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Exposure of Human Bronchial Epithelial Cells to Carbon Nanotubes Caused Oxidative Stress and Cytotoxicity

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Summary

Carbon nanotubes are new members of carbon allotropes similar to fullerenes and graphite. Because of their unique electrical, mechanical and thermal properties, carbon nanotubes are highly important for the development of novel applications in the electronics, aerospace and computer industries. Occupational exposure to both natural and man-made graphite during mining and milling can cause pneumoconiosis with clinical/radiographic manifestations similar to those in coal miners. We investigated the adverse effects of single wall carbon nanotubes (SWCNT) using a cell culture of human bronchial epithelial cells (BEAS-2B). Exposure of BEAS-2B cells to SWCNT caused ultra-structural and morphological changes, cellular toxicity, and apoptosis. In addition, exposure to SWCNT induced oxidative stress as shown by the formation of free radicals, accumulation of peroxidative products, and depletion of cell antioxidants. These data indicated that exposure to unrefined SWCNT may lead to pulmonary toxicity due to accelerated oxidative stress in the lung of exposed workers.

Introduction

Carbon nanotubes represent a relatively newly discovered allotrope of carbon with potential applications within nanotechnology. The manufac-

turing of nanotube material relies on the use of transition metal catalysts. In our case, unrefined SWCNT contained up to 30% iron. Iron overload toxicity has been generally associated with free radical-mediated tissue damage, as well as the development and progression of several pathological conditions, including lung cancers, liver and heart disease, diabetes, hormonal abnormalities, and immune system dysfunctions (Cavaleri and Rogan, 1992; Rockey, 2003; Bing, 2001; Maritim et al., 2003; Ahsan et al., 2003; Fraga and Oteiza, 2002). There is a substantial body of evidence suggesting that ultrafine particles cause pulmonary damage to a greater extend than fine particles of the same composition (Ferin et al., 1992; Oberdorster et al., 1996, Li et al., 1998). Chemical composition, particle number, surface area and surface reactivity are essential factors contributing to ultrafine particle toxicity. The purpose of the current study was to determine whether oxidative stress, free radical generation involved in cytotoxicity, ultra-structural and morphological alterations, and apoptosis occurred in cultured cells exposed to SWCNT.

Materials and Methods

2,2'-azobis(2-aminodinopropane)-dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). AlamarBlue™ was obtained from Alamar Biosciences Inc. (Sacramento, CA). SWCNT production material prior to catalyst removal was provided by NASA-JSC, (Houston, TX) and was produced by the high pressure carbon monoxide (HiPCO®) process developed at Rice University (Bronikowski et al., 2001). The nanotube material was found to contain 30% iron by mass after dissolution in concentrated nitric and perchloric acid and measurement by inductively coupled argon plasma, atomic emission spectroscopy (NIOSH, 1994). The BEAS-2B cell line was purchased from ATCC (Manassas, VA). Fetal bovine serum (FBS), fatty acid-free human serum albumin (HSA), luminol, sodium dodecyl sulfate (SDS), L-ascorbic acid, deferoxamine (DFO), potassium ferrocyanide, xylene, diethylenetriaminepentaacetic acid (DTPA), dubelco's minimum essential medium (DMEM), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), ascorbic acid and glutathione were purchased from Sigma Chemicals Co. (St. Louis, MO). Methanol, ethanol, chloroform, hexane (HPLC grade), and water (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). ThioGlo-1™ was obtained from Covalent Associates Inc. (Woburn, MA). TUNEL assay kits were purchased from Promega (Madison, WI). BODIPY-FL phallacidin was obtained from Molecular Probe (Eugene, OR).

BEAS-2B Cell Culture and Treatment with SWCNT. Human bronchial epithelial cells (BEAS-2B) were plated onto 96-well plates or 75 cm^2 flasks and grown in DMEM with 5% FBS at 37°C in a tissue culture incubator (5% CO_2) until 80% confluent monolayers were obtained. To study oxidative stress, BEAS-2B cells were incubated with various concentrations of SWCNT

(0.06 mg/ml, 0.12 mg/ml or 0.24 mg/ml) in DMEM without phenol red for 18 h at 37°C. After incubation, cells were washed twice with PBS (pH 7.4) and harvested by trypsinization. BEAS-2B homogenates were prepared by freezing at -80°C and thawing the cells.

Preparation of SWCNT Suspension. The mixture of SWCNT and DMEM (without phenol red) was vortexed (5-10 min) and then sonicated to disperse the particles with a Branson Sonifier 450 (Danbury, CT) for 2-3 minutes prior to cell exposure.

ESR Measurements. Electron spin resonance (ESR) spin trapping was used to examine free radical generation by SWCNT in a cell free system and in BEAS-2B cells. Ascorbate and DMPO spin trapping agents were used for radical detection. All measurements were performed using a Bruker EMX with a HS cavity. Instrument settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; conversion time, 0.6 s; time constant, 1.3 s. Hyperfine coupling constants were determined using the WinSim program of the NIEHS public EPR software tools package, which is available over the Internet (http://epr.niehs.nih.gov/).

Scanning Electron Microscopy. This technique was performed using a standard protocol. BEAS-2B cells were grown and treated on cover slips (Fisher Scientific, Pittsburgh, PA), and fixed with Karnovsky's fixative (2.5% gluteraldehyde, 3% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4). Cells were dehydrated with a graded series of ethanol, placed in hexamethyldisalizane and then air dried. The samples were coated using gold-palladium and viewed with a JEOL 6400 scanning electron microscope (Tokyo, Japan).

Transmission Electron Microscopy. BEAS-2B cells after treatment were washed with PBS, and fixed in Karnovsky's fixative (2.5% glutaraldehyde + 3% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4), and post-fixed with osmium tetroxide. Cells were dehydrated in graded alcohol solutions and propylene oxide and embedded in LX-112 (Ladd, Williston, VT). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Transmission electron microscopy (JEOL 1220, Tokyo, Japan).

AlamarBlue™ Viability Assay. The viability of cells following exposure was determined using the AlamarBlue™ bioassay (Alamar Biosciences Inc., Sacramento, CA), as described by Keane et al. (1997). After incubation with AlamarBlue (4 hr, 37°C) fluorescence (λ_{ex} = 530nm, λ_{em} = 580nm) was determined using multi-well plate reader (CytoFluor Series 4000, Perseptive Biosystems, Framingham, MA). Data were analyzed using CytoFluor Version 4.2.1 (PerSeptive Biosystems, Framingham, MA).

Confocal Microscopy of BEAS-2B Cells Incubated with SWCNT. For confocal microscopy, BEAS-2B cells were grown on glass chamber slides (Fischer Scientific, Pittsburgh, PA). The cells were exposed to SWCNT (0.24 mg/ml, 18hr) while control cells were exposed to saline. Following exposure, the treated and control cells were washed with phosphate-buffered saline

(PBS, pH 7.4) and then fixed with 3.7 % paraformaldehyde. Following fixation, cells were incubated with 200 mL of 165 nM BODIPY-FL phallacidin solution (Molecular Probes, Eugene, OR) in the dark (25°C). Fluorescent phallacidins have been shown to stain F-actin within nanomolar range concentrations in cell cultures and tissue sections (Wang et al., 1982; Weiland, T., 1987). The F-actin microfilaments of BEAS-2B cells were visualized using a Sarastro 2000 laser confocal microscope fitted with an argon laser (Molecular Dynamics, Inc., Sunnyvale, CA with a Optiphot-2, Nikon, Inc., Melville, PA). Photomicrographs were recorded though a 40 x lens objective using a 488-nm laser line. Emission spectra above λ_{510} nm was detected by a photo-detector to image F-actin. Reflected light below λ_{510} nm was simultaneously passed to a separate optical pass providing images. Changes in cell morphology were assessed by phase contrast and light microscopy.

Apoptotic Changes in BEAS-2B Cells Exposed to SWCNT. Apoptosis of BEAS-2B cells was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (Wyllie et al., 1980). BEAS-2B cells were treated as described above. After the incubation, cells were washed twice with PBS (pH 7.4), fixed with formalin, and analyzed for apoptosis using a TUNEL assay kit (Promega, Madison, WI). The apoptotic cell nuclei were visualized as a yellow-green fluorescent signal using fluorescence microscopy. Normal cell nuclei counterstained with propidium iodide emitted red fluorescence.

Detection of Iron in BEAS-2B Cells Exposed to SWCNT or $FeCl_3$. BEAS-2B cells ($1x10^6$) grown on chamber slides were incubated with SWCNT (0.24 mg/ml) or $FeCl_3$ (150 ng/ml) in phenol-free DMEM for 18 hr (37°C). After the treatment, cells were washed twice with PBS (pH 7.4) and stained using Mallory's reaction for iron (Luna, 1968). Changes in cell morphology were assessed by light microscopy (Leica Microscopie & System, Leica, Portugal).

Chemiluminescence Measurements of Total Antioxidant Reserve. A water-soluble azo initiator, 2,2'- azobis(2 aminodinopropane)-dihydrochloride (AAPH), was used to produce peroxyl radicals (Niki, 1990). Oxidation of luminol by AAPH derived peroxyl radicals was assayed by chemiluminescence in the presence of luminol. A delay in the chemiluminescence response caused by interaction of endogenous antioxidants with AAPH derived peroxyl radicals was observed upon addition of cell homogenates. Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants was evaluated. The incubation medium contained 0.1 M phosphate buffer (pH 7.4) at 37 °C, AAPH (50 mM), and luminol (0.4 mM). The reaction was started by the addition of AAPH. The luminescent analyzer 633 (Coral Biomedical, Inc., San Diego, CA) was employed for the determination.

HPLC Assay of α-Tocopherol. Extracts of α tocopherol from BEAS-2B cells were prepared using a procedure described by Lang et al. (1986).

A Waters HPLC system (Waters Assosiates, Milford, MA) with a 717 auto sampler, a Hewlett Packard ODS Hypersil column (5 mm, 200 x 4.6 mm), a Waters 600 controller pump and a 474 fluorescence detector was used to measure vitamin E in samples. The wavelengths employed in the assay were 292 nm (excitation) and 324 nm (emission). Eluent was CH_3OH, and the flow rate was 1 ml/min. Under these conditions, the retention time for α tocopherol was 8.2 min. The data acquired were exported from the Waters 474 detector and analyzed using Millennium 2000 software.

Measurements of Peroxidative Products. Accumulation of lipid peroxidation products reacting with 2-thiobarbituric acid (TBARS) in cell homogenates was measured spectophotometrically by absorbance at 535 nm, as described by Buege and Aust (1978). A molar extinction of 1.56×10^5 M^{-1} cm^{-1} was used for calculations. Measurements were made using 2401-PC Shimadzu spectrophotometer (Kyoto, Japan) interfaced with a Dell OptiPlex GX 400 personal computer.

Fluorescence Assay for Low Molecular Weight Thiols and Protein Sulfhydryls. Low molecular weight thiols and protein sulfhydryl concentration in BEAS-2B cells were determined using ThioGlo™-1, a maleimide reagent, which produces a highly fluorescent adduct upon its reaction with SH- groups (Shvedova et al., 2000). Low molecular weight thiol content was estimated by an immediate fluorescence response registered upon addition of ThioGlo™-1 to the cell homogenate. Protein sulfhydryls were determined as an additional increase in fluorescence response after addition of SDS (4.0 mM) to the same cell homogenate. A standard curve was established by addition of GSH (0.04 - 4.0 μM) to 100 mM disodium phosphate buffer (pH 7.4) containing 10 μM ThioGlo™-1 (DMSO solution). A spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan) was employed for the assay of fluorescence using excitation at 388 nm and emission at 500 nm. The data obtained were exported and analyzed using RF-5301PC Personal Fluorescence Software (Shimadzu).

Protein Assay. The protein content in BEAS-2B cells was measured by the Bradford method, using a BioRad assay kit (BioRad, Richmond, CA). Bovine serum albumin was used as a standard.

Statistics. Data were expressed as the mean (SEM) of 3 experiments for each group. A one-way ANOVA test was employed to compare the responses between treatments. Statistical significance was set at $p < 0.05$.

Results

ESR Detection of Free Radicals. To determine the extent to which SWCNT are able to generate free radicals, we utilized ascorbate as a physiologically relevant scavenger of radicals that undergoes one-electron oxidation to produce ESR-detectable ascorbate radicals. Since ascorbate acts as a primary reductant, reacting with a number of different radicals in cells and tissues, we attempted to use this antioxidant as a natural "spin trap" and

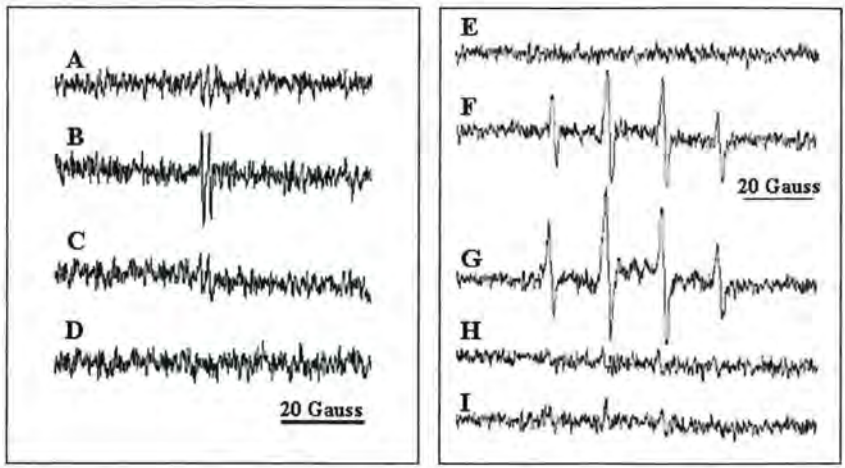

Figure 1. ESR spectra of free radicals generated by SWCNT in a cell free system and BEAS-2B cells. ***A:*** *Ascorbate (10 μM) in a PBS (pH 7.4);* ***B:*** *ESR spectra recorded 5 min after the addition of SWCNT (0.12 mg/ml) to a solution of ascorbate (10 mM) with PBS (pH 7.4);* ***C:*** *Same as B plus deferoxamine (0.2 mM);* ***D:*** *ESR spectra detected 5 min after exposure of SWCNT pre-incubated with DFO to ascorbate.* Pre-incubation with DFO*: SWCNT (0.12 mg/ml) were pre-incubated with DFO (0.2 mM), sedimented and then re-suspended in ascorbate medium.* ***E:*** *BEAS-2B ($2x10^5$ cells/ml) in PBS (pH 7.4) plus 100 mM DMPO;* ***F:*** *ESR spectra recorded 5 min after the addition of SWCNT (0.12 mg/ml) to BEAS-2B ($2x10^5$ cells/ml) in PBS (pH 7.4) plus DMPO (100 mM);* ***G:*** *Same as F plus H_2O_2 (1 mM);* ***H:*** *Same as F plus catalase (20 U/ml);* ***I:*** *Same as F plus DTPA (0.2 mM).* ***Instrument conditions:*** *microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 sec; conversion time, 0.6 sec.*

reporter for oxidative injury. In a cell free system, incubation of SWCNT (0.12 mg/ml) in the presence of ascorbate (10 μM) produced an ESR-detectable signal of ascorbate radicals (Fig.1B). No ESR signal was detected in the medium in the absence of ascorbate. This suggests that SWCNT catalyze one-electron oxidation of ascorbate forming ascorbate radicals detected by ESR. The addition of a metal chelator, deferoxamine (DFO), to the incubation system (SWCNT plus ascorbate) substantially suppressed the ascorbate radical ESR signal (Fig. 1C). Moreover, no ascorbate radical signals were detected when SWCNT were pretreated with DFO, sedimented and then re-suspended in ascorbate-containing medium (Fig. 1D). These results suggest that transition metals play an important role in ascorbate radical formation by SWCNT.

Using the ESR spin trapping technique, we observed formation of free radicals in BEAS-2B cells exposed to SWCNT. After a 5 min exposure of BEAS-2B cells to SWCNT, DMPO radical adducts were recorded (Fig. 1F). The ESR spectra represented superposition of two signals with hyperfine coupling constants (adduct 1: $a^N = 14.9$, $a^H = 14.7$ [80% of the signal] and adduct 2: $a^N = 15.6$, $a^H = 23.0$ [20% of the signal]). The signals have been

simulated and identified as DMPO adducts of hydroxyl (adduct 1) and a carbon-centered (adduct 2) radicals, respectively. Addition of H_2O_2, increased the DMPO/·OH adduct signal (Fig. 1G). Catalase, an H_2O_2 scavenging enzyme, decreased the generation of OH radical (Fig. 1H). A metal chelator, DTPA, also suppressed the DMPO/·OH signal (Fig. 1I). These results suggest that redox-active transition metals in SWCNT, via a Fenton-like reaction, are involved in the generation of highly reactive radicals which may cause oxidative stress, depletion of antioxidants (e.g. GSH, ascorbate) and cell damage.

Scanning/Transmission Electron Microscopy, Confocal Microscopy and Viability of BEAS-2B Cells. Exposure of BEAS-2B cells to SWCNT altered cell surface homogeneity, leading to cell separation from the monolayer, and cytotoxicity (Fig. 2B). The appearance of the nucleus, mitochondria, tonofilaments, and other cytoplasmic organelles in BEAS-2B cells exposed to SWCNT was altered (Fig 2D). Drastic alteration of F-actin structure (Fig. 3B) in the cells exposed to SWCNT was associated with loss of cell viability (Fig 3C). Incubation of BEAS-2B cells with DFO (100 μM) alleviated SWCNT-induced cytotoxicity (Fig.3D) indicating a protective role of iron chelating. SWCNT-induced toxicity was also evident by changes in the cell morphology of BEAS-2B cells showing typical apoptotic nuclear condensation along with apoptotic bodies detected by TUNEL assay 18 h post treatment (Fig. 2D and Fig. 4, respectively).

Detection of iron in BEAS-2B cells exposed to SWCNT or $FeCl_3$. Light

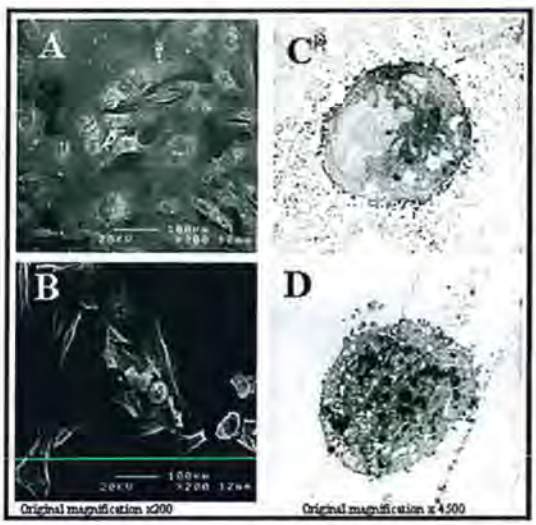

*Figure 2. Scanning (A, B) and transmission (C, D) electron microscopy of BEAS-2B cells exposed to SWCNT. **A, C** – Control cells; **B, D** – Cells after incubation with 0.24 mg/ml of SWCNT (18h). Original magnification: x200 for scanning, and x4500 for transmission electron microscopy.*

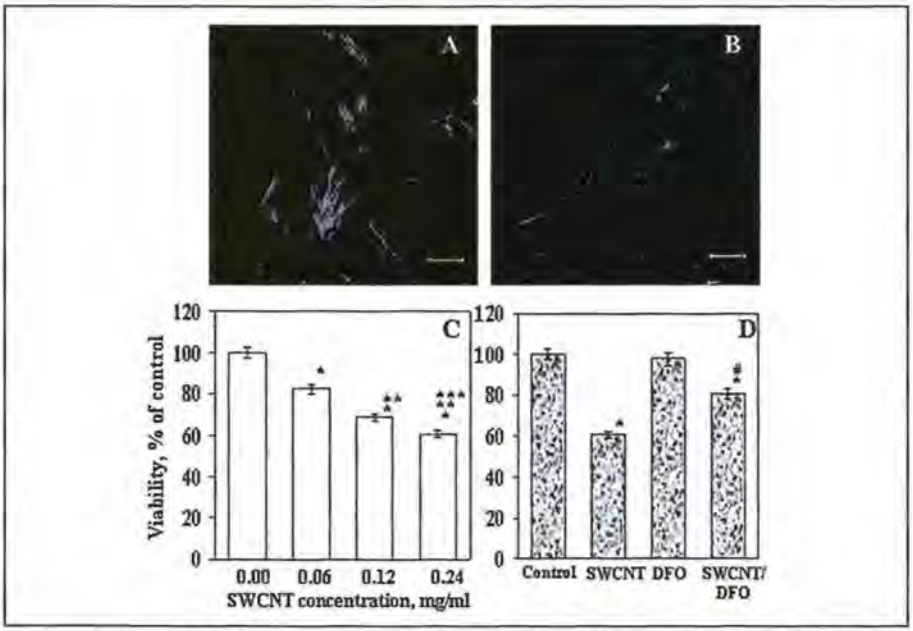

*Figure 3. Confocal photo micrographs (A, B) and viability (C, D) of BEAS-2B cells after exposure to SWCNT. **A** – Control cells; **B** – Cells after incubation with 0.24 mg/ml of SWCNT (18h). **C** – Viability of BEAS-2B cells following exposure to SWCNT. **D** – Viability of BEAS-2B cells following exposure to SWCNT in the presence of deferoxamine. Original magnification x 400. Values are means ±SEM of 3 experiments. *p < 0.05 vs control cells; ** p < 0.05 vs cells exposed to 0.06 mg/ml SWCNT; *** p < 0.05 vs cells exposed to 0.12 mg/ml SWCNT; #p < 0.05 vs cells incubated with DFO (DFO - 0.1 mM; SWCNT – 0.24 mg/ml).*

microscopy of BEAS-2B cells exposed to SWCNT (18 hr) revealed accumulation of dark-black material within cell cytoplasm. To visualize iron in the cytoplasm after treatment with SWCNT (0.24 mg/ml) or $FeCl_3$ (150 ng/ml), the cells were stained with Mallory's reagent. Free iron was not seen in cytoplasm of the cells exposed to SWCNT, while in the cells treated with

*Figure 4. Exposure to SWCNT induced apoptotic cell death. **Conditions:** BEAS-2B cells exposed to SWCNT (0.24 mg/ml, 18 hr) were stained with TUNEL reagent. Apoptotic cells exhibited yellow-green fluorescence, while normal cells counter-stained with propidium iodide showed fluoresced red. Original magnification x 40.*

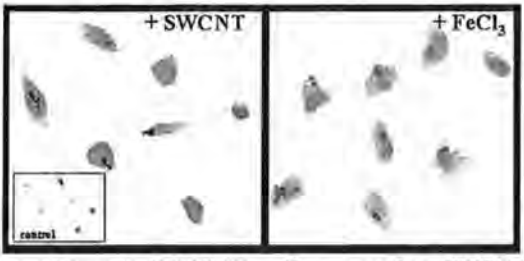

*Figure 5. Detection of iron in BEAS-2B cells exposed to SWCNT or $FeCl_3$. **Conditions:** BEAS-2B cells ($1x10^6$) were incubated (18 hr, 37°C) in DMEM (phenol-free) with SWCNT (0.24 mg/ml) or $FeCl_3$ (150 ng/ml). Incubated cells were washed with PBS (pH 7.4) and stained with Mallory's reagent for intracellular iron detection. Original magnification x 400.*

$FeCL_3$ blue staining of sequestered iron was detected (Fig. 5).

Total Antioxidant Reserve, Lipid Peroxidation (TBARs), and Levels of Vitamin E, GSH and Protein Thiols. As shown in Table 1, exposure of BEAS-2B cells to SWCNT induced a significant concentration-dependent lipid peroxidation, depletion of antioxidant reserves, and decreased levels of vitamin E, GSH, and protein thiols.

Table 1. Biomarkers of Oxidative Stress in the BEAS-2B Cells after Exposure to SWCNT for 18 h

	Control	SWCNT 0.06 mg/ml	SWCNT 0.12 mg/ml	SWCNT 0.24 mg/ml
Total Antioxidant Reserve, nmol/mg protein	112.5±12.9	86.1±3.1*	73.2±3.0*β	39.1±3.5*βα
Vitamin E, pmol/mg protein	42.600±16.995	5.456±4.078*	1.325±0.708*	0.370±0.005*β
GSH, nmol/mg protein	53.2±3.9	37.7±3.7*	26.3±2.6*β	16.3±2.1*βα
Protein Thiols, nmol/mg protein	70.7±3.7	65.9±4.7	62.3±4.1	52.9±2.5*
TBARs, nmol/mg protein	0.014±0.003	0.028±0.007*	0.030±0.005*	0.031±0.004*

* $p < 0.05$ vs control cells;
β $p < 0.05$ vs cells exposed to 0.06 mg/ml SWCNT;
α $p < 0.05$ vs cells exposed to 0.12 mg/ml SWCNT.

Conclusions

Exposure to carbon materials has been reported to cause pneumoconiosis in workers exposed to both natural and man-made graphite (NIOSH, 1978). Severe symptomatic cases with massive pulmonary fibrosis in the past were observed during carbon electrode production (Stellman, 1997). Mixed dust pneumoconiosis caused by long-term occupational exposure to graphite dust is a rare disease (Domej et al., 2002). Screening and clinical examination of 746 graphite workers revealed an elevated occurrence of upper respiratory tract infections, chronic bronchitis and pneumonia (Kasparov et al., 1989). Hypertrophic laryngitis, papillomatous bronchitis and angiofibroma of the larynx, considered to be precancerous lesions, have been reported. However, recent reports emphasized that such exposures are likely to be associated with mixed dusts (Stellman, 1997).

Carbon nanotubes is the material lying between fullerenes and graphite and is a new member of carbon allotropes. Little toxicity data on this novel material are currently available. We observed that exposure to SWCNT caused oxidative stress and cytotoxicity in BEAS-2B cells. It has been shown that commonly used fullerene, C_{60}, (combined with UV or visible light) induced significant lipid peroxidation, damage to proteins and loss of membrane-bound enzymes in rat liver microsomes (Kamat et al., 1998). Free radicals have been implicated in the detrimental effects of ultrafine particles (MacNee et al., 1991, Oberdorster, 1996). Transition metals, e.g. iron (Fe^{2+}) reacting with H_2O_2 via Fenton-like reaction can generate highly reactive OH radicals responsible for triggering oxidative stress in cells and tissues. The addition of catalase, a scavenger of H_2O_2, to the incubation medium inhibited OH radical generation (Fig.1H), indicating that H_2O_2 was produced in the SWCNT-treated cells serving as a precursor for ·OH generation. Incubation of BEAS-2B cells with SWCNT in the presence of iron chelators, DTPA and DFO, decreased the ESR signal intensity (Fig.1I) pointing to a key role of iron in the radical generation.

The manufacturing of nanotube material relies on the use of transition metal catalysts. The health risks associated with nanotube material prior to removal of the catalyst are therefore likely to be associated with both the carbonaceous and the transition metal components. Despite the fact that transition metals were not shown to be primarily involved in ultrafine-particle-mediated generation of free radicals (Brown et al., 2000), we are not fully convinced that the carbonaceous structure of SWCNT per se was responsible for cytotoxicity and formation of free radicals in the cells exposed to nanotubes. We found that exposure of BEAS-2B cells to SWCNT in the presence of DFO or DTPA remarkably reduced cytoxicity (Fig. 3D) indicating redox active iron within the SWCNT matrix was primarily responsible for SWCNT-induced cytotoxicity. Light microscopy data showed the accumulation of black particulates in the cytoplasm of BEAS-2B cells after exposure to SWCNT (18h). Even though SWCNT is engulfed by the cells,

we have not seen free iron in cytoplasm of the cells (Fig. 5). Although we have seen strong indications of oxidative damage in human keratinocytes (Shvedova et al., 2003) and in bronchial epithelial cells (current data) caused by SWCNT, further research is needed to address the mechanisms of nanotube-specific toxicity by testing refined materials. It is also imperative to establish to what extent the observed toxicity of unrefined SWCNT is directly associated with chemistry of the catalyst vs particle size, surface area and surface reactivity of carbon nanotube particles.

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