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Multi-walled carbon nanotubes induce T lymphocyte apoptosis

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Abstract

Carbon nanotubes are a man-made form of carbon that did not exist in our environment until very recently. Due to their unique chemical, physical, optical, and magnetic properties, carbon nanotubes have found many uses in industrial products and in the field of nanotechnology, including in nanomedicine. However, very little is yet known about the toxicity of carbon nanotubes. Here, we compare the toxicity of pristine and oxidized multi-walled carbon nanotubes on human T cells and find that the latter are more toxic and induce massive loss of cell viability through programmed cell death at doses of 400 μg/ml, which corresponds to approximately 10 million carbon nanotubes per cell. Pristine, hydrophobic, carbon nanotubes were less toxic and a 10-fold lower concentration of either carbon nanotube type were not nearly as toxic. Our results suggest that carbon nanotubes indeed can be very toxic at sufficiently high concentrations and that careful toxicity studies need to be undertaken particularly in conjunction with nanomedical applications of carbon nanotubes.

Keywords: Multi-walled carbon nanotubes; T lymphocytes; Jurkat; Apoptosis

1. Introduction

Nanotechnology is the manufacture and science of materials with at least one dimension in the nanometer scale. Many nanomaterials have novel chemical and biological properties and most of them are not naturally occurring. Carbon nanotubes (CNTs) are an example of a carbon-based nanomaterial (Iijima, 1991), which has won enormous popularity in nanotechnology for its unique properties and applications (Dresselhaus et al., 2001). CNTs have physicochemical properties that are highly desirable for use within the commercial, environmental, and medical sectors. The inclusion of CNTs to improve the quality and performance of many widely used products, as well as potentially in medicine, it is likely that occupational and public exposure to CNT-based nanomaterials will increase dramatically in the near future.

Very little is yet known about the toxicity of CNTs, which exist in many different forms and can be chemically modified and/or functionalized with biomolecules. Pristine single-walled CNTs are extremely hydrophobic tubes of hexagonic carbon (graphene) with diameters as small as 0.4 nm and lengths up to micrometers. Multi-walled CNTs consist of several concentric graphene tubes and diameters of up to 100 nm. These pristine

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CNTs are chemically inert and insoluble in aqueous media and therefore of little use in biological or medical applications. Due to the hydrophobicity and tendency to aggregate, they are harmful to living cells in culture (Cui et al., 2005; Monteiro-Riviere et al., 2005).

For many applications, CNTs are oxidized in strong acid to create hydroxyl and carboxyl groups (Liu et al., 1998), particularly in their ends, to which biomolecules or other nanomaterials can be coupled (Bottini et al., 2005). These oxidized CNTs are much more readily dispersed in aqueous solutions and have been coupled to oligonucleotides, proteins, or peptides. Indeed, CNTs have been used as vehicles to deliver macromolecules that are not able pass through the cellular membrane by themselves into cells (Pantarotto et al., 2004; Shi Kam et al., 2004).

Since little is yet known about the toxicity of CNTs, particularly of oxidized CNTs, we compared these two types of CNTs in a number of functional assays with human T lymphocytes, which would be among the first exposed cell types upon intravenous administration of CNTs in therapeutic and diagnostic nanodevices.

2. Materials and methods

2.1. Carbon nanotubes

Carbon black (CB) was generously provided by Solution Dispersion Inc. (Cynthiana, KY, USA). Multi-walled CNTs with outer diameters of 20–40 nm, lengths of 1–5 μm, and a purity of >95%, were purchased from Nano Lab. Inc. (Newton, MA, USA). Oxidized CNTs were generated by refluxing CNTs in concentrated nitric acid (Sigma Inc., St. Louis, MO, USA) for 6 h. The material was washed several times with distilled water by ultracentrifugation (120,000 × *g* for 12 h). Each

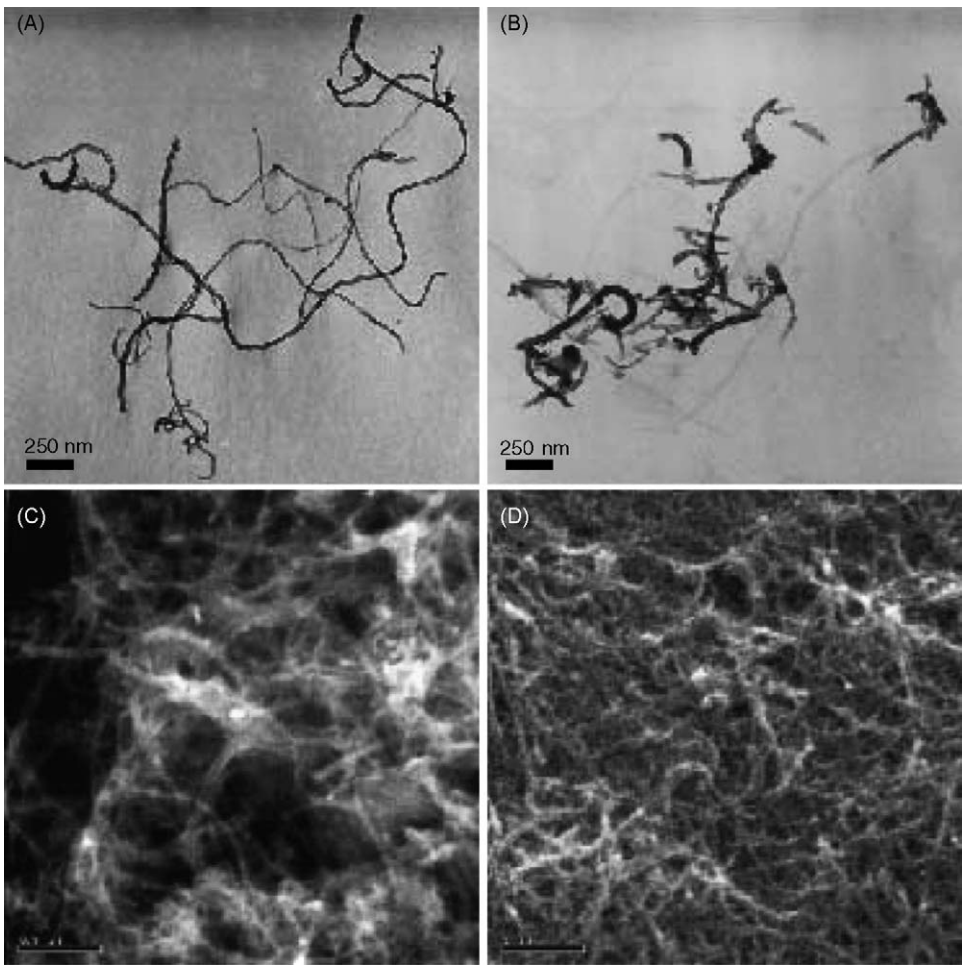

Fig. 1. Transmission electron microscopy (A and B) and scanning electron microscopy (C and D) images of pristine CNTs (A and C) and oxidized CNTs (B and D). Note that the latter appear shorter and straighter.

supernatant was carefully decanted and its pH was recorded. The pH of the last collected supernatant was approximately 6.2, which was not able to affect the pH of the buffered cellular medium. After this treatment, the oxidized CNTs were shorter and straighter than the non-treated (pristine) CNTs when viewed by transmission or scanning electron microscopy (Fig. 1).

2.2. Cell culture

T lymphocytes from healthy human blood donors were isolated as before (Bruckner et al., 2005). Jurkat T leukemia cells were kept in logarithmic growth by culture in RPMI-1640 cell culture medium (Invitrogen Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). Incubation of cells was done by adding 0.5 ml of nanomaterial (CB or CNTs, both pristine and oxidized) dispersion in water, or 0.5 ml water alone, to 4.5 ml of cell suspension containing 2×10^5 cells at 37 °C in 5% CO_2 atmosphere. The final concentrations of the added nanomaterial (NM) were $c_1 = 1$ ng NM/cell and $c_2 = 10$ ng NM/cell.

2.3. Cell viability and proliferation assay

Trypan Blue exclusion was used to evaluate the effects of CNTs on cell viability. Cell aliquots were collected at different time intervals (0, 24, 48, 72, 96, 120 h) and immediately stained for 5 min with Trypan Blue in different ratios. Cell proliferation was measured by counting cells in a Neubauer hemocytometer. Statistics were calculated on three samples for each dilution and relative cell viability was calculated as a ratio between cell viability of treated samples and that of control untreated cells. The standard deviation for relative cell viability was calculated using the following formula: for the standard deviation (Δz) of the ratio $z = a/b$, $\Delta z = ((\Delta a/a)^2 + (\Delta b/b)^2)^{1/2}$, where Δa and Δb were the standard deviation of a and b, respectively. The different treatments were compared using analysis of variance (ANOVA, PRISM 4 for Windows, GraphPad Software Inc., San Diego, CA, USA).

2.4. Cell death assay

The percentage of apoptotic Jurkat or peripheral blood lymphocytes (PBL) was determined at the indicated time points by using annexin V-FITC (BioVision Research Products, Mountain View, CA, USA) according to the manufacturer's protocol, as we have done before and with etoposide as a positive control (Bruckner et al., 2005). Briefly, the cells were washed in PBS, stained with annexin V-FITC for 5 min in the dark, plated onto 8-well coverslip bottom chamber slides that had been coated with 0.1% (w/v) poly-D-lysine, and immediately analyzed by fluorescence microscopy. Cells positive for annexin V-FITC were determined to be apoptotic. The total number of cells in each field was determined by Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) (1 μg/ml) staining.

3. Results

The effects of CNTs on T-cell viability was first evaluated using the Trypan Blue exclusion assay. Viable cells with an intact plasma membrane do not stain with this dye, while dead cells become intensely blue. As shown in Fig. 2, CNTs caused a time-dependent decrease in the viablity of Jurkat T leukemia cells. At 400 μg/ml (equals 10 ng/cell), oxidized CNTs caused a loss of >80% of the cells within 5 days, while pristine CNTs killed less than half. A smaller dose, 40 μg/ml (1 ng/cell), had a much smaller effect, but again the oxidized CNTs reduced cell growth more than pristine CNTs. In comparison, even 400 μg/ml of amorphous carbon, CB, had a minimal effect on cell viability. Thus, we find that pristine CNTs are more toxic than CB at identical chemical amounts of atomic carbon and, furthermore, that oxidized CNTs appear to be more harmful to cells than pristine CNTs.

To determine if the reduced cell viability observed in the presence of CNTs resulted from the induction of

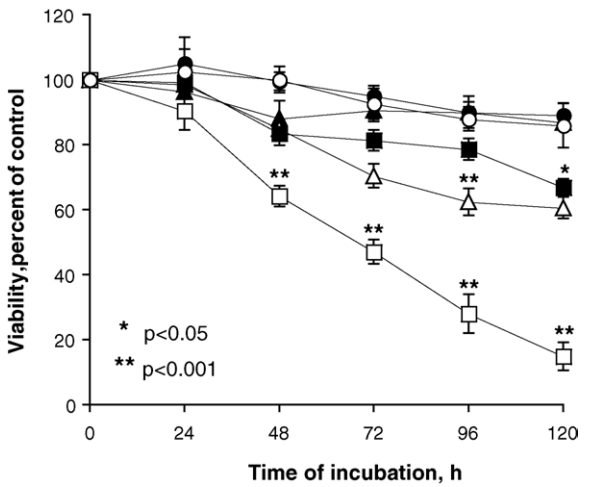

Fig. 2. Dose- and time-dependent reduction in human T-cell viability by CNTs. The graph shows the viability of Jurkat T-cells incubated with 1 ng/cell (closed symbols) or 10 ng/cell (open symbols) of CB (circles), pristine CNTs (triangles) or oxidized CNTs (squares) for the indicated periods of time. The values were calculated as percent of control (untreated cells) and represent the mean ± S.D. for six independent determinations. Statistical significance was calculated using ANOVA and is indicated with (*) for $p < 0.05$ and (**) for $p < 0.001$.

programmed cell death, we used the annexin V binding assay, which measures the loss of the asymmetrical segregation of phosphatidylserine to the inner leaflet of the plasma membrane. Annexin V binds to phosphatidylserine and thus reveals if this phospholipid is exposed on the extracellular surface of cells, a phenomenon known to reflect apoptosis. Indeed, treatment of Jurkat T cells with CNTs increased the number of cells staining with annexin V (Fig. 3A). Higher magnification also revealed chromatin condensation and membrane blebbing (Fig. 3B), two classical hallmarks of cells undergoing apoptosis. The effect of CNTs treatment was both dose-dependent and time dependent (Fig. 3C). Both pristine and oxidized CNTs also induced apoptosis in freshly isolated primary human T lymphocytes (Fig. 3D) in a dose-dependent and time-dependent manner. In all these experiments, oxidized CNTs appeared to be more toxic than the pristine CNTs.

We also tested whether CNTs would affect signal transduction by the T-cell antigen receptor by

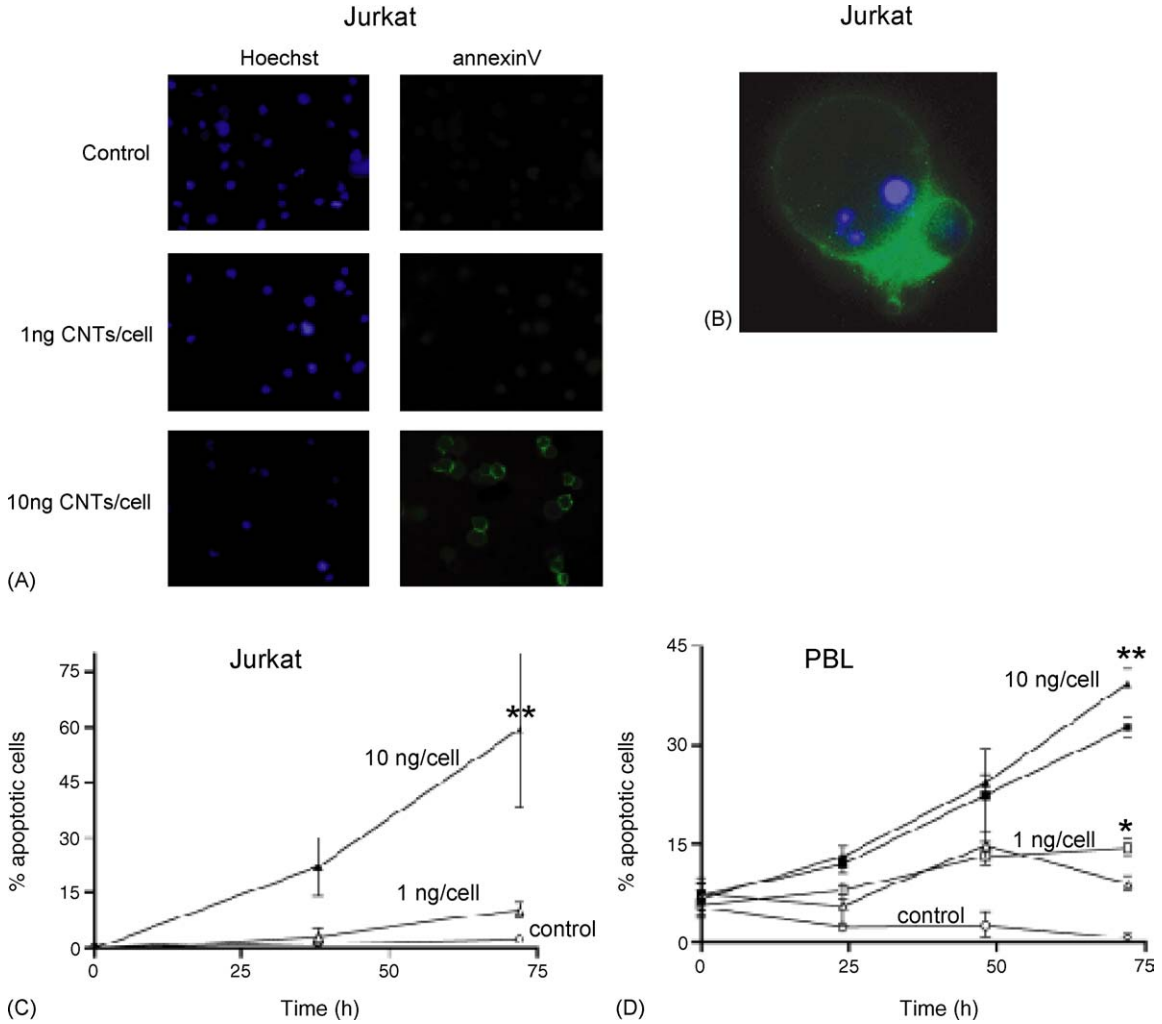

Fig. 3. CNTs induce apoptosis of human T cells. (A) Immunofluorescence images of untreated Jurkat cells (upper panels) and of Jurkat cells treated for 24 h with 1 ng/cell (middle panels) or 10 ng/cell (lower panels) of oxidized CNTs. Right panels show cells stained with annexin V-FITC, left panels shows nuclear staining of same cells with Hoechst 33342. (B) At higher magnification annexin V positive Jurkat cells show pyknotic nuclear DNA condensation and membrane blebbing, two typical features of apoptotic cell death. (C) Time course of Jurkat cell apoptosis after treatment with oxidized CNTs. Graph shows percentage of annexin V positive cells after incubation with 1 ng/cell (open triangles) or 10 ng/cell (filled triangles) of oxidized CNTs. Open circles show percentage of apoptosis of control cells. (D) Time course of human peripheral blood lymphocytes (PBL) apoptosis after treatment with oxidized CNTs. Graph shows percentage of annexin V positive cells at three time points after incubation with 1 ng/cell (open symbols) or 10 ng/cell (filled symbols) of pristine (squares) or oxidized (triangles) CNTs. Open circles show percentage of apoptosis of control untreated PBL. Statistical significance was calculated as in the figure and is indicated with (*) for $p < 0.05$ and (**) for $p < 0.001$.

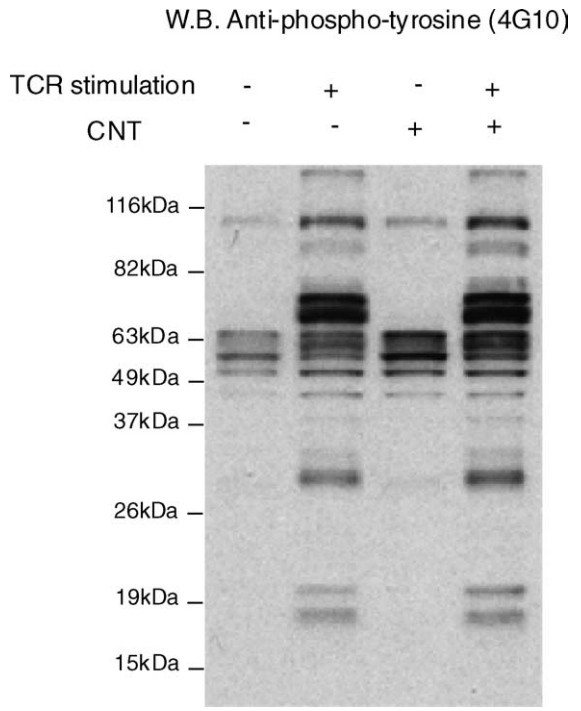

Fig. 4. Effects of CNTs on T-cell antigen receptor signaling. Figure shows an anti-phosphotyrosine Western blot of equal amount of total proteins from untreated Jurkat cells (lane 1 and 2) and Jurkat cells treated with 40 μg/ml of oxidized CNT for 24 h. Lanes 1 and 3 show resting T cells, lane 2 and 4 show cells stimulated with anti-CD3ε plus anti-CD28 mAbs for 5 min.

anti-phosphotyrosine immunoblotting of lysates of cells left untreated or stimulated through the receptor by crosslinking antibodies. As shown in Fig. 4, 40 μg/ml of oxidized CNTs had a small stimulatory effect on the basal level of intracellular tyrosine phosphorylation, but virtually no effect on the receptor-induced increase in this phosphorylation.

4. Discussion

Our results reveal that CNTs indeed can be harmful to cells in a time- and dose-dependent manner. Similar findings were reported for pristine single-walled CNTs on the proliferation of HEK293 kidney epithelial cells (Cui et al., 2005) and pristine multi-walled CNTs on skin epithelial cells (Monteiro-Riviere et al., 2005). Significantly, we find that the physical form of carbon has a major impact on toxicity: CNTs are more toxic than similar chemical amounts of carbon in the form of amorphous carbon black, which is quite non-toxic even at the highest tested concentrations (400 μg/ml). Thus, the molecular structure and topology is essential for the toxicity of a carbonaceous nanomaterials. Furthermore, the more hydrophobic pristine CNTs appear to be less toxic than the oxidized CNTs. The increased toxicity of oxidized CNTs, which are considered better suited for biological applications, may well be because they are better dispersed in aqueous solution and therefore reach higher concentrations of free CNTs at similar weight per volume values. We calculate that the less toxic amount of 40 μg/ml of CNTs under our experimental conditions is equal to approximately 10^6 individual CNTs per cell, based on an average length of 1 μm and a diameter of 40 nm, giving an average molecular mass of 5×10^9 Da. Our results establishes this amount as an upper limit of how many CNTs cells should be exposed to, for example, in applications within the field of nanomedicine.

Since an assay for cell viability may not reveal all harmful effects of a toxic compound, we also examined the function of T cells by measuring the response of these cells to triggering of the T-cell antigen receptor. Ligation of this receptor triggers a complex biochemical signaling cascade that ultimately leads to changes in the expression of many genes, increased metabolism, cytoskeletal rearrangements, activation of the effector functions of the T cell (e.g. secretion of cytokines or killing of target cells) and the initiation of an immune response. The earliest events associated with T-cell antigen receptor signaling include the recruitment of tyrosine kinases and increased tyrosine phosphorylation of many key signaling proteins (Mustelin and Tasken, 2003). We examined this phosphorylation by immunoblotting of cell extracts with monoclonal antibodies against phosphotyrosine, which revealed that 40 μg/ml of CNTs had no detrimental effects on the receptor-induced T-cell activation. In fact, there may even be a slight stimulation of this response. Thus, 40 μg/ml of CNTs did not seem to have any toxic effects on the function of T cells, supporting the notion that this amount does not measurably harm the cells.

Our results do not imply that CNTs should be abandoned for biological or medical purposes, but our study sets an upper limit for the concentration of CNTs that can be safely used. We recommend that CNTs be used a much less than 40 μg/ml (or 1 ng/cell) and that cell viability and well-being be followed carefully with all new forms of CNTs and CNT-containing nanodevices. It is likely that CNT toxicity will depend on many other factors than concentration, including their physical form, their diameter, their length, and the nature of attached molecules or nanomaterials. These considerations should be constantly kept in mind and re-visited frequently during the development of CNT-based devices for nanomedicine, as well as for evaluating hazards of occupational or environmental exposure to nanomaterials.

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