IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK

PAUL E. MOORE,¹ TRUDI L. CHURCH,¹ DAVID D. CHISM,¹ REYNOLD A. PANETTIERI, JR.,² AND STEPHANIE A. SHORE¹

１Physiology Program, Harvard School of Public Health, Boston, Massachusetts 02115; and ²Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received 12 July 2001; accepted in final form 13 November 2001

Moore, Paul E., Trudi L. Church, David D. Chism, Reynold A. Panettieri, Jr., and Stephanie A. Shore. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. Am J Physiol Lung Cell Mol Physiol 282: L847–L853, 2002. First published November 16, 2001; 10.1152/ajplung.00245.2001.—Human airway smooth muscle (HASM) cells express interleukin (IL)-13 and IL-4 receptors and respond to these cytokines with signal transducer and activator of transcription-6 and extracellular signal-regulated kinase (ERK) activation. The purpose of this study was to determine whether IL-13 and/or IL-4 influence eotaxin release in HASM cells and whether the ERK mitogen-activated protein (MAP) kinase pathway is involved in these events. Eotaxin release into HASM cell supernatants was assayed by ELISA, and eotaxin mRNA expression was determined by Northern blot analysis. Pretreatment with either IL-13 or IL-4 resulted in a concentration- and time-dependent release of eotaxin, although IL-4 was more effective. Eotaxin release was approximately twice baseline after treatment with 50 ng/ml IL-13 or IL-4 (P < 0.001). IL-13 and IL-4 also acted synergistically with tumor necrosis factor (TNF)-α to induce eotaxin release: TNF-α alone (10 ng/ml for 24 h) resulted in an approximately fourfold increase in eotaxin release, whereas TNF-α in combination with IL-13 or IL-4 resulted in 10- or 20-fold increases (P < 0.05). Similar results were obtained for eotaxin mRNA expression. Pretreatment with either U-0126 (10 μM) or PD-98059 (30 μM), both inhibitors of MAP/ERK kinase, the enzyme upstream of ERK, inhibited IL-13- or IL-4-induced eotaxin release (P < 0.05). U-0126 also inhibited IL-13, and TNF-α induced mRNA expression. Our results indicate that IL-13 and IL-4 cause eotaxin release in HASM cells through a mechanism that, in part, involves ERK activation and suggest that the smooth muscle may be an important source of chemokines leading to eosinophil recruitment in asthma.

U-0126; PD-98059; tumor necrosis factor-α; asthma; extracellular regulated kinase; interleukin

Asthma is characterized by reversible airway obstruction, bronchial hyperresponsiveness, and airway inflammation, including infiltration with lymphocytes and eosinophils (15). Eotaxin, a C-C (β) chemokine, likely plays an important role in the eosinophilia of asthma. Eotaxin was first identified as an important chemoattractant for eosinophils in antigen-sensitized and -challenged guinea pig lungs (24). Subsequent experiments in murine models of airway inflammation confirmed these findings (13, 43). Importantly, antigen-sensitized and -challenged, eotaxin-deficient mice exhibit a marked decrease in eosinophil recruitment into the airways compared with wild-type mice (42). Furthermore, eotaxin is upregulated in bronchoalveolar lavage fluid and airways of asthmatic patients (25, 34, 44). Expression of eotaxin mRNA and protein was recently shown to be upregulated after segmental allergen challenge in subjects with atopic asthma (29).

Despite the importance of eotaxin in eosinophil recruitment, the regulation of eotaxin expression in the asthmatic airway remains to be established. In vitro studies in epithelial and endothelial cells have demonstrated that the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α increase eotaxin expression (10, 30). The T helper type 2 (Th2) cytokines IL-13 and IL-4, which have been implicated in asthma, have been also shown to induce eotaxin expression in dermal and pulmonary fibroblasts and in airway epithelial cells (36, 46). Animal models also support a role for IL-13 and IL-4 in eotaxin release and eosinophil recruitment. Mice genetically deficient in IL-4 or IL-4 receptors and animals in which endogenous IL-13 or IL-4 is neutralized have reduced eosinophilia on allergen sensitization and challenge (5, 14, 41). Furthermore, exogenous administration of IL-4 or IL-13 (28) or transgenic overexpression of IL-13 in lungs of mice results in increased pulmonary expression of eotaxin (47).

It has recently become apparent that the function of airway smooth muscle is not limited to contraction and subsequent airway narrowing. Rather, airway smooth muscle has a number of other functions, including the production of cytokines and chemokines, such as regulated on activation normal T-cell expressed and secreted (RANTES), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-8, and monocyte che-
moattractant protein (MCP) (20, 40). Using immunohistochemistry of airway sections from subjects with asthma, Ghaffar et al. (11) recently reported that airway myocytes also express eotaxin. They also showed, as have others (6, 39), that human airway smooth muscle (HASM) cells in culture also produce eotaxin constitutively and that eotaxin expression is upregulated by TNF-α and IL-1β. Furthermore, eotaxin accounts for a significant proportion of the eosinophil chemoattractant activity produced by airway smooth muscle cells (11). Hamid et al. (17) observed eosinophils in and around airway smooth muscle in sections of surgically resected lungs from asthmatic subjects. Collectively, these findings suggest that HASM cells can contribute to airway inflammation in asthma through the expression, production, and release of eotaxin (11).

We recently reported that HASM cells express IL-4 and IL-13 receptors and can respond to IL-4 and IL-13 with increases in signal transducer and activator of transcription (STAT)-6 and extracellular signal-regulated kinase (ERK) phosphorylation (27). The purpose of this study was to determine whether IL-4 and IL-13 can also induce eotaxin expression in HASM cells and to begin to examine their mechanism of action. To that end, we examined the effects of IL-4 and IL-13 alone or in combination with TNF-α on eotaxin release in HASM cells supernatants. We also examined the effects of IL-4 and IL-13 on eotaxin mRNA expression. Because both IL-4 and IL-13 induce ERK phosphorylation and because ERK has been demonstrated to be important for other effects of IL-13 in HASM cells (27), we also examined the effect of ERK inhibition on IL-13- and IL-4-induced eotaxin expression.

METHODS

Cell culture. Human tracheas were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Tracheal smooth muscle cells were harvested from the tracheas as previously described (26, 27, 38). Cells were plated in plastic flasks at 10^4 cells/cm² in Ham’s F12 media supplemented with 10% FBS, 10^5 U/ml penicillin, 1 mg/ml streptomycin, 2 mg/ml amphotericin B, 12 mM NaOH, 1.7 μM CaCl_2, 2 mM l-glutamine, and 25 mM HEPES. Culture medium was replaced every 3–4 days. Cells were passaged with 0.25% trypsin and 1 mM EDTA every 10–14 days. Confluent serum-deprived HASM cells in passages 5 to 7 were used in the studies described below. When cells were serum deprived, they were supplemented with 5.7 μg/ml insulin and 5.0 μg/ml transferrin 24 h before use. Twenty-four hours after serum deprivation, cells were treated with mitogen-activated protein (MAP)/ERK kinase (MEK) inhibitors U-0126 (10 μM) (8) or PD-98059 (30 μM) (2, 7) and/or cytokines (IL-13, IL-4, or TNF-α). Because U-0126 and PD-98059 were dissolved in DMSO, control wells were treated with DMSO to yield an equivalent concentration (0.03% in experiments with PD-98059 and 0.01% in experiments with U-0126).

Eotaxin release. The supernatant was removed 24 h after treatment with cytokine and/or MEK inhibitor and stored at −20°C, except in experiments testing the time-dependent release of eotaxin. In those experiments, the supernatant was removed after 12 or 48 h. Eotaxin levels were measured by ELISA by using the Quantikine Human Eotaxin Immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

Eotaxin mRNA expression. Total RNA was extracted 24 h after treatment with cytokine and/or U-0126 by using RNAgent Total RNA Isolation System (Promega, Madison, WI). For Northern analysis, 10 μg of total RNA was subjected to gel electrophoresis on a formaldehyde-1.2% agarose gel and transferred to a nylon membrane (Schleicher & Schuell, Keene, NH). After ultraviolet cross-linking, the membrane was hybridized at 68°C in Express Hyb solution by using the Express Hyb Protocol (Clontech, Palo Alto, CA) and with either a 32P-labeled 400-bp EcoRI fragment of the human eotaxin gene (gift of C. Lilly, Boston, MA) or a 32P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA). The membranes were washed for 40 min at room temperature in 2× SSC-0.05% SDS and then for 40 min at 50°C in 0.1× SSC-0.1% SDS. RNA expression was determined by densitometry, and the control for RNA loading, the hybridization signal obtained for GAPDH was normalized to that obtained for GAPDH with each sample.

Statistics. The effect of cytokotks, ERK inhibitors, or their combination on eotaxin release was assessed by ANOVA by using experimental day and drug treatment as main effects. Follow-up paired t-tests were performed to compare among drug treatment groups. A P value of <0.05 was considered significant.

RESULTS

Pretreatment with IL-13 or IL-4 resulted in a concentration- and time-dependent release of eotaxin in HASM cells (Fig. 1). Concentrations of IL-4 as low as 1 ng/ml resulted in significant eotaxin release above baseline, whereas 30 ng/ml IL-13 was required to elicit this effect (Fig. 1A). At 50 ng/ml, IL-13 and IL-4 each induced an approximately twofold increase in eotaxin release above baseline. Eotaxin release was apparent as early as 12 h after addition of IL-4 and increased progressively with time up to 48 h (Fig. 1B). For IL-13, eotaxin release peaked at 24 h and showed no subsequent increase at 48 h.

IL-13 and IL-4 (50 ng/ml for 24 h) also acted synergistically with TNF-α (10 ng/ml) to induce eotaxin release in HASM cells (Fig. 2). TNF-α alone resulted in an approximately fourfold increase in eotaxin release, consistent with the observations of Ghaffar et al. (11), whereas TNF-α in combination with IL-13 or IL-4 resulted in a 10- or 20-fold increase (P < 0.05). The effects of IL-13 and IL-4 appeared to be the result of increased eotaxin mRNA expression (Fig. 3). Eotaxin mRNA expression was barely detectable in untreated (control) cells but increased significantly in response to
IL-13 or IL-4 (50 ng/ml each for 24 h). TNF-α (10 ng/ml) also increased eotaxin mRNA expression, and IL-4 and IL-13 each had a synergistic effect on TNF-α-induced eotaxin mRNA expression. As was the case for eotaxin release, IL-4 augmented TNF-α-induced eotaxin mRNA to a greater extent than IL-13.

We previously reported that IL-4 and IL-13 induce ERK activation in HASM cells (27). To determine whether the ability of IL-4 and IL-13 to cause ERK phosphorylation is required for their ability to induce eotaxin release, we examined the effect of the MEK inhibitors PD-98059 and U-0126 (Fig. 4). We previously reported that U-0126 and PD-98059 are effective and relatively selective inhibitors of the ability of MEK to phosphorylate ERK in HASM cells (26). Neither U-0126 (10 μM) nor PD-98059 (30 μM) alone had a significant effect on eotaxin release in untreated (control) wells. Both MEK inhibitors caused a significant and substantial reduction in IL-4- and IL-13-induced eotaxin release, but neither drug completely abolished the cytokine-induced release of eotaxin. The role of ERK appears to be upstream of eotaxin message expression, since U-0126 also inhibited the ability of IL-13 to increase eotaxin mRNA (Fig. 5). U-0126 also inhibited the ability of TNF-α to increase eotaxin mRNA (Fig. 6), suggesting that the effects of TNF-α on eotaxin release are also, in part, mediated through ERK. A similar role for ERK in IL-1β-induced eotaxin release has been recently reported by others (16). In contrast, U-0126 had no effect on eotaxin mRNA in untreated cells (Fig. 6B).
DISCUSSION

Our results suggest a mechanism whereby airway smooth muscle cells may contribute to the eosinophilia observed in asthmatic airways. Pretreatment of HASM cells with the Th2 cytokines IL-13 or IL-4, especially in combination with TNF-α, resulted in substantial release of the potent eosinophil chemotactic factor, eotaxin, into cell supernatants (Figs. 1 and 2). Taken together with the observations that IL-4 and IL-13 are present in asthmatic airways, that eosinophils are observed in and around airway smooth muscle in asthmatic airways (17), and that eotaxin accounts for a significant proportion of the eosinophil chemotactic activity produced by HASM cells (11), the results suggest that, in asthma, IL-4 and IL-13 may act on HASM cells to produce eotaxin, which results in eosinophil chemotaxis into the airway smooth muscle microenvironment. The effects of IL-13 and IL-4 appeared to be the result of increased eotaxin mRNA expression (Fig. 3). Our results also suggest that the ERK MAP kinase pathway is involved in eotaxin release, since inhibitors of this pathway significantly reduced IL-13- and IL-4-induced eotaxin release (Fig. 4), as well as IL-13- or TNF-α-induced eotaxin mRNA expression (Figs. 5 and 6).

On IL-4 binding, the IL-4 receptor (IL-4Rα) can dimerize either with the IL-2R common gamma chain (γc) to form the type I IL-4 receptor or with the IL-13 receptor IL-13Rα1 to form the type II IL-4 receptor. Both these receptor dimers are capable of initiating signaling. We have previously shown that HASM express IL-4Rα and IL-13Rα1 but not γc (27). Thus IL-13Rα1 is the sole dimerizing partner for IL-4Rα in these cells. IL-13 also signals through the type II IL-4 receptor. Another IL-13 binding receptor, IL-13RαII, is expressed in HASM cells (27) but has a very short cytoplasmic tail, does not dimerize with either IL-13Rα1 or IL-4Rα, and lacks the ability to initiate cytoplasmic signaling (9). Thus in HASM cells, the effects of both IL-13 and IL-4 appear to occur via the type II IL-4 receptor.

On dimerization of IL-4Rα with IL-13RαI, janus kinases (JAKs), a family of tyrosine kinases that constitutively associate with these receptors, become phosphorylated and activated and subsequently phosphorylate tyrosines on the receptors themselves. Phosphorylation of the IL-4Rα allows monomers of the transcription factor STAT-6 to dock with the receptor and become phosphorylated by JAKs, whereon STAT-6 is released from the receptor, dimerizes with other phosphorylated STAT-6 molecules, and is translocated to the nucleus. IL-13Rα1 does not contain STAT-6-binding domains, so that IL-13-induced activation of
STAT-6 is the result of its ability to induce IL-13Rα1 dimerization and activation of IL-4Rα. We have previously reported that both IL-13 and IL-4 induce STAT-6 activation in HASM cells (27).

IL-13 and IL-4 can also induce activation of other signaling pathways. For example, phosphorylation of the IL-4Rα results in its interaction with insulin receptor substrate 1 and 2 (IRS-1/2), which then also becomes phosphorylated. Phosphorylated IRS-1/2 is capable of interacting with SH2 domains of the adaptor protein Grb2. Grb2 is constitutively complexed to Sos, which activates Ras, leading to Raf activation and subsequent activation of the ERK MAP kinase pathway, although activation of ERK is not observed in all cell types (1, 22), suggesting that IRS/Grb/Sos activation is not sufficient for activation of ERK. We previously reported that both IL-13 and IL-4 induce ERK phosphorylation in HASM cells (27). We also reported that ERK activation appears to be required for IL-13-induced β-adrenergic hyporesponsiveness in HASM cells (27). ERK also appears to be required for IL-4-induced increases in phosphodiesterase 4 in FDCP2 myeloid cells (1) and for IL-4 induced 3β-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase activation in breast cancer cells (12). Consequently, we examined the role of ERK in the ability of IL-13 and IL-4 to induce eotaxin expression in these cells. Our results indicate that the MEK inhibitors U-0126 and PD-98059 significantly reduce but do not abolish IL-4- and IL-13-induced eotaxin release (Fig. 4) and that U-0126 also inhibits IL-13-induced eotaxin mRNA expression (Fig. 5). These results suggest that ERK is involved in eotaxin release caused by IL-13 and IL-4.

We previously reported that at the concentration used in this study, U-0126 effectively abolishes IL-13-induced ERK phosphorylation (26), so that the inability of this agent to completely abolish IL-13- and IL-4-induced eotaxin release is not the result of a lack of efficacy of this compound, although PD-98059 is less effective (26). These results suggest that a pathway other than the MAP kinase pathway likely also contributes to eotaxin release by IL-13 and IL-4. We cannot exclude the possibility that the effects of U-0126 and PD-98059 might be the result of nonspecific effects on enzymes other than MEK1/MEK2. However, other investigators have demonstrated that U-0126 and PD-98059, at the concentrations used in this study, do not inhibit activation of MAP kinase kinase (MKK)-4, protein kinase C, csk2, c-Jun NH2-terminal kinase, MKK-3, or p38 (2, 7, 8). Furthermore, the observation that the two chemically distinct MEK inhibitors had similar effects on eotaxin release makes it less likely that the effects are nonspecific.

We do not know the precise role of ERK in the eotaxin release that is induced by IL-4 and IL-13. However, the observation that U-0126 also inhibited IL-13-induced eotaxin mRNA expression (Fig. 5) suggests that the role of ERK could be at the level of gene transcription. For example, it is possible that ERK induces the activation of transcription factors important for induction of eotaxin expression. Such a mechanism would also account for the effects of U-0126 on TNF-α-induced eotaxin mRNA expression (Fig. 6). The eotaxin promoter contains putative consensus transcription binding sites for activator protein (AP)-1 and nuclear factor for IL-6 (NF-IL6; C/EBPβ) (10, 19). ERK is believed to be capable of activating AP-1 by phosphorylating Elk-1, a transcription factor that leads to the induction of c-fos, one of the components of AP-1 (35). ERK has also been demonstrated to cause the phosphorylation of NF-IL6, an event that may increase its transcriptional activity (37). The promoter of the eotaxin gene contains a STAT-6-binding element that binds STAT-6 after IL-4 or IL-13 stimulation in airway epithelial cells (32, 33), and mutation of this element abolishes the ability of IL-4 to induce luciferase activity in airway epithelial cells transfected with an eotaxin promoter-luciferase reporter construct. Because IL-4 and IL-13 also induce activation of STAT-6 in HASM cells (27), it is possible that STAT-6 binding to the eotaxin promoter accounts for that part of the increase in eotaxin expression that is not inhibited by MEK inhibitors. However, it should be noted that IL-13 and IL-4 are equally effective in inducing STAT-6 phosphorylation in HASM cells (27), whereas IL-4 is clearly more effective in inducing eotaxin release (Fig. 1).

IL-13 and IL-4 not only increased eotaxin expression on their own but also synergized with TNF-α to induce eotaxin release: the amount of eotaxin released in response to the combination of TNF-α and IL-4 or TNF-α and IL-13 was greater than the sum of the eotaxin release caused by TNF-α and IL-13 or IL-4 alone (Fig. 2). It is possible that the synergy between TNF-α and IL-4 or IL-13 is the result of the ability of TNF-α to upregulate IL-4Rα expression, as has been demonstrated in endothelial cells (31). However, in a preliminary report, Hirst et al. (21) were unable to demonstrate any increased expression of IL-4Rα in HASM cells after stimulation with IL-1β, which has a similar though not identical profile of action in these cells. Another preliminary report suggests that TNF-α can increase γc expression in human bronchial smooth muscle cells (45). Although such a mechanism might explain the increased ability of IL-4 to induce eotaxin release in TNF-α-stimulated cells, it could not account for the effects of IL-13, which does not use this receptor.

The marked augmentation of IL-4- and IL-13-induced eotaxin mRNA expression caused by TNF-α (Fig. 3) suggests that the synergistic effects of TNF-α with IL-13 or IL-4 on eotaxin release could be transcriptionally mediated. The STAT-6-binding element in the eotaxin promoter overlaps with a consensus binding site for nuclear factor (NF)-κB (33), and this NF-κB-binding domain appears to be important for TNF-α-induced eotaxin expression in other cell types (23, 33). TNF-α induces NF-κB activation in HASM cells (3), and it is possible that simultaneous binding of both STAT-6 and NF-κB to this region of the eotaxin promoter results in enhanced promoter activity. However, Matsukura et al. (32, 33) have demonstrated only ad-
ditive effects of TNF-α and IL-13 or IL-4 on eotaxin promoter activity in airway epithelial cells. They suggested that posttranscriptional effects might instead contribute to the synergy observed between the two cytokines at the message level (32, 33).

Our results indicate that IL-4 is much more potent at inducing eotaxin release than is IL-13 (Figs. 1–3). Although this is perhaps surprising, given the common signaling receptor for IL-13 and IL-4 in HASM cells, IL-4 and IL-13 have been shown to differ in other effects on HASM cells. For example, we recently demonstrated that IL-13, but not IL-4, significantly reduced β-adrenergic responsiveness in HASM cells (27). Other investigators have shown that IL-4 inhibits IL-1β induced MCP-1 and MCP-2 expression, whereas IL-13 does not (40). IL-4 has been shown to inhibit HASM cell mitogenesis induced by growth factors (18), whereas a recent preliminary report indicates that IL-13 causes mitogenesis (4). We do not yet know whether IL-4 and IL-13 differ in their ability to activate ERK, since our previous data investigated the effects of only one maximally effective concentration of each cytokine (27). However, given the importance of ERK in eotaxin release, such a difference could explain the greater efficacy of IL-4.

In summary, we have demonstrated that the Th2 cytokines IL-13 and IL-4 induce eotaxin release in HASM cells and that TNF-α augments this effect. Indeed, when administered together, TNF-α and IL-4 or IL-13 result in the generation of very substantial concentrations of eotaxin. Although other cell types, such as epithelial cells, are also capable of generating eotaxin, these results suggest that the smooth muscle cell may be an important source of eotaxin in a milieu, such as the asthmatic airway, that is rich in both Th2 and inflammatory cytokines and that HASM cells may contribute to the influx of eosinophils that is observed in asthmatic airways.

This study was supported by National Institute of Heart, Lung, and Blood Grants HL-56383, HL-33009, and HL-04395; the American Lung Association; and the Charles H. Hood Foundation.

REFERENCES


20. Jedrziewicz S, Nakamura H, Silverman ES, Laster AD, Mansharamani N, In KH, Tamura G, and Lilly CM. IL-1β...


