In Vitro Diagnosis and Analysis of the Potential Effects of Carbon Nanotube Exposure on the Human Respiratory System

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ABSTRACT

Recent discoveries of various forms of carbon nanostructure have stimulated research on their applications and hold promise for applications in medicine and many other related engineering areas. While carbon nanotubes (CNTs) are already being produced on a massive scale, few studies have been performed which test the potential harmful effects of this new technology. We used a 3-dimensional *in vitro* model of the human airway using a co-culture of normal human bronchial epithelial cells and normal human fibroblasts for the health risk assessment of CNTs on the human respiratory systems. We measured the production of nitric oxide (NO) as an inflammatory marker and MTT activity for cytotoxic response of the cell layers following exposure of different concentrations of CNTs. Our results indicated that NO production was dramatically increased and cell viability was decreased following exposure of different concentrations of CNTs. Further studies are required to get the transport properties of CNTs across the cells either in monolayer or in co-culture. In addition to inflammatory and cytotoxic responses of cell layers, the changes in physiological functions, such as mucin secretion, tight junction formation, and cilia formation, needs to be measured in the future, following exposure of different concentrations and structures of CNTs. This study will provide a science-based, comprehensive understanding of potential toxicity and ultimately enable the safer use of CNTs and CNT-based materials as novel nano-scale medical tools.

Keywords: SWCNT, nanoparticle(s), 3-D co-culture, tissue-engineered airway, nano-structure

1 INTRODUCTION

The discovery of carbon nanotubes (CNTs) in 1991 initiated a great number of interests in various scientific and technological fields. Owing to their phenomenal, mechanical, electrical, and thermal properties, many potential applications have been proposed for CNTs, including conductive and high-strength composites, energy storage devices, sensors and actuators, field emission displays, nano-scale semiconductor devices, probes, and interconnects [1].

The CNT manufacturing processes and the post processes that transform the raw material into value-added products entail substantial handling of CNTs in powder form and expose the workers to the risk of inhalation or skin contact. Recent studies for nanomaterials indicate: (1) CNTs and fullerenes have produced toxic effects on biological systems; (2) evidence that nanoparticles can translocate to bloodstream; and (3) evidence that nanoparticles can cross blood brain barrier. However, studies are still preliminary, as the current *in vivo* and *in vitro* response data are difficult to extrapolate, and coating (or surface treatment) influences how particles will interact with biological systems.

Two parts of the human anatomy that are especially susceptible to insult by airborne toxic materials are the *respiratory system* and the *skin*. Experimental access to the airways is, in general, very difficult. Post-mortem analysis of the smaller airways and direct bronchial biopsy of the upper airways (approximately generations 2-5) have provided a wealth of information. Unfortunately, supply of these tissues, and experimental control of the system is limited. Monolayer culture of the key individual cell types has also provided abundant fundamental information on the response of these cells (primarily epithelial, fibroblast, and smooth muscle) to external perturbations. These systems are limited by the absence of cell-cell interactions, which are present *in vivo*. More recently, a contracting fibroblast-embedded collagen gel has provided a more dynamic model to simulate human airway [2]. Fibroblasts will naturally contract the extracellular matrix (ECM) to close a wound, and, when placed in a collagen gel, respond in a similar fashion and contract the gel. Another *in vitro* model of airway involves culturing epithelial cells as monolayer on a membrane and fibroblasts as a monolayer a fixed distance away separated by culture media [3]. This model is attractive as it isolates soluble mediators that participate in epithelial-fibroblast communication, but lacks the normal ECM and anatomical dimensions.

Our model of the airway in this study presented several important features: 1) It maintains the normal anatomical arrangement (orientation and dimensions) of epithelial and fibroblast cells. 2) The fibroblast is embedded in collagen I, yet remains anchored. 3) A thin (10 μm) porous polyester membrane separates the epithelial and fibroblast cell allowing communication between the epithelium and

fibroblast, but also clean access to investigate cell-specific protein expression, following exposure to external perturbation [4].

2 MATERIALS AND METHODS

2.1 Characterization of Carbon Nanotubes

The high-purity single-walled carbon nanotubes (SWCNTs) used in this study were produced by Carbon Nanotechnologies Inc, using the HiPCO process. The residual metal content was 3-12% by weight, and the individual nanotubes were 0.8-1.2 nm in diameter and 100-1000 nm in length (manufacturer provided data). The SWCNTs were added to distilled water, and the mixture was sonicated for 60 minutes using Sonicator 3000 manufactured by Misonix (Farmingdale, NY). A nonionic octylphenol ethoxylate surfactant, Triton™ X-100, was added to the dilute aqueous SWCNT solution to facilitate the separation of nanotube ropes into smaller ropes or individual tubes.

The degree of SWCNT dispersion in the aqueous solution was evaluated using an atomic force microscope (AFM) MultiMode II developed by Veeco Digital Instruments Group (Woodbury, NY). Several drops of nanotube suspension were applied on to a silicon substrate and were allowed to dry in open air, leaving nanotube agglomerates. The substrate was observed under the AFM, and the nanotube rope sizes were measured at various locations.

2.2 Anatomy of Human Lung

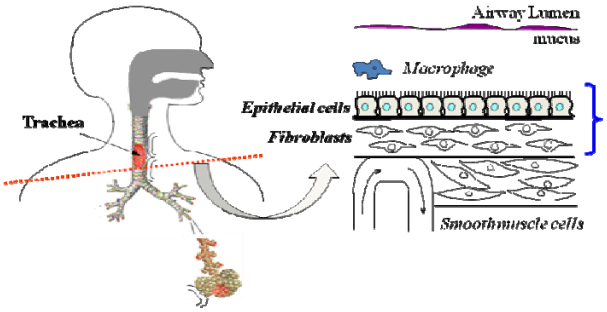


Figure 1. Anatomy of Human Lung

The human airway lining cell layers play a role as a barrier to external stimuli. The airway consists of several cell layers, such as epithelial cells, fibroblasts, and smooth muscle cells, including several inflammatory cells (e.g. macrophages, neutrophiles, mast cells, etc.) (Figure 1). Physiological response to external perturbation is induced by each cell layers and/or the interaction between cell layers. Our *in vitro* co-culture system was established based on the interaction between two cell layers such as epithelial cells and fibroblasts shown in Figure 2.

2.3 3-dimensional *in vitro* System of the Human Lung

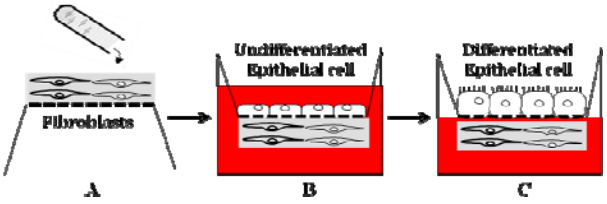


Figure 2. Preparation of tissue engineered airway.

Fibroblast-embedded Collagen I gels are prepared using rat tail tendon collagen (RTTC; Collaborative, Bedford, MA). Normal human lung fibroblasts (NHLFs) are harvested upon reaching 75-80% confluency, and added (seeding density of 5 x 10 fibroblasts/mL of final volume) to an iced mixture of collagen (final concentration 2.0 mg/ml), 5X concentrated DMEM, and 10X reconstitution buffer comprised of $NaHCO^3$, HEPES buffer (Gibco, Grand Island, NY) and NaOH. Aliquots of the mixture are pipetted onto the underside of a Transwell (Costar, Cambridge, MA) polyester membrane (0.4 μm pore). The collagen mixture is then allowed to "gel" (non-covalent cross-link) at 37° C in 5% CO_2 for 10-15 minutes (Figure 2A). Harvested primary human bronchial epithelial (HBE) cells (passage 2-3) are then seeded (1.5 x 10 cells/cm^2) directly on top of the polyester membrane. The entire tissue is submerged in media for 5 days and the epithelium is allowed to attach and become confluent (Figure 2B). For the first 48 hours, the media is basal epithelial growth medium (BEGM, Clonetics) and a low retinoic acid concentration. For days 3-5 (and days 6-21), the media is a 50:25:25 mixture of BEGM:DMEM:Hams F12 with a high retinoic acid concentration. At day 6, an air-liquid interface is established (media maintains a high retinoic acid concentration) and the epithelium is allowed to differentiate for approximately two weeks at which time it is ready for experimentation (Figure 2C).

2.4 Measurement of Transepithelial Electrical Resistance (TER)

Human bronchial epithelial cells were grown at the interface of air and liquid. Culture media was provided from the bottom through the porous membrane. TER of human bronchial epithelial cell with fibroblasts-embeded collagen layers cultured in Transwell™ was monitored using a portable Voltohmmeter (Millipore, Bedford, MA) attached to a dual "chopstick" or transcellular resistance measurement chamber (Millipore, Bedford, MA). Different concentrations of CNTs are exposed to the co-culture layers

for 6 hours. Each of the two electrode systems contains Ag/AgCl electrode for measuring voltage and a concentric spiral of silver wire for passing current across the epithelium. Current can then be passed across the epithelium to measure TER (ohms.cm^2). TER values higher than the background fluid resistance indicate a confluent airway epithelium with tight junctions.

2.5 Cytotoxicity

The MTT assay (Sigma) was used to evaluate mitochondrial activity of cells. Cells were exposed to varying concentrations of CNTs (Table 1). After 48 h, 150 µL of MTT (5 mg/ml) was added to each well and incubated for 4 h. Afterward, 850 µL of the MTT solubilization solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well. The resulting formazan crystals was solubilized in acidic isopropanol and quantified by measuring absorbance at 570 nm. Data were calibrated to the appropriate calibration curve as stated in Sigma protocols.

2.6 Inflammatory Response

Nitric oxide (NO) production was measured to identify the level of inflammation. All media samples were analyzed using Griess Reagent system (Promega Corporation, WI) to detect the level of nitrite (NO^{2-}), one of the two stable oxidized products of NO in the liquid phase.

5% Triton X-100	20 µg/mL of Triton X-100
20% Triton X-100	80 µg/mL of Triton X-100
0% CNT	No Triton X-100 and no CNT
5% CNT	20 µg/mL of Triton X-100 and 2 µg/mL of CNT
10% CNT	40 µg/mL of Triton X-100 and 4 µg/mL of CNT
20% CNT	80 µg/mL of Triton X-100 and 8 µg/mL of CNT

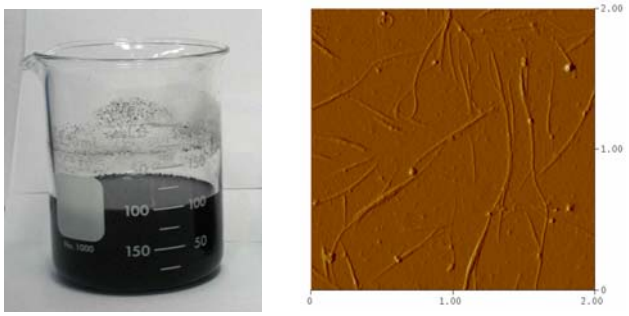
Table 1. Concentrations of CNTs exposed to cell layers

3 RESULTS

TER of our co-culture layers was measured to observe the changes in airway physiological function following exposure of different concentrations of CNTs. We also measured the production of nitric oxide (NO) as an inflammatory marker and MTT activity for cytotoxic response of the cell layers following exposure of different concentrations of CNTs.

3.1 Characterization of Carbon Nanotubes

Figure 3 shows 100 mL of aqueous SWCNT suspension 12 hours after sonication. Dark, uniform solution suggests the nanotubes are well dispersed in the medium. The surfactant-aided nanotube suspension remained stable for at least two months. The AFM image of SWCNTs dried on a silicon substrate is shown in Figure 2 [5]. The average diameter and length of nanotube ropes were about 500 nm and less than 10 nm, respectively. A more detailed study on the distributions of nanotube dimensions using image analysis is discussed in Ref. [5].



(A) (B)

Figure 3. Aqueous SWCNT solution after 12 hours (A). AFM image of SWCNT ropes (B) [4]

3.2 Effect of CNTs on Physiological Function of Airway Epithelial Cells

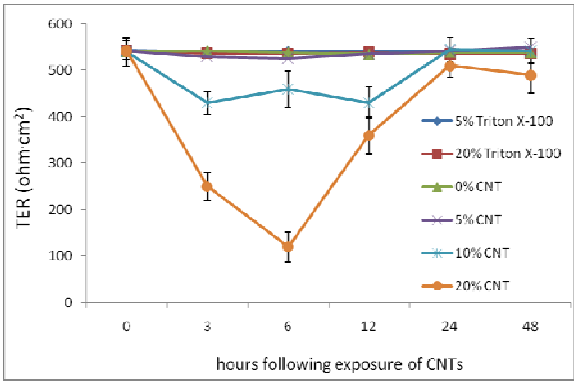


Figure 4. Exposure of CNTs to co-culture layers impacts transepithelial electrical resistance (TER).

Different concentrations of CNTs were exposed to the co-culture layers for 6 hours. The TER of the controls (5% and 20% of Triton X-100 and 0% of CNT) were stable at ~ 500 ohms.cm^2 (resistance of epithelial-free tissue has been subtracted) for 48 hours. 10-20% of CNTs rapidly compromises the barrier function of the epithelium and the TER decreases to ~ 120 ohms.cm^2. After removing CNTs, the TER completely recovered to the control level (Figure 4).

3.3 Inflammatory and Cytotoxic Responses

NO production following exposure of CNTs to epithelial cells was dramatically increased as the concentration of CNTs increased (Figure 5A). At higher

concentrations of CNTs, cells showed cytotoxic response and parts of cell layers were detached (data not shown). Each NO production was normalized by total proteins. Fibroblasts showed inflammatory response, slightly increasing the level of NO following exposure of CNTs (Figure 6A). Cellular metabolic activity was observed following exposure of different concentrations of CNTs to both cell layers. MTT activity was decreased as concentration of CNTs increased, especially for epithelial cells (Figure 5B and 6B).

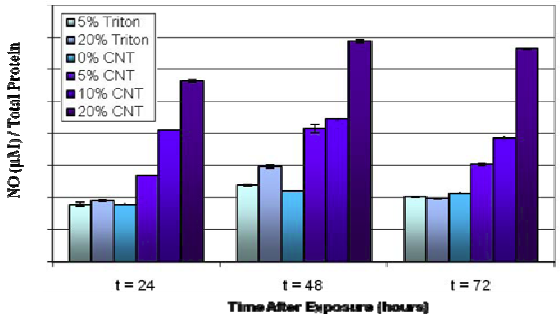


Figure 5A. Effect of CNTs on NO production from epithelial cell layers.

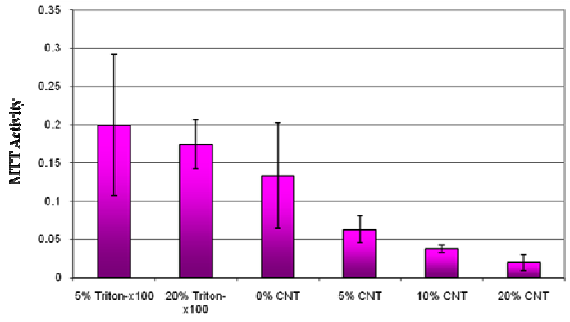


Figure 5B. Effect of CNTs on MTT activity in epithelial cell layers

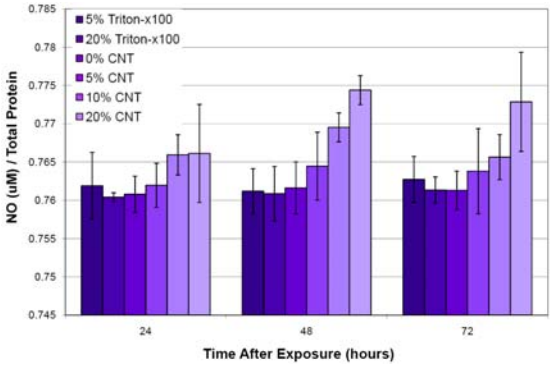


Figure 6A. Effect of CNTs on NO production from fibroblast layers.

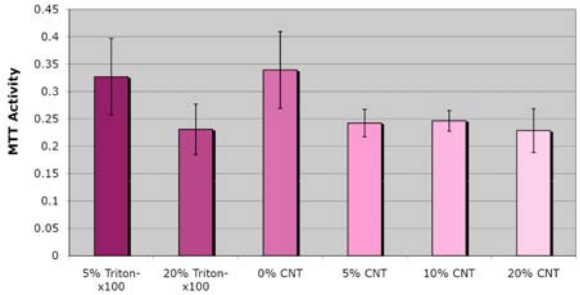


Figure 6B. Effect of CNTs on MTT activity in fibroblast layers

4 DISCUSSION

The complete tissue mimics the normal anatomical arrangement of epithelial cells and fibroblasts in the bronchial mucosa, and the thin porous polyester membrane allows fibroblast-epithelial communications, but also investigation of cell-specific (i.e., epithelial or fibroblast) gene expression in response to external perturbation.

In this study, cytotoxic/inflammatory responses and barrier function of the human lung layers following exposure of CNTs were observed using *in vitro* co-culture system of airway. Further studies on nanoparticle-related cellular toxicity and functional relations between the size or structure of CNTs and the perturbation of cellular or physiological functions are required in the future.

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