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INTERACTIVE EFFECT OF CIGARETTE SMOKE EXTRACT AND WORLD TRADE CENTER DUST PARTICLES ON AIRWAY CELL CYTOTOXICITY

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Rescue workers and residents exposed to the environment surrounding the collapse of the World Trade Center (WTC) on September 11, 2001, have suffered a disproportionate incidence of chronic lung disease attributed to the inhalation of airborne dust. To date, the pathophysiology of this lung disease is poorly understood. The aim of this study was to examine whether airborne dust contaminants recovered from the surrounding area 24–48 h after the collapse of the WTC demonstrate direct cytotoxicity to two airway cell types that were most directly exposed to inhaled dust, airway epithelial and smooth muscle cells. It was also of interest to determine whether the presence of these dusts could modulate the effects of cigarette smoke on these cell types in that some of the individuals who responded to the collapse site were also smokers. Human cultured airway epithelial (BEAS-2B) cells were exposed to 10% cigarette smoke extract (CSE), WTC dust particles (10–53 μm; 0.01–0.5 μg/μl), or a combination of the two for 2–24 h. Cell viability was measured by determining mitochondrial integrity (MTT assays) and apoptosis (poly-ADP-ribose polymerase [PARP] immunoblotting). Conditioned cell culture media recovered from the CSE- and/or WTC dust-exposed BEAS-2B cells were then applied to cultured human airway smooth muscle cells that were subsequently assayed for mitochondrial integrity and their ability to synthesize cyclic AMP (a regulator of airway smooth muscle constriction). BEAS-2B cells underwent necrotic cell death following exposure to WTC dust or CSE for 2–24 h without evidence of apoptosis. Smooth muscle cells demonstrated cellular toxicity and enhanced cyclic AMP synthesis following exposure to conditioned media from WTC- or CSE-exposed epithelial cells. These acute toxicity assays of WTC dust and CSE offer insights into lung cell toxicity that may contribute to the pathophysiology of chronic lung disease in workers and residents exposed to WTC dust. These studies clearly showed that WTC dust (at least the supercoarse particle fraction) or CSE alone exerted direct adverse effects on airway epithelial and smooth muscle cells, and altered the signaling properties of airway smooth muscle cells. In addition the combination of CSE and WTC exerted an interactive effect on cell toxicity. It remains to be determined whether these initial cell death events might account, in part, for the chronic lung effects associated with WTC dust exposure among First Responders and others.

The World Trade Center (WTC) towers' collapse on 9/11 (September 11, 2001) created an enormous dust cloud containing tons of coarse and fine particulate matter (PM) bearing a complex blend of toxic agents (Gavett et al. 2003; McGee et al. 2003; Offenberg et al.

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2003; 2004). On the day of and in the period following 9/11, an estimated 40,000 First Responders, iron and construction workers, and volunteers worked at the site of the towers; others worked at the Staten Island landfill, the primary depository of debris and other remnants from Ground Zero (Herbert et al. 2006; Izbicki et al. 2007). During their time in and around these sites, nearly all individuals were exposed (to varying degrees) to these dusts. Airborne particle densities at the Main Pile at Ground Zero during the first 72-h period post collapse were different from those thereafter (i.e., estimates for 9/11–9/13 = hundreds of mg/m^3; low mg to hundreds of $\mu g/m^3$ after approximately 3 wk; Anonymous 2002; Geyh et al. 2005; Geyh personal communication). As a result, both arrival time and respiratory protection use/non-use became accepted as major factors in studies to relate exposure intensity to subsequent health issues among the workers/volunteers exposed to the WTC dusts (Landrigan et al. 2004; Herbert et al. 2006). The fact that many of these individuals were also smokers—and thus would likely have inhaled a combination of dust particles and cigarette smoke (CS)—has also become an issue in understanding the adverse effects associated with the WTC dusts.

Studies demonstrated that high-level exposures to WTC dusts on 9/11 or in the days/weeks thereafter are associated with development of numerous debilitating and even fatal respiratory diseases (Weiden et al. 2010; de la Hoz et al. 2009; Webber et al. 2009). Among the most commonly reported/documented health issues are persistent "WTC" cough (Kelly et al. 2007; Prezant 2008); upper/lower airway diseases (UAD/LAD) (CDC 2004; Salzman et al. 2004; Herbert et al. 2006; de la Hoz et al. 2008b); large decrements in ventilatory function (Gibbs et al. 2008; 2009; Aldrich et al. 2010); new-onset asthma (Wheeler et al. 2007; Brackbill et al. 2009); "sarcoid-like" granulomatous disease (Izbicki et al. 2007); reactive airways dysfunction syndrome (RADS; associated with increased airway/bronchial hyperreactivity) (Banauch et al. 2005a; 2005b; Weiden et al. 2010); and gastroesophageal reflux disease (GERD) that seems related to lung function abnormalities (de la Hoz et al. 2008a; Webber et al. 2009). To date, the precise cellular mechanism or mechanisms by which the inhalation of these dust particles, by either smokers or nonsmokers among the various exposed individuals, has led to chronic and fatal respiratory disease are still not known. However, one potential plausible mechanism suggested for how the WTC dusts may have induced or contributed to each of the already-noted pathologies is a repeated or persistent induction of respiratory-tract inflammation.

The first cells in the respiratory tree exposed to exogenous pollutants are the airway epithelial cells (King et al. 1998; Asokananthan et al. 2002). These cells, in turn, liberate substances that modulate airway smooth muscle cell function (Asokananthan et al. 2002). The potential direct toxicity of WTC dust particles (in the absence or presence of CS) to airway epithelial cells is unknown. However, combined exposure of such cells to CS and house dust mite allergens enhanced cytokine release, and CS alone was cytotoxic (Rusznak et al. 2001). Liberated substances from epithelial cells (e.g., cytokines and contractile mediators) affect nearby airway smooth muscle cells. Whether WTC dust- or CS extract (CSE)-exposed epithelial cells conferred toxicity or altered important signaling pathways in the smooth muscle cells remains uncertain.

The current studies addressed these points with respect to supercoarse WTC dust particles (10–53 μm) that comprised the major size fraction found in the airborne and settled dust samples (McGee et al. 2003; Maciejczyk et al. 2006) and were most likely entrained into the lungs in significant amounts as a result of mouth breathing by First Responders and others when they labored without protective respiratory equipment at Ground Zero.

MATERIALS AND METHODS

Reagents

M199 cell culture media, fetal bovine serum (FBS), and trypsin were purchased from

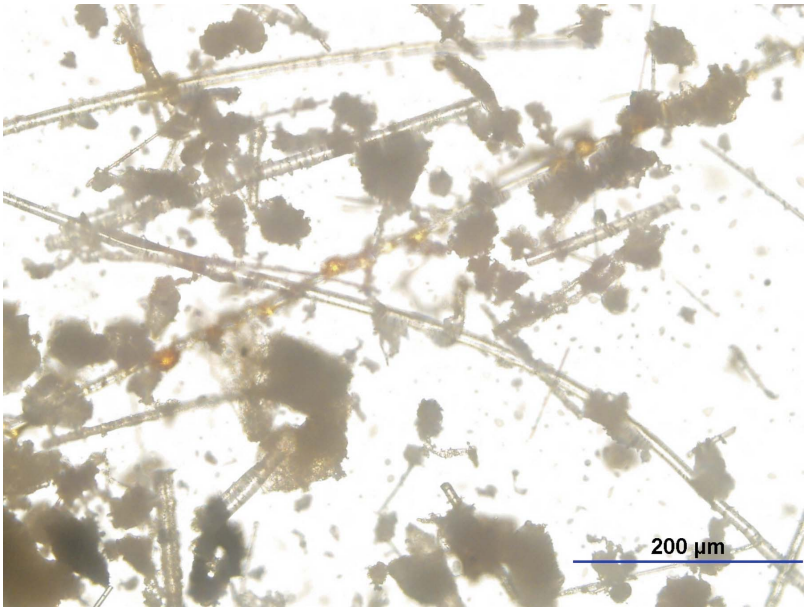

FIGURE 1. Representative light photomicrograph of supercoarse (10–53 µm average diameter) World Trade Center dust particles used in the studies. Dust particles were collected from 120 Broadway on 9/13/01 (color figure available online).

Invitrogen (Carlsbad, CA). BEBM (bronchial epithelial cell basal medium) growth media was obtained from Lonza (Walkersville, MD). Standardized research cigarettes (unfiltered, code 3R4F) were obtained from the University of Kentucky College of Agriculture (Lexington, KY). [^{32}P]-α-ATP was from MP Biomedicals (Solon, OH). Unless elsewise noted, all other reagents were purchased from Sigma (St. Louis, MO).

The World Trade Center dusts used in these studies were collected from two locations (8 Beekman St and 120 Broadway) near the former site of the World Trade Center in New York City on 9/12/01 and 9/13/01, respectively. The parent WTC dust samples from each site were first sieved at 53 µm; particles <53 µm in diameter were then further separated into fractions of 10–53, 2.5–10, and ≤2.5 µm (i.e., supercoarse, coarse, and fine, respectively) aerodynamic diameters using standard protocols (Gavett et al. 2003). For the studies here, the 10- to 53-µm particles were suspended in distilled and deionized water to stock concentrations of 700 µg $WTC_{10–53}$/ml and stored at −80°C until use. These supercoarse particles were selected for use here as they comprised the major size fraction found in the airborne and settled dust samples at Ground Zero and were most likely entrained into the lungs in significant amounts as a result of mouth breathing by First Responders and others as they labored at the WTC site.

To confirm the sizes of the $WTC_{10–53}$ materials used here, samples were analyzed as dry mounts by light microscopy using an inverted IX-70 Olympus microscope and 20× objective lens coupled through a DP71 camera to DP Controller software (version 3.3.1.292) (Olympus, Center Valley, PA) (Figure 1).

Cell Culture

Primary cultures of human airway smooth muscle cells (a kind gift from Reynold A. Panettieri, Jr., University of Pennsylvania) were lines originally established and characterized from lung transplant donors at the University of Pennsylvania (Panettieri et al. 1989). Smooth muscle cells were maintained as primary cultures in M199 medium supplemented with 10% FBS, 0.25 ng/ml EGF (human epidermal growth factor), and 1 ng/ml FGF (human fibroblast growth factor) in the presence of antibiotics (100 U penicillin G/ml, 100 µg streptomycin/ml, 0.25 µg

amphotericin B/ml). Human airway bronchial epithelial (BEAS-2B) cells were cultured in BEBM (bronchial epithelial cell basal medium) with all growth additives as recommended by the manufacturer. Cells were cultured in 24-well plates for exposures prior to MTT or adenylyl cyclase assays. Medium in each well was changed 48–72 h prior to the beginning of any cigarette smoke extract (CSE) or WTC dust exposure.

Preparation of Cigarette Smoke Extract (CSE)

Cigarette smoke extract (CSE) was made fresh immediately before all experimental procedures using protocols outlined by Mercer et al. (2004). Briefly, a 16G needle that penetrated a rubber stopper atop a 500-ml sidearm flask was impaled into the filter of a research cigarette. Following ignition of the cigarette, the stopper was inserted into a 500-ml flask and its sidearm was connected (by tubing through a stopper) to a second 500-ml flask containing 25 ml phosphate-buffered saline (PBS, pH 7.6). This flask, in turn, was connected in series through its sidearm to a third waste flask and then to a house vacuum. The vacuum was adjusted such that the cigarette was completely consumed after 6 min, and the smoke was blown over the surface of the 25 ml PBS in the second flask. The resultant 25-ml extract (CSE in PBS) was adjusted to pH 7.4 and filter sterilized (0.22 μm) and was considered to be 100% CSE. Previous studies in airway epithelial cells have employed final CSE concentrations ranging from 1 to 20% (Thaikoottathil et al. 2009; Gross et al. 2010); as such, for these studies, a 10% CSE final concentration was employed in the cell culture media in all studies. As noted by Kroenig et al. (2008), "Although CSE is arguably not fully representative of 'true' cigarette smoke [CS] exposure, *in vivo*, cells are not exposed to CS, but rather to CS constituents that have been solubilized into biological fluids such as the epithelial and alveolar lining fluid in the lungs." As such, the use of CSE in the studies here is appropriate for modeling the effects of actual cigarette smoke to which some individuals who responded to the WTC site were routinely exposed before, during, and after their time at Ground Zero.

Cytotoxicity Studies of WTC Dust, CSE, or the Combination

Confluent BEAS-2B cells in 24-well plates (culture area/well approximately 1.92 cm^2), last fed 48–72 h prior to any given exposure, had their media removed and replaced with 225 μl/well M199 media (supplemented with 10% FBS, 0.25 ng EGF/ml, 1 ng FGF/ml). Thereafter, 25 μl of vehicle (water), CSE, or WTC dust (10–53 μm average diameter [$WTC_{10–53}$]) at 10× the desired final concentrations was added to dedicated wells for periods of up to 24 h at 37°C in a humidified 5% CO_2/95% air cell culture incubator. Preliminary studies demonstrated that cytotoxicity to a given concentration of WTC dust varied with cell density, time since cells were seeded in cell culture plates, and time since last media change. Therefore, in every protocol, control (untreated) wells were analyzed in parallel.

In an initial concentration-response study, exposure of the BEAS-2B cells to $WTC_{10–53}$ dust was performed using concentrations of 0.01–0.5 μg dust/μl for 24 h. These values equate to treatments of 2.5–125 μg dust/well or 1.3–65.1 μg dust/cm^2. These WTC dust levels were greater than those used in our previous study (approximately 10 μg/cm^2; Wang et al. 2010b) but less than those used in the investigation by Payne et al. (2004) (upward of 100 μg/cm^2). Nonetheless, the value of 65 μg/cm^2 would approximate a dose generated by deposition of approximately 160 mg $WTC_{10–53}$ into the lungs of an individual (not wearing proper respiratory protection and performing mouth breathing during heavy labor) exposed for 4 h to an atmosphere bearing approximately 300 mg dust/m^3 at Ground Zero. Such concentrations were apparently present in the period right after the building collapses (i.e., estimates for 9/11–9/13 = hundreds of mg/m^3; low mg to hundreds of μg/m^3 after approximately 3

wk; Anonymous 2002; Geyh et al. 2005; Geyh personal communication).

To test for the comparative effects from the CSE alone, as well as for potential interactive effects between the CSE and the WTC dust, a follow-up study utilized cells treated for 24 h with the maximal concentration of WTC dust (i.e., 0.5 μg/μl $WTC_{10–53}$ dust) used in the concentration-response studies that did not produce mortality in all cells. In a separate study to identify a time course for cytotoxicity, shorter periods of exposures, i.e., 2 or 6 h, of BEAS-2B cells to WTC dust (at 0.5 μg $WTC_{10–53}$ dust/μl), CSE (at 10%), or the combined agents were examined.

A separate set of studies was conducted to determine whether WTC−, CSE−, or WTC + CSE-exposed epithelial cells released into the culture media (conditioned media) substances that might affect viability or cyclic AMP cell signaling in airway smooth muscle cells. BEAS-2B cells were exposed to 0.5 μg $WTC_{10–53}$ dust/μl, CSE (at 10%), or both agents for 24 h. The culture medium was removed and centrifuged (2400 × g, 6 min) to pellet the dust particles. The supernatant containing cleared conditioned culture media was transferred to (and replaced) the culture media on confluent human airway smooth muscle cells in 24-well plates for a further 24 h of incubation.

Cellular toxicity assessments of the BEAS-2B and airway smooth muscle cells were performed in triplicate in each 24-well cell culture plate and averaged using an MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] colorimetric assay that measures mitochondrial metabolism. Briefly, MTT (dissolved in serum-free M199 medium at 0.5 mg MTT/ml) was added to each culture plate well (0.5 ml/well) and incubated for 3 h at 37°C to permit the mitochondrial dehydrogenase within viable cells to metabolize MTT to insoluble formazan. At the end of the incubation, the medium was removed, the cells were solubilized with 0.5 ml 0.05 *M* HCl in isopropanol, and absorbance was measured at 570 nm using a Beckman DU-640 spectrophotometer (Brea, CA). Absorbance was expressed as percent of the absorbance in untreated control cells in parallel wells.

Adenylyl Cyclase Assays

Cultured human airway smooth muscle cells (in 24-well plates) exposed for 24 h to centrifuged conditioned media recovered from BEAS-2B cells, after their 24-h exposure to WTC dust, CSE, or both agents, were analyzed for forskolin-stimulated adenylyl cyclase activity (Osawa et al. 2007). Adenylyl cyclase enzymes synthesize cyclic AMP (cAMP), an important second messenger in determining airway smooth muscle tone (Barnes 1995). In these studies, after the media was removed, triplicate wells were washed once with 0.5 ml warm (37°C) PBS. An aliquot (100 μl) of PBS (37°C) and 50 μl of 3× adenylyl cyclase buffer (final concentrations of 10 μ*M* forskolin, 50 m*M* HEPES [pH 8], 50 m*M* NaCl, 0.4 m*M* EGTA, 1 m*M* cAMP, 7 m*M* $MgCl_2$, 0.1 m*M* ATP [20 μCi/ml [^{32}P]-α-ATP], 0.1 mg bovine serum albumin [BSA]/ml, 50 U creatine phosphokinase/ml, and 7 m*M* phosphocreatine) were then added to each well (Osawa et al. 2007). The plates were floated in a 37°C waterbath for 15 min before the reaction was stopped by addition of 100 μl stop buffer to each well. All newly synthesized [^{32}P]-cAMP was then separated from precursor [^{32}P]-α-ATP by sequential chromatography over Dowex and alumina columns according Salomon et al. (1974), with column recovery rates of 75–90%.

Immunoblot Analyses of PARP

To differentiate necrotic from apoptotic forms of cell death in the BEAS-2B cells after exposure to the WTC dust, to CSE, or to both agents, immunoblot analyses of fragmented poly-ADP-ribose polymerase (PARP) was performed. PARP is an endogenous DNA repair enzyme cleaved from a 116-kD intact form into 85- and 24-kD fragments as a result of the activity of CPP32 [caspase-3, CASP3] early within hours in the apoptotic process. After exposure of the BEAS-2B cells to $WTC_{10–53}$ dust (0.5 μg/μl) in 24-well plates for 24 h, the medium in each well was removed and 100

μl cell lysis buffer (50 m*M* Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 m*M* NaCl, 1 m*M* ethylenediamine tetraacetic acid [EDTA]) was added and the cells were detached and lysed by trituration. Samples were transferred to microcentrifuge tubes; aliquots were taken for protein determination and samples were frozen at −20°C until immunoblotting. Protein concentrations were determined using the Pierce BCA reagents (Thermo Fisher Scientific, Rockford, IL) using BSA as a control.

Samples were thawed, gel loading buffer was added (final concentrations: 50 m*M* Tris-HCl [pH 6.8], 2.5% SDS, 6% glycerol, 2.5% 2-mercaptoethanol, and 2% bromophenol blue), and the samples were boiled for 10 min. Fifty micrograms of total cellular protein was loaded into wells of a 10% SDS-PAGE gel, electrophoresed at 100 V for 1.5 h, and then transferred at 20 V overnight to PVDF membranes (Osawa et al. 2007). The PVDF membranes were then blocked for 1 h at room temperature with 5% powdered skim milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and then probed with rabbit polyclonal anti-PARP antibodies (Upstate [Millipore], Billerica, MA) (1:1000 in 1% milk in TBST) overnight at 4°C. After washing three times, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:5000; Amersham Biosciences, NA934V, Arlington Heights, IL). Signals from the immunoreactive bands were detected using ECL Plus (Amersham Biosciences) and following the manufacturer's recommendations, and were developed on Kodak Biomax light film (Kodak, Rochester, NY). Each film was developed such that band intensities were within the linear range of film responses; band intensities were then quantified using Quantity One software (Bio-Rad, Hercules, CA).

Statistical Analysis

All MTT and adenylyl cyclase data were analyzed by one-way analysis of variance (ANOVA). Differences in values generating $p < .05$ were considered significant.

RESULTS

BEAS-2B cells exposed to 0.01–0.5 μg/μl $WTC_{10–53\upsilon m}$ dust for 24 h demonstrated a concentration-dependent increase in cytotoxicity, with concentrations as low as 0.03 μg/μl producing significant toxicity (as evidenced by reductions in cell mitochondrial conversion of MTT to its formazan product) ($n = 4$) (Figure 2). The effects from the WTC dust on the airway epithelial cells were compared to those previously documented with CSE, as well as to when airway epithelial cells were concurrently exposed to WTC dust and CSE, to ascertain whether additive effects occurred. BEAS-2B cells that were exposed to 10% CSE or to 0.5 μg $WTC_{10–53}$/μl for 24 h exhibited significant cellular toxicity ($n = 8$) (Figure 3). Cells exposed to a combination of CSE plus the WTC dust exhibited a significantly greater toxicity than cells exposed to CSE alone, but not more so than from the $WTC_{10–53}$ dust alone.

In general, cellular toxicity can result from necrosis or apoptosis, with necrotic cell death usually being more rapid and programmed cell death (apoptosis) typically requiring hours to

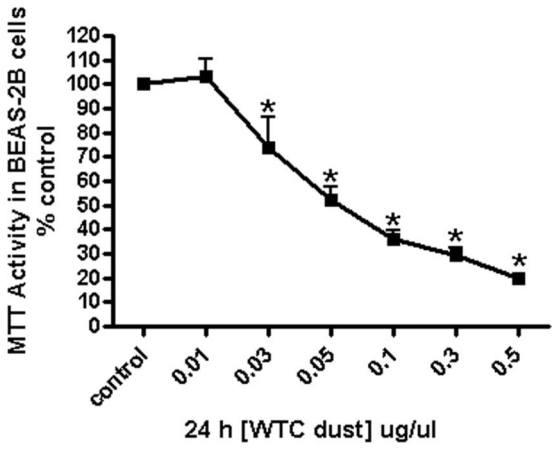

FIGURE 2. Cytotoxicity of human bronchial epithelial cells (BEAS-2B) to WTC dust. Concentration-response toxicity determined by MTT assay after 24 h of exposure to 0.01–0.5 μg $WTC_{10–53}$ dust/μl. Asterisk indicates significant at $p < .05$ vs. control value. Values shown are mean (± SE) from $n = 4$ samples per treatment.

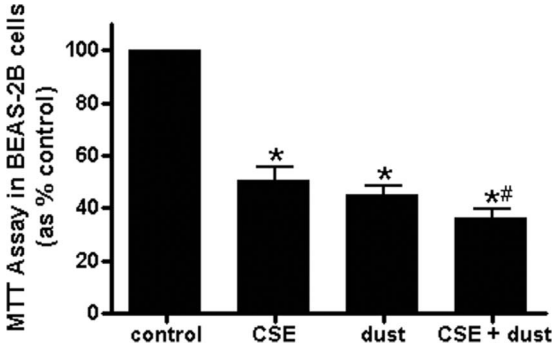

FIGURE 3. Cytotoxicity of human bronchial epithelial cells (BEAS-2B) to WTC dust, cigarette smoke extract (CSE) or the combination. MTT assay after 24 h of exposure to 10% cigarette smoke extract (CSE), 0.5 μg $WTC_{10–53}$/μl dust, or both agents. Asterisk indicates significant at $^*p < .05$ vs. control value; # indicates significant at $p < .05$ vs. CSE value. Values shown are mean (± SE) from $n = 8$ samples per treatment.

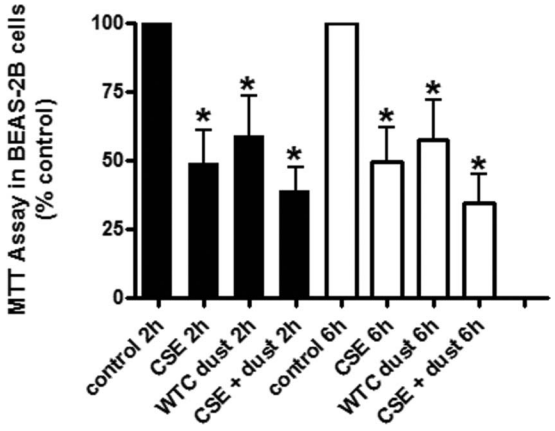

FIGURE 4. Cytotoxicity of human bronchial epithelial cells (BEAS-2B) to WTC dust, cigarette smoke extract (CSE) or the combination. MTT activity after 2 or 6 h of exposure to 10% CSE, 0.5 μg $WTC_{10–53}$/μl dust, or both agents. Double asterisk indicates significant at $p < .05$ vs. control value. Values shown are mean (± SE) from $n = 4$ samples per treatment.

days to occur. Therefore, it was of interest to determine whether shorter exposures (i.e., 2 or 6 h) to the $WTC_{10–53}$ dust would produce cell death and whether levels of PARP (poly-ADP-ribose polymerase, whose activation and cleavage are markers of apoptosis) fragmentation increased over the 24 h period of dust exposure. The results indicated that while exposure of the BEAS-2B cells to 10% CSE, 0.5 μg $WTC_{10–53}$/μl dust, or both agents combined resulted in significant cellular toxicity even after either 2 or 6 h of exposures ($n = 4$) (Figure 4), there was no evidence of enhanced PARP fragmentation within the BEAS-2B cells exposed to CSE, WTC dust, or their combination as compared to control cells ($n = 3$) (Figure 5).

The potential for the BEAS-2B cells to liberate mediators into the cell culture media that could transfer toxicity and/or alter signal transduction pathways in a cell type that is normally their neighbor in the airway wall, i.e., smooth muscle cells, was next examined. To control for the potential carryover of CSE and dust in the media, independent of any liberated BEAS-2B cell products, controls were performed in parallel in which cell culture medium alone (no cells) was incubated with CSE, dust, or both in wells parallel to those containing BEAS-2B cells exposed to the same treatments. As seen in Figure 6, CSE, $WTC_{10–53}$ dust, or their combination incubated with cell culture medium alone for 24 h before transferring to airway smooth muscle cells induced smooth muscle cell toxicity. However, medium removed from BEAS-2B cells resulted in enhanced toxicity in airway smooth muscle cells when the BEAS-2B treatments were either CSE or $WTC_{10–53}$ dust alone ($n = 8$). Media recovered from the combination of $WTC_{10–53}$ dust + CSE in the presence of BEAS-2B cells did not result in greater toxicity in airway smooth muscle cells compared to $WTC_{10–53}$ dust + CSE in the absence of BEAS-2B cells ($n = 8$).

Our earlier studies revealed size-dependent increases in alkalinity of WTC particles; i.e., among on-site dusts collected during 9/12–9/13/01 and then size-fractioned, $WTC_{2.5–10}$ dusts routinely had a pH of 9–10, $WTC_{10–53}$ had a pH of 10–11, and parent (intact) WTC dusts had a pH ≥ 11; only $WTC_{2.5}$ had a near-neutral pH (Maciejczyk et al. 2006). Thus, a concern here was that our outcomes might have been due to a pH-related effect as opposed to mediators associated with the supercoarse dust itself or products released from the treated cells. Since any dramatic (i.e., >0.2 units) change in pH would be reflected in a color change of the

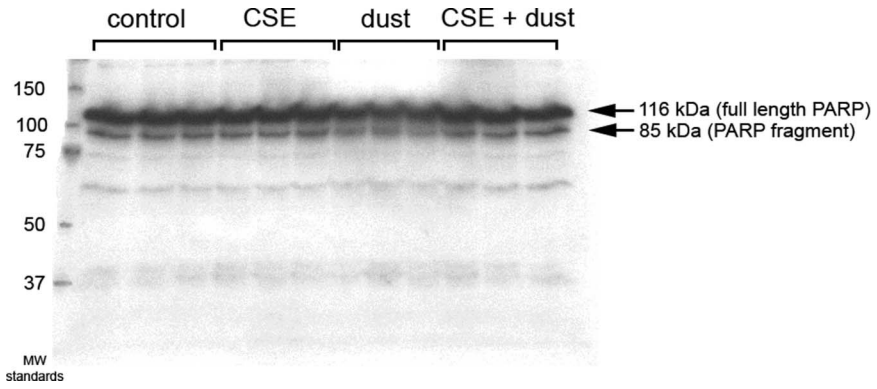

FIGURE 5. Representative image of immunoblot of poly-ADP-ribose polymerase (PARP) fragmentation in human bronchial epithelial cells (BEAS-2B). Lysates of BEAS-2B cells were analyzed for PARP fragmentation after 24 h of exposure to 10% CSE, 0.5 μg $WTC_{10–53}$/μl dust, or both agents. Control levels of fragmented PARP (85 kD) were not increased by CSE or $WTC_{10–53}$ dust. Three independent representative samples of each treatment group are shown.

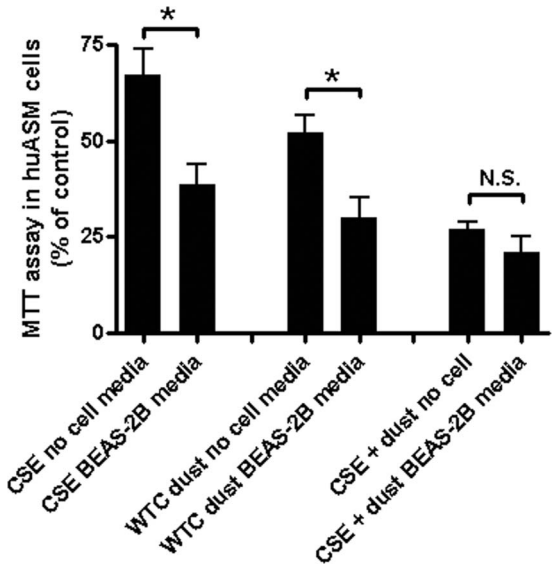

FIGURE 6. Cytotoxic effects of BEAS-2B cell-conditioned media on airway smooth muscle cells. MTT activity was measured in human airway smooth muscle cells that had undergone 24 h exposure to conditioned media obtained from BEAS-2B cells treated for 24 h with 10% CSE, 0.5 μg $WTC_{10–53}$/μl dust, or both agents. "No-cell" controls represent culture media that was incubated for 24 h with 10% CSE, 0.5 μg $WTC_{10–53}$/μl dust, or both agents - without any cells present. Asterisk indicates significant difference at $p < .05$ between the indicated sample values. Values shown are mean (± SE) from $n = 8$ samples per treatment.

indicator that is in the cell culture medium (i.e., phenol red), significant shifts to an alkaline state would have been readily noted. As no color changes occurred, it was concluded that significant change in pH was not an issue and that remediation steps, like neutralization of supernatant before exposing the smooth muscle cells, were not required.

The final series of experiments examined whether media recovered from BEAS-2B cells after 24-h exposures to $WTC_{10–53}$ dust + CSE could induce changes in the synthesis of cyclic AMP in airway smooth muscle cells. As indicated in Figure 7, the ratio of cyclic AMP synthesized for the surviving smooth muscle cells (cAMP/MTT) was significantly enhanced in

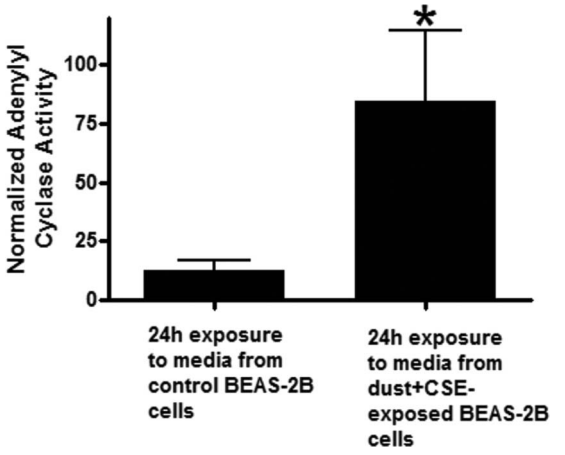

FIGURE 7. Adenylyl cyclase activity in cultured human airway smooth muscle cells. Adenylyl cyclase activity in smooth muscle cells (in terms of mol [^{32}P]-cAMP/well/15 min) was measured in remaining viable cells that had undergone 24 h of exposure to conditioned media obtained from treated (24 h of combined exposure to 10% CSE and 0.5 μg WTC10-53/μl dust) BEAS-2B cells. Asterisk indicates significant difference at $p < .05$ between the indicated sample values. Values shown are mean (± SE) from $n = 5$ samples per treatment.

muscle cells exposed to medium from BEAS-2B cells previously exposed for 24 h to $WTC_{10–53}$ dust + CSE ($n = 5$) as compared to muscle cells exposed to culture medium from control BEAS-2B cells. When analyzed directly, the enzyme activity (based on per well values) was 12.5 ± 4.6 pmol cAMP/well/15 min for the control samples versus 11.8 ± 1.6 pmol cAMP/well/15 min for the dust + CSE values, values that were not significantly different. However, the fact that there were far fewer viable cells left to evince activity indicated that each remaining viable cell in the cells that received medium from BEAS-2B cells previously exposed for 24 h to $WTC_{10–53}$ dust + CSE was exhibiting a greater level of activity.

DISCUSSION

The primary findings of the present study include: (1) human cultured airway epithelial cells (BEAS-2B) exposed for 2, 6, or 24 h to dust (10–53 μm average diameter [$WTC_{10–53}$] specifically) recovered from sites adjoining Ground Zero after the collapse of the WTC, to CSE, or to a combination of the two, undergo necrotic cell death in a concentration-dependent manner; and (2) exposed BEAS-2B cells liberate substance(s) into the cell culture medium that confer both toxicity and increased adenylyl cyclase activity in cultured human airway smooth muscle cells. The acute toxicity observed after the 2- or 6-h exposure to $WTC_{10–53}$ dust, CSE, or their combination, and the lack of fragmentation of the DNA repair enzyme PARP after a 24 h exposure are consistent with a necrotic as opposed to apoptotic method of cell death.

A few in vitro studies provided data to help us understand mechanisms by which WTC dusts may have induced these outcomes. One study showed that primary alveolar macrophages (AM) and Type II epithelial cells exposed to $WTC_{2.5}$ or $WTC_{10–53}$ evinced time- and concentration-related increases in pro-inflammatory cytokine/chemokine (interleukin [IL]-6, IL-8, tumor necrosis factor [TNF]-α; Payne et al. 2004) production. These studies also showed the influence of particle size; $WTC_{2.5}$ always induced the greater response and $WTC_{10–53}$ exerted little impact on type II cell cytokine release. A study by Wang et al. (2010b) examined the role of MAPK signaling pathways in WTC-induced cytokine release. Those studies showed that BEAS-2B cell exposure to $WTC_{2.5}$ for 5 h led to increased IL-6 mRNA expression/protein release; IL-8 and −10 production; and ERK and p38 (but not JNK) pathway activity. Taken together, the findings from these two studies indicate that exposure of lung AM and epithelial cells to fine or supercoarse WTC dust might produce a release of factors that may contribute not only to cell death (see later discussion), but to inflammation/airway remodeling processes in an intact lung.

In the current study, supercoarse WTC dust particles concentration-dependently induced necrotic cell death (as opposed to apoptosis) in epithelial and smooth muscle cells, and co-exposure to CSE exerted an interactive adverse effect. This outcome is important with respect to a potential for development of chronic lung diseases in individuals exposed at Ground Zero. Numerous studies showed that in chronic obstructive pulmonary disease (COPD) patients, lung epithelial cell injury and death were related to an increased presence of both apoptosis and necrosis (Hageman et al. 2003; Yokohori et al. 2004; Liu et al. 2005). While cell death via apoptosis is crucial for removal of damaged cells during inflammatory events/tissue remodeling, necrosis (or excessive apoptosis) produces disrupted cell barrier function, leading to disease. A study by van der Toon et al. (2007) suggested that CS-induced mitochondrial dysfunction (by which depletion of cellular energy causes a shift from early apoptotic states to necrotic cell death) in epithelial cells "may become a new pathophysiological concept in the development of COPD and other diseases." Further, Hageman and colleagues (2003) demonstrated that in patients with COPD, a smoking-related oxidative stress-induced PARP activation seemed critical to disease pathology. Thus, our measures of necrotic cell death and PARP activation/status as a result of exposure to the WTC dusts (with or without

co-exposure to CSE) are important for discerning potential in situ outcomes that may lead to later onset lung diseases.

The Wang et al. (2010b) findings are useful for explaining the results about necrotic versus apoptotic cell death here. In lung epithelial cells, ERK activation appears to help protect against hyperoxia-inducible death (Truong et al. 2004; Xu et al. 2006a). Carvalho et al. (2004) suggested that JNK had a pro-apoptotic role and ERK (and p38) initiated signaling anti-apoptotic roles in determining cell fate in response to hydrogen peroxide. Those results mirrored findings by Buckley et al. (1999) showing that protection against O_2-induced DNA strand breaks and apoptosis in Type II alveolar epithelial cells was mediated via an ERK-dependent pathway. In contrast, lung macrophages with their ERK inhibited show deaths characterized by markers suggestive of both apoptosis (caspase activation) and necrosis (ATP loss) (Monick et al. 2008). Furthermore, JNK activation/p38 deactivation in macrophages resulted in apoptosis (Park et al. 2002; Kim and Sharma 2004; Seimon et al. 2009). Regardless of the findings noted earlier, it remains unclear what the roles of JNK, ERK, and p38 might be with regard to driving lung epithelial cells to select one fate over another in response to WTC dusts.

Increased frequency of death among epithelial cells treated with CSE was in agreement with other studies. Of note, however, there was little indication of apoptosis as the means of death here (i.e., no PARP fragmentation), a pattern that contrasts with results of studies where CSE induced apoptosis in normal and transformed lung epithelial cells (Fu et al. 2006; Liu 2007; Tagawa et al. 2008; Chen et al. 2009). While CSE clearly induces epithelial cell death, there has been an increasing trend in reporting this outcome in terms of autophagy rather than apoptosis (Chen et al. 2008; Kim et al. 2008; Ryter and Choi 2010). As noted by Edinger and Thompson (2004), autophagy is distinct from apoptosis and "autophagy has been suggested as a possible mechanism for non-apoptotic death despite evidence . . . that it represents a survival strategy in times of stress" and that "it is likely autophagy and necrosis often occur in parallel, initiated in response to the same stimuli, but with opposite objectives."

If our suggestion about induction of ERK and p38 pathways is as likely in CSE- as in WTC dust-treated cells, one would anticipate nonapoptotic mechanisms were likely the prevalent form of cell death in our CSE-treated populations. In fact, recent reports by Moretto et al. (2008) and Thaikoottathil et al. (2009) showed that treatment of epithelial cells with CSE results in ERK and p38 pathway activation. Nevertheless, our results here might reflect both nonapoptotic pathways (autophagy and necrosis) that led to cell death after CSE exposure. The problem in distinguishing which form may have been predominant is that viability assessments using MTT detect elevated numbers of cells (specifically macrophages) undergoing caspase-independent death while displaying concurrent increases in autophagy (Xu et al. 2006b). Thus, our measures with CSE-treated epithelial (and likely the dust-exposed) cells here may have been equally as indicative of autophagy as of necrosis. All of our future studies will assess autophagy as another endpoint with the CSE- and WTC dust-treated cells to provide clarification.

As noted earlier, dust from the WTC collapse was previously found to exert acute effects on human lung AM and Type II epithelial cells in vitro. Dust exposure (for 6–24 h) produced an increase in a marker of oxidative stress (GGT) and elevated secretion of several pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) (Payne et al. 2004). Cytokines, in turn, were shown to stimulate adenylyl cyclase activity in airway smooth muscle cells via activation of a raf-1 kinase pathway and phosphorylation of adenylyl cyclase enzymes (Osawa et al. 2007). Thus, in the present study, it is possible that the paracrine mediators in conditioned cell culture media from dust-exposed epithelial cells were cytokines that activated adenylyl cyclase synthesis of cAMP. A rise in cAMP is a known mitogenic (Panettieri 2002) and migratory (Hirakawa et al. 2007) signal in airway smooth muscle that underlies airway

remodeling, a process believed central to the development of the chronic obstructive airway diseases of asthma (Munakata 2006) and chronic obstructive pulmonary disease (COPD) (Puchelle et al. 2003).

The potential for CSE to impart an interactive effect on toxicities induced by WTC dust particles is not unexpected. Several studies demonstrated that CSE/CS co-exposure modified the effects of an array of toxicants in vivo, ex vivo, and in vitro. This is most clear with respect to cancer/assessment of carcinogenicity; Balansky et al. (2000) noted, "as to carcinogenic risk, there is evidence of synergism between CS and exposure to arsenic, asbestos, ethanol, silica, and radiation." Though less well known, interactive effects on noncancer endpoints relevant to our study were also reported (Moszczynski 1992; Morimoto et al. 1993; Moszczynski et al. 1996). Interestingly, among the latter types of studies, TNF-α (endogenous/induced) effects might be modulated by CSE/CS to negatively impact on epithelial cells, macrophages, and other lung cells. Baginski et al. (2006) noted that CSE synergistically increased MUC5AC mucin gene expression and protein production by epithelial cells stimulated with TNFα. Bhavsar et al. (2007) showed that hamster exposure to CS just prior to intratracheal administration of amiodarone led to significant increases in levels of TNF-R1$^+$ AM and apoptotic parenchymal cells compared to those due to drug alone. Most recently, Sarir et al. (2010) showed that a co-presence of CSE with TNF-α led to a significant rise in IL-8 formation/release by macrophages.

Combining the interactive effect studies results with the fact that (1) Payne et al. (2004) and Wang et al. (2010b) demonstrated increases in TNFα/IL-8 release by lung epithelial cells treated with WTC dusts and (2) ERK activation is a key pathway needed for enhanced IL-8 formation/release by macrophages and epithelial cells (Kurosaka et al. 2003; Pace et al. 2008; Wang et al. 2010a) gives us confidence to surmise that in the WTC dust-treated cells, at a minimum, ERK pathways were being activated; no-apoptotic mechanisms (albeit not clear yet as to any domination between autophagy vs. necrosis) were likely the prevalent form of cell death; and, co-exposure to CSE served to amplify each of these parameters so as to increase the incidence of cell death compared to that among cells that received either the dust or CSE alone.

CONCLUSIONS

WTC dust (at least the supercoarse size) and CSE each exert direct adverse effects on airway epithelial and smooth muscle cells, and alter the signaling properties of airway smooth muscle cells. The combination of these two complex mixtures of toxicants also produces an interactive effect upon cell toxicity. It remains to be determined whether these initial cell death events might account, in part, for the chronic lung effects associated with WTC dust exposure among First Responders and others.

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