Abstract

Eotaxin is a potent and specific eosinophil chemoattractant that is mobilized in the respiratory epithelium after allergic stimulation. Pulmonary levels of eotaxin mRNA are known to increase after allergen exposure in sensitized animals. In this study we demonstrate that TNFα and IL-1β induce the accumulation of eotaxin mRNA in the pulmonary epithelial cell lines A549 and BEAS 2B in a dose-dependent manner. Cytokine-induced A549 cell mRNA accumulation was maximal at 4 h and was significantly enhanced when the cells were costimulated with IFNγ. TNFα- and IL-1β-induced increases in eotaxin mRNA were diminished in a dose-dependent manner by the glucocorticoid dexamethasone and were augmented by the protein synthesis inhibitor cycloheximide. Cytokine-induced increases in eotaxin mRNA expression correlated with increased eotaxin protein production and secretion, and dexamethasone inhibition of cytokine-induced eotaxin mRNA augmentation was associated with diminished eotaxin protein secretion. These findings, together with the known kinetics of TNFα and IL-1β mobilization in asthmatic airways and the potent eosinophil chemotactic effects of eotaxin, define a mechanism linking inflammatory cytokine mobilization to eosinophil recruitment that may be relevant to the pathogenesis of asthma. (J. Clin. Invest. 1997; 99:1767–1773.) Key words: chemokine • eosinophil • cytokine • asthma • allergic disease

Introduction

The role of inflammation in asthma and other allergic diseases of the airways is widely appreciated, and airway inflammation is now included as a defining feature of asthma (1, 2). The importance of the presence of eosinophils in the airways of patients with fatal asthma has long been recognized (3), but the mechanisms by which these cells are recruited and retained in the lung are only now being elucidated. The importance of airway eosinophilia in less severe forms of asthma has been documented by studies demonstrating eosinophils in the airways of patients with mild asthma (4–6). These findings and the results of other studies linking eosinophil-derived granular proteins (3), arginine-rich proteins, metalloendopeptidases, and lipid metabolites to airway hyperresponsiveness support the ability of appropriately activated eosinophils to contribute to asthma symptoms. The conclusion that tissue eosinophilia is an important factor in allergic airway disease is based not only on the recognition that eosinophils are present in allergic airways but also on the correlation of the remission of asthma symptoms with the resolution of airway eosinophilia (7–10). These observations suggest that eosinophils may be necessary to the pathogenesis of asthma and make it likely that the mechanisms by which eosinophils are recruited to the airways are relevant to asthma.

An increasing but incomplete body of knowledge defines the mechanisms of eosinophil recruitment, retention, and activation in the airways. Eosinophils respond to a growing list of chemoattractants, including complement fragment C5a; platelet-activating factor (PAF); leukotriene B4; IL-2, -3, -5, and -16; and the chemokines IL-8, macrophage inflammatory protein (MIP)1-1α, monocyte chemoattractant protein (MCP)-2, MCP-3, MCP-4, RANTES, and eotaxin (11). The importance of cytokine mobilization in promoting in vivo airway eosinophil recruitment is demonstrated by the effectiveness of IL-5 antagonists in blocking airway eosinophilia after allergen exposure (12). In addition, IL-5–deficient mice do not develop airway eosinophilia, lung damage, or airway hyperreactivity, all of which follow allergen challenge of normal mice (13). The relevance of eosinophil recruitment to asthma is further suggested by studies in which IL-5–neutralizing monoclonal antibodies administered during the period of sensitization prevent the development of airway eosinophilia and hyperreactivity in rodents and primates (14).

Eotaxin is a CC (double cysteine) chemokine that was originally isolated as the predominant eosinophil chemoattractant in the lung lavage fluid of sensitized guinea pigs after allergic exposure (15). Eotaxin is unique among eosinophil-active chemoattractants in that it specifically attracts eosinophils. This specificity has recently been demonstrated in vitro for both mouse (16–18) and human (19, 20) eotaxin in chemotaxis and calcium flux assays. The in vivo potency and specificity of eotaxin have been confirmed in studies demonstrating tissue eosinophil recruitment following instillation of this chemokine.
into the airways of rodents (17, 18, 21), and following its injection into the skin of rodents (15, 17, 22). Eotaxin is thought to be important in recruiting eosinophils to the airway after allergic stimulation because the time course of its appearance is congruent with that of eosinophil recruitment (15, 18, 21, 22). Although eotaxin can be produced by several cell types, production in airway epithelial cells may be particularly relevant to allergic diseases, as eotaxin immunoreactivity is detectable in the epithelial cells of nasal polyps that are infiltrated with eosinophils (20). It is increasingly clear that the airway epithelium participates in the inflammatory process by altering its phenotype to produce a variety of mediators and cytokines, including prostaglandins (23), platelet-activating factor (24), granulocyte-macrophage colony-stimulating factor (GM-CSF) (25), IL-6 (26), IL-8 (27), MCP-1 (28), and RANTES (29). On the basis of these observations and the known upregulation of eotaxin after allergic pulmonary stimulation, we sought to define the allergic mediators that modulate the expression of eotaxin mRNA, eotaxin protein production, and eotaxin secretion in pulmonary epithelial cells.

Methods

Cell culture
A549 cells, derived from a lung adenocarcinoma with the alveolar type II cell phenotype, were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in F12K medium with 10% heat-inactivated FBS. 24 h before stimulation with cytokines, this medium was exchanged for an identical formulation not containing FBS. BEAS 2B cells, a human bronchial epithelial cell line transformed by hybrid adenovirus SV-40, were cultured in DMEM-F12 with 10% FBS. Epithelial cells grown to confluence were stimulated with geometrically increasing doses of IL-1β, TNFα, and IFNγ, or with 10 nM phorbol PMA, TGFβ, 10 ng/ml or TGFβ (5 ng/ml). In experiments involving dexamethasone or cycloheximide, the agents were added 30 min before cell stimulation. In time course experiments, the cells were harvested 1, 2, 4, 8, 16, 24, 32, and 48 h after stimulation. In concentration-response studies, the cells were harvested at the time of peak expression, which was 4 h after stimulation with IL-1β or TNFα and 2 h after stimulation with IFNγ. Protein synthesis was inhibited by the addition of cycloheximide (10 µg/ml) to the medium. This concentration of cycloheximide inhibited [35S]methionine incorporation into trichloroacetic acid-precipitable protein by > 90%.

RNA analysis
Total RNA was isolated from freshly harvested cells by guanidinium thiocyanate-phenol chloroform extraction (Stratagene Inc., La Jolla, CA). For Northern analysis, 10-20 µg of total RNA was subjected to gel electrophoresis on a formaldehyde-2% agarose gel and transferred to a nylon membrane (Schleicher & Schuell Inc., Keene, NH). After UV cross-linking, the membrane was hybridized at 42°C in 50% formamide buffer (pH 7.5), containing 10% dextran sulfate, 5× SSC, 1× Denhardt’s solution, 1% (wt/vol) SDS, 100 µg of herring sperm DNA/ml, and 20 mM Tris either with a 32P-labeled 1.1-kb HindIII fragment of the human eotaxin gene containing exon 2 and the coding portion of exon 3—with a PCR-generated fragment whose sequence was confirmed to be identical to that reported for human RANTES cDNA—or with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA). The membranes were washed for 40 min at 42°C in 2× SSC-0.1% SDS and then for 40 min at 55°C in 0.2× SSC-0.1% SDS. To control for RNA loading, the hybridization signal obtained for eotaxin was normalized to that of GAPDH for each sample. RNA expression was determined by densitometry as previously reported (22). All experiments involving the quantitation of RNA were performed at least in triplicate and a representative blot is displayed.

Detection of eotaxin protein

Generation of antieotaxin antibodies. BALB/c mice (Taconic Farms, Germantown, NY) and New Zealand White rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA) were immunized with recombinant human eotaxin protein (PeproTech, Rocky Hill, NJ). Spleen cells from immunized mice were fused to the P3X63 hybridoma cell line, and monoclonal antibodies were prepared according to standard techniques. Immune rabbit serum was purified by protein A-Sepharose (Pharmacia, Piscatway, NJ) affinity chromatography according to the manufacturer’s specifications. Immunoblot or ELISA analysis of the antieotaxin monoclonal antibody 2A12 and polyclonal antisera demonstrated that these antibodies were specific for human eotaxin. These antibodies reacted strongly to 100 ng of human eotaxin but did not react to 100 ng of human MCP-1, 2, 3, 4, MIP-1α, MIP-1β, or RANTES.

Figure 1. Eotaxin mRNA expression in cytokine treated airway epithelial cells. (A) Northern blot analysis of 20 µg total RNA isolated from untreated A549 cells or 4 h after stimulation with the indicated cytokines. Molecular size markers appear on the left of each blot. The blots were hybridized sequentially with eotaxin-, RANTES-, and GAPDH-specific gene probes. (B) Northern blot analysis of 10 µg total RNA isolated from untreated BEAS 2B cells 6 or 18 h after stimulation with 10 ng/ml TNFα, 5 ng/ml IL-1α, or both. The blots were hybridized sequentially with eotaxin-, RANTES-, and GAPDH-specific gene probes.
ELISA. Each well of a high-binding efficiency 96-well ELISA plate was coated with 100 ng of a mouse antieotaxin monoclonal antibody, designated 2A12. The plate was blocked with a 3% solution of BSA (Sigma Chemical Co., St. Louis, MO) in PBS with 0.02% sodium azide. After washing with PBS, standards or sample were added; the plate was incubated 2 h at room temperature in a humid environment and was washed again with PBS, and 1 μg of a protein A-purified fraction of a rabbit antieotaxin polyclonal serum was added to each well. The plates were washed twice with PBS after a 2-h room temperature incubation; 50 μl of a horseradish peroxidase-linked anti-rabbit IgG derived from goats (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was diluted 1:1000 in blocking buffer and added to each well. After a 90-min room-temperature incubation, the plates were developed by the 3, 3’-diaminobenzidine microwell-peroxidase substrate method according to the instructions of the manufacturer (Kirkegaard & Perry Laboratories, Inc.). Under these conditions this assay was sensitive to 50 pg/ml. Cell supernatant was harvested from 10^6 cells cultured in the absence of stimulation or 2, 4, 8, 24, or 48 h after stimulation with IL-1β; the amount of eotaxin recovered was calculated from the ELISA concentration and volume of supernatant and expressed as the amount recovered per 10^6 cells.

Figure 2. Time course of cytokine-induced eotaxin mRNA accumulation. Northern analysis of 20 μg of total RNA harvested from A549 cells at various times after treatment with 0.1 ng/ml IL-1β (A), 10 ng/ml TNFα (B), or 100 ng/ml IFNγ (C). A quantitative comparison at each time point between the eotaxin-specific signal and the GAPDH-specific signal is illustrated below A and B.

Figure 3. Dose-response of IL-1β- and TNFα-induced eotaxin mRNA accumulation. Northern analysis of 20 μg of total RNA harvested from A549 cells 4 h after stimulation with increasing concentrations of IL-1β (A) and TNFα (B). Below each blot a quantitative comparison is made at each dose between the eotaxin-specific signal and the GAPDH-specific signal.

Results

Cytokine-induced eotaxin mRNA expression

Eotaxin mRNA expression was not easily detected by Northern analysis in A549 or in BEAS 2B cells in the absence of cytokine stimulation. However, stimulation of the cells with 0.1 ng of IL-1β/ml or 10 ng of TNFα/ml for 4 h induced the accumulation of a distinct eotaxin mRNA species of ~800 bp (Fig. 1A). The addition of 10 nM PMA to the medium was associated with a faint but detectable eotaxin mRNA signal. However, eotaxin mRNA was not detected after 4 h of stimulation with IFNγ (10 ng/ml), TGFβ (10 ng/ml), or TGFβ (5 ng/ml). Similarly, RANTES expression in A549 cells was detectable 4 h after cytokine stimulation with 0.1 ng/ml IL-1β and after 10 ng/ml TNFα (Fig. 1A). Eotaxin mRNA expression in BEAS 2B cells was detectable after stimulation with 5 ng of IL-1α/ml or 10 ng of TNFα/ml with a time course similar to that of A549 cells. In BEAS 2B cells a synergistic effect of these cytokines was observed (Fig. 1B).

Time course

Treatment of A549 cells with 0.1 ng of IL-1β/ml or 10 ng of TNFα/ml induced maximal eotaxin mRNA expression at 4 h; this activity declined over the subsequent 44 h (Fig. 2A and B). When 100 ng of IFNγ/ml was added to the medium, eotaxin mRNA expression was detected as a faint band appearing 2 h after stimulation (Fig. 2C). This is in contrast to RANTES which was maximally induced by IL-1β 24 h after stimulation while maximal expression after TNFα occurred 48 h after stimulation (Fig. 1A; data not shown).

Concentration response characteristic

The addition of geometrically increasing doses of IL-1β to the medium 4 h before harvesting was associated with increasing eotaxin mRNA expression. This effect was maximal at a dose of 1 ng/ml (Fig. 3A). Likewise, the addition of increasing con-
centrations of TNFα to the medium up to a dose of 100 ng/ml was associated with increasing eotaxin mRNA accumulation (Fig. 3 B). Doses of IFNγ below 100 ng/ml produced no signal detectable by Northern analysis (data not shown).

**Effects of IFNγ**

The addition of varying concentrations of IFNγ to A549 cells stimulated with 0.1 ng/ml IL-1β (A) or 10 ng/ml TNFα (B) in the presence of increasing concentrations of IFNγ. Below each blot a quantitative comparison is made at each dose between the eotaxin-specific signal and the GAPDH-specific signal.

**Eotaxin protein levels**

**Time course.** Eotaxin protein was detected in the absence of stimulation in both the cell supernatant and the cell lysate.

**Effects of dexamethasone**

Pretreatment of A549 cells with varying concentrations of dexamethasone was associated with a dose-dependent decrease in IL-1β–induced and TNFα–induced eotaxin mRNA expression (Fig. 5, A and B). Dexamethasone treatment had no effect on cell morphology or viability.

**Effect of protein-synthesis inhibition on dexamethasone effects**

When cycloheximide was used to inhibit protein synthesis in A549 cells, eotaxin mRNA was detectable at low levels in the absence of cytokine stimulation (Fig. 6 A). Eotaxin mRNA accumulation was superinduced when A549 cells were treated with cycloheximide 30 min before IL-1β stimulation; part of the suppressive effect of dexamethasone persisted in the presence of cycloheximide (Fig. 6 A). TNFα–induced eotaxin mRNA expression was likewise enhanced in the presence of cycloheximide and was less inhibited by dexamethasone than IL-1β–induced eotaxin mRNA expression (Fig. 6 B).

**Effects of dexamethasone suppression of cytokine-induced eotaxin mRNA accumulation.** Northern analysis of 20 μg of total RNA harvested from A549 cells 4 h after the indicated treatments with 0.1 ng/ml IL-1β (A), or 10 ng/ml TNFα (B). For cells treated with cycloheximide (10 μg/ml) and/or dexamethasone (1 μM) plus cytokines, the cycloheximide and dexamethasone were added 30 min before the addition of the cytokine.

**Eotaxin protein levels**

**Time course.** Eotaxin protein was detected in the absence of stimulation in both the cell supernatant and the cell lysate.
bronchial epithelial cell lines and that this increase is associated with the intracellular accumulation of eotaxin protein and its secretion into the cell supernatant. These findings, taken together with the known kinetics of TNFα and IL-1β mobilization in asthmatic airways, the kinetics of eosinophil recruitment into human airways after segmental allergen challenge, and the potent eosinophil chemotactic effects of eotaxin, define a mechanism linking cytokine mobilization to eosinophil recruitment that may be relevant to the pathogenesis of asthma.

A growing body of evidence links IL-1β to asthma. Like TNFα, IL-1β is found at increased levels in the lung lavage fluid of patients with asthma, and in situ hybridization studies have implicated airway epithelial cells as well as alveolar macrophages in IL-1β production (30). These cells are known to display surface markers of activation and to produce significant quantities of IL-1β during episodes of nocturnal asthma at a time when eosinophil recruitment correlates with airflow obstruction (31). Histological evaluation of lung biopsy specimens from patients with nocturnal asthma has documented the presence of eosinophils predominantly in distal airway segments, where IL-1β produced by alveolar macrophages are available to epithelial cells (30). Because ciliated epithelial cells are not present in the distal airway epithelium, the alveolar epithelium (as reflected by our findings in alveolar cell lines) may be relevant to eosinophil recruitment. Our finding that IL-1β can act on airway epithelial cells to augment eotaxin mRNA expression, protein production, and secretion supports the hypothesis that IL-1β produced by alveolar macrophages induces airway epithelial cells to produce eotaxin and may explain the association among IL-1β, airway eosinophilia, and nocturnal airway narrowing.

The higher levels of eotaxin immunoreactivity in nasal epithelial cells at sites of active allergic inflammation where cytokines including TNFα and IL-1β are known to be available as opposed to adjacent noninflamed, noncytokine-expressing epithelium imply the existence of a mechanism modulating eotaxin production in allergic airway disease that may involve these cytokines (20). We found that eotaxin protein secreted from A549 cells accumulates over 24 to 48 h in cell supernatant. Because eosinophil recovery from the asthmatic lung is maximal 24–48 h after segmental allergen challenge (32, 33), our findings are compatible with the hypothesis that airway epithelial cell-derived eotaxin contributes to allergen-induced airway eosinophilia.

Although TNFα can cause the characteristic physiological phenotype of asthma, namely airway hyperresponsiveness and eosinophilia, the fact that it is not a potent chemoattractant for eosinophils suggests that its effects on eosinophil recruitment are indirect. Our observation that TNFα significantly enhances eotaxin mRNA expression at 4 h (22) and the observation that TNFα-stimulated A549 cells rapidly mobilize a substance chemotactic for eosinophils (34) are compatible with the notion that TNFα mobilized by allergic stimulation may contribute to eotaxin production and eosinophil recruitment. A549 cells were more sensitive than BEAS 2B cells to TNFα-induced augmentation of eotaxin mRNA, but the responsiveness of both cell types suggests that the eotaxin response to cytokines is not a unique feature of A549 cells. The rapid TNFα-induced mobilization of eotaxin mRNA is congruent with the time course of augmented lung eotaxin mRNA expression following allergen challenge in animal models (15, 22), while the ac-

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**Figure 7.** Eotaxin protein levels. (A) Eotaxin protein was measured in A549 cell lysate harvested at the indicated time points after stimulation with 0.1 ng/ml IL-1β in the presence and absence of 1 μM dexamethasone (n = 5, *P < 0.05). (B) Eotaxin protein was measured at the indicated time points following stimulation with 0.1 ng/ml IL-1β in A549 cell supernatant in the presence and absence of 1 μM dexamethasone (n = 5, *P < 0.05).

Lysate eotaxin protein levels were significantly increased 2 h after stimulation with 0.1 ng/ml IL-1β. They were significantly elevated at all time points measured, and attained their highest level 24 h after stimulation (Fig. 7A; n = 5, P < 0.05, compared with unstimulated cells). Cell supernatant eotaxin levels were elevated 24 h after stimulation and were increased further at 48 h (Fig. 7B; n = 5, P < 0.05, compared with unstimulated cells).

**Dexamethasone effects**

When 1 μM dexamethasone was present in the medium, cell lysate levels of eotaxin in groups of cells stimulated 24 h earlier with 0.1 ng/ml IL-1β were significantly lower than levels in cells not exposed to dexamethasone and were not different from levels in cells that had not been stimulated with IL-1β (n = 5, P < 0.05). Similarly, the presence of 1 μM dexamethasone resulted in a significant decrease in the level of eotaxin in the supernatant of cytokine-treated cells at both 24 and 48 h (n = 5, P < 0.05) compared to cytokine treated cells not exposed to dexamethasone.

**Discussion**

This study demonstrates that TNFα and IL-1β rapidly and transiently induce the accumulation of eotaxin mRNA in

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**Eotaxin in Human Airway Epithelial Cells**

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cumulation of RANTES mRNA in these cells occurs over a longer time course (Fig. 1A). The fact that the time course of RANTES mRNA expression is delayed relative to that of eotaxin implies that they have different roles.

We examined the effects of cycloheximide, given at concentrations sufficient to inhibit protein synthesis in our system, on eotaxin mRNA expression in unstimulated and cytokine-stimulated A549 cells. Protein inhibition resulted in the accumulation of eotaxin mRNA in unstimulated cells and augmented the ability of cytokines to increase eotaxin mRNA expression. These findings suggest that a protein of short half-life inhibits the transcription of eotaxin, which constitutes a mechanism by which epithelial cells prevent the expression on eotaxin. Alternatively, this protein may inhibit epithelial cell eotaxin expression by degrading eotaxin mRNA. The plausibility of the latter scenario is supported by the fact that eotaxin mRNA contains AU-rich motifs in its 3′ untranslated region that are believed to be responsible for decreasing mRNA stability and translational efficiency of cytokine and oncoprotein genes (35, 36). Our data do not allow us to distinguish between these two possibilities.

The synergistic effects of IFNγ on cytokine-induced eotaxin mRNA expression are a mechanism by which IFNγ can promote tissue eosinophilia. These findings imply that TNFα or IL-1β can interact with IFNγ produced by Th1 cells to mobilize airway epithelial eotaxin and to recruit eosinophils through mechanistic pathways that are independent of Th2 lymphocyte products. Our observations support a proallergic role for IFNγ that may be relevant to the airway inflammation and airflow obstruction that follow nonallergic conditions, such as viral infections that are known to induce airway cytokine and IFNγ production.

Glucocorticoids are antiinflammatory steroids that cause a decrement in airway eosinophilia, normalization of airway tone, and resolution of asthma symptoms (7–10). Although glucocorticoids are effective for treating asthma, they have significant adverse effects. It is therefore desirable to dissect the mechanisms of glucocorticoid action relevant to their effectiveness in suppressing the inflammation of asthma that could allow the development of more specific and less toxic effective agents. Glucocorticoids can diminish airway eosinophils by several established mechanisms, including the induction of eosinophil apoptosis (37), limitation of the production of eosinophil growth and differentiation factors (38), and alteration of the expression of cytokines (34, 38–41). The inhibition of cytokine-induced eotaxin mRNA expression and protein production in A549 cells by the glucocorticoid dexamethasone constitutes a novel eosinophil-specific mechanism by which cytokine-induced airway inflammation is suppressed by glucocorticoids. In order to determine whether glucocorticoids directly suppress eotaxin mRNA expression or whether this effect depends on new protein synthesis, we examined the suppressive effects of dexamethasone in the presence and absence of the protein-synthesis inhibitor cycloheximide. Our finding that dexamethasone exerts part of its suppressive effects in the absence of new protein synthesis is compatible with the hypothesis that glucocorticoids act directly to suppress eotaxin mRNA accumulation. The presence of AU-rich sequences in the 3′ untranslated region of eotaxin mRNA suggests that glucocorticoids may decrease mRNA stability as has been described for IFNβ (42). That dexamethasone-induced suppression of eotaxin mRNA expression is associated with suppression of eotaxin protein production and secretion supports the hypothesis that part of the inhibitory effect of glucocorticoids on airway eosinophil presence is due to the inhibition of eotaxin production by airway epithelial cells.

In summary, the proinflammatory cytokines TNFα and IL-1β induce eotaxin mRNA expression, protein production, and secretion in A549 cells, a pulmonary epithelial cell line. The induction of eotaxin by cytokines that are known to be mobilized in asthma and the time course of this induction are compatible with the hypothesis that cytokines released near distal airways drive epithelial-cell eotaxin production, which should cause eosinophil recruitment. Cytokine-induced eotaxin mRNA expression is enhanced by IFNγ and cycloheximide and is suppressed by dexamethasone. The ability of dexamethasone to suppress eotaxin production and secretion in airway epithelial cells defines a novel mechanism of glucocorticoid effect that may be relevant to eosinophilic diseases of the airways. These studies provide a mechanistic link between cytokine mobilization and airway eosinophil recruitment relevant to asthma and support the hypothesis that eotaxin production in airway epithelial cells can be driven by cytokines and suppressed by dexamethasone.

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