Biomaterials 35 (2014) 856–865

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The genotype-dependent influence of functionalized multiwalled carbon nanotubes on fetal development

Xinglu Huang [a], Fan Zhang [a], Xiaolian Sun [a], Ki-Young Choi [a], Gang Niu [a], Guofeng Zhang [b], Jinxia Guo [a], Seulki Lee [a], Xiaoyuan Chen [a,*]

[a] Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, MD 20892, USA
[b] Biomedical Engineering and Physical Science Shared Resource, National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 20 August 2013
Accepted 5 October 2013
Available online 23 October 2013

Keywords:
Carbon nanotubes
Nanotoxicity
Genetic background
Blood-placenta barrier
Fetal development

ABSTRACT

In many cases cancer is caused by gene deficiency that is being passed along from generation to generation. Soluble carbon nanotubes (CNTs) have shown promising applications in the diagnosis and therapy of cancer, however, the potential relationship between cancer-prone individuals and response to CNT exposure as a prerequisite for development of personalized nanomedicine, is still poorly understood. Here we report that intravenous injections of multi-walled carbon nanotubes into p53 (a well-known cancer-susceptible gene) heterozygous pregnant mice can induce p53- dependent responses in fetal development. Larger sized multi-walled carbon nanotubes moved across the blood-placenta barrier (BPB), restricted the development of fetuses, and induced brain deformity, whereas single-walled and smaller sized multi-walled carbon nanotubes showed no or less fetotoxicity. A molecular mechanism study found that multi-walled carbon nanotubes directly triggered p53-dependent apoptosis and cell cycle arrest in response to DNA damage. Based on the molecular mechanism, we also incorporated N-acetylcysteine (NAC), an FDA approved antioxidant, to prevent CNTs induced nuclear DNA damage and reduce brain development abnormalities. Our findings suggest that CNTs might have genetic background-dependent toxic effect on the normal development of the embryo, and provide new insights into protection against nanoparticle-induced toxicity in potential clinical applications.

Published by Elsevier Ltd.

1. Introduction

Carbon nanotubes (CNTs) have attracted increased attention since their discovery because of their unique physical and chemical properties [1]. However, information concerning the potential health hazards of CNTs remains inadequately explored. Previous reports have shown that, under certain inhalation exposures, CNTs either activate cyclooxygenase enzymes in the spleen to suppress systemic immune function [2] or induce subpleural fibrosis [3]. Although inhalation is the most relevant method for determining the toxicity of CNTs, more researchers pay attention to the acute and chronic toxicity of water-soluble, functionalized CNTs when the CNTs enter the bloodstream. This is so for a number of reasons: (i) poor controllability of inhalation exposure; (ii) less cytotoxicity of water-soluble CNTs than non-functionalized particles [4]; and more importantly, (iii) functionalized CNTs have shown exciting bio-applications. For example, engineered carbon nanotubes (CNTs) have been extensively utilized as cancer theranostics [1], serving as composite nanomaterials for cancer imaging [5], drug loading [6,7], and photothermal therapy [8,9].

All cancers arise as a result of alternations that have occurred in the DNA sequence of the genomes of cancer cells [10]. The tumor suppressor p53 gene acts as a major defense against cancer; and it is well-established that over half of all human cancers bear a p53 gene mutation [11]. Information concerning the potential hazards in p53-deficient individuals caused by CNTs exposure is still unclear, despite demonstrations in previous reports that p53-dependent responses occur with other exogenous factors such as radiation [12–14] and environmental pollutant [15]. The p53 heterozygous ($p53^{+/-}$) mouse model is a cancer-prone model, and was originally developed to facilitate the understanding of the role of p53 in protecting cells from exogenous factors induced DNA

* Corresponding author. Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), 31 Center Dr, 31/1C22, Bethesda, MD 20892, USA.
E-mail address: Shawn.Chen@nih.gov (X. Chen).

0142-9612/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.biomaterials.2013.10.027

damage [16]. In addition, embryonic development is extremely sensitive to chemical toxins in water, food and drug formulations [17]. Here, we have chosen fetuses with different p53 genotypes as a model system of cancer-susceptible gene, derived from p53 heterozygous ($p53^{+/-}$) mothers that were injected with surface-modified CNTs during pregnancy, to evaluate potential differences in fetotoxic effects of the nanotubes on fetal development and growth. To investigate the biodistribution and fetotoxicity of particles, short single-walled CNTs (SWCNTs) and short multiwalled CNTs (MWCNTs) of similar surface potential and length–but with different outer diameters of <8 nm (MWCNT-8), 20–30 nm (MWCNT-20) and ~50 nm (MWCNT-50), respectively—were functionalized with PL-PEG-NH_2 by following a previously reported procedure [18], then administered to the pregnant mice.

2. Materials and methods

2.1. Amine-functionalized single-walled and multi-walled carbon nanotubes (CNTs)

Amine-functionalized CNTs were prepared by reacting CNTs with PL-PEG-NH_2 (1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000], Avanti Polar Lipids) as previously reported [5]. Briefly, 20 mg CNTs (Cheap tube Inc, VT) was added to 3-fold excess of PL-PEG-NH_2 in 5 ml distilled water. Then, the solution was dispersed for 1 h via probe sonication using a VCX-750 ultrasonic processor (Sonics & Materials, Newtown, CT). The probe was driven at 40% of the instrument's maximum amplitude in an ice-bath. After sonication, the resulting solution was purified by dialysis membrane (MW 10000) in distilled water for 3 days by changing fresh solution every 6–12 h. Finally, the particles were collected by a centrifugal filter (3 k cutoff, Millipore) and concentrated to 5 mg ml^{-1} in distilled water.

2.2. Cells and mice

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 to 14.5 day embryos using a standard protocol [19] and maintained in DMEM supplemented with 15% FBS. The $p53^{+/-}$ (C57BL/6J) male and female mice were obtained from Jackson Laboratories. All mice were maintained and handled in accordance with the NIH Animal Care and Use Committee. To acquire $p53^{+/-}$ pregnant mice, $p53^{+/-}$ male and female mice at 8–10 weeks old were mated for one day. After 10.5 days, the pregnant mice were identified and the mice with more than 2 g of increased body weight were used for further experiments.

2.3. Fetotoxicity

Different CNTs were injected intravenously through tail vein into pregnant $p53^{+/-}$ mice (n = 6/group) on the indicated days. The body weight of pregnant $p53^{+/-}$ mice was measured each day. All mice were sacrificed after being anaesthetized at gestational day 17.5 (GD 17.5). The fetuses and placenta of each mouse were isolated, examined and weighed. To study the recovery effect of MWCNT-50-NAC on brain deformity, $p53^{+/-}$ pregnant mice were treated with MWCNT-50 or MWCNT-50-NAC intravenously through tail vein on the indicated days. To study the survival rate of offspring, pregnant $p53^{+/-}$ mice were injected with MWCNT-50 at GD 10.5, GD 12.5 and GD 15.5. After natural delivery, the survival percentage of offspring was recorded within 30 days.

2.4. Identification of p53 genotypes

Identification of p53 genotypes was performed by a p53 genotype polymerase chain reaction (PCR) protocol [20]. The primers were listed in supporting information. After running PCR, the products were examined by the agarose gel electrophoresis. The molecular weight of 620 bp and 1069 bp were identified as $p53^{+/+}$ and $p53^{-/-}$, respectively. $p53^{+/-}$ showed both bands in a gel.

2.5. Labeling of radioisotope ^{64}Cu and positron emission tomography (PET) imaging

The radioisotope ^{64}Cu (freshly made from the NIH cyclotron facility) was labeled onto CNTs by introducing a crosslinker DOTA-NHS ester (Macrocyclics Inc, Dallas, TX). Briefly, 2 mg CNTs were reacted with 0.2 mg DOTA-NHS ester in 2 ml borate buffer (pH = 9.0). The mixture was stirred at room temperature for 4 h, and subsequently, was purified by dialysis membrane (MW 3500) in distilled water. The resultant samples were concentrated into 0.5 ml distilled water with a centrifugal filter (3 k cutoff, Millipore). Then, the particles were chelated with 2 mCi ^{64}Cu for 1 h in NH_4Ac buffer (pH = 5.4). The resultant samples were purified and collected by a centrifugal filter. Prior to injection of ^{64}Cu-CNT, the coupling of labeled ^{64}Cu on the particles was confirmed.

Pregnant $p53^{+/-}$ mice were injected with different types of ^{64}Cu-labeled CNTs via tail vein at GD 15.5. PET scans and image analysis were performed using an Inveon microPET scanner (Siemens) at different time points postinjection. The details of small animal PET imaging and the region-of-interest (ROI) analysis have been previously reported [21].

2.6. TEM analysis

At 48 h postinjection of different CNTs, mice were sacrificed under an aesthesia, and the placenta and fetal liver were fixed in 2% gluteraldehyde and 2.5% paraformaldehyde for 2 h. Small pieces of tissue collected from these samples were washed with phosphate buffer, postfixed in sodium cacodylate-buffered 1.5% osmium tetroxide for 60 min at 4 °C, dehydrated using a series of ethanol concentrations, and embedded in Epon resin. The resin was polymerized at 60 °C for 48 h. Ultrathin sections (50–70 nm) obtained with an ultramicrotome were stained with 5% aqueous uranyl acetate and 2% aqueous lead citrate and imaged under transmission electron microscope (TEM).

2.7. Nuclear DNA damage assay

DNA integrity was assessed using a Long PCR (L-PCR) assay as described previously [22]. Briefly, total DNA was extracted from MEFs or mouse liver and placenta using the DNeasy Blood & Tissue Kit (QIAGEN). A 6.5 kb mouse nuclear genome fragment was amplified using GeneAmp XL PCR kit. The primers were shown in the supporting information. Products were quantified by PicoGreen (Molecular Probes) fluorescence detection.

2.8. Western blotting of p21 and Bax expression

Antibody sources were as follows: mouse p21 (Calbiochem), Bax (Cell Signaling), and GADPH (Ambion). Briefly, protein isolated from different p53-status MEF cells were solubilized in cold lysis buffer with protease inhibitor cocktail (Roche), resolved by Tris–glycine SDS PAGE, and transferred to Immobilon-P membrane (Millipore). Standard ECL western blotting was performed (GE Healthcare).

2.9. Real-time PCR of p21 and Bax expression

RNA was isolated from mouse fetal liver and placenta tissues using the RNeasy Fibrous Tissue Mini Kit (QAIGEN) or from different p53-status MEFs by using the poly(dT) magnetic bead system (Invitrogen), reverse transcripted using Superscript II (Invitrogen), and mouse p21 and Bax gene expression were quantified by real-time PCR using SYBR green fluorescence on a 7900HT Sequence Detection System (Applied Biosystems) (RT-PCR). The primers were provided in the supporting information.

2.10. Data and statistical analysis

All graphs were constructed and statistical analysis performed using Graphpad Prism software v.5.00 (GraphPad Software). A one-way ANOVA with a post-hoc Tukey test was used to identify significant differences among treatment groups. Significance was set at $p < 0.05$ unless otherwise stated.

3. Results

3.1. Effect of functionalized CNTs on fetal development and survival

The CNT particles dispersed well in aqueous solutions (Fig. 1). The properties and purities of the particles are characterized in Table 1 and S1. To elucidate the fetotoxicity of SWCNTs and MWCNTs on fetuses of differing p53 genetic backgrounds, $p53^{+/-}$ pregnant mice were prepared by mating $p53^{+/-}$ male and $p53^{+/-}$ female mice prior to injection of particles (Scheme S1). A repeated dose was firstly used to explore whether SWCNTs and MWCNTs affect fetal development during the organogenesis period (gestational day 9.5–16.5) [23,24]. We injected the CNTs intravenously (200 μl, 2 mg kg^{-1}) into different $p53^{+/-}$ pregnant mice at, variously, gestational day 10.5 (GD 10.5), 12.5 (GD 12.5) or 15.5 (GD 15.5), following Timeline 1 of Scheme S2. Compared with untreated mice, the maternal body weight of mice treated with MWCNT-20 and MWCNT-50 decreased after injection ($p < 0.05$), whereas those treated with SWCNTs and MWCNT-8 did not present significant changes in weight (Fig. 2A). At GD 17.5, we euthanized the mice and isolated their fetuses to determine CNTs induced fetotoxicity. As shown in Fig. S2, no significant difference is observed with respect to the number of fetuses per litter in the untreated and CNT-treated groups, while the body weight of fetuses whose pregnant mothers had been treated with MWCNT-20 and MWCNT-50 showed 7.1 ± 5.2% ($p < 0.05$) and 23.2 ± 7.8% ($p < 0.01$) decreases