Cytotoxic Effects of Short Multi-Wall Carbon Nanotubes on Pancreatic Cancer Cells

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ABSTRACT

Although carbon nanotubes (CNTs) are increasingly exploited in diverse industrial and biomedical applications, their health hazard to humans is poorly understood. We have developed many cell models *in vitro* to systematically study the putative cytotoxicity of nanomaterials, including CNTs, on many cell types. Because pancreatic cancer is an aggressive cancer with poor prognosis and current treatments are ineffective, new and/or improved treatments are urgently needed. Since CNTs' putative cytotoxic effects on pancreatic cancer cells are unknown, we examined the effects of functionalized (carboxylated (SMWCNT-COOH)) and non-functionalized SMWCNTs on PANC-1 pancreatic cells. Exposure of PANC-1 cells to SMWCNTs-COOH and SMWCNTs induced dose- and time-related decreases in their survival, the non-functionalized SMWCNTs being more cytotoxic. The non-functionalized SMWCNTs exerted more apoptotic and metabolic effects on these cells than the SMWCNT-COOH. Thus, our findings may have pathophysiological implications in the biocompatibility and health hazard of CNTs. Furthermore, they suggest the cytotoxic effects of CNTs could be productively exploited in designing new drug delivery systems to treat this deadly cancer.

Keywords: Functionalized and non-functionalized carbon nanotubes, cytotoxicity, pancreatic cancer cells, nanotoxicity, anti-cancer drug delivery

1 INTRODUCTION

Extensive recent research in structure and function of carbon nanotubes (CNTs) has elucidated the unique physico-chemical properties of CNTs [1, 2]. Increasingly diverse industrial and biomedical applications of CNTs have exploited their unique properties to some advantages [3-5]. Such applications include, but are not limited to, fabrications of transistors, capacitors, actuators, electrodes, catalysts, and sensors [1, 2]. Their ubiquity in numerous and diverse industries suggests humans are likely to be increasingly exposed to CNTs [3-5]. Nonetheless, the health hazard of exposing humans to CNTs is as yet poorly understood [3-5].

We have developed a variety of cell models *in vitro* to allow us to launch a series of studies to systematically investigate the putative cytotocity of a variety of nanomaterials, including CNTs, on many tumor as well as normal cell types [6-10]. Consequently, employing these models we have been able to make significant advances in understanding the molecular mechanisms underlying the putative cytotoxic effects of a variety of nanomaterials, especially nanoparticles and CNTs [6-10, 13, 14]. Our recent studies of the cytotoxic effects of nanoparticles on several neurotumor cell types, including astrocytoma and neuroblastoma, prompted us to propose that the nanoparticles' cytotoxic effects on cancer cells could be gainfully exploited when the nanomaterials are employed to deliver anti-cancer drugs to target tumors [6-10]. Because CNTs have only been minimally studied in this context comparted to nanoparticles, we have focused this study on the putative cytotoxic effects of short multi-wall carbon nanotubes (SMWCNTs) on pancreatic cancer cells.

Because pancreatic cancer is an aggressive form of cancer with poor prognosis and very low five-year survival rate and chemotherapeutic and radiation treatments are ineffective, there is an urgent need for new and/or improved treatment for this deadly cancer [11, 12].

Based on their unique properties, CNTs have been examined for their potential as drug delivery vehicle and diagnostic agents. Modifications of CNTs with functional groups improves solubility/dispersibility and may serve as attachment site of other molecules and/or drugs [3]. Nonetheless, the putative cytotoxic effects of functionalized and non-functionalized CNTs on pancreatic cancer cells have not been systematically investigated. This study was

initiated to examine the effects of functionalized (namely carboxylated (SMWCNTs-COOH)) and non-functionalized SMWCNTs on PANC-1 and MIA-PaCa-2 pancreatic cancer cells.

2 MATERIALS AND METHODS

2.1 Materials

Thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). The antibodies (monocloncal) employed were obtained from Cell Signaling Technology (Beverly, MA, USA). All chemicals were of analytical grade unless otherwise stated.

2.2 Cell Culture

Human pancreatic cancer PANC-1 cells, obtained from ATCC (Manassas, VA, USA), were cultured in an incubator at 37˚ C and 5 % (v/v) CO_2 in RPMI 1640 medium, supplemented with 10% (v/v) FBS whereas MIA-PaCa-2 pancreatic cancer cells, also obtained from ATCC (Manassas, VA, USA), were cultured in DMEM medium, supplemented with 10% (v/v) FBS.

2.3 Cell viability and Apoptosis Assays

The procedure for cell survival assay was essentially the same as that previously published [8, 9]. Briefly, PANC-1 pancreatic cancer cells were plated onto a 96-well plate at 3000 cells/well. After the cells attached to the bottom of the well, they were either treated with SMWCNTs or SMWCNT-COOH at various concentrations (0-200 μg/ml) for 24, 48 or 72 hours. Cells were then exposed to the MTT dye and incubated for a further 4 hours. The purple formazan crystals formed were dissolved in 100 μl of DMSO and the result solution was then measured at 570 nm in a plate reader.

Apoptosis was determined by flow cytometric analysis. MIA-PaCa-2 pancreatic cancer cells were seeded onto 6-well plates and allowed to attach to the bottom of the wells and then treated with SMWCNT or SMWCNT-COOH (10 or 20 μg/ml) for 24 hours. After the treatment, cells were processed as per the manufacturer's protocol for Annexin V/PI staining. Then flow cytometric analysis was performed employing a Millipore Guava Incyte Cytometer.

2.4 Western Blot Analysis

Expression of proteins of interests was determined by Western blot analysis employing the chemiluminescence technique as described previously [9, 10].

2.5 Sea Horse Metabolic Phenotype Analysis

MIA-PaCa-2 pancreatic cancer cells were treated with SMWCNT or SMWCNT-COOH (10 or 20 μg/ml) seeded in Seahorse 8-well miniplates at a density of 30,000 cells per well and cultured overnight. Then, approximately 60 minutes prior to the assay, the culture medium was replaced with the XF basal medium and the OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate) measurements were taken under baseline and stressed conditions according to the manufacturer's protocol.

2.6 Statistical analysis of data

Experiments were performed at least three times with a minimum of 5 replicates for each set, and all data were recorded as mean ± standard deviation (shown in figures). Data analysis was carried out by one-way analysis of variance, followed by post-hoc Student-Newman-Keuls test for multiple comparisons using KaleidaGraph version 4 (Synergy Software, Reading, PA, USA). Significance level was set at $p<0.05$.

3 RESULTS AND DISCUSSION

Treatment of PANC-1 pancreatic cancer cells with 2-200 μg/ml SMWCNT or SMWCNT-COOH for 24 to 72 hours induced multi-phasic time- and dose-related decreases in survival of the pancreatic cancer cells. The dose-related SMWCNT- and SMWCNT-COOH-induced effects were generally more pronounced during the first 24 hours: by 72 hours, the treated cancer cells showed some trend of recovery as their survival had improved (data not shown). Furthermore, the treatment-induced effects were more pronounced for SMWCNT compared to those of SMWCNT-COOH (data not shown). These observations are consistent with our previous observations that the non-functionalized SMWCNT are more cytotoxic to normal neural cells compared to the functionalized SMWCNT (e.g., SMWCNT-COOH) [13, 14].

Because the SMWCNT-induced effects on lowering the survival of pancreatic cancer cells were more noticeable during the first 24 hours of treatment, we investigated some of the mechanisms underlying these early onset effects of treatment with SMWCNT and SMWCNT-COOH.

Treatment	% Apoptosis
Untreated (i.e., control)	1.5 ± 0.5
SMWCNT (10 μg/ml)	$24.0 \pm 0.3^{*}$
SMWCNT-COOH (10 μg/ml)	$17.0 \pm 0.3^{*+}$
SMWCNT (20 μg/ml)	$16.5 \pm 0.1^{*}$
SMWCNT-COOH (20 μg/ml)	$12.5 \pm 0.05^{*+}$

Table 1. Effects of SMWCNT and SMWCNT-COOH on inducing apoptosis in MIA-PaCa-2 pancreatic cancer cells in 24 hours: $^{*}p<0.05$ vs. control; $^{+}p<0.05$ compared with treatment with SMWCNT at the same treatment dose.

We first investigated the possibility that the SMWCNT-induced effects on lowering the survival of pancreatic cancer cells during the first 24 hours of treatment could be attributed, at least in part, to SMWCNT-induced apoptosis. Indeed, as shown in Table 1, the results are consistent with our hypothesis in that at both doses employed, both SMWCNT and SMWCNT-COOH induced significant increases in apoptosis in MIA-PaCa-2 (which are drug- and radiation-resistant) pancreatic cancer cells in the first 24 hours of treatment. It was also interesting to observe that non-functionalized SMWCNT induced significant higher levels of apoptosis in these pancreatic cancer cells than functionalized SMWCNT (namely, SMWCNT-COOH) at both doses employed (Table 1). Again these observations are compatible with our previous findings that the non-functionalized SMWCNT are more cytotoxic to normal neural cells compared to the functionalized SMWCNT (e.g., SMWCNT-COOH) [13, 14].

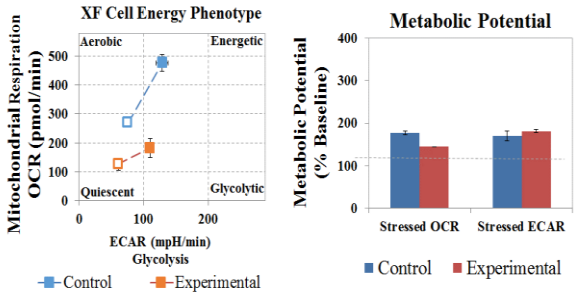

Figure 1. Effects of SMWCNT (10 µg/ml) metabolic phenotype and energetic potential in MIA-PaCa-2 pancreatic cancer cells after overnight treatment as determined using the Seahorse Flux Analyzer. "Control" denotes untreated pancreatic cancer cells while "Experimental" denotes cancer cells treated with SMWCNT.

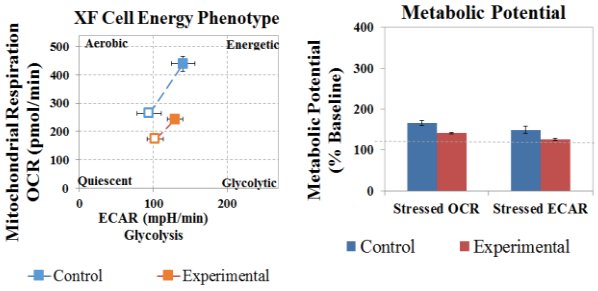

Figure 2. Effects of SMWCNT-COOH (10 µg/ml) metabolic phenotype and energetic potential in MIA-PaCa-2 pancreatic cancer cells after overnight treatment as determined using the Seahorse Flux Analyzer. "Control" denotes untreated pancreatic cancer cells while "Experimental" denotes cancer cells treated with SMWCNT-COOH.

That cancer cells are known to metabolically adapt to enhance their survival and proliferation (the "Warburg effect") [11] prompted us to characterize their metabolic phenotype. In this study, we characterized the metabolic phenotype of MIA-PaCa-2 (which are drug- and radiation-resistant) pancreatic cancer cells after overnight treatment with SMWCNT (Figure 1) or SMWCNT-COOH (Figure 2).

As shown in Figure 1 (left panel), the untreated (i.e., "control") MIA-PaCa-2 pancreatic cancer cells showed substantial basal mitochondrial respiration (OCR) but also significant glycolytic flux as determined by ECAR. Interestingly, when stressed, such cancer cells exhibited some 60% increase in both OCR and ECAR (Figure 1). Upon overnight treatment with non-functionalized SMWCNT, the basal OCR and ECAR of the treated cancer cells were significantly lowered than those in the untreated cancer cells (Figure 1, left panel), their decrease in OCR being more pronounced than their decrease in ECAR suggesting a shift toward glycolysis. When stressed, the OCR of the treated cancer cells dropped to a level below the basal level OCR of the untreated cancer cells while the ECAR of the treated cancer cells decreased only slightly. The comparison plots (Figure 1, right panel) are consistent with these conclusions.

Upon overnight treatment with functionalized SMWCNT (i.e., SMWCNT-COOH), the basal OCR, but not the basal ECAR, decreased by 33% (Figure 2, left panel). When stressed, the OCR of treated cancer cells decreased by 40% while the ECAR in treated cancer cells dropped only slightly compared with corresponding rates in untreated cancer cells (Figure 2, left panel), suggesting a modest shift in favor of glycolytic flux after the treatment of the cancer cells with SMWCNT-COOH. The comparison plots (Figure 2, right panel) are consistent with these conclusions.

When the effects of treatment with non-functionalized SMWCNT were compared with those obtained with functionalized SMWCNT (i.e., SMWCNT-COOH) (compare Figures 1 and 2), it became evident that treatment with non-functionalized SMWCNT induced a decrease in OCR relative to an enhanced increase in ECAR while treatment with functionalized SMWCNT (i.e., SMWCNT-COOH) induced a modest decrease in both OCR and ECAR, indicating that the shift to the glycolytic phenotype induced by non-functionalized SMWCNT was more significant compared to the corresponding shift to the glycolytic phenotype induced by functionalized SMWCNT (i.e., SMWCNT-COOH). Again these observations are compatible with our previous findings that the non-functionalized SMWCNT are more cytotoxic to normal neural cells compared to the functionalized SMWCNT (i.e., SMWCNT-COOH) [13, 14].

4 CONCLUSIONS

As far as we are aware, ours is the first study to report on the putative cytotoxic effects of non-functionalized and functionalized SMWCNT (i.e., SMWCNT-COOH) in pancreatic cancer cells. SMWCNT and SMWCNT-COOH induced differential time- and dose-related decreases in survival of pancreatic cancer cells and apoptosis could, at least in part, account for such effects. As far as we are aware, this is the first study to characterize the metabolic phenotype of pancreatic cancer cells and how treatment with SMWCNT and SMWCNT-COOH induced shifts in the metabolic phenotype of these cancer cells. Clearly, this is an important area that deserves further investigation. Nonetheless, our findings may have pathophysiological implications in the cytotoxicity of carbon nanotubes in pancreatic cancer cells. As such they may have relevance in designing novel anti-cancer drug delivery systems employing carbon nanotubes, whose ultimate goal is for the design of new and/or improved pancreatic cancer therapies.

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