Cytotoxic Effects of Short Multi-Wall Carbon Nanotubes

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ABSTRACT

Applications of carbon nanotubes (CNTs), a novel and important class of nanomaterials, can change paradigms of various research areas. Biomedical applications of CNTs and other classes of nanomaterials warrant a more comprehensive analysis because of their putative toxicity, which has not been systematically addressed. We hypothesized that short multi-wall CNT (MWNT) treatment induces cytotoxicity in different cell phenotypes and functionalization of MWNTs impacts the cytotoxic profile. Our results demonstrate dose-dependent cytotoxicity on treatment with three different types of MWNTs (namely, carboxylated (MWNT-COOH), hydroxylated (MWNT-OH) and non-functionalized MWNTs). Expression of AKT, phosphorylated AKT and beta-actin was strongly inhibited by non-functionalized MWNTs. Taken together MWNTs induce dose-dependent cytotoxicity and modulate signaling pathways.

Keywords: Multi-wall carbon nanotubes, cytotoxicity, functionalization, signaling

1 INTRODUCTION

Carbon nanotubes (CNTs) discovered in 1991 by Dr. Sumio Iijima constitutes one of the novel and important class of nanomaterials [1, 2]. Unique properties of CNTs facilitate their applications in various research areas. Recently biomedical applications of CNTs as novel molecular transporters, drug delivery vehicles and smart nanosensors are rapidly and extensively being investigated [3].CNTs and other classes of nanomaterials in biomedicine warrant a more comprehensive analysis because of their putative toxicity, which has not been systematically addressed [4]. Recent *in vivo* studies with SWNTs demonstrate dose-dependent lung lesions and pulmonary granulomas [5]. *In vitro studies* report dermal toxicity induced by MWNT through secretion of inflammatory cytokines such as IL-8 [6]. Functionally modified CNTs are being investigated for enhanced and site-specific drug delivery in cancer treatments [7]. The type of CNTs, single-wall (SWNT) or multi-wall (MWNT) and functional modifications exert differential effects and exhibit dissimilar cytotoxic profiles [8]. Some researchers argue that CNTs fake a cytotoxic effect by interacting with and quenching the dyes used in cell viability assays [9]. Current literature primarily focuses on cytotoxicity of SWNTs in relation to pulmonary and dermal toxicity and studies on MWNT toxicity are limited. Hence a systematic characterization of MWNT treatment-induced cytotoxicity is essential to resolve discrepancies in the literature. This approach will facilitate efficient utilization of MWNTs as novel molecular transporters and drug delivery systems and as components of smart nanosensors, for drug targeting and treatment in biomedicine.

To address this paucity of information in the literature, we systematically characterized the effects of three different types of MWNTs (namely, carboxylated (MWNT-COOH), hydroxylated (MWNT-OH) and non-functionalized MWNTs) on adriamycin-resistant murine sarcoma cells (S180A10). We hypothesize that MWNT treatment induces a dose-dependent decrease in cell viability. MWNT treatment modulates the signaling pathways such as Akt and MAPK which are highly up-regulated in most cancers. Thus, our studies report a comparative assessment of a cancerous cell line treated with different types of MWNTs, and the modulation of signaling pathways mediating the MWNT treatment induced cytotoxicity.

2 MATERIALS AND METHODS

The three different types of short multi-wall carbon nanotubes (outer diameter <8nm) were purchased from Cheap Tubes Inc. (Brattleboro, VT). Adriamycin resistant murine sarcoma cell line (S180A10) purchased from American Type Culture Collection (Manassas, VA), were cultured in McCoy's 5A Medium supplemented with 10%

(v/v) horse serum both purchased from Atlanta Biologicals (Lawrenceville, GA), and maintained in a humidified atmosphere containing 5% CO_2 in a Forma Scientific tissue culture incubator (Marietta, OH).

WST-1 Assay: Cells were seeded in 96-well plates and treated with various concentrations of MWNTs. At the end of 72 hours, 10µl of premixed WST-1 cell proliferation reagent (Clontech Laboratories Inc, Mountain View, CA) was added to each well and incubated for 2 hours. At the end of the 2-hour incubation, the cell suspension from each well was spun (2000 rpm × 5 min) and the supernatant was re-plated and absorbance was measured at 405 nm using Power Wave X 340, microplate scanning spectrophotometer (Bio-Tek Instruments Inc. Winooski, VT).

Western blotting: S180A10 cells were treated with the three types of MWNTs (1 mg/ml) for 72 hours and cell lysates were prepared. 10µg of the cell lysate protein was separated by SDS-PAGE electrophoresis and western blot analysis was performed using antibodies specific to total Akt and phosphorylated-Akt (p-Akt), respectively. The blots were re-probed with anti-β-actin antibody to ensure equal loading of protein.

The time-dependent change in expression of total Akt, induced by non-functionalized MWNT treatment, was also assessed using Western blotting.

Protein assay: S180A10 cells were treated with 1 mg/ml of the non-functionalized MWNTs for 72 hours. Cell lysates were prepared using a lysis buffer (containing 1% (v/v) Triton X-100, 10 mM Tris base pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% (w/v) sodium azide, 0.5 µM phenyl methyl sulphonyl fluoride, 0.2 µg aprotinin, 0.4 µg leupeptin, 100 µg sodium orthovandate, and distilled water at pH 7.6). The change in the protein concentration at each time point was determined using BioRad reagents, and absorbance was measured at 595 nm using Power Wave X 340, microplate scanning spectrophotometer (Bio-Tek Instruments Inc. Winooski, VT).

Statistical Analysis: SPSS software was used for statistical analysis. One way ANOVA followed by Tukey's post-hoc test were employed to analyze the differences between groups: $p < 0.05$ was considered significant in such comparisons.

3 RESULTS

3.1 Cell viability assay

Treatment with all three types of MWNTs (MWNT-COOH, MWNT-OH, non-functionalized MWNTs) induced a concentration-dependent reduction in cell viability, and a concentration of 3 mg/ml is strongly toxic to the cells (Figure 1). Functionalization of MWNTs did not induce marked differences in cell viability.

We also performed a control experiment to assess dye quenching by the MWNTs, which indicated that a high concentration of MWNTs (3 mg/ml) showed some dye quenching but was not entirely reflective of the decrease in absorbance (data not shown). These results support our hypothesis that MWNT treatment elicits a cytotoxic response in an *in vitro* model.

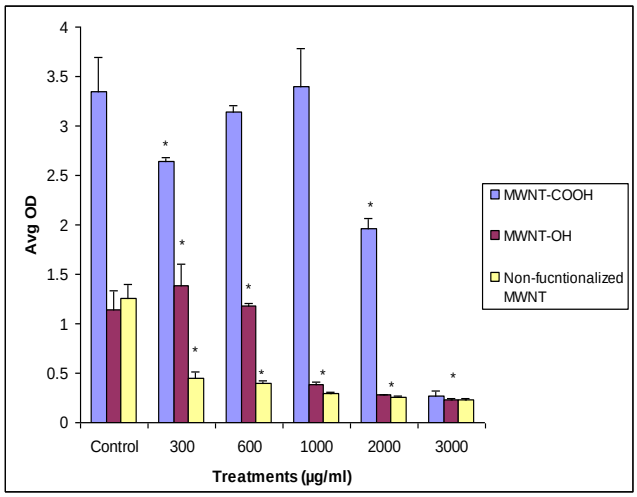

Figure 1: Dose-dependent decreases in viability of S180A10 cells treated with different types of MWNTs for 72 hours. Values represent mean ± SD, (*) indicates $p < 0.05$ compared to control.

3.2 Effect of MWNT treatment on Akt pathway

Modulation of downstream signaling effectors by MWNT treatment particularly in cancer cells is of importance since they are prime therapeutic targets. PI3K/Akt pathway is highly up-regulated in most cancers. It is activated by receptor tyrosine kinases which mediate the phosphorylation of phosphatidylinositol-4, 5 biphosphate (PIP_2) to phosphatidylinositol-3, 4, 5 triphosphate (PIP_3). PIP_3 in turn activates the downstream signaling target Akt (also known as protein kinase B) which regulates different cellular processes like cell proliferation, cell cycle and apoptosis [11].

In this study, we investigated the effects of MWNT treatment on modulation of Akt pathway in S180A10 cells. Our results indicated that treatment with MWNT-COOH and MWNT-OH for 72 hours at 1 mg/ml did not alter the expression of Akt, p-Akt, or β-actin, but that with non-functionalized MWNTs did induce a significant decrease in the expression of Akt, p-Akt, and β-actin (Figure 2).

We also assessed the change in expression of total Akt induced by the non-functionalized MWNTs over 72 hours. Our results indicated that expression of total Akt was decreased after 48 hours of treatment as compared to that in the untreated cells (Figure 3). Thus, functionalization of

MWNTs impacts on their cytotoxicity profile, non-functionalized MWNTs inducing a relatively stronger response.

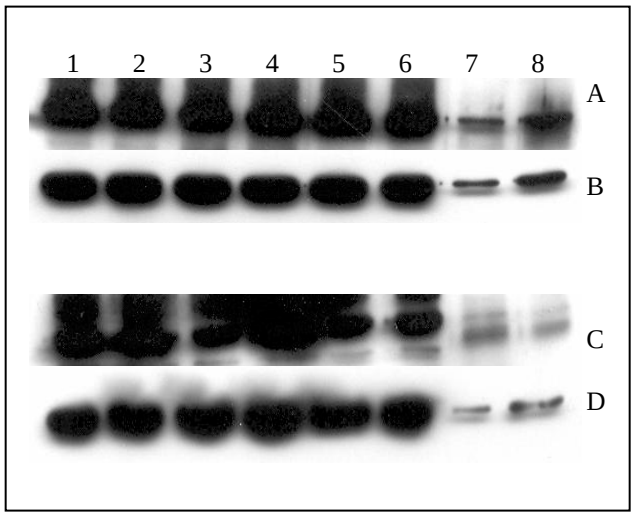

Figure 2: Effect of MWNT treatment for 72 hours on modulation of Akt pathway in S180A10 cells. Blots A, and B represent Akt and p-Akt respectively and blots C, and D represent β-actin. Lanes 1, 2; Control, Lanes 3, 4; MWNT-COOH, 5, 6; MWNT-OH, and 7, 8; non-functionalized MWNTs (1mg/ml)

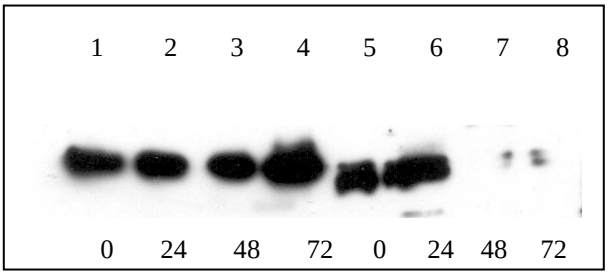

Figure 3: Non-functionalized MWNTs induce a time-dependent decrease in the expression of total Akt compared to the untreated cells. Lanes 1, 2, 3, 4; Control, Lanes 5, 6, 7, 8; Non-functionalized MWNTs (1mg/ml) treated for 0, 24, 48 and 72 hours respectively.

3.3 Effect of non-functionalized MWNTs on the protein content in S180A10 cells

We assessed the effect of changes in protein concentration in the S180A10 cells when treated with non-functionalized MWNTs. Our results indicated that treatment with non-functionalized MWNTs (1 mg/ml) over 72 hours (Figure 4) completely blocked the growth of cells and led to a concomitant decrease in protein content.

4 DISCUSSION

The increasing use of carbon nanomaterials, particularly CNTs in various applications has led to increased concern regarding their effects on the environment and more importantly on human health. Comparative cytotoxicity studies using different types of carbon nanomaterials reveal that CNTs are more toxic as compared to quartz, carbon black or fullerenes, and SWNTs exhibit a stronger cytotoxic response as compared to MWNTs [8]. Most of the studies focus on cytotoxicity of SWNTs and literature on MWNTs is limited. There are some conflicting studies which suggest that CNT treatment does not induce a toxic response [13].

We have systematically investigated the putative cytotoxicity of three different types of MWNTs on S180A10 cells. The results from our cytotoxicity studies are consistent with our hypothesis that aqueous suspensions of different types of MWNTs induce a dose-dependent decrease in cell viability in the S180A10 cells and dye quenching is not responsible for the decrease in OD values in such assays. Thus, MWNT treatment induces dose-dependent cytotoxicity in different cell phenotypes irrespective of the functionalization.

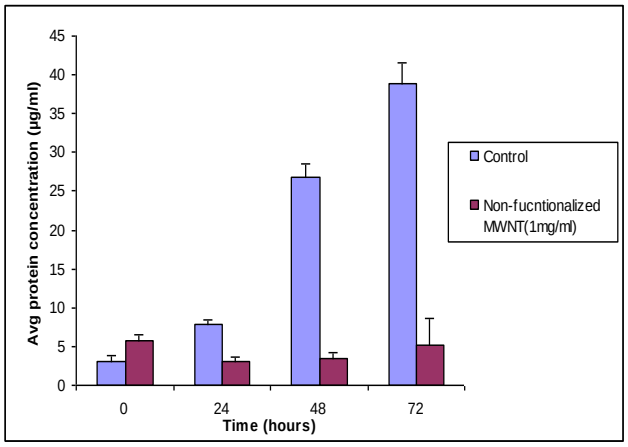

Figure 4: Effect of non-functionalized MWNTs (1 mg/ml) on changes in the protein concentration over 72 hours. The cells treated with non-functionalized CNTs show decrease in protein content as compared to the control

The exact mechanism of induction of apoptosis/necrosis by CNT treatment has not been elucidated, but factors such as size, aspect ratio, hydrophobicity, side-wall functionalization have been attributed to CNT mediated cytotoxicity [12].

CNTs are being extensively investigated for their applications as novel drug delivery vehicles, and diagnostic and therapeutic agents in treatment of diseases such as cancer [14]. Signaling pathways such as PI3K/Akt, MAPK, and Stat3 are highly up-regulated in many cancers and

downstream signaling molecules are prime therapeutic targets. An examination of effect of CNT treatment on expression of downstream effectors will reveal the mechanism mediating CNT-induced cytotoxicity.

Our results indicated a decrease in expression of both total Akt and p-Akt induced by non-functionalized MWNT treatment of S180A10 cells. Treatment with MWNT-COOH or MWNT-OH did not alter the expression levels of Akt or p-Akt. The expression of total Akt showed a time-dependent decrease upon treatment with non-functionalized MWNTs. The protein content also decreased with time in the non-functionalized MWNT-treated cells.

We have also investigated the effects of MWNT treatment on other downstream effectors such as MAPK and Stat3 and observed a similar decrease in their expression with the non-functionalized MWNTs (data not shown).

Functionalization of CNTs is done to overcome the inherent hyrdophobicity and to reduce toxicity, but some have reported that functionalization increases the cytotoxic response [12]. Signaling studies with SWNTs have indicated an up-regulation of apoptosis associated genes, and p38/MAPK cascade as a critical pathway mediating MWNT induced cytotoxicity [15, 16].

Thus, in summary we have demonstrated that different types of functionalized MWNTs induce a dose-dependent decrease in cell viability, and the non-functionalized MWNTs have an impact on expression of total and p-Akt.

Our studies suggest functionalization of MWNTs is a critical factor in determining the toxic response. We have employed *in vitro* models to investigate toxicity, and these results may not reflect the exact response of MWNT treatment *in vivo*. Thorough studies using animal models will further elucidate the biological responses of MWNT treatment and these results will provide critical information for their therapeutic applications. The exact mechanism of induction of apoptosis/necrosis remains to be elucidated. Effect of MWNT treatment on the signaling pathways mediating cell survival, proliferation and apoptosis should be investigated to understand their mechanism of action.

Thus, characterization of toxicity profiles of CNTs, particularly MWNTs, is a prerequisite to address the discrepancies in literature and generate an extensive database which will allow for complete utilization of their potential applications as novel diagnostic and therapeutic agents which would revolutionize biomedical research and provide a promising future for treatment of severe diseases such as cancer.

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