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Potential pulmonary effects of engineered carbon nanotubes: *in vitro* genotoxic effects

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Abstract

The development of novel engineered nano-sized materials is a rapidly emerging technology with many applications in medicine and industry. *In vitro* and *in vivo* studies have suggested many deleterious effects of carbon nanotube exposure including granulomatous inflammation, release of cytosolic enzymes, pulmonary fibrosis, reactive oxygen damage, cellular atypia, DNA fragmentation, mutation and errors in chromosome number as well as mitotic spindle disruption. The physical properties of the carbon nanotubes make respiratory exposure to workers likely during the production or use of commercial products. Many of the investigations of the genotoxicity of carbon nanotubes have focused on reactive oxygen mediated DNA damage; however, the long thin tubular-shaped carbon nanotubes have a striking similarity to cellular microtubules. The similarity of carbon nanotubes to microtubules suggests a potential to interact with cellular biomolecules, such as the mitotic spindle, as well as the motor proteins that separate the chromosomes during cell division. Disruption of centrosomes and mitotic spindles would result in monopolar, tripolar, and quadrapolar divisions of chromosomes. The resulting aneuploidy is a key mechanism in the potential carcinogenicity of carbon nanotubes.

Keywords: *Genotoxic, mitotic spindle, microtubule, carbon nanotube, aneuploid*

Introduction

Carbon nanotubes are currently used in many consumer and industrial products such as electronics, drug delivery devices, protective clothing, sports equipment, and in research of genetically modified crops and space exploration. The nanotechnology industry is a multi-billion dollar industry that is expected to reach a trillion dollars by 2015 (Bradley et al. 2009). The low density and small size of these particles makes significant respiratory exposures likely. Nanotubes are degraded slowly and may stay in the body for long periods of time following exposure. Carbon particles are organic structures which generally have low toxicity based upon chemical composition. However, new technology allows the production of manufactured carbon nanotubes, which are narrow, hollow, fibrous tubes of graphene carbon (Ju-Nam and Lead 2008). Manufactured carbon nanotubes with one layer are known as single-walled carbon nanotubes (SWCNT), while those composed of multiple layers are known as multi-walled carbon nanotubes (MWCNT). The durability and physical characteristics (high aspect ratio) of carbon nanotubes resemble those of asbestos and suggest similar toxicity (Muller et al. 2008). Although occupational exposures to particles are often regulated based upon particle composition, the toxicity of durable inorganic mineral fibers is often determined by particle dimensions rather than chemical composition (Stanton et al. 1981). Both SWCNT and MWCNT can be aerosolized under workplace conditions (Maynard et al. 2004; Aitken et al. 2006; Han et al. 2008; Yeganeh et al. 2008). The numbers of occupational exposures to nanoparticles are likely to increase as their use in manufacturing, electronics, and medicine increases; however, the human health hazards associated with exposure to carbon nanotubes have not been fully investigated, especially their potential for carcinogenicity (Sinha and Yeow 2005; Koyama et al. 2006; Pagona and Tagmatarchis 2006; Malarkey and Parpura 2007; Prakash and Kulamarva 2007; Ju-Nam and Lead 2008).

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Carbon nanotube treatment *in vivo*

Numerous deleterious effects of nanotube exposure have been reported *in vivo*. SWCNT have been reported to cause granulomatous inflammation, release of the cytosolic enzyme lactate dehydrogenase, pulmonary fibrosis, and hypertrophied and hyperplastic bronchiolar and alveolar epithelial cells when administered either by inhalation or pharyngeal aspiration (Shvedova et al. 2005, 2008; Porter et al. 2010). Results from *in vivo* exposure to SWCNT and MWCNT have demonstrated macrophages without nuclei or with multiple nuclei indicating that carbon nanotubes may be capable of inducing errors in cell division *in vivo* either following aspiration or inhalation exposure (Shvedova et al. 2008; Porter et al. 2010). The observation of mutations in the K-ras oncogene in SWCNT-exposed mouse lungs (Shvedova et al. 2008) indicates genotoxicity and the potential to initiate lung cancer. Mutations of the K-ras gene are frequently reported in chemically-induced mouse lung and smoking-induced human lung adenocarcinoma (Pao et al. 2004; Tam et al. 2006; Chan 2007; Hong et al. 2007). Since persistent epithelial proliferation is a feature of the second phase of pulmonary carcinogenesis (promotion), (Pitot et al. 1989; Pitot 1996, 2007; Rubin 2001) and epithelial hyperplasia and cellular atypia were noted in mice exposed to SWCNT and MWCNT *in vivo* (Shvedova et al. 2008; Porter et al. 2010), the potential for carcinogenicity is of particular concern. Recent investigations of MWCNT carcinogenicity have demonstrated that intraperitoneal or intrascrotal injection of MWCNT results in mesotheliomas in p53 +/− transgenic mice and Fischer rats, respectively (Takagi et al. 2008; Sakamoto et al. 2009). In the study described by Takagi et al. (2008), 3 mg of MWCNT were injected into the abdomen of a mouse. However, the high-dose exposure as well as the high agglomeration of the nanotubes used in the Takagi et al. (2008) study have been questioned, since it resulted in a high death rate due to gastrointestinal occlusion (Ichihara et al. 2008; Takagi et al. 2008). The authors reported agglomerated structures as large as 200 micrometers, which are essentially particles rather than fibers (Takagi et al. 2008). Although frustrated phagocytosis could result from the large agglomerates, this would not test whether the nanotubes would induce mesotheliomas similar to asbestos fibers (Donaldson and Poland 2009). The dose of MWCNT was 30,000 times higher than the no observable effect level (NOEL) for an inflammatory response (Poland et al. 2008). If the 3 mg dose were adjusted based on the peritoneal surface area of approximately 600 cm^2, the equivalent pulmonary dose would be 22 mg based on an alveolar surface area of 4400 cm^2 (Oberdörster 2010). However, a recent study found mesothelioma induction following intraperitoneal injection of as little as 50 μg of MWCNT in mice (Kanno et al. 2010). More recently, investigators have demonstrated that well dispersed MWCNT are phagocytosed by macrophages following pulmonary exposure (Porter 2009; Ryman-Rasmussen et al. 2009). The macrophages containing MWCNT can migrate to the subpleural space and enter the intrapleural space as early as as one day post-exposure (Hubbs et al. 2009; Mercer et al. 2010; Porter et al. 2010). The MWCNT penetrate the intrapleural space and induce inflammation and fibrosis in a manner similar to asbestos (Ryman-Rasmussen et al. 2009). This research raises serious concerns about the potential carcinogenesis of carbon nanotubes. Therefore, the route of entry of the carbon nanotubes into the cell, the mechanism of the DNA damage as well as the basis of division errors requires *in vitro* investigation.

Carbon nanotube treatment in vitro

In vivo exposure to carbon nanotubes has demonstrated a wide range of deleterious biological responses. These include increased oxidative stress, inflammation, hypertrophied epithelial cells, interstitial fibrosis (Shvedova et al. 2005), macrophages without nuclei or with multiple nuclei, and mutations in K-ras (Shvedova et al. 2008). *In vitro* studies provide additional insight into the potential mechanism of toxicity of carbon nanotubes. Several studies have demonstrated that SWCNT and MWCNT can enter cells (Monteiro-Riviere et al. 2005; Bottini et al. 2006a, 2006b, 2006c; Worle-Knirsch et al. 2006). Carbon nanotubes can enter cells by both endocytosis and diffusion through the cell membrane (Doak et al. 2009). Nanotubes generate reactive oxygen species and produce oxidative stress and cytotoxicity in exposed cells (Shvedova et al. 2008). SWCNT have been shown to interact with the structural elements of the cell, with apparent binding to the cytoskeleton, and even binding to telomeric DNA (Li et al. 2006b; Porter et al. 2007). In addition, SWCNT also bind to G-C rich DNA sequences in the chromosomes (Li et al. 2006a). The DNA intercalation results in a conformational change which can be stabilized by carboxyl modification of the SWCNT by acid treatment (Li et al. 2006a, 2006b). Intercalating agents can induce chromosome breakage and instability. The damaging effects of carbon nanotubes may be induced by a variety of mechanisms linked in part to the physical and chemical properties of nanotubes. Kisin et al. (2007) have shown DNA damage and marginal

enhancement of multinucleated cells following *in vitro* exposure to 24–96 μg/cm^2 SWCNT.

Later studies demonstrated DNA damage *in vitro* in immortalized bronchial epithelial cells (Pacurari et al. 2008; Lindberg et al. 2009) and primary mouse embryo fibroblasts exposed to 5–100 μg/ml SWCNT (Yang et al. 2009). Although Yang et al. used purified carbon nanotubes, there was evidence of lactate dehydrogenase leakage from cells as well as depletion of the oxidant protective enzymes (glutathione and superoxide dismutase) indicating reactive oxygen species generation. Reactive oxygen species can damage cell membranes, proteins and DNA. The oxidant-induced DNA damage has been observed *in vivo* in both mice and rats following exposure to MWCNT and SWCNT (Folkmann et al. 2009; Jacobsen et al. 2009). However, when the metal contaminants were removed from SWCNT and MWCNT, the generation of intracellular reactive oxygen species was not observed (Pulskamp et al. 2007). Exposure of cancer cell lines to SWCNT or MWCNT has also demonstrated the loss of whole chromosomes indicating a disruption of the mitotic spindle (Muller et al. 2008; Doak et al. 2009; Lindberg et al. 2009). Recent studies have demonstrated induction of micronuclei in primary mouse type II epithelial cells three days following dosing with 1 mg/kg MWCNT (Muller et al. 2008). Micronuclei indicate either a high level of chromosomal breakage or mitotic spindle disruption. Further investigations have shown that *in vitro* treatment of primary small airway epithelial cells and immortalized bronchial epithelial cells with 24–96 μg/cm^2 SWCNT fragmented the centrosomes and induced multiple mitotic spindle poles, anaphase bridges as well as aneuploid chromosome number (Sargent et al. 2009b). The fragmentation of the centrosomes, the disruption of the mitotic spindle and the aneuploidy induced by the SWCNT was greater than the level observed with the positive control, vanadium pentoxide. The SWCNT were observed by Sargent et al. (2009b) within the nucleus, in association with cellular and mitotic tubulin, in the bridge separating dividing daughter cells (midbody) as well as in the DNA potentially disrupting the normal mitotic process shown in Figure 1. A strong association of the carbon nanotubes with the centrosomes was also observed. In some cases the carbon nanotubes were attached to the centrosomes and the DNA (Figure 2). Although the mechanisms of the SWCNT-induced centrosome fragmentation, mitotic spindle damage and aneuploidy are not known, the diameter of SWCNT bundles is comparable to cellular and mitotic microtubules that form the mitotic spindle (Figure 3) (Sargent et al. 2008).

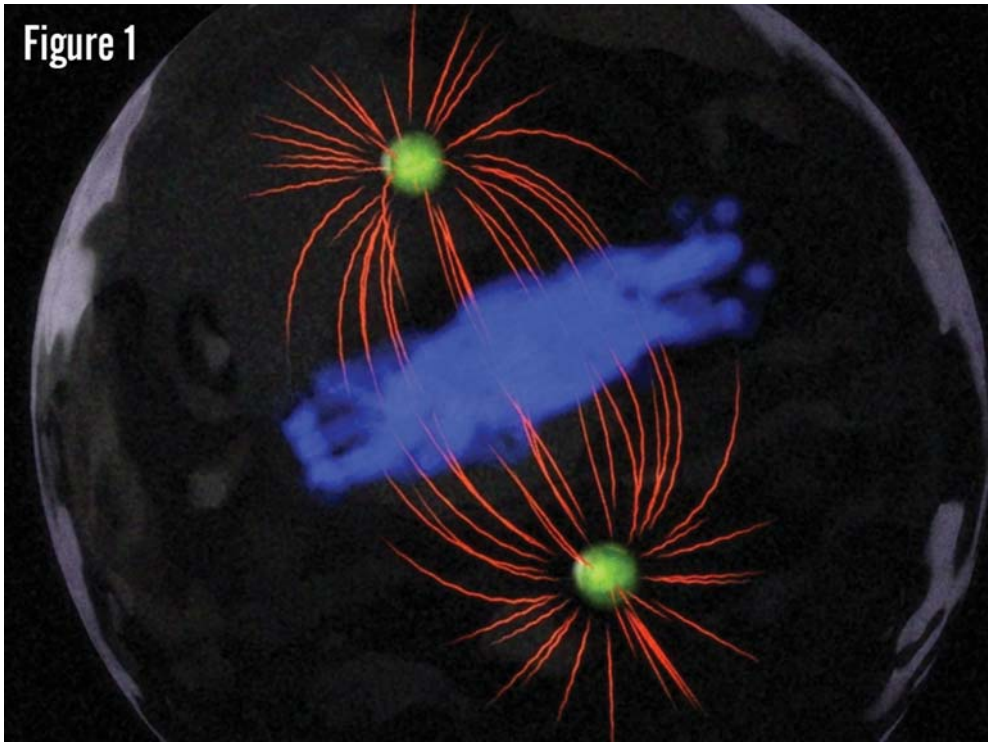

Figure 1. A drawing of a normal mitotic spindle apparatus: The centrosomes (indicated by white arrows) are in green, the microtubules are in red, the DNA is in blue. The cell is in metaphase with the chromosomes lined up in the middle of the mitotic spindle.

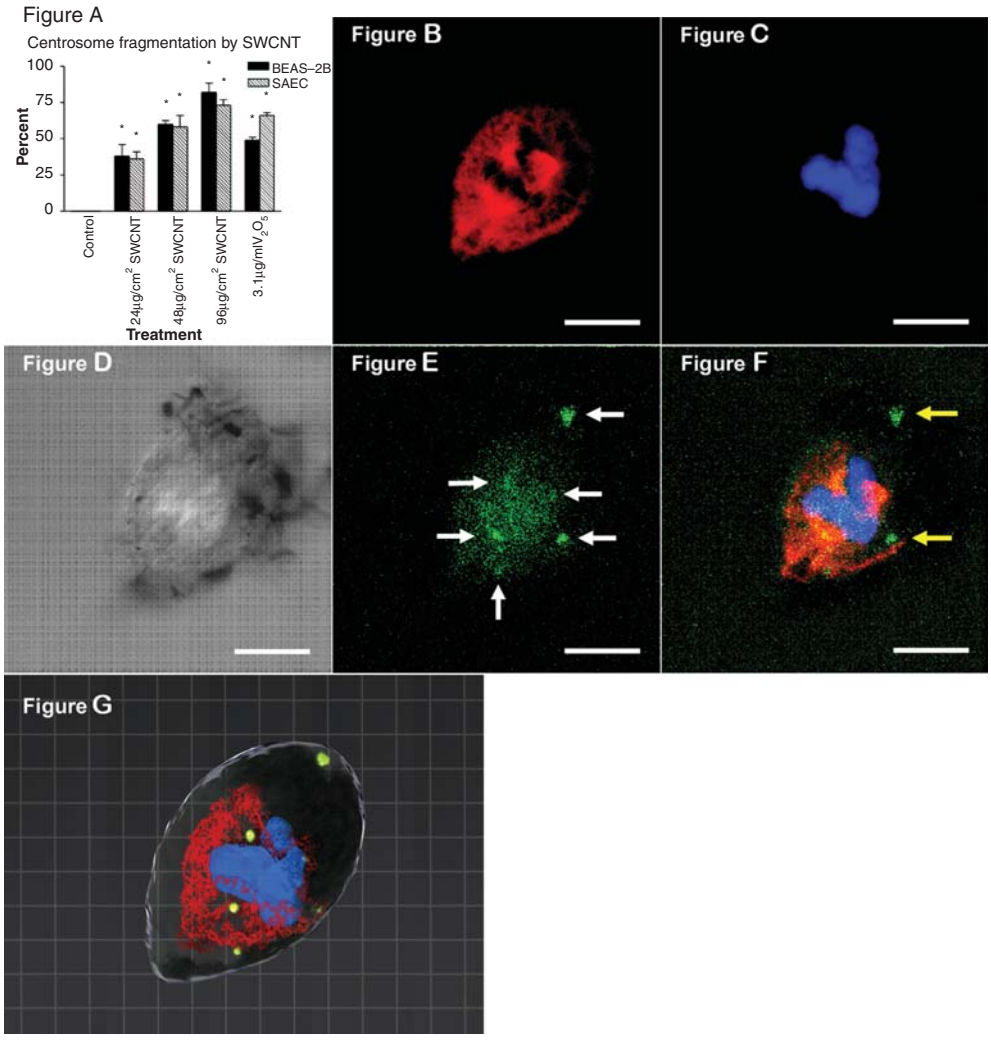

Figure 2. Mitotic spindle and centrosome disruption following SWCNT exposure: (A) The bar graph demonstrates the percent of immortalized bronchial epithelial cells (BEAS-2B, American Type Tissue Culture) and primary small airway epithelial cells (SAEC, Lonza Inc.) with centrosome fragmentation following 24 h exposure to 3.1 μg/cm^2 vanadium pentoxide (positive control) or to 24–96 μg/cm2 SWCNT. The exposure to SWCNT induced centrosome fragmentation in both BEAS-2B and SAEC at all doses of exposure at levels comparable to the positive control V2O5. *indicates significantly different from the unexposed control cells at $P < 0.001$. (B) The figures in (B–G) demonstrate a multipolar mitotic spindle with three poles rather than the two poles that would be expected in a normal cell. The tubulin in (B) was stained red using Spectrum red by indirect immunofluorescence using rabbit anti-beta tubulin (Abcam, La Jolla, CA, USA) and goat anti-rabbit IgG (Abcam, La Jolla, CA). The DNA in C was detected by DAPI and was blue. The nanotubes in (D) were imaged using differential interference contrast and are black. The centrosomes in (E) were stained green by indirect immunofluorescence with mouse anti-centrin (a generous gift from Dr Jeff Salisbury) and secondary goat anti-mouse conjugated with Alexa 488 (Invitrogen). The centrosomes in (E) are indicated by white arrows. In (F) the DNA, tubulin, nanotubes, and centrosome images were merged. The yellow arrows in (F) indicate centrosomes that were in association with nanotubes. In (G), optical sections of 0.1 μ were used to construct a 3-D image of the tripolar mitosis. The reconstructed image shows nanotubes inside the cell in association with the centrosomes, the microtubules, and the DNA. The nanotubes in (B–G) appear to be pulling the DNA in a manner similar to that observed by the mitotic spindle tubulin. The SWCNT appear to be in association with DNA, the centrosomes and the tubulin of the mitotic spindle. The DNA is being pulled toward the spindle poles. In this cell, the three spindle poles, the three unequal DNA bundles, and the disruption of microtubule attachments to two centrosomes suggest major perturbations in cell division. The Figure is reproduced with permission from Sargent et al. (2009b).

Recent laboratory studies indicate that carbon nanotubes can form functional nanotube/tubulin hybrids (Dinu et al. 2009). The hybrid molecules were transported by the cellular motor kinesin that is essential for normal cell division; however, the transport was not as efficient as the transport of cellular microtubules. In addition, spherical nanoparticles less than 40 nanometers in diameter have been shown to inhibit the activity of the kinesin motor further indicating the potential for the disruption of mitosis (Bachand et al. 2005). The physical properties including high tensile strength of carbon nanotubes

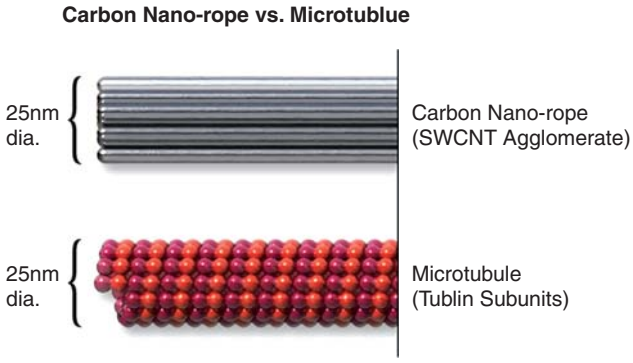

Figure 3. The drawing graphically demonstrates the similarity of the size of single-walled carbon nanotube ropes associated by hydrostatic forces compared to cellular microtubules that form the mitotic spindle apparatus. The microtubules are in red, the carbon nanoropes are in black. The size of the nanoropes is comparable to the size of the microtubules. The similarity in size as well as physical properties may make it possible for the carbon nanotubes to be incorporated into the mitotic spindle apparatus.

are similar to the physical properties of the cellular microtubules that make up the mitotic spindle (Pampaloni and Florin 2008). Displacement of microtubules by carbon nanotubes, or the potential interaction of nanotubes and microtubules as well as interaction with cellular motors may result in the incorporation of nanotubes in the mitotic spindle (Figures 2 and 4). Although it is not known if MWCNT that are greater than 20 nanometers in diameter will form nanotube/microtubule hybrids, MWCNT of 40 nanometers induce micronuclei indicating possible mitotic spindle disruption (Cveticanin et al. 2010). Further research is needed to systematically investigate whether MWCNT induce mitotic spindle damage similar to SWCNT.

Comparison of manufactured carbon nanotubes to microtubules

The cellular microtubules that make up the mitotic spindle are often referred to as 'cellular nanotubes'. Manufactured carbon nanotubes have striking similarities in mechanical behavior to cellular microtubules including bending properties, high resiliency, stiffness, low density and high strength (Pampaloni and Florin 2008). The diameter of single-walled carbon nanotubes is 1–4 nanometers; however, the diameter of nanoropes is comparable to the 25 nanometer diameter of the microtubules (Figure 3) (Huang et al. 2002; Mercer et al. 2008). Microtubules and also carbon

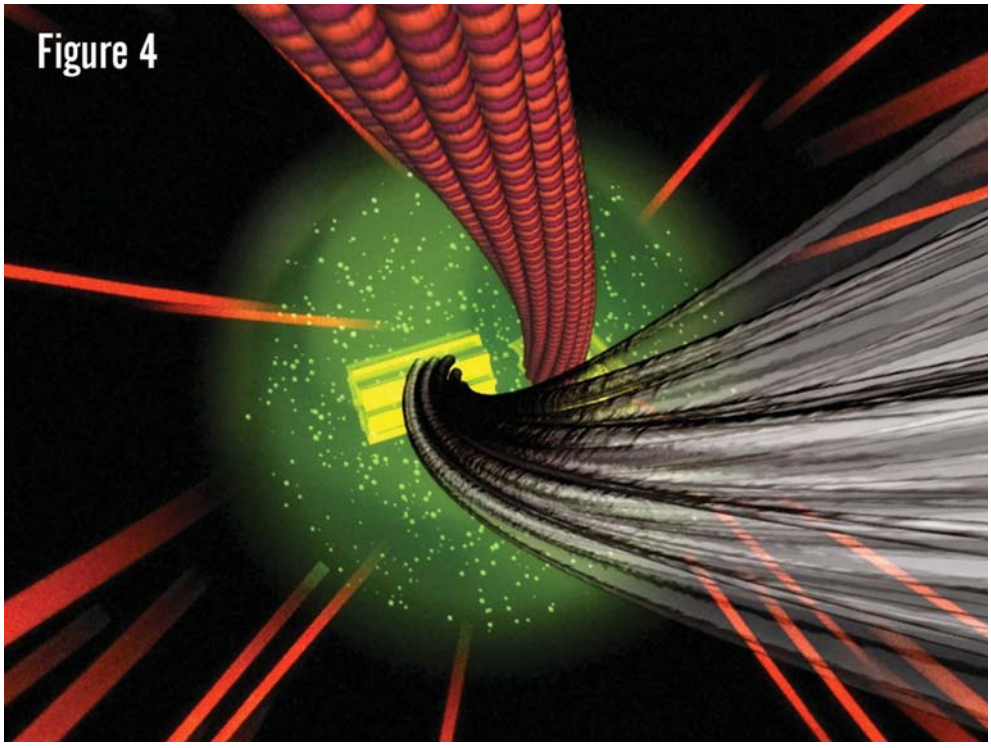

Figure 4. The drawing demonstrates a proposed mechanism of single-walled carbon nanotube attachment to the centrosome. The carbon nanotube bundles are in black. The centrosomes are in yellow. The microtubules of the mitotic spindle are in red. In the proposed model, the carbon nanotubes are attaching to the centrosome in competition with the microtubules. The attachment of the strong carbon nanotubes may result in centrosome fragmentation when the cell divides.

nanotubes dissipate energy by sliding of neighboring cellular or synthetic tubes. Both engineered carbon nanotubes and microtubules form bundles that increase stiffness and resiliency. Engineered carbon nanotubes and microtubules are very strong. Carbon nanotubes are five times stronger than steel. Although the microtubules are 100 times stronger than any other cellular cytoskeletal fibers, their strength is 20 times less than steel and 100 times less than carbon nanotubes (Dalton et al. 2003; Pampaloni and Florin 2008). There are also some distinct chemical differences between microtubules and carbon nanotubes. Microtubules are polymers of alpha and beta tubulin subunits that are bound by non-covalent hydrogen bonds, while carbon nanotubes are composed of covalently-bound carbon molecules rolled into a tube. The microtubules are dynamic structures that polymerize and de-polymerize within the cell to form polar tubes with a plus end and a minus end which is discussed in greater detail later in the manuscript (Yeates and Padilla 2002). Once they are synthesized, individual carbon nanotubes are static in size. The similar size and shape of the carbon nanotubes to the microtubules make it possible for the nanotubes to be incorporated into cellular structures including the mitotic spindle.

Importance of the centrosome in cell division

When cells divide, the microtubules are assembled at the centrosome to form the mitotic spindle apparatus. The mitotic apparatus consists of two mitotic spindle organizing regions, or centrosomes, and the microtubules that direct the segregation of the duplicated chromosomes into two new daughter cells (Figure 1). As cell division progresses, the mitotic spindles elongate and forms a furrow or bridge of cytokinesis that ultimately separates the dividing cell into two new daughter cells. The microtubule dynamics determine mitotic spindle length. The microtubules are polymerized and de-polymerized by the activity of multiple mitotic spindle motors. The formation of a mitotic spindle with two poles as shown in Figure 1 is critical for the proper separation of the chromosomes. The centrosome determines the shape of the mitotic spindle apparatus (Salisbury 2007, 2008). The microtubule dynamics determine the dynamic length of the spindle (Masuda and Cande 1987; Pearson et al. 2006). The similar size and shape of the carbon nanotubes may allow them to displace the cellular nanotubes during mitotic spindle assembly. If this occurs, the carbon nanotubes do not change in size during cell division and would distort the mitotic apparatus. The strength of the carbon nanotubes may prevent separation of the daughter cells during division. Disruption of microtubule assembly, centrosome number or structure results in aberrant mitotic spindles and errors in chromosome number (Hornick et al. 2008; Salisbury 2008). Disruption of centrosome number and structure are common in most cancers (Pihan et al. 1998; Salisbury et al. 2004; Lingle et al. 2005).

Role of kinesin and dynein motors in cell division

The duplication and separation of the centrosome as well as the assembly of the microtubules into the mitotic spindle apparatus is coordinated by the activity of multiple cellular motors (Heald 2000; Zimmerman and Doxsey 2000; McIntosh et al. 2002; Brier et al. 2006). The motors use chemical energy generated by the hydrolysis of ATP to move cellular molecules to duplicate the centrosome, separate duplicated centrosomes (Brier et al. 2006), condense the chromosomes, position the negative end of the microtubules onto the spindle poles, attach the chromosomes to the mitotic spindle microtubules, elongate the mitotic spindle and move chromosomes to opposite poles as the cell divides into two new daughter cells (Heald 2000; Zimmerman and Doxsey 2000; McIntosh et al. 2002). The motors are activated by binding to microtubules. ATP hydrolysis increases 1,000-fold when the motors bind to the microtubules. The motors are divided into two major families: kinesin and dynein. The kinesin motor proteins are further divided into 14 sub-groups based on structural differences in the portion of the motor domain that binds the microtubule and hydrolyses ATP. The kinesin motor structure determines the direction of the motor's movement along the microtubule. The microtubule has a plus end and a minus end (Yeates and Padilla 2002). The plus end directed motors travel toward the negative end of the microtubule while the minus end directed motors travel toward the plus end. Plus end directed kinesin motors have an N-terminal motor domain while minus end directed motors have a motor domain located in the C-terminal region (Hirokawa et al. 1998; Kline-Smith and Walczak 2004). Kinesin-3 (KIF1A) motors, Kinesin-4 proteins (KIF4A and 4B) (Mazumdar and Misteli 2005), Kinesin-5 (Eg5), Kinesin-6 (KIF20A) and Kinesin-7 (CENP-E) are plus end directed motors (Lawrence et al. 2004). The plus end motor Kinsin-13 (KIF2C) does not translocate along microtubules but depolymerizes microtubules and has a central motor domain (Kline-Smith and Walczak 2004). The motor proteins, Ncd, Kar3 and

kinesin-like calmodulin-binding protein (KCBP) are minus end directed kinesins that belong to the Kinesin-14 family (Kikkawa 2008).

Cytoplasmic dynein is important in chromosome segregation, spindle formation and nuclear migration. The form of dynein that is involved in cell division has been identified as one major heavy chain that interacts with multiple subunits, light chains, light intermediate chains and intermediate chains. The composition of the binding partners determines the specific cellular targets that are transported by dynein (Hirokawa et al. 1998).

Mitotic spindle disruption following inhibition of cellular motors

Microtubules activate the cellular motors. Carbon nanotube/microtubule hybrids, as discussed previously, also bind to kinesin and activate transport. The similar physical properties of carbon nanotubes and microtubules may allow the nanotubes to compete with microtubules for binding to the spindle motors. Due to the critical role of the microtubules as well as the cellular motors in cell division, disruption of either the microtubules or the cellular motors can result in errors in the duplication and separation of the centrosome, the formation of the mitotic spindle, the condensation of the chromosomes, the placement of chromosomes on the mitotic spindle, the separation of cells during division or the distribution of chromosomes into daughter cells (Tucker and Preston 1996; Ramirez et al. 1997; Ochi 2002; Caperta et al. 2006). The failure of cytokinesis would result in double the chromosome number (polyploid). In addition, inhibition of the Kinesin-4 motors can result in anaphase mitosis with twisted mitotic spindles that result in daughter cells that are binucleate and/or have micronuclei (Kurasawa et al. 2004; Mazumdar et al. 2004; Mazumdar and Misteli 2005).

The kinesin-5 (Eg5) motor is critically important in the separation of duplicated centrosomes at the onset of mitosis. Coordination of the Eg5 with the dynein motors is required for the separation of the duplicated centrosome. The duplicated centrosome is separated by the pushing action of Eg5 and the pulling action of the deynein motor (Hirokawa et al. 1998). Cooperation of the kinesin-5 and dynein motors is also involved in sliding the microtubules into place with the minus end at the centrosome and the plus end at the chromosome (Kline-Smith and Walczak 2004; Kapitein et al. 2005). Inhibition of the Eg5 motor's ATP hydrolysis by compounds such as monastrol or acrylamide can result in mitotic spindles with only one spindle pole (Brier et al. 2006; Sickles et al. 2007). Inhibition of kinesin motors is observed with spherical nanoparticles; however, the potential for carbon nanotubes to inhibit kinesin has not been investigated (Bachand et al. 2005).

Centrosome assembly requires coordination of both Eg5 and dynein activity. Dynein is the motor primarily responsible for anchoring the minus end of microtubules at the centrosome body (Zhapparova et al. 2007). In addition, dynein is involved in the processing of misfolded proteins in the centrosome body. The ubiquitin ligase, parkin, is transported by dynein to the centrosomal body (Jiang et al. 2008). Inhibition of dynein results in the accumulation of misfolded proteins in the centrosome (Jiang et al. 2008). Vanadium pentoxide has been shown to inhibit the mitotic spindle motor dynein resulting in large centrosomes that fragment during cell division and produce multi-polar mitotic spindles resulting in abnormal chromosome number as well as binucleate cells (Evans et al. 1986; Ramirez et al. 1997; Ehrhardt and Sluder 2005; Sargent et al. 2008).

Potential mechanism of disruption of centrosome and microtubules in nanotube-treated cells

The integrity of the centrosome and the microtubule assembly are critical to the proper distribution of the chromosomes during cell division. Carbon nanotubes are very strong and do not de-polymerize like cellular microtubules. These physical properties of carbon nanotubes may explain the fragmentation of the centrosome, the multi-polar mitotic spindles as well as the aneuploidy that was observed in the SWCNT treated cells (Figure 2). Centrosomes were observed in a strong association with SWCNT (Sargent et al. 2009b). Separating centrosomes that were attached to carbon nanotubes could be held together by the strong nanotubes. When the centrosomes attempt to separate, the nanotubes may hold them together resulting in centrosome fragmentation. In addition, the nanotubes that were observed in the bridge separating dividing cells would not be taken apart (de-polymerize) like microtubules, but would remain intact. The strength of the nanotubes may then prevent the separation of the daughter cells resulting in abnormal chromosome number or aneuploidy (Sargent et al. 2009b). In addition, the physical properties of the carbon nanotubes may make it possible for nanotubes to attach to the cellular motors that assemble the mitotic spindle. Inhibition of mitotic spindle motors by nanotube attachment could also be responsible for the SWCNT-induced centrosome fragmentation, aneuploidy and mitotic spindle

aberrations. If the function of the spindle motors is inhibited or the microtubules that form the mitotic spindle do not direct the segregation of the chromosomes to each pole of the cell equally, cells will be formed with an abnormal number of chromosomes.

Comparison of carbon nanotubes to asbestos

The similarity in durability and aspect ratio of carbon nanotube fibers to asbestos fibers has been a subject of concern. Evidence of the migration of nanotubes to the intrapleural space as seen with asbestos is also a concern (Donaldson and Poland 2009; Hubbs et al. 2009; Ryman-Rasmussen et al. 2009; Porter et al. 2010). Lastly, the finding that inhalation of SWCNT cause mutation of the K-ras gene in lung tissue has raised concern about the carcinogenic as well as the mutagenic potential of carbon nanotubes (Shvedova et al. 2008). An earlier paper by Mangum et al. (2006) observed SWCNT bridging dividing cells. The carbon bridges indicated that the nanotubes may inhibit the separation of dividing cells similar to chrysotile asbestos fibers. Both carbon nanotubes and chrysotile asbestos fibers induce multi-polar mitotic spindles and errors in chromosome number (Cortez and Machado-Santelli 2008; Sargent et al. 2009a). The long, thin chrysotile asbestos fibers of less than 18–30 nanometers in diameter and greater than 8000 nanometers in length are more genotoxic and carcinogenic than larger diameter shorter chrysotile fibers (Stanton et al. 1981; Cortez and Machado-Santelli 2008). Time lapse photography and confocal microscopy has shown that during the initial stages of cell division when the chromosomes condense and the centrosomes divide to migrate and form separate poles, chrysotile fibers are excluded from the mitotic spindle by a protective keratin cage that prevents cellular organelles from entering the division apparatus (Mandeville and Rieder 1990). Later in division when the chromosomes line up in the middle of the cell (metaphase), the keratin cage collapses and the chrysotile fibers gain access to the spindle apparatus (Ault et al. 1995). Although the association with DNA is rare during interphase, three-dimensional imaging of mitotic cells demonstrated chrysotile fibers in association with the chromosomes and mitotic spindle during metaphase and anaphase as well as in the intercellular bridge separating daughter cells during cell division (Figure 5). Chrysotile asbestos has been shown to induce amplification of the centrosome number possibly due to inhibition of cell separation during division; however, the fibers do not associate with the centrosome (Cortez and Machado-Santelli 2008). By contrast, SWCNT have been shown to enter the cell by both passive diffusion and endocytosis (Doak et al. 2009). They are smaller than the nuclear pore and have been observed in the nucleus in interphase cells (Pantarotto et al. 2004; Sargent et al. 2008). Although carbon nanotubes were observed in the bridge of cytokinesis and induced multi-polar mitotic spindles, the centrosomes were not amplified, but fragmented during cell division. In contrast to asbestos, the SWCNT have a strong association with the centrosome as well as the DNA (Li et al. 2006a, 2006b; Martin et al. 2008; Sargent et al. 2009b). Although chrysotile fibers have low tensile strength and are brittle (Langer and Nolan 1994), carbon nanotubes can sustain a great deal of force before breaking (Pampaloni and Florin 2008).

Design of nanotubes for spindle motor transport

As discussed previously, molecular cargos such as proteins, centrosomes, chromosomes and microtubules, are transported by the kinesin and dynein motors through a chemical transport system. The active transport by the cellular motors is very efficient in terms of the conversion of ATP into energy (Kapitein et al. 2005; Kobayashi and Murayama 2009). The cellular motors are capable of transporting synthetic materials that are nano-sized (Hess and Tseng 2007). The motor's efficient use of energy and the ability to transport nano-sized cargo make the cellular motors useful for the design of novel biosensors (Ramachandran et al. 2006), nanomachines (Du et al. 2005; Taira et al. 2008), diagnostics (Huh et al. 2005), and drug delivery systems (Yinghuai et al. 2005; Taira et al. 2008). The similarity of carbon nanotubes to the microtubules and the efficiency of the cellular motors make it possible to design hybrid machines. The properties of carbon nanotubes that make them attractive for biohybrid machines also present a potential health hazard. Current research to produce carbon nanotubes that self-assemble in a solution thus generating an even greater similarity to cellular microtubules should involve a careful evaluation of the toxicity and genotoxicity of the new nanomaterial.

Conclusion

In vitro and *in vivo* studies suggest the potential for carbon nanotubes to induce granulomatous inflammation, oxidative stress, pulmonary fibrosis,

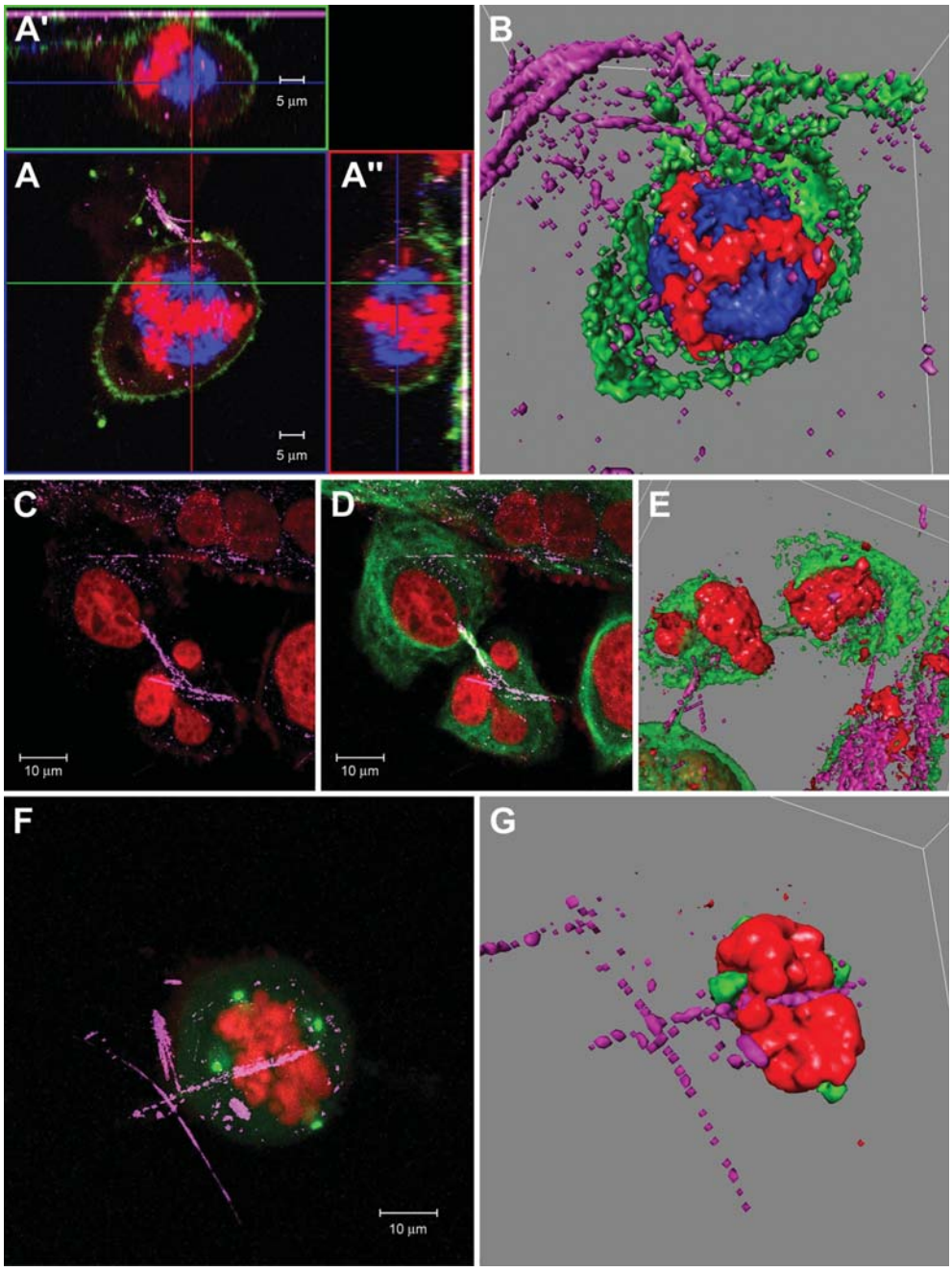

Figure 5. The Figure shows the association of chrysotile with the mitotic spindle. The microtubules are in blue, the DNA is in red, the actin filaments are in green, the chrysotile fibers are in pink. The laser scanning confocal microscope images of HK2 cells (ATTC) after a 48 h treatment of chrysotile and 24 h recovery. (A) A tripolar mitotic spindle with small fragments of chrysotile inside the cell indicated in pink; (A′) and (A″) orthogonal projections showing the intracellular localization of chrysotile fibers; (B) The 3-D reconstruction of optical sections of the same tripolar cell in A, A′ and A″. In (C–E) cells in late telophase have chrysotile fibers in the furrow separating the dividing cells. (C) Image of nuclei in red and chrysotile fibers in pink. (D) The merged image of the microtubules in green appear to be normally organized. The midbody in the furrow of the dividing cells appears normal; however, chrysotile fibers are evident. (E) Optical sections of the cell in (D) have been reconstructed to form a 3-D image. The 3-D image shows interaction of the chrysotile fibers inside the cell and in the bridge separating the dividing daughter cells. (F) and (G) the confocal image shows four centrosomes detected by anti-gamma tubulin (green) and red stained "condensed mitotic chromosomes". The chrysotile fibers (pink) are evident inside the cell in confocal image. (G) The 3-D reconstruction demonstrates chrysotile fibers in association with the chromatin. With permission from: Cortez and Machado-Santelli (2008). Chrysotile effects on human lung cell carcinoma in culture: 3-D reconstruction and DNA quantification by image analysis (Cortez and Machado-Santelli 2008).

hypertrophied and hyperplastic bronchiolar and alveolar epithelial cells, cellular atypia, DNA binding, DNA damage, micronuclei, mutations and disruption of the mitotic spindle as well as errors in chromosome number. Errors in chromosome number and mutations lead to the loss of tumor suppressor genes as well as duplication and activation of oncogenes that contribute to the development of cancer (Aardema et al. 1998; Baker et al. 2009). Research suggesting that carbon nanotubes can migrate to the subpleural tissue and intrapleural space (Hubbs et al. 2009; Porter et al. 2009; Ryman-Rasmussen et al. 2009) raises concerns that carbon nanotubes may induce lung cancer similar to asbestos. MWCNT have been shown to induce mesotheliomas after intraperitoneal and intrascrotal injections (Takagi et al. 2008; Sakamoto et al. 2009). Although these studies have been criticized due to the route of exposure and high dose (Ichihara et al. 2008), intraperitoneal injections of long but not short MWCNTs have been reported to cause asbestos-like acute lesions (Poland et al. 2008). Similarities of the carbon nanotube to microtubules may explain the interaction with the centrosome and mitotic spindles reported by Sargent et al. (2009) rather than the physical interference of the spindle that occurs with fibers such as asbestos (Cortez and Machado-Santelli 2008; Sargent et al. 2009b). Further *in vitro* and *in vivo* research is needed with exposure to the target organs to determine the diameter, length, and chemical properties that are necessary to generate a genotoxic response. The current research indicates caution should be used to control exposures during the production and processing of carbon nanotubes.

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