Differential regulation of eotaxin expression by TNF-α and PMA in human monocytic U-937 cells

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Nakamura, Hidetoshi, Kathleen J. Haley, Toshiko Nakamura, Andrew D. Luster, and Craig M. Lilly. Differential regulation of eotaxin expression by TNF-α and PMA in human monocytic U-937 cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L601–L610, 1998.—Regulation of eotaxin expression was investigated in U-937 cells, a human monocytic cell line. Eotaxin mRNA was induced by tumor necrosis factor-α (TNF-α; 0.1–100 ng/ml) and phorbol 12-myristate 13-acetate (PMA; 0.01–1 µM). PMA-induced eotaxin mRNA expression was of greater magnitude and was maximal at a later time point than TNF-α-induced expression (16 h vs. 2 h after stimulation), which was consistent with eotaxin protein expression detected by immunocytochemistry. Dexamethasone (0.01–10 µM) decreased eotaxin mRNA expression in both TNF-α- and PMA-stimulated U-937 cells. PMA-induced eotaxin mRNA expression was inhibited by cycloheximide (10 µg/ml), whereas TNF-α-induced expression was not. The protein kinase C (PKC) inhibitor staurosporine (10–50 nM) inhibited PMA-induced eotaxin mRNA expression, whereas TNF-α-induced expression was enhanced by this reagent. These results suggest that eotaxin expression can be induced by more than one mechanism: the PMA-triggered pathway is mediated by PKC activation and requires new protein synthesis, whereas the TNF-α-triggered pathway is independent of PKC and protein synthesis. TNF-α- and PMA-induced pathways are both associated with nuclear factor-κB, because its binding activity was enhanced in the presence of these stimuli, and both pathways were limited by its inhibitor, diethylthiocarbamate.

- Monocytic cells are known to be an important source of chemokines such as IL-8 and MCP-1 (5, 21). Alveolar macrophages are the dominant mononuclear cell form in the air spaces and are an important source of cytokines that are associated with airway inflammation and allergic disease of the airways (4). Recent reports have demonstrated that, in the inflamed human airway, eotaxin is produced by both epithelial cells and mononuclear cells present in the subepithelial layer (16, 24). We have recently reported the regulatory mechanisms of eotaxin expression in human lung epithelial cells (17), but the regulation of eotaxin expression in monocytic cells has not yet been elucidated. We therefore studied the mechanisms by which eotaxin expression is regulated in a human monocytic-like cell line.

- The mechanisms that govern the regulation of eotaxin expression in inflammatory cells are relevant to the pathophysiology of eosinophilic inflammation. Differences in the mechanisms that drive eotaxin expression at distinct tissue loci may be important for the creation of chemotactic gradients of eotaxin that govern eosinophil distribution in the tissues. We have demonstrated that proinflammatory cytokines including IL-1β and tumor necrosis factor-α (TNF-α) induce eotaxin expression in human lung epithelial cells, which is enhanced in the presence of interferon-γ (IFN-γ) and suppressed by dexamethasone (17). In addition to these cytokines, lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) are known to be potent inducers of cytokines in monocytic cells (21, 22). PMA is also a well-established protein kinase C (PKC) activator that can modulate cytokine expression (2). To determine differences among airway epithelial cells and other mononuclear cell types known to produce eotaxin in the human airways, we examined the mechanisms by which these cytokines and agents influence eotaxin expression in U-937 cells.

Eosinophil recruitment into tissues is known to be a feature of parasitic infection and allergic inflammation (3, 32). Advances in eosinophil biology have included the discovery of cytokines that are chemotactic for eosinophils: regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein (MCP)-3, MCP-4, and interleukin (IL)-16 (9, 13, 26). Most of these cytokines are not specific for eosinophils because they act through receptor systems that are present on a variety of cell types (13, 14). Eotaxin is a unique member of the C-C family of chemokines that selectively recruits eosinophils into the skin of guinea pigs and primates (7, 24). The mechanism of this selective effect on eosinophils is thought to relate to the high affinity of eotaxin for the chemokine receptor-3 (CCR-3), which is expressed predominantly on eosinophils (8, 14).

MATERIALS AND METHODS

- U-937 cell culture. U-937 cells, which are derived from human histiocytic lymphoma cells and have monocytic-like characteristics (1), were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) at a concentration of 2 × 10⁹ cells/ml. The cells were then stimulated with geometrically increasing doses of TNF-α, PMA, IL-1β, IFN-γ, IL-4, or LPS. In experiments involving dexamethasone, cycloheximide, staurosporine, or diethylthiocarbamate (DETC), the agents were added 30 min before cell stimulation. Viability was >95% in the cells treated with dexamethasone, cycloheximide, staurosporine,
or DETC after culture for 16 h. In the experiments with cycloheximide, >90% of radiolabeled methionine uptake was inhibited. In the time-course experiments, cells were harvested 1, 2, 4, 8, 16, 24, and 48 h after stimulation. In concentration-response studies, the cells were harvested at the determined times of peak expression, which were 2 h after stimulation with TNF-α and 16 h after stimulation with PMA. All cell stimulation experiments were performed at least in duplicate.

Human monocyte and epithelial cell culture. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of three healthy platelet donors by density gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, MO). This procedure yielded ~5 × 10^8 cells from each donor, with a PBMC purity of >98%. None of the subjects had allergic disease or peripheral blood eosinophilia (eosinophil percentages were <5%), and all gave informed written consent with the prior approval of the appropriate institutional review board. PBMCs were cultured in RPMI 1640 with 10% type AB human serum (Sigma) on 10-cm culture plates (Falcon 3003, Becton Dickinson Laboratories, Lincoln Park, NJ) overnight at a concentration of 5 × 10^6 cells/ml. After nonadherent cells were removed, adherent cells were washed three times with PBS and designated as monocytes. Monocyte purity was judged to be >80% by microscopic examination.

A549 cells, derived from a lung adenocarcinoma with the alveolar type II cell phenotype, were obtained from the American Type Culture Collection. The cells were cultured in F-12K medium with 10% FBS. Twenty-four hours before stimulation when the cells were grown to confluence, the medium was exchanged for an identical formulation not containing FBS.

Monocytes and A549 cells were cultured in the absence or presence of TNF-α (10 ng/ml) and PMA (0.1 µM) for 4 h. In experiments involving cycloheximide (10 µg/ml), staurosporine (50 nM), or DETC (1 mM), the agents were added 30 min before cell stimulation. Viability was >95% in the cells treated with these reagents for 4 h. In the experiments with cycloheximide, >90% of radiolabeled methionine uptake was inhibited in PBMCs and A549 cells. All cell stimulation experiments were performed at least in duplicate.

TNF-α immunoassay. TNF-α concentrations in the culture supernatant of U-937 cells (2 × 10^6/ml) stimulated with 0.1 µM PMA were measured by a sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The minimum detectable concentration was 4.4 pg/ml. The culture supernatant was collected 0, 1, 2, 4, 8, 16, 24, and 48 h after stimulation with PMA (n = 4).

Neutralization of TNF-α. U-937 cells were stimulated with 0.1 µM PMA for 16 h in the absence or presence of an anti-human TNF-α neutralizing antibody or a control monoclonal antibody (both mouse IgG1, R&D Systems) in duplicate. The antibodies (1 µg/ml) were added to the cells simultaneously with PMA. The 50% effective neutralizing dose (ND50) of this neutralizing antibody is reported in the manufacturer’s instructions to be 0.02–0.04 µg/ml for 0.25 ng TNF-α/ml in murine L929 cells.

To confirm the neutralizing ability of the antibody for TNF-α-induced eotaxin expression in U-937 cells, the neutralizing or control antibody (1 µg/ml) was incubated with TNF-α (1 or 10 ng/ml) for 1 h at 37°C. After incubation, the cells were stimulated with the mixture for 2 h. Isolated total RNA was then subjected to Northern analysis.

RNA analysis. Total RNA was isolated from freshly harvested cells by guanidinium-thiocyanate-phenol chloroform extraction (Stratagene, La Jolla, CA). For Northern analysis, Fig. 1. Northern blot analyses of eotaxin mRNA expression in U-937 cells. A: effects of various stimuli on eotaxin mRNA expression 4 h after stimulation. B: time courses of eotaxin mRNA expression after stimulation with tumor necrosis factor (TNF-α) and phorbol 12-myristate 13-acetate (PMA). C: concentration (Conc)-response effects of TNF-α and PMA on eotaxin mRNA expression. Cells were harvested 2 h after stimulation with TNF-α and 16 h after stimulation with PMA in these concentration-response studies. Each blot was hybridized sequentially with eotaxin- and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA probes. A representative series of blots is shown in A and B (n = 3) and C (n = 2). LPS, lipopolysaccharide; IL, interleukin; IFN, interferon; US, unstimulated.
20 µg of total RNA were subjected to gel electrophoresis on a formamide-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 ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) with a 32P-labeled 0.35-kb cDNA probe containing the entire coding region of the human eotaxin gene (10), a 0.8-kb cDNA probe of the 10-kDa IFN-γ-inducible protein (IP-10) (18), or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech). The membranes were washed for 10 min at room temperature in 2× saline-sodium citrate (SSC; 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)-0.05% SDS and then for 20 min at 50°C in 0.2× SSC-0.1% SDS. To control for RNA loading, the hybridization signal obtained for eotaxin or IP-10 was normalized to that for GAPDH in each sample.

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared as previously described by Takeshita et al. (29). U-937 cells were cultured for 2 or 8 h in the absence or presence of TNF-α (10 ng/ml) and PMA (0.1 µM). After culture, the cells were washed with PBS and incubated with buffer A [10 mM HEPES (pH 7.9), 5 mM dithiothreitol (DTT), 0.3 M sucrose, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] containing 1 µg/ml of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A (Sigma) on ice for 15 min. After centrifugation, the cells were resuspended in 1 ml of buffer A containing the protease inhibitors and were subjected to Dounce homogenization (20 strokes). The homogenates were microcentrifuged for 30 s, and nuclei were resuspended in 200 µl of buffer B [20 mM HEPES (pH 7.9), 0.5 mM DTT, 5 mM MgCl2, 300 mM KCl, 25% glycerol, 0.2 mM EGTa, and 0.5 mM PMSF] and rocked at 4°C for 30 min. After microcentrifugation, the supernatants were dialyzed against 200 ml of buffer D [20 mM HEPES (pH 7.9), 0.5 mM DTT, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, and 0.5 mM PMSF] at 4°C overnight. After microcentrifugation, protein concentrations of the supernatants were measured by the Coomassie blue protein assay (Pierce, Rockford, IL).

Electrophoretic mobility shift assay (EMSA) was performed by standard methods (29). Briefly, 2 µg of nuclear extract under each condition were incubated for 20 min at room temperature in 20 µl of binding buffer [10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, and 40 mM NaCl] containing 50 pg of γ-32P-labeled NF-κB consensus oligonucleotide probe [35,000 counts/min (cpm), 5′-AGTTGAGGGGACTTTCCCAGGC-3′, Santa Cruz Biotechnology, Santa Cruz, CA] and 0.5 µg of poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ). A competition assay was performed by the addition of 100-fold molar excess unlabeled probe to nuclear extracts 10 min before the binding reaction with the labeled probe was begun. DNA-protein complexes were separated in a 4% polyacrylamide gel and analyzed by autoradiography.

Immunocytochemical staining. Cytospin slides were prepared with U-937 cells cultured under the following conditions: unstimulated (8 h), TNF-α stimulated (10 ng/ml, 8 h), unstimulated (48 h), and PMA stimulated (0.1 µM, 48 h). For eotaxin identification, a rabbit polyclonal anti-human eotaxin antibody was used. The cytospins were fixed in 4% formaldehyde for 10 min and then treated with trypsin for 5 min. Nonspecific immunoglobulin binding was blocked with 10% normal goat serum. The primary rabbit polyclonal antibody, diluted 1:400 in PBS with 2% bovine serum albumin, was applied to the samples and incubated at 4°C overnight. The slides were then incubated in the secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA) at 4°C for 2 h. Endogenous peroxidase activity was quenched with methanol containing 1% hydrogen peroxide. Avidin-biotin complex standard (Vector Laboratories) was applied to the samples and incubated at 4°C overnight. The slides were then incubated in the secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA) at 4°C for 1 h. Immunopositivity was localized with the chromagen diaminozobenzidine (0.025%) in PBS and 0.1% hydrogen peroxide. As a negative control, rabbit IgG (Vector Laboratories) was substituted for the primary antibody. The rabbit polyclonal anti-human eotaxin antibody reacted strongly to 100 ng of human eotaxin but did not react to 100 ng of human...
MCP-1, -2, -3, and -4; MIP-1α; MIP-1β; or RANTES. All experiments were performed at least in duplicate.

RESULTS

Time courses of eotaxin mRNA expression by TNF-α and PMA. Eotaxin mRNA expression in U-937 cells (Fig. 1A) was detectable 4 h after stimulation with TNF-α (10 ng/ml) and PMA (0.1 µM) but not with LPS (1 µg/ml), IL-1β (10 ng/ml), IL-4 (10 ng/ml), or IFN-γ (10 ng/ml). Treatment of U-937 cells with 100 ng TNF-α/ml induced maximal eotaxin mRNA expression at 2 h, which declined over the subsequent 6 h (Fig. 1B). In contrast, 0.1 µM PMA induced maximal eotaxin mRNA expression 16 h after stimulation and significant but declining expression in the subsequent 32 h (Fig. 1B). LPS (1 µg/ml), IL-1β (10 ng/ml), IL-4 (10 ng/ml), and IFN-γ (10 ng/ml) did not induce significant eotaxin mRNA expression at any of these time points (data not shown). Neither did 4 h of incubation with LPS (0.01, 0.1, 1, and 10 µg/ml), IL-1β (0.1, 1, 10, and 100 ng/ml), IL-4 (0.1, 1, 10, and 100 ng/ml), or IFN-γ (0.1, 1, 10, and 100 ng/ml) induce significant eotaxin mRNA expression in this cell line (data not shown). The following experiments were performed as a control for the effects of these stimuli on monocytic cells. Biological effects of IFN-γ (1–100 ng/ml) on IP-10 expression in U-937 cells were demonstrated (see Fig. 3). IL-4 (10 ng/ml) decreased cytokine-induced eotaxin mRNA expression, and LPS (1 µg/ml) and IL-1β (10 ng/ml) induced IL-8 expression in human monocytes (data not shown).

Concentration-response effects of TNF-α and PMA on eotaxin mRNA expression. The concentration-response effects of TNF-α and PMA on eotaxin mRNA expression are shown in Fig. 1C. The addition of increasing doses from 0.1 to 10 ng/ml of TNF-α to the medium was associated with a concentration-dependent increase in eotaxin mRNA expression, with decreasing eotaxin expression observed at the highest concentration (100 ng/ml). Doses of 0.01–1 µM PMA induced significant eotaxin expression, with a striking elevation in expression between 0.001 and 0.01 µM. Cells were harvested after 2 h of stimulation with TNF-α and 16 h with PMA. RNA size markers, shown in Fig. 1C, demonstrate a 0.8-kb transcript of eotaxin gene and a 1.3-kb transcript of GAPDH gene.

Effects of dexamethasone and IFN-γ. Pretreatment of U-937 cells with increasing concentrations of dexamethasone was associated with a concentration-dependent decrease in TNF-α (100 ng/ml)- and PMA (0.1 µM)-induced eotaxin mRNA expression (Fig. 2, A and B). Coincubation of U-937 cells with 1, 10, or 100 ng/ml of IFN-γ had no significant effect on TNF-α and PMA-induced eotaxin mRNA expression, whereas IP-10 mRNA expression was enhanced in the presence of IFN-γ (Fig. 3, A and B). Our IP-10-specific probe detected a single 1.3-kb band.

Effects of protein synthesis inhibitor cycloheximide. TNF-α-induced eotaxin mRNA expression was slightly decreased when protein synthesis was inhibited by 10 µg/ml of cycloheximide (Fig. 4A). In contrast, PMA-induced eotaxin mRNA expression could not be detected in the presence of cycloheximide (Fig. 4B). IP-10 mRNA expression was slightly superinduced by cycloheximide with or without TNF-α or PMA stimulation (data not shown).

TNF-α concentrations in the culture supernatant. TNF-α concentrations in the culture supernatant of U-937 cells were less than 5 pg/ml 1, 2, 4, 8, 16, and 24 h after stimulation with PMA (0.1 µM). The concentration was 11.0 ± 1.0 pg/ml (mean ± SE; n = 4) 48 h after stimulation, which was less than the minimum exogenous concentration required to induce eotaxin expression as determined in the concentration-response experiment.

Effects of TNF-α neutralization. PMA-induced eotaxin mRNA expression was not significantly inhibited in the presence of anti-TNF-α neutralizing antibody (0.1 and 1 µg/ml) (Fig. 4C). The anti-TNF-α antibody (1 µg/ml) neutralized >90% of TNF-α effects on eotaxin mRNA expression in U-937 cells when the TNF-α concentration was 1 ng/ml and >70% when it was 10 ng/ml (data not shown).
Effects of protein kinase C inhibitor staurosporine. Pretreatment of U-937 cells with increasing concentrations of the PKC inhibitor staurosporine was associated with a concentration-dependent increase in TNF-α-induced eotaxin mRNA expression (Fig. 5A). In contrast, PMA-induced eotaxin expression was inhibited by staurosporine in a concentration-dependent manner (Fig. 5B). Staurosporine (50 nM) itself did not induce eotaxin mRNA expression 2 and 16 h after its addition to U-937 cell supernatant (data not shown).

Effects of nuclear factor-κB inhibitor DETC. Pretreatment of U-937 cells with 0.001–1 mM of the nuclear factor-κB (NF-κB) inhibitor DETC was associated with a concentration-dependent decrease in TNF-α-induced eotaxin mRNA expression (Fig. 6A). PMA-induced eotaxin expression was inhibited by 0.1–1 mM DETC,
and a steep decrease in eotaxin expression was observed between 0.01 and 0.1 mM (Fig. 6B).

NF-κB binding activity in U-937 cells. Enhanced NF-κB binding activity was demonstrated in U-937 cells after 2 h of stimulation with TNF-α and 8 h of stimulation with PMA by EMSA (Fig. 6C). These results were consistent with the kinetics of eotaxin mRNA expression induced by TNF-α and PMA. Specific binding of the NF-κB consensus probe to the nuclear extracts was suggested by the competition experiment with unlabeled probe.

Immunocytochemical staining. Minimal eotaxin immunoreactivity was detectable in unstimulated U-937 cells (Fig. 7, A and C). Compared with the unstimulated cells, increased eotaxin immunoreactivity was observed in U-937 cells after incubation with TNF-α for 8 h and PMA for 48 h (Fig. 7, B and D). These observations were consistent with the eotaxin mRNA induction after stimulation with TNF-α or PMA.

Regulation of eotaxin mRNA expression in monocytes and A549 cells. TNF-α induced significant eotaxin mRNA expression in monocytes and A549 cells, whereas PMA induced only faint eotaxin expression in both cell types (Fig. 8, A and B). TNF-α-induced eotaxin mRNA expression was enhanced by staurosporine and inhibited by DETC in monocytes, which was similar to the findings in U-937 cells, although the expression was diminished in the presence of cycloheximide in monocytes (Fig. 8A). In contrast, TNF-α-induced eotaxin mRNA expression was markedly enhanced in the presence of cycloheximide in A549 cells. The expression was inhibited by staurosporine and DETC in this cell line, which suggests that TNF-α-induced eotaxin expression is partially mediated by PKC as well as by NF-κB (Fig. 8B). PMA-induced eotaxin mRNA expression was also markedly enhanced by cycloheximide in A549 cells.

**DISCUSSION**

We demonstrated that PMA-induced eotaxin mRNA expression was of greater magnitude and occurred later than TNF-α-induced eotaxin expression in U-937 cells. Eotaxin protein was also detected by immunocytochemistry in both TNF-α- and PMA-stimulated U-937 cells. The protein synthesis inhibitor cycloheximide markedly diminished PMA-induced eotaxin mRNA expression, although TNF-α-induced expression was not significantly decreased by this reagent. The PKC inhibitor staurosporine inhibited PMA-induced eotaxin mRNA expression but enhanced TNF-α-induced expression.
These results suggest that TNF-α and PMA augment eotaxin expression in U-937 cells by distinct mechanisms.

Staurosporine is a well-described inhibitor of PKC, the activity of which is related to its ability to bind the catalytic domain of PKC (19). One of the important actions of PMA is its ability to activate PKC. We demonstrated concentration-dependent inhibition of PMA-induced eotaxin mRNA expression at moderate levels of staurosporine in U-937 cells. PMA-induced eotaxin expression therefore appears to be mediated by PKC activation. When the cells were stimulated with TNF-α, eotaxin expression was enhanced in a concentration-dependent manner by similar levels of staurosporine. We determined that staurosporine alone did not significantly affect U-937 cell eotaxin mRNA expression, thus suggesting that it increases TNF-α-induced eotaxin mRNA expression by an indirect mechanism. One explanation for this increase relates to the ability of staurosporine to increase the presence of TNF receptors on the cell surface (33). These observations indicate that TNF-α can induce eotaxin mRNA through PKC-independent pathways in U-937 cells. TNF-α-induced eotaxin mRNA expression was also enhanced by staurosporine in human monocytes, whereas the expression was inhibited by this agent in the epithelial cell line A549. These results suggest that the TNF-α-triggered pathway may be regulated by PKC-independent mechanisms in human monocytes as well as in U-937 cells but that TNF-α can induce eotaxin mRNA expression at least partially through PKC-dependent mechanisms in epithelial cells.

An NF-κB response element has been identified in the human eotaxin promoter region near the transcription initiation site (11). EMSA demonstrated enhanced NF-κB binding activity in U-937 cells 2 h after stimulation with TNF-α and 8 h after stimulation with PMA. These observations suggest that PMA- and TNF-α-induced eotaxin expression is mediated by NF-κB activation. The mechanisms by which these stimuli lead to NF-κB activation are complex, but PKC activation and reactive oxygen intermediates are thought to be involved in these pathways (20, 28). A previous study suggested that TNF-α can activate NF-κB by PKC-independent mechanisms (20), which is consistent with our finding that TNF-α-induced eotaxin mRNA expression does not depend on PKC activation in U-937 cells. DETC is an NF-κB inhibitor with antioxidative proper-

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**Fig. 7. Immunostaining for eotaxin in U-937 cells:** unstimulated cells cultured for either 8 h (A) or 48 h (C) demonstrate minimal eotaxin immunoreactivity, whereas cells cultured with TNF-α for 8 h (B) or PMA for 48 h (D) demonstrate induction of eotaxin immunostaining. Magnification, ×100. A representative series of photographs is shown (n = 3).
ties and is a chelator of heavy metals (15, 27). Its inhibitory effects on NF-κB activation are thought to be related to the inhibition of the release of the inhibitor IKb from the NF-κB-IκB complex in the cytoplasm (12). DETC inhibited eotaxin mRNA expression in both PMA- and TNF-α-stimulated U-937 cells at 0.1 and 1 mM, which is consistent with our notion that NF-κB activation is a common element in the mechanistic pathways triggered by PMA and TNF-α that induce eotaxin expression in U-937 cells. These observations may be applicable to human monocytes and epithelial cells because DETC also inhibited TNF-α-induced eotaxin mRNA expression in these cell types.

We found that PMA-induced eotaxin mRNA expression was dependent on protein synthesis, and it is known that PMA can increase TNF-α expression in U-937 cells (6). To determine whether PMA induced eotaxin mRNA expression by TNF-α synthesis, we measured PMA-induced TNF-α protein levels in the culture supernatant and examined the effects of anti-TNF-α neutralizing antibody on PMA-induced eotaxin mRNA expression. The levels that we detected do not appear to be sufficient to account for the magnitude of PMA-induced eotaxin mRNA expression that we observed. Because TNF-α concentrations close to the cell surface may have been higher than those we detected in cell supernatants, we examined the effects of anti-TNF-α neutralizing antibody on PMA-induced eotaxin expression. The ability of this antibody to block TNF-α but not PMA-induced eotaxin expression indicates that the PMA pathway is independent of TNF-α production in U-937 cells. The dependence of this pathway on new protein synthesis suggests that de novo synthesis of transcription factors or RNase-repressive factors is involved in PMA-induced eotaxin mRNA expression in this cell line. Similarly, TNF-α-induced eotaxin mRNA expression was dependent on protein synthesis in human monocytes.

Although different mechanisms are involved in eotaxin mRNA expression by TNF-α and PMA in U-937 cells, inhibitory effects of dexamethasone were observed in both pathways. Because NF-κB activation is thought to be a common element, the effects of glucocorticoids could relate to the well-described ability of the glucocorticoid receptor to inhibit NF-κB binding to its response element (25). Alternatively, dexamethasone may decrease the stability of eotaxin mRNA. Because the ATTATA sequences were identified in the 3′-untranslated region of the human eotaxin gene, glucocorticoids can promote the effects of RNase, which degrades mRNA that contains AU-rich sequences (10, 23, 31). It is also possible that dexamethasone effects are mediated by the human eotaxin promoter glucocorticoid response element (11). In any case, part of the known eosinophil-suppressive effects of glucocorticoids may relate to this ability to suppress eotaxin mRNA expression.

TNF-α induced eotaxin mRNA in human monocytes by protein synthesis-dependent mechanisms. PMA induced prolonged and enhanced eotaxin expression in U-937 cells, which was also dependent on new protein synthesis. In contrast, TNF-α and PMA-induced eotaxin mRNA was superinduced by cycloheximide in a human epithelial cell line, A549. Cytokine-induced eotaxin mRNA expression, enhanced in the presence of IFN-γ in A549 cells, was thought to be mediated by IFN-γ response elements identified in the human eotaxin promoter region (11, 17). However, these synergistic effects of IFN-γ on eotaxin mRNA expression were not observed in U-937 cells despite the findings that

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Fig. 8. Effects of cycloheximide (Cy), staurosporine (Stauro), and DETC on TNF-α- and PMA-induced eotaxin mRNA expression in human monocytes (A) and A549 cells (B). Monocytes and A549 cells were stimulated for 4 h with TNF-α (10 ng/ml) or PMA (0.1 µM). Cycloheximide (10 µg/ml), staurosporine (50 nM), and DETC (1 mM) were added to cells 30 min before stimulation. A and B: representative series of blots (n = 3 and 2, respectively). Below each blot is a quantitative comparison between eotaxin- and GAPDH-specific signals.
these cells could respond to IFN-γ by increasing the levels of IP-10 mRNA (30). These results suggest that human monocyte cells can express eotaxin mRNA as well as epithelial cells, but the expression is regulated differently in these two cell types. Differential expression of eotaxin may be important for establishing eotaxin gradients in tissue that allow it to function as an eosinophil chemoattractant.

In summary, we have demonstrated that eotaxin expression is regulated by different mechanisms in TNF-α- and PMA-stimulated U-937 cells; the PMA-triggered pathway is mediated by de novo protein synthesis and PKC activation, whereas the TNF-α-triggered pathway does not depend on either of these steps. However, NF-κB activation is thought to be a common downstream element in these two distinct cascades and may also contribute to eotaxin mRNA induction in human monocytes and epithelial cells, as suggested by the response to the NF-κB inhibitor Detc.

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