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

Tomoko Suzuki[1], Hirokazu Arakawa[1], Takahisa Mizuno[1], Kazuhiro Muramatsu[1], Hiromi Tadaki[1], Takumi Takizawa[1], Hiroyuki Mochizuki[1], Kenichi Tokuyama[2], Satoshi Matsukura[3], and Akihiro Morikawa[1]

[1]Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine, Gunma, Japan; [2]Department of Pharmacy, Takasaki University of Health and Welfare, Gunma, Japan; and [3]Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan

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.

(Received in original form September 16, 2007 and in final form December 29, 2007)

This work was supported by a grant from Research on Eye and Ear Sciences, Immunology, Allergy and Organ Transplantation, Japan.

Correspondence and requests for reprints should be addressed to Hirokazu Arakawa, M.D., Ph.D., Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine, 3-39-15, Showa-machi, Maebashi, Gunma 371-8511, Japan. E-mail: harakawa@showa.gunma-u.ac.jp

Am J Respir Cell Mol Biol Vol 38. pp 707–714, 2008
Originally Published in Press as DOI: 10.1165/rcmb.2007-0337OC on January 18, 2008
Internet address: www.atsjournals.org

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

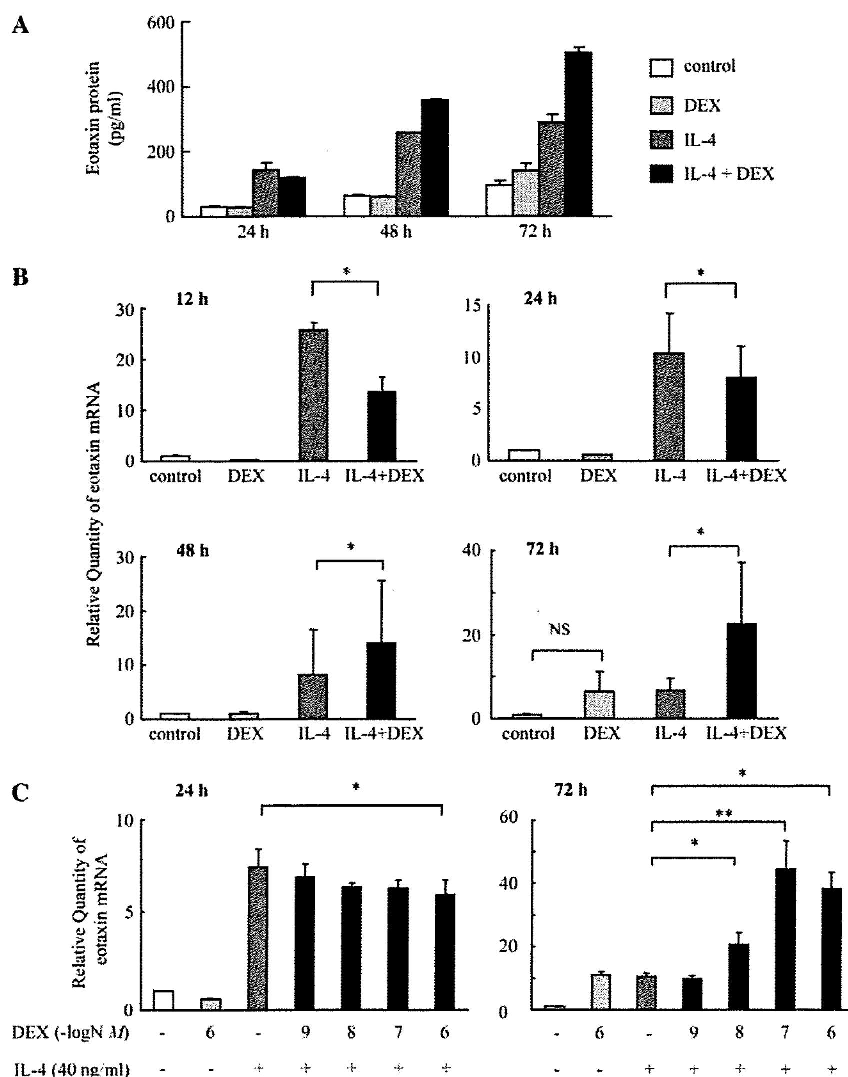

Figure 1. Effect of dexamethasone (DEX) and IL-4 on the production of eotaxin protein and mRNA in normal human lung fibroblast (NHLF) cells. (*A*) Cells were incubated with or without IL-4 (40 ng/ml) and/or DEX (10^{-6} M) for 24, 48, and 72 hours, and the concentration of eotaxin protein in the medium 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 (*$P < 0.05$, **$P < 0.01$).

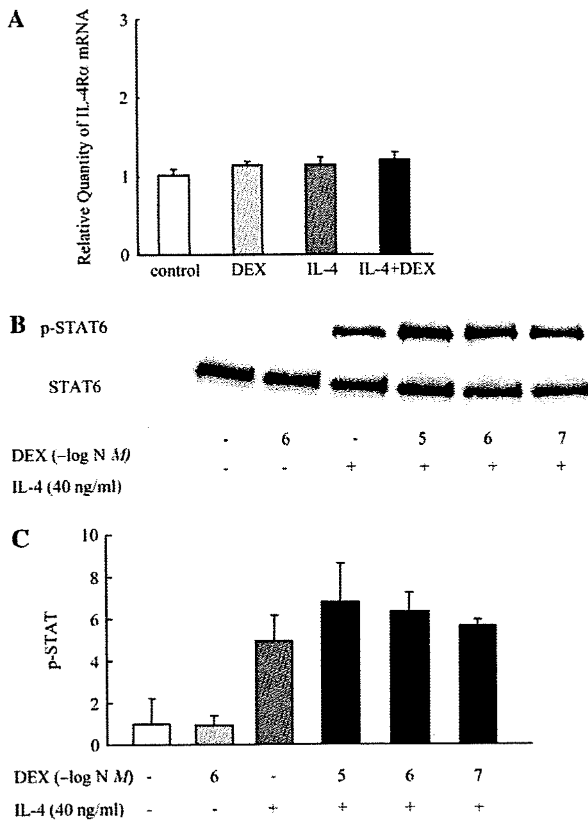

Figure 2. IL-4Rα mRNA analysis and representative Western blots. (*A*) Forty-eight hours after stimulation with or without IL-4 (40 ng/ml) and DEX (10^{-6} M), IL-4Rα mRNA expression was determined by quantitative real-time PCR. Data are presented as the mean ± SD of four independent experiments. Results 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

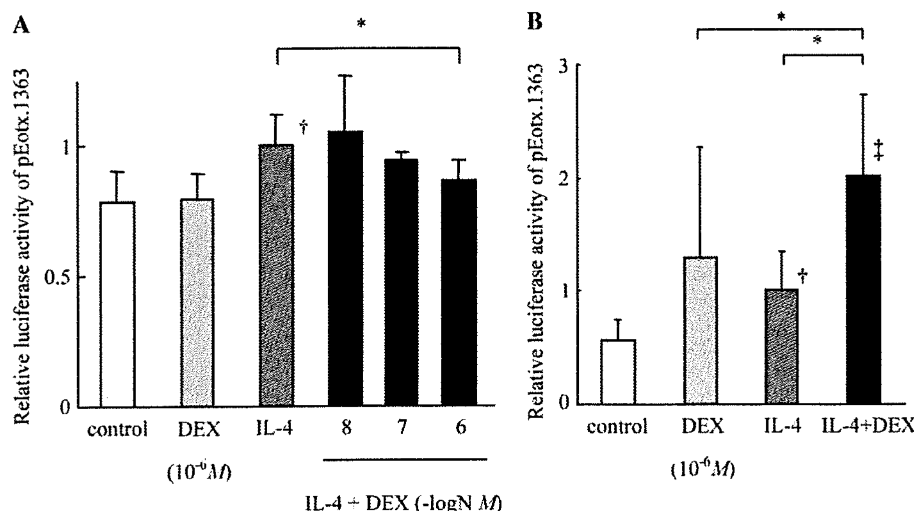

Figure 3. Activation of an eotaxin promoter-luciferase reporter plasmid (pEotax.1363) by IL-4 and/or DEX in NHLF cells. IL-4 (40 ng/ml) and/or DEX (10^{-6}–10^{-8} M) was added to the media, and after 8 hours (*A*) luciferase assay was performed. Forty-eight hours after coincubation with IL-4 (40 ng/ml) 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 ($^{*}P < 0.05$). $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ are compared with the value of control.

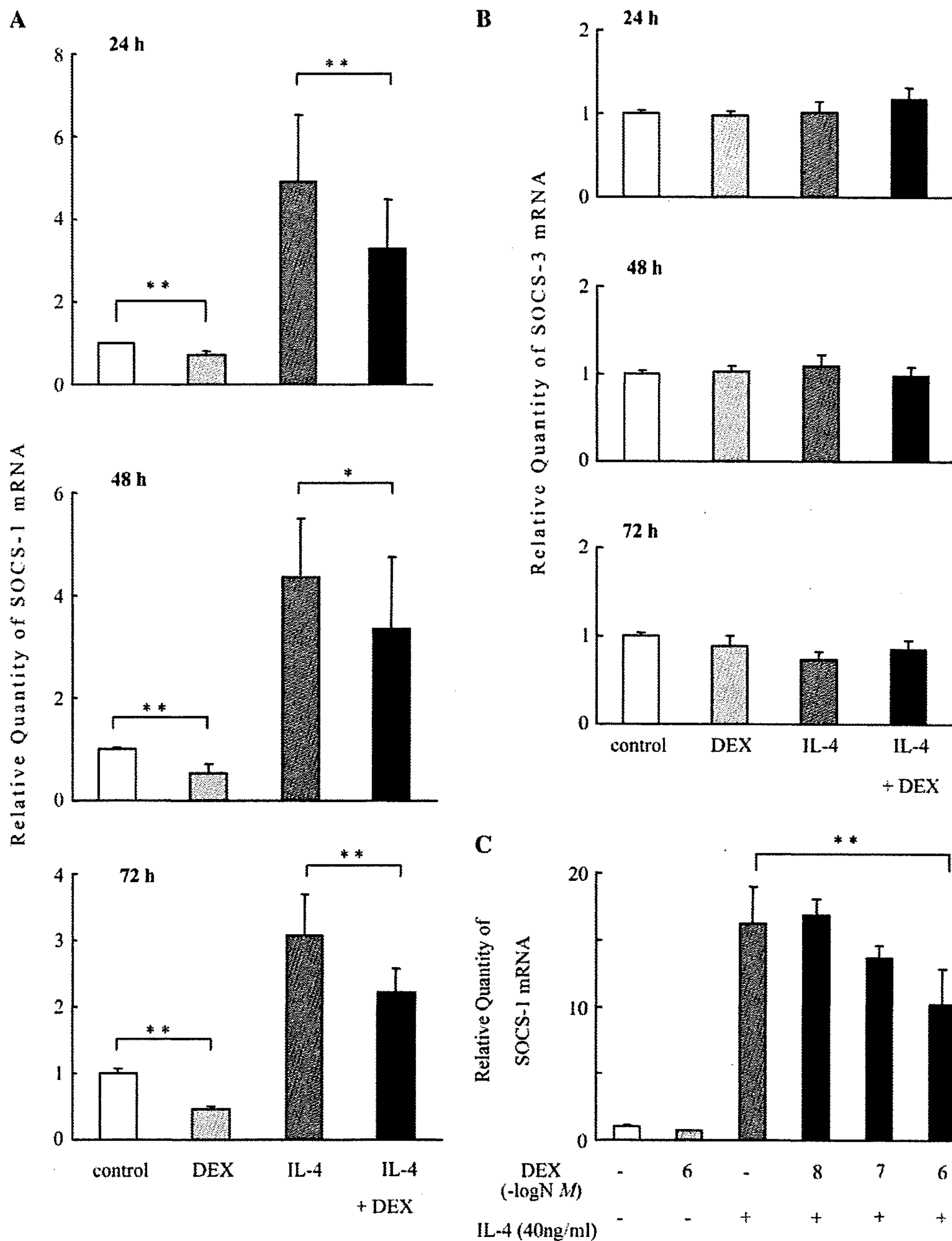

Figure 4. SOCS-1 and SOCS-3 mRNA analysis. (*A*) Quantitative real-time PCR assessment of the fold changes in SOCS-1 mRNA at 24, 48, and 72 hours after coincubation with IL-4 (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity of eotaxin 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

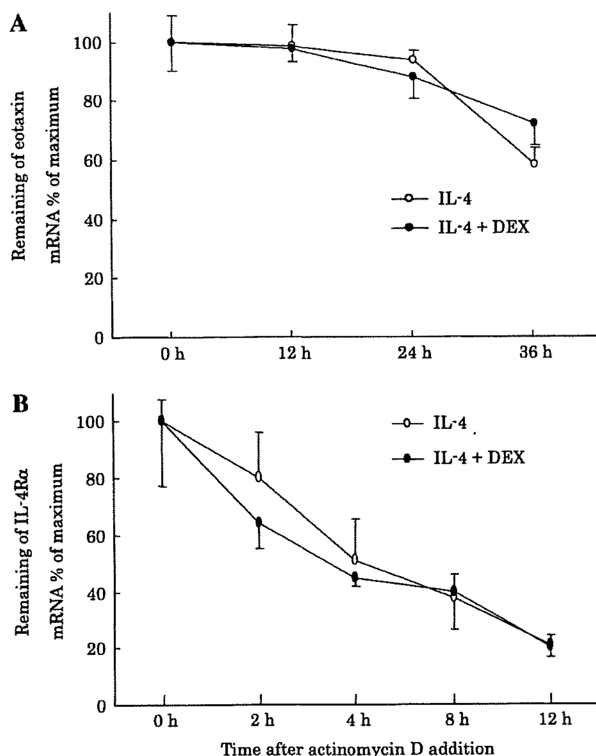

Figure 5. Eotaxin and IL-4Rα mRNA stability. After induction of eotaxin mRNA (*A*) and IL-4Rα mRNA (*B*) by 36 hours of incubation with 40 ng/ml IL-4 and/or DEX (10^{-6} M), cells were treated with actinomycin D (1 μg/ml). After treatment with actinomycin D, eotaxin or IL-4Rα mRNA expression was analyzed 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).

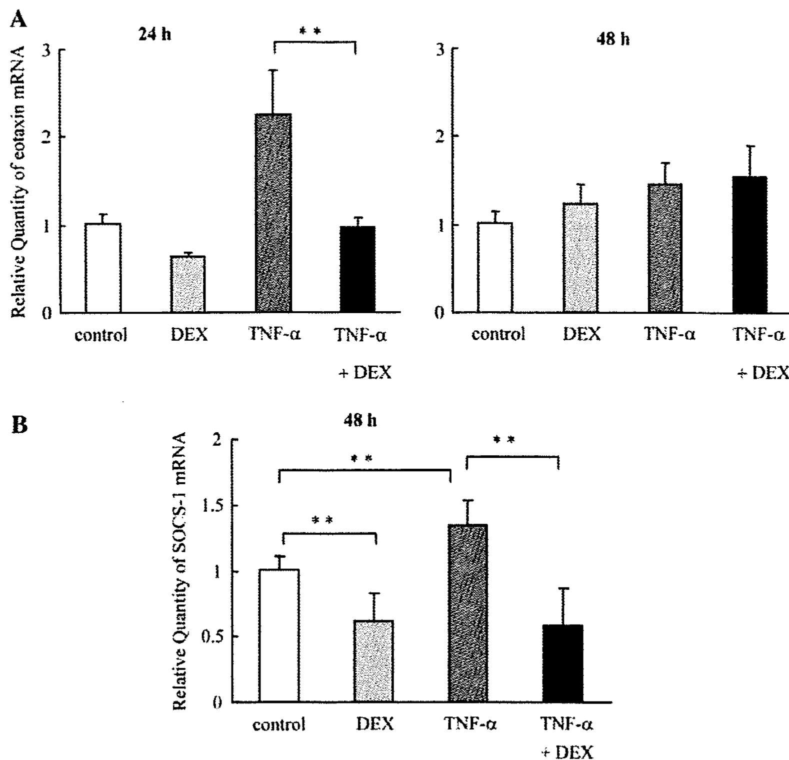

Figure 6. (*A*) Effect of DEX on TNF-α–induced eotaxin mRNA expression. Real-time PCR assessment of the fold changes in eotaxin mRNA at 24 and 48 hours after coincubation with TNF-α (40 ng/ml) and/or DEX (10^{-6} M) or the unstimulated values (control). Results are expressed as the relative quantity 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

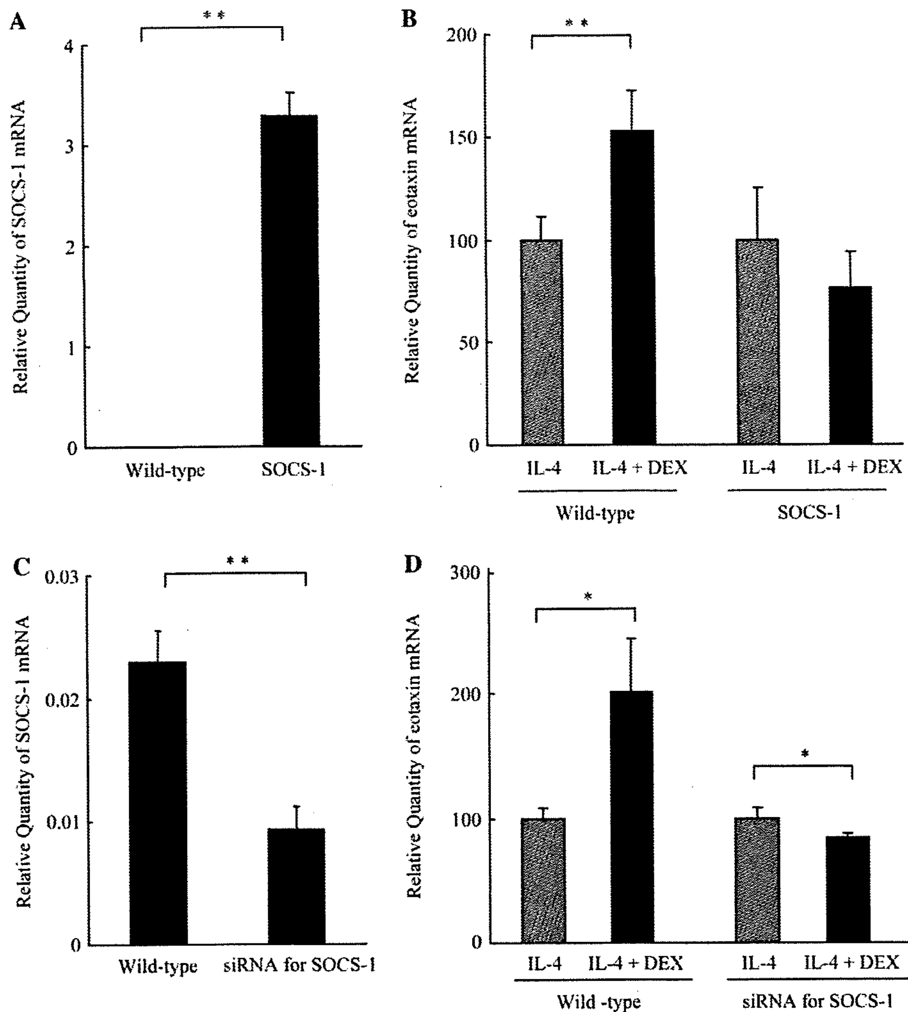

Figure 7. Effect of SOCS-1 expression plasmids cloned into pAcGFP1-C1 vector (*A* and *B*) and siRNA for SOCS-1 (*C* and *D*) on SOCS-1 mRNA and IL-4 (40 ng/ml) induced eotaxin mRNA enhanced by DEX (10^{-6} M) 48 hours after coincubation. Results are expressed in relative quantity of SOCS-1 mRNA (*A* 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.

Acknowledgments: The authors thank Tomoko Endo and Chinori Iijima for their excellent technical assistance.

References

1. Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350–362.
2. Wenzel S. Severe asthma in Adults. *Am J Respir Crit Care Med* 2005; 172:149–160.
3. Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 2003;111: 215–225.
4. Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. *Immunol Cell Biol* 2001;79:376–384.
5. Hamid Q, Tulic' MK, Liu MC, Moqbel R. Inflammatory cells in asthma: mechanisms and implications for therapy. *J Allergy Clin Immunol* 2003;111:S5–S12.
6. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol* 1998;114:137–146.
7. Puxeddu I, Bader R, Piliponsky AM, Reich R, Levi-Schaffer F, Berkman N. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. *J Allergy Clin Immunol* 2006; 117:103–110.
8. Stellato C, Matsukura S, Fal A, White J, Beck LA, Proud D, Schleimer RP. Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and the glucocorticoid budesonide. *J Immunol* 1999;163:5624–5632.
9. Matsukura S, Stellato C, Georas SN, Casolaro V, Plitt JR, Miura K, Kurosawa S, Schindler U, Schleimer RP. Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT-6-dependent mechanism. *Am J Respir Cell Mol Biol* 2001;24:755–761.
10. Kraft M, Lewis C, Pham D, Chu HW. IL-4, IL-13, and dexamethasone augment fibroblast proliferation in asthma. *J Allergy Clin Immunol* 2001;107:602–606.
11. Matsukura S, Kokubu F, Kurokawa M, Kawaguchi M, Kuga H, Ieki K, Odaka M, Suzuki S, Watanabe S, Takeuchi H, *et al.* Molecular mechanisms of repression of eotaxin expression with fluticasone propionate in airway epithelial cells. *Int Arch Allergy Immunol* 2004;134:12–20.
12. Lilly CM, Nakamura H, Kesselman H, Nagler-Anderson C, Asano K, Garcia-Zepeda EA, Rothenberg ME, Drazen JM, Luster AD. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J Clin Invest* 1997;99:1767–1773.
13. Matsukura S, Kokubu F, Kuga H, Kawaguchi M, Ieki K, Odaka M, Suzuki S, Watanabe S, Takeuchi H, Adachi M, *et al.* Differential regulation of eotaxin expression by IFN-γ in airway epithelial cells. *J Allergy Clin Immunol* 2003;111:1337–1344.
14. Yamamoto S, Kobayashi I, Tsuji K, Nishi N, Muro E, Miyazaki M, Zaitsu M, Inada S, Ichimaru T, Hamasaki Y. Upregulation of interleukin-4 receptor by interferon-γ enhanced interleukin-4-induced eotaxin-3 production in airway epithelium. *Am J Respir Cell Mol Biol* 2004;31:456–462.
15. Heller NM, Matsukura S, Georas SN, Boothby MR, Rothman PB, Stellato C, Schleimer RP. Interferon-γ inhibits STAT6 signal transduction and gene expression in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2004;31:573–582.
16. Lamkhioued B, Renzi PM, Abi-Younes S, Garcia-Zepada EA, Allakhverdi Z, Ghaffar O, Rothenberg MD, Luster AD, Hamid Q. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol* 1997;159:4593–4601.
17. Hebenstreit D, Luft P, Schmiedlechner A, Duschl A, Horejs-Hoeck J. SOCS-1 and SOCS-3 inhibit IL-4 and IL-13 induced activation of eotaxin-3/CCL26 gene expression in HEK293 cells. *Mol Immunol* 2005;42:295–303.
18. Sato T, Saito R, Jinushi T, Tsuji T, Matsuzaki J, Koda T, Nishimura S, Takeshima H, Nishimura T. IFN-γ-induced SOCS-1 regulates STAT6-dependent eotaxin production triggered by IL-4 and TNF-α. *Biochem Biophys Res Commun* 2004;314:468–475.
19. Paul C, Seiliez I, Thissen JP, Le Cam A. Regulation of expression of the rat SOCS-3 gene in hepatocytes by growth hormone, interleukin-6 and glucocorticoids. *Eur J Biochem* 2000;267:5849–5857.
20. Atasoy U, Curry SL, Silanes IL, Shyu AB, Casolaro V, Gorospe M, Stellato C. Regulation of eotaxin gene expression by TNF-α and IL-4 through mRNA stabilization: Involvement of the RNA-binding protein HuR. *J Immunol* 2003;171:4369–4378.
21. Goleva E, Hauk PJ, Boguniewicz J, Martin RJ, Leung DY. Airway remodeling and lack of bronchodilator response in steroid-resistant asthma. *J Allergy Clin Immunol* 2007;120:1065–1072.

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2009;149(suppl 1):45–50
DOI: 10.1159/000210653

Published online: June 3, 2009

Intracellular Protein Phosphorylation in Eosinophils and the Functional Relevance in Cytokine Production

Koichi Yamamura Tetsuya Adachi Tomoko Masuda Yasuhiro Kojima
Asae Hara Takako Toda Hiroyuki Nagase Ken Ohta

Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan

Key Words
Chemokines · Cytokines · Eosinophils · Protein kinases/phosphatases · Signal transduction

Abstract
Background: Eosinophils play a pivotal role in the pathogenesis of asthma. Thus, it is of paramount importance to investigate the mechanism of eosinophil activation. Although a number of factors including cytokines/chemokines activate eosinophils, the potency of each stimulus to phosphorylate intracellular molecules and activate eosinophils remains to be elucidated. In the present study, we performed inclusive analyses of protein phosphorylation in eosinophils and studied the functional relevance of such phosphorylation in cytokine production. ***Methods:*** Blood eosinophils were purified using Percoll and anti-CD16 antibody-coated magnetic beads. Purified eosinophils were stimulated with various stimuli. The eosinophil lysates were subjected to phosphoprotein analysis using the Luminex system. In some of these experiments, we studied the effect of a few signaling inhibitors on cytokine production from eosinophils. ***Results:*** We found that several factors such as IL-5, eotaxin, platelet-activating factor (PAF), and PGD_2 phosphorylated Akt, ERK1/2, p38 MAPK, and glycogen synthase kinase-3 (GSK-3) in the eosinophils. Because eotaxin most potently induced the production of various cytokines, we performed the inhibition study using eotaxin-stimulated eosinophils. Eotaxin-induced production of IL-1β, IL-6, and MIP-1β was significantly reduced by the MEK inhibitor PD98059, p38 MAPK inhibitor SB203580, or PI3K inhibitor LY294002. In contrast, the GSK-3 inhibitor SB216763 blocked only IL-1β production from the eosinophils. ***Conclusions:*** In terms of the phosphorylation of intracellular signaling molecules, we could quantify the potency of various stimuli that activate eosinophils. We are the first to demonstrate the role of GSK-3 in cytokine production from eosinophils. The Luminex system aids in examining the mechanism of eosinophil activation.
Copyright © 2009 S. Karger AG, Basel

Introduction

The pathogenesis of asthma is characterized by tissue infiltration with inflammatory cells such as eosinophils, mast cells, and T cells. Several mediators released by these cells cause epithelial damage, leading to enhanced bronchial hyperresponsiveness and airway obstruction [1]. Although eosinophils are considered to be the most im-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1018–2438/09/1495–0045$26.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Tetsuya Adachi
Department of Internal Medicine, Teikyo University School of Medicine
2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605 (Japan)
Tel. +81 3 3964 1211, Fax +81 3 3964 1291
E-Mail tadachi@med.teikyo-u.ac.jp

Table 1. Phosphorylation of intracellular molecules in eosinophils

	PMA	Ionomycin	fMLP	PAF	PGD_2	Eotaxin	IL-5
Akt	–	+	+	+*	+	+	+
ATF-2	+	+	+	+†	+†	+	+
CREB	+	–	+	+†	+†	+	+
ERK1/2	+	+	+	+‡	+§	+	–
GSK-3	+	–	+	+†	+†	+	+
Hsp27	–	+	+	+	+	+	–
IRS-1	–	–	–	–	–	–	–
JNK	–	+	+	+	+	–	–
NF-κB	–	+	–	–	–	–	–
p38 MAPK	–	+	+	+†	+†	+	–
p53	–	–	–	–	–	–	–
p70 S6 kinase	+	–	–	+	+	–	–
p90 RSK	+	–	+	+†	+†	+	–
STAT3	–	–	–	–	–	–	+

Eosinophils were stimulated with PMA, ionomycin, fMLP, PAF, PGD_2, eotaxin, or IL-5 for 3 min and then lysed. The lysate was subjected to phosphoprotein measurement using a Bio-Plex suspension array and the Luminex system (n = 8).

+ $p < 0.05$ vs. control; * $p < 0.05$ vs. PGD_2, eotaxin, or IL-5; † $p < 0.05$ vs. eotaxin or IL-5; ‡ $p < 0.05$ vs. PGD_2 or eotaxin; § $p < 0.05$ vs. eotaxin (Wilcoxon signed-ranks test).

portant cells in this process, the results of a clinical trial using an anti-IL-5 antibody have raised questions about the role of eosinophils in bronchial hyperresponsiveness [2]. A subsequent study demonstrated that treating asthmatics with anti-IL-5 antibody reduces airway eosinophil numbers and the deposition of extracellular matrix proteins in the bronchial subepithelial basement membrane, suggesting the involvement of eosinophils in airway remodeling [3]. In support of this finding, eosinophil-deficient mice exhibit decreased subepithelial fibrosis and smooth muscle hyperplasia [4]. Thus, the targeting of eosinophils is considered to be an effective strategy for treating asthma.

Eosinophils express several surface receptors against platelet-activating factor (PAF), complements, cytokines/chemokines, and so on [5]. Once activated by these ligands, eosinophils elicit effecter functions such as degranulation, superoxide production, and cytokine production. PAF, fMLP, complement 5a (C5a), and secretory IgA (sIgA) potently induce degranulation and superoxide production, whereas the effect of eosinophil-specific cytokines/chemokines, IL-5 or eotaxin is relatively weak. One may explain that the functional difference induced by each stimulus is due to the specific signaling pathway or activation profile of intracellular molecules. Although the IL-5 or eotaxin signaling pathway in eosinophils is well elucidated [6–11], little is known about the signaling potency among the stimuli.

In this study, we compared the stimulating potency by quantifying intracellular phosphoproteins in eosinophils. Furthermore, we investigated their functional relevance in cytokine/chemokine production from eosinophils.

Materials and Methods

Eosinophil Purification

Peripheral venous blood was obtained from healthy subjects with slight eosinophilia. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradients, modified from the method of Hansel et al. [12]. The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The eosinophils (>99% purity) were then suspended in HBSS with 1% FCS in tubes coated with 3% human serum albumin.

Preparation of Cytosolic Cell Extracts

Eosinophils were incubated with PMA (Sigma-Aldrich, St. Louis, Mo., USA), ionomycin (Sigma-Aldrich), PAF (Sigma-Aldrich), fMLP (Sigma-Aldrich), PGD_2 (Sigma-Aldrich), IL-5 (R&D, Minneapolis, Minn., USA), and eotaxin (R&D) for 3 min at 37°C. In some of the experiments, the cells were preincubated with the MEK inhibitor PD98059 (Calbiochem, San Diego, Calif., USA), p38 MAPK inhibitor SB203580 (Calbiochem), glycogen synthase kinase-3 (GSK-3) inhibitor SB216763 (BIOMOL, Plymouth Meeting, Pa., USA), or PI3K inhibitor LY294002 (Calbiochem) for 30 min. The reaction was then terminated by adding 9 vol of ice-cold HBSS containing 1 mM Na_3VO_4. The cells were lysed using a Bio-Plex cell lysis kit (BioRad Laboratories, Hercules, Calif., USA) for further quantification of protein phosphorylation performed using a Bio-Plex phosphoprotein panel (BioRad) and Luminex 200 (Luminex Corp., Austin, Tex., USA). Data were shown as a percentage of the mean fluorescence intensity of unstimulated control.

Production of Cytokines or Chemokines

Purified eosinophils (10^6 cells/ml) were suspended in RPMI 1640 with 10% FCS. After treatment with or without the inhibitors for 30 min at 37°C, eosinophils were stimulated with PMA, ionomycin, PAF, fMLP, PGD_2, IL-5, and eotaxin in the presence or absence of 5 mg/ml cytochalasin B for 24 h. The measurement of cytokines/chemokines in the supernatants was performed using a Bio-Plex cytokine panel (BioRad) and Luminex 200 (Luminex Corp.).

Statistical Analysis

Results were expressed as mean ± SEM. The data were analyzed for statistical significance using the Wilcoxon signed-ranks test.

Results

Phosphorylation of Intracellular Proteins in Eosinophils

We measured the phosphorylation of Akt, activating transcription factor-2 (ATF-2), CREB, ERK1/2, GSK-3, Hsp27, IRS-1, JNK, NF-κB, p38 MAPK, p53, p70 S6 kinase, p90 ribosomal S6 kinase (RSK), and STAT3. Stimulation of eosinophils with fMLP (1 μM), PAF (1 μM), PGD_2 (1 μM), or eotaxin (100 nM) elicited the phosphorylation of Akt, ATF-2, CREB, ERK1/2, GSK-3, Hsp27, p38 MAPK, and p90 RSK (table 1). Especially, PAF was the most potent in phosphorylating Akt, ERK1/2, p38 MAPK, and GSK-3 compared to the other stimuli. Although IL-5 (100 ng/ml) induced the phosphorylation of Akt, ATF-2, CREB, GSK-3, and STAT3, we did not observe significant phosphorylation of ERK1/2 and p38 MAPK, which is contrary to previously reported findings [7].

Cytokine/Chemokine Production from Eosinophils

We measured cytokine/chemokine production from eosinophils incubated with the various stimulants for 24 h. As cytokine production by C5a or fMLP and degranulation by chemokine is dependent on cytochalasin B, we pretreated eosinophils with the compound before stimulation with the ligands for seven-transmembrane receptors such as PAF, eotaxin, and PGD_2 [13, 14]. Eotaxin potently induced production of IL-1β, IL-6, IL-8, IFN-γ, GM-CSF, G-CSF, MIP-1α/β, and VEGF, whereas IL-7, IL-8, IL-12, G-CSF, MIP-1α/β, and VEGF were released after stimulation with IL-5 (table 2). In contrast, we could hardly observe the production of any cytokine from eosinophils stimulated with fMLP, PAF, or PGD_2 despite their potency for phosphorylating intracellular proteins.

Table 2. Production of cytokines from eosinophils

	PMA	Ionomycin	fMLP	PAF	PGD_2	eotaxin	IL-5
IL-1β	–	+	–	–	–	++	–
IL-1ra	–	–	–	–	–	–	–
IL-2	–	–	–	–	–	–	–
IL-4	–	–	–	–	–	–	–
IL-5	–	+	–	–	–	–	
IL-6	–	–	–	–	–	++	–
IL-7	–	–	–	–	–	–	+
IL-8	+	+	+	–	–	++	++
IL-9	–	–	–	–	–	–	–
IL-10	–	–	–	–	–	–	–
IL-12	–	+	–	–	–	–	+
IL-13	–	–	–	–	–	–	–
IL-15	–	–	–	–	–	–	–
IL-17	–	–	–	–	–	–	–
IFN-γ	–	–	–	–	–	+	–
GM-CSF	–	–	–	–	–	+	–
G-CSF	+	+	+	+	+	+	+
TNF-α	–	–	–	–	–	–	–
Eotaxin	–	–	–	–	–		–
RANTES	++	+	–	–	–	–	–
MCP-1	–	+	–	–	–	–	–
MIP-1α	–	+	–	–	–	+	+
MIP-1β	–	++	–	–	–	++	++
IP-10	–	–	–	–	–	–	–
FGF basic	–	–	–	–	–	–	–
PDGF-BB	–	–	–	–	–	–	–
VEGF	–	+	–	–	–	++	+

Eosinophils were cultured for 24 h in the presence of PMA, ionomycin, fMLP, PAF, PGD_2, eotaxin, or IL-5. In the experiments using fMLP, PAF, PGD_2, and eotaxin, eosinophils were pretreated with cytochalasin B. The concentration of cytokines/chemokines in the culture supernatant was measured by a Bio-Plex suspension array and the Luminex system (n = 9).

+ $p < 0.05$ vs. control; ++ $p < 0.01$ vs. control (Wilcoxon signed-ranks test).

Effect of Signal-Specific Inhibitors on Cytokine/Chemokine Production

In order to analyze the signaling pathway involved in eosinophil function, we attempted to block the activity of ERK1/2, p38 MAPK, GSK-3, and PI3K, whose phosphorylation was induced by eotaxin. For this purpose, we utilized the inhibitors for MEK (PD98059), p38 MAPK (SB203580), GSK-3 (SB216763), and PI3K (LY294002). As expected, eotaxin-induced phosphorylation of ERK1/2 (downstream of MEK), p38 MAPK, and Akt (downstream of PI3K) was significantly inhibited by PD98059, SB203580, and LY294002, respectively (data not shown). SB216763 is known to reduce the activity of downstream molecules of GSK-3 such as glycogen synthase without affecting GSK-3 phosphorylation. We observed significant inhibition of GSK-3 phosphorylation by PD98059 instead of by SB216763 (data not shown).

Next, we investigated the effect of signaling inhibitors on the production of IL-1β, IL-6, IFN-γ, GM-CSF, and MIP-1α/β. PD98059 and SB203580 significantly inhibited the production of all the cytokines except IFN-γ and GM-CSF, respectively. SB216763 blocked only IL-1β production, whereas LY294002 was effective in the release of IL-1β, GM-CSF, and MIP-1β (fig. 1a–f).

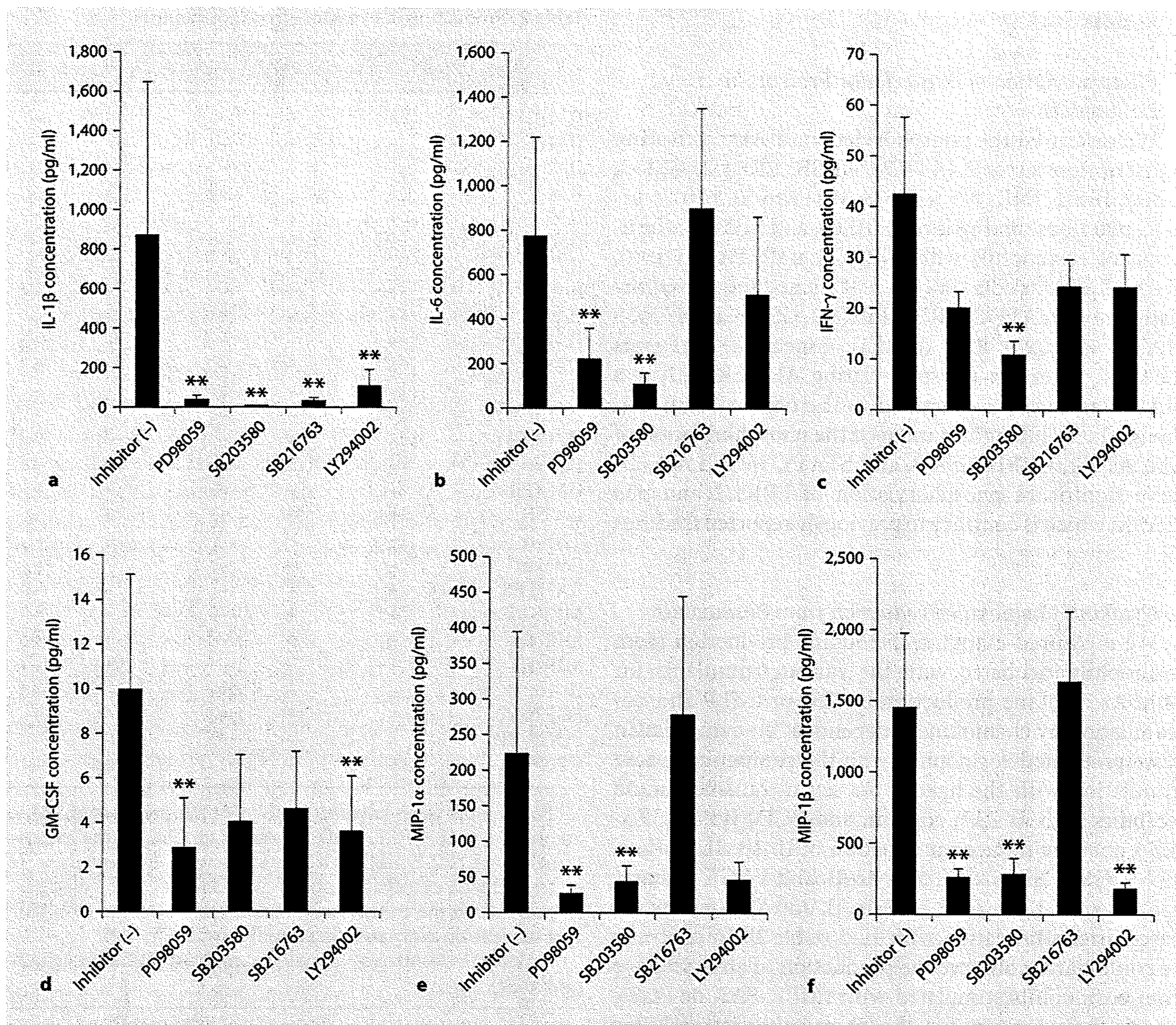

Fig. 1. Effect of signal-specific inhibitors on cytokine production from eosinophils stimulated with eotaxin. Eosinophils were pretreated with 50-μM PD98059, 10-μM SB203580, 10-μM SB216763, or 25-μM LY294002 in the presence of cytochalasin B for 30 min, and then stimulated with eotaxin for 24 h. The concentration of IL-1β (**a**), IL-6 (**b**), IFN-γ (**c**), GM-CSF (**d**), MIP-1α (**e**), and MIP-1β (**f**) in the culture supernatant was measured by a Bio-Plex suspension array and the Luminex system. Data are expressed as mean ± SEM (n = 8); ** $p < 0.01$ vs. control (Wilcoxon signed-ranks test).

Discussion

Eosinophils are activated by various factors such as immunoglobulins, complements, metabolites of arachidonic acid, cytokines/chemokines, and so on. Especially, the signaling pathway of IL-5 or eotaxin has been well elucidated. IL-5 propagates signals via the Lyn–Ras–Raf-1-MEK–ERK1/2 and JAK2–STAT pathways [6]. An alternative pathway involves PI3K that activates Akt/PKB [8]. The signal through CCR3, a specific receptor for eotaxin, activates ERK1/2 and p38 MAPK via the α_i subunit of G protein [9]. Furthermore, another pathway including Rho–ROCK or MAPK–MLCK appears to be involved in eotaxin signaling [10, 11]. The PAF receptor is a seven-

transmembrane receptor as well as CCR3 and couples with both $G\alpha_i$ and $G\alpha_{q/11}$ [15]. PGD_2 acts on both DP1 and CRTH2 that are coupled with $G\alpha_s$ and $G\alpha_i$, respectively [16]. Although eosinophil-activating factors are likely to propagate specific signals, there are only few studies that compare the signaling potency of different stimuli. We therefore investigated the profile of intracellular phosphoproteins in eosinophils. Stimulation with fMLP, PAF, PGD_2, eotaxin, or IL-5 induced significant phosphorylation of Akt, ERK1/2, p38 MAPK, GSK-3, p90 RSK, and p70 S6 kinase. In the comparison of the tested stimulants, PAF was the most potent in phosphorylating intracellular proteins. Although the mechanism is not clear, the overlapping pathways through $G\alpha_i$ and $G\alpha_{q/11}$ may potentiate the activation of signaling molecules.

It has been shown that growth factors phosphorylate GSK-3α and GSK-3β on Ser^{21} and Ser^{9}, respectively [17]. Once phosphorylated, GSK-3 becomes functionally inactive. Several signaling molecules, such as p90 RSK, p70 S6 kinase, PKA, PKB, and PKC, are involved in the regulation of GSK-3 function. IL-5 phosphorylates GSK-3 via PI3K in eosinophils, and GSK-3 is active during IL-5 starvation [18]. In contrast, GSK-3 phosphorylation is regulated by p90 RSK and Akt in neutrophils stimulated with fMLP [19]. In this study, we showed that eotaxin-induced GSK-3 phosphorylation is inhibited by PD98059. Although the main action of PD98059 is to inhibit ERK1/2 activation, it partially blocks p90 RSK function as well [20]. Thus, the effect of PD98059 on GSK-3 phosphorylation shown in our results is possibly mediated via p90 RSK rather than ERK1/2. In support of our data, ERK1/2 does not regulate Ser^{9} phosphorylation despite inhibiting GSK-3β activity in the model of apoptosis of murine central nerve neurons [21].

It has been shown that the effect of PAF, fMLP, C5a, and sIgA on eliciting eosinophil degranulation and superoxide production is more potent than that of cytokines such as IL-5 and eotaxin [5]. However, our results reveal that eosinophils produce the largest amount of cytokines when stimulated with eotaxin, whereas PAF potently induces intracellular protein phosphorylation. The reason for the poor effect of PAF on cytokine production is that eosinophils may undergo apoptosis after PAF stimulation. Eosinophils produce a number of cytokines including IL-1, IL-6, IFN-γ, GM-CSF, and MIP-1 [22–26], which were analyzed in this study. These cytokines play various roles in the pathogenesis of asthma: IL-1 in adhesion molecule expression of endothelium and epithelium, IL-6 in immune regulation during viral infection, IFN-γ in Th1 inflammation, GM-CSF in maintenance of eosinophil survival, and MIP-1 in Th1 cell recruitment [1]. Although it is important to study the mechanism of cytokine production in eosinophils, little is known about the specific signaling pathway. Adachi et al. [7] have reported that C5a regulates MIP-1α production through ERK1/2 and p38 MAPK in eosinophils. In our study, either ERK1/2 or p38 MAPK or both were involved in the production of IL-1, IL-6, IFN-γ, GM-CSF, and MIP-1, which were stimulated by eotaxin. Furthermore, the signals to produce IL-1, GM-CSF, and MIP-1 were mediated through PI3K.

GSK-3 is known to be active in the resting condition, and it becomes inactive once phosphorylated [17]. In TLR4 signaling of peripheral mononuclear cells, SB216763 blocked IL-1β production by LPS stimulation despite the inactivation of glycogen synthase mediated by LPS-induced GSK-3 phosphorylation [27]. In support of these findings, we found that SB216763 reduced IL-1β production from eotaxin-stimulated eosinophils in spite of GSK-3 phosphorylation. These results suggest that the activity of phosphorylated GSK-3 considerably, but not completely, decreases, and that the residual activity contributes to IL-1β production. Interestingly, however, non-phosphorylated/activated GSK-3 participates in the inhibition of eosinophil apoptosis during IL-5 starvation [18]. Thus, GSK-3 is a unique molecule regulating eosinophil function both in active and inactive forms.

In conclusion, we investigated intracellular protein phosphorylation in eosinophils and its functional relevance in cytokine production. Utilization of the Luminex system enabled us to perform inclusive analysis from a small amount of samples, especially in terms of molecular screening of signal transduction. In particular, we are the first to demonstrate the role of GSK-3 in cytokine production from eosinophils. Further studies are necessary to elucidate the detailed signaling complex around GSK-3 in eosinophils, the outcome of which may lead to the development of new molecular targeting therapies.

Acknowledgment

This work was funded in part by grants for scientific research supported by the Ministry of Health, Labor and Welfare.

Disclosure Statement

The authors declare that no financial or other conflicts of interest exist in relation to the content of the article.

References

1 Holgate ST, Lemanske RF, O'Byrne PM, Kakumanu S, Busse WW: Asthma Pathogenesis; in Adkinson NF, Bochner BS, Busse WW, Holgate ST, Lemanske RF, Simons FER (eds): Allergy: Principles & Practice. Philadelphia, Mosby Elsevier, 2009, pp 893–919.

2 Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. Lancet 2000;356:2144–2148.

3 Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB: Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. J Clin Invest 2003; 112:1029–1036.

4 Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C: A critical role for eosinophils in allergic airways remodeling. Science 2004;305:1776–1779.

5 Moqbel R, Lacy P, Adamko DJ, Odemuyiwa SO: Biology of eosinophils; in Adkinson NF, Bochner BS, Busse WW, Holgate ST, Lemanske RF, Simons FER (eds): Allergy: Principles & Practice. Philadelphia, Mosby Elsevier, 2009, pp 295–310.

6 Adachi T, Alam R: The mechanism of IL-5 signal transduction. Am J Physiol 1998;275: C623–C633.

7 Adachi T, Choudhury BK, Stafford S, Sur S, Alam R: The differential role of extracellular signal-regulated kinases and p38 mitogen-activated protein kinase in eosinophil functions. J Immunol 2000;165:2198–2204.

8 Coffer PJ, Schweizer RC, Dubois GR, Maikoe T, Lammers JW, Koenderman L: Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. Blood 1998;91: 2547–2557.

9 Kampen GT, Stafford S, Adachi T, Jinquan T, Quan S, Grant JA, Skov PS, Poulsen LK, Alam R: Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. Blood 2000;95:1911–1917.

10 Adachi T, Vita R, Sannohe S, Stafford S, Alam R, Kayaba H, Chihara J: The functional role of rho and rho-associated coiled-coil forming protein kinase in eotaxin signaling of eosinophils. J Immunol 2001;167:4609–4615.

11 Adachi T, Stafford S, Kayaba H, Chihara J, Alam R: Myosin light chain kinase mediates eosinophil chemotaxis in a mitogen-activated protein kinase-dependent manner. J Allergy Clin Immunol 2003;111:113–116.

12 Hansel TT, de Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C: An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J Immunol Methods 1991;145: 105–110.

13 Miyamasu M, Hirai K, Takahashi Y, Iida M, Yamaguchi M, Koshino T, Takaishi T, Morita Y, Ohta K, Kasahara T: Chemotactic agonists induce cytokine generation in eosinophils. J Immunol 1995;154:1339–1349.

14 Fujisawa T, Kato Y, Nagase H, Atsuta J, Terada A, Iguchi K, Kamiya H, Morita Y, Kitaura M, Kawasaki H, Yoshie O, Hirai K: Chemokines induce eosinophil degranulation through CCR-3. J Allergy Clin Immunol 2000;106:507–513.

15 Kato M, Kimura H, Motegi Y, Tachibana A, Minakami H, Morikawa A, Kita H: Platelet-activating factor activates two distinct effector pathways in human eosinophils. J Immunol 2002;169:5252–5259.

16 Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K: Prostaglandin D_2 selectively induces chemotaxis in T-helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001;193:255–261.

17 Doble BW, Woodgett JR: GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 2003;116:1175–1188.

18 Rosas M, Dijkers PF, Lindemans CL, Lammers JWJ, Koenderman L, Coffer PJ: IL-5-mediated eosinophil survival requires inhibition of GSK-3 and correlates with β-catenin relocalization. J Leukoc Biol 2006;80:186–195.

19 De Mesquita DD, Zhan Q, Crossley L, Badwey JA: p90-RSK and Akt may promote rapid phosphorylation/inactivation of glycogen synthase kinase 3 in chemoattractant-stimulated neutrophils. FEBS Lett 2001;502:84–88.

20 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 1995;270:27489–27494.

21 Hetman M, Hsuan SL, Habas A, Higgins MJ, Xia Z: ERK1/2 antagonizes glycogen synthase kinase-3-induced apoptosis in cortical neurons. J Biol Chem 2002;277:49577–49584.

22 Weller PF, Rand TH, Barrett T, Elovic A, Wong DT, Finberg RW: Accessory cell function of human eosinophils: HLA-DR-dependent, MHC-restricted antigen-presentation and IL-1α expression. J Immunol 1993;150: 2554–2562.

23 Hamid Q, Barkans J, Meng Q, Ying S, Abrams JS, Kay AB, Moqbel R: Human eosinophils synthesize and secrete interleukin-6, in vitro. Blood 1992;80:1496–1501.

24 Costa JJ, Matossian K, Resnick MB, Beil WJ, Wong DT, Gordon JR, Dvorak AM, Weller PF, Galli SJ: Human eosinophils can express the cytokines tumor necrosis factor-α and macrophage inflammatory protein-1α. J Clin Invest 1993;91:2673–2684.

25 Lamkhioued B, Gounni AS, Aldebert D, Delaporte E, Prin L, Capron A, Capron M: Synthesis of type 1 (IFN-γ) and type 2 (IL-4, IL-5, and IL-10) cytokines by human eosinophils. Ann NY Acad Sci 1996;796:203–208.

26 Kita H, Ohnishi T, Okubo Y, Weiler D, Abrams JS, Gleich GJ: Granulocyte/macrophage colony-stimulating factor and interleukin-3 release from human peripheral blood eosinophils and neutrophils. J Exp Med 1991;174:745–748.

27 Martin M, Rehani K, Jope RS, Michalek SM: Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 2005;6: 777–784.

帝京医学雑誌 33(1)：39～50 (2010)

■原　著■

喘息コントロール状態と非侵襲的気道炎症マーカーとの関連

原　　麻　恵

帝京大学医学部内科学講座　呼吸器・アレルギー学

背景・目的：気管支喘息管理において，気道炎症モニタリングの有用性が示されており，非侵襲的手法の確立が求められている。呼気一酸化窒素濃度（FeNO），呼気凝縮液（EBC）解析の臨床的有用性を，網羅的手法を用いて検証し，気道炎症マーカーを抽出することを目的とした。

対象・方法：気管支喘息患者64名を対象とし，FeNO測定とEBC収集を行った。EBC中の27分子の濃度はLuminex®を用いて測定し，喘息コントロールテスト（ACT）との関連を検討した。ACT 25点満点のコントロール良好群をH群，20-24点をM群，20点未満をL群として分類し検討した。

結果：FeNO，IL-1β，IL-1raはL群で高い傾向を示し，3分子間には有意な正相関を認め，コントロール不良マーカーであることが示唆された。これらはコントロール不良を予測する陰性的中率が高く，低値であればコントロール良好である可能性が約0.7であった。一方，IP-10，VEGFは，L群で有意に低値で，2分子間には有意な正相関を認め，コントロール良好マーカーであることが示唆された。これらはコントロール良好を予測する陽性的中率が高く，高値であればコントロール良好である可能性が約0.9であった。

結論：網羅的検討によりFeNOやEBC解析の臨床的有用性が示唆されるとともに，コントロール良好群と不良群では異なる気道炎症プロファイルが存在することが示された。

キーワード：気管支喘息，呼気凝縮液，呼気一酸化窒素濃度，IP-10，IL-1β

はじめに

近年，気管支喘息の有病率は全年齢層で増加している。小児においては，ISAAC（International Study of Asthma and Allergies in Childhood）[1]において，1995年および2002年に世界共同疫学調査が行われ，喘息の有病率は増加していることが示されている。また，成人においてもATS-DLD（American Thoracic Society for Division of Lung Disease）[2]やECRHS（European Community Respiratory Health Survey）[3]による調査で，喘息有病率は1％から3％程度まで増加したと推定されている。わが国における藤枝市の調査でも有病率は，1986年と1999年の間に3.14％から4.15％に増加したことが報告されており[4]，その至適コントロールは社会的要請である。

気管支喘息病態には，アレルギー性気道炎症が中心的に関与しており，炎症細胞としては，特に好酸球の関与が重要であるとされている。治療薬としては，抗炎症作用を有する吸入ステロイド薬の普及により，喘息死や入院患者数は減少傾向にある[5]。現状の喘息コントロールは，喘息コントロールテスト（Asthma Control Test：ACT）などの自覚症状に基づいた問診票やピークフローモニタリングを用いて行われている。しかしながら，より病態に本質的に関与する可能性がある気道炎症

(指導：大田健教授，長瀬洋之准教授)
学位申請論文

を定量的に評価することはいまだに困難であり，実際の臨床現場では実現していない。研究レベルでは，気道炎症を指標とした治療を行う有用性も報告されはじめており，気道炎症を評価する臨床的指標を確立する必要がある。現在，その評価方法としては，気管支鏡を用いた気道粘膜生検，気管支肺胞洗浄（Broncho-alveolar lavage：BAL）が行われてきているが，侵襲性が高く，繰り返し行う事が困難である。実際の臨床に応用が近いものとしては，喀痰中好酸球比率，呼気一酸化窒素濃度（Fraction of exhaled nitric oxide：FeNO），呼気凝縮液（Exhaled breath condensate：EBC）などの解析が期待されている。

なかでも，今回われわれはFeNOおよびEBCに注目してコントロール状態との関連を検討した。方法論としては，少量の検体で多数の分子濃度を同時かつ網羅的に測定できるサイトカインアレイが開発されてきたため，気管支喘息患者のEBCの解析に応用可能かどうかを検討した。コントロール良好群と不良群における気道炎症プロファイルの差異を網羅的かつ非侵襲的に明らかにし，臨床指標として有用な分子を抽出する事を目的とした。

対象および方法

帝京大学内科呼吸器・アレルギー外来に通院中で，文書にて解析の同意を得た喘息患者64名（**表1**）を対象とした。喘息コントロール状態はACTを用いて評価した。ACTは自己記入式の問診票であり，高得点ほどコントロール状態が良好である事を示し，25点満点である。25点をH群（n=13），20点以上24点以下をM群（n=29），20点未満をL群（n=22）の3群に分類し比較検討した。喘息重症度分類は本邦のガイドラインに準じて決定した[5]。

1）EBCの解析：EcoScreen®(Jaeger, Hoehberg, Germany）を用いてEBCを収集した。ノーズクリップを装着し，15分の安静換気にて収集し，収集後はただちに−70℃で凍結保存した。解析前にフリーズドライを行い，10倍濃縮した。濃縮後のEBC中の液性因子濃度は，Luminex® system（Hitachi, Tokyo, Japan）を用いて，既報の通り測定した[6]。測定はトリプリケートで行い，平均値を測定値とし，結果は実測値を0.1倍し原液濃度で示した。測定には27種の分子の蛋白濃度を測定できるHuman Cytokine 27-plex Panel Kit (BIO-RAD, Hercules, USA）を用いた。測定した分子は，炎症性サイトカイン：IL-1β，IL-1ra，IL-6，IL-7，IL-15，IL-17，TNF-α，IFN-γ，Th1サイトカイン：IL-2，IL-12 (p70)，Th2サイトカイン：IL-4，IL-5，IL-9，IL-13，Tregサイトカイン：IL-10，ケモカイン：Eotaxin，RANTES，

表1 患者背景

		L群 (n=22)	M群 (n=29)	H群 (n=13)
年齢	歳	56.5±3.3	51.0±3.3	51.5±5.2
性別	男：女(%)	7：15 (31.8：68.2)	8：21 (27.6：72.4)	6：7 (46.2：53.9)
喫煙歴	C：Ex：N(%)1)	5：9：8(22.7：40.9：36.4)	7：4：18(24.1：13.8：62.1)	1：5：7(7.7：38.5：53.9)
罹病期間	年	26.2±3.0	15.7±2.6*	15.9±3.9
重症度	Step	3.8±0.1	3.5±0.1	2.8±0.3**
ICS使用量	FP換算(mg)	527.3±52.0	527.6±45.3	430.8±67.7
OCS	人(%)	4 (18.2%)	6 (20.7%)	0 (0%)
テオフィリン薬†	人(%)	20 (90.9%)	17 (58.6%)	7 (53.8%)
LABA	人(%)	19 (86.4%)	24 (82.8%)	8 (61.5%)
LTRA †	人(%)	13 (59.1%)	15 (51.7%)	2 (15.4%)

L群と比較して，$^{*}p<0.05$，$^{**}p<0.01$で有意差あり。
カイ2乗検定で，†$p<0.05$で群間に有意差あり。
ACTスコア：L群：20点未満，M群：20-24点，H群25点
1) C：Ex：N：現喫煙：過去の喫煙歴有り：非喫煙
ICS：吸入ステロイド薬，FP：フルチカゾン，OCS：経口ステロイド薬，LABA：サルメテロール吸入，LTRA：ロイコトリエン受容体拮抗薬

MIP-1α，MIP-1β，MCP-1，IL-8，IP-10，FGF basic，増殖因子：PDGF-BB，VEGF，G-CSF，GM-CSF の合計 27 種である。各ターゲットに対する特異的一次抗体には，ターゲット毎に異なる蛍光強度で標識された 27 種類のビーズが結合しており，ビオチン結合特異的二次抗体，ストレプトアビジン-PE でターゲットの濃度を検出した。すなわち，濃縮した EBC をビーズ結合一次抗体と 30 分反応させ，その後二次抗体と 30 分反応，さらにストレプトアビジン-PE と 10 分間反応させ，LUMINEX® system で測定した。

2）FeNO 測定：FeNO は，標準化された方法に準じて[7]，呼気流速 50 ml/s で，ノーズクリップを装着せずに測定した。機器は，NO Analyzer（Model 280i NOA，Sievers）を用いた。

3）統計学的解析：有意差検定には一元配置分散分析法（One-factor ANOVA）を用い，有意差を認めた場合，Tukey-kramer の HSD 検定で群間比較を行った。結果は平均値±標準誤差で示した。いずれの検定においても $p<0.05$ をもって有意と判定した。また，相関解析についてはピアソン積率相関係数とその有意確率の結果を示した。各指標の閾値については，ROC（Receiver operating characteristic）曲線を描き，感度＋特異度が最良となる閾値を設定した。

結　果

対象患者の群別の背景を**表 1** に示す。年齢，性，喫煙歴については，群間で有意差を認めなかったが，罹病期間は，L 群では M 群に比して有意に長かった。また，喘息重症度 step は，H 群では，L 群に比して，有意に低く軽症であった。治療としては，全例で吸入ステロイド薬を使用中であったが，群間で吸入用量に有意差はなかった。気管支拡張薬については，テオフィリン薬，ロイコトリエン受容体拮抗薬の内服人数において群間に有意差を認め，L 群では高率に処方されていた。また，5 例で上気道症状を有し感染の合併が疑われたが，人数分布に群間有意差は認めなかった。

次に FeNO の結果を**図 1** に示す。H 群で低値傾向を認めたが，3 群間で有意差は認めなかった（**図 1A**）。ROC 曲線による解析の結果，FeNO の閾値を 55.7 ppb と定めた（**図 1C**）。ACT 20 点未満であるコントロール不良状態を予測する際の特異度は 0.857 と高く，陰性的中率 0.720 であることから（**表 2**），FeNO 低値の場合，コントロール良好である可能性が高いことが示唆された。

次に，呼気凝縮液の Luminex による解析結果を示す。27 分子中 12 分子は，20 % 以上の症例で測定可能であった（**表 3**）。測定可能率が 1-20 % の分子は 11 分子であり，4 分子は全例で測定不能であった。この中で，測定可能率が 20 % 以上であり，かつ測定平均値が 0.5 pg/ml を超える，IL-1ra，IL-1β，IP-10，VEGF についてさらに検討した。

IL-1ra（**図 2A**）および，IL-1β（**図 3A**）は，H 群で低値傾向は示したが，有意差は認めなかった。ROC 曲線により IL-1ra の閾値は 0.31 pg/ml，IL-1β の閾値は 0.23 pg/ml と定めた。双方ともコントロール不良を予測する感度は低いが，特異度，陰性的中率は約 0.7 であった（**表 2**）。

一方 IP-10 は，L 群に比して H 群で有意に高値を示し（**図 4A**），また VEGF は，L 群に比して M 群で有意に高値を示した（**図 5A**）。IP-10 については ACT スコアと有意な正の相関を示した（**図 4B**）。IP-10 の閾値を 0.78 pg/ml とすると，コントロール良好を予測する特異度，陽性的中率は 0.9 を超えており，IP-10 高値の場合，コントロール良好である可能性が極めて高い事が示された。VEGF の閾値は 0.05 pg/ml とされ，やはりコントロール良好に対する特異度，陽性的中率は 0.8 を超えた。

以上のように L 群で高値傾向をとった指標は FeNO，IL-1ra，IL-1β であり，この 3 分子間では有意な正相関を認めた（**表 4**）。また。M 群，H 群で高値傾向をとった指標は IP-10，VEGF であり，この 2 分子間では有意な正相関を認めた（**表 4**）。ACT スコアへの寄与の強さを検討するために，ACT スコア分類（コントロール良好：20 点以上，不良：20 点未満）を目的変数，IL-1ra，IL-1β，VEGF，IP-10，FeNO を説明変数として，ロジスティック回帰分析を行った。Wald カイ 2 乗 p 値は，IP-10 が最も低く（$p=0.1610$），寄与が大きいことが示された。

また，これらの因子に及ぼす治療薬の影響も検討したが，経口ステロイド薬，テオフィリン薬，

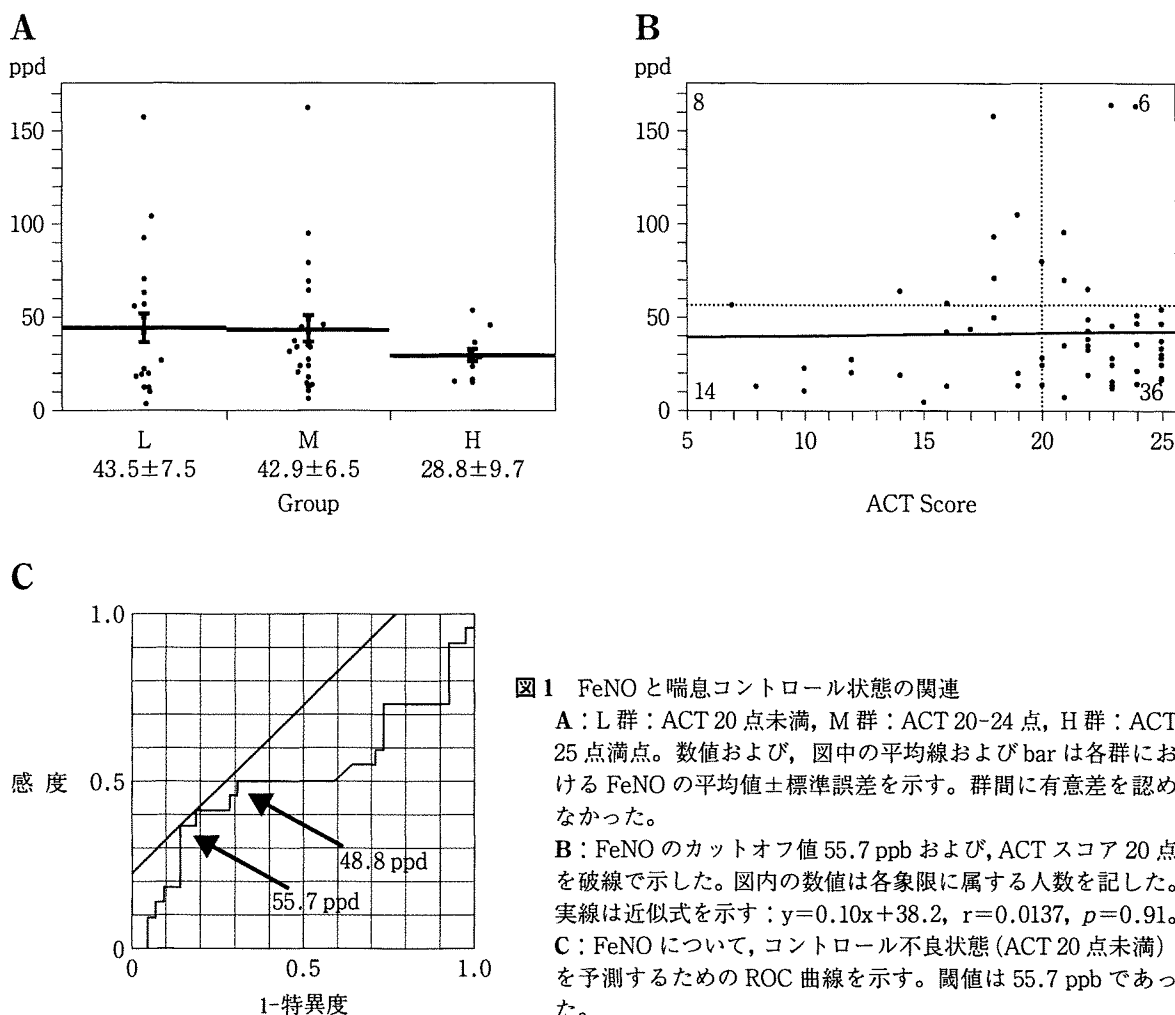

図 1　FeNO と喘息コントロール状態の関連

A：L 群：ACT 20 点未満，M 群：ACT 20-24 点，H 群：ACT 25 点満点。数値および，図中の平均線および bar は各群における FeNO の平均値±標準誤差を示す。群間に有意差を認めなかった。

B：FeNO のカットオフ値 55.7 ppb および，ACT スコア 20 点を破線で示した。図内の数値は各象限に属する人数を記した。実線は近似式を示す：$y=0.10x+38.2$，$r=0.0137$，$p=0.91$。

C：FeNO について，コントロール不良状態（ACT 20 点未満）を予測するための ROC 曲線を示す。閾値は 55.7 ppb であった。

表 2　各指標の閾値による喘息コントロール状態の予測

	指標	閾値	感度	特異度	陽性的中率	陰性的中率
FeNO	コントロール不良	55.7 ppb	0.364	0.857	0.571	0.720
IL-1ra		0.31 pg/ml	0.455	0.691	0.435	0.707
IL-1β		0.23 pg/ml	0.318	0.753	0.700	0.722
IP-10	コントロール良好	0.78 pg/ml	0.357	0.955	0.938	0.438
VEGF		0.05 pg/ml	0.429	0.864	0.857	0.442

コントロール不良：ACT 20 点未満
コントロール良好：ACT 20 点以上

長時間作用型吸入 β_2 刺激薬，ロイコトリエン受容体拮抗薬の使用の有無で，測定値に有意差は認めなかった。また，上気道症状の有無についても，各指標で有意差は認めなかった。

考　察

気管支喘息患者における非侵襲的な気道炎症マーカーとして，FeNO，EBC 中の液性因子濃度を網羅的に解析し，喘息コントロール状態との関連を解析した。FeNO および，EBC 中 IL-1β，IL-1ra はコントロール不良群で高値傾向を示し，3 分子間には有意な正相関を認めたことから，これらの指標は喘息コントロール不良マーカーと考えられた。一方，EBC 中 IP-10，VEGF はコントロー

表 3　Luminex 測定可能率（%）

測定可能率（%）	サイトカイン	%	ケモカイン	%	増殖因子	%
50<	IL-1β	93.8				
	IL-1ra	73.4				
	IL-12 (p70)	54.7	MIP-1α	51.6		
20～50	IL-6	35.9	MIP-1β	37.5	VEGF	43.8
	IL-7	35.9	IP-10	34.4	FGF basic	28.1
			Eotaxin	20.3	PDGF-BB	25.0
1～20	IL-15	17.2				
	IL-9	12.5				
	IFN-γ	10.9				
	IL-17	7.8				
	IL-2	6.3	IL-8	6.3		
	IL-10	4.7				
	IL-4	1.6	RANTES	1.6		
	IL-13	1.6				
	GM-CSF	1.6				
0	TNF-α	0				
	IL-5	0	MCP-1	0		
	G-CSF	0				

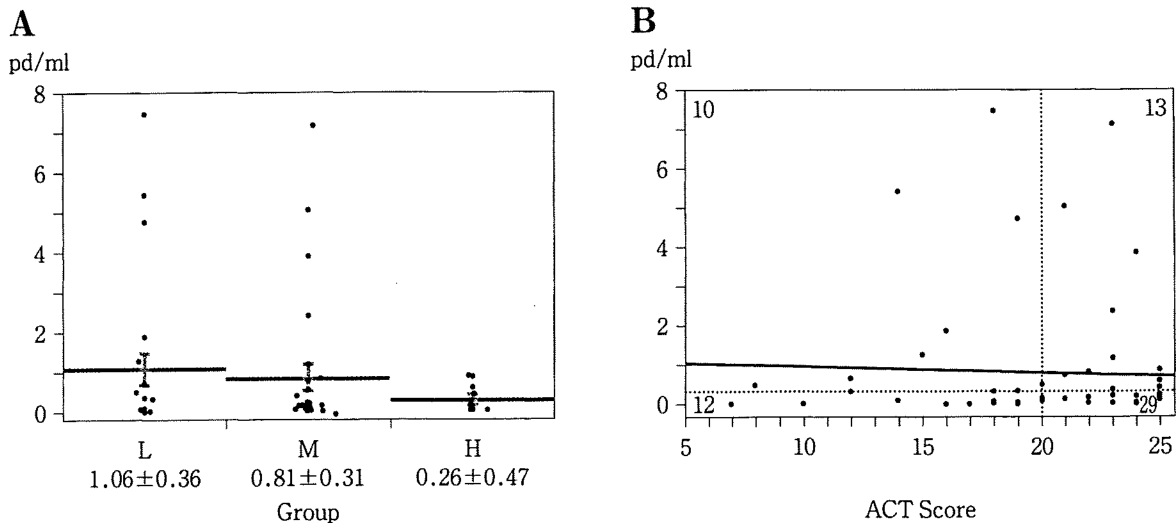

図 2　EBC 中 IL-1ra 濃度と喘息コントロール状態の関連
A：L 群：ACT 20 点未満，M 群：ACT 20-24 点，H 群：ACT 25 点満点。数値および，図中の平均線および bar は各群における IL-1ra 濃度の平均値±標準誤差を示す。群間に有意差を認めなかった。
B：IL-1ra のカットオフ値 0.31 pg/ml および，ACT スコア 20 点を破線で示した。図内の数値は各象限に属する人数を記した。実線は，近似式を示す：$y=-0.016x+1.11$，$r=-0.045$，$p=0.72$。

ル不良群で有意に低値であり，IP-10 濃度は ACT スコアと有意な正相関を示し，さらに 2 分子間には有意な正相関を認めたことから，これらの分子は喘息コントロール良好マーカーと考えられた。

FeNO 測定は非侵襲的であり，測定は 3 分程度で終了し，結果が即時に得られることから，日常

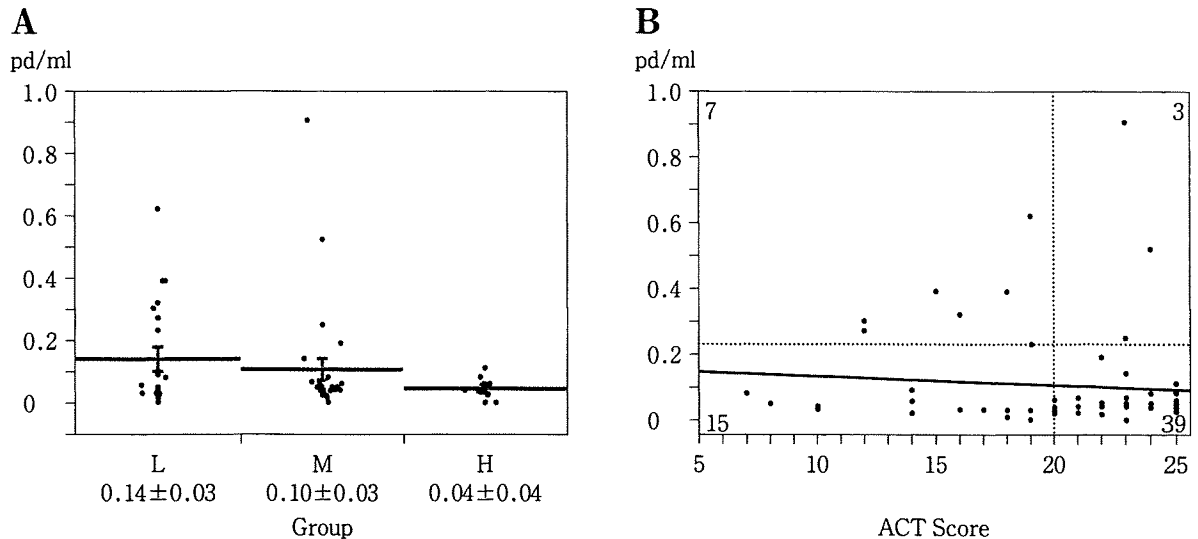

図3 EBC中IL-1β濃度と喘息コントロール状態の関連
A：L群：ACT 20点未満，M群：ACT 20-24点，H群：ACT 25点満点。数値および，図中の平均線およびbarは各群におけるIL-1β濃度の平均値±標準誤差を示す。群間に有意差を認めなかった。
B：IL-1βのカットオフ値0.23 pg/mlおよび，ACTスコア20点を破線で示した。図内の数値は各象限に属する人数を記した。実線は近似式を示す：y=－0.0028x+0.16，r=－0.08，p=0.52。

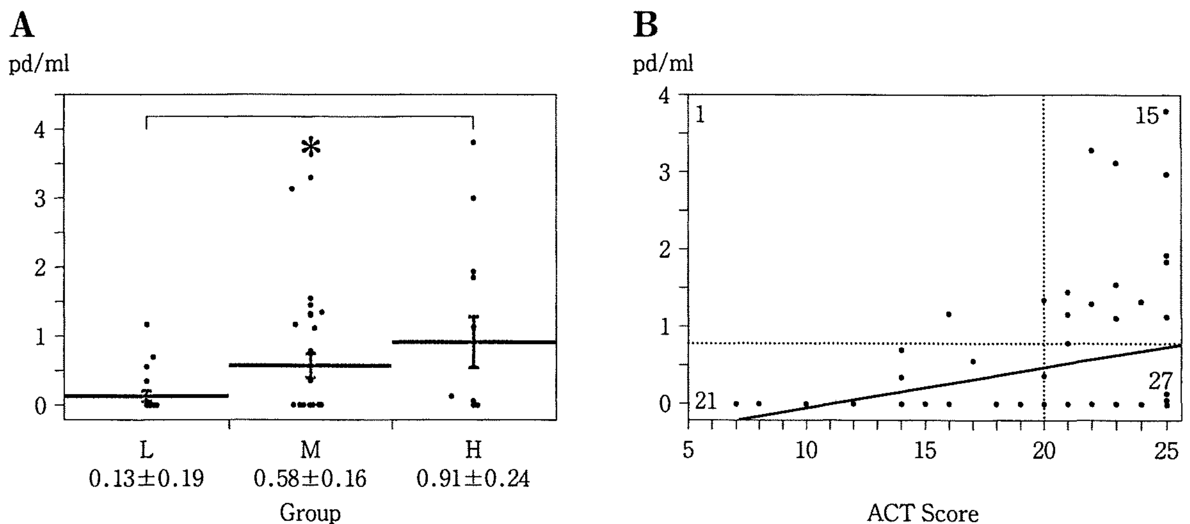

図4 EBC中IP-10濃度と喘息コントロール状態の関連
A：L群：ACT 20点未満，M群：ACT 20-24点，H群：ACT 25点満点。数値および，図中の平均線およびbarはIP-10濃度の平均値±標準誤差を示す。L群に比しH群で有意に高値であった。*p<0.05。
B：IP-10のカットオフ値0.78 pg/mlおよび，ACTスコア20点を破線で示した。図内の数値は各象限に属する人数を記した。IP-10はACTスコアと有意な正の相関を示した。実線は近似式を示す：y=－0.053x+0.58，r=0.270，*p=0.032。

臨床への応用が最も期待されている指標である。NOは，L-アルギニンからNO合成酵素（nitric oxide synthase：NOS）の作用によって産生される。FeNOは，未治療の喘息患者において健常者よりも有意に高く，喘息診断のためのカットオフ値は20-40 ppbとされている[8~10]。また，吸入ステロイド薬によりFeNO値は減少する事も報告されている[11~13]。さらに，FeNOを指標として吸入