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Carbon nanotubes provoke inflammation by inducing the pro-inflammatory genes IL-1β and IL-6

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ABSTRACT

Carbon nanotubes (CNTs) are largely produced and widely used because of their novel features, and the annual yield is expected to increase dramatically in the near future. Meanwhile, adverse health influences from exposure to CNTs are widely concerned, partially due to their asbestoid characteristics. In the current study, to assess the inflammatory responses and related mechanisms, we established a mouse model of chronic exposure to CNTs using intraperitoneal injection of single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs). Our results demonstrated the fibre-like pathogenic behaviors of CNTs, reflected by increased total protein content in the lavageate from peritoneal cavities and increased serum levels of inflammatory cytokines, IL-1β and IL-6. The pro-inflammatory effects of CNTs were further validated with exposure to *in vitro* cultured monocyte-macrophage cells, J774A.1, as SWNTs and MWNTs significantly increased the expression levels of pro-inflammatory genes IL-1β and IL-6. Collectively, our data demonstrate that SWNTs and MWNTs provoke considerable inflammation presumably due to their fibre-like shape, and further confirm the length- and size-related structure-activity relationship for CNTs in stimulating inflammatory responses.

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1. Introduction

With huge application related to nanomaterials, the chance of human exposure to them increases dramatically (Simko and Mattsson, 2010). Carbon nanotubes (CNTs) represent a family of important products of nanotechnology. Due to their novel and distinct characteristic (e.g. extraordinarily electrical and thermal conductivity), CNTs are highly produced and the yield is presumably to climb all over the world. CNTs comprise of single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) according to the number of stacked cylinders. Due to the high aspect ratio, CNTs are suspected to provoke inflammatory response similar to asbestos fibers (Poland et al., 2008). Previous animal studies have suggested significant asbestoid toxicity of carbon nanotubes, as their retention in the pleural (chest) and peritoneal (abdominel) cavities triggered inflammation and cancer of the pleura (mesothelioma) (Poland et al., 2008; Jessica P et al., 2009).

The fiber-like structure of CNTs renders them to trigger asbestos-like pathology, such as oxidative stress, inflammation and pulmonary toxicity (Mitchell et al., 2007; Elgrabli et al., 2008; Poland et al., 2008; Mitchell et al., 2009). Recent investigations also suggested potential genotoxicity of CNTs (Muller et al., 2008a, 2008b; Lindberg et al., 2009). However, most previous studies investigated acute biological impacts of CNT exposure, and results from different experiments seemed inconsistent, and even contradictory because of differences in materials, surface modifications, doses and experimental systems (Helland et al., 2008; Ostrowski et al., 2009). Thus, the toxicity of CNTs keeps inconclusive based on the current literature and the mechanisms implicated in CNT-mediated effects have not yet been elucidated.

In the current study, we therefore investigated systemic inflammatory response in mice in response to chronic exposure with MWNTs and SWNTs, and shed light on the mechanism responsible for CNT-stimulated inflammation using *in vitro* cultured cells. And to better evaluate adverse effects of CNTs *per se*, we here used unmodified MWNTs and SWNTs. Our data together suggest that CNTs trigger great inflammation by inducing pro-inflammatory genes IL-1β and IL-6, which is presumably due to their asbestos-like characteristics.

Abbreviations: CNTs, carbon nanotubes; SWNTs, single-walled carbon nanotubes; MWNTs, multi-walled carbon nanotubes; ANOVA, one-way analysis of variance; IP, intraperitoneal; nAg, nanosliver; QDs, quantum dots.

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2. Materials and methods

2.1. Chemicals and reagents

SWNTs and MWNTs were purchased from Shenzhen Nanotech Port Co., LTD, China. Both SWNTs and MWNTs used in this study did not carry any modification (such as organic compounds) and functional coating. CdSe QDs and nAg without any modification were obtained from Wuhan Jiayuan Quantum Dots Co., LTD., China, and Shanghai Huzheng Nanotechnology Co., LTD., China, respectively. All these nanomaterials were characterized prior to experiments. Their physical features were summarized in Table 1. LPS was purchased from Sigma, USA.

Prior to administration in animals and *in vitro* cultured cells, the CNT particles were dissolved in ultra pure water followed by sonication for 5 min and then were centrifuged at 20,000 rpm for 20 min. The supernatant was discarded to remove potential metal contaminants and the pellet was re-suspended in 0.1% Tween 80 followed by sonication for 5 min to obtain stable dispersion. QDs and nAg particles were dissolved in borate buffer and sodium citrate buffer, respectively.

2.2. Animal experiments

Adult Kunming mice were maintained under aseptic sterile conditions. IP injection was administrated in mice at various concentrations as described in Table 1. Nanoparticles were given 3 times a week for 6 weeks, and the volume for each injection was 200 μl. The vehicle control mice received buffers corresponding to each type of nanoparticles, and the blank control mice received PBS only. Mice were sacrificed at 24 h post the final injection, and blood, spleen, heart, kidney and liver samples were collected. The peritoneal cavity was lavaged 2× using 2 ml cold PBS, which was then pooled together for future analyses.

2.3. Cell culture

Mouse J774A.1 cells were stored and cultured as described previously (Chen et al., 2010). For cell treatments, all nanomaterials were spun down and then re-dissolved in PBS. The control cells received PBS only.

2.4. Protein concentration determination and ELISA assay

Protein concentration of the lavageate from peritoneal cavities was determined with the Bradford method as previously described (Liu et al., 2007). Serum IL-1β, IL-6 and TNF-α from mice were measured by ELISA according to the instruction from R&D systems.

2.5. RT-PCR assay

Total RNA samples were extracted from cells using the Trizol reagent (Invitrogen, USA). RT-PCR analysis was carried out as described previously (Liu et al., 2009). GAPDH was used as an internal control. The primer sequences for PCR analysis were shown below, IL-1β: forward, 5′-cccaagcaatacccaaagaagaag-3′, reverse, 5′-ggaaacaacagtggtcaggaca-3′; IL-6: forward, 5′-tagtccttcctaccccaatttcc-3′, reverse, 5′-ttggtccttagccactccttc-3′; TNF-α: forward, 5′-cgagtgacaagcctgtagcc-3′, reverse, 5′-gtctactcccaggttctcttcaa-3′; GAPDH: forward, 5′-tgaccacagtccatgccatc-3′, reverse, 5′-gacggacacattgggggtag-3′.

Table 1
The physical characteristics of nanoparticles used in this study.

	Diameter	Length/shape	*In vivo* treatment (in 200 μl)
SWNTs	<5 nm	5–15 μm	25, 50, 100 μg/ml
MWNTs	20–30 nma (outside diameter)	10–30 μm	62.5,125,250 μg/ml
QDs	About 4 nm	Sphere	0.96, 1.91, 3.82 ng/ml
nAg	About 24 nm	Sphere	0.54, 1.08, 2.16 ng/ml

2.6. Statistical analysis

The SPSS Statistics 17.0 package was utilized to analyze the data. The difference between two groups was assessed using the independent *t*-test, and the one-way analysis of variance (ANOVA) was used to analyze the mean difference among groups with nanoparticle treatment compared to blank or vehicle control. All results were presented as mean ± SEM, and the $P<0.05$ was considered statistically significant.

3. Result and discussion

To evaluate the stimulatory influence of CNTs on inflammation, we exposed mice with intraperitoneal (IP) injection of SWNTs and MWNTs at various concentrations. Although the main exposure routes for dry CNTs are inhalation into lung and direct dermal contact (Flahaut, 2011), we utilized a way of mesothelium exposure based on the concern that nanomaterials might be bio-persistent in the biological settings, which renders them to distribute via circulation into mesothelium and other tissues with great odds (Flahaut, 2011).

The innate response to foreign or pathogenic particles is recruitment of inflammatory cells (such as leukocytes and monocytes) and exudation of proteins (such as inflammatory cytokines). We first assessed the total protein levels in the lavageate from peritoneal cavities. In mice exposed to different concentrations of SWNTs, the total protein levels in the lavageate were higher than those in the vehicle control (0.1% Tween 80) and the PBS control ($p<0.01$, one way-ANOVA test), especially for mice treated with 50 or 100 μg/ml SWNTs ($P<0.05$, independent *t-test*) (Fig. 1A). Similarly, MWNTs also caused large increase of total protein content in the peritoneal cavities compared to the vehicle control and the PBS control ($p<0.05$, one way-ANOVA test) (Fig. 1B). We did not observe a clear concentration-dependent manner for inflammation induction by SWNTs or MWNTs, suggesting that the bioavailability of CNTs might be saturated at certain concentration in the peritoneal cavity.

To further assess whether CNTs could induce systemic inflammatory responses, we determined principal serum inflammatory cytokines IL-1β, IL-6 and TNF-α by ELISA. Regarding serum IL-1β, SWNTs significantly elevated its levels compared to the PBS control and the vehicle control ($P<0.05$, one way-ANOVA test) (Fig. 2A), especially at the highest concentration (100 μg/ml) (>2 fold, Fig. 2A). A clear induction of serum IL-1β was also observed in mice upon MWNT exposure ($P<0.05$, Fig. 2B). In parallel to IL-1β, serum IL-6 levels were also greatly induced upon exposure to both SWNTs and MWNTs (Fig. 3), which is consistent with a previous study (Crouzier et al., 2010) . In contrast, the TNF-α level was not significantly altered upon SWNTs or MWNTs (data not shown), in agreement with a previous observation (Crouzier et al., 2010).

To better understand the nature of inflammation induced by SWNTs or MWNTs, we used ultrafine spheric nanoparticles, nanosliver (nAg) and quantum dots (QDs), as the size-dependent control. No significant inflammation was observed, indicated by the total protein content in the lavageate from the peritoneal cavities and serum inflammatory cytokines (data not shown). These results together demonstrate that the long-fibre-like properties of CNTs determine their asbestoid effects in terms of arousing inflammation (Yamashita et al., 2010), and ultrafine nanoparticles in sphere shape surely lack these effects similar to graphite (Poland et al., 2008). In addition, no toxicity was demonstrated to organs collected from mice upon treatment with all nanoparticles used in this study through histological examination (data not shown).

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