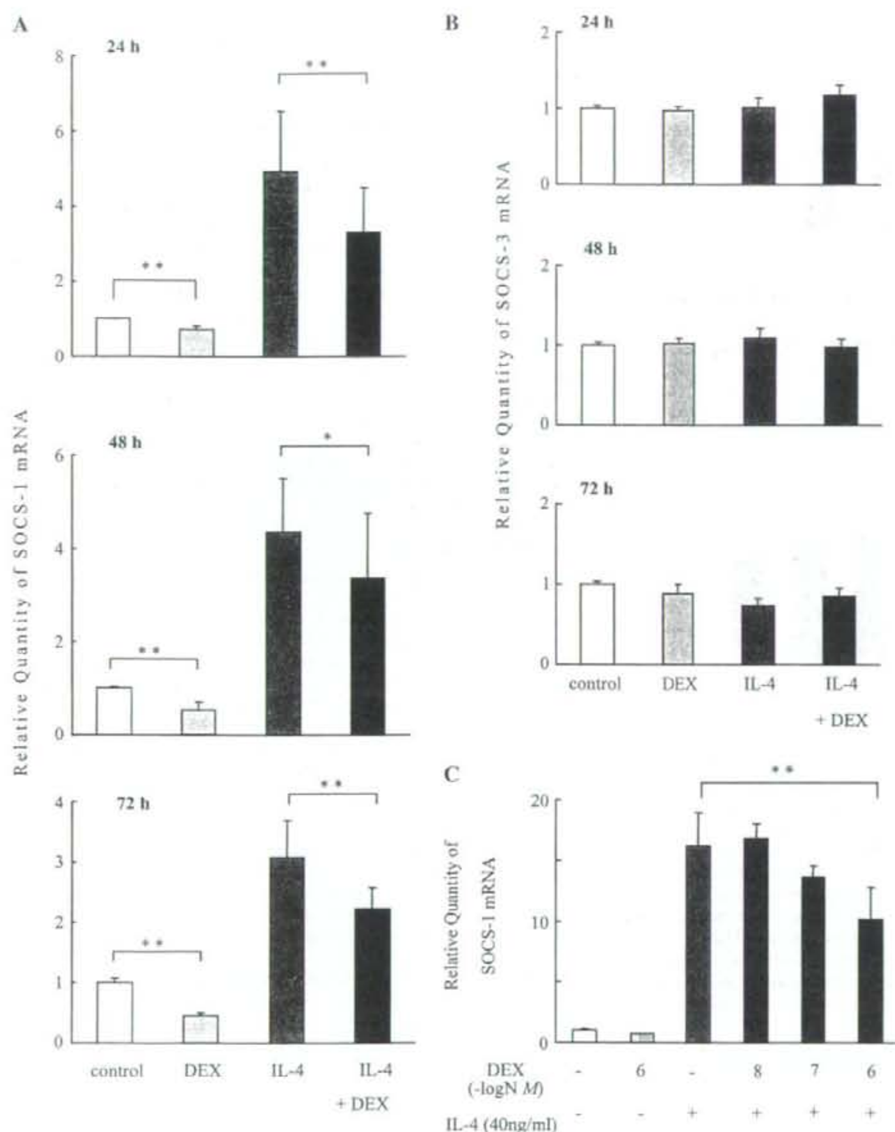

Figure 4. SOCS-1 and SOCS-3 mRNA analysis. (*A*) Quantitative real-time PCR assessment of the fold changes in SOCS-1 mRNA at 24, 48, and 72 hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). Data are presented as the mean ± SD of six independent experiments (*$P < 0.05$, **$P < 0.01$). (*B*) Quantitative real-time PCR assessment of the fold changes in SOCS-3 mRNA at 24, 48, and 72 hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). Data are presented as the mean ± SD of six independent experiments. (*C*) Concentration-dependent effects of DEX on the expression of SOCS-1 mRNA. Quantitative real-time PCR assessment of the fold changes in SOCS-1 mRNA 48 hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6}–10^{-8} M). Results are expressed as the relative quantity of SOCS-1 mRNA (= fold over control). Data are presented as the mean ± SD of four independent experiments (**$P < 0.01$).

School of Medicine, Northwestern University, Chicago, IL), is a 1,363-bp fragment of the promoter region of the eotaxin gene (site 1,363 to 1) (16). NHLF cells were seeded into 12-well plates and allowed to grow to 50 to 70% confluence. Cells were transfected with 0.75 μg of reporter plasmids and 10 ng of a control Renilla luciferase vector pRL-TK (Promega Corporation, Madison, WI) using 1.5 μl of Fugene 6 transfection reagent (Roche Diagnostics Co., Indianapolis, IN) and incubated for 12 hour in 1 ml medium. Eight or 48 hours after coincubation with or without dexamethasone (10^{-6}–10^{-8} M) and IL-4 (40 ng/ml), cells were washed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), solubilized by incubation in 250 μl of lysis buffer for 20 minutes, transferred to microtubes, and then centrifuged to pellet cellular debris. The supernatants were measured for luciferase activity using a Dual-Luciferase Assay System (Promega Corporation). The firefly luciferase activity of the eotaxin promoter-reporter plasmid was normalized using Renilla luciferase activity.

Eotaxin and IL-4Rα mRNA Stability

NHLF cells were treated for 36 hours with IL-4 (40 ng/ml) and DEX (10^{-6} M). Cells were subsequently harvested at time 0 (as control) or further treated with actinomycin D (ACD, 1 μg/ml) (Sigma) for each specified time to block further transcription of mRNA. Eotaxin mRNA expression was analyzed 12, 24, and 36 hours after ACD was added, and IL-4Rα mRNA expression at 2, 4, 8, and 12 hours after ACD was added, as mentioned above.

Cloning of SOCS-1 Expression Vector and Transfection into NHLF Cells

A DNA fragment of the coding sequence of SOCS-1 was amplified by PCR using cDNA from IL-4–treated NHLF cells. The purified PCR product was digested with *Bgl*II and *Eco*RI and cloned into the pAcGFP1-C1 vector (Clontech Laboratories Inc., Shiga, Japan). The

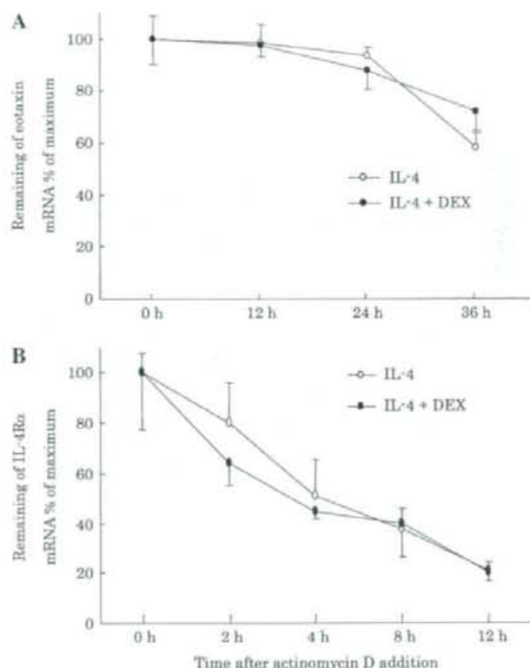

Figure 5. Eotaxin and IL-4Rα mRNA stability. After induction of eotaxin mRNA (*A*) and IL-4Rα mRNA (*B*) by 36 hours of incubation with 40 ng/ml IL-4 and/or DEX (10^{-6} M), cells were treated with actinomycin D (1 μg/ml). After treatment with actinomycin D, eotaxin or IL-4Rα mRNA expression was analyzed at the indicated time intervals by real-time PCR. Results are expressed as 100% of maximum (eotaxin or IL-4Rα mRNA expression at time 0). Data are presented as the mean ± SD of four to six independent experiments.

plasmid was analyzed by digestion with restriction enzymes and DNA sequencing. Plasmids for transfection were purified with HiSpeed Plasmid Maxi Kit (QIAGEN Sciences, Germantown, MD).

NHLF cells were seeded into 6-well plates and allowed to grow to 50% confluence. Cells were transfected with 4 μg of expression vector with 10 μl Lipofectamine 2000 (Promega) and grown in fibroblast cell basal medium containing FGM-2 Single Quots without antibiotics. After 24 hours, the medium of the cells was changed to fibroblast cell basal medium with antibiotics; 30 mg/ml gentamicin, and 15 μg/ml amphotericin. Then, cells were exposed to IL-4 (40 ng/ml) or DEX (10^{-6} M) alone, or a combination of both. Forty-eight hours after coincubation, eotaxin or SOCS-1 mRNA expression was evaluated.

Knockdown of Gene Expression with short interfering RNA

Pre-designed short interfering RNA (siRNA) for SOCS-1 (catalog #45060) was purchased from Ambion (Tokyo, Japan). NHLF cells were seeded into 6-well plates and allowed to grow to 50% confluence. Cells were transfected with 16.5 nM of siRNA with 5 μl Lipofectamine 2000. Then, the same procedures described for the knockdown of gene expression were performed. Scrambled siRNA was used as non-specific negative control of siRNA (Ambion).

Statistical Analysis

Data are expressed as means ± SD. Statistical differences were determined by ANOVA first before confirming significance with a paired Student's *t* test. Data were analyzed with Dr. SPSS II (SPSS Japan Inc., Tokyo, Japan). *P* values less than 0.05 were considered statistically significant.

RESULTS

Eotaxin Protein Production and mRNA Expression

The minimum level of eotaxin protein was detected in the medium of unstimulated NHLF (Figure 1A). Coincubation with DEX (10^{-6} M) alone until 48 hours did not interfere with eotaxin production or mRNA expression in NHLF. DEX incubation for 72 hours slightly increased eotaxin mRNA expression and protein, although it did not reach significance for eotaxin mRNA expression ($P = 0.056$) (Figures 1A and 1B). At all of the time points monitored, stimulation with IL-4 (40 ng/ml) alone increased eotaxin production and mRNA expression. The combination of IL-4 (40 ng/ml) and DEX (10^{-6} M) partially inhibited eotaxin production and mRNA expression at 12 and 24 hours after stimulation, while at 48 and 72 hours it increased eotaxin production and mRNA expression compared with incubation with IL-4 alone. Coincubation of IL-4 (40 ng/ml) and DEX (10^{-6}–10^{-9} M) for 24 hours slightly, but concentration-dependently, suppressed the expression of eotaxin mRNA. Coincubation of IL-4 and DEX for 72 hours markedly increased eotaxin mRNA in a concentration-dependent manner compared with IL-4 alone (Figure 1C).

Effect of DEX on IL-4Rα mRNA Expression

We next investigated IL-4Rα mRNA expression stimulated with IL-4 (40 ng/ml) and DEX (10^{-6} M) in NHLF. IL-4Rα mRNA expression was not enhanced after 48 hours of coincubation with IL-4 and DEX (Figure 2A).

DEX Enhanced IL-4-Induced STAT6 Activation

STAT6 and phospho-STAT6 levels were investigated by Western blot analysis using specific monoclonal antibodies for them. The cell lysates 36 hours after stimulation with IL-4 contained substantial amounts of phospho-STAT6, as shown by the appearance of an intense band detected at 105 kD (Figure 2B, *lane 3*) that was not present in the cell lysates from unstimulated and DEX-treated cells (*lanes 1* and *2*). When cultured with IL-4, phospho-STAT6 generation was enhanced by DEX in a concentration-dependent manner (Figure 2C, *lanes 4–6*).

Transient Transfection and Luciferase Assay

After 8 hours, IL-4 alone enhanced induction of the eotaxin promoter, pEotx.1363 (Figure 3A). Coincubation with DEX and IL-4 inhibited induction of the eotaxin promoter, and this effect was significant and concentration-dependent. By contrast, after 48 hours, coincubation with DEX and IL-4 significantly enhanced the activities of the eotaxin promoter, pEotx.1363, compared with either stimulus alone (Figure 3B).

Effect of DEX on IL-4-Induced SOCS-1 and SOCS-3 mRNA Expression

At all of the time points monitored, the expression of SOCS-1 mRNA was significantly inhibited by DEX (10^{-6} M) alone. Stimulation with IL-4 (40 ng/ml) alone increased SOCS-1 mRNA expression. The combination of IL-4 and DEX significantly inhibited SOCS-1 mRNA expression compared with stimulation with IL-4 alone at all time points (Figure 4A). By contrast, IL-4 (40 ng/ml) alone did not increase SOCS-3 mRNA expression, and DEX (10^{-6} M) had no effect on IL-4–induced SOCS-3 mRNA expression at any of the time points (Figure 4B). Coincubation with IL-4 (40 ng/ml) and DEX (10^{-6}–10^{-8} M) for 48 hours concentration-dependently suppressed the expression of SOCS-1 mRNA, with significant suppression found at a concentration of 10^{-6} M of DEX (Figure 4C).

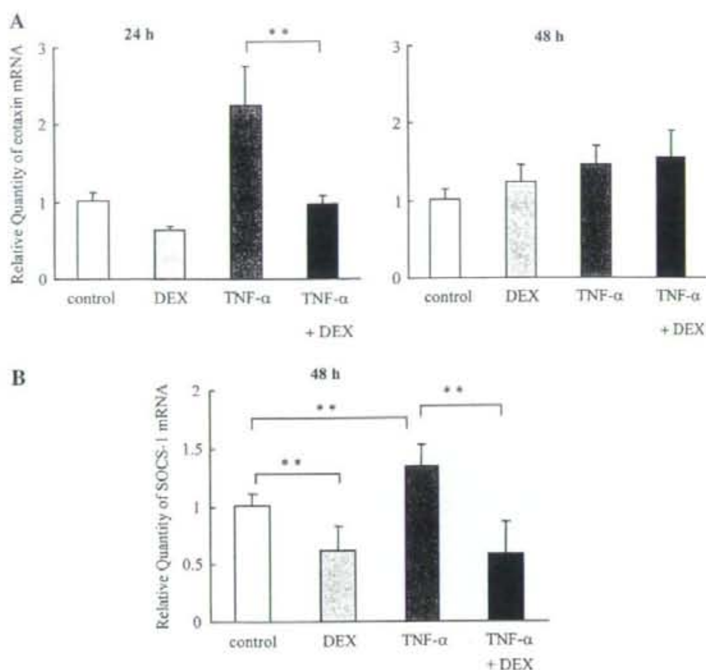

Figure 6. (*A*) Effect of DEX on TNF-α–induced eotaxin mRNA expression. Real-time PCR assessment of the fold changes in eotaxin mRNA at 24 and 48 hours after coincubation with TNF-α (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity of eotaxin mRNA (= fold over control). Data are presented as the mean ± SD of four independent experiments (**$P < 0.01$). (*B*) Effect of DEX on suppressor of cytokine signaling (SOCS)-1 mRNA expression. Real-time PCR assessment of the fold changes in SOCS-1 mRNA 48 hours after coincubation with TNF-α (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed in relative quantity of SOCS-1 mRNA (= fold over control). Data are presented as the mean ± SD of eight independent experiments (**$P < 0.01$).

Effects of DEX on IL-4-Induced Eotaxin and IL-4Rα mRNA Stability

There was no difference in the stabilities of eotaxin mRNA and IL-4Rα mRNA expressions between coincubation with IL-4 alone and combination with IL-4 and DEX (Figures 5A and 5B).

TNF-α–Induced Eotaxin and SOCS-1 mRNA

TNF-α (40 ng/ml) increased the expression of eotaxin mRNA at 24 hours after stimulation, while the combination of TNF-α (40 ng/ml) and DEX (10^{-6} M) significantly inhibited eotaxin mRNA. Unlike IL-4, an enhancing effect was not observed at 48 hours with the combination of TNF-α and DEX (Figure 6A). The expression of SOCS-1 mRNA was significantly inhibited by DEX alone and the combination of TNF-α and DEX at 48 hours after stimulation, as was the case with IL-4 (Figure 6B).

Effect of Expression Vector and siRNA for SOCS-1

Levels of SOCS-1 mRNA were significantly enhanced in the cells transfected with SOCS-1 expression plasmids cloned into pAcGFP1-C1 vector compared with wild-type cells, while reduced in the cells with siRNA for SOCS-1 (Figures 7A and 7C). Enhanced expression of eotaxin mRNA by dexamethasone (DEX, 10^{-6} M) at 48 hours after coincubation with IL-4 (40 ng/ml) in wild-type cells was completely diminished in the cells transfected with either expression vector or siRNA for SOCS-1 (Figures 7B and 7D).

DISCUSSION

A variety of cell types are responsible for eotaxin production, including macrophages, T lymphocytes, bronchial epithelial cells, and endothelial cells (1, 2, 6, 16). We found that eotaxin was produced by stimulation with IL-4 in normal human lung fibroblast (NHLF) cells, indicating that Th2 cytokines may regulate eotaxin production in lung fibroblasts. We also demonstrated here that glucocorticoid inhibited the eotaxin production induced by IL-4 at 24 hours after coincubation, which is in agreement with previous reports (8, 11). Interestingly, coincubation with IL-4 and DEX significantly enhanced eotaxin production at 48 and 72 hours after stimulation. Thus, DEX exhibited dual effects on IL-4–induced eotaxin production in NHLF. Such dual effects were also observed in other glucocorticoids, including hydrocortisone and prednisolone (data not shown).

Glucocorticoids suppress inflammatory genes by many different molecular mechanisms. Matsukura and coworkers (11) demonstrated that fluticasone inhibited eotaxin expression in airway epithelial cells (BEAS-2B) in part through repression of eotaxin transcription, and that this mechanism may not depend on the direct inhibition of transcription factors, such as NF-κB or STAT6. Lilly and colleagues (12) reported that TNF-α and IL-1β induced the accumulation of eotaxin mRNA with a maximum at 4 hours in the pulmonary epithelial cell lines A549 and BEAS-2B. In addition, DEX diminished TNF-α– and IL-1β–induced increases in eotaxin mRNA in a concentration-dependent manner. In the present study, we found that DEX repressed eotaxin production and mRNA expression 24 hours after stimulation with DEX in NHLF, as was the case with epithelial cells. In addition, DEX inhibited activation of the eotaxin promoter-luciferase reporter plasmid pEotx.1363 induced by IL-4, indicating that the transcriptional regulation may be related to the repression of eotaxin gene expression by glucocorticoids in lung fibroblasts.

Studies were performed to investigate the mechanisms of the observed enhancement in IL-4–induced eotaxin production and mRNA expression at 48 and 72 hours after incubation with DEX. One possible mechanism of synergy between IL-4 and DEX might be the up-regulation of IL-4 receptors or the activation of STAT-6 by DEX. Yamamoto and coworkers found that enhanced IL-4–induced eotaxin-3 production by

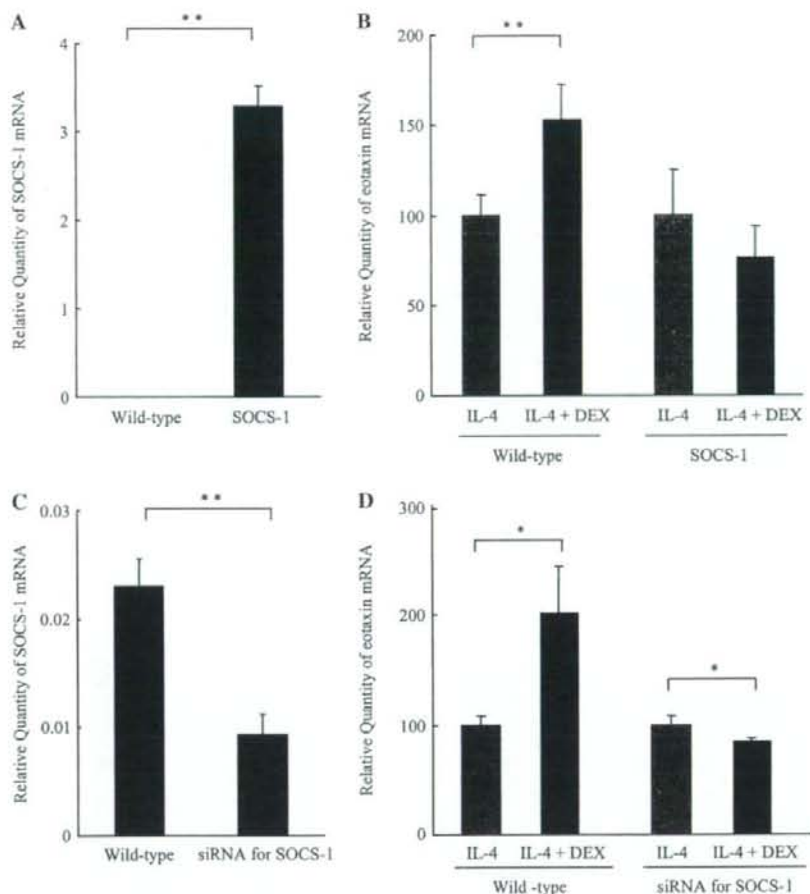

Figure 7. Effect of SOCS-1 expression plasmids cloned into pAcGFP1-C1 vector (*A* and *B*) and siRNA for SOCS-1 (*C* and *D*) on SOCS-1 mRNA and IL-4 (40 ng/ml) induced eotaxin mRNA enhanced by DEX (10^{-6} M) 48 hours after coincubation. Results are expressed in relative quantity of SOCS-1 mRNA (*A* and *C*). Eotaxin mRNA levels were expressed as 100% of value stimulated with IL-4 (*B* and *D*). Data are presented as the mean ± SD of four to six independent experiments (*$P < 0.05$, **$P < 0.01$). Levels of SOCS-1 mRNA were enhanced significantly in the cells transfected with SOCS-1 expression plasmids cloned into pAcGFP1-C1 vector (*A*), while reduced in the cells with siRNA for SOCS-1 (*C*). Enhanced expression of eotaxin mRNA by dexamethasone at 48 hours after coincubation in each wild-type cells was completely diminished in the cells transfected with either expression vector (SOCS-1) (*B*) or siRNA for SOCS-1 (*D*).

IFN-γ may be due to up-regulation of IL-4Rα in airway epithelium (14). In the present study, we could not find mRNA expression of IL-4Rα enhanced by coincubation of IL-4 and DEX. The effect of DEX on STAT6 phosphorylation by IL-4 was examined by Western blotting for the latent and activated forms of the STAT6 protein at 36 hours after coincubation with DEX and IL-4. IL-4–induced phosphorylation of STAT6 and p-STAT6 generation was enhanced by DEX in a concentration-dependent manner.

In the present study, eotaxin promoter activity was enhanced 48 hours after coincubation with DEX and IL-4, whereas it was inhibited at 8 hours, suggesting that the transcription of eotaxin gene exhibits dual effects similar to the response of eotaxin production and mRNA expression. DEX alone induced an increase in eotaxin promoter activity at 48 hours after stimulation, although it did not reach significance. This may explain the slight increase in eotaxin protein production and mRNA expression at 72 hours after stimulation with DEX alone. Thus, it implies that transcriptional regulation is involved in the enhanced expression of eotaxin by DEX, which was further confirmed by the effect of actinomycin D.

The suppressors of cytokine signaling (SOCS) represent a recently discovered family of proteins engaged in the negative regulation of cytokine signaling, primarily signaling associated with the Jak-STAT pathway (17). Sato and colleagues provided evidence that SOCS-1 can negatively regulate IL-4– and IL-13–induced eotaxin-1 expression (18). These findings are in agreement with a recent study demonstrating the regulation of eotaxin-3 by SOCS-1 and SOCS-3 but not SOCS-2 (17). To test whether SOCS proteins play an important role in the regulation of eotaxin expressions by DEX, we analyzed SOCS-1 and SOCS-3 mRNA expressions. We detected the induction of mRNA for SOCS-1, but not SOCS-3, from 24 to 72 hours of treatment of NHLF cells with IL-4. We found that SOCS-1, which serves to down-regulate cytokine signaling, was suppressed by DEX alone and in combination with IL-4 stimulation, suggesting that up-regulation of eotaxin was likely to be due to down-regulation of SOCS-1 by DEX. To confirm that the effects seen with increasing eotaxin are in fact due to changes in SOCS-1, we have performed some studies using overexpressing SOCS-1 or siRNA for SOCS-1 in NHLF cells then treating with DEX. We found that reversal or diminished SOCS-1 levels prevented up-regulation of eotaxin in cells transfected with expression vector or siRNA for SOCS-1 after DEX treatment. Our results may be partly supported by the findings of Paul and coworkers showing that glucocorticoids strongly inhibit both basal and IL-6–induced rat SOCS-3 mRNA synthesis in hepatocytes (19). They also found the negative regulation of SOCS-3 promoter by glucocorticoids caused by a glucocorticoid response element–independent pathway. Thus, taken together with these previous studies, our results suggest that SOCS-1 suppression by DEX may possibly

be because of the negative regulation of SOCS-1 promoter activity. Furthermore, SOCS-1 suppression could enhance the level of phospho-STAT6 and up-regulate transcription, subsequently enhancing eotaxin production in response to IL-4.

To confirm that the enhancing effect of DEX was not caused by a generalized enhancement of cellular responses, we analyzed the effect of DEX on TNF-α–induced eotaxin production in NHLF cells. TNF-α induced a small but significant increase in eotaxin mRNA expression, and this response was not enhanced 48 hours after coincubation with TNF-α and DEX. Although DEX also suppressed SOCS-1 mRNA expression in this study, it could not induce the enhancing of eotaxin mRNA expression. Thus, taken together with the IL-4 stimulation study, suppression of SOCS-1 by DEX may have an important role for the enhancement of IL-4–induced eotaxin production.

Atasoy and colleagues, using an actinomycin D–based assessment, demonstrated that TNF-α and IL-4 significantly increase eotaxin mRNA stability (20). While in unstimulated cells eotaxin mRNA is short-lived (with a half-life of ~ 2 h), they showed treatment with either TNF-α or IL-4 induced up to a 3-fold extension of the eotaxin mRNA half-life. In the present study, DEX had no effect on IL-4–induced eotaxin mRNA expression. This suggested that eotaxin mRNA stability was unrelated; implying that post-transcriptional regulation such as eotaxin mRNA stability may be not involved in the enhanced expression of eotaxin by DEX.

Finally, since Kraft and coworkers (10) found that IL-4 and DEX significantly increased fibroblast proliferation in the biopsy specimens from subjects with mild asthma, we hypothesized that the numbers of NHLF coincubated with IL-4 and/or DEX increased compared with the numbers of untreated cells. However, we did not find any differences in the numbers of viable cells with or without DEX at 24 to 72 hours after coincubation (data not shown).

In conclusion, DEX induced dual effects on the expression and production of eotaxin in lung fibroblasts. The mechanisms of the later enhanced production may depend on the prolonged transcriptional activity of the eotaxin gene, in part due to inhibition of SOCS-1 expression. Our findings showing a lesser antiinflammatory effect of glucocorticoids in fibroblasts may be relevant to the relatively-insensitive-to-steroid therapy for difficult-to-treat asthma with increased progression of airway remodeling (21). Because glucocorticoids are recommended as a first-line therapy for asthma, further study regarding the molecular mechanisms of glucocorticoids and cytokine modulation of fibroblast function is required to determine whether the currently available therapies for asthma provide long-term benefits for patients.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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ORIGINAL ARTICLE

Age-Related Changes of Transforming Growth Factor *β*1 in Japanese Children

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ABSTRACT

Background: Transforming growth factor β1 (TGFβ1) is an important factor in immunomodulation. The expression of TGFβ1 has been shown to be influenced by the C-509T polymorphism in the TGFβ1 gene. We investigated age-related changes of plasma TGFβ1 levels in a birth-cohort study. In addition, the genotypes of the C-509T polymorphism were investigated in allergic and non-allergic subjects.

Methods: Sixty-four neonates who met the following criteria were enrolled in this cohort study: 1) full-term vaginally delivery; 2) underwent DNA polymorphism analysis; and 3) questionnaire forms were filled out by parents at 0, 6 and 14 months of age. The umbilical cord blood at 0 months and peripheral blood at 6, and 14 months were collected. Plasma TGFβ1 levels were measured at 0, 6 and 14 months of age. Genomic DNA was extracted from their umbilical cord blood. The genotype of the subjects was examined for the presence of C-509T.

Results: The plasma TGFβ1 level at 6 months was the highest of the 3 measurements (at 0, 6, and 14 months of age). The TGFβ1 levels at 14 months in allergic subjects were significantly higher than those in non-allergic subjects ($p = 0.03$). All subjects with bronchial asthma ($n = 3$) had the TT genotype of the C-509T polymorphism.

Conclusions: The plasma TGFβ1 levels change with age. In addition, TGFβ1 may play a role in the pathogenesis of bronchial asthma.

KEY WORDS

bronchial asthma, single nucleotide polymorphism (SNP), transforming growth factorβ1 (TGFβ1), umbilical cord blood

INTRODUCTION

Transforming growth factorβ1 (TGFβ1) is a 25 kDa disulfide-linked homodimeric multifunctional cytokine. TGFβ1 may be the most important growth factor related to immunomodulatory effects because knockout mice died of massive inflammatory lesions.[1] The role of TGFβ1 in allergic diseases have been reported.[2-4] The levels of TGFβ1 concentration were found to be higher in the bronchoalveolar lavage fluid of patients with asthma compared with subjects without asthma.[3] The expression of TGFβ1 was found to be influenced by polymorphisms in the TGFβ1 gene, some of which may be associated with bronchial asthma and other diseases.[4,5] In particular, the T allele of the C-509T polymorphism within the TGFβ1 gene was associated with elevated serum TGFβ1 levels and also associated with elevated levels of total IgE in allergic asthma patients.[5,6]

In this study, we investigated the age-related changes of TGFβ1 levels in a birth-cohort study. In addition, C-509T within the TGFβ1 gene in subjects with asthma was compared with non-allergic subjects.

METHODS

SUBJECTS

Sixty-four neonates born at Iwasa Maternity Hospital in Gifu Prefecture between November 2005 and July

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Table 1 Characteristics of subjects

Gender	Male	32	Female	32
Birth body weight (g)	Mean±SD	3137.1 ± 400.5 (range: 2410−4026g)		
Gestational week	Mean±SD	39w3d ± 1w2d (range: 37w6d−41w1d)		
Family history of allergy	Father	With allergy 25		
		Without allergy 32		
		No record 7		
	Mother	With allergy 34		
		Without allergy 24		
		No record 6		
Family smoking history with 1 or both parents still smoking	Present	16	Absent	48

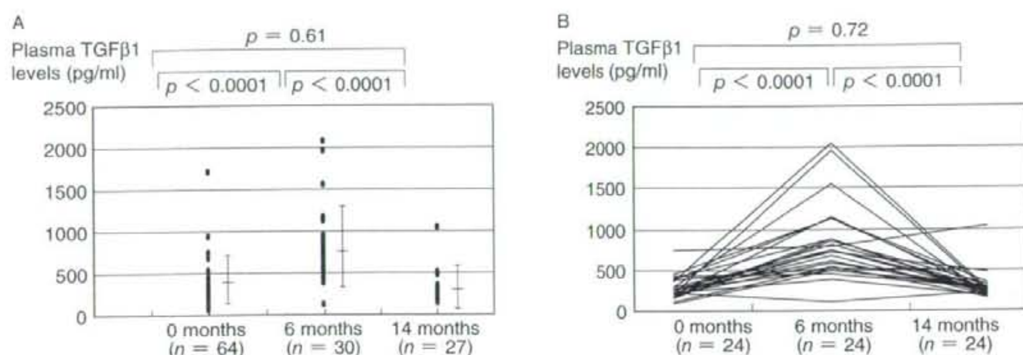

Fig. 1 (**A**) Plasma TGFβ1 levels at 0, 6, and 14 months of age. (**B**) Changes of plasma TGFβ1 levels in 24 subjects whose data were available at all three points.

2006, and satisfying the following criteria were enrolled in this cohort study: 1) full-term vaginally delivery; 2) underwent DNA polymorphism analysis; and 3) questionnaire forms were filled out by parents at 0, 6 and 14 months of age. Informed consent, including DNA analysis, was obtained from the parents of the neonates during their stay in the hospital. This cohort study was approved by the Ethical Committee of the Graduate School of Medicine of Gifu University. Umbilical cord blood samples were collected from 64 neonates and peripheral blood samples were also collected for analysis from 30 subjects at 6 months and from 27 subjects at 14 months of age. Subjects who were given a diagnosis of atopic dermatitis and bronchial asthma during the follow-up over a period of 14 months after birth, were classified as having those diseases. Allergic subjects were defined as those who were given a diagnosis of atopic dermatitis and/or bronchial asthma and non-allergic subjects were defined as those having neither atopic dermatitis nor bronchial asthma during the 14-month observation period. Some data of the recruited population are summarized in Table 1.

ASSAYS FOR PLASMA IgE LEVELS AND PLASMA TGF*β*1 LEVELS

Plasma samples obtained from heparinized blood were kept at -30℃. Plasma IgE levels were determined by chemiluminescent enzyme immunoassay and plasma TGFβ1 levels were measured with a human ELISA kit (R & D Systems, Mineapolis, MN, USA); the detection range was 31.2-2000 pg/ml.

DETECTION OF C-509T WITHIN THE TGF*β*1 GENE

Genomic DNA was extracted from the heparinized umbilical cord blood using a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The TGFβ1 gene was amplified and sequenced using the PCR technique and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The primers used for amplification of the DNA fragments were the forward primer at position -737- -718 (5'-CAGACTCTAGAGACTGTCAG-3') and the reverse primer at position -320- -338 (5'-GTCACCAGAGAAAGAGGAC-3').

STATISTICAL ANALYSES

The relations between age and plasma in TGFβ1 lev-

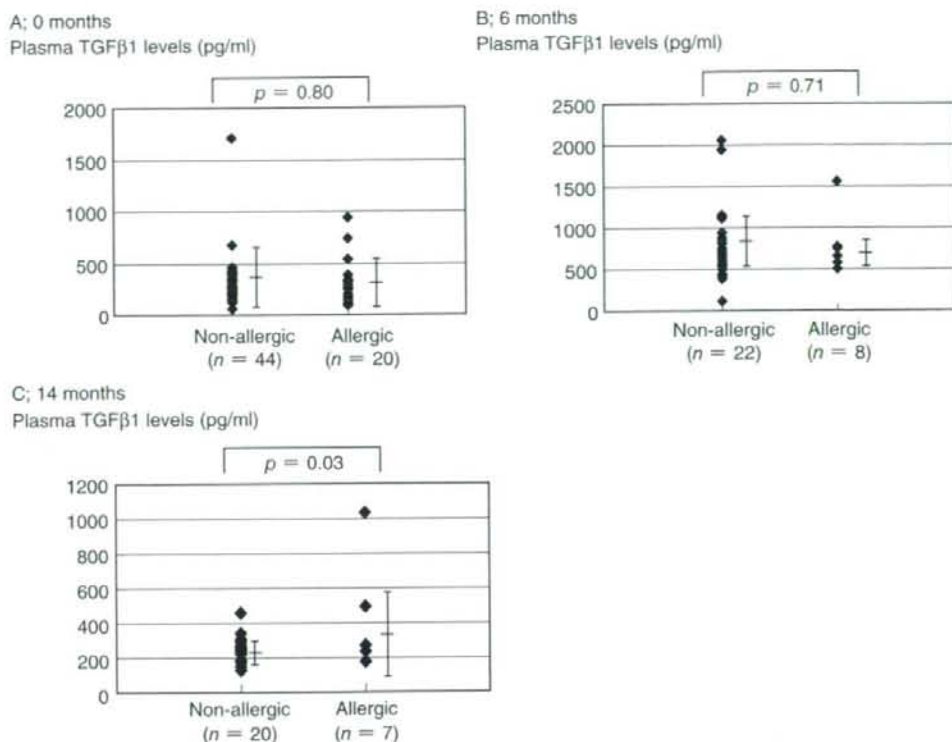

Fig. 2 Plasma TGFβ1 levels of non-allergic and allergic subjects at 0 months (**A**), 6 months (**B**) and 14 months (**C**) are shown. The plasma TGFβ1 levels at 14 months of age in allergic subjects were significantly higher than those in non-allergic subjects ($p = 0.03$, **C**).

els were determined by using the Mann-Whitney unpaired U-test. Pearson's correlation coefficient was used for statistical analysis of plasma IgE levels and TGFβ1 levels. The chi-square test was performed for statistical analysis of allergic symptoms and C-509T within the TGFβ1 gene. Probability (p) values of less than 0.05 were considered to indicate statistical significance.

RESULTS

ALLERGIC DISORDERS AND SYMPTOMS IN SUBJECTS

By the age of 14 months, 20 of the 64 subjects were allergic and 44 were non-allergic as defined in the Methods. Of the 64 subjects, 19 had atopic dermatitis and 3 had bronchial asthma, while two subjects suffered from both atopic dermatitis and bronchial asthma. Peripheral blood samples were collected at 6 months of age in 30 subjects and at 14 months of age in 27 subjects.

CHANGES IN PLASMA TGF*β*1 LEVELS BY AGE

Plasma TGFβ1 levels at 0, 6, and 14 months of age are shown in Figure 1A. Plasma TGFβ1 levels were 306.2 ± 230.9 pg/ml (M ± SD) at 0 months ($n = 64$), 805.3 ± 428.4 pg/ml at 6 months ($n = 30$), and 273.6 ± 175.8 pg/ml at 14 months ($n = 27$). Plasma TGFβ1 levels at 6 months of age were significantly ($p <$ 0.0001 for each) higher than those in both 0 months and 14 months.

TGFβ1 levels were measured at all 3 points in all 24 subjects (data shown in Fig. 1B). The plasma TGFβ1 levels at 6 months of age were significantly ($p <$ 0.0001 for each) higher than at both 0 and 14 months. This pattern was observed in 22 of the 24 subjects whose plasma TGFβ1 levels were measured at all 3 points.

RELATIONSHIP BETWEEN PLASMA TGF*β*1 LEVELS AND ALLERGIC DISEASES OR PLASMA IgE LEVELS

Figure 2A, B, C show plasma TGFβ1 levels at 0, 6, and 14 months of age in non-allergic and allergic subjects. The plasma TGFβ1 levels at 14 months of age in allergic subjects were significantly higher than those in the non-allergic subjects ($p = 0.03$, Fig. 2C).