Size Fractions of Ambient Particulate Matter Induce Granulocyte Macrophage Colony–Stimulating Factor in Human Bronchial Epithelial Cells by Mitogen-Activated Protein Kinase Pathways

Joan Reibman, Yanshen Hsu, Lung Chi Chen, Asok Kumar, Wei Cheng Su, Wanda Choy, Anita Talbot, and Terry Gordon

Division of Pulmonary and Critical Care Medicine, Department of Medicine; Department of Environmental Medicine, Nelson Institute of Environmental Medicine; and Department of Pathology, New York University School of Medicine, New York, New York

Environmental pollutants, including ambient particulate matter (PM), increase respiratory morbidity. Studies of model PM particles, including residual oil fly ash and freshly generated diesel exhaust particles, have demonstrated that PM affects inflammatory airway responses. Neither of these particles completely represents ambient PM, and therefore questions remain about ambient particulates. We hypothesized that ambient PM of different size fractions collected from an urban environment (New York City air), would activate primary culture human bronchial epithelial cells (HBECs). Because of the importance of granulocyte-macrophage colony–stimulating factor (GM-CSF) on inflammatory and immunomodulatory processes, we focused our studies on this cytokine. We demonstrated that the smallest size fraction (ultrafine/fine; < 0.18 μm) of ambient PM (11 μg/cm^2), upregulated GM-CSF production (2-fold increase). The absence of effect of carbon particles of similar size, and the day-to-day variation in response, suggested that the chemical composition, but not the particle itself, was necessary for GM-CSF induction. Activation of the extracellular signal-regulated kinase and the p38 mitogen-activated protein kinase was associated with, and necessary for, GM-CSF release. These studies serve to corroborate and extend those on model particles. Moreover, they emphasize the role of the smallest size ambient particles in airway epithelial cell responses.

The biologic plausibility of adverse respiratory health effects due to particulate matter (PM) is supported by an increasing number of epidemiologic analyses (1–4). The characteristics of the particles and the mechanisms by which PM influences the development and severity of respiratory diseases remain incompletely clarified. Particulate pollutants may participate in respiratory effects by the induction of acute inflammatory responses in the airway. There is also increasing evidence for an immunomodulatory role for PM (5). The properties of ambient pollutants that mediate these effects remain to be elucidated.

(*Received in original form December 10, 2001 and in revised form April 9, 2002*)

Address correspondence to: Joan Reibman, M.D., New York University School of Medicine, Division of Pulmonary and Critical Care Medicine, 550 1st Avenue, Room NB7N24, New York, NY 10016. E-mail: reibmj01@gcrc.med.nyu.edu

Abbreviations: antigen-presenting cells, APC; diesel exhaust particles, DEP; enzyme-linked immunosorbent assay, ELISA; extracellular signal-related kinase, ERK; granulocyte-macrophage colony–stimulating factor, GM-CSF; intercellular adhesion molecule, ICAM; interleukin, IL; mitogen-activated protein kinase, MAPK; mass median aerodynamic diameter, MMAD; primary culture human bronchial epithelial cells, HBECs; particulate matter, PM; residual oil fly ash, ROFA; tumor necrosis factor-α, TNF-α.

Am. J. Respir. Cell Mol. Biol. Vol. 27, pp. 455–462, 2002
DOI: 10.1165/rcmb.2001-0005OC
Internet address: www.atsjournals.org

Inflammatory and immune effects of PM have been most studied using model systems such as residual oil fly ash (ROFA) or freshly-generated diesel exhaust particles (DEP). ROFA, a byproduct of oil combustion, induces human and animal cells from the lung to produce reactive oxygen species as well as cytokines and chemokines (6–9). In animal models, ROFA upregulates ovalbumin-induced airway responsiveness as well as sensitization to house dust mite (7, 10). Freshly collected DEP have also been used extensively to model effects of ambient particulates (5). Exposure of bronchial epithelial cells to freshly collected DEP induces the release of proinflammatory cytokines including RANTES, granulocyte-macrophage colony–stimulating factor (GM-CSF), intercellular adhesion molecule-1 (ICAM-1), and interleukin (IL)-8 (11–13). Animal models and human studies have demonstrated that DEP induce and exacerbate pulmonary and nasal inflammation and induce IgE (5, 14–16) .

Although both ROFA and DEP are model particles, neither completely represents ambient PM. Indeed, metal concentrations in ROFA are significantly higher than those in typical fine ambient PM. In addition, whereas diesel exhaust is a major component of ambient air pollution, it is only one of many different combustion products. Therefore, studies on ambient urban particles are needed to corroborate studies of model particles.

Bronchial epithelial cells are one of the first targets for ambient pollutants. These cells synthesize and secrete proinflammatory and immunomodulatory cytokines and chemokines, including GM-CSF (17). GM-CSF, a 23-kD protein, has well-described antiapoptotic effects (18). Most intriguing, GM-CSF is a critical factor for the functional maturation of dendritic cells (DC) into potent antigen-presenting cells (APC) (19). In the lung, elevated epithelial levels of GM-CSF have been demonstrated in association with the accumulation of DC (20). Moreover, compartmentalized transgene expression of GM-CSF to epithelial cells facilitates the development of an antigen-specific, eosinophilic inflammatory response (21). Thus, GM-CSF production in the airways is pertinent for diseases such as asthma, as it serves to modify the functions of effector cells such as eosinophils, as well as initiator cells such as dendritic cells.

In this study we used size fractions of ambient PM derived from an urban environment to investigate mechanisms by which ambient PM activates epithelial cells, and focused our studies on GM-CSF production. Primary culture human bronchial epithelial cells (HBECs) were used as the relevant target cell. We demonstrated that the smallest size fraction of ambient PM serves as a potent stimulus

for GM-CSF release by these cells. We also describe signal transduction pathways by which GM-CSF release is regulated by ambient PM. Our study describes a mechanism by which ambient PM may disrupt the airway balance of inflammatory and immunomodulatory mediators.

Materials and Methods

Reagents

Basal cell culture medium (BEBM) was obtained from Clonetics (San Diego, CA) and for routine cell growth was supplemented with the following compounds: basal epithelial growth medium hEGF (0.5 ng/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 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). Human recombinant tumor necrosis factor-α (TNF-α) was obtained from R&D (Minneapolis, MN). Antibodies directed against phosphorylated and nonphosphorylated forms of extracellular signal-related kinase (ERK)1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and antibodies directed against p38 were obtained from Cell Signal (Beverly, MA). PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA) and were dissolved in dimethyl sulfoxide (Me_2SO). The final concentration of Me_2SO did not exceed 0.1% (vol/vol). ECL Plus kits were obtained from Amersham Pharmacia (Buckinghamshire, UK). Carbon particles were generated by passing a small quantity of acetylene in argon into a high temperature silicon carbide furnace maintained at 1,098°C. The acetylene underwent a thermal decomposition and produced carbon black particles. These carbon particles were collected using a cascade impactor. Mount St. Helen particles were a gift of Kevin Dreher (U.S. EPA, Research Triangle Park, NC).

Size-fractionated samples of ambient PM were collected from New York City air using four stages of a cascade impactor (micro-orifice uniform deposition impactor [MOUDI]; MSP Corp., St. Paul, MN) located in downtown Manhattan, New York City (8th floor of a building located on 26th St. and 1st Ave.). The MOUDI is a cascade impactor that employs micro-orifice nozzles to extend the cut sizes of the lower stages without going to low pressures or creating excessive pressure drops across the impactor stages. A sealed transport cover for impaction plates and filter holders was used to allow transport without contamination. A pre-impactor screen prevented insects or rain/snow droplets from entering the impactor. The first stage removes nuisance particles and was not used for biologic assays. The particles collected on filters, and the after-filter particles do not strictly correspond to PM_{10} and $PM_{2.5}$ definitions. For the purposes of the study, we defined sizes to closely approximate standard definitions as follows: ultrafine (UF)/fine, < 0.18 μm; fine, 0.18–1.0 μm; intermediate, 1–3.2 μm; and coarse, > 3.2 μm.

Samples were collected (14 d) on inert filters (Nucleopore or Teflon filters) used as impactor substrates (endotoxin-free). Impactor substrates and after-filters were weighed before and after sampling on a Cahn electrobalance (1 μg sensitivity) (Cahn Division, Ventron Instruments, Cerritos, CA) and PM removed by ultrasonification (20 min) into sterile medium in a sterile 30-ml conical polypropylene tube followed by ultrasonification (10 s) with a Virsonic 50 ultrasonicator (VirTis, Gardiner, NY). Samples were suspended in medium used for growth of HBECs. HBECs were exposed to varying concentrations of size-fractionated ambient PM (25–100 ug/ml, 18 h, 37°C), the maximal dose of which corresponded to 11 $\mu g/cm^2$. Cultures were examined visually using an inverted microscope for changes in morphology and adhesion. Gross alterations were not detected in cellular morphology or adhesion in UF/fine PM–exposed cells compared with control cells. Toxicity was measured by trypan blue exclusion and cells were $> 90\%$ viable.

Cell Culture

Culture of HBECs from bronchial brush biopsies was performed as previously described (22). Briefly, cells were obtained during bronchoscopy of normal human subjects (New York University IRB-approved protocol). Bronchial brushing was performed with a disposable brush (Model BC-15C; Olympus Industrial America Inc., Nanuet, NY). The cells obtained by brushing were collected into serum-free, hormonally supplemented medium (BEGM; Clonetics) containing amphotericin and gentamicin. The cells were plated in uncoated T25 tissue culture flasks and incubated (37°C, 5% CO_2) for 7–10 d, during which time the cells were fed every 2 d. 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 antihuman cytokeratin antibodies. For some experiments, HBECs were purchased from Clonetics and were cultured in the same manner and used at passage 3.

Enzyme-Linked Immunosorbent Assay

Cells were grown to near confluence at passage 3 and stimulated with the specified agents (18 h, 37°C). Supernatants were subsequently collected, centrifuged (1,000 rpm, 10 min), diluted appropriately, and concentration of GM-CSF determined by enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, MA). Measurements were performed in duplicate and quantified at 450 nm (Bio-Rad microplate reader; Bio-Rad, Richmond, CA).

Immunoblotting with Phosphospecific Antibody Probes

Activated mitogen-activated protein kinase (MAPK) species were detected using phosphospecific antibodies 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 Na_3VO_4, 50 mM NaF, 1 mM EGTA, 50 μg/ml aprotinin, 50 μg/ml chymostatin, 25 μg/ml pepstatin). Lysates were centrifuged (1,000 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% sodium dodecyl sulfate-Tris Glycine gels and resolved proteins transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked (5% nonfat dried milk/Tris-buffered saline/0.25% Tween 20) and probed with anti-phospho ERKs 1 and 2 (ERK1/2; 1:200) or anti–phospho-p38 (1:200) or anti-ERK2, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,500). Bound antibodies were visualized using the ECL Western blot detection system according to the manufacturer's instructions. Equal loading of samples was checked by using stripped blots for immunodetection of ERK2 with phosphorylation state-independent pan-antibodies.

Electron Microscopy

HBECs were fixed in 2.5% gluteraldehyde and post-fixed in 1% OsO_4, dehydrated in ascending series of ethylene alcohol, and embedded in Embed-812 (Electronmicroscopy Sciences, Fort Washington, PA). Ultrathin sections were cut on a Reichert ultracult S,

stained with saturated uranyl acetate and Reynold's lead citrate, and photographed on a Zeiss EM 10 (Carl Zeiss, Inc., Thornwood, NY).

Statistical Analysis

All data were examined by analysis of variance. A Greenhouse-Geisse adjustment *post hoc* analysis with simple contrast was used to adjust for multiple pairwise comparisons between groups.

Results

PM Collection

To characterize the distribution of the particle sizes collected by the MOUDI, we analyzed the mass distribution of each size collected over 2-wk periods (n = 15) (Figure 1). The mass median aerodynamic diameter (MMAD) of all particle samples collected was 0.41 ± 0.03. On a daily mass basis, the PM collected by the MOUDI contained predominantly particles of the UF/fine and fine fraction with UF/fine and fine particles representing 32.3 ± 0.02 and 40.8 ± 0.02%, respectively, of the total mass. When analyzed by season, we did not detect a significant difference in the MMAD or mass particle distribution (data not shown).

Response of HBECs to Ambient PM

To determine whether PM derived from size-fractions of ambient PM upregulated GM-CSF release from bronchial epithelial cells, HBECs were exposed (18 h) to increasing concentrations of size-fractionated ambient PM. Medium was removed and assayed for GM-CSF release by ELISA. UF/fine PM elicited a dose-dependent increase in GM-CSF release (Figure 2A). No dose–response was detected for the larger fractions (data not shown). The maximal dose tested (100 μg/ml; 11 μg/cm^2) was consistent with *in vitro* exposures commonly used in published reports (8, 23, 24). This dose stimulated a significant increase in GM-CSF release (2.0 ± 0.8 ng/10^6 cells above background, n = 5, P < 0.04; background release = 0.51 ± 0.22 ng/10^6 cells). This response was equivalent to 43.0 ± 0.06% of that induced by a high dose of TNF-α (100 U/ml, mean ± standard error of the mean, n = 4).

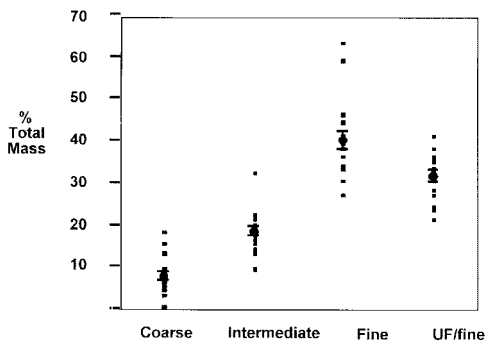

Figure 1. Daily mass distribution of ambient PM size fractions. Samples were collected from filters of a cascade impactor (MOUDI). Impactor substrates and after-filters were weighed before and after sampling on a Cahn electrobalance (1 μg sensitivity). The mass distribution of each size collected over the two-week periods (n = 15) was calculated and is presented as the % of the total daily total mass collected.

To determine whether size fractions of PM elicited a difference in GM-CSF release, GM-CSF released in HBEC supernatants was compared after stimulating cells with the equivalent mass of each size fraction. When compared on an equivalent mass basis in cells cultured from the same donors, UF/fine PM, but neither the fine nor intermediate fractions, elicited a significant increase in GM-CSF release by HBECs (Figure 2B). We therefore focused all subsequent studies on UF/fine ambient PM.

Daily Variability

To test whether the response to UF/fine PM was a result of a general particle effect, we determined whether the response of HBECs to ambient PM would be reproduced by exposure of cells to elemental carbon of a similar size. Using a technique previously developed in our laboratory, carbon particles were generated by the pyrolysis of acetylene. Briefly, carbon particles were generated by passing a

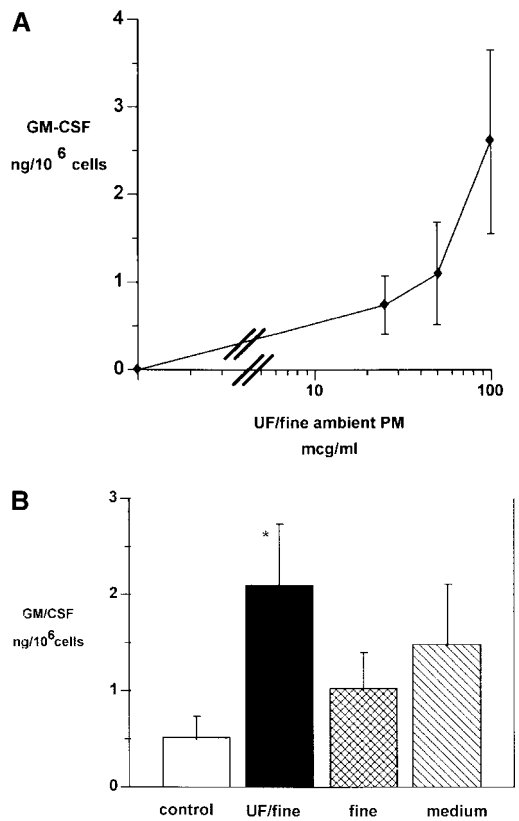

Figure 2. GM-CSF release in response to ambient PM. HBECs were prepared from bronchial brush biopsies and used at passage 3. Cells were exposed (18 h) to increasing concentrations of size fractions of ambient PM. Medium was removed and assayed for GM-CSF release by ELISA. The maximal dose tested (100 μg/ml) corresponded to 11 μg/cm^2. Data are presented as ng GM-CSF release/10^6 cells after subtraction of background release from each experiment (background release = 0.51 + 0.30 ng/10^6 cells, n = 3). (*A*) Cells were exposed to increasing concentrations of UF/fine ambient PM (n = 5, P < 0.04). (*B*) HBECs derived from the same donors were exposed to the equivalent mass of each size fraction and GM-CSF determined by ELISA (n = 3, *P < 0.04, UF/fine compared with control).

small quantity of acetylene in argon into a high-temperature silicon carbide furnace maintained at 1,098°C. The acetylene underwent a thermal decomposition and produced carbon black particles. These particles were collected using the same MOUDI system, thus resulting in particles with the same size distribution as the ambient particles used for exposure. Carbon particles of UF/fine and fine sizes (100 μg/ml, 18 h, 37°C) failed to elicit a significant increase in the release in GM-CSF from HBECs (Figure 3A). Absence of stimulation was also detected in response to exposure to volcanic ash derived from Mt. St. Helen, which contains particles with an MMAD greater than that of ambient urban PM (Figure 3A).

Ambient PM used in experiments was collected over discrete two-week periods. Day to day variation in the chemical components of ambient PM may vary depending on the vehicular traffic and industrial waste. Because of the possibility that the chemical components of PM may vary, the effect of UF/fine particles collected from multiple sampling periods was tested on the release of GM-CSF by a single donor. As shown in the representative experiment in Figure 3B, samples collected during different time periods elicited GM-CSF production with a > 2-fold induction. However, there was variation in the induction of GM-CSF by samples collected over different time periods, suggesting the possibility that the chemical composition of the samples was important for the induction of GM-CSF.

Because ambient particles may be associated with endotoxin, we tested whether this ambient contaminant stimulated the release of GM-CSF from HBECs. HBECs were exposed to lipopolysaccharide (1 μg/ml, 18 h, 37°C) and GM-CSF release monitored. Exposure of cells to endotoxin failed to stimulate the release of GM-CSF (data not shown), suggesting that this component was not responsible for the observed upregulation of GM-CSF.

Electron Microscopy

Macrophages, professional phagocytic cells, are well described as cells that endocytose and phagocytose particles. The process by which epithelial cells respond to particles is less well described. To visualize the interaction between airway epithelial cells and particles, HBECs were exposed to UF/fine PM (18 h, 100 μg/ml) at the time and concentration to which they had previously been demonstrated to display a maximal response. Transmission electron microscopy of HBECs is shown in Figure 4. Epithelial cells dis-

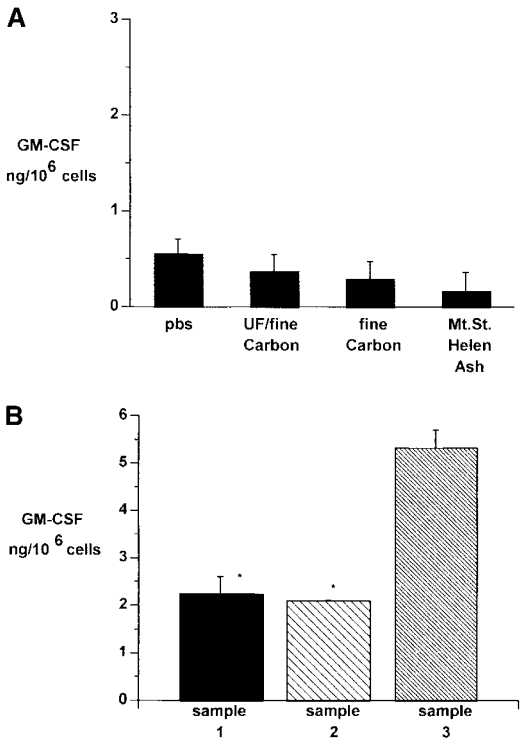

Figure 3. GM-CSF release by HBECs in response to carbon particles and particles derived from different collection periods. (*A*) HBECs were exposed to size-fractionated carbon particles (100 μg/ml, 18 h) derived by thermal decomposition, or ash derived from Mt. St. Helen (MSH) and GM-CSF assayed from the medium. Data are presented as ng GM-CSF release/10^6 cells ($n = 3$; background release = 0.54 + 0.16 ng/10^6 cells). (*B*) HBECs derived from the same donor were exposed to UF/fine samples obtained from three different collection periods. Data are presented as ng GM-CSF release/10^6 cells after subtraction of background release (Samples 1 and 2 were tested in triplicate, $*P < 0.05$. Sample 3 was tested twice and the range is presented).

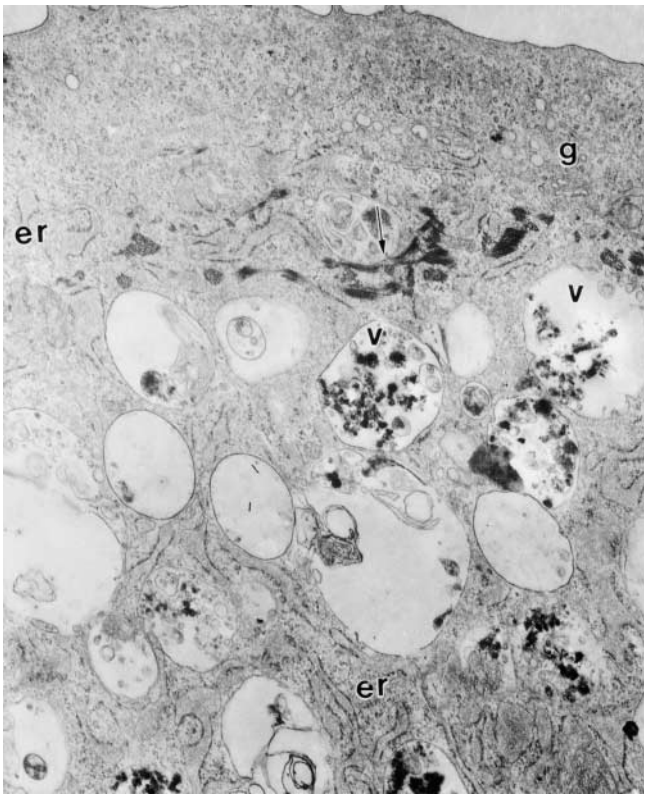

Figure 4. Electron microscopy of HBECs and UF/fine ambient PM. HBECs showing bundles of tonofilaments (*arrow*), well-developed Golgi apparatus (g), and few stacks of endoplasmic reticulum (er) in the cytoplasm of the cell. Multivesicular bodies (v) containing particles can be visualized. *Original magnification:* ×20,000.

played few microvilli, and as expected from the culture technique used, had not differentiated into ciliated cells. Bundles of tonofilaments were seen in the cytoplasm. Golgi apparatus and rough endoplasmic reticulum were consistent with active protein synthesis in these cells. Epithelial cells could be detected in various stages of interaction with particles. Particles were evident in multivesicular bodies inside the cytoplasm. The presence of additional cytoplasmic vesicles, some of which were empty, suggested that these vesicles might have previously harbored particles.

MAPK Activation by UF/Fine Ambient PM in HBECs

MAPK cascades are involved in cell proliferation, differentiation, and apoptosis (25). We have previously demonstrated that the activation of ERK1/2 is both associated with and necessary for the production of phorbol myristate acetate (PMA) and TNF-α–induced GM-CSF in HBECs. In these studies we also confirmed that ERK1/2 phosphorylation detected in the Western blots correlated with actual ERK1/2 activity (22). We therefore tested whether the upregulation of GM-CSF by the UF/fine fraction of ambient PM was associated with the activation of ERK1/2. Activation of ERK1/2 was determined by immunoblot analysis of lysates of HBECs using an antibody that recognizes the dually phosphorylated, active state of ERK1/2 (p-ERK1/2). As demonstrated in a representative study in Figure 5A, p-ERK1/2 was barely detectable in resting HBECs. Upregulation of p-ERK1/2 was detected in HBECs derived from a single donor exposed to samples obtained from three additional different collection periods. These effects were not due to changes in total ERK1/2, as stripped blots subsequently exposed to an antibody that does not distinguish between the active (phosphorylated) and inactive state of ERK1/2 (pan-ERK) did not show a change in total levels. Exposure of HBECs derived from different donors to UF/fine PM (60 min) elicited a 6.3 ± 3.8-fold increase in pERK as determined by densitometry (n = 6 donors). UF/fine carbon particles (100 μg/ml, 60 min, 37°C) that had been generated by pyrolysis of acetylene and collected using the MOUDI system failed to upregulate ERK1/2 (Figure 5A), and even decreased p-ERK1/2 expression.

The p38 MAPKs are activated by a variety of stress-related signals such as heat, osmotic shock, UV irradiation, and proinflammatory cytokines (25). Because activation of p38 MAPK has been demonstrated in HBECs (26), we also examined whether UF/fine ambient PM induced the expression of phosphorylated p38. Activity of p38 MAPK was analyzed with an anti–phospho-p38 MAPK antibody that is specific for p38 and does not crossreact with ERK1/2. As demonstrated in the representative figure (Figure 5B), HBECs constitutively expressed activated p38 as determined by the presence of phosphorylated p38 (p-p38). Upregulation of p-p38 was detected in HBECs in response to TNF-α, and only minimally increased in the same cells derived from a single donor and exposed to samples obtained from three different collection periods. In multiple experiments using different donors (n = 3), TNF-α (60 min) induced a 2-fold induction of activated p38, whereas p-p38 was minimally increased in response to UF/fine PM samples (1.4 ± 0.070-fold increase).

We subsequently asked whether the activation of the ERK1/2 and p38 MAPK pathways was necessary for the release of GM-CSF by HBECs stimulated with UF/fine ambient PM. The cell permeant molecule PD98059 is a selective inhibitor of the upstream MAPK extracellular-regulated kinases (MEK1/2), which phosphorylate and activate ERK1/2 (25). We have previously demonstrated that PD98059 (40 μM) inhibits ERK1/2 activity in HBECs stim-

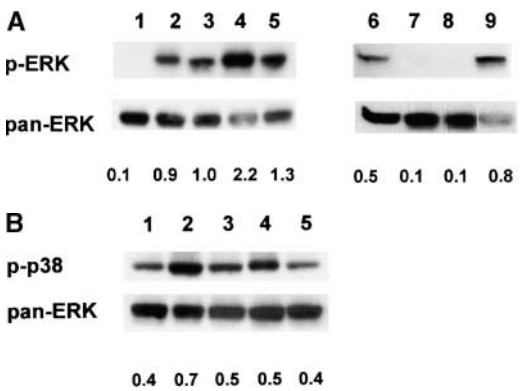

Figure 5. Activation of MAPK pathways in HBECs. (*A*) Lysates were prepared from resting HBECs (*lane 1*), HBECs stimulated with TNF-α (100 U/ml, 1 h, *lane 2*), or UF/fine ambient PM (100 μg/ml, 1 h) collected over three different collection periods (*lanes 3–5*). In a separate experiment, HBECs were exposed to UF/fine carbon (100 μg/ml, 1 h, *lanes 7* and *8*) or TNF-α (100 U/ml, 1 h, *lane 9*). After SDS-PAGE and transfer to PDVF membranes, immunoblotting was performed with phosphospecific (p-ERK1/2) or control (pan-ERK2) probes. (*B*) Lysates were prepared from resting HBECs (*lane 1*), HBECs stimulated with TNF-α (100 U/ml, 1 h, *lane 2*), or UF/fine ambient PM (100 μg/ml, 1 h) collected over three different collection periods (*lanes 3–5*). Immunoblotting was performed with phosphospecific p38 (p-p38) or control pan-ERK2 probes. Numbers at the bottom of the lanes are the densitometry ratio of phosphorylated over control antibody to correct for variations in protein loading.

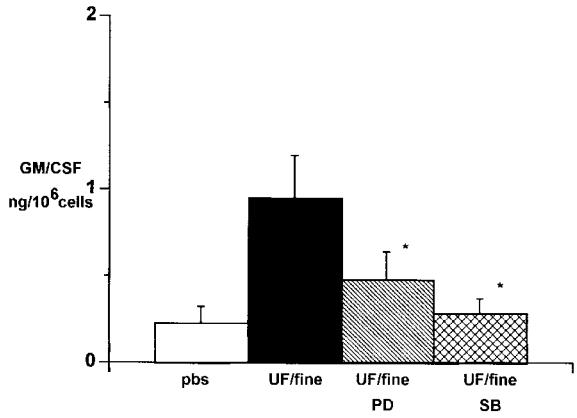

Figure 6. Inhibition of ERK1/2 or p38 MAPK activation and GM-CSF production induced by UF/fine ambient PM. HBECs were stimulated with UF/fine ambient PM (100 μg/ml, 18 h) in the absence or presence of PD98059 (40 μM) or SB203580 (0.1 μM). Supernatants were removed and GM-CSF release determined by ELISA. Data are presented as ng GM-CSF release/106 cells (n = 6, *P < 0.01 compared with stimulated cells).

ulated by PMA or TNF-α (27). We therefore asked whether this agent would inhibit GM-CSF production in HBECs stimulated by UF/fine ambient PM using concentrations that have previously been demonstrated to be relatively specific and efficacious for the inhibition of these kinases (35). HBECs were grown to near-confluence and stimulated with UF/fine ambient PM in the presence or absence of PD98059 (40 μM). As shown in Figure 6, PD98059 significantly inhibited GM-CSF release induced by UF/fine ambient PM (54.0 ± 0.12% UF/fine response, $n = 7$, $P \leq 0.01$). Exposure of cells to SB203580 (0.1 μM), an inhibitor of p38 MAPKs, also decreased UF/fine-stimulated GM-CSF release (39.1 ± 13% UF/fine response, $n = 6$, $P \leq 0.01$). Neither PD nor SB, at the doses used in these studies, inhibited basal release of GM-CSF ($n = 6$–7, data not shown). These data are consistent with a role for both MAPK pathways in activation of HBECs induced by UF/fine ambient PM.

Discussion

The role of ambient PM as a cause of adverse health effects has come under increasing scrutiny. The plethora of epidemiologic studies suggesting an association with respiratory morbidity and PM invites investigation into the characteristics of the particles and the mechanisms by which adverse effects are mediated. The studies now reported serve to further expand the information available on the cellular effects of ambient PM. Using an environmentally relevant particle and cell model, we now demonstrate that ambient PM upregulates GM-CSF production in primary culture HBECs. We have demonstrated that the upregulation is associated with particle size of ambient PM, with an important role for smaller particles. We suggest that the chemical component of ambient PM, rather than the particle itself, is responsible for cell activation, and have demonstrated intracellular incorporation of UF/fine particles. Finally, we have demonstrated that upregulation of multiple MAPK pathways is associated with, and necessary for, GM-CSF production in response to UF/fine ambient PM. The results of our studies using ambient particles were remarkably similar to those reported for DEP (12, 13, 27). Our studies now serve to support these studies using an ambient particle model.

The epithelial cell serves as a barrier between the environment and the cells of the airway. In this position, epithelial cells have an extraordinary capacity to modify cell responses in the airway via their production of cytokines. We investigated epithelial-cell release of GM-CSF in particular because of the critical importance of this cytokine in mediating acute inflammatory responses via effects on eosinophils, and in mediating immune responses via effects on dendritic cells. The ability of GM-CSF to mediate airway inflammatory effects and airway sensitization has been elegantly demonstrated in murine models (21). These studies, and the presence of increased GM-CSF expression in the human airway in diseases such as asthma, support the importance of GM-CSF as an important component of the complex signal that mediates airway sensitization and inflammation.

Normal HBECs were used for the studies reported in this paper. These cells were cultured from a variety of donors and thus provided an expected variation in responses. The variation reflected individual biologic diversity and was found in response to all stimuli used, including TNF-α. Our study did not use differentiated cells and we cannot rule out that polarized, differentiated cells might display a different response.

We focused our studies on ambient PM as a relevant model of PM and collected ambient PM from a well-traversed street in a large urban center. We chose this model of an ambient particle to test whether we would corroborate studies using DEP. Moreover, DEP comprise a significant but incomplete source of urban ambient PM. The particle sizes tested were all < 3.2 μm, correlating most closely with the $PM_{2.5}$ fraction that is commonly monitored. Indeed, most of the total measured annual PM_{10} concentration consists of the fine particle fraction (< 2.5 μm) (28). $PM_{2.5}$ is derived from regional as well as mobile sources. In a recent study of six U.S. cities, the $PM_{2.5}$ fraction was derived predominantly from coal as well as mobile sources (29). In New York City streets, simultaneous monitoring of $PM_{2.5}$ and elemental carbon (EC) suggested broad regional as well as local sources (30). Thus, mobile sources are a predominant but not exclusive source of smaller particles of ambient PM.

Our studies demonstrated that the smallest component of ambient PM that was collected, the UF/fine fraction, was the most proficient at eliciting GM-CSF when compared on a mass basis. Particle size is important for determining the site and efficiency of pulmonary deposition. Importantly, particle size is also a surrogate for the source and composition of particles. Although we have not yet fully characterized the components of these ambient UF/fine particles, our data suggest that the chemical components are critical determinants for cell activation. We base this conclusion on the finding that carbon particles generated of the same size failed to stimulate GM-CSF production. We also used ambient PM collected over various time periods. Because of industrial source, weather, and traffic pattern variations, it would be expected that the components of the particles collected would also display some variation. Indeed, when tested on a single donor, particles collected from multiple sampling periods had a similar effect, although the magnitude of the effect differed, suggesting some variation in components of the particles. The effect of particle size and component has been studied on the release of IL-1, IL-6, and TNF-α production by epithelial cells and has also been demonstrated to be dependent on these characteristics (31). Our data serve to confirm recent studies of PM_{10} (32) and further demonstrate that different size PM fractions may have discrete cellular effects. The differences in size effects may be due in part to variations in chemical composition.

The important role of endotoxin in biologic effects of particles has been well described (33). Our cells failed to respond to lipopolysaccharide, a finding consistent with a requirement for serum-derived sCD14 for the induction of a lipopolysaccharide response in epithelial cells (34). Our cells were cultured in serum-free medium and thus lacked this component. The data suggest that although endotoxin may participate in biologic responses induced by ambient particles, there are additional components involved in the process.

The process by which ambient particles interact with epithelial cells is only beginning to be investigated. To begin to elucidate the interaction between ambient particles and epithelial cells, we performed transmission electron microscopy of primary culture epithelial cells exposed to a stimulatory dose of UF/fine ambient PM. HBECs internalized clusters of particles and contained multiple vacuoles filled with particles. These findings are similar to those of Boland and coworkers, who describe endocytosis of DEP (12). The studies raise a multitude of questions for further study, including inquiries about whether internalization is required for cell activation, the identity of the endocytic receptors, and the characteristics of the vesicle.

The mechanisms by which ambient particles activate epithelial cells are also only just beginning to be elucidated. Activation of the network of MAPK cascades induces a myriad of cell functions including cell differentiation, proliferation, and death (25). Although there are distinct MAPKs regulated by upstream three-kinase modules, there is also overlap between the functions of some of these proline-directed, serine/threonine kinases. Because mammalian cells possess multiple MAPK pathways that respond to inflammation and stress, we began our investigation with two parallel MAPK-signaling cascades. Because we previously demonstrated that activation of the ERK1/2 MAPK pathway is associated with, and necessary for, GM-CSF production by HBECs, we began our investigations of the signaling pathways activated in response to UF/fine ambient PM with an investigation of this cascade. Our data show that ambient UF/fine PM upregulates ERK1/2 activation and that this activation is required for GM-CSF production. The requirement for ERK1/2 activation was demonstrated using the MEK1/2 inhibitor PD98059. Although this compound has little inhibitory activity toward other protein kinase pathways, it is also capable of inhibiting MEK5, a kinase upstream of ERK5, and we cannot rule out a role for this pathway.

The stress-activated p38 MAPK consists of four identified genes, the most studied of which are the p38α and β isoforms (25). We demonstrated constitutive activation of this kinase in HBECs, and variable and often only minimal upregulation of the p38 pathway by UF/fine ambient particles. Using the pyridinyl imidazole SB203580, an inhibitor of p38α and β activity, we demonstrated inhibition of GM-CSF release induced by UF/fine ambient PM. Although SB203580 is relatively specific for p38α and β, it has also been demonstrated to have additional, albeit less potent, effects on additional protein kinases including c-Raf and LCK (35). The studies raise the possibility that the inhibition of UF/fine-induced GM-CSF by SB203580 may be due to downregulation of constitutively activated p38 or may be mediated by additional pathways. Our data are in agreement with the pathways recently described to be activated in epithelial cell lines (16HBE140− cells) in response to DEP (27). Our studies are also in accord with models using ROFA as well as benzo[a]pyrene adsorbed on carbon black (36, 37). Interestingly, our data differ from that of Hashimoto and colleagues, in which the effect of DEP was studied on the transformed epithelial cell line, BET-1A. In these studies, DEP stimulated IL-8 and RANTES production and was associated with activation of p38 MAPK but not ERK (26). These differences in the findings may in part be explained by differences in the epithelial cells.

These data, using relevant particle and cell models, serve to demonstrate that ambient particulates, particularly the smallest size fraction, induce GM-CSF, a critical proinflammatory and proimmunomodulatory cytokine in epithelial cells. Moreover, these particles act via intracellular signaling pathways that are associated with physiologic stimuli. Our investigations serve to expand upon studies being described for DEP, and further elucidate mechanisms of toxicity of ambient pollutants.

Acknowledgments: This study was supported by grants NIH R01 ES10187-01A1, NIEHS 5 P30 ES00260, EPA R826244, EPA CR827164, and NIH CCR MO1 00096.

References

1. Bascom, R., P. A. Bromberg, D. A. Costa, R. Devlin, D. W. Dockery, M. W. Frampton, W. Lambert, J. M. Samet, F. E. Speizer, and M. Utell. 1995. Health effects of outdoor air pollution. *Am. J. Respir. Crit. Care Med.* 153:3–50.
2. Dockery, D. W., C. A. Pope, X. Xu, J. D. Spengler, J. H. Ware, M. E. Fay, B. G. Ferris, and F. E. Speizer. 1993. An association between air pollution and mortality in six US cities. *N. Engl. J. Med.* 329:1753–1759.
3. Thurston, G. D., K. Ito, D. Hayes, D. V. Bates, and M. Lippmann. 1994. Respiratory hospital admissions and summertime haze air pollution in Toronto, Ontario: consideration of the role of acid aerosols. *Environ. Res.* 65:271–290.
4. Samet, J. M., F. Dominici, F. C. Curriero, I. Coursac, and S. L. Zeger. 2000. Fine particulate air pollution and mortality in 20 US cities, 1987–1994. *N. Engl. J. Med.* 343:1742–1749.
5. Diaz-Sanchez, D. 2000. Pollution and the immune response: atopic diseases are we too dirty or too clean? *Immunology* 101:11–18.
6. Dye, J. A., K. B. Adler, J. H. Richards, and K. L. Dreher. 1997. Epithelial injury induced by exposure to residual oil fly-ash particles: role of reactive oxygen species. *Am. J. Respir. Cell Mol. Biol.* 17:625–633.
7. Gavett, S. H., S. L. Madison, M. A. Stevens, and D. L. Costa. 1999. Residual oil fly ash amplifies allergic cytokines, airway responsiveness, and inflammation in mice. *Am. J. Respir. Crit. Care Med.* 160:1897–1904.
8. Carter, J. D., A. J. Ghio, J. M. Samet, and R. B. Devlin. 1997. Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol. Appl. Pharmocol.* 146:180–188.
9. Goldsmith, C.-A., A. Imrich, H. Danaee, Y. Ning, and L. Kobzik. 1998. Analysis of air pollution particulate-mediated oxidant stress in alveolar macrophages. *J. Toxicol. Environ. Health* 54:529–545.
10. Lambert, A. L., W. Dong, D. W. Winsett, M. K. Selgrade, and M. I. Gilmour. 1999. Residual oil fly ash exposure enhances allergic sensitization to house dust mite. *Toxicol. Appl. Pharmocol.* 158:269–277.
11. Ohtoshi, T., H. Takizawa, H. Okazaki, S. Kawasaki, N. Takeuchi, K. Ohta, and K. Ito. 1998. Diesel exhaust particles stimulate human airway epithelial cells to produce cytokines relevant to airway inflammation in vitro. *J. Allergy Clin. Immunol.* 101:778–785.
12. Boland, S., A. Baeza-Squiban, C. Houcine, C. Guennou, and F. Marano. 1999. Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am. J. Physiol.* 276:L604–L613.
13. Devalia, J. L., H. Bayram, M. M. Abdelaziz, R. J. Sapsford, and R. J. Davies. 1999. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int. Arch. Allergy Immunol.* 118:437–439.
14. Diaz-Sanchez, D., A. Tsien, A. Casillas, A. R. Dotson, and A. Saxon. 1996. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. *J. Allergy Clin. Immunol.* 98:114–123.
15. Diaz-Sanchez, D., M. Hyrala, D. Ng, A. Nel, and A. Saxon. 2000. *In vivo* nasal challenge with diesel exhaust particles enhances expression of the CC chemokines Rantes, MIP-1alpha, and MCP-3 in humans. *Clin. Immunol.* 97:140–145.
16. Fujieda, S., D. Diaz-Sanchez, and A. Saxon. 1998. Combined nasal challenge with diesel exhaust particles and allergen induces *in vivo* IgE isotype switching. *Am. J. Respir. Cell Mol. Biol.* 19:507–512.
17. Polito, A. J., and D. Proud. 1998. Epithelial cells as regulators of airway inflammation. *J. Allergy Clin. Immunol.* 102:714–718.
18. Metcalf, D. 1999. Cellular hematopoiesis in the twentieth century. *Semin. Hematol.* 36:S5–S12.
19. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245–252.
20. Tazi, A., F. Bouchonnet, M. Grandsaingne, L. Boumsell, A. J. Hance, and P. Soler. 1993. Evidence that granulocyte macrophage-colony-stimulating fac-

tor regulates the distribution and differentiated state of dendritic cells/langerhans cells in human lung and lung cancers. *J. Clin. Invest.* 91:566–576.
21. Stamplfli, M. R., R. E. Wiley, G. S. Neigh, B. U. Gajewska, X.-F. Lei, D. P. Snider, X. Xing, and M. Jordana. 1998. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest.* 102:1704–1714.
22. Reibman, J., A. Talbot, D. Nilson, J. Jover, G. Ou, M. Pillinger. 2000. Regulation of expression of granulocyte-macrophage colony stimulating factor in human bronchial epithelial cells: role of protein kinase C and MAP kinases. *J. Immunol.* 165:1618–1625.
23. Samet, J. M., J. Stonehuerner, W. Reed, R. B. Devlin, L. A. Dailey, T. P. Kennedy, P. A. Bromberg, and A. J. Ghio. 1997. Disruption of protein tyrosine phosphate homeostasis in bronchial epithelial cells exposed to oil fly ash. *Am. J. Pathol.* 272:L246–L432.
24. Timblin, C., K. Berube, A. Churg, K. Driscoll, T. Gordon, D. Hemenway, E. Walsh, A. B. Cummins, P. Bacek, and B. Mossman. 1998. Ambient particulate matter causes activation of the c-*jun* kinase/stress-activated protein kinase cascade and DNA synthesis in lung epithelial cells. *Cancer Res.* 58:4543–4547.
25. Kyriakis, J. M., and J. Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81:807–869.
26. Hashimoto, S., Y. Gon, I. Takeshita, K. Matsumoto, I. Jibiki, H. Takizawa, S. Kudoh, and T. Horie. 2000. Diesel exhaust particles activate p38 MAP kinase to produce interleukin 8 and RANTES by human bronchial epithelial cells and N-acetylcysteine attenuates p38 MAP kinase activation. *Am. J. Respir. Crit. Care Med.* 161:280–285.
27. Boland, S., V. Bonvallot, T. Fourneir, A. Baeza-Squiban, M. Aubier, and R. Marano. 2000. Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. *Am. J. Pathol.* 278:L25–L32.
28. NYS Department of Environmental Conservation. 2001. Technical reports and documents. NYS Department of Conservation, Statewide Air Quality Trends 1995–1996 and Contraventions of Air Quality Standards 1996, www.dec.state.ny.us/website/dar/reports/98annrprt/98ar_trd.html.
29. Laden, F., L. M. Neas, D. W. Dockery, and J. Schwartz. 2000. Association of fine particulate matter from different sources with daily mortality in six US cities. *Environ. Health Perspect.* 108:941–947.
30. Kinney, P. L., M. Aggarwal, M. E. Northridge, N. A. Janssen, and P. Shepard. 2000. Airborne concentrations of PM2.5 and diesel exhaust particles on Harlem sidewalks: a community-based pilot study. *Environ. Health Perspect.* 108:213–218.
31. Finkelstein, J. N., C. Johnston, T. Barrett, and G. Oberdorster. 1997. Particulate-cell interactions and pulmonary cytokine expression. *Environ. Health Perspect.* 105:1179–1182.
32. Fujii, T., S. Hayashi, J. C. Hogg, S. F. Van Eeden. 2001. Particulate matter induces cytokine expression in human broncial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 25:265–271.
33. Ning, Y., A. Imrich, C. A. Goldsmith, G. Qin, L. Kobzik. 2000. Alveolar macrophage cytokine prduction in response to air particles in vitro: role of endotoxin. *J. Toxicol. Environ. Health* 11:165–180.
34. Pugin J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R.J. Ulevitch, P.S. Tobias. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc. Natl. Acad. Sci. USA* 90:2744–2748.
35. Davies, S. P., H. Reddy, M. Caivano, and P. Cohen. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 351:95–105.
36. Silbajoris, R., A. J. Ghio, J. M. Samet, R. Jaskot, K. L. Dreher, and L. E. Brighton. 2000. In vivo and in vitro correlation of pulmonary MAP kinase activation following metallic exposure. *Inhal. Toxicol.* 12:453–468.
37. Chin, B. Y., M. E. Choi, M. D. Burdick, R. M. Strieter, T. H. Risby, and A. M. K. Choi. 1998. Induction of apoptosis by particulate matter: role of TNF-alpha and MAPK. *Am. J. Physiol.* 275:L942–L949.