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Regulation of Expression of Granulocyte-Macrophage Colony-Stimulating Factor in Human Bronchial Epithelial Cells: Roles of Protein Kinase C and Mitogen-Activated Protein Kinases

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GM-CSF has a major role in the immune and inflammatory milieu of the airway. Airway epithelial cells (AEC) are among the first targets of environmental stimuli and local cytokines, in response to which they can produce GM-CSF. The regulation of GM-CSF is only minimally understood in AEC. We hypothesized that GM-CSF expression in AEC would result from activation of protein kinase C (PKC) and subsequent activation of the extracellular signal-regulated kinase (MAPKerk1/2) pathway, so we investigated signal transduction pathways in human primary culture bronchial epithelial cells (HBECs). TNF-α, IL-1β, and PMA induced the release of GM-CSF in HBECs. The robust response to PMA was not detected in SV40 adenovirus-transformed normal human bronchial epithelial cells (BEAS-2B). PMA and TNF-α stimulation of GM-CSF required activation of PKC (inhibition by staurosporine and bisindolylmaleimide I). GM-CSF expression was up-regulated by a nonphorbol PKC activator, but not by an inactive PMA analogue. PMA-induced GM-CSF production in HBECs did not require a Ca2+ ionophore and was not inhibited by cyclosporin A. Activation of MAPKerk1/2 via PKC was associated with and was required for GM-CSF production induced by PMA and TNF-α. The data demonstrate regulation of GM-CSF in HBECs by PKC pathways converging on the MAPKerk1/2 pathway and further define cell-specific regulation critical for local airway responses. The Journal of Immunology, 2000, 165: 1618–1625.

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irway epithelial cells (AEC) are primary targets for inhaled infectious agents, aerosolized Ags and pollutants, as well as locally released cytokines. In response to these stimuli, AEC synthesize and secrete proinflammatory and immunomodulatory cytokines, including GM-CSF, a 23-kDa glycoprotein with pleiotropic effects on myeloid cell function (1–6). Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses.

Elevated levels of GM-CSF have been well described in bronchoalveolar lavage, endobronchial biopsy, and sputum samples from asthmatics (1, 7–12). Elevated levels of GM-CSF, largely derived from epithelial cells, have been demonstrated to increase eosinophil activation and survival in asthmatics (13–15). GM-CSF stimulates the recruitment and activation of eosinophils via β integrin-mediated adhesion to epithelial and endothelial cells (16) and prolongs eosinophil survival via inhibition of apoptosis (5, 17, 18). Moreover, GM-CSF stimulates activation of the 5-lipoxygenase pathway (19). In addition, murine models of asthma and diesel-induced hyper-responsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyper-responsiveness (20, 21).

GM-CSF may also participate in immune mechanisms of asthma via an effect on dendritic cells (DC), which are abundantly distributed in the submucosa and intraepithelial regions of the airway (22–24). DC along with other local but less potent APC are required for the initiation and perpetuation of the T cell activation involved in asthma. GM-CSF is a critical factor for the maturation of DC and enhances the expression of accessory molecules such as ICAM-1, B7-1 (CD80), and B7-2 (CD86) (22). In the human lung, elevated epithelial levels of GM-CSF have been demonstrated in association with the accumulation of DC (25). Murine models of asthma with epithelial cell transgene expression of GM-CSF describe DC cell recruitment in the airway as well as that of inflammatory cells (20, 26). In murine models, DC have been demonstrated to be essential for presenting inhaled Ag to previously primed Th2 cells, and thus for chronic eosinophilic airway inflammation (20, 27).

Despite the potential critical importance of epithelial cell-derived GM-CSF, little is known about the regulation of GM-CSF production by these cells. In general, regulation of expression of GM-CSF is cell and stimulus specific and can be controlled at multiple levels. Most studies of GM-CSF regulation have been performed in lymphocytes or transformed, transfected cells over-expressing constitutively active or dominant negative signaling
components, the regulation of which may differ from that of primary cells (28, 29). AEC release GM-CSF in response to physiologic stimuli relevant for asthma, including dust mite proteolytic allergens, human rhinovirus-14, respiratory syncytial virus, and histamine (30–32). AEC have also been demonstrated to secrete GM-CSF in response to cytokines such as TNF-α, IL-1, IL-4, and IL-13 (2–4, 33). These disparate stimuli act via a multitude of signaling pathways and therefore fail to provide clear clues to deciphering pathways that may lead to optimal production of GM-CSF.

In view of the easy access of AEC to relevant stimuli and the importance of locally released GM-CSF in airway immune and inflammatory homeostasis, it is critical to understand the regulation of GM-CSF in these cells. We investigated whether regulation of GM-CSF production in primary culture human bronchial epithelial cells (HBECS) involved activation of PKC and subsequent activation of the MAPKerk1/2 pathways.

Materials and Methods
Reagents
Basal cell culture medium was obtained from Clonetics (San Diego, CA) and for routine cell growth was supplemented with the following components: human epidermal growth factor (0.5 ng/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), gentamicin 50 μg/ml, amphotericin B (50 ng/ml), bovine pituitary extract (13 μg/ml), and retinoic acid (0.1 ng/ml) (34). LHC-9 was obtained from Biofluids (Rockville, MD) and was similarly supplemented. PMA and A23187 were obtained from Sigma (St. Louis, MO). Human recombinant TNF-α and IL-1 were obtained from R&D (Minneapolis, MN). TRIZol reagent was obtained from Life Technologies (Gaithersburg, MD). Quik Hyb hybridization solution and Ran-dom Primer Labeling Kit Prime-It II were obtained from Stratagene (La Jolla, CA). The 751-bp IGM-CSF cDNA probe was provided by Dr. Steven Clark (Cambridge, MA). A cDNA probe to a fragment GAPDH was generated by RT-PCR. All Abs directed against phosphorylated and nonphosphorylated forms of MAPKerk1/2, MAPKerkβ, and MAPKerkδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclosporin A, mezeirein, 4α-phorbol 12,13-didecanoate, bisindolylmaleimide I, staurosporine, PD98059, and SB203580 were purchased from Calbiochem (La Jolla, CA) and were dissolved in DMSO (Me2SO). The final concentration of Me2SO did not exceed 0.1% (v/v). ECL Plus kits were obtained from Amersham Pharmacia (Aylesbury, U.K.).

Cell culture
Culture of normal HBECs from bronchial brush biopsies was performed using a modification of the methods of Wu and Turner (35), Kelson et al. (36), and Nakamura et al. (35). Briefly, cells were obtained during bronchoscopic examination of normal human subjects (New York University review board-approved protocol). Bronchial brushing was performed with a disposable bronchoscope of normal human subjects (New York University review board-approved protocol). Bronchial brushing was performed with a disposable brush (model BC-15C, Olympus, New Hyde Park, NY), which was introduced via the bronchoscope into subsegmental bronchi and rubbed against the epithelial surface with 10 strokes. The cells obtained by brushing were collected into serum-free, hormonally supplemented medium (Clonetics) containing amphotericin and gentamicin. The cells were plated in uncoated T25 tissue culture flasks and incubated (37°C, 5% CO2) for 7–10 days, during which time the cells were fed every 2 days. When cells reached 70% confluence, they were passaged into appropriate tissue culture plates required for specific experiments. All experiments were performed at passage 3, as additional passaging led to increased constitutive release of GM-CSF. Hydrocortisone, retinoic acid, and epinephrine (known to suppress GM-CSF production) were removed from the medium 24 h before each experiment. Epithelial cell phenotype was confirmed by appropriate staining with anti-human cytokeratin Abs (positive staining for CK7 and CK18, absence of CK20). For some experiments, HBECs were purchased from Clonetics, cultured in the same manner, and used at passage 3.

SV40 adenovirus-transformed normal human bronchial epithelial cells (BEAS-2B) were obtained from American Type Culture Collection (Ma-nassas, VA), grown in LHC-9, and used between passages 14–25.

ELISA
Cells were grown to near confluence at passage 3 and stimulated with the appropriate agents (18 h, 37°C). Supernatants were subsequently collected, centrifuged (1000 rpm, 10 min), and diluted appropriately, and the concentration of GM-CSF was determined by ELISA (Endogen, Cambridge, MA). Measurements were performed in duplicate and were quantitated at 450 nm (microplate reader, Bio-Rad, Richmond, CA).

RNA isolation and Northern analysis
Cells were grown to near confluence at passage 3 and were stimulated with the appropriate agent (4 h, 37°C). Total RNA was extracted with 4 M guanidinium-HCl and isolated by CsCl centrifugation (5.7 M CsCl and 0.01 M EDTA, pH 7.5) or alternatively by TRIzol reagent. Total RNA (20 μg/lane) was electrophoresed overnight on a 2% agarose-formaldehyde gel (22 V/cm). RNA was transferred onto Nytran filters (Schleicher & Schuell, Keene, NH) by capillary action, and filters were cross-linked (Stratagene cross-linker). Filters were prehybridized (4 h, 65°C), followed by hybridization with a [α-32P]dCTP random primer-labeled cDNA probe for GM-CSF or GAPDH. Filters were subjected to three stringent washes, placed between intensifying screens, and exposed to x-ray film (Fuji, Tokyo, Japan; −70°C). Densitometry was performed with an Ul-trascan XL densitometer (LKB, Bromma, Sweden).

Immunoblotting with phosphospecific Ab probes
Activated MAPK species were detected using phosphospecific Abs directed against the dually phosphorylated forms of the protein. Cells were incubated in basal medium (4 h) before stimulation with defined agents for the times indicated in the figure legends. Lysates were prepared by treating cells with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.5 M PMSF, 2 mM Na3VO4, 50 mM NaF, 1 mM EGTA, 50 μg/ml aprotinin, 50 μg/ml chymostatin, and 25 μg/ml pepstatin). Lysates were centrifuged (13,000 rpm, 30 min) to sediment the particulate material. The protein concentration of the supernatant was measured by the BCA protein assay method (Pierce, Rockville, IL). Equal amounts of protein (50 μg/lane) were electrophoresed in 10% SDS-Tris glycine gels, and resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked (0.5% nonfat dried milk) and probed with anti-phospho-MAPKerk1/2 (1/200) or anti-MAPKerk1/2 (1/200) followed by incubation with the appropriate HRP-conjugated secondary Ab (1/1000). Bound Abs were visualized using the ECL Western blot detection system according to the manufacturer’s instructions. Equal loading of samples was checked by using parallel blots or stripped blots for immunodetection of MAPKerk2 with phosphorylation state-independent pan-Abs, and reprobing with anti-MAPKerk2.

In vitro kinase assay for MAPKerk1/2
HBECS were lysed (20 mM Tris-HE (pH 7.4), 1% Triton X-100, 10 mM β-glycerophosphate, 0.5 mM PMSF, 2 mM Na3VO4, 20 mM NaF, 1 mM EGTA, 1 mM DTT, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 25 μg/ml pepstatin). Cell lysates were preincubated with protein A-Sepharose beads, and immunoprecipitations were performed with anti-MAPKerk1/2 and MAPKerk2 together in the presence of protein A-Sepharose beads. Beads were incubated (30 min) with 20 μCi of [γ-32P]ATP and myelin basic protein (500 μM) as peptide substrate in buffer (20 mM HEPES (pH 7.6), 200 mM MgCl2, 20 μM ATP, 2 mM DTT, 100 μM sodium orthovanadate, and 25 μM β-glycerophosphate). Reactions were stopped by the addition of 15% formaldehyde. The beads were washed, and supernatants were spotted onto phosphocellulose papers. Filters were washed, and [γ-32P]PMB was quantified by scintillation counting. Duplicate assays in the absence of MBP peptide were performed to determine non-MAPK background kinase activities, which was subtracted for the MBP assays to determine true MBP phosphorylation.

Data analysis
Student’s t tests with statistical significance at the 0.05 level or, when appropriate, ANOVA for multiple comparisons were used for data analysis. Results are expressed as the mean ± SEM.

Results
Production of GM-CSF protein and mRNA in AEC
To evaluate the regulation of GM-CSF production in AEC, we first confirmed the response of primary culture HBECS to cytokines previously reported to enhance GM-CSF production in AEC. HBECS were grown to near confluence, and the release of GM-CSF in the supernatant was determined by ELISA under resting or stimulated conditions. As shown in Fig. 1a, stimulation of HBECS
with TNF-α elicited a dose-dependent increase in GM-CSF production; these levels continued to increase at doses as high as 0.3 μM. The increase in GM-CSF production at this concentration was ~400% above that in unstimulated controls (1.24 ± 0.20 ng/10^6 cells; n = 12; p < 0.004). Stimulation of HBECs with IL-1 also induced a dose-dependent stimulation of GM-CSF production, with maximal activity observed at 0.6–6 μM. The maximal observed increase in GM-CSF production at 0.6 μM was ~300% above the unstimulated control value (1.11 ± 0.39 ng/10^6 cells; n = 5; p < 0.5).

Because the responses of some cell types to TNF-α and IL-1 may be dependent upon the activation of PKC, we also tested the effects of PMA, an analogue of diacylglycerol and a direct activator of PKC. PMA elicited a dose-dependent increase in GM-CSF production even greater than that stimulated by TNF-α and IL-1, with maximal activity at 5 ng/ml (8 nM; Fig. 1b). At this concentration, PMA resulted in a >14-fold increase in GM-CSF release (14.35 ± 5.0 ng/10^6 cells; n = 6; p < 0.03). These observations established the capacity TNF-α and IL-1 to stimulate GM-CSF release in HBECs. Moreover, these studies suggested the capacity of PKC to signal the production of GM-CSF in these cells, raising the possibility that GM-CSF production in response to IL-1 and TNF-α may be PKC dependent.

To confirm that increases in GM-CSF protein release were associated with similar increases in gene expression, we determined the effects of PMA (8 nM) and TNF-α (0.3 μM) on steady-state levels of GM-CSF mRNA in HBECs. As shown in the representative Northern blot in Fig. 1c, steady-state GM-CSF mRNA was barely detectable in unstimulated HBECs. Consistent with the ability of PMA to stimulate a larger GM-CSF release than TNF-α, the amount of message observed was greater in PMA-treated cells than in TNF-α-treated cells.

Because of the difficulties involved in obtaining and culturing primary bronchial epithelial cells, many studies of airway epithelium employ BEAS-2B cells, an SV-40 adenovirus-transformed cell line originally derived from normal human bronchial epithelial cells. To determine whether BEAS-2B cells respond in a manner similar to that of HBECs, and thus whether they can serve as an adequate model for epithelial cell GM-CSF production, the release of GM-CSF in response to stimuli was determined for BEAS-2B cells (passages 14–25). Exposure of BEAS-2B to either TNF-α (0.3 μM) or IL-1 (0.6 μM) resulted in a roughly 2-fold increase in GM-CSF above the unstimulated control value (233 ± 11 and 148 ± 20% above unstimulated control, respectively; n = 3; p < 0.05). In contrast to the exuberant response to PMA demonstrated in HBECs, the addition of PMA (8 nM) to BEAS-2B cells resulted in negligible GM-CSF (0.32 ± 0.1 ng GM-CSF/10^6 cells above background; n = 3). Thus, BEAS-2B cells failed to respond in a manner similar to that of HBECs. In view of the difference in response between HBECs and BEAS-2B cells, we focused all subsequent studies on HBECs. Because the effect of TNF-α was similar to that of IL-1, all further cytokine studies were performed with TNF-α.
release by TNF-α-stimulated HBECs was also inhibited by staurosporine. These data support the importance of PKC activation as a critical step in the upstream signaling pathway leading to GM-CSF production by HBECs.

Role of calcium in PKC-dependent GM-CSF production by HBECs

In T cells and endothelial cells stimulated with PMA, GM-CSF mRNA production occurs only in the presence of a second stimulus, consisting of a Ca\(^{2+}\) ionophore (28, 29, 41, 42). Moreover, cyclosporin A (CsA), which blocks the activity of the calcium-binding protein calcineurin, inhibits increases in GM-CSF mRNA in some cell types (43). We therefore tested to what extent PMA-stimulated GM-CSF production in HBECs was dependent upon the ionophore-induced increase in intracellular Ca\(^{2+}\) (Fig. 3a). As noted previously, stimulation of HBECs with PMA in the absence of a Ca\(^{2+}\) ionophore elicited a dramatic increase in GM-CSF production. In contrast, stimulation of HBECs with the Ca\(^{2+}\) ionophore A23187 (10 μM) in the absence of PMA elicited a minimal and nonsignificant increase in the release of GM-CSF above background levels. The addition of A23187 to PMA resulted in a small increase in GM-CSF production relative to PMA alone, an effect that failed to achieve statistical significance.

The PMA-induced expression of GM-CSF mRNA was also monitored in the absence or the presence of A23187 by Northern blot analysis. As shown in Fig. 3b, the addition of A23187 to PMA resulted in a small, but statistically insignificant, increase in GM-CSF message (160% PMA response; \(n = 3\); not significant).

The above studies were extended by testing the effects of CsA on GM-CSF protein expression in HBECs. Cells were stimulated with PMA with or without A23187 (18 h) in the presence or the absence of CsA, and GM-CSF release was determined by ELISA (Fig. 3c). CsA (0.1–1 μM) failed to inhibit GM-CSF production stimulated by PMA alone (98.3 ± 1.2 and 99.2 ± 20.3% of control value; \(n = 3\)). GM-CSF release elicited by the combination of PMA and A23187 was modestly inhibited by the higher dose (1.0 μM) of CsA (66.3 ± 4.4% stimulated release; \(n = 3\); \(p < 0.01\)).

We next tested the effect of CsA (0.1 μM) on steady-state mRNA in cells stimulated with PMA and A23187. As demonstrated in Fig. 3, CsA did not decrease steady-state mRNA for GM-CSF in HBECs when data were normalized to GAPDH. Thus, in contrast to data reported for T cells and endothelial cells, up-regulation of GM-CSF in response to PMA in HBECs did not appear to require the addition of a calcium costimulus.
presented are representative of three separate experiments. The control value for each data point as determined by densitometry. Data analysis was performed with phosphospecific (p-MAPKerk1/2) or control (MAPKerk2) probes. The numbers at the top of the lanes are time in minutes. The numbers in the middle of the blot are the increase over the unstimulated controls (MAPKerk2) probe. The level of PMA stimulation at 30 min was similar to that observed at 15 min, although the power of our analysis did not allow us to detect a significant difference from baseline. TNF-α-induced activation of MAPKerk1/2 was detected by 60 min.

The ability of both TNF-α and PMA to stimulate MAPKerk1/2 activity in HBECs together with our observation that GM-CSF production stimulated by TNF-α was PKC dependent suggested that the capacity of TNF-α to stimulate MAPKerk1/2 activation might also be PKC dependent. We therefore tested the effects of PKC inhibitors on MAPKerk1/2 activation in response to TNF-α. As shown in Fig. 5, both staurosporine (20 nM) and bisindolylmaleimide (5 μM) inhibited TNF-α-stimulation of MAPKerk1/2 phosphorylation, as determined by immunoblot. Taken together, these data indicate that TNF-α retained the capacity to activate MAPKerk1/2 in HBECs via a PKC-dependent pathway.

Role of MAPKerk1/2 in GM-CSF production

We next asked whether the activation of MAPKerk1/2 was necessary for the stimulation of GM-CSF production/release by HBECs. The cell-permeant molecule PD98059 is a selective inhibitor of the MAPK kinases (MKK) MEK1/2 (MKKerk1/2), proximal activators of MAPKerk1/2 (46). As shown in Fig. 5, preincubation of HBECs with PD98059 (40 μM) resulted in marked inhibition of MAPKerk1/2 activity induced by PMA or TNF-α. The availability of a MAPKerk1/2 inhibitor thus allowed us to test the effects of MAPKerk1/2 inhibition on GM-CSF production. HBECs were grown to near confluence and stimulated with PMA or TNF-α in the presence or absence of staurosporine (stauro), bisindolylmaleimide I (bis), or PD98059 (PD). After SDS-PAGE and transfer to PVDF membranes, immunoblotting was performed with phosphospecific (p-MAPKerk1/2) or control (pan-MAPKerk2) probe.

To confirm that MAPKerk1/2 phosphorylation detected in the Western blots correlated with actual MAPKerk1/2 activity after brief exposures, we used an in vitro kinase assay to evaluate whether PMA and TNF-α-induced activation of MAPKerk1/2 in HBECs were grown to near confluence and stimulated with PMA (8 nM) or TNF-α (300 nM) in the presence or the absence of staurosporine (stauro), bisindolylmaleimide I (bis), or PD98059 (PD). After SDS-PAGE and transfer to PVDF membranes, immunoblotting was performed with phosphospecific (p-MAPKerk1/2) or control (pan-MAPKerk2) probe.

FIGURE 4. Activation of MAPKerk1/2 in HBECs. Lysates were prepared from HBECs stimulated with PMA (8 nM) or TNF-α (300 nM) for the defined times. a, After SDS-PAGE and transfer to PVDF membranes, immunoblotting was performed with phosphospecific (p-MAPKerk1/2) or control (MAPKerk2) probes. The numbers at the top of the lanes are time in minutes. b, In vitro phosphorylation assay with MBP to directly measure MAPKerk1/2 kinase activity. Lysates from stimulated (0 – 60 min) cells were immunoprecipitated with rabbit polyclonal Ab directed against MAPKerk1/2. Activity of MAPKerk1/2 in the immunoprecipitates was determined as the phosphorylation of MBP (n = 3–6; p ≤ 0.01).

FIGURE 5. Effect of PKC or MAPKerk1/2 inhibitors on PMA- or TNF-α-stimulated MAPKerk1/2 in HBECs. Lysates were prepared from HBECs stimulated (60 min) with PMA (8 nM) or TNF-α (300 nM) in the presence or the absence of staurosporine (stauro), bisindolylmaleimide I (bis), or PD98059 (PD). Western blots correlated with actual MAPKerk1/2 activity after brief exposures, we used an in vitro kinase assay to evaluate whether PMA and TNF-α-induced activation of MAPKerk1/2 in HBECs were grown to near confluence and stimulated with PMA (8 nM) or TNF-α (300 nM), and lysates were immunoprecipitated with rabbit polyclonal Ab directed against MAPKerk1/2. Kinase activities in the immunoprecipitates were determined as their capacity to phosphorylate MBP. As demonstrated in Fig. 4b, PMA induced rapid activation of MAPKerk1/2 at 15 min, consistent with the Western blot information. The level of PMA stimulation at 30 min was similar to that observed at 15 min, although the power of our analysis did not allow us to detect a significant difference from baseline. TNF-α-induced activation of MAPKerk1/2 was detected by 60 min.

The ability of both TNF-α and PMA to stimulate MAPKerk1/2 activity in HBECs together with our observation that GM-CSF production stimulated by TNF-α was PKC dependent suggested that the capacity of TNF-α to stimulate MAPKerk1/2 activation might also be PKC dependent. We therefore tested the effects of PKC inhibitors on MAPKerk1/2 activation in response to TNF-α. As shown in Fig. 5, both staurosporine (20 nM) and bisindolylmaleimide (5 μM) inhibited TNF-α-stimulation of MAPKerk1/2 phosphorylation, as determined by immunoblot. Taken together, these data indicate that TNF-α retained the capacity to activate MAPKerk1/2 in HBECs via a PKC-dependent pathway.

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PMA and TNF-α MAPK erk1/2 and tested the ability of PD98059 (40 μM) to inhibit TNF-α-stimulated GM-CSF release profoundly (12.7 ± 6.0% TNF-α response; n = 3; p < 0.003). A selective inhibitor of the MAPK erk1/2 pathway, SB203580 (0.1–10 μM), failed to inhibit PMA-induced GM-CSF production by HBECs, although SB203580 (0.1 μM) moderately inhibited TNF-α-stimulated GM-CSF release (57.3 ± 11.2% TNF-α response; n = 3; p < 0.02; data not shown).

To confirm that the decrease in GM-CSF protein in response to inhibition of the MAPK erk1/2 pathway was also manifest at the level of GM-CSF mRNA, we used PMA (8 nM) to stimulate MAPK erk1/2 and tested the ability of PD98059 (40 μM) to inhibit GM-CSF mRNA, as determined by Northern analysis and densitometry. As demonstrated in Fig. 6b, steady-state GM-CSF mRNA induced in response to PMA was decreased in the presence of PD98059 (25.5 ± 4.4% PMA response; n = 3; p < 0.001). Thus, PMA and TNF-α stimulation of GM-CSF message expression and protein production in HBECs correlated with and appeared to depend upon the activation of MAPK erk1/2 by these agents.

Discussion

AEC have been demonstrated to release GM-CSF and are available for stimulation by a wide array of cytokines. Because of the important role of GM-CSF in mediating the responses of both eosinophils and dendritic cells, this cytokine is likely to play a critical role in airway diseases such as asthma. Moreover, since the effects of GM-CSF are transmitted locally, an understanding of its regulation in the local airway environment is important. Human AEC have been demonstrated to generate GM-CSF in response to a variety of stimuli that in other cell types elicit responses via complex and disparate signaling mechanisms. Little is known about intracellular signals required for GM-CSF stimulation in AEC. We now demonstrate that primary culture human bronchial epithelial cells have the potential to release GM-CSF in response to multiple stimuli and suggest a critical role for PKC and MAPK erk1/2 in these pathways.

Our experiments focused on primary culture epithelial cells. This model was used because of the demonstration that transformed HBEC (BEAS-2B) responded in a manner different from that of primary culture HBECs. Both BEAS-2B cells and HBECs released a similar quantity of GM-CSF protein and up-regulated GM-CSF mRNA in response to TNF-α and IL-1β, a response in accordance with that reported by Nakamura et al. (33). In contrast, the response to PMA differed markedly between HBECs and BEAS-2B cells; HBECs, but not BEAS-2B cells, produced abundant (nanogram) quantities of GM-CSF protein in response to PMA. Differences in the response between primary culture and transformed bronchial epithelial cells have also been demonstrated for additional stimuli such as IL-4 (33). In addition, studies of integrin expression demonstrate a disparity between the expression of β5, β6, and α6β1 in HBECs and BEAS-2B cells (47).

Thus, our data served again to reinforce the need to evaluate findings in primary cell cultures as well as in transformed cell lines.

Among the agents we tested, PMA was a potent stimulus for expression of GM-CSF mRNA and protein. PMA activates conventional and novel isozymes of PKC leading to activation of the Ras and Raf-1 pathways that result in up-regulation of MAPK erk1/2 (44). Since the intracellular pathways stimulated by phorbol esters have been well described, the use of this agent provided us with an instrument to begin to dissect signaling pathways in HBECs. In addition to multiple PKC isozymes, recent studies have demonstrated that PMA has high affinity for and can also activate additional signaling proteins, including the GTPase-activating proteins n-chimaerin and Vav (39, 40). However, PMA stimulation of GM-CSF in our studies was unlikely to be due to these alternative pathways, because the use of multiple PKC inhibitors blocked GM-CSF production, and the nonphorbol PKC agonist stimulated GM-CSF. Finally, the inactive PKC analogue had no effect. These data support the importance of PKC activation in GM-CSF release in HBECs; the role of specific isozymes awaits further analysis.

Although the ability of PMA to signal via PKC pathways has been abundantly described, the pathways stimulated by cytokines are complex, and the interaction with PKC isozymes is less clear. Our data suggest a role for TNF-α-induced PKC activation in GM-CSF production in primary culture cells. In particular, inhibition of PKC by staurosporine inhibited TNF-α-induced GM-CSF production. TNF-α triggers biologic effects via engagement of two receptors: p55(TNFFR-1) and p75 (TNFR-II), both of which have been described in airway cells (48–51). Biologic responses induced by TNF are due to the interaction of the cytoplasmic domains of multimerized receptors with families of cytoplasmic proteins (49). The variation in responses induced by ligation of TNF depends on cell type, state of cell differentiation, and transformation. TNF-α induced migration of bovine epithelial cells and mucin secretion in guinea pig tracheal epithelial cells have been demonstrated to be associated with PKC activation (51, 52). Our studies thus suggest an additional association of TNF and PKC in epithelial-type cells.

The requirement of PKC activation for GM-CSF induction in HBECs together with the ability of PKC to activate MAPK cascades suggested that one or more of these MAPK cascades might lie downstream of PKC in GM-CSF signaling. The common motif of MAPK pathways consists of three kinases that are sequentially activated (53). In the MAPK erk1/2 cascade, PKC activates Ras and c-Raf-1, leading to stimulation of MKK mek1/mek2 and subsequent...
activation of MAPK<sup>erk1/2</sup> (44, 53, 54). Our studies extend the function of this pathway into primary culture cells and, using two methods for determination of MAPK<sup>erk1/2</sup> activation, confirm that PKC activation stimulated the MAPK<sup>erk1/2</sup> pathway. It is unlikely that either the MAPK<sup>jak</sup> or the MAPK<sup>p38</sup> pathway was similarly involved in PMA-stimulated GM-CSF release in HBECs, because 1 PMA failed to elicit phosphorylation of MAPK<sup>jak</sup> and MAPK<sup>p38</sup> at time points during which MAPK<sup>erk1/2</sup> was observed; and 2 PMA-induced GM-CSF release was not inhibited by SB203580, an agent that inhibits MAPK<sup>p38</sup> at low concentrations and has recently been reported to inhibit MAPK<sup>jak</sup> at higher ones (>10 μM).

The ability of TNF receptor family members to activate the MAPK<sup>erk1/2</sup> pathway has been recently reported, but is less well established (45). Our data demonstrated that TNF-α elicited up-regulation of MAPK<sup>erk1/2</sup> in HBECs. The response was slightly delayed compared with that induced by PMA, but was clearly present by 60 min. Moreover, the activation of MAPK<sup>erk1/2</sup> by TNF-α was also associated with and necessary for GM-CSF release. The upstream signals by which TNF receptors activate MAPK<sup>erk1/2</sup> have not been fully described. Whereas the MAPK<sup>erk1/2</sup> pathway is associated with Ras/Raf-1 activation, the interaction of TNF signaling with this pathway is not as well described and may be cell type specific. The TNF receptor has been observed to interact with the adaptor protein Grb2 and the exchange factor SOS in response to TNF-α, linking the TNF-R1 to c-Raf-1 kinase (55). TNF-α has also been suggested to activate the MAPK<sup>erk1/2</sup> pathway through sequential activation of ceramide-activated protein kinase and Ras-1 (56, 57). Recently, a novel serine-threonine kinase, RIP2 has been described to interact with members of the TNF receptor family and interact cooperatively with Ras, Raf-1, and MAPK<sup>erk1/2</sup> (58). Our studies raise the tantalizing possibility that the TNF-α-induced activation of MAPK<sup>erk1/2</sup> in HBECs is mediated by the cooperative activity of this pathway.

Engagement of the TNF-α receptors has been well described to activate MKKK<sup>meck1/3</sup> and MKK<sup>mek</sup>4,7, and subsequently isoforms of MAPK<sup>jak</sup> or MAPK<sup>p38</sup> in a TRAF-dependent, Ras/Raf-1-independent manner (53, 59, 60). In contrast to our findings with PMA, our studies do not preclude the involvement of MAPK<sup>jak</sup> or MAPK<sup>p38</sup> pathways in TNF-α-induced GM-CSF production, because GM-CSF production was moderately decreased in the presence of the MAPK<sup>p38</sup> inhibitor. However, the degree of the effects observed suggest that the MAPK<sup>erk1/2</sup> pathway is probably of primary importance.

In T cells and endothelial cells, activation of PKC via PMA is not sufficient for GM-CSF production (28, 29, 41, 42). Rather, the delayed compared with that induced by PMA, but was clearly present by 60 min. Moreover, the activation of MAPK<sup>erk1/2</sup> by TNF-α was also associated with and necessary for GM-CSF release. The upstream signals by which TNF receptors activate MAPK<sup>erk1/2</sup> have not been fully described. Whereas the MAPK<sup>erk1/2</sup> pathway is associated with Ras/Raf-1 activation, the interaction of TNF signaling with this pathway is not as well described and may be cell type specific. The TNF receptor has been observed to interact with the adaptor protein Grb2 and the exchange factor SOS in response to TNF-α, linking the TNF-R1 to c-Raf-1 kinase (55). TNF-α has also been suggested to activate the MAPK<sup>erk1/2</sup> pathway through sequential activation of ceramide-activated protein kinase and Ras-1 (56, 57). Recently, a novel serine-threonine kinase, RIP2 has been described to interact with members of the TNF receptor family and interact cooperatively with Ras, Raf-1, and MAPK<sup>erk1/2</sup> (58). Our studies raise the tantalizing possibility that the TNF-α-induced activation of MAPK<sup>erk1/2</sup> in HBECs is mediated by the cooperative activity of this pathway.

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