Cytotoxicity of single-walled carbon nanotubes to human lung carcinoma cells: the influence of N-acetylcysteine

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SUMMARY

Background: *Single-walled carbon nanotubes (SWCNTs) have been reported to induce cytotoxicity in different cell lines. Although the mechanisms underlying cytotoxicity are not fully understood, accumulation of reactive oxygen species (ROS) and oxidative damage is considered to be a likely contributing factor.*

Methods: *Human lung carcinoma cells, A549, and human fetal lung fibroblasts, MRC-5 were used to assess the cytotoxicity of SWCNT in the presence and absence of a redox status regulator, N-acetylcysteine (NAC), via the MTT assay.*

Results: *SWCNT induced a nearly three-fold greater loss of viability in A594 vs. MRC-5 cells at ≤250 μg/ml. SWCNT cytotoxicity at higher concentrations was similar for both cell lines, while NAC alone was non-toxic. The cytotoxicity of SWCNT (250 μg/ml) in combination with NAC to A549 cells was significantly decreased at the lowest NAC concentration (1.5 μg/ml), and was similar to NAC treatment alone at that concentration. Higher concentrations of NAC in combination with SWCNT (250 μg/ml) resulted in increased cytotoxicity in both A549 and MRC-5 cells.*

Conclusion: *A549 malignant lung cells are more susceptible to low concentrations of SWCNT vs. normal lung cells, and low concentrations of N-acetylcysteine appear to be cytoprotective, possibly due to its antioxidant properties.*

Key words: *Nanotubes, Carbon; Acetylcysteine; Oxidative Stress; Cell Line, Tumor*

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INTRODUCTION

Carbon nanotubes (CNTs) are a distinct molecular form of carbon atoms. They were described in 1991 by Sumio Lijima as a tube-shaped, well-ordered, flat network of carbon (1). There are two major classes of carbon nanotubes: single-walled nanotubes (SWNT) composed of a single graphite layer with diameters ranging from 1 to 10 nm and multi-walled nanotubes (MWNT) containing several concentrically arranged cylindrical layers (2). The unique and diverse properties of carbon nanotubes, in addition to the wide range of functionality afforded by chemical modification, make this nanomaterial suitable for many applications including electronics (3), biosensor design (4), drug development and as biomolecule carriers (5).

While earlier research suggested that CNTs have no apparent cytotoxicity (6), recent studies have shown that CNTs could be harmful to human health (7, 8). In addition, the fibrous morphology and high surface energy of CNTs raises other health concerns, due to their structural similarities with asbestos fibers (9), which are classified as a group I (one) human carcinogen by the International Agency of Research in Cancer (10, 11). Several *in vitro* studies have demonstrated that CNTs exhibit substantial cytotoxicity, possibly via induction of oxidative stress, inhibition of cellular proliferation, and induction of apoptosis/necrosis (12, 13).

Therefore, assessment of reactive oxygen species (ROS) production by cells exposed to carbon nanoparticles is an essential part of *in vitro* cytotoxicity testing. ROS are oxygen-containing molecules with unpaired electrons, and therefore are highly reactive. ROS molecules can damage DNA, proteins and lipids, compromising normal cell function, and production of ROS by cells can be indicative of a stress response. ROS detoxification and regulation of the cellular redox state is achieved by enzymatic and/or non-enzymatic antioxidants, such as glutathione (GSH) and thioredoxin. Oxidative stress occurs when there is an imbalance between oxidants and antioxidants that favors the presence of ROS, due to the excessive production of oxidant species and/or depletion of antioxidants.

Based on research reports that cell damage caused by nanoparticle-induced oxidative stress could be reduced by antioxidant treatment (14), we used the GSH precursor N-acetyl-L-cysteine (NAC) as a regulator of thiol redox status (15) to verify whether the modification of the cellular redox status have a protective effect on the *in vitro* cytotoxicity of single-walled carbon nanotubes in A549 malignant human lung epithelial cells.

MATERIAL AND METHODS

Cell lines

The cell lines used in the present study were A594 (human lung carcinoma, ATCC, CCL 185) and MRC-5 (human fetal lung fibroblasts, ATCC, CCL 171). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose supplemented with 10% fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/ml of penicillin and 100 μg/ml of streptomycin (ICN Galenika). All cell lines were cultured in flasks (Sarstedt, 25 cm^2) at 37°C in a 100% humidity atmosphere and 5% CO_2. Cells were sub-cultured twice a week and a single cell suspension of adherent cells was obtained using 0.25% trypsin in EDTA (Serva). Exponentially growing cells were used throughout the assays. Cell density and the percentage of viable cells were monitored using a dye exclusion test with Trypan Blue. The viability of cells used in assays in the present study was over 95%.

Tested substances

Single-walled carbon nanotubes (SWCNTs, ALDRICH), and *N*-acetyl cysteine (NAC, SIGMA) were tested for cytotoxicity against A594 and MRC5 cells.

Substances used in the MTT assay were applied at a concentration range of 10^{-8}M to 10^{-4}M. SWCNT and NAC were tested alone, and SWCNT (250 μg) was tested in combination with five NAC concentrations.

MTT assay

Cytotoxicity was evaluated using the tetrazolium colorimetric MTT assay (SIGMA). The MTT assay is based on cleavage of the tetrazolium salt MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan by mitochondrial dehydrogenases in viable cells (15).

Cells were plated in 96-well microtiter plates (Costar) at a volume of 90ml per well. Cells were plated in complete medium at an optimal seeding density of $5x10^3$ cells per well to assure a logarithmic growth rate throughout the assay period. Tested substances were added to all wells (except controls) at a concentration ranging from 10^{-8}M to 10^{-4}M.

Plates were incubated at 37°C for 48h. Three hours before the end of the incubation period, 10 μl of MTT solution (5mg/ml) was added to all wells, and plates were incubated for 3 hours at 37°C. Medium and MTT were then removed by aspiration. The resulting formazan product was then solubilized in 100 μl of 0.04 M HCl in isopropanol. After a few minutes incubation at room temperature, plates were read using a spectrophotometer plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. Wells without cells, but containing complete medium and MTT only acted as a reference.

Cytotoxicity, expressed as percent cytotoxicity, was calculated according the following formula:

$$\% C = 1-(OD_{test}/OD_{control}) \times 100.$$

Data analysis

Two independent experiments were conducted with quadruplicate wells for each concentration of test compound. IC_{50} values were used to define the dose of test substance that inhibits cell growth by 50%. IC_{50} values of test substances were determined by Median effect analysis.

RESULTS

Single-walled carbon nanotubes displayed varying degrees of cytotoxicity against A549 cells and MRC-5 cells in a concentration dependent manner (Figure 1).

SWCNT treatment resulted in a nearly three-fold greater loss of cell viability for malignant A594 cells (at doses ≤250 μg/ml) versus non-cancerous MRC-5 control cells. At higher concentrations of SWCNT (>250 μg/ml), cytotoxicity was similar in both cell lines.

The cytotoxicity of SWCNT (250 μg/ml) in combination with NAC for A549 cells was significantly decreased at the lowest NAC concentration (1.5 μg/ml), and was similar to NAC treatment alone (Figure 2). At higher NAC concentrations, treatment with SWCNT (250 μg/ml) resulted in increased cytotoxicity in A549 cells compared to that induced by the individual substances alone. In MRC-5 cells, the lowest concentration of NAC reduced SWCNT toxicity, in contrast to NAC treatment alone at the same concentration.

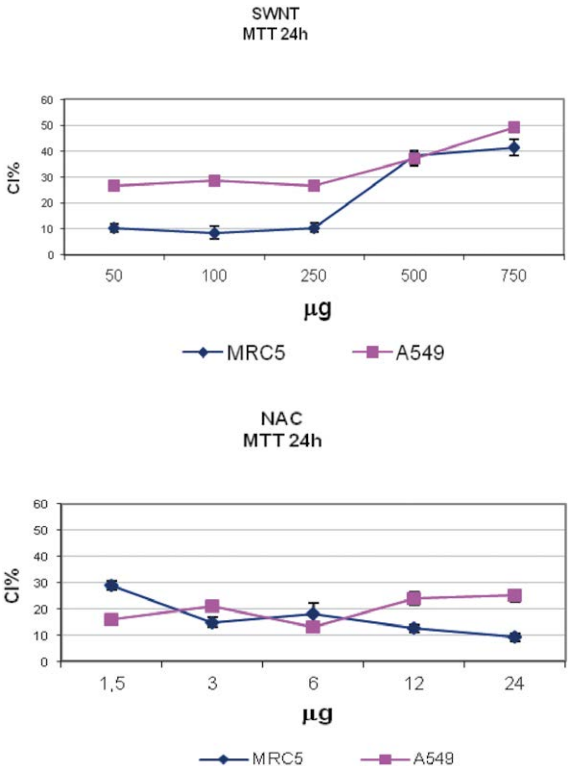

Figure 1. Cytotoxic activity of SWCNT and NAC tested in A549 and MRC-5 cells

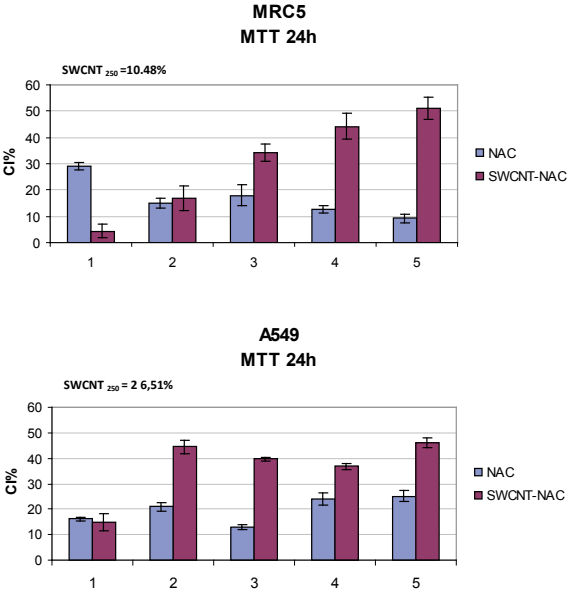

Figure 2. Cytotoxic activity of SWCNT, NAC and SWCNT in the presence of NAC tested in A549 and MRC-5 cells. Cytotoxicity of SWCNT (250μg) was evaluated in combination with various concentrations of NAC: 1.5 μg (1), 3 μg (2) and 6 μg (3), 12 μg (4) and 24 μg (5) and compared to SWCNT or NAC cytotoxicity alone

DISCUSSION

Numerous studies have reported notable cytotoxicity for carbon nanotubes in mammalian cells (17, 18). Our results confirm that SWCNTs are cytotoxic to human lung carcinoma A549 cells.

Among the suggested cytotoxicity mechanisms for SWCNTs, including oxidative stress, rupture of cell membranes and interfering with

intracellular metabolic routes, oxidative stress is considered to be the most plausible mechanism of SWCNT cytotoxicity. In fact, it was found that these nanoparticles induce increased ROS production and oxidative stress both *in vivo* and *in vitro* (19, 20). However, our present understanding of the mechanisms of carbon nanotube-induced cytotoxicity and the discovery of the enzymatic biodegradation of carbonaceous nanomaterials by peroxidases (21) indicates that "oxidative stress" may not be a major mechanism that is responsible for the toxic effects of nanoparticles, but should be viewed as an array of modifiable oxidative reactions. For example, in addition to increased ROS production and the cell damage caused by their actions, ROS participate in cellular signaling, leading to apoptosis and cell cycle arrest.

The increased toxicity to malignant A549 cells versus non-cancerous control cells (MRC-5 cells) observed in our study may indicate an imbalanced antioxidant status in malignant cells under the conditions of pro-oxidant activity induced by the presence of carbon-nanotubes. Our aim was to confirm that oxidative stress could underlie the cytotoxicity of SWCNTs by simultaneous incubation of cells with NAC as a redox modulator. Interestingly, we found that the lowest NAC concentration was protective to A549 cells. N-acetyl-cysteine is an acetylated cysteine molecule and a major contributor to the maintenance of cellular glutathione level, which is important for the protective response of cells to oxidative stress (22). We speculated that specific interactions of SWCNT with NAC or other cellular components might modulate the oxidative response and oxidative damage. In addition, it is interesting that NAC alone was more toxic to non-cancerous MRC-5 cells at the lowest concentration compared to higher concentrations, but in the presence of SWCNT, the toxicity of NAC was reduced. It is possible that NAC induces adaptive changes in MRC-5 cells, but in the presence of SWCNT, this was prevented.

CONCLUSION

The present study shows that malignant lung cells (A549) are more susceptible to SWCNTs at low concentrations than normal, non-cancerous lung cells (MRC-5), while the presence of low concentrations of N-acetylcysteine (NAC) as a regulator of redox status was found to be protective, indicating that single-walled carbon nanotubes (SWCNT) may act as pro-oxidant species.

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Conflicts of Interest

We declare no conflicts of interest.

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