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RESEARCH ARTICLE

Single-walled carbon nanotubes impair human macrophage engulfment of apoptotic cell corpses

Erika Witasp[1], Anna A. Shvedova[2], Valerian E. Kagan[1,3], and Bengt Fadeel[1]

[1]Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, [2]Pathology/Physiology Research Branch, Health Effects Laboratory Division (HELD), National Institute for Occupational Safety and Health (NIOSH), Morgantown, West Virginia, USA, and [3]Center for Free Radical and Antioxidant Health, Graduate School of Public Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Abstract

Single-walled carbon nanotubes (SWCNT) are being produced in increasing quantities and the application of these materials in a large number of new technologies and consumer products necessitates studies of their potential impact on human health and the environment. To determine whether SWCNT affect viability or function of macrophages, important components of the innate immune system, we performed in vitro studies using primary human monocyte-derived macrophages (HMDM). Our findings show that SWCNT with a low content of metal impurities do not exert direct cytotoxic effects on HMDM. However, SWCNT suppressed chemotaxis of primary human monocytes in a standard chemotaxis assay. Moreover, macrophage engulfment of apoptotic target cells was significantly impaired following pre-incubation of HMDM with SWCNT at non-cytotoxic concentrations. These results are in line with previous studies showing that ultrafine carbon particles and carbon nanotubes may impair alveolar macrophage ingestion of microorganisms, and suggest that tissue homeostasis may be compromised by SWCNT due to suppressive effects on macrophages.

Keywords: *Single-walled carbon nanotubes; human monocyte-derived macrophages; phagocytosis; chemotaxis; cytotoxicity*

Introduction

Carbon nanotubes were discovered more than 15 years ago and have become a focus of intensive research and development due to the unique physicochemical properties of these materials. The exceptional features of this class of engineered nanomaterials in combination with increasing applications of carbon nanotubes in a wide range of areas raise concerns regarding their environmental and ecological impact as well as potential adverse effects on human health (Donaldson et al., 2006; Fadeel et al., 2007). Our previous work has demonstrated that SWCNT cause an unusual and robust inflammatory response in rodents with very early termination of the acute phase and rapid onset of chronic fibrosis (Shvedova et al., 2005). More recent studies have disclosed a role for a functional NADPH oxidase in phagocytes in determining the course of the pulmonary response to SWCNT (Shvedova et al., 2008b).

Macrophage recognition and clearance of apoptotic cell corpses, a process referred to as programmed cell clearance, is essential for maintenance of tissue homeostasis, and an impairment of this process may lead to chronic inflammation, autoimmune disease, and other pathological conditions (Witasp et al., 2008). Loss of plasma membrane phospholipid asymmetry with externalization on the cell surface of the anionic phospholipid phosphatidylserine (PS) is a common event during apoptosis and serves as an important “eat-me” signal for phagocytes (Kagan et al., 2003). Moreover, serum factors or “bridging molecules” that bind to PS and/or other ligands on the surfaces of apoptotic cells and phagocytes are also important for efficient clearance of dying cells by macrophages (Witasp et al., 2008).

In the present study, we studied whether purified SWCNT affect two important functions of macrophages, chemotaxis (migration) and phagocytosis of apoptotic cells. Our findings

Address for correspondence: Bengt Fadeel, MD, PhD, Cell Death Research Group, Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet, 171 77 Stockholm, Sweden. E-mail: bengt.fadeel@ki.se

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demonstrate that SWCNT do not induce a loss of cell viability in primary human macrophages at the doses and time points tested, but macrophage internalization of apoptotic target cells is compromised.

Materials and methods

SWCNT procurement and characterization

SWCNT (CNI, Inc., Houston, TX) produced by the high-pressure CO disproportionation process (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor (Bronikowski et al., 2001), and purified by acid treatment to remove metal contaminates (Gorelik et al., 2000), were used in this study. Chemical analysis of total elemental carbon and trace metal (iron) in SWCNT was performed at the Chemical Exposure and Monitoring Branch (DART/NIOSH, Cincinnati, OH). Elemental carbon in SWCNT was assessed according to the *NIOSH Manual of Analytical Methods* (NMAN) (Birch, 2003), while metal content (iron) was analyzed using nitric acid dissolution and inductively coupled plasma–atomic emission spectrometry (ICP-AES) performed according to NMAM method 7300 for trace metals. For purity assessment of HiPco SWCNT, several standard analytical techniques were used, including thermogravimetric analysis with differential scanning colorimetry (TGA-DSC), thermoprogramming oxidation (TPO), Raman spectroscopy, and near infrared (NIR) spectroscopy (Arepalli et al., 2004; Dresselhaus et al., 2004). Specific surface area was measured at −196°C by the nitrogen absorption–desorption technique (Brunauer Emmet Teller method, BET) using an SA3100 surface area and pore size analyzer (Beckman Coulter, Inc., Fullerton, CA).

Transmission electron microscopy

The sample of purified SWCNT was diluted in double-distilled filtered water. No stabilizing agent was added to the solution. The solution was then mixed and a drop was placed on a Formvar-coated copper grid and allowed to air dry. Images were photographed on a JEOL 1220 transmission electron microscope (TEM) (JEOL, Ltd., Tokyo).

Macrophage isolation and culture

Human mononuclear cells were prepared from buffy coats obtained from adult blood donors by density-gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). Monocytes were separated by adhesion to tissue culture plastic for 1 h at 37°C and non-adherent cells were washed off with phosphate-buffered saline (PBS). Cells were then grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To generate activated macrophages, human monocyte-derived macrophages (HMDM) were cultured in the presence of recombinant macrophage colony-stimulating factor (M-CSF) (50 ng/ml) (R&D Systems, Minneapolis, MN) for 3 days.

Jurkat cell culture and treatment

Human Jurkat T lymphoblastic leukemia cells (European Cell Culture Collection, Salisbury, UK) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 m*M* glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. To induce apoptosis, Jurkat cells were incubated overnight (19 h) with the chemotherapeutic agent, etoposide (Bristol-Myers Squibb, Stockholm, Sweden).

Cell viability assessment

Trypan blue exclusion was used to assess viability of macrophages incubated with SWCNT for the indicated time points. HMDM were cultured in the absence of M-CSF, and triplicate samples were assessed for each condition.

PS externalization

PS exposure was determined by flow cytometry using the annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA). Cells (0.5×10^6) were co-stained with propidium iodide (50 μg/ml) before analysis with a FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488-nm argon laser. Ten thousand events were collected and analyzed using the CellQuest software (BD Biosciences). Cell debris was gated out prior to analysis based on light scattering properties.

Chemotaxis assay

Monocyte chemotaxis toward SWCNT was evaluated using a standard two-chamber system in a 24-well plate format (BD Biocoat Laminin Inserts; BD Biosciences, Bedford, MA). The upper chamber consisted of an insert equipped with a laminin-coated membrane with a pore size of 3 μm. The inserts were blocked with 0.1% FBS prior to the assay. Cell culture medium containing SWCNT was added to the lower compartment. Recombinant human monocyte-chemoattractant protein-1 (MCP-1) (300 ng/ml) (R&D systems, Abingdon, UK) was used as a positive control, and cell culture medium alone as negative control. Monocytes (4.0×10^5) in cell culture medium were added to upper compartment, and the chambers were incubated for 4 h at 37°C. The inserts were then removed and both chambers were washed thoroughly with ice-cold 5 m*M* ethylenediamine tetraacetic acid (EDTA). The chemotactic index was calculated as the ratio of the number of monocytes migrated toward MCP-1 or SWCNT and the number of monocytes migrated toward cell culture medium alone.

Phagocytosis assessment

Phagocytosis of apoptotic target cells was performed essentially as described previously (Kagan et al., 2002). In brief, Jurkat cells were prestained with 5-carboxy-tetramethyl-rhodamine-*N*-hydroxy-succinimide ester (TAMRA) (Sigma, St. Louis, MO) and were then incubated in medium alone or with etoposide to trigger apoptosis, as described earlier. Jurkat cells were subsequently added to HMDM in 24-well tissue culture plates at a ratio of 10:1. Macrophages were pre-incubated for 4 h with SWCNT or PBS alone and washed

3 times prior to co-cultivation with target cells. Following co-cultivation for 1 h, non-engulfed cells were washed off and the remaining cells were fixed in 4% paraformaldehyde, followed by visualization of cell nuclei with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Phagocytosis was evaluated by counting at least 500 macrophages per experimental condition in visual light and thereafter counting macrophage-engulfed cells under ultraviolet (UV) illumination using an inverted Nikon ECLIPSE TE2000-S fluorescence microscope (Nikon Corporation, Kanagawa, Japan) equipped with a Nikon Digital Sight DS-U2 camera and operating with NIS-Elements F software (Nikon Corporation).

Statistical analysis

Data are expressed as mean values ± SD. Changes in variables for different assays were measured using Student's *t*-test, and differences among mean values were considered significant when $p < .05$.

Results

Cell viability

Representative images of purified SWCNT used in the present study are shown in Figure 1A. The average length of SWCNT was estimated to range from 500 nm to 1–2 μm, as determined by TEM. SWCNT displayed a mean diameter of 1–4 nm, and surface areas of purified SWCNT were 1040 m^2/g. Administration of purified SWCNT (99.7wt% elemental carbon and 0.23wt% iron) suspended in PBS (0.1 mg/ml) (Kagan et al., 2006) to HMDM for 6 and 24 h did not affect cell viability as evidenced by vital dye (trypan blue) exclusion (Figure 1B).

Chemotaxis

Migration of monocytes from blood to tissues at inflammatory sites is crucial for the resolution of inflammation. Apoptotic cells can secrete chemotactic signals that stimulate the attraction of monocytes and macrophages (Witasp et al., 2008). Here, we evaluated the ability of primary human monocytes to migrate towards SWCNT. Recombinant monocyte chemotactic protein-1 (MCP-1) (Sozzani et al., 1991) was used as a reference. As expected, MCP-1 stimulated migration of monocytes in this model (Figure 2). The carbon nanotubes, on the other hand, appeared to suppress chemotaxis of human monocytes in this model.

Phagocytosis

A primary function of macrophages is to engulf and dispose of apoptotic cell corpses/cell debris (Witasp et al., 2008). Here, we pre-incubated HMDM with SWCNT suspended in PBS (0.1 mg/ml) for 4 h, followed by extensive washing and subsequent incubation of HMDM and fluorescence-labeled apoptotic cells for 1 h. Figure 3A shows the increase in PS externalization on target cells following apoptosis induction. Extensive washing was performed following co-culture to remove unengulfed target cells, and the degree of phagocytosis of target cells by macrophages was determined. As seen in Figure 3B, pre-incubation with SWCNT at non-cytotoxic doses (Figure 1B) resulted in a significant impairment of engulfment of apoptotic cells by macrophages.

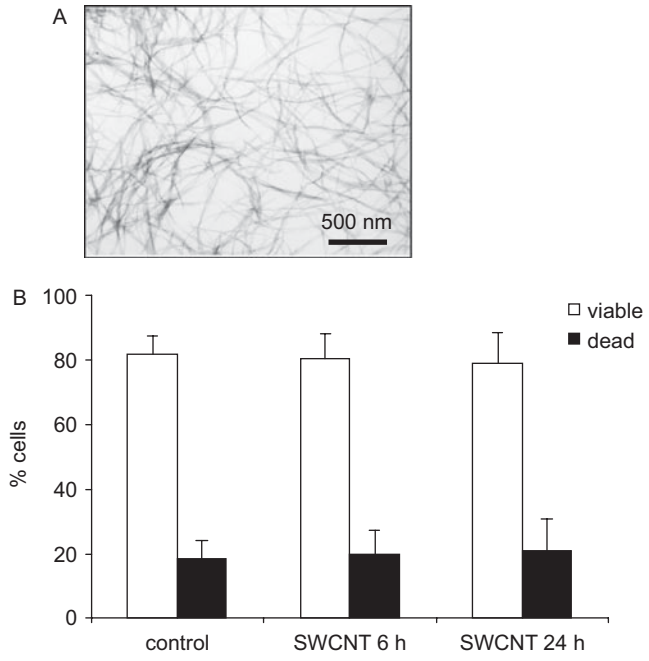

Figure 1. SWCNT are non-cytotoxic to primary human monocyte-derived macrophages. (A) TEM of SWCNT dispersed in dH_2O. (B) Viability of HMDM incubated for 6 and 24 h with SWCNT (0.1 mg/ml) in RPMI 1640 medium with 10% FBS, as assessed by Trypan blue exclusion. Data are shown as mean ± SD of three independent experiments.

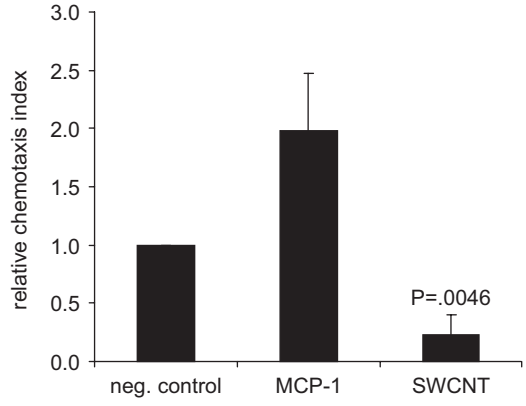

Figure 2. SWCNT suppress chemotaxis of primary human monocytes in a standard in vitro assay. The chemotactic response of monocytes to MCP-1 (300 ng/ml) or SWCNT (0.1 mg/ml) after 4 h of incubation was assessed using a two-chamber system with a laminin-coated membrane. Migration toward cell culture medium alone was used as negative control. The response is calculated as the ratio of the chemotaxis indices of monocytes migrated toward MCP-1 or SWCNT over negative control. Data are shown as mean ± SD of three independent experiments. For reference, the absolute numbers of migrated cells were 40 ± 5.3 for the negative control, 80 ± 29.4 for MCP-1, and 9.7 ± 7.5 for SWCNT.

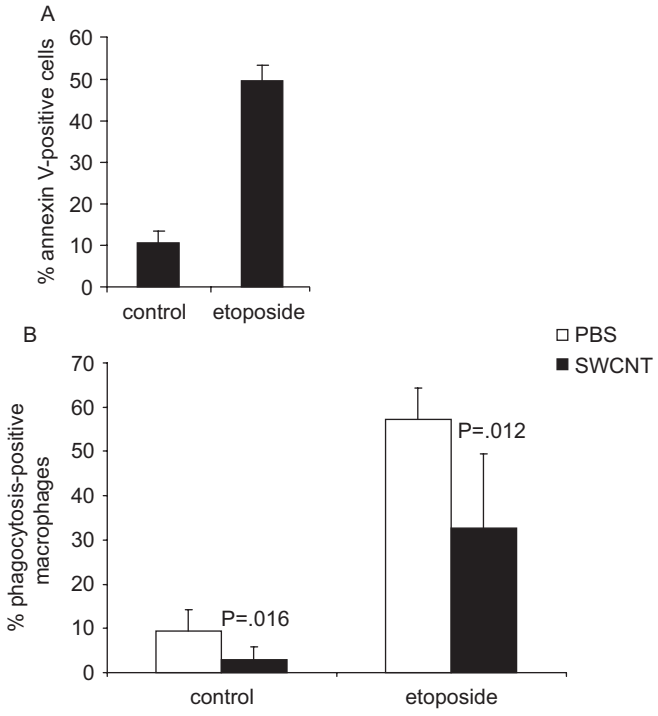

Figure 3. SWCNT impair engulfment of apoptotic target cells by primary human monocyte-derived macrophages. (A) PS externalization, indicative of apoptosis, recorded for Jurkat target cells incubated overnight in cell culture medium alone or with etoposide (6 μg/ml). (B) HMDM pre-incubated for 4 h with or without SWCNT (0.1 mg/ml) in cell culture medium supplemented with FBS were co-cultivated for 1 h with TAMRA-labeled Jurkat cells, rendered either non-apoptotic (control) or apoptotic (etoposide), as indicated earlier. The percentages of macrophages positive for uptake of TAMRA-labeled target cells were then determined using fluorescence microscopy. Data are shown as mean ± SD of three independent experiments.

Discussion

Our data show that purified SWCNT impair engulfment of apoptotic target cells by primary human macrophages in the absence of the induction of macrophage cell death. Previous studies on the cytotoxic potential of SWCNT on macrophages have generated divergent results. Jia et al. (2005) reported that SWCNT are toxic to primary alveolar macrophages ex vivo with a concomitant reduction in the capacity of SWCNT-exposed macrophages to ingest latex beads; interestingly, single-walled CNTs were found to be more toxic than multiwalled CNTs, while fullerenes were nontoxic in this model. Other investigators demonstrated that murine J774.1A macrophages can ingest SWCNT without evidence of toxicity (Cherukuri et al., 2004). Of course, differences between different in vitro studies could be explained by different sensitivities of different cell types (primary cells and cell lines), as well as the use of different forms of carbon nanotubes with varying degrees of trace metals and other impurities. Indeed, we have previously shown that the redox-dependent responses of murine RAW264.7 macrophages to SWCNT are related to iron contaminants of non-purified SWCNT (26wt% of iron), whereas purified SWCNT (0.23wt% of iron) are nontoxic (Kagan et al., 2006). Similar observations were made in a recent study of rat NR8383 macrophages and human A549 lung cancer cells exposed to various purified (acid-treated) and non-purified samples of single-walled and multiwalled CNTs (Pulskamp et al., 2007). Furthermore, functionalization of CNTs may also affect their cytotoxic potential (Sayes et al., 2006). One general conclusion from the studies cited earlier is that "carbon nanotubes" should be considered not as a single type of nanomaterial but rather as a class of nanomaterials with distinct physicochemical characteristics and, hence, engendering different biological responses following interactions with cells and tissues.

Renwick et al. (2001) reported that ultrafine titanium dioxide and carbon black particles impair phagocytosis of latex beads by the murine J774.2 macrophage cell line, in the absence of a decrease in cell viability. Of note, ultrafine particles impaired macrophage phagocytosis to a greater extent than fine particles compared on a mass basis. These authors also observed that instillation of ultrafine particles, but not fine particles into the lungs of rats resulted in enhanced alveolar macrophage chemotaxis (Renwick et al., 2004). Our current in vitro studies indicate that SWCNT are not chemotactic for human monocyte-derived macrophages. Instead, we observed a decrease in chemotaxis that could perhaps be explained by the usurpation of serum factors required for chemotaxis of these cells. Indeed, recent studies have indicated that SWCNT can induce an indirect cytotoxicity in vitro by depletion of the cell culture medium in which they have been dispersed (Casey et al., 2008). This mechanism should be considered when evaluating results of cell culture experiments, and for this reason, we are hesitant to draw definitive conclusions based on our current in vitro studies of chemotaxis.

Lundborg et al. (2001) showed that alveolar macrophage phagocytosis of fluorescent silica beads was impaired by ultrafine carbon particles. Human alveolar macrophage ingestion of microorganisms was also shown to be impaired by ultrafine particles (Lundborg et al., 2006). The latter observations are in line with our recent studies demonstrating that SWCNT suppress murine alveolar macrophage uptake of bacteria both in vitro and in vivo (Shvedova et al., 2008a). Together, these findings suggest that inhalation of ultrafine particles or carbon nanotubes, which may occur in an occupational setting (Donaldson et al., 2006), could contribute to increased susceptibility to infections and perhaps also to exacerbations of preexisting lung disease (asthma and chronic obstructive pulmonary disease). Furthermore, the current studies are the first to demonstrate that macrophage uptake of apoptotic cells is decreased following in vitro exposure to engineered nanomaterials (carbon nanotubes). Macrophage clearance of apoptotic cells is a fundamental biological process that is required for the maintenance of tissue homeostasis (Henson & Tuder, 2008; Witasp et al., 2008), and our observations thus indicate that SWCNT could interfere with the normal process of cell clearance. We have also observed increased numbers of apoptotic cells in the lungs of NADPH oxidase-deficient mice following administration of SWCNT (Shvedova et al., 2008b), and this phenomenon

could be related, at least in part, to a decreased capacity for clearance of apoptotic cells by macrophages. By affecting clearance of apoptotic cells, SWCNT could block the timely resolution of inflammation and dangerously extend the duration of the acute inflammatory phase.

The current studies were performed using non-functionalized SWCNT that are not readily taken up by murine macrophages (Shvedova et al., 2005) or primary human macrophages (Witasp et al., unpublished observations) according to careful electron microscopic examination of cells incubated with SWCNT. Therefore, the effects on macrophages observed for SWCNT in the current study are not likely to be related to macrophage internalization of these nanomaterials. However, we recently demonstrated that functionalization of SWCNT with the phospholipid recognition signal PS facilitates specific uptake of SWCNT by several classes of professional phagocytes, including primary human monocyte-derived macrophages and dendritic cells, and quenches the pro-inflammatory cytokine responses of zymosan-activated murine macrophages (Konduru et al., 2009). The latter observations suggest a potential strategy for the mitigation of toxic responses induced by carbon nanotubes.

In conclusion, the current studies show that exposure of primary human macrophages to SWCNT decreases their capacity to ingest apoptotic cells. The mechanism underlying SWCNT-mediated impairment of macrophage functions remains to be elucidated, but could be related to cytoskeletal dysfunction (Möller et al., 2002), as cytoskeletal reorganization in macrophages is required for chemotaxis as well as engulfment processes. On the other hand, SWCNT were shown to cause a depletion of serum factors required for cell viability in cultured cell lines (Casey et al., 2008), and therefore the possibility that SWCNT may adsorb specific serum factor(s) needed for chemotaxis of macrophages as well as engulfment of dying cells by macrophages also merits attention. Further studies are needed to assess the relevance of these in vitro observations for our understanding of in vivo responses following exposure to carbon nanotubes.

Declaration of interest: The current work was presented, in part, at the 11th International Inhalation Symposium (INIS) on Benefits and Risks of Inhaled Engineered Nanoparticles, June 11–14, 2008, Hannover, Germany. Supported by the Swedish Research Council, NIOSH OH008282, NIH HL70755, NORA 927000Y, and the 7th Framework Programme of the European Commission (NANOMMUNE). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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