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DIRECT FIBROGENIC EFFECTS OF DISPERSED SINGLE-WALLED CARBON NANOTUBES ON HUMAN LUNG FIBROBLASTS

Liying Wang[1,2], Robert R. Mercer[1], Yon Rojanasakul[2], Aijun Qiu[1], Yongju Lu[2], James F. Scabilloni[1], Nianqiang Wu[3], Vincent Castranova[1,2]

[1]National Institute for Occupational Safety and Health, HELD/PPRB, Morgantown, West Virginia
[2]Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia
[3]Department of Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia, USA

Nanomaterials, including single-walled carbon nanotubes (SWCNT), are being developed for a variety of commercial products. However, adverse health effects attributed to these new materials are not well understood. Recent reports showed that exposure of mice to dispersed SWCNT (DSWCNT) produced a rapid and progressive interstitial lung fibrosis without persistent inflammation. To understand the mechanism underlying this unusual fibrogenicity of DSWCNT, the present investigation focused on the direct bioactivity of DSWCNT using a cell culture of lung fibroblasts that represent a major cell type resident in the lung interstitium and responsible for the production of collagen matrix. At concentrations relevant to those used *in vivo*, *in vitro* exposure of lung fibroblasts to DSWCNT stimulated cell proliferation and induced collagen production without producing cell damage. One of the major matrix metalloproteinases (MMP), MMP-9, which is known to be involved in lung fibrosis, was also elevated by DSWCNT treatment both *in vitro* and *in vivo*. Taken together, these results suggest that direct stimulation of fibroblasts by DSWCNT translocated into the interstitium may play a significant role in DSWCNT-induced lung fibrosis. Our data also suggest that the dispersion status and/or size of the SWCNT structures is a critical factor in determining nanoparticle fibrogenicity and that MMP-9 may be involved in the fibrogenic process. The results obtained may aid in the development of *in vitro* models for rapid screening of the potential fibrogenicity of carbon nanotubes, which are lacking and urgently needed.

Nanomaterials, including single-walled carbon nanotubes (SWCNT), have the potential for wide use in various commercial and biomedical applications. Nanoparticles, used to produce nanomaterials, are currently defined as single particles with at least one dimension being less than 100 nm. Agglomerates of nanoparticles may be larger than 100 nm in diameter but may be de-agglomerated with weak mechanical forces or after dispersal in a solvent (Yang et al., 2008). As a class of small-scale (<100 nm) substances formed by molecular-level engineering, nanoparticles exhibit a great potential to become aerosolized, leading to the potential for worker exposure. For example, exposure to nanomaterials may occur when transferring, weighing, blending, or spraying SWCNT, which releases fine particles into the air (Maynard et al., 2004). Early evidence from mammalian toxicity studies indicates that inhalation exposure to this new class of engineered nanoparticles may

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Address correspondence to Liying Wang, MD, PhD, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505, USA. E-mail: lmw6@cdc.gov

produce adverse pulmonary effects (Maynard & Kuempel, 2005).

The lung is a major target organ for airborne nanoparticle exposure. Classically, the inhaled particles, after entering the lung, interact with specific lung cells, such as alveolar macrophages and epithelial cells, to produce diverse biological and toxicological effects, including inflammation, oxidative stress, tissue damage, and slow development of lung fibrosis. However, pulmonary aspirated or instilled carbon nanotubes are processed differently by lung cells and exhibit different toxicities depending on their size and preparation method (Mercer et al., 2008; Shvedova et al., 2005; Mangum et al., 2006; Nimmagadda et al., 2006; Donaldson et al., 2006; Lam et al., 2004). For example, our previous studies showed that pharyngeal aspirated large agglomerates of SWCNT (~1.5 μm diameter) deposit in the terminal bronchioles and proximal alveoli and induce development of granulomatous lesions encased by epithelioid macrophages. In contrast, dispersed SWCNT (DSWCNT) with a mean aggregate diameter of 0.69 μm rapidly enter the alveolar interstitial space to induce a progressive interstitial fibrotic response with minimal lung inflammation (Mercer et al., 2008). Shvedova et al. (2005) compared the fibrotic effect of silica at the same concentrations (10–40 μg/mouse) and found silica without an effect, which suggested a unique fibrotic effect of SWCNT. Thus, the ability of small DSWCNT particles to enter lung interstitium and interact with local fibroblasts might be a key determining factor in the induction of interstitial fibrosis by DSWCNT. Furthermore, fibroblasts represent one of the major cell types for production of metalloproteinases, such as matrix metalloproteinase (MMP)-9, which is a known fibrogenic mediator in the lung (Wang et al., 2002; Scabilloni et al., 2005). Therefore, the overall objective of this study was to determine the direct interaction of DSWCNT with target lung fibroblasts using a cell culture system and evaluate the biological consequences of such interactions.

Typically, the biological activity of particles increases as their particle size decreases. Smaller particles occupy less volume, resulting in a larger number of particles with a greater surface area per unit mass and increased potential for biological interactions (Cassee et al., 2002; Oberdorster 1996; Sager et al., 2008). Nanotubes suspended in physiological saline tend to form micrometer-sized agglomerates. Generation of an aerosol of SWCNT from dry material produces more dispersed structures than suspension in saline. Shvedova et al. (2008) reported that although pulmonary responses were qualitatively similar, exposure to smaller SWCNT structures by inhalation of a dry aerosol was more potent than aspiration of an equivalent mass of a SWCNT suspension containing micrometer-sized agglomerates in producing acute pulmonary inflammation and persistent interstitial fibrosis. In order to reduce agglomeration, efforts were made in the present study to ensure that SWCNT samples were well dispersed to more closely simulate structure sizes observed with airborne exposure.

As new nanotechnology-based materials are rapidly being developed and used, it will be essential to evaluate and predict their toxicities, particularly in light of their potential to induce lung fibrosis, which is usually progressive and without effective treatment (Selman et al., 2004; Studer & Kaminski., 2007). Since lung fibrosis induced by DSWCNT occurred rapidly without a persistent inflammatory or oxidative stress response (Mercer et al., 2008; Shvedova et al., 2005; Mangum et al., 2006), traditional *in vitro* screening assays based on cytotoxicity and oxidative stress might not predict the fibrogenic effects of SWCNT. Thus, an alternative *in vitro* assay that would more reliably predict the fibrogenic potential of nanomaterials is needed. Since fibroblast proliferation and increased production of collagen matrix are hallmarks of lung fibrosis, an *in vitro* model system was developed employing lung fibroblasts and cell proliferation and collagen production determined in response to SWCNT exposure.

MATERIALS AND METHODS

Preparation of Dispersed Single-Walled Carbon Nanotubes (DSWCNT)

SWCNT were purchased from Carbon Nanotechnology (CNI, Houston, TX). The nanotubes were synthesized by the high-pressure carbon monoxide disproportionate process (HiPCO) and treated to remove a significant portion of the contaminating metal catalysts. The purified SWCNT contained less than 2 wt% of contaminants (mainly iron). Dispersed single-walled carbon nanotubes (DSWCNT) were prepared as described previously (Mercer et al., 2008). Briefly, SWCNT were initially suspended in distilled water and sonicated for 10 min. The solution was then expanded with acetone and placed in an ultrasonic bath for 24 h. After filtering using two stages of a nylon mesh with a 4-μm pore size and a 0.1-μmPTFE filter, DSWCNT were collected and washed several times with distilled water to remove residual acetone. The filter was dried overnight under vacuum and weighed to determine the quantity of DSWCNT. Prior to use, the filter containing the DSWCNT was placed in phosphate-buffered saline (PBS), and the DSWCNT were released by brief sonication. For suspension of poorly dispersed SWCNT, non-acetone-treated SWCNT were placed in PBS with brief sonication.

Dynamic Light Scattering (DLS)

The dynamic light scattering (DLS) technique was used to evaluate the particle size distributions of the poorly dispersed SWCNT and DSWCNT samples. DLS measures Brownian motion and relates this movement to the size of the particles. When a laser light with a known frequency is directed to suspended particles with Brownian motion, the light is scattered and subject to the frequency shift. The shift in the frequency of light is quantitatively related to the size of the particles that undergo Brownian motion. Therefore, the particle size can be derived from the frequency shift of the scattered light. In the present study, DLS was performed on the poorly dispersed SWCNT and DSWCNT samples suspended in PBS (10 μg/ml) using a Zetasizer Nanoseries Instrument (Malvern Instrument, UK). Two-milliliter suspensions of SWCNT and DSWCNT were sonicated for 1 min and then placed in a disposable plastic cuvette. Ten measurements were carried out and the final results were averaged. The refractive index of the suspension medium was accounted for in these measurements.

Field Emission Scanning Electron Microscopy (FESEM)

SWCNT samples were dispersed in distilled water, placed on carbon planchets, dried, and carbon coated. The samples were then examined with a Hitachi model S-4800 field emission scanning electron microscope (FESEM) at 5–20 kV.

Animal Model

Pathogen-free C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for animal studies. Animals were housed in an AAALAC-accredited, specific-pathogen-free, environmentally controlled facility and allowed to acclimate at least 1 wk prior to use. Mice were kept in laminar-flow cages, which were provided HEPA-filtered air, with Alpha-Dri virgin cellulose chips and hardwood Beta-Chips for bedding. Food and tap water were given ad libitum. The animals were exposed by pharyngeal aspiration (Rao et al., 2003). This method of pulmonary administration is well established and widely used in small laboratory animals, since it is noninvasive and provides deep lung deposition as well as even distribution of the administered particles. Briefly, animals were anesthetized by an intraperitoneal (ip) injection of ketamine and xylazine (45 and 8 mg/kg) and placed on a board in the supine position. The animal's tongue was extended with padded forceps. A suspension of dispersed single-walled nanotubes (DSWCNT), dispersed ultrafine carbon black (DUFCB) (both at 10 μg/100 μl per mouse), or sterile phosphate-buffered saline (PBS) was placed on the back of the tongue. A slight pull of the tongue results in a reflex gasp and aspiration of the droplet. The tongue was held and the animal monitored for a few breaths after

aspiration. All mice recovered by this procedure within minutes. Mice were sacrificed at indicated time points after the treatment, and bronchoalveolar lavage was conducted to evaluate markers of lung damage or lung tissue was lysed for matrix metalloproteinase measurement by Western blot, respectively, as described later.

Bronchoalveolar Lavage (BAL)

At selected time intervals, a group of treated mice were weighed (mean body weight 25.0 g) and euthanized with an ip injection of sodium pentobarbital (>100 mg/kg). An intratracheal cannula was inserted, and the lungs were lavaged with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The first lavage at a volume of 0.8 ml was kept separate and 1 ml of Ca^{2+}- and Mg^{2+}-free PBS was used for the subsequent 4 lavages. Pooled BAL from each mouse was centrifuged at 600 × g for 10 min at 4°C and cell pellets were resuspended in 1 ml PBS for determination of cell counts and differentials using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL). The acellular first lavage fluid was used to measure lactate dehydrogenase (LDH) activity, a marker of cell damage.

Cytotoxicity

In vivo cytotoxicity was determined by LDH assay using acellular BAL fluids. Mice were treated with DSWCNT (10 μg/mouse) or PBS by pulmonary aspiration. At 1, 7, or 30 d post treatment, animals were sacrificed and bronchoalveolar lavage was performed. The first BAL fluid from each treated mouse was used to determine LDH activity by monitoring the LDH-catalyzed oxidation of pyruvate coupled with the reduction of NAD at 340 nm using a commercial kit and a Cobas Mira Plus transfer analyzer (Roche Diagnostics System, Montclair, NJ). For *in vitro* experiments, cell supernatants from control and treated experiments were collected and analyzed for LDH activity as already described.

Cell Culture

Lung fibroblasts, CRL1490 cells, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). This fibroblast cell line was derived from normal human lung fibroblasts, making it a suitable model for *in vitro* lung fibrogenesis studies. The cells were cultured in ATCC complete growth medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fibroblasts were maintained in a 5% CO_2 humidified atmosphere at 37°C.

In Vitro Concentrations of DSWCNT

Lung fibroblasts were exposed to 0–0.24 μg/cm^2 of DSWCNT. This concentration range of nanoparticles is much lower than frequently reported in the nanotoxicology literature for *in vitro* experiments. It was chosen to be relevant to *in vivo* exposure doses for DSWCNT of 10–40 μg/mouse reported by Mercer et al. (2008). Briefly, lung burdens per alveolar epithelial surface area, i.e., 500 cm^2/mouse lung (Stone et al., 1992), correspond to *in vitro* concentrations of 0.02–0.08 μg/cm^2.

Cell Proliferation

Proliferation of cultured fibroblasts was determined by using metallothionein (MTT) assay kit I from Roche Molecular Systems (Alameda, CA) and by quantification of cell counts using a hemocytometer. The MTT assay is a colorimetric assay that measures short-term cell-dependent metabolic activity. MTT is reduced by mitochondrial enzymes in viable cells to form insoluble purple formazan products. This conversion is directly proportional to the number of viable cells and is monitored by spectrophotometry. Cells were seeded onto 96-well plates at a density of 2×10^3 cells per well. After remaining overnight in culture, the cells were treated with 0.02, 0.06, or 0.2 μg/cm^2 of DSWCNT for 2 d. MTT reagent was then added to each well, and cells were incubated for an additional 4 h. After adding a solubilization solution, cells were kept in an incubator at 37°C overnight. Absorbance was determined using a microplate reader (Spectra Max, Sunnyvale, CA) at 595 nm. For hemocytometry, cells (2×10^4/well) in a 24-well plate were incubated in culture medium in the presence or absence of DSWCNT for 2 d, after which they were trypsinized and suspended in 100 μl culture medium. Ten microliters of the cell

suspension was used to determine cell number using a hemocytometer.

Western Blot Analysis of Collagen and Matrix Metalloproteinase Content

Treated and control lung fibroblasts or mouse lung tissues were prepared for lysis in cold lysis buffer containing 50 m*M* Tris/HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM EGTA, and 1% protease inhibitor cocktail (Roche Molecular Biochemicals) for 30 min. After insoluble debris was precipitated by centrifugation at 14,000 × g for 15 min at 4°C, the supernatants were collected and analyzed for protein content by the bicinchoninic acid assay. Equal amounts of protein per sample were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The transferred membrane was blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, and 0.05% Tween-20) and incubated with appropriate primary antibodies at 4°C overnight. After 3 washes with TBST, the membrane was incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature and washed with 0.05% Tween-20 in PBS. Immunoreactive proteins were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL).

Statistical Analysis

Analysis of variance and Duncan's multiple comparison tests were used to evaluate the significance between measurements. Most tests were two-sided. Data were presented as means ± SD. For all analyses, the criterion for significance was set at $p < .05$.

RESULTS

Dispersion of Single-Walled Carbon Nanotubes

Purified SWCNT were used in this study. Poorly dispersed SWCNT and DSWCNT were prepared (see Methods section) and suspended in PBS. A field emission scanning electron micrograph (FESEM) of poorly dispersed SWCNT showed the typical tangled configuration of nanotubes with significant variations in diameter (Figure 1A). Size and size distribution of non-dispersed and dispersed SWCNT in suspension (0.1 mg/ml) were analyzed by light microscopy (Figure 1, B and C). The non-dispersed SWCNT in PBS exhibited large micrometer-sized agglomerates as compared to the acetone-sonication dispersed form. Dynamic light scattering measurements of non-dispersed SWCNT (10 μg/ml in PBS) showed agglomerated structures (Figure 1D). In contrast, DSWCNT showed some degree of dispersion into nanometer-sized structures (Figure 1E). Because of the fibrous shape of SWCNT, this method is not ideal for particle size analysis but it supports the light microscopy result (Figure 1, B and C). Previous SEM data showed that the distribution of average area equivalent diameter of the DSWCNT structures in suspension was approximately 0.69 μm (Mercer et al., 2008).

In Vivo Pulmonary Response

Acellular bronchoalveolar lavage (BAL) fluid from DSWCNT-treated mice showed no significant change in LDH activity as compared to PBS-treated control mice at all time points evaluated (Figure 2). BAL cell counts showed a transient rise in inflammatory cells, such as neutrophils, at 1 d postexposure, which returned to the baseline level by 7 d postexposure to DSWCNT (data not shown), consistent with our previous finding (Mercer et al., 2008). These results indicate the absence of significant cytotoxicity or persistent inflammation following pulmonary exposure of mice to DSWCNT at this relatively low administered dose. Although pulmonary exposure to DSWCNT did not produce persistent inflammation or cell damage, our previous findings indicated that significant interstitial fibrosis occurred that is rapid in onset (7 d postexposure) and progresses through 30 d postexposure (Mercer et al., 2008).

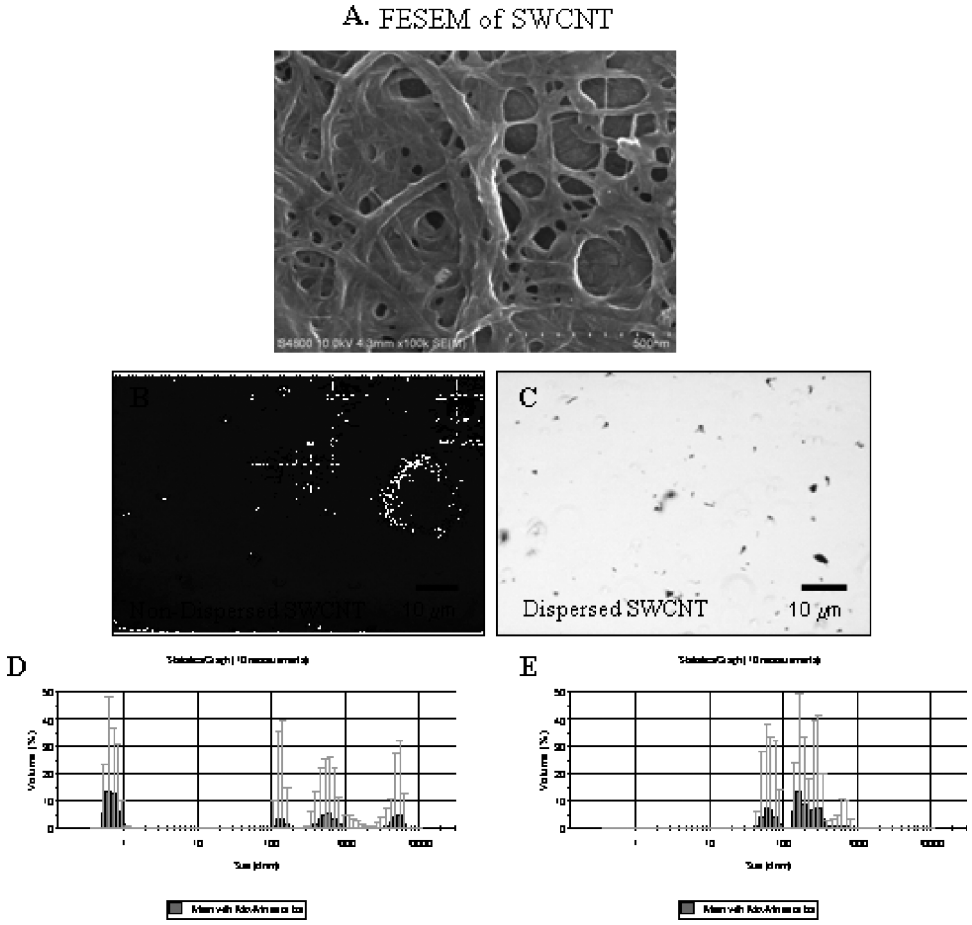

FIGURE 1. Dispersion of single-walled carbon nanotubes (SWCNT). Representative field emission scanning electron micrograph of a poorly dispersed SWCNT sample used in this study (A). The micrograph demonstrated the typical tangled configuration of the nanotubes with significant variations in diameter. The non-dispersed SWCNT and acetone/sonication-dispersed SWCNT (DSWCNT) were suspended in PBS (0.1 mg/ml). Each particle sample was viewed using light microscopy at a magnification of 10×. Non-dispersed SWCNT exhibited large compact agglomerates (B), while DSWCNT exhibited smaller sizes (C). The size distributions of non-dispersed SWCNT and DSWCNT structures were also estimated by the dynamic light scattering method (D and E).

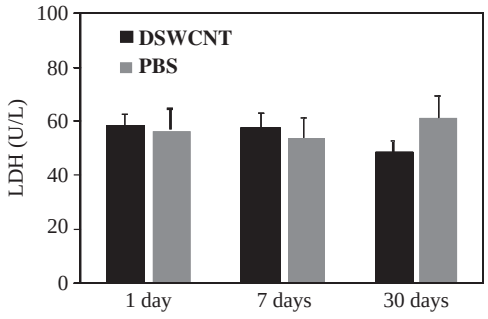

FIGURE 2. Cytotoxicity of aspirated DSWCNT. Mice were exposed to DSWCNT (10 μg/mouse) by pharyngeal aspiration. At the indicated times after the treatment, bronchoalveolar lavage (BAL) was performed. Acellular BAL fluid from this action was analyzed for cell damage using the LDH assay. There was no significant difference in LDH activity between DSWCNT treatment and PBS control groups.

Effect of DSWCNT on Human Lung Fibroblast Cell Growth *In Vitro*

Although previous *in vivo* studies demonstrated that pulmonary exposure of mice to DSWCNT resulted in a rapid and progressive interstitial fibrosis, the mechanism for this fibrotic response does not fit the persistent inflammation paradigm. Incorporation of gold-labeled DSWCNT into the interstitial lung tissue was detected using a silver enhancement of lung tissue sections (Mercer et al., 2008). Since fibroblasts are resident in the interstitium and represent a major cell type responsible for the production of collagen matrix, direct interaction of translocated DSWCNT with resident

fibroblasts may be a key contributor to DSWCNT-induced lung fibrosis. To determine whether there is a direct effect of DSWCNT on lung fibroblasts, *in vitro* studies were performed using cultured human lung fibroblast CRL1490 cells exposed to various concentrations of DSWCNT, which were relevant to doses used in the *in vivo* study. The cell proliferation effect of DSWCNT on the fibroblasts was determined by MTT assay and by direct cell counting. At low exposure concentrations (0.02–0.2 μg/cm^2) DSWCNT were not cytotoxic to cultured fibroblasts. Rather, DSWCNT significantly enhanced fibroblast proliferation (Figure 3A). Microscopic study of the treated cells shows a more elongated and fibrous morphology, suggesting of cell activation, upon treatment with DSWCNT as compared to non-treated cells (Figure 3B). Cell count measurements (Figure 3C) and LDH measurements (Figure 3D) confirmed the MTT results, indicating that at low concentrations, DSWCNT exhibit no cytotoxicity and induce proliferation of lung fibroblasts *in vitro*. Taken together, these studies indicated that DSWCNT may exert a direct concentration-dependent stimulatory

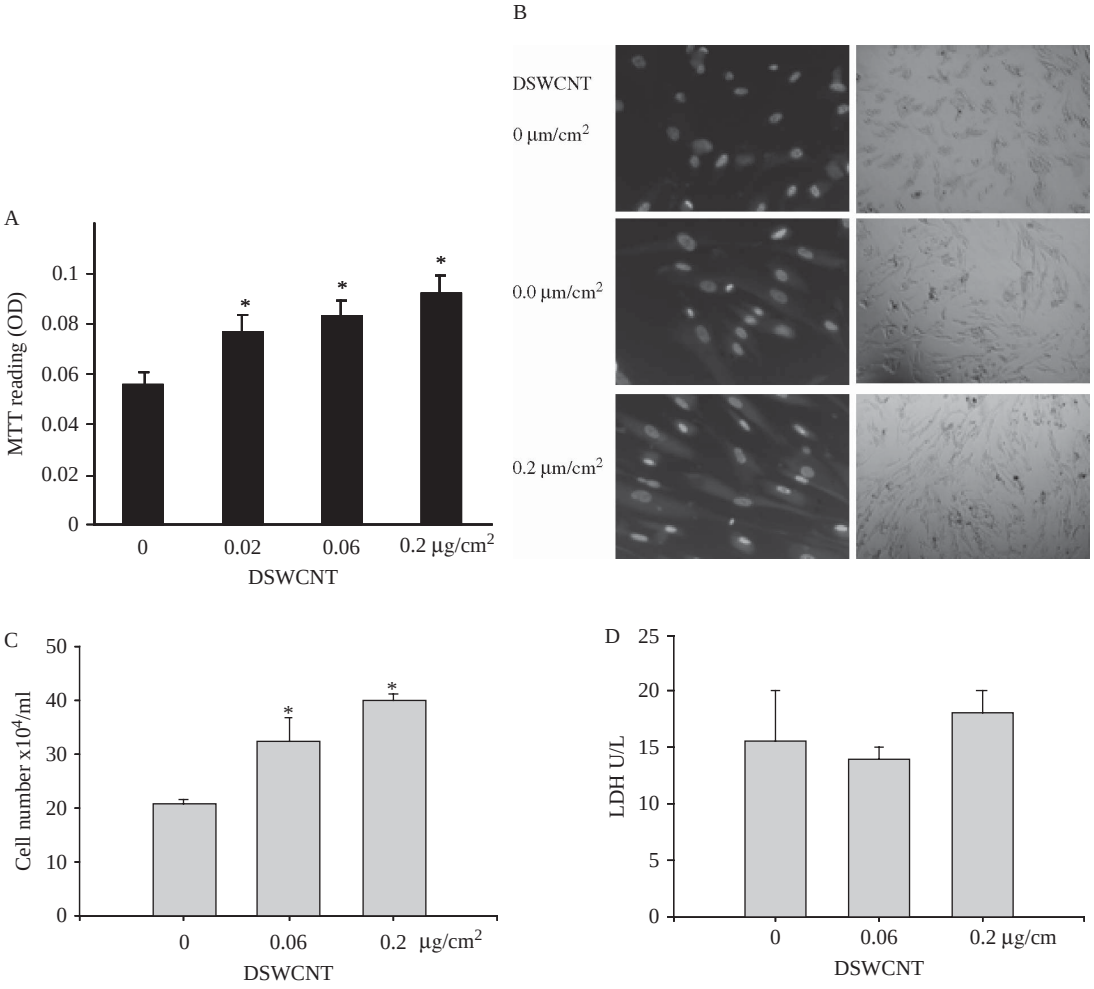

FIGURE 3. Proliferative and cytotoxic effects of DSWCNT on lung fibroblasts. Lung fibroblast CRL1490 cells were treated with DSWCNT (0.02, 0.06, or 0.2 μg/cm^2). At 2 d post treatment, fibroblast cell growth was analyzed by the MTT assay (A). Plots are mean ± SD, $n = 3$. Asterisk indicates a significant increase from control level ($p < .05$). DSWCNT induced morphologic changes of lung fibroblast (B) as observed by light microscopy (right panel) and fluorescence microscopy (left panel) at 6 h post treatment. The proliferative effect of DSWCNT was also demonstrated by cell counting (C). Measurement of LDH activity of fibroblasts treated with DSWCNT found no significant difference from the control, suggesting a noncytotoxic effect of DSWCNT under these experimental conditions (D).

effect on lung fibroblasts, which might be an important factor contributing to the development of interstitial lung fibrosis induced by DSWCNT *in vivo*.

Effect of DSWCNT on Collagen Production in Lung Fibroblasts *In Vitro*

To test whether DSWCNT may exert a direct stimulatory effect on collagen production of lung fibroblasts, which is a hallmark of pulmonary fibrosis, human lung fibroblast CRL1490 cells were directly treated with various concentrations of DSWCNT. At 2 d post DSWCNT treatment, collagen content of treated cells was analyzed by Western blotting. The extensive collagen family is composed of several chain types, with type I and type III representing the two most abundant proteins of the extracellular matrix (Brinckerhoff & Matrisian, 2002; Pardo & Selman, 2005). In this study, anti-human antibodies specific to collagen type I and type III were employed (Santa Cruz Biotechnology, Inc.). Two clear bands of collagen protein were observed when each antibody was used, which indicated the multiple isoforms of collagen or its processed product. For example, the molecular mass of collagen type I provided by the company is in the range of 140–210 kD. As a nanoparticle control, dispersed ultrafine carbon black (DUFCB), which does not show fibrogenic bioactivity *in vivo* (Shvedova et al., 2005), was used. Figure 4 shows that DSWCNT were able to induce the production of collagen type I and type III, whereas DUFCB was ineffective at the same treatment concentrations. The induction of collagen by DSWCNT was independent of its effect on fibroblast cell growth since analysis of the collagen content was performed using equal total cellular protein per sample. Data suggest that direct stimulation of collagen production by lung fibroblasts may contribute to the fibrogenic effect of DSWCNT *in vivo*.

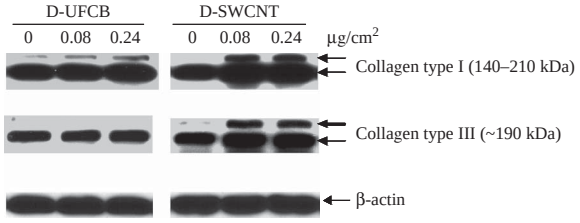

FIGURE 4. Western blot of collagen induced by DSWCNT *in vitro*. CRL1490 cells were treated with 0.08 or 0.24 μg/cm^2 of DSWCNT or DUFCB as a nanoparticle control. At 2 d post treatment, cells extracts were prepared and separated on 10% polyacrylamide–SDS gels, transferred, and probed with anti-human collagen type I (molecular mass 140-210 kD) and type III (molecular mass 190 kD) antibodies. β-Actin was used as a loading control.

Effect of DSWCNT on Matrix Metalloproteinase-9 (MMP-9) Production *In Vitro* and *In Vivo*

By Western blot analysis, the pro-form (latent form) of human MMP-9 (92 kD) from DSWCNT- or DUFCB-treated fibroblast cell lysates was identified (Figure 5). Increased MMP-9 production was observed from DSWCNT-treated fibroblasts as compared to that from the DUFCB-treated cells. This result was also validated by *in vivo* data (Figure 6). Figure 6 shows elevated mouse MMP-9 protein (105 kD) by immunoblotting of DSWCNT-treated mouse lung as compared to that from the PBS- and DUFCB-treated groups.

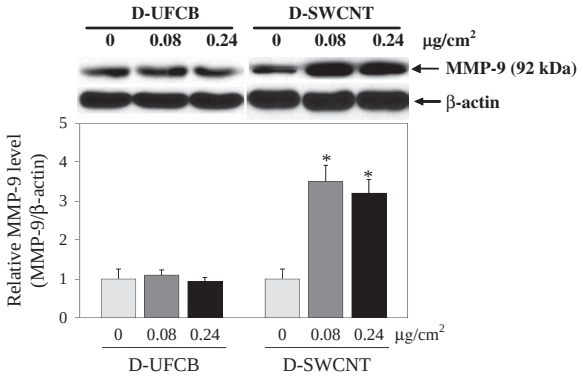

FIGURE 5. DSWCNT-induced matrix metalloproteinase-9 (MMP-9) expression *in vitro*. CRL1490 cells were treated with 0.08 or 0.24 μg/cm^2 of DSWCNT or DUFCB. At 2 d post treatment, cells extracts were prepared and analyzed for MMP-9 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry using UN-SCAN-IT digitizing software (Silk Scientific, Orem, UT). Mean densitometry data from independent experiments (one of which is shown here) were normalized to the results obtained from DUFCB-treated control cells. Values are means ± SD (n = 3). Asterisk indicates significant at $p < .05$ versus control.

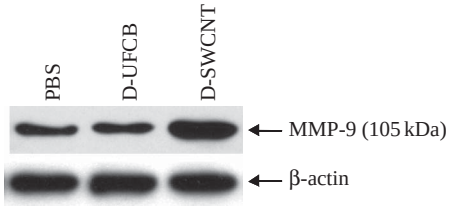

FIGURE 6. DSWCNT-induced matrix metalloproteinase-9 (MMP-9) expression *in vivo*. Three groups of mice were pharyngeal aspirated with 10 μg/mouse of DSWCNT, DUFCB, or PBS. At 2 wk post treatment, mice were sacrificed and the lungs were isolated. Lung tissue lysates were prepared and separated on polyacrylamide–SDS gels, transferred, and probed with MMP-9 antibody. Blots were reprobed with β-actin antibody to confirm equal loading of samples.

DISCUSSION

Knowledge of the biological effects of nanoparticles on biological systems is limited due to the relative novelty of the materials. A current paradigm in environmental epidemiology is that toxicity of particulates, found in air pollution and some workplaces, may be driven by the ultrafine particle fraction, indicating that exposure to materials in the nanosize range may result in significant public health problems, such as pulmonary and skin diseases (Donaldson et al., 2005; Powell and Kanarek, 2006a, 2006b; Heyder et al., 1986; Shvedova et al., 2003a, 2003b).

The results of this study demonstrate that DSWCNT directly stimulated fibroblast cell growth and collagen production after 2 d of exposure to non-cytotoxic concentrations of DSWCNT in culture. In addition, the fibrotic mediator MMP9 is induced at relevant DSWCNT concentrations both *in vitro* and *in vivo* without inducing significant cellular damage. These observations are consistent with previous findings (Mercer et al., 2008; Shvedova et al., 2005; Mangum et al., 2006) showing that SWCNT induced interstitial lung fibrosis as early as 1 wk postexposure in mice without persistent inflammation. These findings led to the hypothesis that carbon nanotubes, due to their unique properties such as small size and fibrous shape, penetrate interstitial tissues to form a matrix that induces fibrogenesis by directly stimulating fibroblasts. Such a mechanism for interstitial fibrosis is distinct from the pulmonary reaction to "traditional" fine particles in which pulmonary fibrosis is driven by persistent inflammation and oxidant damage resulting in a slow development of lung scarring (fibrosis).

In general, aerosol particles measuring about 1 μm in aerodynamic diameter would be exhaled up to 80% after inspiration (Heyder et al., 1986; Heyder & Rudolf, 1984). However, the deposition of particles in the lung is bimodal, and ultrafine particles (less than 100 nm) appear to deposit at high efficiency in the alveolar region. Previous studies showed that dispersed and nondispersed SWCNT are processed differently by the lung (Mercer et al., 2008). Large agglomerates of poorly dispersed SWCNT deposited mainly near the proximal alveolar region and formed granulomas, while smaller SWCNT structure deposited the deep lung and rapidly penetrated the alveolar epithelial wall into the interstitium. Evidence indicates that the dispersion of nanomaterials plays a key role in determining their tissue distribution and biological activity.

The direct action of DSWCNT on lung fibroblast was tested *in vitro* using physiologically relevant doses. The doses were calculated based on previously published *in vivo* studies at levels that were shown to penetrate the alveolar epithelium and enter the interstitium (Mercer et al., 2008; Shvedova et al., 2005, 2008). Both *in vivo* and *in vitro* LDH data (Figures 2 and 3D) showed that DSWCNT were relatively nontoxic at the exposure concentrations and times used in this study. This result is consistent with recent CNT toxicity studies using non-MTT cytotoxicity assays (Davoren et al., 2007; Worle-Knirsch et al., 2006; Pulskamp et al., 2007). These studies indicated that CNT bind MTT and reduce its absorbance, producing a false positive (cytotoxicity) reading. In the present study, low concentrations of DSWCNT were used and an increase rather than artifactual decrease in MTT reading in DSWCNT-treated cells was observed, which indicated a direct proliferative effect of DSWCNT on fibroblasts. Direct cell

count measurements (Figure 3C) further confirmed this observation. Data suggest that DSWCNT directly augmented fibroblast cell growth in a concentration-dependent manner. The use of an *in vitro* cell system allows detection of the growth rate of a specific cell type, i.e., fibroblasts, which could not be detected accurately *in vivo* because of tissue complexity and presence of numerous cell types in the lung.

Extracellular matrix is a complex network of macromolecules consisting of collagens and other fibrous materials. Extracellular matrix remodeling that involves deposition and accumulation of collagens in the interstitium is a key characteristic of pulmonary interstitial fibrosis (Mott & Werb, 2004, Corbel et al., 2001). Matrix metalloproteinases (MMP), enzymes involved in degradation of extracellular matrix, play a significant role in the pulmonary structural remodeling. In the early stage of lung fibrosis, gelatinolytic activity of MMP-9 probably contributes to the disruption of alveolar epithelial basement membrane and enhances fibroblast invasion to alveolar spaces (Woessner, 1991). In silica-induced lung fibrosis, MMP-9 was elevated during the fibrotic developmental process (Scalbiloni et al., 2005). To test whether DSWCNT might affect these two key fibrotic factors, production of collagen and production of MMP-9 in lung fibroblasts exposed to DSWCNT were measured in this study. Both collagen and MMP-9 were upregulated by the DSWCNT treatment. These results indicated a direct effect of DSWCNT on the fibrogenic activity of lung fibroblasts, which may be responsible for the *in vivo* fibrogenic effect of DSWCNT. Indeed, *in vivo* results of DSWCNT-induced lung fibrosis (Mercer et al., 2008) and MMP-9 induction (Figure 6) validate the *in vitro* observations. DUFCB, which was shown to produce minimal lung response and exert no fibrogenic effect *in vivo* (Lam et al., 2006; Shvedova et al., 2005), did not induce collagen or MMP-9 in cultured lung fibroblasts or MMP-9 *in vivo* as compared to DSWCNT (Figures 5 and 6). This observation may reflect a specific property-dependent effect of DSWCNT, such as structure of fibrous carbon nanotubes (SWCNT) versus spherical carbon black. Therefore, data support the use of the *in vitro* lung fibroblast model system as a potential screening method for prediction of the fibrogenicity of carbon nanomaterials.

Fibrogenicity testing of nanomaterials is currently performed using animals. However, due to the presence of an uncountable number and variety of nanomaterials currently being used or under development, it is impractical to test them all using animals, since this method of testing is laborious, time-consuming, costly, and technically difficult. *In vitro* assay of oxidant generation and oxidant stress was proposed as a useful screening test for nanoparticle toxicity (Nel et al., 2006). However, SWCNT, purified to remove contaminating catalytic metals, do not generate oxidants or generate significant oxidant stress *in vitro* (Shvedova et al., 2003a, 2005). In addition, *in vivo* data indicate that SWCNT-induced fibrosis is not dependent on persistent inflammation and damage (Mercer et al., 2008; Shvedova et al., 2005). Therefore, an alternative *in vitro* testing method that is predictive of the *in vivo* fibrogenicity of SWCNT would be of great value. The present study describes one such method that could potentially be used to screen a large number of nanomaterials in a relatively short time. Since this method could be adapted to high-throughput assays, e.g., in multi-well plate format, it would allow automation and rapid analysis of the potential adverse effects of nanomaterials. Such a screening assay would also allow investigation of which physicochemical properties of the nanomaterials are critical to fibrogenicity and may lead to identification carbon nanotubes with low fibrogenic potential. In addition, this *in vitro* model system may be used to conduct detailed mechanistic investigations of the fibrogenic effects of nanomaterials that are not achievable *in vivo*.

In summary, the present study demonstrated that (1) DSWCNT do not produce significant cell and tissue damage as indicated by BAL LDH assays *in vivo* when administered at doses that induced persistent lung fibrosis, (2) DSWCNT stimulated fibroblast proliferation and collagen production *in vitro* with no

detectable cytotoxicity at relevant concentrations, and (3) DSWCNT increased the expression of MMP-9 both *in vitro* and *in vivo*. These findings support our hypothesis that dispersed single-walled carbon nanotubes rapidly enter the interstitial tissues and form a matrix that exerts a direct stimulatory effect on lung fibroblast growth and collagen production. This direct interaction of DSWCNT with fibroblast represents a novel mechanism for fibrogenesis that differs from the classical paradigm of persistent inflammation and oxidant stress.

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