

Ambient Particulate Matter Causes Activation of the *c-jun* Kinase/Stress-activated Protein Kinase Cascade and DNA Synthesis in Lung Epithelial Cells¹

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Abstract

Numerous epidemiological studies have demonstrated a positive association between ambient air pollution and adverse health effects including respiratory morbidity, asthma, and lung cancer. It has been suggested in some experimental studies that airborne particulate matter (PM) can produce inflammatory effects, but nothing is known about the possible proliferative and carcinogenic effects of these particles on cells of the lung. We show here that exposure of pulmonary epithelial cells, a cell type affected in acute lung injury, asthma, and lung carcinomas, to nontoxic concentrations of PM *in vitro* results in increases in *c-jun* kinase activity, levels of phosphorylated cJun immunoreactive protein, and transcriptional activation of activator protein-1-dependent gene expression. These changes are accompanied by elevations in numbers of cells incorporating 5'-bromodeoxyuridine, a marker of unscheduled DNA synthesis and/or cell proliferation. Data here are the first to demonstrate that interaction of ambient PM with target cells of the lung initiates a cell signaling cascade related causally to aberrant cell proliferation and carcinogenesis.

Introduction

Epidemiological evidence indicates that exposure to PM,³ a component of urban air pollution, is linked to increased acute and chronic respiratory morbidity (1), possible elevations in lung cancer risk (2), and episodes of asthma (3), chronic bronchitis (4), and pneumonia (5) in predisposed individuals. These observations have engendered intense scrutiny by regulatory agencies and health assessment panels. In experimental animals, PM evokes an inflammatory response. However, nothing is known of the critical molecular and pathogenic effects of PM on lung epithelium.

Ambient PM is generated primarily during the combustion of fossil fuels and is composed of organic or elemental carbon aggregates containing various metals, acid salts, organic pollutants, and biological contaminants such as endotoxin. Samples of PM may differ somewhat depending upon the site and method of collection. However, an association between high airborne concentrations of PM and increases in adverse respiratory effects are observed regardless of geographic site or time of year. Recent attention has

focused on fine PM_{2.5}, *i.e.*, particles of an aerodynamic diameter <2.5 μm, because the United States Environmental Protection Agency has introduced new standards for PM_{2.5} particles in addition to retaining the present standards for larger particles of PM₁₀ (<10-μm diameter). One premise for these new policies is that fine, and especially ultrafine, particles of submicrometer diameters may be more pathogenic and/or present in high numbers in PM samples (although they comprise a minor component of the total particulate mass). Ultrafine particles also have a greater surface area per unit weight and may be retained more efficiently than larger particles in the peripheral lung.

A critical cell type encountering inhaled particles after inhalation and affected in a number of respiratory diseases is the epithelial cell of the airways and alveoli. Previous work in our laboratory has demonstrated that epithelial cells in various compartments of the lung exhibit increased *c-jun* expression, DNA synthesis, and hyperplasia after inhalation or *in vitro* exposures to pathogenic minerals such as asbestos (6-8). The proto-oncogene, *c-jun*, is a member of the immediate-early response, multigene family transiently expressed in response to a variety of external stresses. *c-jun* and *c-fos* members encode protein subunits (Jun/Jun homodimers or Jun/Fos heterodimers) that comprise the AP-1 transcription factor involved in the transition of the G₁ phase and entry into the S phase of the cell cycle (9). In epithelial and mesothelial cells exposed to the fibrogenic and carcinogenic mineral fiber, asbestos, increased *c-jun* mRNA levels are accompanied by elevated cJun protein and increased AP-1 to DNA binding activity (6, 10). These changes are not observed after exposure of cells to nonpathogenic particles. Moreover, overexpression of *c-jun* in tracheal epithelial cells causes increased cell proliferation and morphological transformation (11), supporting a causal role of cJun in mitogenesis and carcinogenesis. Links between cJun expression and the development of lung cancers have also been suggested from studies evaluating immunoreactive cJun protein in hyperplasias and metaplasias from human lungs (12). In this study, we examined the hypothesis that PM initiates a cell signaling cascade causing increased expression of *c-jun* and transactivation of AP-1-dependent genes in epithelial cells of the lung. We speculated that these early molecular changes would lead to increased cell proliferation, a critical event in the pathogenesis of a number of respiratory disorders and diseases.

Materials and Methods

Preparations and Characterization of PM, Asbestos, and TiO₂ for *in Vitro* Studies. PM₁₀ samples were collected on filters from the Burlington, VT monitoring station using a Wedding collection apparatus. To prepare a PM_{2.5} fraction, PM-containing filters were placed in sterile centrifuge tubes, immersed in endotoxin-free double-distilled H₂O, and sonicated to release particles. Particle samples representative of PM_{2.5} were obtained by filtration through a 2.5-μm diameter pore size, polycarbonate filter. Samples were concentrated by lyophilization and stored at -80°C until use. A total of 5 VT PM preparations were used in experiments in duplicate. Fine and ultrafine TiO₂ samples were a generous gift from Dr. Gunter Oberdörster from the

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³ The abbreviations used are: PM, particulate matter; AP-1, activator protein-1; TEM, transmission electron microscopy; EPXMA, electron probe X-ray microanalysis; RLE, rat lung epithelial; NBS, newborn calf serum; β-gal, β-galactosidase; BrdUrd, 5'-bromodeoxyuridine; JNK, *c-jun* NH₂-terminal kinase; ERK, extracellular signal-related kinase.

University of Rochester (Rochester, NY). A reference sample of the National Institute of Environmental Health Sciences processed crocidolite asbestos was obtained from the Thermal Insulation Manufacturers Association fiber repository (Littleton, CO) as characterized previously (6, 10). The size and inorganic elemental surface chemistry of VT PM_{2.5} and TiO₂ samples were determined using a combination of TEM and EPXMA using standard techniques (13). Briefly, for both techniques, particle suspensions were aliquoted onto pioloform-coated, nickel TEM grids and allowed to air dry at room temperature inside sterile Petri dishes. Nickel grids were chosen for EPXMA because of the lack of nickel in PM and TiO₂ samples, as established with preliminary analyses using titanium grids. Furthermore, the presence of nickel in the X-ray spectrum did not interfere with the detection of other elements commonly found in such particles. For particle sizing (*n* = 600), equivalent spherical diameters were determined by image analysis of TEM micrographs (collected at an accelerating voltage of 80kV) using a Quantimet 500 MC image processing and analysis system (Leica, UK). Particle surface chemistry was assessed using a combination of TEM-EPXMA [JOEL JEM 1210 TEM attached to a Link ATW Pentafet detector (138eV resolution) and a Link "Isis" analyzer (Oxford Instruments, UK)], thus permitting the correlation of structural information with chemical content. Static probe analyses (*n* = 10) were performed on randomly selected areas per particle/grid preparation. The scope was operated at an accelerating voltage of 80 kV, "fine probe" mode, spot size 1, 1000 counts/second, and total acquisition time of 100 s.

The physicochemical analyses of VT PM_{2.5} and TiO₂ fine/ultrafine particles revealed that preparations (*n* = 2) of PM_{2.5} had a mean (± SE) equivalent spherical diameter of 38.8 ± 0.75. Preparations of the fine and ultrafine TiO₂ particles had equivalent spherical diameters of 159 ± 2.2 and 37 ± 0.50 nm, respectively. Thus, the VT PM_{2.5} and TiO₂ UF fractions have similar mean equivalent spherical diameters. Static probe analysis of particle surfaces (initial atomic layers) revealed that PM_{2.5} has a complex surface chemistry (C, O, Na, Si, P, S, Cl, K, Ca, Mg, Al, Cu, Zn, Fe) denoted by the presence of a range of metals. In contrast, TiO₂ surface chemistry is less complex, exhibiting only C, O, Si and Ti for both the ultrafine and fine particles, with the inclusion of Zn in the latter. These analyses are consistent with work by other investigators.

Addition of Particulates to RLE Cells *in Vitro*. RLE-6TN cells are a spontaneously derived cell line of alveolar type II epithelial cells that have been characterized previously (14). Cells were maintained and passaged in DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 7% NBS. At confluence, cells were switched to 0.5% serum containing medium for 24 h prior to the addition of PM at 1, 2.5, 5, or 10 μg/cm² dish. These concentrations were nontoxic, as assessed by the trypan blue exclusion technique and viable cell counts at 24 and 48 h after addition of PM preparations to RLE cells. All particles were weighed and suspended in 1

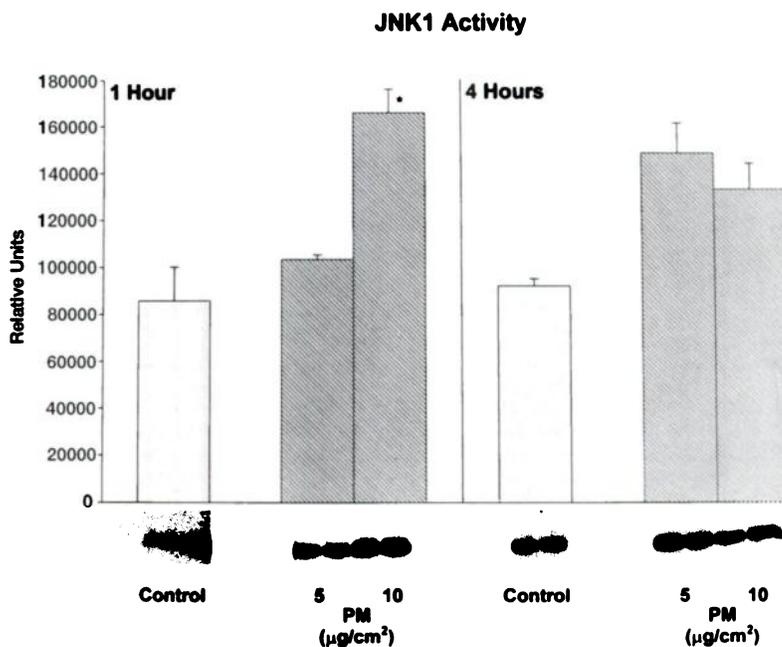
mg/ml in HBSS (Life Technologies, Inc., Grand Island, NY) before addition at final concentrations above to cell cultures (6, 10). Crocidolite asbestos and TiO₂ were processed identically after sterilization at 225°C for 16 h in a dry oven to remove possible microbial contaminants and added at final concentrations of up to 5 μg/cm² dish. For each experiment using methods described below, duplicate or triplicate dishes of untreated or particulate-exposed cells were assayed, and all experiments were performed in duplicate.

***In Vitro* Kinase Activity Assays.** Assays for JNK1 and ERK2 were performed using an immunoprecipitation assay as described previously (15, 16), using either a rabbit polyclonal anti-ERK2 (C12; 0.1 μg/μl) or anti-JNK1 antibody (both from Santa Cruz, CA) at a 1:100 dilution. Glutathione *S*-transferase-c-Jun (kindly provided by Dr. Roger Davis, University of Massachusetts, Worcester, MA) and myelin basic protein (Sigma Chemical Co., St. Louis, MO) were used as substrates for JNK1 and ERK2 kinase activities, respectively. Incorporation of ³²P into substrate was detected by autoradiography, and data were quantitated using a phosphorimager (Bio-Rad, Hercules, CA).

Preparation of Nuclear Extracts and Western Blot Analysis for Phosphorylated cJun. Nuclear extracts were prepared as described previously (17), and the amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad). Ten μg of each sample were then mixed with an equal volume of 2× sample buffer [5 mM EDTA, 4% SDS, 40% β-mercaptoethanol, 20% glycerol, 0.2 M Tris-base (pH 6.8), and saturated bromophenol blue], electrophoresed in 12% SDS polyacrylamide gels, and transferred to nitrocellulose using a semidry transfer apparatus (Ellard Instrumentation, Ltd., Seattle, WA). Blots were incubated overnight at 4°C in Tris-buffered saline (TBS) containing 5% nonfat powdered milk. After a 1-h incubation at room temperature in TBS/5% milk plus 0.05% Tween 20 (Sigma), blots were incubated at room temperature with a mouse monoclonal anti-cJun antibody specific to the phosphorylated protein (Sc-822; Santa Cruz, CA) for 45 min. They then were washed three times with TBS/0.05% Tween 20 and incubated with an anti-mouse peroxidase-conjugated secondary antibody for 30 min at room temperature. Blots were washed three times in TBS/0.05% Tween 20 and once with TBS, and protein bands were visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Autoradiographs were quantitated by densitometric analysis using a Microtex scanner and HP Deskscan and Sigma Scan 3.0 analysis software.

Assay for Transcriptional Activation of *c-jun*-dependent Gene Expression. The reporter plasmid, *jun*-luciferase, which contains 249 bp of the *c-jun* promoter (from position -70 to +170 bp encompassing a single AP-1 binding sequence) coupled to the luciferase reporter gene, was used to determine transcriptional activation of AP-1-dependent genes by various agents. This plasmid (*jun*-luciferase) and the empty expression vector (RSV-O), used as a control in all studies,

Fig. 1. Activation of the *c-jun* kinase, JNK1, by ambient PM in RLE cells using an immunoprecipitation assay. *, *P* < 0.05 in comparison with sham controls. Bars, SE.



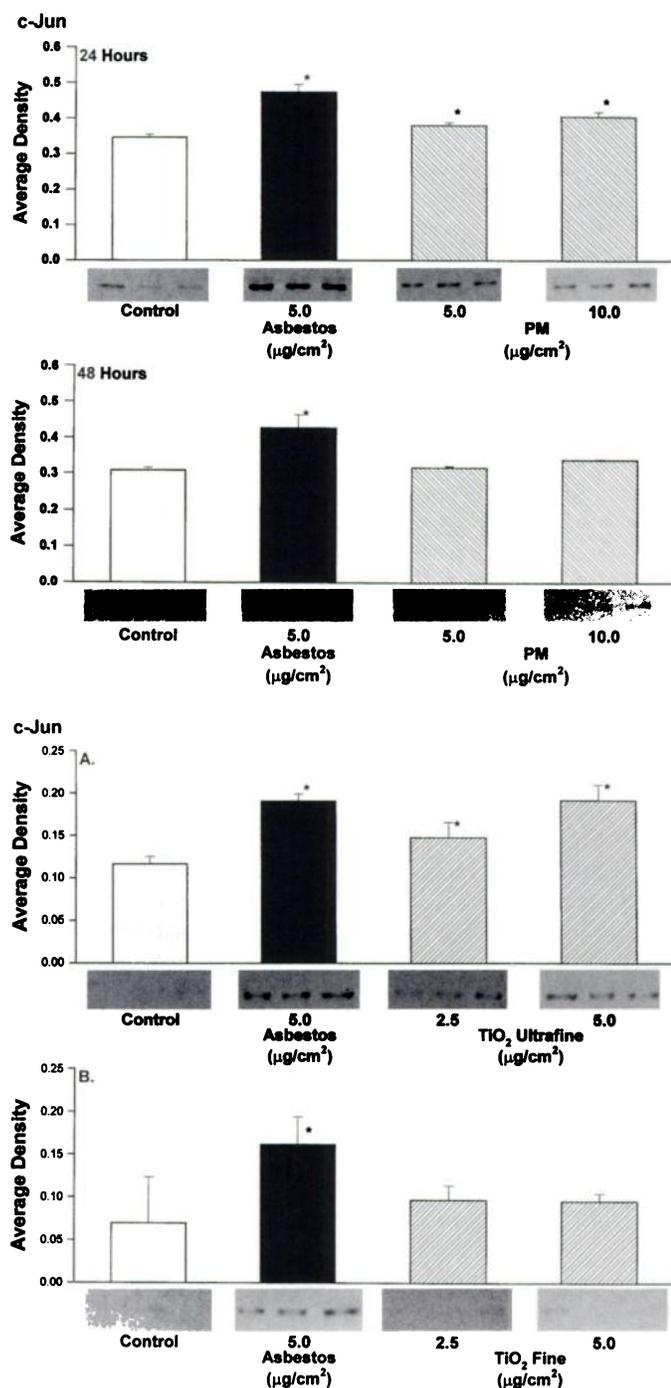


Fig. 2. Increases in phosphorylated cJun protein as shown by Western blot analysis. *Upper panels*, RLE cells exposed to PM or crocidolite asbestos. Note that cJun is increased at both 24 and 48 h in RLE cells exposed to asbestos; bars, SE. *Lower panels*, increases in phosphorylated cJun protein at 24 h in RLE cells exposed to ultrafine (A), but not fine (B), TiO_2 particles; bars, SE. Both *upper* and *lower panels* are separate experiments that were repeated in duplicate. *, $P < 0.05$ in comparison to sham controls.

were obtained from Dr. Michael Karin (University of California at San Diego, San Diego, CA). Cells were cotransfected with pSV β -gal to control for transfection efficiency by electroporation using a Bio-Rad Gene Pulser and manufacturer-recommended protocols. After transfection, RLE cells were plated and allowed to recover overnight in 7% NBS-containing culture medium and incubated in 0.5% NBS-containing medium before addition of particulates for 18 h. Total cell extracts were then prepared and assayed for luciferase activity (Luciferase Assay System; Promega Corp., Madison, WI), β -gal activity, and total cellular protein as described previously (11). The amount of luciferase activity was expressed as total luciferase/ β -gal/ μg protein.

Assays for Determination of Increased DNA Synthesis. Incorporation of the thymidine analogue, BrdUrd was used to indicate RLE cells in S phase as determined by two complementary techniques. For *in situ* detection of cells incorporating BrdUrd by computer-assisted cell imaging, cells were plated on glass coverslips, grown to confluency, and exposed to particulates as described above for 48 h, *i.e.*, the time of asbestos-induced increases in BrdUrd incorporation (18). After a 24-h pulse with BrdUrd (10 μM ; Sigma), coverslips were then fixed in 4% paraformaldehyde and incubated with a primary mouse anti-BrdUrd antibody (5 $\mu\text{g}/\text{ml}$ calcium and magnesium-free PBS, CMFPBS; DAKO, Carpinteria, CA), followed by a Cy3-conjugated, affinity-purified, donkey antimouse secondary antibody (4 $\mu\text{g}/\text{ml}$ CMFPBS; Jackson Immunoresearch Laboratories, West Grove, PA) as described previously (18). Coverslips were also counterstained with a YOYO dye (1:3000 CMFPBS; Molecular Probes, Eugene OR) to detect nuclear DNA and examined using a dual fluorescence detection technique (18). Numbers of BrdUrd-positive cells per total numbers of YOYO-positive cells were determined after assessment of 10 random fields per coverslip in duplicate at $\times 400$, and data were expressed as an average percentage value (mean \pm SE).

Flow cytometry was used to determine the fraction of cells incorporating BrdUrd in the S phase of the cell cycle. Confluent RLE cultures were exposed to particulates for 48 h as described above. Twenty-four h prior to harvest, BrdUrd (10 μM) was added to both sham and particulate-exposed cell cultures. Cells were harvested by trypsinization, washed once with PBS/5% NBS, resuspended at 1×10^6 cells/ml in freeze solution [250 mM sucrose, 40 mM sodium citrate (pH 7.6), and 5% v/v DMSO], and stored in aliquots at -70°C until analysis. Aliquots were then thawed, pelleted at 6000 rpm for 1 min, washed one time with PBS/1% BSA, resuspended in 150 μl PBS/1% BSA, and stained with 300 μl of lysis-stain solution (0.1% Triton X-100, 20 $\mu\text{g}/\text{ml}$ propidium iodide, 0.2 mg/ml RNase A, and 0.5 mM EDTA) for 15 min at 4°C on a rocking platform. The DNA was denatured by the addition of 75 μl of 1 N HCl for 30 min at room temperature, normalized with 225 μl of 1 M Tris-base for 5 min on ice, and incubated with a mouse monoclonal fluorescein-conjugated anti-BrdUrd antibody (0.013 $\mu\text{g}/\mu\text{l}$ concentration; Boehringer Mannheim, Indianapolis, IN) for 15 min at 4°C on a rocking platform. In some experiments, PM preparations were pretreated with 10 $\mu\text{g}/\text{ml}$ polymyxin B (Sigma) for 30 min before addition to cell cultures. The distribution of cells in the various cell cycle compartments was determined as described previously (19) using a Coulter Epics Elite flow cytometer (Coulter Corporation, Miami, FL). Data were expressed as the percentage of BrdUrd-positive cells (mean \pm SE) in the S-phase fraction.

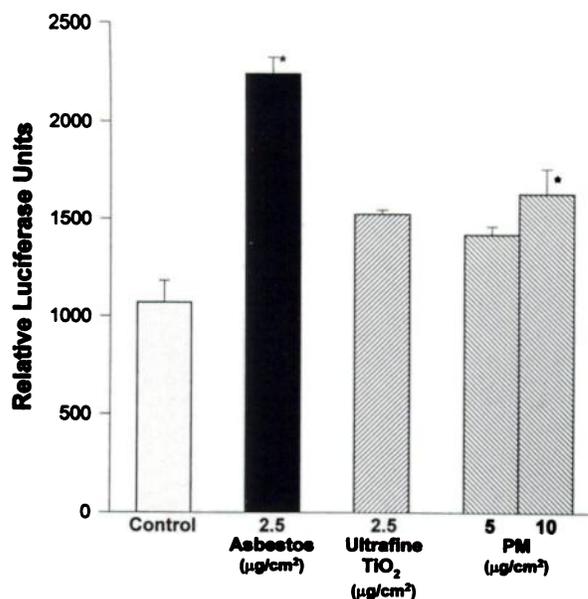
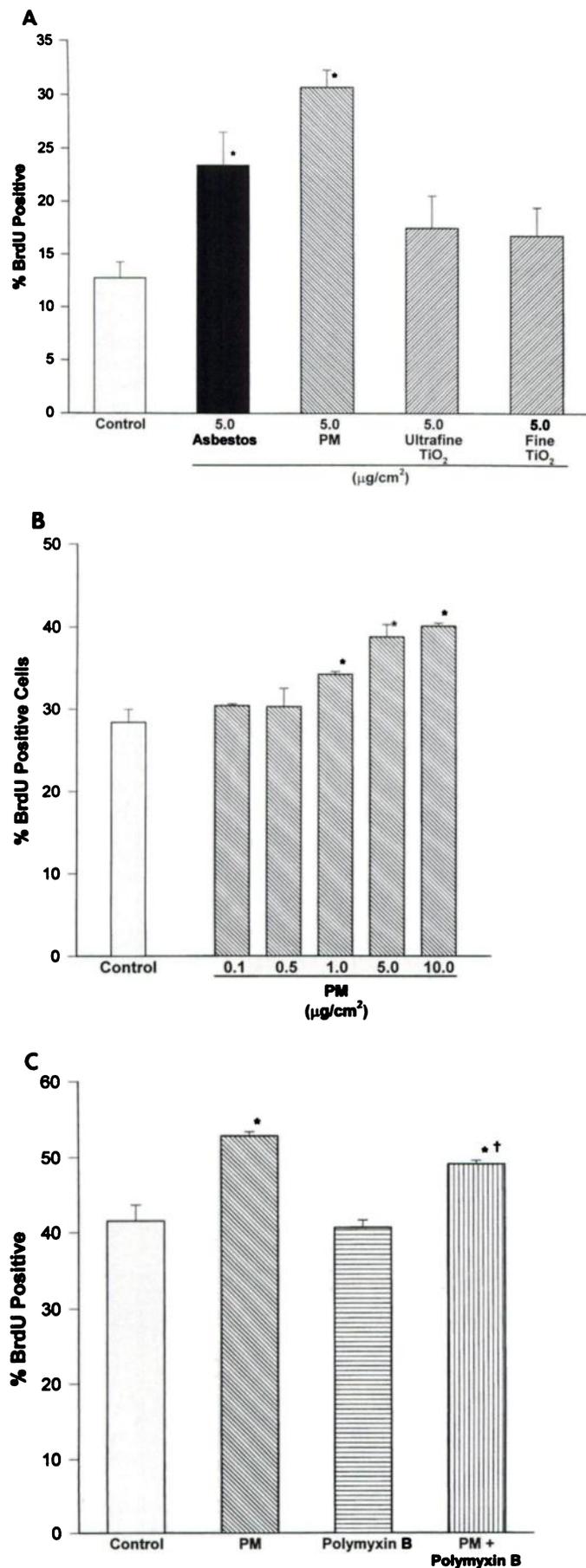


Fig. 3. Transcriptional activation of AP-1-dependent genes by asbestos and PM, but not ultrafine TiO_2 , in RLE cells. RLE cells were cotransfected with a *jun*-luciferase construct and pSV β -gal by electroporation. The amount of luciferase activity was expressed as total luciferase/ β -gal/ μg protein and expressed as relative units. *, $P < 0.05$ in comparison with sham controls.



Statistical Analysis. All data were examined by ANOVA using the Student-Newman Keuls procedure to adjust for multiple pairwise comparisons between groups.

Results and Discussion

We first tested the hypothesis that PM initiated signaling events in lung epithelium associated with transactivation of AP-1-dependent genes. In Fig. 1, we demonstrate the activation of *c-jun*-NH₂-terminal/stress-activated protein kinases, which lead to phosphorylation and activation of cJun (9) by PM. After addition of nontoxic concentrations of PM samples to confluent RLE cells, assays for JNK1 or ERK2 were performed using an immunoprecipitation assay as described previously using H₂O₂, which induces approximately 10-fold increases in JNK1 and ERK2 activity in these cell types (data not shown), as a positive control (15). At 1 and 4 h after addition to cells, PM induced significant increases ($P < 0.05$ in comparison to sham controls) in JNK1 (Fig. 1) but not ERK2 activity (data not shown). Whether RLE cells exhibited increased amounts of cJun protein after exposure to PM was then evaluated using an antibody specific to phosphorylated cJun and Western blot analysis (17). At 24 h, PM induced significant increases ($P < 0.05$) in phosphorylated cJun, but unlike asbestos, a mineral inducing protracted increases in cJun over time (10, 17), elevations in cJun did not persist for 48 h (Fig. 2, *upper panel*). To determine whether particle size played a role in the induction of phosphorylated cJun, RLE cells were exposed for 24 h to identical concentrations of ultrafine TiO₂ or fine TiO₂. Ultrafine, in contrast to fine, TiO₂ caused significant increases ($P < 0.05$) in amounts of phosphorylated cJun when compared with sham controls (Fig. 2, *lower panel*). To examine whether PM caused transcriptional activation of *c-jun*, we next transiently transfected RLE cells with a *jun*-luciferase reporter construct that contains a single AP-1 binding sequence (Ref. 11; Fig. 3). RLE cells transfected with the *jun*-luciferase construct showed increased luciferase activity after exposure to PM ($P < 0.05$) in comparison to sham or ultrafine TiO₂-exposed cells as did the positive control, asbestos. These results demonstrate that PM and asbestos activate AP-1-dependent gene transcription.

AP-1 plays a central role in mediating cellular proliferation in response to extracellular signals (9). Using a recently described *in situ* dual fluorescence technique for measuring proliferation and apoptosis (18), we next evaluated whether PM induced these phenotypic end points in RLE cells. Cells were examined after 48-h exposure to asbestos (a mineral causing both increases in numbers of cells incorporating BrdUrd and apoptosis at this time point; Refs. 18 and 19) PM, ultrafine TiO₂, or fine TiO₂. Significant elevations ($P < 0.05$) in numbers of epithelial cells incorporating BrdUrd were observed after exposure to asbestos or PM but not to TiO₂ particles (Fig. 4A). No increases in apoptosis were observed in response to these concentrations of PM or TiO₂, and trypan blue exclusion assays indicated no decreases in cell numbers at concentrations of PM as high as 10 µg/cm² (data not shown). Using flow cytometry, dose-related increases in BrdUrd incorporation after exposure to PM was confirmed. In these experiments, concentrations of ≥ 1 µg/cm² PM caused significant elevations in numbers of RLE cells incorporating

Fig. 4. PM-induced increases in DNA synthesis in RLE cells. In A, PM and asbestos cause elevations in numbers of RLE cells incorporating BrdUrd at 48 h after initial exposure to particulates, as demonstrated using computer-assisted cell imaging. These increases are not observed after addition of ultrafine or fine titanium dioxide particles; bars, SE. In B, dose-related increases in numbers of RLE cells incorporating BrdUrd as demonstrated by flow cytometry at 48 h after addition of PM; bars, SE. In C, increases in numbers of RLE cells incorporating BrdUrd by PM are observed in the absence of endotoxin. PM preincubated in the absence or presence of polymyxin B (10 µg/ml), which inactivates endotoxin, was added to RLE cells for 48 h and analyzed by flow cytometry. *, $P < 0.05$ in comparison with sham controls. †, $P < 0.05$ in comparison with PM group.

BrdUrd (Fig. 4B). Because endotoxin, a contaminant of PM samples, has been shown to stimulate proliferation of pulmonary epithelial cells both *in vitro* and *in vivo*, we next examined whether elevations in cells incorporating BrdUrd could be induced by PM samples in the absence of endotoxin. In Fig. 4C, we show, using flow cytometry, that exposure of RLE cells to either native PM or PM preincubated with polymyxin B, an inhibitor of endotoxin activity, causes increases in numbers of RLE cells incorporating BrdUrd. These data indicate that PM in the absence of endotoxin can induce cell proliferation. However, numbers of cells incorporating BrdUrd were significantly reduced ($P < 0.05$) in the polymyxin B-pretreated PM group in comparison with cultures exposed to native PM, indicating some role of PM-associated endotoxin in induction of DNA synthesis.

Data in concert suggest a model whereby inhaled PM initiates the *c-jun* kinase cell signaling cascade after interaction with and/or uptake by epithelial cells of the respiratory tract. This then leads to phosphorylation of cJun and transcriptional activation of AP-1-regulated genes, which may be important in cell proliferation. For example, a number of reports have indicated that *c-jun* induces cell proliferation and/or plays a role in cell cycle progression (9, 11, 12). Noteworthy here is the early demonstration of JNK activity in RLE cells exposed to PM before increases in cells in S phase are observed at 48 h. In addition to its role in carcinogenesis and tumor promotion, compensatory cell proliferation often follows epithelial cell damage, a key feature of acute lung injury, asthma, and pulmonary fibrosis. Thus, activation of cJun and increases in AP-1-related gene transactivation by PM may be intrinsic to the pathogenesis of a number of respiratory disorders and diseases.

Several other aspects of studies here deserve further comment. Ultrafine, but not fine, TiO₂ induced significant increases in phosphorylated cJun that were not accompanied by either elevations in luciferase activity in *jun* promoter-reporter transfection assays or increased cell proliferation. These observations support the possibility that additional posttranslational changes may be necessary for AP-1-dependent gene transactivation by ultrafine particles. The increased potency of ultrafine in comparison to fine TiO₂, which failed to induce molecular and mitogenic effects in RLE cells, is consistent with results showing increased inflammatory, carcinogenic, and fibrogenic effects by ultrafine particles after inhalation by rodents (20). For example, preparations of TiO₂ used here have been evaluated comparatively in subchronic inhalation studies using rats. At similar airborne concentrations, ultrafine, but not fine, particles elicited oxidative stress, increases in neutrophils, lymphocytes, and alveolar macrophages in lavage fluids and elevations in lung hydroxyproline, an indicator of pulmonary fibrosis (21).

Although studies here suggest certain parallels in responses of respiratory epithelial cells to PM or asbestos, some differences also exist: (a) asbestos stimulates the ERK arm of the mitogen-activated protein kinase cascade, and not JNK activity, in pleural mesothelial cells (15, 16); (b) asbestos causes more persistent increases in steady-state mRNA levels of *c-jun* (as well as *c-fos* and *c-myc*) and cJun protein in mesothelial and epithelial cells (6, 10, 11). In experiments here, PM caused elevations in phosphorylated cJun at 24 h, but asbestos-induced increases persisted for 48 h; (c) asbestos instigates increases in cell proliferation, necrosis, and apoptosis in mesothelial and epithelial cells *in vitro* and *in vivo* (7, 8, 18, 19). In contrast, increased numbers of cells undergoing DNA synthesis in the absence of necrosis or apoptosis occur after *in vitro* exposures to PM; and (d) the time frame of JNK activation by PM, in

contrast to ERK activation by asbestos (4–8 h), is different. Whether earlier cell signaling and other molecular events by PM are due to soluble factors and/or free radical reactions, which are linked causally to the pathogenesis of asbestos-related diseases (22), is presently under investigation.

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