Appalachian Mountaintop Mining Particulate Matter Induces Neoplastic Transformation of Human Bronchial Epithelial Cells and Promotes Tumor Formation

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SUPPORTING INFORMATION

Supporting Information includes Supplementary Table S1 and S2 and Supplementary Materials and Methods.

Supplementary Table

Table S1. PM mass following extraction

Tube Number	Tube Weight		PM Mass (mg)
	Pre-Weight	Post-Weight	
PM_{MTM}			
A30	1812.5661	1813.5236	0.9575
A31	1804.91545	1805.782	0.8665
A32	1796.1867	1797.174	0.9873
PM_{CON}			
C42	1807.9917	1808.3369	0.3452
C43	1769.6169	1796.9458	0.3289
C44	1801.0517	1801.4122	0.3605
C45	1811.1372	1811.5933	0.4561
C46	1790.7088	1791.1664	0.4576

Table S2. PM_{MTM} analysis of organic/elemental carbon, metals, and sulphate

Element	% Weight
Aluminum	3.15
Calcium	2.08
Iron	1.38
Potassium	1.48
Magnesium	0.48
Sodium	0.63
Titanium	0.22
Zinc	0.04
Sulphate	0.15
Organic carbon (OC)	18.90
Elemental carbon (EC)	2.88

Supplementary Materials And Methods

Cells and Reagents

Human bronchial epithelial BEAS-2B cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, Gaithersburg, MA). Non-small cell lung cancer H460 cells were obtained from ATCC and were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% FBS, 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and molybdenum (Mo) were obtained from Sigma-Aldrich (St. Louis, MO). Crystalline silica (Si, [Min-U-Sil]) was obtained from U.S. Silica (Berkeley Springs, WV) and micronized to less than 5 μm with an Accucut Particle Classifier (Donaldson Majal Division, St. Paul, MN). Trypan blue and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, CA).

Collection of MTM and control particulate matters

Air samples were taken at two rural residential sites located within 1 mile of an active MTM site in Edwight, WV, USA during summertime (July−August 2011). For control, air was similarly sampled from selected rural areas in Green Bank, WV, which does not have coal mining[S1] (*see* Ref. S1 for summary of sampling site characteristics). PM_{MTM} and PM_{CON} were collected on 35-mm, 5 μm-pore size PTFE fiber-backed filters (Whatman, Springfield Mill, UK) for 2-4 weeks. Air flow rate across the filters averaged 12 L/min. Following collection, the filters were stored at room temperature (20-25°C) and ambient humidity (10-30%) in the dark prior to extraction of PM, according to the

method previously described (see Table S1 for PM mass).[S2] Briefly, the filters were gently agitated in 15 mL of ultrapure water in a glass jar for 96 hours. Aliquots of the particle suspension were dried down in the pre-weighed 2 mL cryovials for 18 hours in a Speedvac (Savant, Midland, MI) and the total particle weight was determined by a microbalance (Mettler-Toledo, Columbus, OH). It is worth noting that this method of PM collection could not preserve the volatile organic compounds. Scanning Electron Microscope-Energy-Dispersive X-ray Spectroscopy (SEM-EDX), which was limited to the analysis of inorganic compounds, was further used to perform PM compositional analysis (RTI International, Research Triangle Park, NC). In comparison with PM_{CON}, Si and Mo were found to be the main inorganic chemical elements in PM_{MTM} with the % weight average of 48.15 ± 26.91% and 28.90 ± 4.16% respectively for Si and Mo *vs.* 23.75 ± 15.07% and 0.00 ± 0.00% of the elements in PM_{CON}. Analysis of organic carbon, and elemental carbon (OC/EC), metals, and sulphate using carbon analyzer, SF-ICP-MS, and ion chromatography, respectively was shown in Table S2.

Cytotoxicity Assay

Cell viability was determined by MTT assay. Bronchial epithelial BEAS-2B cells were seeded in 96-well plates and treated with various concentrations of PM_{MTM}, PM_{CON}, Si or Mo for 48 hours. All particles were suspended in phosphate buffer saline (PBS) containing 5% bovine serum albumin (BSA) and were lightly sonicated prior to use to disperse the particles. After specific treatments, cells were incubated with 500 μg/mL of MTT for 4 hours at 37°C. The intensity of formazan product was then measured at 550 nm using a microplate reader. The absorbance ratio of treated and non-treated cells was calculated and presented as relative cell viability.

Chronic Particle Exposure

Subconfluent cultures of bronchial epithelial BEAS-2B cells were continuously exposed to non-cytotoxic concentration (1 μg/mL) of PM_{MTM} or PM_{CON} in 6-well plates for 3 months. The cells were passaged biweekly at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen). PM_{MTM}- and PM_{CON}-exposed BEAS-2B cells were designated as B-PM_{MTM} and B-PM_{CON} cells and were cultured in complete medium (without treatment) for at least 10 passages prior to further experiments to rule out any reversible effects. Parallel culture grown with the same background level of dispersant provided a passage-matched (non-treatment) control (B-NTX cells). To study the effect of PM_{MTM} inorganic chemical elements, cells were similarly exposed to a non-cytotoxic concentration (1 μg/mL) of Si or Mo. After 3 months of exposure, the cells (designated as B-Si and B-Mo cells) were cultured in complete medium (without treatment) for at least 10 passages prior to experiments.

Dosage Calculation and Human Extrapolation

PM exposure dose of 1 μg/mL in the 6-well plates (growth area ~ 10 cm^2) at the total volume of 1 mL corresponds to the surface area dose of 0.1 μg/cm^2. Based on the reported rat lung surface area of 5000 cm^2,[S3] this exposure dose is equivalent to a bolus exposure of PM at 0.5 mg in the rats, which was previously shown to induce pathological changes.[S4] Extrapolation to human exposure scenarios in the residential areas can be evaluated by the earlier described approach being used by the National Institute for Occupational Safety and Health (NIOSH) with slight modifications.[S5,S6] Assuming the pulmonary surface area in humans of 100 m^2, the human burden is equal to 100 mg/lung. Considering a respiratory deposition of ~40%[S1] and an adult inhalation rate of ~16 m^3/day,[S7] the experimental dose could be reached within 8.5 years of human inhalation

exposure at 5 μg/m^3 (average total PM mass concentration in Edwight and Green Bank, WV).[S1]

Soft Agar Colony Formation Assay

The chronic exposed cells at 3×10^4 cells per 24-well plate were mixed with culture medium containing 0.5% agar. The resulting cell suspensions were immediately plated onto dishes coated with 0.5% agar in culture medium. After 2 weeks, cell colonies were visualized under a phase contrast microscope (Leica Microsystems, Bannockburn, IL), and diameters were measured using Leica Application Suite Software. Colonies larger than 50 μm in diameter were scored as positive for growth.[S8]

Cell Counting

The exposed cells (3×10^4 cells) were seeded in 24-well plates and cultured in complete medium for up to 5 days. The cells were stained with 0.4% trypan blue in 0.85% saline (Invitrogen) (to indicate dead cells) and healthy cell number was scored using Countess® automated cell counter (Invitrogen) at 2 and 5 days.

Proliferative Index

The chronic exposed cells (2×10^6 cells) were labeled with CellVue® Claret Far Red Fluorescent Cell Linker (Sigma) at 2 μmol/L for 5 minutes according to the manufacturer's protocol. After 4 days of culture, the cells were harvested, fixed in 0.4% paraformaldehyde, and far red fluorescence intensity was determined using BD LSRFortessa™ flow cytometry (BD Biosciences, San Jose, CA) with a 655-nm excitation beam and a 675-nm band-pass filter. Cell proliferative index was determined

from 50,000 events for each sample using FSC Express 4 flow cytometry software (De Novo Software, Los Angeles, CA).

Cell Cycle Analysis

The chronic exposed cells were serum starved for 12 hours and incubated in the complete medium for 8 hours. The cells were harvested and fixed in ice cold 80% ethanol at 4°C for 15 minutes. Fixed cells were stained with 20 µg/mL PI in PBS containing 1% FBS, 0.05% Triton X-100, and 50 µg/mL RNase for 30 minutes at room temperature. The samples were analyzed by BD LSRFortessa^{TM} flow cytometry and the percentage of cells in the different phases of cell cycle (G1, S and G2/M) was determined by FSC Express 4 Flow Cytometry software.

Migration Assay

Cell migration was determined by wound healing or scratch assay as previously described.[S9] Briefly, a monolayer of chronic exposed cells was cultured in 24-well plate and a wound space was created with a 1-mm width tip. After rinsing with PBS, the cell monolayers were incubated in complete medium and allowed to migrate for 24 hours. Micrographs were taken under a phase contrast microscope (Leica Microsystems), and wound spaces were measured from 10 random fields of view using Leica Application Suite software. Quantitative analysis of cell migration was performed using an average wound space from the random fields of view, and percent changes in the wound space were calculated using the formula: % change = (average space at time 0 hour) − (average space at time 24 hours)/(average space at time 0 hour) × 100.

Xenograft Mouse Model

Animal care and experimental procedure described in this study were in accordance with the Guidelines for Animal Experiments at West Virginia University approved by the Institutional Animal Care and Use Committee (IACUC #12-0502). Immunodeficient NOD/SCID gamma mice, strain NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG; Jackson Laboratory, Bar Harbor, ME), were maintained under pathogen-free conditions within the institutional animal facility.[S10,S11] Food and water were given ad libitum. Mice were injected subcutaneously (SC) with 3×10^5 luciferase (Luc2; Capital Biosciences, Rockville, MD)-labeled lung cancer H460 cells and 6×10^5 PM-exposed cells (1:2 ratio) suspended in 100 μL of ExtraCel® hydrogel (Advanced BioMatrix, San Diego, CA). The mice were inspected daily for any signs of distress such as weight loss, hunching, failure to groom, and red discharge from the eyes. Tumor growth of luciferase-labeled cells was monitored weekly using IVIS® bioimaging (Perkin Elmer, Waltham, MA). At the end of experiments, mice were euthanized and SC tumors were dissected and weighted. Metastasis of tumor cells to neighbor tissues was analyzed by IVIS® imaging after removal of primary SC tumors.

Statistical Analysis

The data represent means ± SD from three or more independent experiments as indicated. Statistical analysis was performed by Student's t test at a significance level of $P < 0.05$.

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