Single-walled Carbon Nanotubes Induce Fibrogenic Response by Promoting Epithelial/Fibroblast-to-Myofibroblast Transition: An in vitro Molecular Toxicity Pathway Based Risk Assessment

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ABSTRACT

Single-walled carbon nanotubes (SWCNT) are novel material with unique electronic and mechanical properties. Several reports have shown that pulmonary exposure to SWCNT caused pulmonary fibrosis in rodents, although the molecular mechanisms of SWCNT-induced pulmonary toxicity remain largely unaddressed. Here, we report that SWCNT exert multiple effects on human lung cells. First, SWCNT induced mitochondrial inner membrane potential depolarization and generate ROS in human bronchial epithelial BEAS-2B cells, alveolar type II A549 cells, and fibroblast WI38-VA13 cells at sub-toxic doses. Second, SWCNT induced fibrogenic cytokine TGF-β1 in BEAS-2B and macrophage RAW264.7 cells. Third, conditioned media from SWCNT-treated macrophages promoted epithelial/fibroblast-to-myofibroblast transiition, a key molecular event in the pathogenesis of lung fibrosis. This work provides a simple and rapid method for mechanism-based risk assessment of nanomaterial fibrogenicity.

Keywords: single-walled carbon nanotubes (SWCNT), oxidative stress, fibrosis, epithelial/fibroblast to myofibroblst trasition, toxicity assessment.

INTRODUCTION

Single-walled carbon nanotubes (SWCNT) are molecular-scale tubes of graphitic carbon with unique electrical, chemical, and physical properties (*1*). These cylindrical carbon molecules are valuable for electronics, optics, bioengineering and other fields of materials science and nanotechnology. Conversely, the extremely small size and low density of SWCNT makes them easily become airborne and inhaled into human lungs. The high length-to-width ratio and large surface area may lead to toxic effects similar to those of asbestos fibers such as mesothelioma and fibrosis (*2*, *3*). Evaluating the safety of SWCNT and other nanomaterials in humans would help avoid the potential harms of exposing SWCNT to workers and general population (*4*). Lung fibrosis is a progressive, irreversible and usually fatal end stage condition of various lung diseases. Many cellular events are associated with lung fibrosis, such as ROS generation (*5*), inflamation (*6*) and release of pro-inflammatory/fibrogenic cytokines and growth factors (*7*). Patient with idiopathic pulmonary fibrosis and some animal models of pulmonary fibrosis have shown increased ROS generation and elevated TGF-β1 production (*8*). Bleomycin, an anticancer drug and potent fibrogenic agent, induces differentiation of lung fibroblasts into myofibroblasts, characterized by α-smooth muscle actin (αSMA) expression and associated with increased ROS generation and TGFβ1 expression (*9*). The present study was undertaken to assess the cellular toxicities of SWCNT and determined the molecular mechanisms of action. We tested the ROS generation and mitochondrial membrane depolarization associated with SWCNT toxicity and analyzed the TGF-β1 production and epithelial/fibroblast to myofibroblast transition in response to SWCNT stimulation. In addition, we developed a simple and rapid mechanism-based toxicity assessment model that can be used for high throughput screening of nanomaterial fibrogenicity.

MATERIALS AND METHODS

Materials:

SWCNT (2 nm × 2 μm, 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 was used to produce the SWCNT. The nanomaterial was then treated with diluted hydrochloric acid to remove the catalyst and other metals.

Dispersion of SWCNT:

Two mg of SWCNT were dispersed in 1 ml of cell culture medium supplemented with 1% fetal bovine serum (FBS). The dispersion was vortexed and sonicated in a tissue culture hood using a sterile sonicating probe 3 times, 30 sec each. The stock solution was then diluted with culture medium to obtain the desired concentrations. Prior to use, the preparation was resonicated as described.

Cell culture:

BEAS-2B, a human normal bronchial epithelial cell line transformed with SV40, was cultured in BEBM medium along with additives from Lonza/Clonetics Corporation (Walkersville, MD). A549, a human lung carcinoma epithelial cell line, was cultured in DMEM medium with 10% FBS. WI38-VA13, a human normal lung fibroblast cell line, was cultured in αMEM medium with 10% FBS. RAW264.7, a mouse leukemic monocyte-macrophage cell line, was grown as a monolayer in DMEM medium with 10% FBS. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Mitochondrial membrane potential:

Tetramethylrodamine ester (TMRE, Sigma-Aldrich) and Mitotrack deep red 633 (Invitrogen) are fluorescent probes that specifically accumulate in the mitochondrial matrix in an inner-membrane-potential-dependent manner. Cells were cultured in 8-well chamber slides (for confocal microscopy) or 6-well plates (for flow cytometry) and were treated with SWCNT for 16 h. Forty-five minutes prior to the end of each treatment, 50 nM TMRE and 1 μM Mitotrack deep red 633 were added to the culture medium. Cells were washed three times with ice cold PBS. For confocal microscopy, cells were fixed with 4% paraformaldehyde in the chamber slides and were mounted with mounting solution containing DAPI to counterstain the nucleus. Images were taken with a Zeiss LSM510 confocal microscope. For flow cytometry, cells were collected and analyzed for fluorescence intensity using FACSCalibur at 585 nm (FL-2) and 670 nm (FL-3).

Immunofluorescence staining:

Cells grown in 8-well chamber slides were treated with SWCNT for 16 h, after which they were fixed with 4% paraformaldehyde and permeabilized with 0.5% triton X-100. The cells were stained with primary antibody in the culture medium containing 1% FBS overnight at 4°C. The cells were washed three times with PBS. An Alexia 488 or 594-conjugated second antibody was diluted (1:500) in the culture medium with 1% FBS and incubated with the cells for 1 h avoiding light. After washing with PBS three times, the cells were mounted with mounting solution containing DAPI. Images were taken with a Zeiss LSM510 confocal microscope. For detection of fibroblast-to-myofibroblast transformation, RAW264.7 macrophages were treated with SWCNT for 16 h. The culture medium was collected and centrifuged to remove remaining cells, cell debris, and SWCNT. This cell/SWCNT-free medium was designated as SWCNT-conditioned medium. Human lung epithelial BEAS-2B and fibroblast WI38-VA13 cells grown in 8-well chamber slides were cultured with the SWCNT-conditioned medium for 24 h. The cells were then fixed, permeabilized, and stained with anti-αSMA or anti-E-cadherin antibodies.

Real-time qPCR:

Cells were treated with SWCNT at 10 μg/ml for 24 h. IL-1β (Sino Biological Inc) at 5 ng/ml was used as a positive control. Total RNA was isolated using Qiagen RNA mini kit. Two μg of total RNA were reverse transcribed into cDNA using reverse transcriptase III (Invitrogen). The cDNA samples were analyzed by real-time PCR performed on ABI 7900HT (SABiosciences) using ABsolute Blue qPCR SYBR Green Rox Master Mix (Thermo Scientific) following standard procedures. In brief, for each reaction, cDNA temple, forword and reverse primers (10 μM each), PCR master mix, and water were added to make a final volume of 25 μl. Thermal cycling was performed as follows: 95°C for 15 min as initial denaturing condition, followed by 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 30s. Threshold cycles (Ct values) were determined using ABI 7900 Fast system SDS software. Relative DNA amounts were culculated from Ct values for each sample and normalized with GAPDH control. The sequences of primer sets used for real-time PCR are as follows: TGFβ1, F-5'GGACATCAACGGGTTCACTA, R-5'GCCATGAGAAGCAGGAAAG; E-cadherin (CHD1), F-5'TGAGTGTCCCCCGGTATCTTC, R-5' CAGTATCAGCCGCTTTCAGATTTT, and smooth muscle actin (ACTA2), F-5'TCAATGTCCCAGCCATGTAT, and R-5'CAGCACGATGCCAGTTGT.

ROS detection:

Cells were cultured in 8-well chamber slides and treated with SWCNT for 16 h. Thirty minutes prior to the end of treatment, dihydroethium (hydroethidine or DHE), (Invitrogen, CA) was added at 5 μM as a fluorescent probe for ROS detection. Cells were washed with ice cold PBS three times to remove free dye. They were then fixed with 4% paraformaldehyde and mounted with DAPI-containing mounting solution for nuclear counterstaining. Images were collected using a Zeiss LSM510 confocal microscope with rhodamine-DAPI setting.

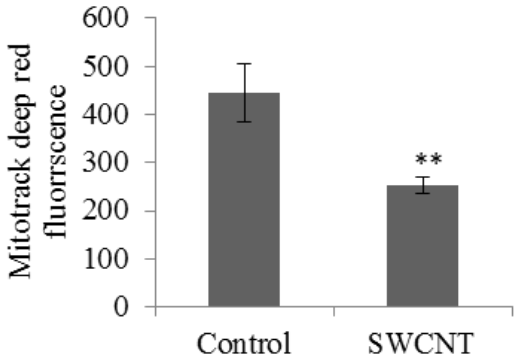
Statistics:

Image quantification was performed using ImageQuant program (Molecular Dynamics, San Jose, CA). Statistical analysis was performed by standard t-test using Microsoft Excel program.

RESULTS

SWCNT-induced mitochondial depolorization and ROS production:

A



B

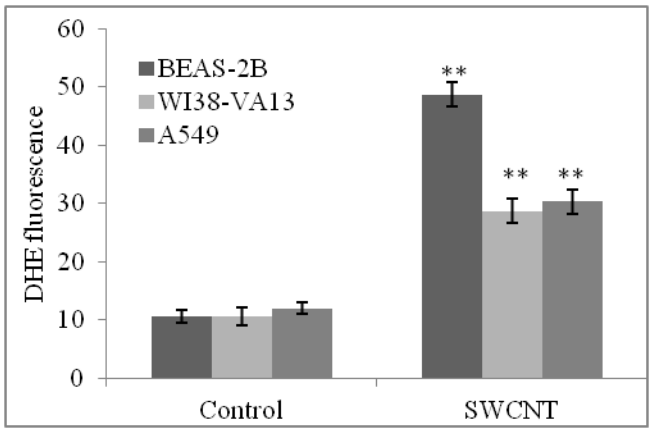


Figure 1. A. Mitochondrial depolarization.
BEAS-2B cells were treated with SWCNT for 16 h. Mitochondrial membrane potential was detected by flow cytometry using Mitotrack deep red 633. Data represent means ± SD of three measurements. **, p<0.01.
B. ROS production: Human A549, BEAS-2B and WI38-VA13 cells were treated with 20 μg/ml of SWCNT for 16 h. ROS production was measured by confocal fluorescence microscopy using the fluorescent probe DHE. Data represent means ± SD of fluoresence intensity measurements from 6 images.

SWCNT-induced fibrogenic TGFβ1 expression

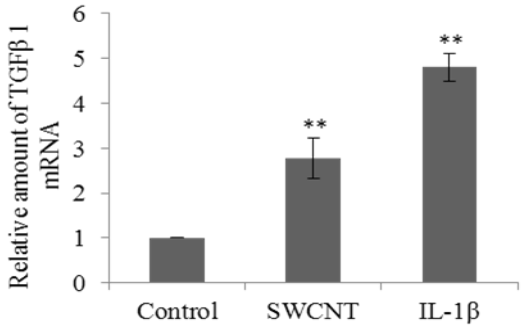
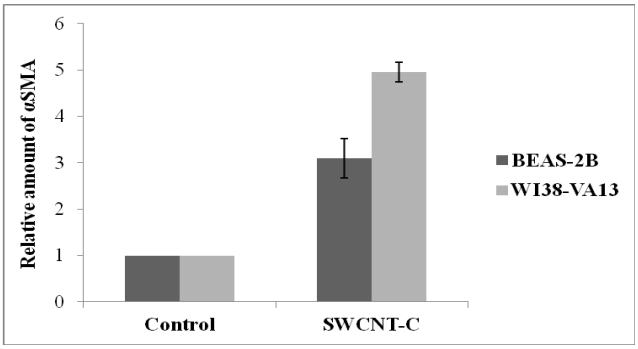


Figure 2. Induction of TGFβ1 expression.
BEAS-2B cells were treated with 20 μg/ml of SWCNT or 5 ng/ml of IL-1β for 24 h. TGFβ1 mRNA expression was detected by real-time PCR. Data represent means ± SD of three measurements. **, p<0.01.

SWCNT-induced epithelial/fibroblast-to-myofibroblast transition

A



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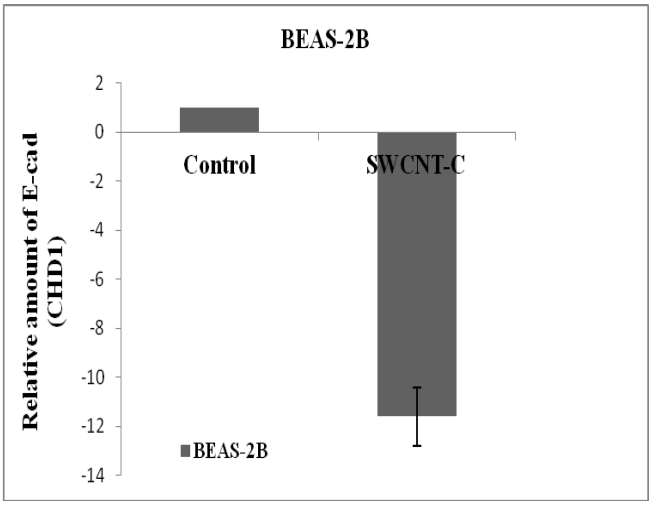

C

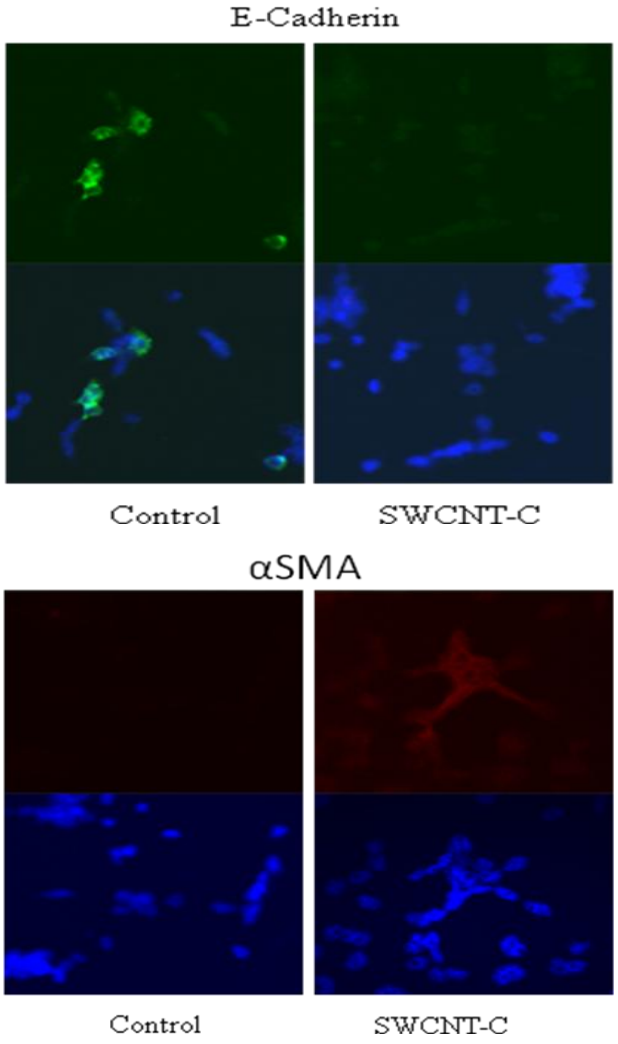

Figure 3. Induction of epithelial/fibroblast to myofibroblast transformation by SWCNT. RAW264.7 cells were treated with 20 μg/ml of SWCNT for 16 h. Cell/SWCNT-free culture medium was collected and used as SWCNT conditioned medium.

A. Induction of α smooth muscle actin (αSMA) mRNA expression. BEAS-2B and WI38-VA13 cells were cultured with SWCNT-conditioned medium and the expression of αSMA gene ACTA2 was measured by real-time PCR.
B. E-cadherin (CHD1) mRNA expression. BEAS-2B cells were cultured with SWCNT-conditioned medium and E-cadherin (CHD1) gene expression was measured by real-time PCR.
C. E-cadherin protein expression. BEAS-2B cells were cultured with SWCNT-conditioned medium for 24 h. E-cadherin protein expression was detected by confocal microscopy using anti-E-cadherin antibody followed by Alexia-488-conjugated secondary antibody. The nuclei were stained with DAPI.
D. The expression of αSMA in BEAS-2B cells.

CONCLUSIONS

1. SWCNT altered mitochondrial function by altering mitochondrial membrane potential and inducing ROS production in human lung epithelial cells and fibroblasts.
2. SWCNT upregulated profibroenic TGFβ1 protein expression in lung cells.
3. SWCNT induced transition of epithelial/fibroblast cells to myofibroblasts.

These results indicate that SWCNT elicits molecular signaling events involving oxidative damage, fibrogenic cytokine TGF-β1 expression, and epithelial/fibroblast-to-myofibroblast transition which potentially underlie SWCNT-induced fibrogenesis in human lungs.

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