Single-Walled Carbon Nanotubes Induce Fibrogenic Effect by Disturbing Mitochondrial Oxidative Stress and Activating NF-κB Signaling

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Abstract

Single-walled carbon nanotubes (SWCNTs) are newly discovered material of crystalline carbon that forms single-carbon layer cylinders with nanometer diameters and varying lengths. Although SWCNTs are potentially suitable for a range of novel applications, their extremely small size, fiber-like shape, large surface area, and unique surface chemistry raise potential hazard to humans, including lung toxicity and fibrosis. The molecular mechanisms by which SWCNTs cause lung damage remain elusive. Here we show that SWCNTs dose and time-dependently caused toxicity in cultured human bronchial epithelial (BEAS-2B), alveolar epithelial (A549), and lung fibroblast (WI38) cells. At molecular levels, SWCNTs induced significant mitochondrial depolarization and ROS production at subtoxic doses. SWCNTs stimulated the secretion of proinflammatory cytokines and chemokinesTNFα, IL-1β, IL-6, IL-10 and MCP1 from macrophages (Raw 264.7), which was attributed to the activation of the canonical signaling pathway of NF-κB by SWCNT. Finally, SWCNTs stimulated profibrogenic growth factors TGFβ1 production and fibroblast-to-myofibroblast-transformation. These results indicate that SWCNTs has a potential to induce human lung damage and fibrosis by damaging mitochondria, generating ROS, and stimulating production of proinflammatory and profibrogenic cytokines and growth factors.

Keywords: Single-walled carbon nanotubes; Human bronchial epithelial; Alveolar epithelial; Canonical signaling


Introduction

Single-walled carbon nanotubes (SWCNTs) are molecular-scale tubes of graphitic carbon with unique electrical, chemical, and physical properties [1]. These cylindrical carbon molecules are valuable for nanotechnology, electronics, optics and other fields of materials science and technology. Conversely, the extremely small size (nano scale in diameter) and fiber-like shape of SWCNTs makes them easily become airborne and inhaled into the human lung. The high length-to-width ratio and large surface area may lead to toxic effect similar to those of asbestos fibers (mesothelioma) and silica (interstitial fibrosis) [2,3]. Evaluating the safety of SWCNTs and other nano materials in humans would help avoid the potential harms of exposing SWCNTs to workers and general population [4].

Human toxicity data concerning SWCNTs are scarce, whereas animal data are limited, and in some cases, contradictory, partly due to the fact that many parameters of SWCNTs, such as structure, size distribution, surface area, surface chemistry, charge, agglomeration state, and purity considerably affect the reactivity of carbon nanotubes with the human body. Nevertheless, current data indicated that carbon nanotubes (CNTs) including SWCNTs can enter human cells causing cell death, penetrate tissue structures to migrate and cause lesions in remote area, and induce fibrotic reactions resulting in fibrosis or mesothelioma in the lungs [5-11]. The molecular mechanism involved in the toxicities of SWCNT has not been addressed.

Although many fibrogenic particles and fibers, such as asbestos fibers and silica, cause profound fibrotic responses in the lungs of humans and animals, these materials do not appear to damage lung cells directly. Instead, they may stimulate lung epithelial, fibroblast, and macrophage cells to produce various substances that in turn induce lung lesions and fibrotic reactions. One potential culprit is reactive oxygen species (ROS). Particles and fibers may stimulate the production of ROS via three mechanisms: (a) activated macrophages and neutrophiles produce large amounts of ROS and other radicals from their respiratory burst during phagocytosis; (b) the agents damage the mitochondria to increase ROS production-mitochondria are the major organelle for oxygen consumption and ROS production under normal and many pathological conditions in mammalian cells; and (c) the surface chemistry of many particles and fibers promote ROS production [12]. Whether SWCNTs induce ROS production as a mechanism of fibrosis is currently uncertain.

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NFκB is a family of transcription factors that plays critical roles in inflammation, immune response, apoptosis, and cell proliferation [13,14]. A large number of diverse external stimuli, such as infection, UV light, ROS, and cytokines lead to activation of NF-xB [15]. In unstimulated cells, NF-xB is bound with its inhibitory protein (IκB) and retained in the cytoplasm. Upon stimulation, IκB was phosphorylated and degraded by proteasomes, and thereby release NF-xB from its inhibitor protein, results in the nuclei translocation of NF-xB and where it binds to specific sequences in the promoter regions of target genes. The activation of NF-xB therefore leads to a coordinated increase in the expression of many genes whose products mediate inflammatory, immune, and fibrotic responses. Among the proinflammatory and profibrogenic cytokines and growth factors induced through the NF-xB pathway during inflammation and fibrosis are TNFα, IL-1β, IL-6, MCP1, and TGFβ1. Whether SWCNTs activate the NF-xB pathway to influence the toxicity of SWCNTs is unclear at the present.

Mild or appropriate inflammatory response will help tissue repair, while extensive and persistent activation of inflammatory response in pulmonary will induce aberrant matrix production in interstitial and cause lung fibrosis [16]. Lung fibrosis is a progressive, irreversible and usually fatal end stage condition of various lung diseases. Many factors associate with lung fibrosis, TGFβ1 has been considered as a critic potenter regulator of extra cellular matrix formation and tissue remodeling [17]. Patient with idiopathic pulmonary fibrosis and some animal models of pulmonary fibrosis have shown increased lung TGFβ1 production [18]. Bleomycin, an anticancer drug and potent profibrogenic drug in humans, induces lung fibroblast cell differentiated into myofibroblast cells, characterized by α-smooth muscle actin (α-SMA) expression, is associated with increased expression of TGFβ1 [19]. The potential effect of SWCNTs on TGFβ1 expression and a myofibroblast function has not been addressed.

This study was conducted to identify toxicities of SWCNTs in vitro and their molecular mechanism of action. We tested the ROS generation and mitochondrial membrane potential in the contribution of SWCNT-induced cell toxicity, and investigated cytokine release, activation of the NF-xB pathway, and the fibrogenic factor TGFβ1 in the process of lung fibrosis. The purpose of the study are 1) evaluate the toxicity of SWCNTs and identify the molecular mechanism of action of SWCNTs and 2) establish a simple and quick molecular mechanism based toxicity assessment method which can be used for high throughput screening for inflammatory/fibrogenic toxicants in vitro.

Materials and Methods

Materials

SWCNTs (2nm×2μm, purity>90%) was purchased from Sigma-Aldrich (Milwaukee, WI). A patented process that involves the decomposition of carbon monoxide over a Co-MgO catalyst at about 600°C produces SWCNTs. The material is then treated in diluted hydrochloric acid to remove of the catalyst and other metals.

Dispersion of SWCNTs

Two mg of SWCNTs were dispersed into 1 ml of the cell culture medium supplemented with 1% fetal bovine serum (FBS). The solution was mixed by vortexing for 3 times, 2 min each, followed by sonication in a tissue culture hood using a sterile sonicating probe for 3 times, 30 sec each. The 2 mg/ml stock solution was then diluted with the culture medium to appropriate concentrations and was sonicated as described above just before the experiment.

High Resolution Transmission Electron Microscopy (HRTEM)

SWCNTs were dissolved into ethanol with sonication. The samples were deposited on a 200 mesh copper TEM grid with lacy carbon film. The grid was examined using the JEOL 2100 F STEM at an accelerating voltage of 200 keV. Images were obtained at various magnifications in a bright field TEM mode.

Cell culture

BEAS-2B, a human normal bronchial epithelial cell line transformed with SV40, was cultured with a BMEM medium along with all the additives from Lonza/Clonetics Corporation (Walkersville, MD). A549, a human lung carcinoma epithelial cell line, was cultured with the DMEM medium with 10% FBS. WI38-VA13, a human normal lung fibroblast cell line, was cultured in the αMEM medium with 10% FBS. RAW 264.7, a mouse leukemic monocyte-macrophage cell line, was grown as a monolayer in the Dulbecco's modified Eagle's medium with 10% FBS. All above cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Endotoxin detection

SWCNTs were prepared as suspension in water at a concentration of 2 mg/ml. The suspension was rocked on a platform for 24 h at room temperature. Samples were centrifuged for 10 min and supernatants were collected and filtered to remove remaining particles. Detection of endotoxin was performed by using the Toxin Sensor Chromogenic limulus amebocyte lysate assay kit according to the manufacturer's instructions (GenScript, Piscataway, NJ).

Cytotoxicity assay (MTT based assay)

The CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) was used to examine cell proliferation/cytotoxicity. About 1×10^5 cells were seeded in 96-well plates for overnight. Cells were treated with SWCNTs by replacing the medium with 100 μl of the fresh medium containing varying concentrations of SWCNTs for 1, 2, and 4 days. Twenty μl of the Cell Titer 96 AQueous One Solution Reagent was added to each well. The plates were incubated for 3 h at 37°C in a humidified incubator with 5% CO₂. Absorbance at 490 nm was recorded by using a 96-well plate reader. Parallel wells with no cells but having the medium containing SWCNTs at the same concentration as treated wells were used as the reference control for SWCNTs. Mean absorbance was calculated from three replicates of each exposure and subtracted from blank controls.

Reporter assay

Raw 264.7 cells stably transfected with a NF-xB binding site /Luc reporter construct was described previously [20]. Cells were treated with SWCNTs for 16 h as indicated in the figure legend. The cells were lysed with the passive reporter lysis buffer (Promega, Madison, WI) and cell lysates were vortexed and centrifuged briefly to remove cell debris. 20 μl of supernatant were mixed with 100 μl of the luciferase reagent (Promega). Luciferase activities were detected by using TD 20/20 luminometer (Agilent Technologies, Santa Clara, CA) and were normalized with protein concentrations.

Immunoblotting

Cells were lysed on ice with the RIPA buffer containing protease and phosphatase inhibitors for 30 min. Cell lysate was sonicated briefly and was centrifuged at 14,000xg for 20 min to remove cell
Cells were treated with SWCNTs for 20 µg/ml for 16 h or LPS at 1µg/ml for 5 h. Nuclear extracts were prepared by using the Nuclei EZ PREP reagents from Sigma. 10 µg of nuclear lysates were added to an ELISA plate pre-coated with NF-κB - binding-site dsDNA. Bound NF-κB proteins were detected with an anti-NF-κBp65 antibody following the instructions from Cayman (Ann Arbor, MI).

Results

Characterization of SWCNTs

The SWCNTs used in this study have a purity of large than 90% on carbon basis by X-ray diffraction. Independent laboratory tests further verified the quality of this material. High-resolution transmission electron microscopy (HRTEM) revealed the distinctive crystalline structure of a bundle of SWCNTs with each tube diameter around 2 nm and purity large than 90% (Figure 1). Elemental analyses indicated that the material contains trace amounts (µg/mg sample) of calcium.
13.9, cobalt 4.3, chromium 4.8, magnesium 1.9, zinc 1.47, molybdenum 0.65, and manganese 0.19, with total metal contaminants of about 4.5%. Endotoxin contamination of the SWCNTs was determined to be below the level of detection (maximum sensitivity of assay was 0.005 EU/ml).

To disperse SWCNTs into solution, 2 mg of SWCNTs was suspended in PBS, a dispersal medium (DM) [21], or a culture medium with 1% FBS, respectively. Agglomeration status of SWCNTs was analyzed by TEM. Large agglomerates of SWCNTs were found in the PBS suspension, whereas SWCNTs suspended in the DM or the 1% FBS culture medium were well dispersed (data was not shown). Since the culture medium with 1% FBS is compatible with the cell culture conditions, SWCNTs dispersed in a culture medium with 1% FBS were used for all the following experiments.

**Cytotoxicity, ROS production, and mitochondrial damage**

Bronchial epithelial, alveolar epithelial and interstitial fibroblast cells are first lines of cells to encounter inhaled stimuli. We first characterized the overall toxicity of SWCNT in human normal bronchial epithelial BEAS-2B cells, human lung alveolar epithelial A549 cells, and human lung fibroblast WI-38-VA13 cells. A modified MTT assay was used to measure cell death and proliferation. Parallel controls using the culture medium containing the same concentrations of SWCNTs as the treatment groups but no cells were used as reference to correct for light absorption from SWCNTs. As shown in Figure 2A-C, the three human lung cell lines exhibited varying, dose and time-dependent responses to SWCNTs. BEAS-2B is the most sensitive: with the 2 µg/ml SWCNTs treatment, 82% of the cells survived after 1 day of treatment and ~80% survived after 4 days; at 200 µg/ml, only 12.5% cells survived after 1 day and no cells survived after 4 days; the 20 µg/ml treatment caused toxicities in the middle range between 2 and 200 µg/ml (Figure 2A). A549 cells were resistant to SWCNTs: after 4 days, cells survival was more than 90, 80, and 60% for 2, 20, and 200 µg/ml treatments, respectively (Figure 2B). The toxicity of SWCNTs in WI38-VA13 cells was less severe than in BEAS-2B similarly to that in A549 cells (Figure 2C).

Inducing cellular oxidative stress is a major cause of cell toxicity by fibers and particles, even though many of them do not appear to generate ROS in vitro [22,23]. We examined whether ROS production is a potential mechanism of cell toxicity by SWCNTs. Fluorescent staining experiments using a ROS-sensitive probe DHE revealed that exposure to SWCNTs at 20 µg/ml for 16 h induced production of ROS in all of the three cell lines, among which more ROS was generated in BEAS-2B cells than in A549 and WI38-VA13 cells (Figure 2D).
SWCNTs activate the NF-κB pathway as a molecular mechanism for induction of the cytokines. Macrophages stably transfected with a synthetic 5x NF-κB binding site/luc reporter plasmid construct were treated with SWCNTs for 16 h. SWCNTs induced strongly expression of the reporter luciferase (Figure 5A). This result indicates that SWCNTs could activate NF-κB promoter for gene transcription. In the canonical pathway of NF-κB signaling, NF-κB is retained in the cytoplasm by its inhibitor protein IκB in unstimulated cells and is translocated into the nucleus upon stimulation. We found that SWCNTs induced a sharp reduction in the level of IκB protein at 30 min (Figure 5B). Immunofluorescent confocal microscope results indicated that SWCNT treatment for 16 h induced a strong green fluorescence staining of NF-κB in the nucleus colocalized with DAPI stained nucleus (Figure 5C). Using a consensus NF-κB DNA-binding sequence ELISA assay, SWCNTs at 20 µg/ml for 16 h were shown to induce NF-κB binding to DNA (Figure 5D). Taken together, these results revealed that SWCNTs activate the canonical NF-κB signaling pathway to mediate the induction of proinflammatory cytokines and chemokines in macrophages.

**Induction of TGFβ1 and fibroblast-to-myofibroblast transformation**

A common response to many inhaled particles like silica and asbestos is the fibrogenic reaction that leads to fibrosis or mesothelioma or mesothelioma. We examined whether SWCNTs have a fibrogenic effect on lung cells. It has been reported that profibrogenic growth factors, such as transforming growth factor (TGF) β1, plays an important role in fibroblast differentiation, type I collagen deposition, and lung fibrosis. We found that the mRNA and protein levels of

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TGFβ1 were significantly increased in BEAS-2B (Figures 6A and 6B) and macrophage (data not shown) cells treated with 20 µg/ml SWCNTs for 24 h; the increases were comparable to those treated with IL-1β, a known inducer of TGFβ1 [24].

The de novo synthesis of α-smooth muscle actin (αSMA) by lung myofibroblasts is a hallmark of fibroblast-to-myofibroblast transformation that can be induced by profibrogenic factors released from activated macrophages or epithelial cells through a paracrine mechanism. Incorporation of αSMA into contractile fibers increases the contractile force of myofibroblasts to enhance matrix remodeling and scar formation. We analyzed the expression of αSMA in lung myofibroblasts under the treatment of SWCNTs. We first treated macrophages with SWCNTs (20 µg/ml for 16 h) or LPS (1 µg/ml for 5 h, positive control). The culture medium free of cells and SWCNTs was collected and designed as SWCNT-conditioned medium. WI 38VA-13 cells were then cultured in the SWCNT-conditioned medium for 24 h. Expression of αSMA was measured by using fluorescent microscopy with a specific antibody against αSMA. As shown in Figures 6C and D, a significant increase in the fluorescence intensity of αSMA was observed in fibroblasts cultured in the SWCNT-conditioned medium, indicating that SWCNTs stimulated macrophages to secret soluble profibrogenic factors, such as TGFβ1, which in turn function as paracines to promote differentiation of the fibroblasts into myofibroblasts.

Discussion

Pulmonary exposure to fibrogenic agents causes damage to the lungs that eventually progresses into lung fibrosis or mesothelioma [25-28]. Mechanistic studies implicated that oxidative stress, inflammation, and production of growth factors are three major factors contributing in lung injury/repair and development of lung fibrosis [29,30]. SWCNTs are a newly discovered form of crystalline carbon, which forms cylinders of carbon having a diameter in the nanometer scale and a variable length [31,32]. Recent research indicated that carbon nanotubes such as SWCNTs could penetrate through lung tissues causing damage to the lungs and inducing lung fibrogenic lesions [8,33,34]. We use cell based molecular model to address whether SWCNTs has a fibrogenic effect and the underlining molecular events involved in the process.

ROS are produced as by-products of cellular respiration in the mitochondria or through damaging mitochondria by microorganism infection or chemical insults [12]. ROS are also formed by various particles via radicals on the surface or from the surface bound chemicals such as quinines that can undergo redox cycling to generate superoxide anions and hydrogen peroxide [29]. Last but not the least; activated macrophages and neutrophiles can produce ROS through respiratory burst [35]. Excessive ROS results in oxidative stress that promotes cell death and activates specific signaling pathways, both have been implicated in the pathogenesis of lung fibrosis [12]. We found here that SWCNTs induced significant ROS production in bronchial epithelial, alveolar epithelial, fibroblast, and macrophage cells. ROS production correlated with SWCNT-induced cell toxicity. Parallel to ROS production, SWCNTs treatment for 16 h could induce depolarization of the mitochondrial inner membrane. Our previous published result of MWNTs [36] shown the similar toxicity pattern as SWCNTs, while the toxicity of SWCNTs are appeared earlier and more severe than the
MWCNTs. Adding antioxidant, such as NAC and GSH counteract SWCNTs induced ROS in cultured cells [37,38]. Fenoglio et al. [22] used a cell-free system to show that CNTs do not directly generate free radicals. Together, these findings suggest that mitochondrial damage is likely a common mechanism by which SWCNTs induce oxidative stress and cause toxicity to lung cells.

Inhalation of CNTs induces pulmonary inflammation and fibrosis dose dependently in animals [6,39-41]. In one study, pulmonary fibrosis was observed at 7 days and granulomatous inflammation persistently through 56-day post-exposure period [42]. Pro-inflammatory cytokines were induced in mice treated with MWCNTs [43,44]. Some studies indicated that SWCNTs induced quick interstitial fibrosis with only a transient inflammation [41]. We showed here that SWCNTs induced a panel of proinflammatory cytokines/chemokines including TNFa, IL-1β, IL-6, IL-10, and MCP1, which are commonly elevated during lung inflammation and fibrosis. We demonstrated that induction of the inflammatory mediators by SWCNTs is mediated through the canonical pathway of NF-κB signaling, as shown by the evidence that SWCNTs (1) stimulated the NF-κB response element controlled transcription of reporter gene; (2) reduced the IkB protein level in the cytoplasm; (3) increased the nuclear accumulation of the NF-κBp65 protein; and (4) promoted binding of NF-κB to NF-κB

CNTs appear to potently induce lung interstitial fibrosis and mesothelioma in rodent models [45]. Myofibroblasts are the primary collagen-producing cells characterized by expression of αSMA and collagen I [46]. Pulmonary mesenchymal (fibroblasts and epithelial cells) can transform into myofibroblast under certain pathological conditions. Many factors, such as growth factors TGFβ1 and PDGF, and proinflammatory cytokines TNFa and IL-1β, promote the fibroblast-to-myofibroblast transformation and subsequent matrix synthesis by myofibroblasts [19,47]. Our study using in vitro cell based model indicates that SWCNTs up-regulated TGFβ1 expression in lung fibroblast, epithelial cells, and macrophages. Furthermore, we observed that SWCNT-induced growth factors stimulate the differentiation of fibroblast into myofibroblast cells, as evidenced by the de novo synthesis of αSMA in the cells. These results demonstrate that SWCNTs is a lung inflammation and fibrogenicity inducers, which could cause lung damage and fibrosis.

Because of the extreme paucity of human data and very limited animal models for CNT safety evaluation, and of the potential high demand of the new material for the new economy worldwide, the need for rapid and efficient evaluation of CNT toxicity is apparent. Our molecular toxicity pathway based in vitro study avoided using large amount of animals, and provides a simple and quick molecular mechanism based toxicity assessment method, which can be used for high throughput screening of inflammatory/fibrogenic toxicants, such as SWCNTs, in vitro, in the future.

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References


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