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In vitro evaluation of cytotoxicity of engineered carbon nanotubes in selected human cell lines

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ABSTRACT

In this study, we used a systematic approach to study and compare the *in vitro* cytotoxicity of selected engineered carbon nanotubes (CNTs) to test cell lines including human skin keratinocytes, lung cells and lymphocytes. Results of fluorescein diacetate (FDA) uptake in T4 lymphocyte A3 cells indicated cytotoxicity caused by single-walled carbon nanotubes (SWCNTs) at concentrations of 2, 5 and 10 ppm. At 2 ppm, the SWCNT treatment group retained 71.3% viability compared to the PBS control group. At 10 ppm, cellular viability further decreased to 56.5% of the PBS control group. In the skin keratinocyte HaCaT cells and lung MSTO-211H cells, the SWCNT did not demonstrate any cytotoxicity at concentrations of 2 and 5 ppm but slightly inhibited HaCaT cells and caused significant toxicity to MSTO-211H cells at 10 ppm. Multi-walled carbon nanotube (MWCNT) testing showed significant cytotoxicity to A3 cells in a dose-dependent manner. At 10 ppm the viability of the cells decreased to 89.1% compared to the PBS control. In MSTO-211H cells, MWCNT caused significant toxicity at concentrations of 2 ppm and higher. By comparison, HaCaT cells were inhibited significantly only at 10 ppm. Overall, the test CNTs inhibited cellular viabilities in a concentration, cell type, and CNT type-dependent pattern. The viabilities of the MWCNT-impacted cells are higher than the corresponding SWCNT groups. We speculate that on a per volume basis, the greater availability of defects and contaminants for cellular interaction may contribute to the higher cytotoxicity of SWCNT in this study. The interaction between the SWCNTs and A3 lymphocytes was also observed by scanning electron microscopy. The mechanism for causing cell death in this study was attributed to apoptosis and necrosis after physical penetration by CNTs and oxidative stress *via* formation of reactive oxygen species.

1. Introduction

Advances in the engineering of nanostructures with exquisite size and shape control, elucidation of their unique properties, and demonstration of their broad applications have made nanotechnology an exciting research area (Fischer and Chan, 2007). Due to their unique properties, carbon nanotubes (CNTs) have been used in various consumer, medical, and industrial applications (Iijima, 1991). Consequently, this expanding usage may lead to widespread human exposure *via* skin contact, ingestion, intravenous injection (in medical application) or by inhalation. The growing use of CNTs has put forward the agenda to establish a paradigm for accurately assessing the toxicity of CNTs. Moreover, divergent literature reports of CNTs' toxicity make it difficult to conclude if any health risks are associated with CNTs exposure. A quick screening test in assessing potential CNT toxicology is the utilizing of various mammalian cells to determine viability or increase/decrease in a designated biological pathway against chosen engineered CNTs. Nanomaterials can enter the human body via the lungs, the intestines and, probably to a lesser extent, the skin, because the skin and lungs are in direct contact with the environment (Hoet et al., 2004; Di Sotto et al., 2008). The small size and the high surface area define the chemical reactivity of CNTs and induce changes in permeability or conductivity of biological membranes (Rotoli et al., 2008). Therefore, engineered CNTs pose high health risk because of their ability to reach every part of the organs and tissues and their interaction with cellular functions. Despite these concerns, very few studies have actually been conducted with various human cell lines simultaneously to assess the health effects of different CNTs.

CNTs and functionalized CNTs are considered ideal materials for applications such as ultra-strong fibers, field emission displays, vaccine delivery, drug delivery, bone implants, neural tissue regeneration and protein transporters (Baughman et al., 2002; Martin and Kohli, 2003; Ericson et al., 2004; Milne et al., 2004; Bianco et al., 2005) because of their great tensile strength, high conductivity and unique electronic features. SWCNTs have a diameter of 1–2 nm and a length of up to 100 μm. MWCNTs consist of several layers of carbon cylinders with diameters up to 10–30 nm. There have been a few reports on

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toxic effects of CNTs with *in vivo* (Lam et al., 2004; Shvedova et al., 2005) or *in vitro* studies (Shvedova et al., 2003; Lam et al., 2004; Cui et al., 2005; Jia et al., 2005; Wörle-Knirsch et al., 2006; Pulskamp et al., 2007). However, the results have been divergent. Some scientists have suggested that metal traces associated with the commercial nanotubes are responsible for the cytotoxicity and that CNTs show no sign of acute toxicity (Pulskamp et al., 2007). Pristine CNTs are insoluble in almost all solvents; therefore, evaluation and characterization of their cytotoxic potential need special approaches.

A key challenge to developing green CNT technology is the achievement of more precise control of the assembly for the engineering process to produce the desired physical and chemical properties, thus minimizing potential health risks in their applications. This study aimed to provide new information on nanotoxicity of SWCNT and MWCNT by using three human cell lines (the skin cells, lung cells and lymphocytes) to simulate different exposure/uptake routes. Their relative toxicity was also compared.

2. Materials and methods

2.1. Chemicals and instruments

The SWCNTs and MWCNTs were supplied by Cheap Tubes, Inc. (Brattleboro, VT). They were prepared without further purification in distilled water to reach concentrations of 2, 5 and 10 ppm. Tween 80 at the final concentration of 0.04% (w/v) was added to increase the solubility and avoid aggregation of the CNTs. The physical dimensions of the SWCNT were a diameter of 1–2 nm and a length of 5–30 μm. The MWCNT had a diameter of 20–30 nm and length of 10–30 μm. Fluorescein diacetate, hydrogen peroxide (H_2O_2; 30% w/w), and phosphate-buffered saline (PBS; 1×, Mg^{2+}, Ca^{2+} free) were purchased from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO), bacterium *Escherichia coli* (Migula) Castellani & Chalmers (ATCC #25254), the A3 lymphocyte cell line and the MSTO-211H lung cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, fetal bovine serum, trypsin-ethylenediaminetetraacetic acid (EDTA; 25%), penicillin, and streptomycin were purchased from Gibco-Invitrogen (Carlsbad, CA). The HaCaT human keratinocyte cell line, at passage 34, was a generous gift from Norbert E. Fusenig of the German Cancer Research Center (Heidelberg, Germany).

A Triad LT microplate reader supplied by Dynex Technologies (Chantilly, VA) was used to measure the fluorescence of the samples in the FDA test (Hu et al., 2007).

2.2. Cytotoxicity of the SWCNT and MWCNT to cell lines

The human keratinocyte HaCaT cells at passage number 34 were grown in T-25 Falcon flasks (Becton Dickinson Labware, Bedford, MA) at 37 °C in a 95% air/5% CO_2 humidified incubator (Isotemp; Fisher Scientific, Houston, TX) using the recommended DMEM medium (Pfeifer et al., 2005). The T4 lymphocytes, A3, and lung cells (MSTO-211H), were cultured in RPMI 1640 medium recommended by the ATCC. The A3 cells and the MSTO-211H cells used for the experiments were between passages 14–16 and passages 2–8, respectively (Hu et al., 2007).

2.3. Treatment of cells with various concentrations of SWCNT and MWCNT

HaCaT cells and MSTO-211H cells were harvested by spreading 1 mL of 0.25% trypsin over the stationary cultures and incubating at 37 °C for 5 min. Then, 4 mL of medium was added to stop the reaction. The A3 cells were collected directly. All of the three types of cells were collected by centrifugation at 129×*g* for 5 min at 4 °C. The pellet was washed twice with 1× PBS. The viability of cells processed in this way was over 95% as determined by the Trypan blue dye method (Jolanda et al., 2003). The cell density was adjusted to 1×10^6/mL with 1× PBS. One hundred μL aliquots of HaCaT cells, A3 cells and MSTO-211H cells (1×10^5 cells) were seeded into the wells of 96-well plates. Then, 100 μL of different concentrations of either SWCNT or MWCNT in Tween 80 (final Tween 80 concentration of 0.04%) were added to each well to achieve the final test concentrations of 0, 2, 5 and 10 ppm. The concentration range of CNTs was chosen according to their solubility in PBS (containing 0.04% Tween 80). The concentration range of CNTs was chosen according to their solubility in PBS (containing 0.04% Tween 80; see lines 120–121). According to the instruction of the Comet Assay reagent kit (Trevigen; catalog #4250-050-K), the prerequisite for conducting the genotoxicity test is that cell viability should be at least 75% viability or higher to rule out the false genotoxic effect caused by other mechanisms such as necrosis and apoptosis (Zheng et al., 2004). For cross comparison with genotoxicity assessment in the future, therefore, we selected final candidate solvents that retained at least 75% of the viability of the control. Because of these limitations, a typical L_{C50} toxicity curve was not obtained in this study. A solvent control containing PBS with 0.04% Tween 80 was included in all the experiments. Positive controls, cultures treated with 100 μM H_2O_2, also were included to assure the sensitivity of the assays. Each treatment concentration was assayed in eight replicate wells. The plates were covered with aluminum foil to avoid ambient irradiation.

2.4. Fluorescein diacetate uptake (FDA) test

The effect of CNTs on cell proliferation was evaluated and conducted by the widely established FDA test (Zheng et al., 2004). According to the instruction of the Comet Assay reagent kit, the prerequisite for conducting the genotoxicity test is that cell viability should be at least 75% viability or higher to rule out the false genotoxic effect caused by other mechanisms such as necrosis and apoptosis (Zheng et al., 2004). For cross comparison with genotoxicity assessment in the future, therefore, we selected final candidate solvents that retained at least 75% of the viability of the saline control.

2.5. Visualization of interaction between SWCNTs and A3 cells by scanning electron microscopy (SEM)

To identify and visualize the effect of SWCNTs on A3 lymphocytes, the suspension of SWCNTs and cells was washed 3 times with distilled water containing 2% glutaraldehyde to remove the salt which can obscure the SWCNTs presence during SEM analysis. Then, a few droplets of a suspension of the CNTs and cells were deposited on a support (Si Wafer) after 30-min incubation. SEM imaging was performed on a FEI Quanta 200 (FEI, Hillsboro, OR) microscope, which was operated under a high vacuum condition.

2.6. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). For the FDA test, FDA uptake was measured by the fluorescence intensity of FDA, and the fluorescence intensity of treated cells was compared to the corresponding PBS control group (containing 0.04% Tween 80), whose fluorescence intensity was set as 100%. The experiment was conducted at least 3 times, and each treatment had eight replicates.

The SAS System for Windows, V8 (SAS Institute, Gary, NC) was used for statistical evaluations. Means ± SD were calculated for normalized FDA uptake. Differences among treatment and control groups were tested by one-way analysis of variance (ANOVA), followed by pair-wise comparisons between groups using Tukey's