Entry of Short Multi-Wall Carbon Nanotubes into Dorsal Root Ganglion (DRG) Neurons Induces Cell Death

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ABSTRACT

Carbon nanotubes (CNTs) have found their way into diverse industrial and biomedical applications because of their unique physico-chemical properties. However, the human health impact of exposure to CNTs is unknown. Our recent and ongoing studies focus on the putative cytotoxicity of CNTs in neural cells as neurotoxicity of CNTs has not been reportedly studied. We have therefore investigated the putative cytotoxic effects of two functionalized, namely carboxylated and hydroxylated, and non-functionalized short multi-wall carbon nanotubes (SMWCNTs) on dorsal root ganglion (DRG) neurons, which constitute an excellent model *in vitro* of neurons derived from the peripheral nervous system (PNS). We hypothesized that treatment with CNTs induces a dose-related decrease in the viability of DRG neurons and functionalization of CNTs modulates the cytotoxicity profile of CNTs. Employing confocal microscopy, we found that exposure of DRG neurons to SMWCNTs resulted in the entry of the SMWCNTs into the cytoplasm of the neurons. Apparently, concomitant with the entry of SMWCNTs into DRG neurons, their survival was greatly decreased dependent on the levels of SMWCNTs, the non-functionalized being more cytotoxic than the functionalized SMWCNTs. Thus, our results may have pathophysiological implications in how exposure to SMWCNTs impacts on the structure and function of the PNS.

Key words: carbon nanotubes, cytotoxicity of carbon nanotubes, peripheral nervous system, DRG neuron, biocompatibility, nanotoxicity

1 INTRODUCTION

Advances in research in structure and function of carbon nanotubes (CNTs) have revealed that they possess unique physico-chemical properties. Diverse industrial and biomedical applications of CNTs have clearly exploited their properties to some advantage. Such applications of CNTs include fabrications of transistors, capacitors, actuators, electrodes, catalysts, and sensors although this list of their applications is ever-increasing. [1-3]. Consequently, their escalating uses in multiple and diverse industries imply that humans are likely to be increasingly exposed to CNTs. However, the health hazard of exposure of humans to CNTs is as yet poorly understood [1-4]. Moreover, the effects of CNTs on the nervous system and/or neural cells are virtually unknown [3].

We have developed several cell models *in vitro* to allow us to launch a series of studies to systematically investigate the putative cytotoxicity of various nanomaterials, including CNTs. [3,5-15]. As the putative neurotoxicity of CNTs has not been reportedly investigated [3,4], we have determined the putative cytotoxic effects of two functionalized, namely carboxylated and hydroxylated, and non-functionalized short multi-wall carbon nanotubes (SMWCNTs) on dorsal root ganglion (DRG) neurons, which constitute an excellent model *in vitro* of neurons derived from the peripheral nervous system (PNS) [5-15]. We hypothesized that treatment with CNTs induces a dose-related decrease in the viability of DRG neurons and functionalization of CNTs modulates the cytotoxicity profile of CNTs.

2 MATERIALS AND METHODS

2.1 Materials

50B 11 DRG neurons were kind gifts from Dr Hoke's Laboratory at Johns Hopkins School of Medicine. 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). All chemicals were of analytical grade unless otherwise stated.

2.2 Cell Culture

Doral root ganglion (DRG) neurons were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

2.4 MTT Assay

Cell survival and growth was determined using the MTT assay [8,9]. DRG neurons were cultured as described in the cell culture subsection and were treated with specified concentrations of carbon nanotubes for specified times. At the end of the treatment periods, MTT dye (0.5%, w/v, in PBS) was added to each well and the plates were incubated for an additional 4 hours at 37°C. Purple-color insoluble formazan crystals in viable cells were dissolved using DMSO, and the subsequent absorbance of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA) [8,9].

2.5 Other Methods

The assay of lactate dehydrogenase (LDH) release from cells (a marker of necrotic damage or necrotic cell death) and Western blot analysis for detection of protein expression were carried out as we had described previously [9,12].

2.6 Statistical Analysis of Data

Results are presented as mean ± standard error of the mean (S.E.M.) of 6 determinations in each experiment. Replicate experiments showed essentially the same patterns of results.

3 RESULTS AND DISCUSSION

Employing the MTT assay, we compared the effects of non-functionalized short multi-wall carbon nanotubes (SMWCNTs) with those of two functionalized, namely hydroxylated and carboxylated, SMWCNTs on dorsal root ganglion (DRG) neurons [16]. As we previously reported [16], exposure of DRG neurons to non-functionalized SMWCNTs induced a concentration- and time-related decreases in su liferation, and/or growth of the neurons.

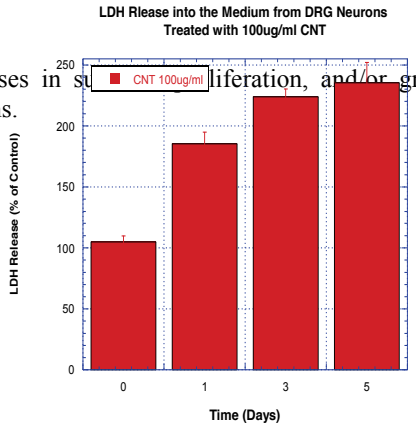

Figure 1: Effect of non-functionalized short multi-wall carbon nanotubes (SMWCNTs) on lactate dehydrogenase (LDH) release from dorsal root ganglion (DRG) neurons into the medium. DRG neurons were treated with SMWCNTs at 100 µg/mL; at the end of the specified time, the medium was collected and kept frozen at -70°C until they were used to assay the LDH activity therein. The LDH released into the medium was normalized with respect to that obtained at zero time (i.e., the 100%, at the start of the experiment). Values are the mean ± S.E.M. of at least three separate determinations.

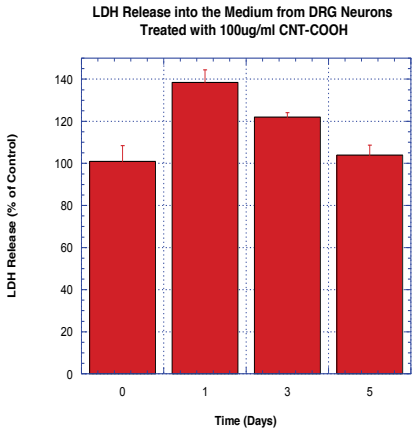

Figure 2: Effect of carboxylated short multi-wall carbon nanotubes (SMWCNTs) on lactate dehydrogenase (LDH) release from dorsal root ganglion (DRG) neurons into the medium. Other details were the same as those in Figure 1

except carboxylated SMWCNTs were used. Values are the mean ± S.E.M. of at least three separate determinations.

Exposure of DRG neurons to hydroxylated or carboxylated SMWCNTs also induced a concentration- and time-related decreases in survival, proliferation, and/or growth of the neurons [16]. However, while the effects of the functionalized SMWCNTs were somewhat similar, the effects of the non-functionalized SMWCNTs were generally more pronounced than those of the functionalized SMWCNTs, especially at the higher treatment concentrations of SMWCNTs [16]. Those results [16] suggested that non-functionalized SMWCNTs are generally more cytotoxic to DRG neurons compared to the functionalized SMWCNTs.

Because the SMWCNTs clearly exerted cytotoxic effects on DRG neurons [16], we were interested to determine whether the CNTs exerted their effects upon entering the cytoplasm of the DRG neurons or they exerted their effects via interactions with the DRG neurons on their cell surface. Our initial light microscopic observations suggested after DRG neurons had been treated with SMWCNTs, there were some internalization of the CNTs by the neurons (data not shown). We then employed confocal microscopy to help ascertain that our conclusion was tenable. Based on co-localization criteria, our confocal microscopic observations strongly suggest exposure of DRG neurons to SMWCNTs resulted in the entry of the SMWCNTs into the cytoplasm of the neurons (data not shown). Apparently, concomitant with the entry of the SMWCNTs into DRG neurons, their survival was greatly decreased dependent on the levels of SMWCNTs.

To further elucidate some of the putative mechanisms underlying the cytotoxic effects of functionalized and non-functionalized SMWCNTs on DRG neurons, we investigated the effect of the nanotubes on lactate dehydrogenase (LDH) release (a marker of necrotic damage and/or necrotic cell death) by DRG neurons into the medium when the neurons were treated with the non-functionalized SMWCNTs. When DRG neurons were treated with 100 μg/mL of non-functionalized SMWCNTs, they showed time-related increases in the release of LDH into the medium (Fig. 1), suggesting that necrosis may at least be one cell death mechanism responsible for lowering the survival of DRG neurons induced by the non-functionalized SMWCNTs. Although carboxylated SMWCNTs also induced some time-dependent LDH release into the medium by DRG neurons (Fig. 2), the effect of the carboxylated SMWCNTs was quite modest compared to that noted in non-functionalized SMWCNTs (Fig. 1), suggesting that necrosis played only a minor role in lowering the survival of DRG neurons induced by carboxylated SMWCNTs.

To investigate the possibility that other than necrosis, other molecular mechanisms could also be induced by non-functionalized SMWCNTs in lowering the survival of DRG neurons, we have examined the effects of non-functionalized SMWCNTs on the expression of cell signaling proteins in DRG neurons. Results of our ongoing studies suggest that the expression of Akt and phospho-Akt was decreased in DGR neurons treated with 100 μg/mL of non-functionalized SMWCNTs (data not shown). Akt and phosphor-Akt are important signaling protein involved in cell survival and/or proliferation [see 12 and references therein]. Thus, our observation suggests that another mechanism whereby the non-functionalized SMWCNTs induced in DRG neurons in lowering their survival was through decreasing their survival and/or proliferation signaling mechanisms such as those involving Akt and phospho-Akt.

4 CONCLUSIONS

SMWCNTs induced time- and concentration-related decreases in survival, proliferation and/or growth of DRG neurons, the non-functionalized ones being more cytotoxic than the functionalized ones, especially at the higher treatment concentrations. The necrotic damage and cell death induced by non-functionalized SMWCNTs in DRG neurons was much more marked compared to that induced by carboxylated SMWCNTs. Our confocal microscopic observations strongly suggest exposure of DRG neurons to SMWCNTs resulted in the entry of the SMWCNTs into the cytoplasm of the neurons. Apparently, concomitant with the entry of the SMWCNTs into DRG neurons, their survival was greatly decreased dependent on the levels of SMWCNTs. Additionally, results from our ongoing studies suggested that DRG neurons treated with a high concentration of non-functionalized SMWCNTs exhibited decreased expression of phospho-Akt, a cell survival and proliferation signal. Thus, our results may assume pathophysiological importance in determining how exposure to SMWCNTs alters the structure and function of the peripheral nervous system. Furthermore, they may also have implications in neurodegenerative mechanisms induced by exposure to SMWCNTs. Nevertheless, we need to emphasize that our understanding of the putative cytotoxic and neurotoxic effects of carbon nanotubes is still very minimal.

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