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Single-walled carbon nanotube-induced mitotic disruption☆

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ABSTRACT

Carbon nanotubes were among the earliest products of nanotechnology and have many potential applications in medicine, electronics, and manufacturing. The low density, small size, and biological persistence of carbon nanotubes create challenges for exposure control and monitoring and make respiratory exposures to workers likely. We have previously shown mitotic spindle aberrations in cultured primary and immortalized human airway epithelial cells exposed to 24, 48 and 96 μg/cm^2 single-walled carbon nanotubes (SWCNT). To investigate mitotic spindle aberrations at concentrations anticipated in exposed workers, primary and immortalized human airway epithelial cells were exposed to SWCNT for 24–72 h at doses equivalent to 20 weeks of exposure at the Permissible Exposure Limit for particulates not otherwise regulated. We have now demonstrated fragmented centrosomes, disrupted mitotic spindles and aneuploid chromosome number at those doses. The data further demonstrated multipolar mitotic spindles comprised 95% of the disrupted mitoses. The increased multipolar mitotic spindles were associated with an increased number of cells in the G2 phase of mitosis, indicating a mitotic checkpoint response. Nanotubes were observed in association with mitotic spindle microtubules, the centrosomes and condensed chromatin in cells exposed to 0.024, 0.24, 2.4 and 24 μg/cm^2 SWCNT. Three-dimensional reconstructions showed carbon nanotubes within the centrosome structure. The lower doses did not cause cytotoxicity or reduction in colony formation after 24 h; however, after three days, significant cytotoxicity was observed in the SWCNT-exposed cells. Colony formation assays showed an increased proliferation seven days after exposure. Our results show significant disruption of the mitotic spindle by SWCNT at occupationally relevant doses. The increased proliferation that was observed in carbon nanotube-exposed cells indicates a greater potential to pass the genetic damage to daughter cells. Disruption of the centrosome is common in many solid tumors including lung cancer. The resulting aneuploidy is an early event in the progression of many cancers, suggesting that it may play a role in both tumorigenesis and tumor progression. These results suggest caution should be used in the handling and processing of carbon nanotubes.

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1. Introduction

Carbon nanotubes are currently used in many consumer and industrial products. Current uses include electronic and drug delivery products, protective clothing, sports equipment, and space exploration. The multi-billion dollar nanotechnology industry is expected to reach a trillion dollars by 2015 [1]. Carbon nanotubes are available commercially in two major forms: single-walled carbon nanotubes (SWCNT); and the more rigid, multi-walled carbon nanotubes (MWCNT). The low density and small size of carbon nanotubes makes respiratory exposures likely, with the highest exposures expected to occur occupationally, either during production or through incorporation into various products. Although the industry is expanding rapidly, the associated human health hazards have not been investigated fully.

The durability, narrow width and proportionally greater length of the carbon nanotube are characteristics shared with asbestos

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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and are a reason for concern [2]. While some carbon nanotubes can be degraded by myeloperoxidase in neutrophils under specific conditions [3], they may stay in the body for long periods of time following exposure. Previous investigations have demonstrated that both SWCNT and MWCNT can enter cells [4–7], and cause a variety of inflammatory, cytotoxic, proliferative and genetic changes *in vitro* and *in vivo* through a variety of mechanisms [8,9]. Nanotube exposure induced the generation of reactive oxygen species, oxidative stress and cytotoxicity [9–12]. SWCNT interacted with the structural elements of the cell, with apparent binding to the cytoskeleton [13–15], telomeric DNA [16], and G–C rich DNA sequences in the chromosomes [17]. The intercalation of SWCNT with the DNA causes a conformational change [17]. Destabilization of the DNA structure can induce chromosome breakage. *In vitro* investigations have shown SWCNT-induced DNA damage in established cancer cell lines, immortalized bronchial epithelial cells as well as primary mouse embryo fibroblasts and human mesothelial cells [18–20]. Micronuclei have been observed in significant numbers following *in vitro* treatment with SWCNT or MWCNT indicating disruption of the mitotic spindle apparatus [19,21]. The presence of chromosome centromeres in the micronuclei indicates the loss of whole chromosomes.

In vivo studies have shown that SWCNT exposure results in macrophages without nuclei as well as dividing macrophage daughter cells connected by nanotubes, indicating SWCNT are capable of inducing errors in cell division *in vivo* [8,22]. Exposure of rodents to the larger diameter MWCNT (11.3 nm) results in micronuclei in Type II epithelial cells indicating either a high level of chromosomal breakage or mitotic spindle disruption [2]. The integrity of the mitotic spindle and chromosome number are critical because mitotic spindle disruption, centrosome damage and aneuploidy may lead to a greater risk of cancer [23–25].

Worker exposure in laboratories is likely during mixing and processing [26,27]. In commercial processing there is a potential for even higher exposures during production and processing if proper engineering controls are not used [28]. Although workplace exposures are difficult to measure, direct reading instrumentation, and filter-based methods have been used to evaluate nanoparticle concentrations and emissions to the outdoor environment of unbound engineered nanoparticles [29]. Accurate exposure assessment will be critical in evaluating the risk of nanotube exposures in workers.

The current exposure limit for carbon nanotubes falls in the class of 'particles not otherwise regulated' and is 5 mg/m^3 [30]. Recently, much lower exposure limits have been proposed for carbon nanotubes but are not yet recommended [31]. We, therefore, examined whether exposure to SWCNT has the potential to induce aneuploidy, mitotic spindle aberrations or disruption of the cell cycle in normal and immortalized human respiratory epithelial cells at levels that are possible in the workplace under current regulations for particulates not otherwise regulated.

2. Methods

2.1. Particles for all experiments

SWCNT (CNI Inc., Houston, TX) used in this study were produced by the high pressure CO disproportionation process (HiPco), employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor, and were purified by acid treatment to remove metal contaminates [32]. Chemical analysis of total elemental carbon and trace metal (iron) in SWCNT was performed at the Chemical Exposure and Monitoring Branch (DART/NIOSH, Cincinnati, OH). Elemental carbon in SWCNT (HiPco) was assessed according to the NIOSH Manual of Analytical Methods [33], while metal content (iron) was determined using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES, NMAM #7300). The purity of HiPco SWCNT was assessed by several standard analytical techniques including thermo-gravimetric analysis with differential scanning calorimetry, Raman spectroscopy and near-infrared (NIR) spectroscopy [34]. The specific surface area was measured at −196 °C by the nitrogen absorption–desorption technique (Brunauer Emmet Teller method, BET) using a SA3100 Surface Area and Pore Size Analyzer (Beckman Coulter Inc., Fullerton, CA), while diameter and length were measured by transmission electron microscopy (TEM). The diameter and length of the purified SWCNT were 1–4 nm and 0.5–1 μm respectively. The surface area of purified SWCNT was 1040 m^2/g. The chemical analysis was assessed at DATA CHEM Laboratories Inc. using plasma-atomic emission spectrometry where the SWCNT were defined as 99% elemental carbon and 0.23% iron. A more detailed analysis of the chemical composition has been reported previously [35]. The same lot of SWCNT was utilized for all experiments reported.

2.2. Culture of cells

Both immortalized and primary human respiratory epithelial cell populations were used to examine the potential genetic damage due to SWCNT exposure. Primary human respiratory epithelial cells (SAEC; Lonza, Walkersville, MD) isolated from the small airway of a normal human donor were examined to determine the response of a normal cell population to SWCNT exposure. The primary SAEC cells exhibited a cobblestone epithelial morphology that was free of fibroblasts during the culture period. Cells of a single lot were cultured and used between passages 1 and 6. In addition, the primary cells were examined by electron microscope and cytokeratin 8 and 18 staining to confirm the Type II phenotype. The primary cells have a normal diploid karyotype, which was necessary for the determination of potential aneuploidy induction following exposure. The primary cell cultures double every 20–24 h, which makes it possible to analyze a potential change in chromosome number and centrosome morphology of cells that have divided during a 24–72 h exposure. The mitotic index is the number of cells in mitosis when the cells are fixed. Although cells have gone through mitosis during the period of exposure, the analysis of the mitotic spindle must be performed on cells that are in division at the time of fixation. The mitotic index of the SAEC cells was 0.5% which prevented analysis of mitotic spindle integrity in this cell population.

Normal human bronchial epithelial cells (BEAS-2B) from a human donor (ATCC, Manassas, VA 20108) were immortalized with an adenovirus 12-SV40 (Ad 12SV40) as described previously [36]. BEAS-2B cells were cultured in DMEM media supplemented with 10% fetal bovine serum (Thermo Scientific, Rockford, IL), while SAEC were obtained and cultured following manufacturer's directions using Cabrex media (Lonza, Walkersville, MD). Immortalized human bronchial epithelial cell (BEAS-2B) cultures in the serum-enriched media double every 18–20 h and have normal mitotic spindle morphology (ATCC, Manassas, VA). The mitotic index of the BEAS-2B cells was 9.0 + 4.0%. The proliferation rate, the high mitotic index and the integrity of the mitotic spindle of BEAS-2B cells make it possible to examine a minimum of 100 mitotic spindles of good morphology for each of three replicate cultures for each treatment combination.

2.3. Treatment protocol

Immortalized BEAS-2B and the primary SAEC were exposed in parallel culture dishes to single-walled carbon nanotubes (SWCNT) or to the spindle poison (positive control), vanadium pentoxide (Sigma, St. Louis, MO). Vanadium pentoxide fragments the centrosome and also inhibits the assembly of microtubules resulting in aberrant spindles, aneuploidy, polyploid and binucleate cells [37]. The dose of SWCNT was based on *in vivo* exposures that demonstrated epithelial cell proliferation and abnormal nuclei at 20 μg/mouse, and is equivalent to an exposure predicted in workers of 40 h per week for 20 weeks at the OSHA particle exposure limit (PEL) of 5 mg/m^3 for particles less than 5 μm in diameter [8,30]. The 20 μg/mouse *in vivo* dose was adjusted to the alveolar surface area of a mouse of 500 cm^2/mouse lung [38]. The adjusted dose for *in vitro* exposure was 0.02–0.08 μg/cm^2 of culture surface area. SWCNT were suspended in media and sonicated over ice for 5 min. The dispersion of the carbon nanotubes in culture media was evaluated by TEM. Vanadium pentoxide was suspended in media and sonicated over ice in the cold room for 30 min. Specifically, cultured cells were exposed to 0.024, 0.24, 2.4 or 24 μg/cm^2 SWCNT or to 0.031, 0.31 or 3.1 μg/cm^2 vanadium pentoxide. Twenty-four and 72 h after exposure, SAEC and BEAS-2B cells were prepared for analysis of apoptosis and necrosis. The SAEC cells were analyzed for centrosome integrity and chromosome number. The BEAS-2B cells were prepared for analysis of the mitotic spindle. Three independent replicates were performed for each exposure of the SAEC and BEAS-2B.

2.4. Mitotic spindle and centrosome morphology analysis

BEAS-2B and SAEC were cultured in 1-mL chamber slides. Dual chambers were prepared for each treatment and cell type. Three independent replicates were prepared for each cell type and treatment. After exposure, the media was removed and the cells were washed twice for 5 min each with 2 mL of calcium and magnesium free Dulbecco phosphate buffered saline (DPBS) + 0.1% Tween 20 (Invitrogen, Carlsbad, CA). The cells were then fixed with 100% methanol. Spindle integrity was examined using dual-label immunofluorescence for tubulin and centrin to detect the mitotic spindle and the centrosomes following methods described previously [25]. Primary antibodies were rabbit anti-beta tubulin (Abcam, La Jolla, CA, USA) and mouse anti-centrin (Salisbury Laboratory). Secondary antibodies were Rhodamine Red goat anti-rabbit IgG and Alexa 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Small aggregates of SWCNT (carbon nanoropes) appeared as black structures in differential interference contrast (DIC) imaging due to absorbance of light [39–41].