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The effects of carbon nanotubes on the proliferation and differentiation of primary osteoblasts

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Abstract
The effects of single-walled carbon nanotubes (SWNTs), double-walled carbon nanotubes (DWNTs) and multi-walled carbon nanotubes (MWNTs) on the proliferation, differentiation, adipocytic transdifferentiation and mineralization of primary osteoblasts were investigated. SWNTs, DWNTs and MWNTs with the same mean length of 5–15 μm and various diameters were shown to reduce the viability of osteoblasts and inhibit the adipocytic trans-differentiation in a time- and dose-dependent manner. The order of inhibition effect is SWNTs > DWNTs > MWNTs. Carbon nanotubes (CNTs) inhibited the formation of mineralized nodules greatly and dose-dependently during the final stage of osteoblast differentiation, revealing 50% decrease in the formation of mineralized nodules at the concentration of 50 μg ml\(^{-1}\). The expression of important proteins such as Runx-2 and Col-I in osteoblasts was also greatly inhibited upon interacting with CNTs. Transmission electron microscope (TEM) results revealed that the effects on cellular behavior may be exerted by the CNTs from inside and outside the cells.

(Some figures in this article are in colour only in the electronic version)

1. Introduction
Carbon nanotubes (CNTs) have potential applications in biosensors, tissue engineering, and biomedical devices, because of their unique electronic, chemical and mechanical properties [1–4]. Recent studies have also suggested that carbon-based nanomaterials may be present in the atmospheric environment [5]. Experimental evidence over the past few years showed that ultra-fine nanomaterials with mean diameters <100 nm in the atmosphere are potentially toxic [6]. Therefore, with the envisaged broad application of CNTs, the potential effects of CNTs on both the living system and the environment should be thoroughly evaluated [7].

Considerable efforts have been made to investigate the in vivo and in vitro toxicity of CNTs. It has been reported that SWNTs were mainly accumulated in the stomach, kidneys, bones and other organic tissues and could shuttle freely in different organs of mice, regardless of administration modes [8]. Investigations on pulmonary toxicity of SWNTs in rats showed that SWNTs induced non-dose-dependent series of multifocal granulomas [9] and dose-dependent interstitial granulomas [10]. In vitro studies also showed that carbon nanotubes exhibited cytotoxicity towards human keratinocyte cells [11] and alveolar macrophage [12], inhibited the growth of embryonic rat-brain neuron cells [13], induced cell
apoptosis and decreased cellular adhesion ability of HEK293 cells [14], and decreased the activities of DNA polymerase and restriction endonuclease [15]. Though carbon nanofibres have been proposed as a possible orthopaedic/dental implant material [16], no study has been carried out to systematically investigate and compare the biological effects of CNTs on bone cells using a primary osteoblasts model.

Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which build bone, and osteoclasts, which resorb bone. Primary osteoblast cell cultures offer several advantages, particularly for studying cell growth control mechanisms and differentiation in the context of a mineralizing matrix. An osteoblast is a mononucleate cell that is responsible for bone formation and mineralization of the osteoid matrix. Primary osteoblast cells have been shown to be a model system for revealing biological effects of nanomaterials because their susceptibility to nanomaterials is similar to that in vivo [17–19]. We herein investigated the biological effects of CNTs, including SWNTs, DWNTs and MWNTs, on proliferation, differentiation, adipocytic transdifferentiation and mineralization of primary osteoblasts. The results showed that SWNTs, DWNTs and MWNTs can reduce the viability of osteoblasts and inhibit the adipocytic transdifferentiation of osteoblasts in a time- and dose-dependent manner. The order of inhibition effect is SWNTs > DWNTs > MWNTs. Oxidized CNTs were generated by refluxing in a mixture of concentrated sulfuric and nitric acids (3:1, 98%, 69%, respectively, Sigma) at 70 °C for 4 h, as described in detail elsewhere [20, 21]. Oxidized CNTs were washed several times with distilled water by ultracentrifugation (10000g × 30 min) until the pH reached 7.0, which could not affect the pH of the buffered cellular medium. After this process, CNTs were modified with carboxylic acid groups. Figures 1(A)–(C) show the transmission electron microscope (TEM) images of an SWNT, DWNT and MWNT, respectively (obtained with a Phillips Tecnai 12 instrument). X-ray photoelectron

2. Materials and methods

2.1. Carbon nanotubes (CNTs)

SWNTs (diameter < 2 nm), DWNTs (diameter < 5 nm), and MWNTs (diameter < 10 nm) with the same mean length of 5–15 μm, prepared by the chemical vaporization deposition (CVD) method, were obtained from Nanotech Port (Shenzhen, China). Oxidized CNTs were generated by refluxing in a mixture of concentrated sulfuric and nitric acids (3:1, 98%, 69%, respectively, Sigma) at 70 °C for 4 h, as described in detail elsewhere [20, 21]. Oxidized CNTs were washed several times with distilled water by ultracentrifugation (10000g × 30 min) until the pH reached 7.0, which could not affect the pH of the buffered cellular medium. After this process, CNTs were modified with carboxylic acid groups. Figures 1(A)–(C) show the transmission electron microscope (TEM) images of an SWNT, DWNT and MWNT, respectively (obtained with a Phillips Tecnai 12 instrument). X-ray photoelectron
spectroscopy (XPS) measurements confirmed the presence of carboxylic groups on CNTs, where the peaks at 288.6 and 286.3 eV of C 1s spectra could be ascribed to C=O and C– O (figure 1d), respectively. The CNT suspensions used in the experiments (100, 50, 10, 1, 0.1 μg ml⁻¹) were prepared by suspending CNTs with minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (Gibco Invitrogen, USA). In addition, quartz particles (Merk, Silica gel 60, 63–200 μm) was used as a positive control and dispersed in α-MEM.

2.2. Isolation and culture of primary osteoblasts
Primary osteoblasts were prepared from three-day-old NIH mice calvarias (Guangzhou University of Traditional Medicine, China) following the sequential enzymatic digestion method [22]. In brief, skulls (frontal and perietal bones) were dissected; then the endosteum and periosteum were stripped off, and the bone was cut into approximately 1–2 mm² pieces and sequentially digested with trypsin (2.5 g l⁻¹) for 30 min and collagenase II (1.0 g l⁻¹, Sigma, St Louis, MO, USA) twice for 1 h each time. The cells were collected and cultured in α-MEM with 10% heat-inactivated fetal bovine serum, 100 U/ml interferon-γ (IFN-γ), then replaced with fresh medium. The cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37 °C.

2.3. Osteoblast viability and proliferation assay
Osteoblast viability and proliferation was determined by testing the mitochondrial enzyme function according to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method as described previously [23]. In brief, osteoblast cells were seeded in 96-well tissue culture plates at the density of 2 × 10³ cells per well and incubated overnight to allow the attachment of CNT suspensions of different concentrations, then cultured for another 24, 48 and 72 h. Cells with SiO₂ and cells without CNT treatment were used as positive control and negative control respectively, and wells without cells were set as blanks. After treatment, 20 μl of MTT (5.0 mg ml⁻¹, Sigma, St Louis, MO, USA) was added to each well and incubated for another 4 h at 37 °C. Then, the supernatant was removed and DMSO was added, and the absorbance at 570 nm was recorded on a microplate spectrophotometer (Bio-rad Model 680, USA). The relative cytotoxicity was expressed as percentage of [ODsample−ODblank]/[ODcontrol−ODblank]. Each experiment was performed in triplicate.

2.4. Assay for alkaline phosphatase activity
Osteoblast cells (20000 cells per well) were seeded in 48-well tissue culture plates and cultured overnight at 37 °C in a 5% CO₂ humidified incubator. CNTs were added to culture medium at final concentrations of 1, 10, 50 μg ml⁻¹ and cultured for a further three days. The plates were washed twice with ice-cold PBS and lysed by two cycles of freezing and thaw. Aliquots of supernatants were subjected to alkaline phosphatase activity and protein content measurement by an alkaline phosphatase activity kit (Nanjing Jiancheng Biological Engineering Institute, China) and a micro-Bradford assay kit (Beyotime Biotechnology, China). All results were normalized by protein content. Unit definition: one unit will convert 1 g of tissue protein to 1 μg p-nitrophenol and phosphate in 15 min at 37 °C.

2.5. Assay for adipocytic transdifferentiation of osteoblasts
Osteoblast cells (20000 cells per well) were seeded in 48-well tissue culture plates, and were cultured for ten days. The adipogenic supplement (10 mg l⁻¹ insulin, 1.0 × 10⁻⁵ mol l⁻¹ dexamethasone, Sigma, St Louis, MO, USA) and CNTs (final concentrations of 1, 10, 50 μg ml⁻¹) were added to the culture medium. Fat droplets within transdifferentiated adipocytes from osteoclast cells were evaluated by the oil red O staining method developed by Sekiya et al [24]. Cells monolayers were washed by PBS twice, then stained by 0.6% (w/v) oil red O (Sigma, St Louis, MO, USA) solution (60% isopropyl alcohol, 40% water) for 15 min at room temperature. For quantification of oil red O content, the cells were washed with distilled H₂O three times to remove background staining, and isopropyl alcohol was added to resolve oil red O. Absorbance at 510 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA).

2.6. Assay for mineralized matrix formation
Osteoblast cells (30000 cells per well) were seeded in 24-well tissue culture plates and cultured overnight at 37 °C in a 5% CO₂ humidified incubator. The medium was then changed to differentiation medium containing 10 mM β-glycerophosphate (BBI) and 50 μg ml⁻¹ ascorbic acid (Sigma, St Louis, MO, USA) in the presence or absence of 0.1, 1, 10, 50 μg ml⁻¹ of CNTs for 21 days. The formation of mineralized matrix nodules was determined by alizarin red S staining. Briefly, the cells were fixed in 70% ethanol for 1 h at room temperature. The fixed cells were washed with PBS and stained with 40 mM alizarin red S (Sigma, St Louis, MO, USA), pH 4.2, for 30 min at room temperature. Quantitation of alizarin red S staining was performed by elution with 10% (w/v) cetylpyridinium chloride (Sigma, St Louis, MO, USA) for 10 min at room temperature and measuring the absorbance at 570 nm [25]. Results were expressed as moles of alizarin red S per milligram of total cellular protein.

2.7. Statistical analysis
Data were collected from three separate experiments and expressed as means ± standard deviation (SD). The statistical differences were analyzed by a paired Student’s t-test. P values less than 0.05 were considered to indicate statistical differences.

2.8. Western blotting analysis
Cells were washed with cold PBS and lysed in cold 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 4.3 M Urea and 1% Triton X-100 (USB Corporation, Japan). Protein concentrations were determined using the method developed by Bio-Rad Laboratories, Inc. (Hercules, CA). Proteins were subjected to SDS-PAGE using 10% gel for detection of Runx-2, type-I collagen (COL-I) and transferred onto a nitrocellulose membrane (Amersham Biosciences, UK). The membrane was blocked overnight at 4 °C with 5% bovine serum albumin
Concentration g/ml

1d  2d  3d

B. DWNTs

Concentration g/ml

1d  2d  3d

C. MWNTs

Figure 2. Time- and dose-dependence of SWNTs (A), DWNTs (B), and MWNTs (C) on the viability and proliferation of osteoblasts. Results are mean ± SD of the triplicate experiments, $p < 0.05$. 10 and 50 $\mu$g ml$^{-1}$ SiO$_2$ were used as positive controls.

3. Results and discussion

Bone consists mainly of collagen proteins and hydroxyapatite, where both the size and the orientation of the hydroxyapatite crystals ($10–50$ nm length and $10$ nm in width) are dictated specifically by the collagen template. The precise structural relationship between the collagen and hydroxyapatite is critical to the bone’s resilience and strength [26]. Because nanomaterials have dimensions similar to hydroxyapatite crystals and collagen fibres found in bone [19], it is necessary to investigate the biocompatibility of nanomaterials to bone cells and determine whether they will interfere with the biological process of osteoblast and osteoclast differentiation and function. We thus investigated the effects of CNTs, including SWNTs (diameter < 2 nm), DWNTs (diameter < 5 nm) and MWNTs (diameter < 10 nm), on the proliferation, differentiation, and adipocytic transdifferentiation of primary osteoblasts.

3.1. Effects of CNTs on the viability and proliferation of osteoblasts

The cytotoxic effect of CNTs on primary osteoblasts was evaluated by the widely used MTT assay [23, 27]. The method was based on the fact that living cells are capable
of reducing light color tetrazolium salts into an intense color formazan derivative. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death. The results are shown in figures 2(A)–(C), where the treatment of osteoblasts with a series of dilutions (0.1, 1, 10, 50, 100 μg ml⁻¹) of CNTs generated a time- and dose-dependent decrease in cell viability with the maximum inhibition effect on day 3 at the concentration of 100 μg ml⁻¹. There is a tendency that the cytotoxic effects of SWNTs increased more greatly than those of DWNTs and MWNTs over time. Besides, CNTs exhibited more potent cytotoxic effects than SiO₂ particles at the same concentration, which was consistent with a previous study on alveolar macrophage [12]. Cell morphology was also changed greatly upon treatment with CNTs (figures 3(A)–(D)); most of the cells treated with CNTs were round and less elongated, also tended to grow separately, and detached from the cell populations when compared to the control cells. These cell images also showed that CNTs were aggregated together and surrounded by osteoblasts, which has been previously observed on HEK293 cells [14]. It was suggested that small molecular proteins secreted by cells when interacting CNTs should be responsible for the aggregation of CNTs [14].

3.2. Effects of CNTs on the differentiation of osteoblasts

Osteoblast differentiation is a crucial aspect of bone formation and remodeling, a process that is severely compromised in osteoporosis. Potential effects of CNTs on osteoblast differentiation were herein investigated by alkaline phosphate (ALP) activity assay, which is an early marker of osteoblast differentiation. The results suggested that CNTs exhibited complex effects on the differentiation of osteoblasts without any obvious dose-dependence (figure 4), which might be caused by the different differentiation states of the osteoblast cells. Upon treatment with 50 μg ml⁻¹ SiO₂ particles, the differentiation of osteoblasts was inhibited obviously and consistently through the whole experiment time. In the first 48 h, the ALP activities dropped greatly in the presence of 50 μg ml⁻¹ SiO₂ particles, 1–50 μg ml⁻¹ DWNTs and 50 μg ml⁻¹ MWNTs, while 1 μg ml⁻¹ SWNTs and 1 μg ml⁻¹ MWNTs slightly increased the ALP activity. On day 3, the ALP activity of cells treated by all types of CNTs was obviously decreased compared with day 2. Thus, the CNTs inhibited the ALP activity of primary osteoblasts in a time-dependent manner without evident dose-dependence. A previous study also showed that there was no

Figure 3. Morphological changes of osteoblasts in the presence of CNTs. (A) Cells without CNT treatment; (B) cells treated with SWNTs; (C) cells treated with DWNTs; (D) cells treated with MWNTs. Dotted arrows indicate osteoblast cells, and solid arrows show the aggregated CNTs. Original magnification = 200 ×; bar = 40 μm.

Figure 4. Effects of carbon nanotubes on the ALP activity of osteoblasts. Results are mean ± SD of the triplicate experiments, *p < 0.05. 50 μg ml⁻¹ SiO₂ was used as a positive control.
apparent correlation between nanotube dose and the change in expression of specific genes [28].

3.3. Effects of CNTs on the adipocytic transdifferentiation of osteoblasts

Transdifferentiation is a process whereby a cell type commits to and progresses along a specific developmental lineage by switching into another cell type of a different lineage through genetic reprogramming [29]. The adipocytic transdifferentiation rates of osteoblasts in the absence and presence of CNTs were thus determined by specifically staining intracytoplasmic lipids with oil red O. Figures 5(A)–(F) show primary osteoblasts in the absence and presence of CNTs stained by oil red O. The results indicated that CNTs could reduce the adipocytic transdifferentiation of primary osteoblasts in a dose-dependent manner on day 10, and the effect was increased in the order of SWCNT > DWCNT > MWCNT (figure 6). Compared with SiO₂ particles, CNTs showed less inhibitory effects on adipocytic transdifferentiation of osteoblasts, except for SWNTs. In addition, DWNTs and MWNTs did not influence the adipocytic transdifferentiation of primary osteoblasts on day 6.

3.4. Effects of CNTs on the formation of mineralized matrix nodules

The formation of mineralized bone nodules is the sign for the final stage of osteoblast differentiation. These nodules can easily be detected by staining fixed cultures with alizarin red S (ARS, Sigma). There was an earlier appearance and persistent increase in the number of alizarin red staining nodules in control cultures when compared with those treated with CNTs (figures 7(A)–(F)). Counting the number of mineralization nodules revealed a sharp decrease under the treatment of CNTs (figure 8(A)). Elution of the ARS bound to the nodules confirmed this reduction in mineralized matrix formation (figure 8(B)). CNTs inhibited the formation of mineralized nodules greatly and dose-dependently. Coupling the number count with quantitation of ARS deposition revealed a greater than 50% decrease in the formation of mineralized nodules upon CNTs treatment at the concentration of 50 μg ml⁻¹, which was more significant than SiO₂ particle treatment. The difference in diameter was not reflected in the inhibitory effects of CNTs at most concentrations. However, at the concentration of 50 μg ml⁻¹ the inhibitory effect was in the order of SWNTs > DWNTs > MWNTs.

Osteoblasts progress through a three-stage process of differentiation: proliferation, matrix maturation, and mineralization [30]. The present study showed that the treatment of osteoblasts with a series of concentrations (100, 50, 10, 1, 0.1 μg ml⁻¹) of CNTs generated a time- and dose-dependent decrease in cell viability of primary cultured osteoblasts cells. This observation is comparable with
reports that carbon nanotubes were much more toxic than quartz in alveolar macrophages [12]. During the subsequent differentiation process, a well characterized temporal and spatial expression pattern of extracellular bone matrix proteins and other genes occurs. As the appearance of ALP activity is an early phenotypic marker for osteoblast differentiation and mineralized nodule formation is a phenotypic marker for the last stage of mature osteoblasts, our results clearly indicated that 10 and 50 μg ml⁻¹ CNTs strongly suppressed the mineralization of primary osteoblasts, although CNTs showed complex effects on the ALP activity without dose-dependence. Adipocytes express a number of proteins and lipid-derived products contributing to osteoclast-like cells and osteoclast differentiation [31, 32]. Meanwhile, the evidence for the reciprocal transdifferentiation between osteoblasts and adipocytes is accumulating [33, 34]. In our experiment, 1, 10, 50 μg ml⁻¹ CNTs slightly inhibited the adipocytic transdifferentiation of primary osteoblasts in a dose-dependent manner on day 10. However, the inhibition on adipocytic transdifferentiation was not strong enough to reverse the cytotoxicity and suppression of CNTs on viability and mineralization of primary osteoblasts.

3.5. Effects of CNTs on the protein expression in osteoblasts

A western blot can be used to detect a specific protein expression in a given sample. The results from the western blot showed the marked reduction of Runx-2 and Col-I protein expression after osteoblasts were exposed to 50 μg ml⁻¹ CNTs (figure 9). Runx-2 (also called cbfa1, PEBPα2A) is not only a differentiation factor but also a well known gene product that regulates the level of bone
3.6. Mechanisms of interaction between CNTs and osteoblasts

Previous studies on the toxicity of nanomaterials have suggested several possible mechanisms. The main characteristics of nanomaterials, such as small size and large surface area, may be responsible for the material interactions that could result in toxicological effects [7]. Oxidative stress induced by transition-metal catalyst residues in nanomaterials when interacting with biological systems may cause inflammatory cytokines and cytotoxic cellular response [11–14]. However, in our experiments the concentration of transition-metal catalyst residue in CNT was greatly decreased after the treatment with a mixture of concentrated sulfuric and nitric acids to generate CNT-COOH, as evidenced by ICP-MS (inductively coupled plasma mass spectrometry) measurements (data not shown), which indicated that oxidative stress mechanism did not contribute to the toxicity of CNTs in our study. There are also evidences indicating that aggregates of nanomaterials or the release of toxic chemicals under ultraviolet (UV) radiation can induce in vivo and in vitro toxicity [37, 38]. Our recent study using SEM and energy dispersive spectroscopy (EDS) confirmed that the silicon nanowires (SiNWs) aggregating on HepG2 cell surface could affect cell adhesion and spreading, thus decreasing cell proliferation [39]. In this study, we used a TEM to investigate the internalization of carbon nanotubes and ultrastructural changes of osteoblasts. TEM images clearly showed that although most of SWNTs and MWNTs did remain in the extracellular space throughout the 24 h exposure time, there were a limited number of carbon nanotubes taken up by osteoblasts (figures 10(A) and (D)). From figures 10(B) and (C), it can be observed that CNTs aggregated around osteoblasts and interacted with the cell membrane of osteoblasts, which may have a further impact on the permeability and integrity of the cell membrane. Based on the present experiment results (figures 2, 3 and 10), the cell metabolism may be affected by the CNTs inside and outside the cells. As the diameter of CNTs decreases, their surface energy increases, which results in the formation of CNT aggregates in the order of SWNTs > DWNTs > MWNTs. This order is also consistent with the observed inhibitory effects of CNTs on the viability of
osteoblasts and the adipocytic transdifferentiation (figures 2, 6 and 8).

4. Conclusions

In summary, the treatment of CNTs could reduce the viability of primary osteoblasts and inhibit the mineralization of osteoblasts in a dose-dependent manner. CNTs also reduced the adipocytic transdifferentiation of osteoblasts, but the inhibition was not strong enough to reverse the cytotoxicity and suppression on viability and mineralization of primary osteoblasts. The expression of important proteins such as Runx-2 and Col-I in osteoblasts was greatly attenuated upon interacting CNTs. TEM results revealed that the cell metabolism may be affected by the CNTs from inside and outside the cells. This study demonstrated that CNTs with diameters ranging 2–10 nm exhibited complicated effects on the biological process of primary osteoblast cells, and further study on the molecular mechanisms of such effects is needed in order to provide a better understanding on the impact of CNTs on biological systems and important information for future safe applications of CNTs and the design of biocompatible nanomaterials.

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