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Cytotoxicity Evaluations of Pristine Graphene and Carbon Nanotubes in Fibroblastic Cells

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This study concentrates upon investigating differential cellular responses to carbon nanoparticles (CNPs) such as pristine graphene flakes, single-walled carbon nanotubes (SWCNTs) and multi-walled CNTs (MWCNTs), in human dermal fibroblasts (HDFs) *vs.* the L-929 fibroblast cell line. Characterizing the surface morphology of each CNP by using scanning electron microscopy, we determined their cytotoxicity profiles by quantifying the mitochondrial activity. Graphene was found to have an irregular flake-like shape with various lengths within the ranges of 50 $\sim$ 1500 nm. The estimated sizes of the SWCNTs and the MWCNTs were about 400 nm and 1000 nm in diameter, respectively and several μm in length. All the CNPs tested here exerted adverse effects on the viability of HDFs even at 15.6 ppm, through intracellular uptake whereas except MWCNTs, they did not show any cytotoxicity against L-929 cells at 250 ppm. This result suggests that CNPs can have a differential influence on normal fibroblasts *vs.* immortalized ones.

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I. INTRODUCTION

Over the last decade, nanobiotechnology has been rapidly growing and has provided potential breakthroughs in biomedical applications such as cellular imaging, diagnosis, therapeutics and drug delivery by using various functional nanomaterials [1,2]. Nevertheless, the long-term fate and potential toxicity of functional nanomaterials should be well examined before any novel nanomaterials can be translated into the clinic. Human beings will inevitably be exposed to carbon nanoparticles (CNPs), especially when the particles are utilized as diagnostic and therapeutic tools to better understand, detect, and treat human diseases. CNPs have been shown to reach systemic circulation after inhalation, ingestion or intravenous injection, with further distribution and accumulation in several organs such as the lung, liver, spleen, kidneys, brain or heart [3–5]. A previous study reported that in rats exposed to ultrafine CNPs by inhalation, the liver accumulated a significant amount of these CNPs within 24-h post-exposure, with little detectable deposition in other extrapulmonary organs [6].

A number of *in vitro* studies have been performed to evaluate the cytotoxicity of CNPs with different mechanistic endpoints. Single-walled carbon nanotubes (SWCNTs) have been shown to induce oxidative stress and loss of cell viability in human epidermal keratinocytes with ultrastructural and morphological alterations [7]. Another study demonstrated that water-soluble multi-walled CNTs (MWCNTs), which were able to enter into the cells and were mainly accumulated in the cytoplasm, induced variations in cell proliferation and the cell cycle [8]. The present study was aimed at evaluating the potential cytotoxicity of pristine graphene, SWCNTs and MWCNTs in two different types of fibroblastic cells, *e.g.*, primary cultured fibroblasts and the fibroblast cell line.

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Fibroblastic cells have been well characterized for their relevance to *in vitro* toxicity studies [9,10].

II. MATERIALS AND METHODS

1. Preparation and Characterization of Carbon NPs

Graphene and SWCNTs were grown by using chemical vapor deposition (CVD), as previously described [11,12]. MWCNTs were prepared using spray pyrolysis combined with a subsequent thermal CVD process, as previously described [13,14]. All the CNPs were suspended in phosphate-buffered saline (PBS, pH 7.4) with a final concentration of 1000 ppm and were then sonicated for homogenous dispersions under mild conditions by using a water bath sonicator with a bath temperature of 25 °C overnight [15]. The surface morphology of each CNP was observed by using scanning electron microscopy (SEM). In brief, the particles were coated with an ultrathin layer of gold/platinum by an ion sputter (E1010, Hitachi, Tokyo, Japan) and were then observed with a scanning electron microscope (Hitachi S-800) at an accelerating voltage of 15 kV. For the cytotoxicity assay, the suspension of each CNP was diluted with 2× Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Co, St Louis, MO) and then treated to the cultured monolayer of either human dermal fibroblasts (HDFs) or L-929 cells.

2. Cell cultures and Conditions

HDFs from neonatal dermis were kindly provided by Dr. Dong Kyun Rah (Department of Plastic and Reconstructive Surgery, Yonsei University College of Medicine, Seoul, Korea) [16]. A murine fibroblast cell line (L-929 cells from mouse subcutaneous connective tissue) was obtained from the American Type Culture Collection (ATCC CCL-1^{TM}, Rockville, MD). Both cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich Co) and a 1% antibiotic antimycotic solution (including 10,000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL, Sigma-Aldrich Co) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Studies were performed with HDFs and L-929 cells within 10 and 30 passages, respectively.

3. Cytotoxicity Assay

The number of viable cells was quantified indirectly by using highly water-soluble tetrazolium salt (WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono sodium salt; Dojindo Lab., Kumamoto, Japan), reduced to a water-soluble formazan dye by mitochondrial dehydrogenases. The cell viability was found to be directly proportional to the metabolic reaction products obtained in WST-8. Briefly, the WST-8 assay was conducted as follows: Either HDFs or L-929 cells were treated with increasing concentrations (0.5 $\sim$ 500 ppm) of each CNP and were then incubated with WST-8 reagent for the last 4 h of the culture period (24 h) at 37 °C in the dark. Parallel sets of wells containing freshly-cultured non-treated cells were regarded as negative controls. The absorbance was determined at 450 nm by using an ELISA reader (SpectraMax® 340, Molecular Device Co., Sunnyvale, CA). The relative cell viability was determined as the percentage ratio of the optical density in the medium (containing NPs at each concentration) to that of fresh control medium.

4. Transmission Electron Microscopic Observation

Transmission electron microscopy (TEM) was performed to obtain information regarding the intracellular ultrastructure. After treatment with 100 ppm of each CNP, the cells were immediately fixed with 2% glutaraldehyde, rinsed with PBS, and then post-fixed in 1% sodium-cacodylate-buffered osmium tetroxide (OsO_4). The fixed cell cultures were subsequently dehydrated with a graded series of ethanol and finally embedded in situ by covering them with a layer of Spurr epoxy resin (Polysciences Inc., Warrington, PA), which was allowed to polymerize. The prepared blocks were sectioned using a diamond knife mounted in an Ultracut-Reichert microtome (Leica, Heidelberg, Germany). Ultrathin sections (70 – 80 nm) were contrasted with uranyl acetate and lead citrate and observed using an electron microscope (CM-120, Philips, Eindhoven, Netherlands) at 80 kV.

III. RESULTS AND DISCUSSION

Figure 1 shows the SEM images of pristine graphene, SWCNTs and MWCNTs. All the CNPs were well dispersed in the culture medium (DMEM) with serum. A few tens of graphene flakes with lateral sizes of around 50 $\sim$ 200 nm have been observed while several tens of flakes showed larger sizes within the range of 500 $\sim$ 1500 nm [17,18]. SWCNTs and MWCNTs partially formed bundles of 200 $\sim$ 600 nm and 800 $\sim$ 1200 nm in diameter, respectively, and several μm in length in the suspension [11,14].

The cell viability was determined to estimate the toxicity of CNPs quantitatively by using the WST-8 assay where the formation of formazan dye depends on the

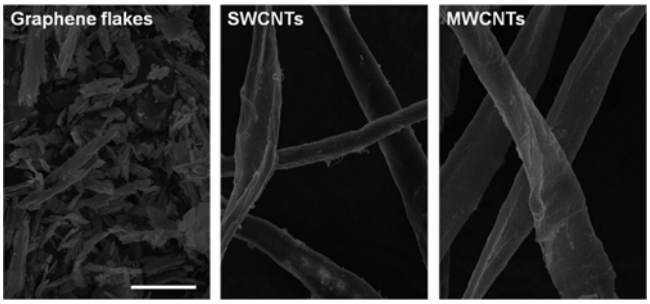

Fig. 1. SEM image of the surface morphologies of graphene flakes, SWCNTs and MWCNTs. The scale bar is 1000 nm.

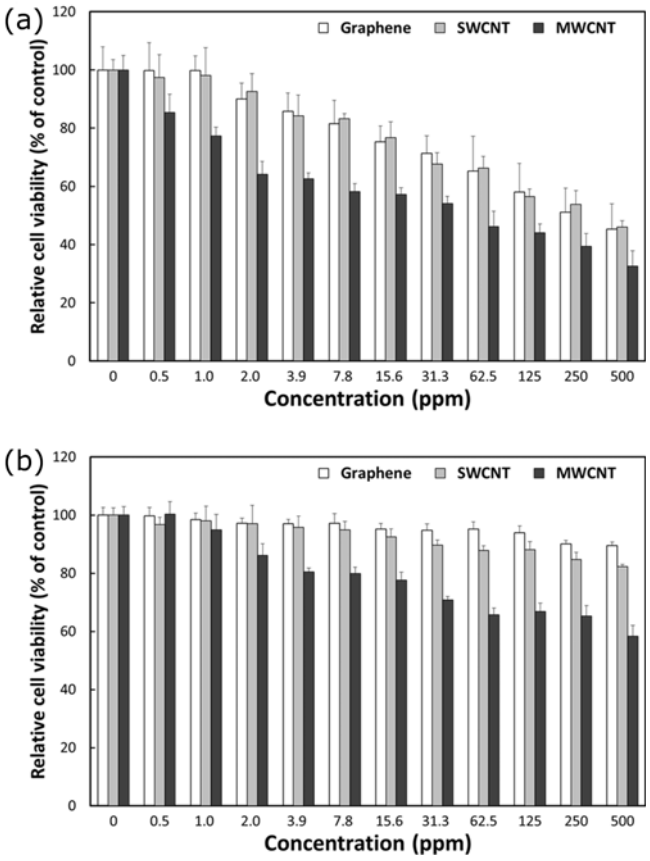

Fig. 2. Effects of graphene flakes, SWCNTs and MWCNTs on mitochondrial activities of (a) HDFs and (b) L-929 cells. Relative cell viabilities of both types of cells exposed for 24 h to increasing concentrations (0 ∼ 500 ppm) of graphene flakes, SWCNTs and MWCNTs was evaluated using the WST-8 assay.

mitochondrial activity. As shown in Fig. 2(a), the viability loss of nNFs was dose-dependent after exposure to increasing concentrations of each CNP. Graphene and SWCNTs showed significant cytotoxicity at concentrations above 15.6 ppm and triggered about 54% inhibition in the cell viability at the top concentration tested (500 ppm) in comparison to unexposed controls. Recently, graphene and SWCNTs were reported to induce cytotoxic effects against PC-12 neural cells, and those effects were concentration- and shape-dependent [19]. Additionally, a previous study reported that graphene oxide showed stronger hemolytic activity against red blood cells than aggregated graphene sheets whereas compacted graphene sheets were more damaging to mammalian fibroblasts than less densely packed graphene oxide [20]. On the contrary, MWCNTs induced appreciable cytotoxicity even at 2 ppm, with approximately 36% inhibition in comparison to untreated controls. MWCNTs have been reported to show dose-dependent cytotoxicity to human dermal fibroblasts and to induce massive loss of cell viability through DNA damage and programmed cell-death in the range of 40 ∼ 400 μg/mL (ppm) [21].

Following exposure of L-929 cells to graphene and SWCNTs, no significant cytotoxicity was observed, not even at 250 ppm [Fig. 2(b)]. However, significant cytotoxicity started to be recorded from 15.6 ppm of MWCNTs, which resulted in a 23% inhibitory effect in comparison to controls. A recent study demonstrated that in human dermal fibroblasts and NIH 3T3 murine fibroblasts, MWCNT treatment induced dose-dependent cytotoxicity, arrested the cell cycle in the G_1 phase, downregulated the expression level of adhesion-related genes, and simultaneously caused cytoskeleton damage and disturbance of actin stress fibers, thereby inducing dramatically adverse effects on the cell's physiological functions, such as cell spreading, adhesion, migration, and wound healing ability [22]. The culture period had little influence on the viability. Similar results were obtained from 24- and 72-h exposures (data not shown here).

The uptake of the CNPs into HDFs and L-929 cells was studied using TEM images (Fig. 3). The mechanisms of uptake are very complex. Generally, NPs penetrate into cells by endocytosis such as phagocytosis, pinocytosis, nonspecific endocytosis, receptor-mediated endocytosis and so on [9,23]. Although further accurate measurements for the cellular uptake mechanism were not examined here, some other studies have indicated that larger particles (100 ∼ 200 nm) penetrate the cell membrane more easily by endocytosis than smaller ones (∼10 nm) [10,24]. In our case, because some specific ligands for receptors on the cell membrane were not covered on the surface of CNPs, it is suggested that graphene and SWCNTs with sizes of 50 ∼ 200 nm can permeate into the cell by pinocytosis or nonspecific endocytosis and that MWCNTs with sizes of 700 nm can do that by phagocytosis or nonspecific endocytosis.

The mode of cytotoxic effects of CNPs is multifaceted. the underlying mechanisms of CNPs are already known to depend on their concentration, exposure time, size, morphology and *etc.*, [25]. Some of the suggested mechanisms are as follows:

1) Graphene or graphene oxide induces cytotoxicity by affecting the mitochondrial activities of cells, cell-cycle arrests leading to apoptosis and oxidative stress through the generation of intracellular reactive oxy-

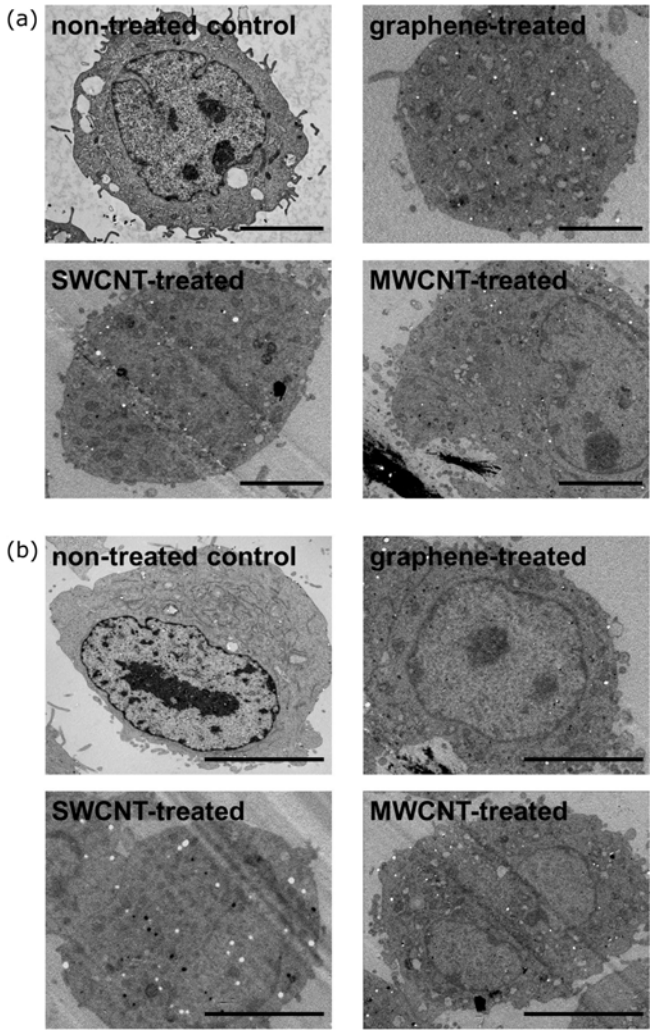

Fig. 3. TEM images of intracellular ultrastructures of (a) HDFs and (b) L-929 cells exposed to 100 ppm of graphene flakes, SWCNTs and MWCNTs for 24 h. The scale bar is 10 μm. The electron micrographs shown in this figure are representative of six independent experiments with similar results.

gen species in concentration- and time-dependent manners [26,27].

2) CNTs result in cytotoxicity by inducing free-radical generation, oxidative stress, cell-cycle arrest and inflammation, and by interfering with metabolic activity and membrane integrity [28,29].

Although CNPs are considered to have weak detrimental effect on tissues, long-term toxicity is possible. If minute quantities of CNPs are ingested, they will accumulate over a long period and may cause unexpected diseases. Therefore, how to decrease or eliminate the toxicity of CNPs is still a challengeable task for *in vivo* biomedical applications. Further work will focus on investigating the possible mechanism of the interaction between CNPs and immune cells or tissues.

IV. CONCLUSION

In conclusion, our results demonstrate that CNPs might exert a differential influence on normal cells *vs.* their immortalized counterparts. We suggest that CNPs, indeed, can be very toxic at sufficiently high concentrations and that careful monitoring of toxicity studies is essential for risk assessment.

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