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A Dissertation for the Degree of Doctor of Philosophy

Toxicity mechanism of multi-walled carbon nanotubes in murine lung

다중벽탄소나노튜브의 마우스 폐에서의 독성 기전

February 2013

By

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Toxicity mechanism of multi-walled carbon nanotubes in murine lung

By Ji-Eun Kim, D.V.M.

Supervised by Professor Myung-Haing Cho, D.V.M., Ph.D.

A Dissertation submitted to the Faculty of the Graduate School of Seoul National University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Interdisciplinary Program in Nano–Science and Technology

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Abstract

Toxicity mechanism of multi-walled carbon nanotubes in murine lung

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Graduate School of Seoul National University

Multi-walled carbon nanotubes (MWCNTs) have gained great interest owing to their ultrahigh elasticity and tensile strength, which make them promising reinforcements for composite materials. However, the high aspect ratio of CNTs, a feature they share with asbestos, is suspected to be the key factor for their adverse effects in humans. In the current study, we compared the toxicological differences between pristine MWCNTs and MWCNTs after post-treatment, which permitted the elucidation of toxicological distinctions according to the specific physicochemical characteristics of the material.

To clarify the distinctive systemic response and clearance of pristine (PMWCNTs) and acid-treated MWCNTs (TMWCNTs), both types of MWCNTs were administered intratracheally into murine lungs and the potential toxic effects were observed.
PMWCNTs induced a more severe acute inflammatory response than TMWCNTs and showed delayed clearance compared to that for TMWCNTs. PMWCNTs caused tumors in the lungs of mice after 1 year of administration, whereas TMWCNTs did not. PMWCNT-induced lung tumors were associated with increased protein expression of cathepsin D and Bcl-2 and increased VEGF and PCNA expression also supported tumorigenicity in PMWCNT-treated mice.

In addition to performing systemic studies, we also compared cellular ATP amounts for eight different MWCNTs that were generated by different synthetic methods and post-treatments. From this, we found major physicochemical characteristics determinants of toxicity among various factors. Creation of binding sites on tube walls by breaking the C–C bonds had a pivotal role in the increased toxicity, since π-orbital misalignment between adjacent carbon atoms has an influence on increment of overall reactivity that may attack important cellular molecules. This C-C bond break could be clearly described and expected by the Raman G peak shift as well as $I_D/I_G$ ratio.

The results of this study suggest that physicochemical determinants affect toxicity and may be considered as criteria when using MWCNTs in industries and for predicting their toxicity.

**Keywords:** multi-walled carbon nanotube, toxicity determinants, differential tumorigenic effect, lung cancer, physicochemical characteristics

**Student number:** 2007-22882
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<tr>
<td>A-MWCNT</td>
<td>Acid-treated multi-walled carbon nanotube (CM-100)</td>
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<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>AS</td>
<td>Alveolar space</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer–Emmett–Teller</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>CCD</td>
<td>Charge coupled device</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotubes</td>
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<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
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<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
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<td>EC50</td>
<td>Effective concentration 50</td>
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<tr>
<td>EF-TEM</td>
<td>Energy-filtering transmission electron microscope</td>
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<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FE-SEM</td>
<td>Field emission scanning electron microscopy</td>
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<tr>
<td>I0/Ig ratio</td>
<td>Peak intensity of defect/graphite ratio</td>
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<tr>
<td>H &amp; E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>H-MWCNT</td>
<td>Heat-treated multi-walled carbon nanotube</td>
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<td>HA-MWCNT</td>
<td>Heat and acid treated multi-walled carbon nanotube</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma-atomic emission spectroscopy</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IC50</td>
<td>Inhibitory concentration 50</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>LC</td>
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<tr>
<td>MNGC</td>
<td>Multi-nucleated giant cell</td>
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<td>MTD</td>
<td>Maximum tolerated dose</td>
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<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
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<td>PCA</td>
<td>Principal component analysis</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PMWCNT</td>
<td>Pristine multi-walled carbon nanotube</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<td>TGA</td>
<td>Thermo gravimetric analysis</td>
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<td>Acid-treated multi-walled carbon nanotube (CM-95)</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>Water-soluble tetrazolium salts</td>
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<td>XRD</td>
<td>X-ray powder diffraction</td>
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GENERAL INTRODUCTION

Multi-walled carbon nanotubes (MWCNTs) are made up of unique graphitic sheets nested together in a tubular multilayer structure. MWCNTs can be synthesized by three major methods: laser ablation, arc-discharge, and chemical vapor deposition (CVD); the first two methods are based on the evaporation of graphite due to high temperatures. Evaporated carbon condenses successively in the form of MWCNTs and other structures, producing longer, less graphitized forms and amorphous carbons than materials obtained by other methods. CVD is the most important method for the industrial manufacturing of CNTs because it allows for large-volume production, i.e., in tons (Brand et al., 2009). On the other hand, the arc-discharge method has been shown to be an interesting method for the mass production of the highly graphitized form of MWCNTs.

Owing to the interesting electrical, mechanical, and thermal properties of MWCNTs, their mass production is expected to be useful in the industries. Future applications include energy storage devices (Niu et al., 1997), high-resistance composites (Kota et al., 2007; Spitalsky et al., 2010), electronic devices (Cheng and Zhou, 2003), and biomedical applications (Huang et al., 2011; Karchemski et al., 2012). Among these, reinforcement composite materials and drug delivery carriers may be potential fields for the use of MWCNTs. The attractiveness of MWCNTs for application in these areas comes from the possibility of functionalizing them with peptides, proteins, or specific target molecules to develop delivery systems that can move through the body to target...
cells (Bianco et al., 2005) or to build tissue scaffolds to promote cell proliferation and differentiation (Zanello et al., 2006) and to prepare homogeneous mixtures with polymers (Cadek et al., 2004; Spitalsky et al., 2010). These potential applications call for thorough studies on biocompatibility. Additionally, toxicological studies are required to prevent possible health hazards among workers involved in the research and manufacture of these materials and in the future for the general public (Lam et al., 2006).

In general, nanotoxicity, i.e., the toxicity of certain nanoparticles such as MWCNTs is known to be related to their surface area, length, degree of agglomeration, water solubility, metal content, and surface charge (Donaldson et al., 2006). However, studies focused on determining which physicochemical factors play a dominant role in toxicity, according to synthetic methods and following acid and thermal treatment, have not provided detailed descriptions of these factors (Aillon et al., 2009). In fact, studying the toxicity of MWCNTs is a challenge for scientists because of their complex nature, for example, the specific dispersion, the presence of metal catalyst rests, and the tendency for agglomeration. Consequently, in terms of restricting the particle conditions for comparative experiments, the length of MWCNTs is difficult to control during synthesis, and the MWCNTs prepared tend to be contaminated with impurities such as metal catalyst particles and amorphous carbons and exhibit inherent viability, broad diameter, and chirality distribution (Peng and Wong, 2009). Therefore, in this study, we examined the toxicity of MWCNTs synthesized by various methods and subjected to post-treatments, rather than restricting the conditions to be evaluated.
In the case of MWCNTs, from a toxicological point of view and owing to their possible mass usage in industries, concerns over the unanticipated effects of MWCNTs have been emphasized, especially in terms of cancer risks. Like asbestos, which is classified as a group I human carcinogen (O'Reilly et al., 2007), MWCNTs have a high aspect (length to width) ratio. Recently, several in vitro studies have demonstrated the possible cancer risk of MWCNT exposure by inducing key molecular events involved in asbestos-induced lung cancer and mesothelioma (Kim et al., 2012; Wang et al., 2011). In vivo studies using intraperitoneal injection for heterozygous p53\(^{+/−}\) mice have also indicated that MWCNTs can induce mesothelioma in practice (Takagi et al., 2012). The low clearance rate from the interstitium is also expected to lead to biopersistence of CNTs in the lungs, a critical factor in the paradigm of hazardous fibers (Pezerat, 2009). Long biopersistent fibers can continuously generate free radicals that directly damage DNA because of the difficulties encountered in clearing such fibers, leading to long residence time and accumulation of free radical concentrations, as well as interactions with cells of the immune system. In addition, iron that is integrated into the crystalline structure of asbestos can contribute to redox-cycling reactions, leading to the production of damaging hydroxyl radicals (Lund and Aust, 1992), and MWCNTs also contain these metals. Consequently, although yet there are no reports demonstrating the occurrence of lung cancer or mesothelioma in normal mouse inhalation models, long term in vivo studies are needed to determine the tumorigenic potential of MWCNTs.

Although there have been many in vivo and in vitro toxicity studies on MWCNTs,
their results are controversial (Nagai et al., 2011; Saxena et al., 2007; Tong et al., 2009). Anticipating the toxicity of various types of MWCNTs is important because MWCNTs are not a homogeneous material, and it is not feasible to test every newly synthesized batch of MWCNTs. Consequently, uncovering the toxic physicochemical determinants of MWCNTs is important in new toxicity studies. Then, the results and methods of the study may be used in the benign design of MWCNTs and in predicting the toxicity of newly synthesized MWCNTs.

Based on this current state of knowledge, we compared the inflammogenicity and tumorigenicity of pristine MWCNTs (PMWCNTs) and acid-treated MWCNTs (TMWCNTs) in the murine lung in order to determine the mechanisms mediating the toxicity of MWCNTs. Because the lung is a major route of exposure for airborne MWCNTs, we focused on lung exposure by tracheal administration. In addition to systemic studies, we conducted comparison analysis using a normal lung cell line with different MWCNTs synthesized by different methods and subjected to acid and thermal post-treatments. In this study, we also determined which physicochemical properties had a greater impact on toxicity and which characterization methods were useful for the prediction of the toxicity of various types of newly synthesized MWCNTs.
Chapter I

Toxicity and clearance of intratracheally administered multi-walled carbon nanotubes from murine lung

(Published in 2010, *J Toxicol Environ Health A*)
ABSTRACT

Carbon nanotubes (CNT) are known to have widespread industrial applications; however, several reports indicated that these compounds may be associated with adverse effects in humans. In this study, multi-walled carbon nanotubes were administered to murine lungs intratracheally to determine whether acute and chronic pulmonary toxicity occurred. In particular, pristine multi-walled carbon nanotube (PMWCNT) and acid treated multi-walled carbon nanotube (TMWCNT) were used in this study. In bronchoalveolar lavage fluid (BALF) cell analysis, PMWCNT induced more severe acute inflammatory cell recruitment than TMWCNT. Histopathologically, both PMWCNT and TMWCNT induced multifocal inflammatory granulomas in a dose-dependent manner. The observed granulomas were reversible with TMWCNT-induced granulomas diminishing faster than PMWCNT-induced granulomas. Although the area of granuloma reduced with time, hyperplasia and dysplastic characteristics such as mitotic figures, anisokaryosis, and anisocytosis were still observed. These findings demonstrate that MWCNT induces granulomatous inflammation and the duration and pattern of inflammation seem to vary depending upon the types of MWCNT to which mice are exposed. Therefore, toxicity studies on various types of CNT are needed as the responsiveness to these compounds differs.
1.1. INTRODUCTION

Nanotechnology is an emerging science involving manipulation of materials at the nanometer level and has already yielded new materials, products, and devices that are believed to be beneficial for many industrial purposes and medicine (Bianco et al., 2008). Multi-walled carbon nanotubes (MWCNT) are of increasing interest because of their use in biological and industrial applications as well as medical research (Iijima, 1991; Liu et al., 2006; Ebbesen and Ajayan, 1992; Robertson, 2004; Yang et al., 2007). However, concerns regarding adverse effects on human health have been raised because of its physicochemical resemblance to asbestos. Several in vivo studies demonstrated that MWCNT induced mesothelioma (Takagi et al., 2008), fibrosis (Shvedova et al., 2005; 2008b), and other biochemical/toxicological changes in lungs or human pulmonary cells (Knaapen et al., 2004; Lam et al., 2004; Porter et al., 2007; Poland et al., 2008; Tabet et al, 2009). Moreover, there are complex interactions between oxidative stress and inflammatory responses that synergistically amplify each other to produce enhanced pulmonary toxicity following exposure to MWCNT (Kagan et al., 2006; Shvedova et al., 2008a). MWCNT are biopersistent (Schipper et al., 2008) and have ability to produce reactive oxygen species (ROS) generation (Pulskamp et al., 2007; Ye et al., 2009) similar to asbestos. Therefore, the present study was conducted to determine the potential toxicity produced by MWCNT and clearance from murine lung.
It is well-known that the pathogenicity of inhaled particles is governed by their physical and chemical features (Guthrie, 1997; Fubini and Are’án, 1999). Different types of MWCNT are produced with a variety of physical and chemical properties depending upon the method of synthesis and post-synthetic modifications (Xie et al., 2006). Pristine MWCNT (PMWCNT) may be contaminated by amorphous carbon and by catalyst remnants (usually Fe, Al, Co, Ni, or Mo), which appear as metallic clusters entrapped within the PMWCNT or as metal residues existing at the surface of the PMWCNT (Bello et al., 2009). Post-synthetic functionalization may alter PMWCNT characteristics, including aspect ratio (ratio of long to short dimension) surface reactivity, hydrophilicity, adsorptive nature, purity and interaction with intracellular molecules (Xie et al., 2006). Each of these properties may therefore contribute to toxicity of MWCNT independent of or associated with other characteristics (Fubini et al., 1999; Muller et al., 2008b). Bello et al (2009) noted that metallic contaminants play a role in carbon nanotube-induced toxicity. The presence of metal trace impurities contained within CNT was shown to increase reactive oxygen species (ROS) levels and decrease mitochondrial membrane potential in rat NR8383 macrophages and human A549 lung cells (Pulskamp et al., 2007). Sato et al (2005) also reported that the length of CNT influence tissue reactivity. The degree of inflammatory response to 220 nm-long CNT was less than that noted for 880 nm-long CNT after implantation in subcutaneous tissue of rats. The acute influence of surface chemistry and aspect ratio was also investigated by Magrez et al. (2006) and Muller et al.(2008b) who found that thermal treatment of CNT reduced acute pulmonary toxicity and genotoxicity; thus,
suggesting that the intrinsic toxicity of CNT may be mediated by the presence of
defective sites in carbon frame. TMWCNT are precursors for the synthesis of various
functionalized PWCNT (Prato et al., 2007). Therefore, in this study, the potential
differences of toxicity and clearance between pristine multi-walled carbon nanotube
(PMWCNT) and acid-treated PMWCNT (TMWCNT) were investigated.
1.2. MATERIALS AND METHODS

1.2.1. Preparation of multi-walled carbon nanotubes

MWCNT CM-95\textsuperscript{TM} (diameter: 12.5 ± 2.5 nm, purity > 95\%) were purchased from Hanhwa Nanotech (Seoul, Korea). These MWCNT were synthesized by chemical vapor deposition (CVD). Non-treated MWCNT were termed as pristine-MWCNT (PMWCNT) while acid treated MWCNT were designated as treated-MWCNT (TMWCNT). To obtain TMWCNT, PMWCNT were mixed in a mixture of H\textsubscript{2}SO\textsubscript{4}/HNO\textsubscript{3} (3:1 = v/v) at room temperature. Subsequently, mixed PMWCNT were subjected to ultrasound in a bath for 10 min. Samples were heated for 1 hr in 120°C and, then, sonicated for additional 1 hr. This solution was filtered through a 0.22 mm cellulose acetate membrane and then washed several times using deionized water until the pH reached 5.5. These TMWCNT were dried at 120°C for 1 hr (Osorio et al., 2008). Hot air sterilization was performed for 1 hr at 150°C before dispersion of PMWCNT and TMWCNT using normal sterilized saline prior to experimentation.

1.2.2. Characterization of PMWCNT and TMWCNT

Field emission scanning electron microscopy (FE-SEM) images of PMWCNT and TMWCNT were obtained using JSM-6700F (JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV. The samples were prepared by loading a droplet of sample solution on a silicon wafer and then dried on hot plate. Energy-filtering transmission electron microscope (EF-TEM) images of MWCNT were obtained using a LIBRA 120 (Carl
Zeiss, Oberkochen, Germany) at an acceleration voltage of 120 kV. The samples were prepared by loading a droplet of sample solution on a copper grid, which were dried at room temperature. The size distributions of each sample were an average of 6 measurements for accuracy. The Raman scattering signal was collected in 180° scattering geometry and detected by a spectrometer equipped with a thermoelectrically cooled charge coupled device (CCD) detector in this Raman system (LabRam 300, JY-Horiba, Edison, NJ, USA). A 514.5 nm laser line from a continuous wave Ar ion laser 35-MAP-321 (CVI Melles Griot, Albuquerque, NM, USA) was used as a photoexcitation source with a laser power of approximately 2 mW at the sample.

1.2.3. Animals and tracheal instillation

Male C57BL/6 mice (6 weeks old, about 20-22 g) were purchased from ORIENT BIO (Seongnam, Korea) and quarantined one week prior to the experiment. The animals were kept in the lab animal facility with temperature and relative humidity maintained at 23 ± 2°C and 50 ± 20%, respectively, under a 12 hrs light/dark cycle. All methods used in this study were accepted by the Animal Care and Use Committee at Seoul National University (SNU-080215-1). Total 50 µl PMWCNT or TMWCNT suspensions were administered by intratracheal instillation to mice at doses of 10 or 100 µg/mouse. Vehicle control mice received 50 µl of sterilized saline. The animals were then sacrificed at varying times of 24 hrs; or 1, 2 or 4 weeks; or 4 months following injection (n = 6/group). After anesthetization with ketamine/xylazin solution (ketamine/xylazin; 150/15 mg/kg mixture, i.p.), the trachea was exposed by a
1 cm incision on the ventral neck skin for validation of successful intubation using 24 gauge catheter and the catheter was intubated into trachea. 10 µl of TMWCNT, PMWCNT suspension and normal saline were instilled by catheter drop by drop 5 times (total 50 µl). The instillation procedures for mice reported by other authors (Lam et al., 2004) were modified to ensure that instilled material was delivered into the lungs with a good distribution. The mice were sutured and allowed to recover.

1.2.4. Bronchoalveolar lavage fluid analysis

The trachea was cannulated and lungs were lavaged 5 times with 1 ml (total volume of 5 ml) sterilized 0.9% NaCl following anesthetization with ketamine/xylazine solution. First and second aliquots were combined and centrifuged (400 g, 10 min and 4°C), and the cell-free supernatant was stored at -70°C for further analysis. Other aliquots were combined, and supernatant was discarded after centrifugation. All pellets were resuspended in 200 µl of MEM media and the total bronchoalveolar lavage fluid (BALF) inflammatory cell number was measured with a hemocytometer (Marienfeld, Lauda-Königshofen, Germany) after staining with vital stain trypan blue (Invitrogen, Carlsbad, Ca, USA). Differential cell counts (minimum of 300 cells/slide) were performed on cytopsin-prepared slides (Fisher Scientific, Milwaukee, IL, USA) with cytocentrifuge (900 g x 10 min, 4°C) and stained with Diff-Quik (Sysmex, Kobe, Japan). Two replicate slides were used for each data point, and every experiment was performed thrice.
1.2.5. Hematoxylin and eosin (H&E) staining

The lung tissues were fixed in 10% neutral buffered formalin, paraffin processed, and sectioned at 3 μm. For histologic analysis, tissue sections were deparaffinized in xylene and rehydrated through alcohol gradients and then stained with H&E (Sigma-Aldrich, St Louis, MO, USA). Then, coverslips were mounted using faramount aqueous mounting medium (Dako Cytomation, Copenhagen, Denmark), and slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY, USA). Three mice per group were used.

1.2.6. Granuloma area analysis

The granulomatous lesions present with each section was quantified using serial images taken at 200 magnification using In Studio version 3.01 program (Pixera, San Jose, CA, USA) and the mean lesion area established using Image-Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA). Three different areas were captured from one sample and three different slides were used for quantifying mean granulomatous lesion.

1.2.7. Immunohistochemistry (IHC)

For IHC, formalin-fixed, paraffin-embedded tissue were sectioned at 3 μm and transferred to plus slide (Fisher Scientific, Pittsburgh, PA, USA). The tissue sections were deparaffinized in xylene and rehydrated through alcohol gradients, washed and incubated in 3% hydrogen peroxide (H₂O₂) (AppliChem, Darmstadt, Germany) for 30
min to quench endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the tissue sections were “ringed” with a DAKO-pen (DAKO, Carpinteria, CA, USA). After that, sections were incubated with proteinase K in 37°C for antigen retrieval, then rinsed with 1xTBS (100 mM Tris-Cl, 150 mM NaCl, pH 7.5), and fixed with an ice-cold methanol-acetone mixture (v/v = 1:1) for 10 min. Tissue sections were washed with 1 × TBS, rinsed for 10 min in 0.025% Triton X-100 in 1 × TBS to reduce surface tension, and incubated with 3% BSA in 1 x TTBS (Tris buffered saline including 1% Tween 20) for 1 hr at room temperature to block nonspecific binding sites. Tissue sections were washed with 1 x TTBS for 10 min. Primary antibodies were applied on tissue sections overnight at 4°C. The following day, tissue sections were washed and incubated with secondary horseradish peroxide conjugated antibodies (v/v = 1:50) for 2 hrs at room temperature. The sections were rinsed with 1 x TTBS and then developed with 0.075% diaminobenzidine (DAB) (DAKO, Carpinteria, CA, USA) with 0.008% H₂O₂ in 1 x TTBS for 6 min. After washing with tap water, tissue sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St Louis, MO, USA) for 0.5 min, washed again in running tap water for 10 min, dehydrated, and immersed in xylene. Coverslips were mounted using Permount (Fisher Scientific, Waltham, MA, USA), and slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY, USA). Three mice samples per group were used in this experiment. Quantification of staining intensity of PCNA was performed using In Studio version 3.01 program (Pixera, San Jose, CA, USA). Staining intensity was assessed by counting the number of positive cells in randomly selected fields viewed with
appropriate magnification of objective lens.

1.2.8. Statistical analysis

Results are presented as the mean ± S.E.M. of three experiments. Statistical analyses were performed following analysis of Student’s t-test when the data consisted of only two groups. The differences between groups were considered significant at \( p^* \) < 0.05 as indicated. Quantification of Western blot analysis was performed using Multi Gauge version 3.0 program (Fujifilm, Tokyo, Japan).
1-3. RESULTS

1.3.1. Characteristics of PMWCNT and TMWCNT

Comparison of FE-SEM and EF-TEM images of PMWCNT (Figure 1-1A and 1-1C) and TMWCNT (Figure 1-1B and 1-1D) clearly show that TMWCNT were well dispersed in distilled water compared to PMWCNT. The mean length and diameter of PMWCNT were $15 \pm 5 \mu m$, $13.5 \pm 1.5 \text{ nm}$, respectively and TMWCNT were $400 \pm 99.4 \text{ nm}$ in length and $7.5 \pm 2.5 \text{ nm}$ in diameter. Raman spectra acquired from the PMWCNT and TMWCNT are illustrated in Figure 1-1E. Dresselhaus et al (2002) reported that the spectra demonstrate a number of well characterized CNT resonances; in particular, the radial breathing modes (RBM) in the $100 - 300 \text{ cm}^{-1}$ region, the D-band at $1306 \text{ cm}^{-1}$ and the G-band in the $1550 - 1605 \text{ cm}^{-1}$ region. Higher $I_D/I_G$ ratio of TMWCNT (Figure 1-1F) supports functionalization of PMWCNT by acid treatment (Maultzsch et al., 2002).
Figure 1-1 | Characteristics of MWCNTs. FE-SEM image of (A) pristine multi-walled carbon nanotube (PMWCNT), (B) acid treated multi-walled carbon nanotube (TMWCNT). The Raman spectra analysis shows different intensities and peak centers for each sample. The table below summarizes the Raman shift values for D and G peaks.
(TMWCNT) and EF-TEM image of (C) PMWCNT, (D) TMWCNTs. These images clearly show that TMWCNT are well dispersed in distilled water compared to PMWCNTs (E) Raman peak of PMWCNT and TMWCNT. (F) Higher $I_D/I_G$ ratio reveals a higher grade of hydrophilicity.

1.3.2. Distribution of PMWCNT and MWCNT after tracheal instillation

To determine the distribution of MWCNT through lobes of the lung, the pictures of total lung lobes are presented (Figure 1-2A, 1-2B, 1-2C and 1-2D). Black spots indicate PMWCNT (Red arrow) in the lung. PMWCNT showed wide distribution patterns through the lung lobes (Figure 1-2D). The lungs of mice in the group treated with TMWCNT, compared to PMWCNT displayed broader distributed patterns through the lung (Figure 1-2F and 1-2G).
Figure 1-2 | Lungs of intratracheally instilled mice. (A) Control (Normal saline), (B) Low (0.01 mg/mouse), (C) High (0.1 mg/mouse). Red arrows indicate visible PMWCNT spots. (D) Image of whole lung (0.1 mg/mouse) shows distribution of tracheally instilled PMWCNT through the lung lobes. TMWCNT treated lungs of mice. (E) Control (Normal saline), (F) Low (0.01 mg/mouse), (G) High (0.1 mg/mouse). TMWCNT distributed end side part of the lung lobes. Red arrows indicate distributed small TMWCNT spots. Scale bar represents 3 mm.
1.3.3. **Bronchoalveolar lavage cell count and morphology**

To determine inflammatory responses induced by MWCNT, total cell number of BALF was counted. Dose-dependent increase and time-related decrease in total cell number were clearly observed (Figure 1-3A). 24 hr post exposure, acute inflammation was evidenced by tissue influx of large number of neutrophils. Most intense neutrophilic inflammation had subsided two weeks post instillation (Figure 1-3C) while sustained granulomatous inflammation were detected during the treatment period (Figure 1-4A and 1-4B). TMWCNT and PMWCNT increased number of multinucleated giant cells (MNGC) in a dose-dependent manner (Figure 1-3B); however, PMWCNT recruited quantitatively more inflammatory cells than TMWCNT (Figure 1-3A).
Figure 1-3 | Bronchoalveolar lavage fluid (BALF) cell analysis of tracheal instilled MWCNTs. (A) Total inflammatory cell counts of 24 hrs, 1 week, 2 weeks, 1 month and 4 months after tracheal instillation of sterilized saline (vehicle control), 0.01 mg/mouse (low) and 0.1 mg/mouse (high) of pristine MWCNT (PMWCNT) and acid treated MWCNT (TMWCNT). Data represent mean ± S.E.M. (n = 3). Significant differences compared to each corresponding vehicle control are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001. #P < 0.05 denotes significant differences compared to PMWCNT group treated with equal concentration of TMCNT and same time interval. (Continue-next page)
(Continued) **Figure 1-3** | Bronchoalveolar lavage fluid (BALF) cell analysis of tracheal instilled MWCNT. (B) Dose-dependent cell morphology of bronchoalveolar lavage fluid (BALF) of TMWCNT. Original magnification, x 1000, scale bar represents 5 μm. (AM: Alveolar macrophage, LC: Lymphocyte, Neu: Neutrophil, MNGC: Multi-nucleated giant cell) (C) Differential cell count of inflammatory cells in BALF of mouse treated with TMWCNT.
1.3.4. MWCNT-induced granulomatous inflammation & dysplastic characteristics

The main histopathological changes following TMWCNT or PMWCNT administration were pulmonary inflammation, bronchial epithelial cell hyperplasia, hypertrophy, mitotic figures and other characteristics of dysplasistic lesions including anisocytosis and anisokaryosis. Compared to vehicle control, 2 weeks post exposure groups showed granulomatous inflammation (Figure 1-4A, dotted circle) accompanied by bronchial epithelial cell hyperplasia (Figure 1-4A, red arrow). The site of macrophage aggregation was manifested mainly in bronchoalveolar duct junction and peribronchiolar region. The multi-focal interstitial granuloma was maintained for 4 months even though the mean area of granuloma had largely decreased (Figure 1-4B and 1-6A). Despite time-dependent reduction of granuloma mean area, microgranuloma (Figure 1-4B-High, Red solid circle), type II cell hyperplasia still remained after 4 months. Furthermore, in the lungs of 1 month post exposure group, clear mitotic figures (Figure 1-4C and 1-4D) and binucleated cells (Figure 1-4E, red filled arrow) were observed in bronchial epithelial cells. Other characteristics of dysplasia such as anisocytosis (Figure 1-4F, red filled arrow) and anisokaryosis (Figure 1-4F, red empty arrow) were also detected in hyperplastic areas in both group of lungs treated with TMWCNT or PMWCNT. IHC analysis of PCNA revealed more PCNA positive cells in peribronchiolar and granulomatous region (Figure 1-5B and 1-5C) compared to control (Figure 1-5A) in the lungs of mice instilled with high concentration of PMWCNT (Figure 1-5B) and TMWCNT (Figure 1-5C).
Figure 1-4 | Histopathology of the lung. (A) C57BL/6 mice were tracheally instilled with TMWCNT and sacrificed after 2 weeks. Left : TMWCNT induced granuloma (Dotted red circle). (Continue-next page)
(Continued) **Figure 1-4 | Histopathology of the lung.** (B) C57BL/6 mice were tracheally instilled with TMWCNT and sacrificed after 4 months. Left: TMWCNT induced granuloma (Dotted red circle). Original magnification, x200, Scale bar represents 50 μm. Right: Magnified image of blue square. Original magnification, x400, Scale bar represents 20 μm. (Continue-next page)
(Continued) **Figure 1-4 | Histopathology of the lung.** Characteristics of dysplasia were observed in lung of C57BL/6 mice tracheally instilled with TMWCNT and sacrificed after 1 month. Mitotic figures (C), (D) and binucleated cells (E) were observed. Anisocytosis (F, Red empty arrow), anisokaryosis (F, Red filled arrow), epithelial cell hyperplasia (G) also have been observed. Original magnification, x1000, Scale bar represents 5 μm. (AS: Alveolar space, B: Bronchiole)
Figure 1-5 | Immunohistochemical analysis of PCNA. Lungs from saline control (A), PMWCNT (B) and TMWCNT (C) treated mice were fixed and incubated with PCNA antibody. Dark brown represents positive signal. Black represents MWCNT. Original magnification, x200, Scale bar represents 50 μm. (D), (E) and (F) are magnified image of blue square in (A), (B) and (C) respectively. Original magnification, x400, Scale bar represents 20 μm.
1.3.5. Clearance of TMWCNT and PMWCNT through lymphatic system

After 4 months of post exposure, the mean area of granuloma had largely decreased (Figure 1-4B and 1-6A) and mediastinal lymph nodes of mice treated with high concentrations of TMWCNT (0.1 mg/mouse) displayed remarkably dark color and were distended due to cellular proliferation and edema compared to control (Figure 1-6B and 1-6C). In mediastinal lymph node, TMWCNT engulfed cells were mainly located in medullary cords where macrophages and activated B cells migrate from the cortex as plasma cells and enter the medullary sinuses (Figure 1-6E, left and right). Similar pattern of mediastinal lymph nodes changes as detected in the lungs TMWCNT treated mice was also observed in the lungs of mice treated with PMWCNT (data are not shown).
Figure 1-6 | Clearance of MWCNTs through lymphatic system. (A) Time-dependent decrease of area of granuloma was observed in lungs of mice treated with TMWCNT and PMWCNT for 4 months. *$P < 0.05$ indicates significant differences compared to lung of PMWCNT 4 months post instillation. (Continue-next page)
Gross lesion of tracheobronchial mediastinal lymph node of mice treated with high concentration (0.1 mg/mouse) of TMWCNT 4 months after post exposure. (C) Comparison of tracheobronchial mediastinal lymph node (LN) between LN from control (left) and mice treated with high concentration (0.1 mg/mouse) of TMWCNT 4 months after post exposure (Right). LN from mice treated with high concentration is
bigger in size and darker in color than control. Scale bar represents 2 mm. (D), (E) Histopathology of the tracheobronchial mediastinal lymph node was observed in C57BL/6 mice tracheally instilled with TMWCNT and sacrificed after 4 months. Original magnification of (D), (E) is x200 and scale bar represents 50 μm. (D-Right) represents magnified image of blue square and its original magnification is x400 and scale bar represents 20 μm. (E-Right) represents magnified image of blue square of (E). Original magnification of (E-Right) is x1000 and scale bar represents 5 μm. Data represent mean ± S.E.M. (n = 3).
1.4. DISCUSSION

Several studies showed that environmental and occupational exposures to nanoparticles with a size dimension < 100 nm are associated with skin damage, respiratory diseases, and lung cancer (Shvedova et al., 2003; Knaapen et al., 2004). MWCNT have been functionalized according to specific purposes (Singh et al., 2006; Prato et al., 2007; Wepasnick et al., 2010), and TMWCNT are a predecessor of this functionalized form (Prato et al., 2007). The significant difference between TMWCNT and PMWCNT is that they appear to differ with respect to hydrophilicity and hydrophobicity, which affects dispersion (Osorio et al., 2008). Although several physicochemical features of MWCNT have been found to modulate their toxicity (Sato et al., 2005; Magrez et al., 2006), clearance of MWCNT following chronic exposure and influence of hydrophilic MWCNT on lung functions in mice have not been investigated systemically. Therefore, in the present study, time and dose-dependent in vivo toxicity following PMWCNT and TMWCNT was investigated in murine lungs.

A number of different methods have been used to disperse PMWCNT. Among them, a multistep acid treatment using both hydrochloric and nitric acid was carried out in this study (Saito et al., 2002; Osorio et al., 2008). TMWCNT are well dispersed in distilled water compared to PMWCNT (Figure 1-1C and 1-1D). Porter et al. (2009) reported that acid treatment introduced carboxyl acid groups at the ends of tube and, possibly, at defects on the sidewalls of PMWCNT, which modified surface properties of the PMWCNT by generating defects that break the end caps. Consequently, this
modification progressively converts the hydrophobic surfaces of PMWCNT to hydrophilic ones. The Raman peaks for PMWCNT and TMWCNT also showed that hydrophilic functional groups were introduced at defects on the side walls of PMWCNT (Figure 1-1E). The peaks most appropriate for observation of functionalization are the D band (approximately 1306 cm\(^{-1}\)) and the G band (1550–1605 cm\(^{-1}\)) (Maultzsch et al., 2002). When the letter I indicates the peak intensity of the feature, the higher I\(_D\)/I\(_G\) ratio revealed the higher grade of side-wall damage of MWCNT (Wepasnick et al., 2010). Data (Figure 1-1F) thus also support a functionalization of PMWCNT by acid treatment.

The properties of TMWCNT induced by acid treatment, like higher hydrophilicity, shortened length, and less metal catalyst contents, resulted in less recruitment of inflammatory cells in 24 hrs compared to PMWCNT (Figure 1-3A). As reported earlier, water-soluble SWCNT induce neither cell death nor activation of lymphocytes and macrophages, and do not disturb cell functions. The inability of acute TMWCNT treatment to recruit inflammatory cells compared to PMWCNT was also observed in this study, which is in agreement with previous findings (Kagan et al., 2003; Dumortier et al., 2006). Furthermore, among the inflammatory cells, the major cell population recruited in 24 hr was neutrophils (Figure 1-3C). Data suggest that TMWCNT may produce less severe acute inflammatory reactions than PMWCNT, as less neutrophils were recruited for inflammatory responses (Bassett et al., 2000; Knaapen et al., 2006). As fibers were reported to directly interfere with chromosomes and mitotic spindles during mitosis (Hesterberg and Barrett, 1985; Knaapen et al., 2004), interaction of
MWCNT with mitotic spindle may induce chromosomal aberrations in dividing cells, producing dysplasia.

Dysplasia is a non-adaptive change in cell appearance due to a loss of uniformity of individual cells and a loss of architectural orientation (Thomas et al., 1999). Previous studies demonstrated that MWCNT and SWCNT induced clastogenic, aneugenic events and genotoxicity (Kisin et al., 2007; Muller et al., 2008a). In our study, clear mitotic figures (Figure 1-4C and 1-4D), binucleated cells (Figure 1-4E), and other characteristics of dysplasia-like anisocytosis (Figure 1-4F, red empty arrow) and anisokaryosis (Figure 1-4F, red filled arrow) were observed in bronchial epithelial cells. Epithelial hyperplasia was also observed (Figure 1-4G). These types of changes and inflammatory hyperplasia can be categorized as a physiologic hyperplasia that occurs in response to a known stimulus and ceases when the stimulus is removed (Cullen et al., 2002).

Our data indicated that PMWCNT and TMWCNT did not induce carcinogenic effects at doses lower than 0.1 mg/mouse, since removal of granuloma was achieved through lymphatic system and no apparent neoplastic characteristics were noted in lungs of mice after 4 months. However, not all dysplastic lesions result in neoplasia; thus, it is possible that effects of MWCNT may create synergistic effects when combined with various cancer-predisposing factors including smoking, chemical exposure, and radiation (Berry et al., 1972; Jackson et al., 1987; Dodson et al., 2002).

In conclusion, the present study demonstrated that tracheal instillation of TMWCNT induced less severe acute inflammation compared to PMWCNT. Granulomatous
lesions of lung induced by instillation of TMWCNT or PMWCNT were reduced time-dependently after 4 months. Only the group treated with high concentrations of PMWCNT or TMWCNT showed dysplastic changes, and there were no remarkable differences between these groups as evidenced by histopathology. All these findings suggest that chronic and repeated exposure studies using different types of MWCNT are needed to determine whether the potential for carcinogenesis development exists.
Chapter II

Carcinogenicity of multi-walled carbon nanotubes (MWCNTs) in murine lung: a comparison study of pristine- and acid-treated MWCNTs
ABSTRACT

Carbon nanotubes (CNTs) have gained great research interest owing to unique and superb properties such as ultra-high Young’s modulus of elasticity and tensile strength that make them promising reinforcement materials. However, the high aspect ratio of CNTs is a key factor in potential toxicity. The aim of this study was to compare pulmonary carcinogenicity in 2 types of multi-walled carbon nanotubes (MWCNTs): pristine (PMWCNTs) and acid treated (TMWCNTs). TMWCNTs are used as composite materials because highly dispersible acid-functionalized MWCNTs are preferred for homogeneous blending with polymers. This study showed that PMWCNTs caused tumors in murine lung 1 year after administration, whereas TMWCNTs did not. PMWCNTs increased protein expression levels of cathepsin D and Bcl-2 compared to those accompanying TMWCNTs. Increased vascular endothelial growth factor and proliferating cell nuclear antigen expression also seemed to be partially responsible for lung tumors in PMWCNT-administered mice. These results suggest that PMWCNTs are more tumorigenic than TMWCNTs.
2.1. INTRODUCTION

Multi-walled carbon nanotubes (MWCNTs) have unique mechanical, electrical, and thermal properties (Berber et al., 2000) that have led to their use in the development of the next generation of materials for mechanical reinforcement in lightweight composite systems. Given the possibility of the widespread use of MWCNTs in industrial fields, concerns about unanticipated effects, especially cancer risks, have been emphasized. Like asbestos, a group I human carcinogen (O'Reilly et al., 2007), MWCNTs have a high aspect (length to width) ratio. Recently, several in vitro studies have reported possible risks of MWCNT exposure, which induces key molecular events involved in asbestos-induced lung cancer and mesothelioma (Kim et al., 2012; Wang et al., 2011). In vivo studies of intraperitoneal injection using heterozygous p53+/- mice have also indicated that MWCNTs can induce mesothelioma in practice (Takagi et al., 2012).

The study of MWCNT toxicity is challenging owing to their complex nature, which is hard to control. For example, MWCNTs contain various metal catalyst rests and have a tendency to agglomerate because of their geometry and hydrophobic surfaces (Wick et al., 2007). Furthermore, many fundamental studies and technological applications of carbon nanotubes (CNTs) require a population of tubes with high crystallinity and identical chirality (metal/semiconductor form) that current syntheses cannot provide (Hersam, 2008). Consequently, MWCNTs as a composite material may be a realistic industrial application in practice (Spitalsky et al., 2010). In this case,
agglomeration has negative effects on the applicability of carbon nanotubes because dispersion becomes important when nanotubes are homogeneously blended into polymers. Nanotubes tend to remain as entangled agglomerates, and therefore, homogeneous dispersion is not easily obtained. Surface modification of CNTs is the most common strategy used to achieve better dispersity; increasing nanotube–polymer interactions and decreasing filler self-aggregation in turn improves load transfer (Spitalsky et al., 2010). The chemical degradation of MWCNTs, which uses strong acids and oxidants (such as mixtures of sulfuric acid and hydrogen peroxide) to modify nanotube surfaces, is the most common approach for surface functionalization and for the addition of functional groups (Osorio et al., 2008).

Based on the current state of MWCNT study and application, we compared lung tumorigenicity in 2 kinds of MWCNTs: pristine (PMWCNTs) and acid treated (TMWCNTs). PMWCNTs are raw MWCNTs in purchased form, whereas TMWCNTs are MWCNTs made dispersible through a multistep acid treatment with both hydrochloric and nitric acids. Because the number of CNT researchers and workers who handle highly dispersible, functionalized MWCNTs is increasing, we used TMWCNTs as test materials (Gasnier et al., 2012; Sun et al., 2013). In a previous study, we have shown that TMWCNTs clear faster than PMWCNTs (Kim et al., 2010). We anticipate that a lower clearance rate from the interstitium leads to longer biopersistence of PMWCNTs compared to that of TMWCNTs in the lung, a critical factor in the paradigm of hazardous fibers (Pezerat, 2009).

Although inhalation is most ideal method with which to test airborne materials,
generating and characterizing the exposure atmosphere is difficult, especially in the case of fibrous MWCNTs. Therefore, direct instillation of a test material into the lungs was used as an alternative to inhalational exposure in this study. Tracheal instillation in mice has certain advantages over inhalation—for example, it is a quantitatively reliable method for the comparison of different materials because the dose delivered to the lungs can be defined accurately (Driscoll et al., 2000; Warheit et al., 2005). Exploiting such advantages, we compared the tumorigenic effects of PMWCNTs and TMWCNTs in murine lung 1 year after tracheal instillation.
2.2. MATERIALS AND METHODS

2.2.1. Preparation of MWCNTs

CM-95™ MWCNTs were synthesized via chemical vapor deposition and purchased from Hanwha Nanotech (Seoul, Korea). MWCNTs used as purchased were called pristine MWCNTs (PMWCNTs), and acid-treated MWCNTs were called treated MWCNTs (TMWCNTs). To make TMWCNTs, we immersed PMWCNTs in a mixture of sulfuric acid/nitric acid (v/v = 3:1) at room temperature. Immersed PMWCNTs were then treated in an ultrasound bath for 10 minutes. After heating for 1 hour at 120°C, this solution was sonicated for 1 hour and filtered with a 0.22-mm cellulose acetate membrane. Samples were washed several times using deionized water until the pH reached 5.5, at which point they were dried at 120°C for 1 hour (Osorio et al., 2008). PMWCNTs and TMWCNTs were sterilized with dry heat for 1 hour at 150°C before dispersion in normal sterilized saline for the animal experiment.

2.2.2. Characterization of PMWCNTs and TMWCNTs

Energy-filtering transmission electron microscopy (EF-TEM) measurement of MWCNTs was performed at 120 kV on a LIBRA 120 (Carl Zeiss, Oberkochen, Germany) after the sample solutions were dropped onto a copper grid (Samchang Commercial Co., Ltd., Seoul, Korea) and air dried at room temperature. Field emission scanning electron microscopy (SEM) images of PMWCNTs and TMWCNTs were
obtained using a JSM-6700F (JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV. Ten microliters of aqueous sample solutions were loaded onto a silicon wafer and dried on a hot plate. The size distributions of each sample were averaged from ~100 SEM images of MWCNTs and measured 6 times for accuracy. Raman scattering was collected in 180° scattering geometry and detected with a spectrometer (LabRam 300, JY-Horiba) equipped with a thermoelectrically cooled charge-coupled device and 514.5-nm laser line from a continuous-wave argon ion laser (35-MAP-321, Melles Griot, Albuquerque, NM, USA) as a photo excitation source. The laser power was ~2 mW at the samples. Metal catalyst remnants were measured using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Briefly, MWCNTs were aspirated into the flame of an atomic emission spectrometer and subjected to nebulization, desolation, liquefaction, vaporization, atomization, excitation, and ionization using an Optima-4300 DV (PerkinElmer, Waltham, MA, USA). The emission wavelength during the atomization and excitation stages was measured at the characteristic wavelength for the element of interest. Samples were analyzed for the presence of the following transition metal elements: manganese, cobalt, nickel, copper, zinc, aluminum, iron, titanium, and platinum.

2.2.3. Dose determination for PMWCNTs and TMWCNTs

To determine the dose to be used for the experiment examining the effects of 1 year of chronic exposure to MWCNTs, we used data from the previous 3 months of our subchronic exposure experiment (Kim et al., 2010). During this time, mice treated with
0.1 mg PMWCNTs and TMWCNTs lost approximately 10% body weight compared to those in the control group (Supplementary Figure S2-1). The 10% weight loss dose was assessed and defined as the maximum tolerated dose of PMWCNTs and TMWCNTs. This determination was based on studies in which the highest subtoxic dose tolerated by test animals over a long period of time was appropriate for carcinogenicity bioassays (Carr and Kolbye, 1991).

2.2.4. Animals and tracheal instillation

Six-week-old male C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea) and acclimated for 1 week before the experiment. The mice were housed in polycarbonate cages (5 mice per cage) in the laboratory animal facility with temperature and relative humidity maintained at 23 ± 2°C and 50 ± 20%, respectively, under a 12-hour light/dark cycle. Mice were given food and filtered water ad libitum. All methods used in this study were approved by the animal care and use committee at Seoul National University (SNU-080215-1). After being anesthetized with ketamine/xylazine solution (ketamine/xylazine; 150/15 mg/kg mixture, i.p.), mice were intubated with a 24-gauge catheter, and 50 µL MWCNT suspension was placed in the catheter via intratracheal instillation in doses of 0.01 and 0.1 mg/mouse. Vehicle control mice received 50 µL sterilized saline. The animals were killed 1 year after injection (n = 5/group).
2.2.5. Histopathological examination and immunohistochemistry (IHC)

Tissue was fixed in 10% neutral buffered formalin, processed, embedded in paraffin blocks, and sectioned at 3 μm. For histologic analysis, the tissue sections were deparaffinized in xylene (10 minutes, twice), rehydrated through alcohol gradients (each 5 minutes), and stained with hematoxylin and eosin (Sigma-Aldrich, Saint Quentin Fallavier, France). Cover slips were then mounted using Faramount aqueous mounting medium (Dako Cytomation, Copenhagen, Denmark), and the slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY, USA). For immunohistochemistry, paraffin-embedded tissue was sectioned at 3 μm and transferred to Plus slides (Fisher Scientific, Pittsburgh, PA, USA). The lung sections were deparaffinized and rehydrated as previously described. Sections were boiled for 10 minutes in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval and then rinsed with 1x Tween 20 in Tris-buffered saline (TTBS; 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 10 minutes to quench endogenous peroxidase activity. After being washed with phosphate-buffered saline, the tissue sections were “ringed” with a DAKO Pen (DAKO, Carpinteria, CA, USA). Nonspecific binding sites were blocked for 1 hour with 3% bovine serum albumin in 1x TTBS at room temperature and incubated overnight with proliferating cell nuclear antigen (PCNA) primary antibody (mouse monoclonal IgG2a; sc-56, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% bovine serum albumin (v/v = 1:20) at 4°C. The next day, the tissue sections were washed 3 times with 1x TTBS and incubated with secondary horseradish peroxidase
conjugated antibodies (v/v = 1:50) for 3 hours at room temperature. The sections were rinsed with 1x TTBS, and 3, 3-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) was applied accordingly to the manufacturer protocol. After being washed with tap water, tissue sections were counterstained with Mayer’s hematoxylin (DAKO) for 10 minutes, washed again in running tap water for 10 minutes, dehydrated, and immersed in xylene. Cover slips were mounted using Permount (Fisher Scientific, Pittsburgh, PA, USA), and the slides were examined with a light microscope (Carl Zeiss, Thornwood, NY, USA). Staining was assessed by counting the number of positive cells in randomly selected field images viewed with ×400 magnification using In Studio version 3.01 (Pixera, San Jose, CA, USA). Images’ counting was performed using Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.2.6. Western blot analysis

Lungs were homogenized with Passive Lysis Buffer (Promega, Madison, WI, USA), and protein concentrations were measured with a Bradford kit (Bio-Rad, Hercules, CA, USA). An equal amount of protein (25 μg) was loaded and separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 1x TTBS containing 5% skim milk for 1 hour at room temperature and incubated overnight at 4°C with primary antibody. Vascular endothelial growth factor (VEGF, mouse monoclonal immunoglobulin G2a [IgG2a], sc-7269), Bcl-2 (mouse monoclonal IgG2a, sc-7382), cathepsin D (goat polyclonal
IgG2a, sc-6486), and PCNA (mouse monoclonal IgG2a, sc-56) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abfrontier (Seoul, South Korea), and alpha-tubulin antibody (cat. no. 2144s) was purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies conjugated with horseradish peroxidase (HRP) (Invitrogen, Carlsbad, CA, USA) diluted 1:500 (v/v) were incubated 3 hours at room temperature in 5% skim milk. Bands of interest were visualized with an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan) and quantified using the Multi Gauge version 2.02 program (Fujifilm, Tokyo, Japan).

2.2.7. Statistical analysis

Results are shown as means ± S.E.M. of 5 experiments. Statistical analyses were performed with the Student’s t-test using GraphPad software version 4.02 (GraphPad Software, San Diego, CA, USA). *P < 0.05 was considered significant and **P < 0.01 and ***P < 0.001 were considered highly significant compared to corresponding control values.
2.3. RESULTS

2.3.1. Characteristics of multi-walled carbon nanotubes

The diameter and length of the PMWCNTs were 12.5 ± 2.5 nm and ~20 μm, respectively, and those of the TMWCNTs were 11.3 ± 3.5 nm and ~2 μm, respectively. EF-TEM (Figure 2-1A and 2-1B) and SEM (Figure 2-1C and 2-1D) images of the PMWCNTs and TMWCNTs clearly showed that the latter were well dispersed, whereas PMWCNTs were aggregated. Raman spectra acquired from the PMWCNTs and TMWCNTs are shown in Figure 2-1C. The graphite/defect ratio of the TMWCNTs was higher than that of the PMWCNTs (Figure 2-1E, Table 2-1). The purity of the PMWCNTs was ~95% carbon and ~5% impurities such as iron and aluminum, and (ICP-AES) results showed that most of metal catalyst remnants were removed after acid treatment (Table 2-2).
Figure 2-1 | Characteristics of 2 types of MWCNTs. (A and B) Energy-filtering transmission electron microscopy (EF-TEM) and (C and D) scanning electron microscopy (SEM) images of pristine MWCNTs (PMWCNTs) and acid-treated MWCNTs (TMWCNTs) clearly show that TMWCNTs, but not PMWCNTs, are well dispersed. (E) Raman spectra acquired from PMWCNTs and TMWCNTs.
Table 2-1. Result of Raman spectroscopy

<table>
<thead>
<tr>
<th></th>
<th>Intensity (D)</th>
<th>Intensity (G)</th>
<th>$I_D/I_G$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMWCNT</td>
<td>1563</td>
<td>1552</td>
<td>1.007</td>
</tr>
<tr>
<td>TMWCNT</td>
<td>2554</td>
<td>2489</td>
<td>1.026</td>
</tr>
</tbody>
</table>

Table 2-2. Metal catalyst contents in PMWCNTs and TMWCNTs

<table>
<thead>
<tr>
<th></th>
<th>PMWCNT (ppm)</th>
<th>TMWCNT (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Co</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ni</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cu</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Zn</td>
<td>nd</td>
<td>9.26</td>
</tr>
<tr>
<td>Al</td>
<td>8690.7</td>
<td>588.43</td>
</tr>
<tr>
<td>Fe</td>
<td>11809.0</td>
<td>1149.31</td>
</tr>
<tr>
<td>Ti</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Pt</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*nd: not detected

ppm: parts per million

Inductively coupled plasma atomic emission (ICP-AES) results shows that most of metal catalyst remnants were removed after acid treatment.
2.3.2. Effects of MWCNTs on mouse body weight and dosage determination

Direct instillation of a test material into the lungs has been employed for alternative exposure to inhalation for a qualitatively reliable method for comparing PMWCNTs and TMWCNTs. 10% weight loss dose was assessed in 3 months of subchronic study and defined here as the maximum tolerated dose (MTD) of PMWCNTs and TMWCNTs, and according to this, dosage for 1 year exposure of MWCNTs was determined to 0.1 mg/mouse (Figure S2-1). The initial dosages of previous subchronic study, 0.01 mg/mouse (low concentration) and 0.1 mg/mouse (high concentration) were inferred from in vitro cell viability IC₅₀ (Figure S2-2) on the basis of mouse lung area (Mund et al., 2008; Soutiere et al., 2004).
Supplementary Figure S2-1 | Effects of MWCNTs on mouse body weight gain. At 92 days post-exposure, the average body weight of the control group was $31.7 \pm 0.81$ g. By contrast, those of the high-dose PMWCNT group and high-dosage TMWCNT group were $29.59 \pm 0.56$ and $29.59 \pm 0.56$ g, respectively. Each group consisted of 5 mice. The control group was treated with 50 μL saline. The low-dose group was treated with 0.01 mg/mouse MWCNTs in 50 μL saline, whereas the high-dose group was treated with 0.1 mg/mouse of MWCNTs in 50 μL saline. Average weight at the start point was approximately 20 g. No toxicity (assessed and defined here as 10% weight loss) was observed up to a dose of 0.1 mg/mouse TMWCNTs and PMWCNTs. Therefore, the maximum tolerated dose of PMWCNTs and TMWCNTs was established as 0.1 mg/mouse.
Supplementary Figure S2-2 | Water-soluble tetrazolium salts (WST) assay of PMWCNTs and TMWCNTs. WST assay of PMWCNTs and TMWCNTs using 16HBE14o- normal lung epithelial cell line days of 24 hours (Figure S2-2A) and 48 hours (Figure S2-2B). Figure S2-2C is IC$_{50}$ of each time point of PMWCNTs and TMWCNTs.
2.3.3. Tumor occurrence

At 1 year post-instillation, adenoma was observed in mice treated with PMWCNTs. Tumor incidence was observed in 2 of the 5 mice that received 0.1 mg/mouse and 1 of the 5 mice that received 0.01 mg/mouse (Table 2-3, Figure 2-2A). Hematoxylin and eosin staining demonstrated that the tumors induced in the lungs of mice treated with 0.1 mg/mouse PMWCNTs were adenomas (Figure 2-2B) with several mitotic figures (Figure 2-2C and 2-2D), Mallory’s hyaline (Figure 2-2E), and epithelial hyperplasia (Figure 2-2F). The morphological changes summarized in Figure 2-2C–F are typical indicators of chronic inflammation.

Table 2-3. Summary of tumor incidence 1 year after tracheal instillation of pristine multi-walled carbon nanotubes (PMWCNTs)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Adenoma Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td>Low</td>
<td>5</td>
<td>1/5</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

Control group: Normal saline, Low: 0.01 mg/mouse of PMWCNT, High: 0.1 mg/mouse of PMWCNT.
Figure 2-2 | Tumor incidence and histologic features 1 year post-PMWCNT instillation. (A) Representative photograph of PMWCNT-treated mouse lung 1 year after tracheal instillation. Dotted circle indicates tumor. (B) Hematoxylin and eosin staining of lung sections from mice exposed to saline and PMWCNTs at a dose of 0.01 and 0.1 mg/mouse showing adenoma formation 1 year after tracheal instillation at a dose of 0.1 mg/mouse PMWCNTs. (Continue-next page)
(Continued) **Figure 2-2 | Tumor incidence and histologic features 1 year post-PMWCNT instillation.** (C and D) Mitotic figures. (E) Alveolar epithelial cells containing eosinophilic material that resembles Mallory’s hyaline. (F) Epithelial hyperplasia in images of lung from mice receiving 0.1 mg/mouse PMWCNTs.
2.3.4. Western blot analysis of cathepsin D and Bcl-2

Western blot analysis showed that cathepsin D levels were increased in mice in which 0.1 mg/mouse PMWCNTs was instilled, whereas no dose-dependent pattern was observed in TMWCNT-treated murine lung (Figure 2-3A). Densitometric analysis clearly confirmed the western blot results (Figure 2-3B). The expression of anti-apoptotic protein Bcl-2 was increased in both treatment groups in a dose-dependent manner; however, the increment of increase in lung from mice treated with PMWCNTs was more prominent than that in mice treated with TMWCNTs (see Figure 2-3A and 2-3C).
Figure 2-3 | Differences in distinguishable cellular organelle protein expression level in lung homogenates. (A) Expression of cathepsin D and Bcl-2 1 month post-instillation of MWCNTs. (B) The cathepsin D band was further analyzed with densitometry. The intensity of the cathepsin D band was divided by the intensity of the α-tubulin band. Each bar represents the mean ± S.E.M. (n = 4). (C) The Bcl-2 band was also analyzed with densitometry. Each bar represents the mean ± S.E.M. (n = 5).
2.3.5. Western blot analysis and IHC of VEGF and PCNA

The expression of VEGF, an angiogenesis-related protein, was increased in mice treated with PMWCNTs, whereas mice treated with TMWCNTs showed no increase (Figure 2-4A and 2-4B). IHC analysis also demonstrated that VEGF expression was increased according to dosage in tissue from PMWCNT-treated mice (Figure 2-4C, upper panel), whereas no VEGF expression change in lung tissue was induced by TMWCNT (see Figure 2-4C, lower panel). The same difference in MWCNT type-dependent protein expression was also observed in PCNA IHC analysis: the number of positive cells increased dose dependently in the PMWCNT-treated group, but a dose-dependent pattern was unclear in the TMWCNT-treated group (Figure 2-4D). Dose-dependent PCNA staining was further confirmed by counting PCNA-positive cells (Figure 2-4E).
Figure 2-4 | Increased protein expression level of proliferating cell nuclear antigen (PCNA) and vascular endothelial growth factor (VEGF) in the PMWCNT-treated group. (A) Expression level of VEGF and glyceraldehyde 3-phosphate dehydrogenase
in lung homogenate. (B) The bands of interest were further analyzed with densitometry. Each bar represents the mean ± S.E.M. (n = 5). (C and D) Immunohistochemical analysis of VEGF and PCNA, respectively. (E) Comparison of PCNA labeling indexes. Each bar represents the mean ± S.E.M. (n = 5). **Statistically different (P < 0.01) compared to control group. ***Statistically different (P < 0.001) compared to control group. #Statistically different (P < 0.05) between 2 indicated groups. Original magnification, ×400; scale bar represents 20 μm.
2.4. DISCUSSION

The present study was designed to evaluate the potential differential effects of acid treatment/functionalization on the tumorigenicity of 2 types of MWCNTs. For most technical applications, MWCNTs of high purity are needed. A high degree of dispersity is also required, especially in applications as composite reinforcement material (Loos and Schulte, 2011; Zhu et al., 2003). Consequently, studying the effects of acid functionalization on the toxicity of MWCNTs is necessary if MWCNTs are to be applied broadly and properly in industrial fields. Conventional purification is based on acidic and optional oxidative treatment, which endows enhanced dispersity in MWCNTs (Loos and Schulte, 2011; Toyokuni, 2009). EF-TEM (see Figure 2-1A and 2-1B) and SEM (see Figure 2-1C and 2-1D) images of PMWCNTs and TMWCNTs clearly showed that TMWCNTs were well dispersed and had shorter lengths and smaller diameters. The higher graphite/defect ratio in TMWCNTs (see Figure 2-1E and Table 2-1) confirms the functionalization of PMWCNTs through multistep acid treatment (Mases et al., 2011). Acid treatment reportedly introduces carboxyl acid groups at the ends of tubes as well as possible defects on the sidewalls of PMWCNTs, consequently modifying surface properties (Kagan et al., 2010; Osorio et al., 2008). This modification progressively converts the hydrophobic surface of PMWCNTs to a hydrophilic one. In addition, ICP-AES results showed that most of metal catalyst remnants, which accelerate reactive oxygen-mediated cell damage, were removed by acid treatment (see Table 2-2). Generally, the toxicity of nanoparticles is associated
with surface area (Oberdorster et al., 2005), and acid functionalization greatly reduces the surface area of CNTs by rebundling the nanotubes (Chakraborty et al., 2006). This effect may be partially responsible for the lower tumorigenicity of TMWCNTs compared with that of PMWCNTs.

Several studies have been conducted to reveal the effects of acid functionalization on CNT toxicity. Saxena et al. (2007) have reported that acid-functionalized CNTs are more cytotoxic, and Tong et al. (2009) have also demonstrated that acid treatment increases cardiopulmonary toxicity. Their findings oppose our results. The main reason for this difference may be variation in the acid-treatment processes. Saxena et al. (2007) and Tong et al. (2009) adopted a simple process of treating CNTs with a 1:1 mixture of nitric/sulfuric acid in addition to microwave cooking and dialysis. In contrast to that treatment, ours was an extensive multistep acid treatment (see the materials and methods section) that may have increased solubility and enhanced clearance, possibly making TMWCNTs less tumorigenic than PMWCNTs, as shown in Figure 2-2 and Table 2-3. Our results strongly suggest that extensive multistep acid treatment may provide a safe way to use MWCNTs in practice. Moreover, MWCNTs treated with this process may have faster clearance. In fact, we have concluded from previous study that TMWCNTs clear faster than PMWCNTs (Kim et al., 2010). Our hypothesis is further supported by a recent publication showing that a low clearance rate from the interstitium leads to the biopersistence of PMWCNTs in the lung, a critical factor in the paradigm of hazardous fibers (Pezerat, 2009).

Cathepsin D belongs to the family of aspartic peptidases and is overexpressed in many
malignant tumors and chronically inflamed organs (Fan et al., 2012; Malik et al., 2011). Moreover, it has mitogenic activity independent of its proteolytic activity, and it attenuates the anti-tumor immune response of decaying chemokines by inhibiting dendritic cell function (Nomura and Katunuma, 2005). Bcl-2 is a membrane-bound protein that strongly inhibits apoptosis, enhances cell survival, and has been shown to suppress apoptosis (Malik et al., 2011). We believe that the increased expression of both cathepsin D and Bcl-2 (see Figure 2-3A-C), aided by the overexpression of VEGF (see Figure 2-4A-C) and PCNA (see Figure 2-4D), which are indicative markers for tumorigenesis, is partly responsible for PMWCNT-induced lung tumorigenesis.

Together, these results suggest that PMWCNTs may be more tumorigenic than TMWCNTs because they (1) are deposited in the lung longer (Kim et al., 2010), (2) contain 10 times the amount of iron catalyst (see Table 2-2) (McDonald et al., 1982), (3) are longer and larger in diameter (see Figure 2-1D) (Kim et al., 2010; Nagai et al., 2011), and (4) induce more chronic inflammation of longer duration (see Figures 2-2C-F, 2-3A and B). As demonstrated in our previous study (Kim et al., 2010), the long biopersistence of PMWCNTs may chronically damage lung tissue in a way that enhances inflammation. Moreover, as shown in Table 2-2, a multistep acid treatment process reduced aluminum and iron significantly. The elevated iron content in PMWCNTs may contribute to redox-cycling and trigger tumorigenesis. Our finding is further supported by several lines of evidence that iron-containing asbestos fiber has comparatively greater carcinogenicity (McDonald et al., 1982). Recently, iron regulation has been shown to play a key role in diverse diseases including lung cancer,
supporting our results (Jomova and Valko, 2011). Moreover, Lu et al. (2009) have demonstrated that instillation of aluminum causes significant inflammation in rat lungs, suggesting that the increased amount of aluminum in PMWCNTs is also partly responsible for lung tumorigenesis. The chemical degradation of PMWCNTs through an extensive multistep acid treatment may improve clearance owing to its effective degradation of CNTs. PMWCNTs and TMWCNTs demonstrated differential tumorigenicity such that PMWCNTs were more tumorigenic than TMWCNTs.

Our results suggest that multistep acid treatment of MWCNTs reduces their tumorigenic effects compared with those of PMWCNTs, and in the matter of tumorigenicity, changing characteristics of MWCNTs using acid treatment may be a safety criterion for the use of MWCNTs as composite materials in industrial fields.
Chapter III

Physicochemical determinants of multi-walled carbon nanotubes on cellular toxicity: influence of synthetic method and post-treatment
ABSTRACT

Since the discovery of carbon nanotubes (CNTs), extensive studies related to nanotubes in the fields of materials science, physics, and electronic engineering have been performed. Because MWCNTs are not homogeneous materials and because it is not feasible to test every newly synthesized MWCNT, this study was aimed at investigating the physicochemical properties that primarily determine the cellular toxicity. This study analyzed the relationship between cell viability and physicochemical characteristics following exposure to eight different MWCNTs. We generated eight different MWCNTs by different synthetic methods and post-treatments. From this analysis, we sought to identify the major physicochemical determinants that could be used to predict the cellular toxicity of MWCNTs, regardless of the synthetic methods and post-treatment conditions. Creation of binding sites on the tube walls by breaking C–C bonds played a pivotal role in increasing toxicity and was most clearly demonstrated by a Raman G peak shift and $I_D/I_G$ ratio. In addition to this, several factors were uncovered to be strongly related to cellular toxicity; that was surface charge in case of chemical vapor deposition method based MWCNTs, and were surface area and EPR intensity in case of arc-discharge based MWCNTs. The results and methods of this study could be applied for the benign design of and for predicting the toxicity of newly synthesized MWCNTs.
3.1. INTRODUCTION

Polymer nanocomposites based on multiwalled carbon nanotubes (MWCNTs) have attracted great interest owing to their remarkable mechanical (Yakobson et al., 1996), thermal, and electrical properties. Compared to conventional particles such as carbon black or nanoclays, the high aspect ratio of CNTs allows for enhancement of the properties of these CNTs at lower concentrations (Andrews et al., 2002), demonstrating the lower percolation threshold for application of polymer/CNT nanocomposites as conductive, strong, and yet lightweight materials (Kota et al., 2007). However, the MWCNTs prepared exist as highly entangled clusters because of their extremely long lengths, and large amounts of unnecessary carbonaceous particles such as fullerenes, nanoparticles, and amorphous phases are always present in the soot (Yan et al., 2011). Since high purity and reproducible dispersity are general requirements for further delicate measurement and practical applications of MWCNTs as composite materials, it is very necessary to disperse highly entangled MWCNTs uniformly in fluids or in polymer melts without impurities.

In general, there are largely two types of impurities found in prepared MWCNTs: catalytic metallic impurities and carbonaceous materials. Thus, the purification of raw products through various post-treatment processes, including chemical or thermal oxidation, is a prerequisite to various MWCNT applications, especially in the area of composite applications. In addition to removing impurities, improvement of dispersity can also be achieved by these post-treatments. The acid treatment of nanotubes is a
well-known method for removing catalytic impurities and shortening the length of CNTs under simultaneous sonication. Acid treatment is also well known to generate functional groups on the opened ends or sidewalls of CNTs to promote easy dispersion of CNTs in solution (Wang et al., 2003). On the other hand, thermal treatment above 1800°C is generally used for annealing or enhancing CNT surface crystallization and removing amorphous carbon (Behler et al., 2006; Park et al., 2005). Thermal oxidation of CNTs up to 400°C will improve hydrophilicity by creating oxygen-terminated surfaces (Behler et al., 2006). As the temperature increases, removal of smaller diameter tubes can occur through a thermal oxidation route (Osswald et al., 2005). Single-walled CNTs (SWCNTs) are the first species to oxidize, followed by double- and triple-walled CNTs, and finally small-diameter MWCNTs (Osswald et al., 2005; Zhou et al., 2001). In addition, the thermal oxidation of tubes generates binding sites on the tube walls by breaking C–C bonds (Wiltshire et al., 2004). By doing this, composite materials may benefit greatly by utilizing tubes of a narrower diameter distribution, which is free of amorphous carbon and contains more binding sites for better functionalization.

The aim of the present work was to reveal the role of the physicochemical characteristics of MWCNTs in cell viability, according to the synthetic method and the thermal or acid post-treatment. In this study, before their use in biological assays, all samples were thoroughly characterized in terms of diameter, length, surface chemistry, surface area, and metal impurities. MWCNT samples were finally tested in intracellular ATP assays to evaluate the relationship between cellular toxicity and
physicochemical characteristics by comparing these results.
3.2. MATERIALS AND METHODS

3.2.1. Post-treatment of MWCNTs and preparation of eight different types of MWCNTs

Two different MWCNTs were used as starting materials for post-treatment procedures. The first was synthesized via chemical vapor deposition (CVD), and the second was synthesized via arc-discharge. These two different commercially synthesized MWCNT powders were purchased from Hanwha Nanotech Inc. (Incheon, Korea), and each pristine MWCNT (PMWCNT) was treated using two processes: thermal treatment and acid treatment. In thermal treatment, MWCNTs were heated in air at 500°C for 2 h to oxidize amorphous carbons in PMWCNTs (H-MWCNTs). In acid treatment, MWCNTs were mixed with H$_2$SO$_4$ and HNO$_3$ (3:1 = v/v) to attach functional group on the surface of the MWCNTs (A-MWCNTs). Following acid treatment, the carbon nanotubes were washed with deionized water. After treating the MWCNTs using these two processes, eight types of MWCNTs were prepared from the two different starting materials (CVD-PMWCNTs, CVD-A-MWCNTs, CVD-H-MWCNTs, CVD-HA-MWCNTs, Arc-PMWCNTs, Arc-A-MWCNTs, Arc-H-MWCNTs, and Arc-HA-MWCNTs).

3.2.2. Preparation of MWCNTs for in vitro applications

The eight different MWCNTs were prepared as dry powders. Each type of
MWCNT was weighed in a 10-mL glass vial in the fume hood on an analytical balance and was dry-heat sterilized at 200°C for 1 h. They were suspended in a medium containing 10% fetal bovine serum (FBS) at a final concentration of 1 mg/mL in 10-mL glass vials. These suspensions were sonicated for 15 min in a water sonicator bath (5510-DTH; Branson, Danbury, CT, USA) and used as the stock solution for treatment in cell culture media. An appropriate amount of each stock solution was added to the desired final concentration in cell culture flasks. To prevent morphological or physical changes in MWCNTs, the stock solution of each MWCNT was prepared immediately before each cell experiment.

3.2.3. Transmission electron microscopy (TEM)

MWCNTs were sonicated in ethanol for a few minutes. A drop of the solution was placed on a formvar/carbon-film-coated 400 mesh Cu TEM grid (Samchang Commercial Co., Ltd., Seoul, Korea), and the morphology and size of MWCNTs were analyzed using energy-filtering TEM (EF-TEM) on a LIBRA 120 instrument (Carl Zeiss, Oberkochen, Germany) with an operating voltage of 120 kV.

3.2.4. Field-emission scanning electron microscopy (FE-SEM)

FE-SEM images of MWCNTs were obtained using a JSM-6700F (JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV. The samples were prepared by loading a droplet of each MWCNT solution on a silicon wafer and then drying the droplet on a hot plate.
3.2.5. Raman

Raman measurement was performed using a confocal microscope Raman system (LabRAM 300; JY-Horiba, Edison, NJ, USA) equipped with an optical microscope (Olympus, Tokyo, Japan). In this system, Raman scattering signals were collected in a 180° back-scattering geometry and detected by a spectrometer equipped with a thermoelectrically cooled (-70°C) charge-coupled device (CCD) detector. Focusing of the excitation laser and collection of the Raman signal was conducted using a ×100 objective lens (NA 0.90; Olympus, Tokyo, Japan). The excitation source was a 647-nm Kr laser (Innova I-301; Coherent, CA, USA), and the power of the laser was approximately 1.2 mW at the samples. Raman signals were collected on the selected point for 1 s.

3.2.6. Electron paramagnetic resonance (EPR) spectrometer

EPR signals were measured by a Bruker EMX/Plus spectrometer (Bruker, Stuttgart, Germany). Measurements were performed at the following settings: temperature, 298 K; modulation amplitude, 5 G; modulation frequency, 100 kHz; microwave power, 0.94 mW; microwave frequency, 9.6 GHz. For quantitative analysis, 5 mg of each of the eight different MWCNTs was placed into an EPR quartz tube and was analyzed by EPR.

3.2.7. Thermogravimetric analysis (TGA)

The total metal content of MWCNTs was measured by TGA using a Q-5000IR
instrument (TA instruments, Brussels, Belgium). CVD-PMWCNTs MWCNTs were heated to 800°C and Arc-PMWCNTs were heated to 1000°C at a heating rate of 10°C/min in air atmosphere.

3.2.8. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES)

MWCNTs were exposed to the flame of an atomic emission spectrometer and subjected to nebulization, desolation, liquefaction, vaporization, atomization, excitation, and ionization using an Optima-4300 DV (PerkinElmer, Waltham, MA, USA). During the atomization and excitation stages, the emission wavelength was measured at the characteristic wavelength for the elements of interest. Samples were analyzed for the presence of the following transition metal elements: manganese, cobalt, nickel, copper, zinc, aluminum, iron, titanium, and platinum.

3.2.9. X-ray powder diffraction (XRD)

The crystallinity of MWCNTs was analyzed using an X-ray diffractometer (M18XHF-SRA, MAC Science Co., Chiba, Japan) operating at 40 kV and 200 mA. The scan rate and angle were fixed at 4° per minute and 5–90, respectively.

3.2.10. Dynamic light scattering (DLS)

The hydrodynamic diameter was obtained by dynamic light scattering (ELS-8000; Otsuka Electronics Co., Ltd., Osaka, Japan). MWCNTs were dispersed in
dimethylformamide (DMF) and sonicated for 15 min. DLS measurement of MWCNTs was performed after sufficient dispersion.

3.2.11. Brunauer-Emmett-Teller (BET) analysis

BET surface area analysis of MWCNTs was performed by N\textsubscript{2}-adsorption-desorption isotherm nanoPOROSITY-XQ (MiraeSi, Gwangju, Korea). The samples were out-gassed at 150°C for 12 h before the adsorption test, reaching a final pressure of 10\textsuperscript{-6} mbar.

3.2.12. ATP assay

Normal human bronchial epithelial 16HBE14o- cells were maintained in DMEM/F-12 (Gibco, Carlsbad, CA, USA) with 10% heat-inactivated FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO\textsubscript{2} incubator. For ATP assay, 16HBE14o- cells were seeded in 96-well white tissue culture plates at 1 × 10\textsuperscript{4} cells/well. Twenty-four hours later, the culture medium was replaced with new medium, and MWCNTs were added at two-fold serial dilutions (from 1000 µg/mL to 1 µg/mL). Cells were then incubated for 48 h. The medium was removed, and ATP was measured using CellTiter-Glo (Promega, Madison, WI, USA) on a luminometer (Titertek Berthold, Pforzheim, Germany) according to the manufacturer’s instructions.
3.2.13. Principal component analysis (PCA)

PCA was performed using FacroMineR package (released from the website of Institute for Statistics and Mathematics of the Vienna University of Wien) using the covariance matrix. A data frame was constructed with the average value of each physicochemical factor for the eight different MWCNT materials. In case of factor “length”, results by manual scaling method, length from the SEM image analysis were used for PCA.

3.2.14. Statistical analysis

Results are shown as the mean ± S.D. of repetitive experiments. Statistical analyses were performed using the SPSS 12K program. Factors including “defect”, “surface charge”, “length”, “diameter”, “Raman shift (G)”, and “Raman shift (D)” were supposed to follow normality and were statistically analyzed. Equal variances were tested after finding significance in ANOVA. The Duncan test was used in cases of equal variance, and Dunnett’s T test was performed in cases that did not satisfy equal variance. In case of factor “length”, results by manual scaling method were used for ANOVA test.
3.3. RESULTS

3.3.1. Schematic categorization of eight different MWCNTs

According to the starting materials and the acid and thermal post-treatments employed, a total of eight different MWCNTs were generated (Figure 3-1).

![Flowchart of the treatment process of MWCNTs](image)

Figure 3-1 | Flowchart of the treatment process of MWCNTs. (A) As-purchased
pristine MWCNTs (PMWCNTs) were used as the starting material and were treated by thermal treatment or acid treatment. (B) Two types of MWCNTs were used as starting materials: One was synthesized via chemical vapor deposition (CVD) and the other was synthesized via arc-discharge (Arc). After these two post-treatment processes, eight MWCNTs were generated: CVD-PMWCNT, CVD-A-MWCNT, CVD-HMWCNT, CVD-HA-MWCNT, Arc-PMWCNT, Arc-A-MWCNT, Arc-H-MWCNT, and Arc-HA-MWCNT.

3.3.2. Morphology of the eight different MWCNTs

There were obvious differences between CVD-PMWCNTs and Arc-PMWCNTs as starting materials. The morphology of arc-discharge-based PMWCNTs (Figure 3-2B upper left) was straight and sharp in shape compared to that of CVD-based PMWCNTs (Figure 3-2A upper left). This tendency was found in all treated MWCNTs derived from the CVD method, as opposed to the arc-discharge method (Figure 3-2A and 3-2C versus Figure 3-2B and 3-2D). Sharp and clean XRD peaks of arc-discharge-based MWCNTs also support this (Figure S3-1). After thermal post-treatment, there was no significant difference in morphology (Figure 3-2A–D, lower left panel). On the other hand, acid post-treatment of MWCNTs obtained by both synthetic routes resulted in A-MWCNTs exhibiting smaller diameters and shorter lengths as compared to those of PMWCNTs (Figure 3-2A–D, upper right panel).
Figure 3-2 | Images of eight different materials. TEM (A and B) and SEM (C and D) images of eight different MWCNTs. Compared to CVD-based MWCNTs (A and C), arc-discharge-based MWCNTs (B and D) showed high crystallinity and sharp and straight fibers. After thermal treatment, CVD-H-MWCNTs (A, lower left and C, lower left) shortened as compared to CVD-PMWCNTs (A, upper left and C, lower left), while Arc-H-MWCNTs (B, lower left and D, lower left) did not. Acid post-treatment, in both synthetic types of MWCNTs, the diameter and length of A-MWCNTs became smaller and shorter than those of PMWCNTs (B, upper right compared to B, upper left; D, upper right compared to D, upper left). CVD-HA-MWCNTs showed the shortest form among all CVD-based MWCNTs, while Arc-HA-MWCNTs did not show a significant difference.
Supplementary Figure S3-1 | XRD peak of eight different MWCNTs. XRD peaks of arc-discharge-based MWCNTs are sharper than that of CVD-based MWCNTs. This shows higher crystallinity of arc-discharge-based MWCNTs compared to that of CVD-based MWCNTs.
3.3.3. Raman spectroscopy of the eight MWCNTs

The use of Raman spectroscopy allowed for the identification of many characteristics of carbon materials, especially nanotubes. The Raman spectra of MWCNTs showed two major peaks: a D-band at 1320 cm\(^{-1}\) assigned to carbonaceous compounds and the tangential mode, a so-called G band at 1580 cm\(^{-1}\), which served as a measure of the structural defects in MWCNTs (Figure 3-3A). Similar to D-bands, the D’-peak at 1615 cm\(^{-1}\) was a double-resonance Raman feature induced by disorders and defects (Figure 3-3A). This D’-band was caused by a defect in C–C bond and was observed for all CVD-based MWCNTs in addition to Arc-H-MWCNTs (Figure 3-3A, square with black dotted line). The intensity ratio of the D-band to the G-band (I\(_D\)/I\(_G\)) was higher in A-MWCNT than in PMWCNTs, implying that PMWCNTs were less defective than A-MWCNTs (Figure 3-3B). In addition, the I\(_D\)/I\(_G\) ratio of H-MWCNTs was higher than that of PMWCNTs, indicating that H-MWCNTs contained more binding sites for better functionalization on the side walls (Figure 3-3B).
Figure 3-3 | Raman spectroscopy of the eight MWCNTs. (A) Raman spectra of each MWCNTs showed two major peaks in the high frequency range; the tangential mode or so-called G band at 1601 cm\(^{-1}\) and D-band at 1289 cm\(^{-1}\) assigned to carbonaceous compounds or defects in MWCNTs. The inset shows the dissociation of the D’ peak from G peak, which was observed in CVD-based MWCNTs (Black lined square) and
in Arc-H-MWCNT (Red lined square). The intensity ratio of D-band to the G-band ($I_D/I_G$) in A-MWCNT is higher than that in PMWCNTs, implying that PMWCNTs were less defective than A-MWCNTs. The $I_D/I_G$ value of H-MWCNT was higher than that of PMWCNTs, indicating that H-MWCNTs contained more defects that could act as bonding sites for better functionalization on the side wall.
<table>
<thead>
<tr>
<th></th>
<th>Raman shift (D)(cm$^{-1}$)</th>
<th>Raman shift (G)(cm$^{-1}$)</th>
<th>Intensity (D)</th>
<th>Intensity (G)</th>
<th>$I_D/I_G$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CVD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMWCNT</td>
<td>1318</td>
<td>1572</td>
<td>694.6</td>
<td>376.6</td>
<td>1.83 ± 0.19*</td>
</tr>
<tr>
<td>H-MWCNT</td>
<td>1317</td>
<td>1571*</td>
<td>720.0</td>
<td>356.8</td>
<td>2.04 ± 0.32</td>
</tr>
<tr>
<td>A-MWCNT</td>
<td>1322*</td>
<td>1579</td>
<td>704.9</td>
<td>332.5</td>
<td>2.14 ± 0.15</td>
</tr>
<tr>
<td>HA-MWCNT</td>
<td>1321*</td>
<td>1574</td>
<td>758.0</td>
<td>366.3</td>
<td>2.07 ± 0.06</td>
</tr>
<tr>
<td><strong>Arc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMWCNT</td>
<td>1320</td>
<td>1565</td>
<td>53.6</td>
<td>578.4</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>H-MWCNT</td>
<td>1332*</td>
<td>1578*</td>
<td>219.1</td>
<td>818.6</td>
<td>0.28 ± 0.08*</td>
</tr>
<tr>
<td>A-MWCNT</td>
<td>1322</td>
<td>1568</td>
<td>62.3</td>
<td>565.7</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>HA-MWCNT</td>
<td>1319</td>
<td>1566</td>
<td>111.9</td>
<td>727.4</td>
<td>0.16 ± 0.03</td>
</tr>
</tbody>
</table>

Positive Raman shifts of D- and G- band is predominant in Arc-H-MWCNTs implying the splitting of G band. CVD-A-MWCNT and CVD-HA-MWCNT also shows positive Raman shift of D- and G- peak. (ANOVA, *$p < 0.05$)
3.3.4. Singlet electrons on the surfaces of the eight types of MWCNTs

The EPR signals of MWCNTs in Figure 3-4 show quantitative EPR signal detection. In CVD-based MWCNTs, singlet electrons were not detected (Figure 3-4A), while MWCNTs synthesized using the arc-discharge method showed positive EPR signals (Figure 3-4B). EPR results strongly depended both on the degree of purity of the sample and the method of preparation (Kosaka et al., 1994).
Figure 3-4 | Quantitative electron paramagnetic resonance (EPR) signals of MWCNTs. (A) CVD-based MWCNTs did not show positive EPR signal. (B) MWCNTs synthesized from the arc-discharge method showed positive EPR signals. Arc-HA-MWCNTs (peak intensity: 117.0) showed the strongest signal, followed by Arc-A-MWCNTs (peak intensity: 39.3), Arc-H-MWCNTs (peak intensity: 33.6), and Arc-PMWCNTs (peak intensity: 17.9) in decreasing order.
3.3.5. Amount of metal catalyst remnants of 8 kinds of MWCNTs

In thermogravimetric analysis, CVD-PMWCNTs and CVD-H-MWCNTs showed 14.75% and 15.05% metal catalyst remnants, respectively. Acid post-treatment removed metal catalyst remnants in PMWCNTs (Figure 3-5A compared to Figure 3-5C), while thermal treatment concentrated the amount of metal catalysts owing to the removal of carbonaceous particles. After thermal treatment, the residual mass increased because heating in air at 500°C for 2 h could not remove metal contents, but instead burned amorphous carbon in PMWCNTs. Consequently, for MWCNTs of the same weight, H-MWCNT contained more metal content than did PMWCNTs. Sample purity was tabulated as residual mass after TGA analysis (Table 3-2). The burn-out temperature of arc-discharge-based MWCNTs was higher than that of CVD-based MWCNTs, indicating that arc-discharge-based MWCNTs were more effectively crystallized than were CVD-based MWCNTs. The burn-out temperatures of CVD-PMWCNTs, CVD-H-MWCNTs, CVD-A-MWCNTs, and CVD-HT-MWCNTs were 600.25°C, 597.57°C, 528.18°C, and 643.72°C, respectively (Figure 3-5A–D and Figure 3-5I, orange diamond). In the case of arc-discharge-based MWCNTs, the oxidation temperatures of Arc-PMWCNTs, Arc-H-MWCNTs, Arc-A-MWCNTs, and Arc-HA-MWCNTs were 803.65°C, 804.41°C, 727.88°C, and 777.12°C, respectively (Figure 3-5E–H and Figure 3-5I, blue square). The concentrations of metal elements in MWCNTs are shown in Table 3-3. Using this, the quantity of each metal element was estimated by multiplication of the metal catalyst remnants resulting from TGA (Figure 3-5A–H and Table 3-2).
Figure 3-5 | Total metal contents and burn-out temperatures of MWCNTs.
(Continue-next page)
(Continued) **Figure 3-5 | Total metal contents and burn-out temperatures of MWCNTs.** Metal contents of MWCNTs were measured by a thermogravimetric analyzer (TGA). (A) CVD-PMWCNTs showed oxidation temperatures of amorphous carbon contaminants and SWCNTs (150–200°C and 350–500°C, respectively) in addition to 14.75% residual mass and a peak oxidation temperature of 600.25°C. (B) CVD-H-MWCNTs showed 15.05% residual mass and a 597.57°C oxidation temperature. (C) CVD-A-MWCNTs exhibited 0.98% residual mass and a 528.18°C oxidation temperature. (D) CVD-HA-MWCNTs showed 0.92% residual mass and a 643.72°C oxidation temperature. Arc-discharge-based MWCNTs (E–H) contained less metal catalysts than did CVD-based MWCNTs (A–D). (I) The pattern of residual mass and burn-out temperature was the same in CVD-based MWCNTs.
### Table 3-2. Metal catalyst remnant after TGA analysis

<table>
<thead>
<tr>
<th>Catalyst remnants (%)</th>
<th>PMWCNT</th>
<th>H-MWCNT</th>
<th>A-MWCNT</th>
<th>HA-MWCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD</td>
<td>14.75</td>
<td>15.05</td>
<td>0.98</td>
<td>0.92</td>
</tr>
<tr>
<td>Arc</td>
<td>0.32</td>
<td>0.87</td>
<td>0.19</td>
<td>0.36</td>
</tr>
</tbody>
</table>

### Table 3-3. Concentrations of each metal element in samples by ICP-AES

<table>
<thead>
<tr>
<th></th>
<th>CVD (CM-100)</th>
<th>Arc-discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMWCNT</td>
<td>H-MWCNT</td>
</tr>
<tr>
<td>Mn</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Co</td>
<td>568.47</td>
<td>4927.68</td>
</tr>
<tr>
<td>Ni</td>
<td>nd</td>
<td>6.56</td>
</tr>
<tr>
<td>Cu</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Zn</td>
<td>14.59</td>
<td>15.85</td>
</tr>
<tr>
<td>Al</td>
<td>4220.04</td>
<td>9572.47</td>
</tr>
<tr>
<td>Fe</td>
<td>721.76</td>
<td>4648.10</td>
</tr>
<tr>
<td>Ti</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Pt</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

i) ICP-AES

. Model : OPTIMA 4300DV, Perkin-Elmer(USA)
. Source : Argon plasma(6000K)
. Spectral range : 167-782nm
. Resolution : Better than 0.006nm at 200nm
. Detection limit : 10 ppb – X00 ppb
3.3.6. The change of length, diameter and surface area of 8 kinds of MWCNTs after post-treatment

In the case of CVD-based MWCNTs, PMWCNTs were the longest, followed by H-PMWCNTs, A-PMWCNTs, and HA-PMWCNTs for both scaling methods (Figure 3-6A). Arc-discharge-based MWCNTs did not show big differences in length after post-treatment (Figure 3-6A). DLS and manual scaling method results showed the same pattern, though the actual length appeared longer when measured using the manual scaling method (Figure 3-6A). For correlation PCA and ANOVA test between the physic-chemical factors and EC\(_{50}\), result by manual scaling method, length from the SEM image analysis was used. Diameter from SEM images analysis did not show any significant change (Figure 3-6B). The change of surface area after acid-treatment of arc-discharge-based MWCNTs and CVD-based MWCNTs represents conflicting result. Several reports also show controversy result after acid-treatment (Chakraborty et al., 2006; Kim et al., 2011). Although the reason for this controversy need to be studied, Figure 3-7 shows the “diameter” used in this correlation analysis study. The overall characteristics are summarized in Table 3-4.
Figure 3-6 | Length and diameter of the eight types of MWCNTs. (A) The lengths of MWCNTs were measured by 2 different methods. Thermal treatment shortened the length of CVD-based MWCNTs whereas not of the arc-discharge-based MWCNTs. Acid treatment shortened length of MWCNTs created from both synthetic methods. (B) The diameter of MWCNTs measured from SEM images.
Surface area (m$^2$/g)

Figure 3-7 | Surface areas of the eight types of MWCNTs. CVD-A-MWCNTs showed approximately half the surface area of CVD-PMWCNTs. The surface area of Arc-H-MWCNTs was greater than that of Arc-PMWCNTs.
Table 3-4. Overall characteristics of 8 kinds of MWCNTs

<table>
<thead>
<tr>
<th></th>
<th>CVD (CM-100)</th>
<th>Arc-discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMWCNT</td>
<td>H-MWCNT</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G.S.D.)</td>
<td>47.87 nm</td>
<td>28.26 nm</td>
</tr>
<tr>
<td></td>
<td>(1.18)</td>
<td>(1.18)</td>
</tr>
<tr>
<td>Length (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G.S.D.)</td>
<td>1.33 μm*</td>
<td>1.10 μm*</td>
</tr>
<tr>
<td></td>
<td>(1.62)</td>
<td>(2.03)</td>
</tr>
<tr>
<td>Degree of</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>dispersion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(in water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purity (by TGA)</td>
<td>85.25%</td>
<td>84.95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_D/I_G$</td>
<td>1.83*</td>
<td>2.04</td>
</tr>
<tr>
<td>Surface area</td>
<td>243.38</td>
<td>235.78</td>
</tr>
<tr>
<td>(m²/g)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G.S.D.: Geometric Standard Deviation, (ANOVA, *$p < 0.05$)
3.3.7. Intracellular ATP levels of cells treated with the eight types of MWCNTs

ATP levels of MWCNT-treated cells were assessed using the CellTiter-Glo luciferase assay (Figure 3-8A and Figure 3-8B), and the half-maximal effective concentration (EC$_{50}$; Figure 3-8C) was calculated from data presented in Figure 3-8A and Figure 3-8B. In general, the EC$_{50}$ of CVD-MWCNT-treated cells was lower than that of Arc-MWCNT-treated cells (Figure 3-8C). The effects of acid treatment on intracellular ATP levels were clear in CVD-MWCNTs, which changed from an EC$_{50}$ of 195.74 µg/mL with CVD-PMWCNTs to an EC$_{50}$ of 28.25 µg/mL with CVD-A-MWCNTs (Figure 3-8C). However, this toxicity potentiation effect by acid treatment was not observed in arc-discharge-based MWCNTs (Figure 3-8C). The toxicity of heat-treated MWCNTs was also higher than PMWCNTs in both CVD and arc-discharge methods, although the potentiation effect was not as substantial as that of acid treatment of CVD-MWCNTs (Figure 3-8C). For both heat and acid treatment, the EC$_{50}$ correlated with the treatment that endowed higher toxicity (Figure 3-8C).
Figure 3-8 | Effects of the eight types of MWCNTs on cellular ATP amount.

16HBE14o- cells were seeded in 96 well plates and maintained in DMEM/F-12 media
supplemented with 10% fetal bovine serum. Cell viability was measured after 48 h under the treatment of CVD-based MWCNTs (A) and arc-discharge-based MWCNTs (B) at the indicated concentrations using the CellTiter-Glo luminescent Cell viability assay. (C) The half-maximal effective concentration (EC_{50}) was calculated from the data presented in A and B.
3.3.8. Correlation analysis between the physicochemical factors and cellular toxicity

The correlation analyzed into three ways. To find general factor correlated with toxicity, integrated data of 8 different MWCNTs applied to the PCA (Figure 3-9). In addition to this, correlation was analyzed using data of CVD-based MWCNTs (Figure 3-10) and arc-discharge-based MWCNT (Figure 3-11) each. In integrated analysis using data of all of 8 MWCNTs, the factor “Raman shift (G)”, which projected to the side opposite the factor “EC$_{50}$” had the greatest relevance because the EC$_{50}$ and toxicity is inversely related (Figure 9-8A and 9-8B). The graph shows different clusters of 8 different MWCNTs (Figure 9-8A). The first dimension (52.3% of the total inertia) opposed Arc-H-MWCNTs (high intensity ratings in “Raman shift (D)”) to CVD-HA-MWCNTs and CVD-A-MWCNTs (high intensity ratings in “Raman shift (G)” and “Defect”); the second dimension (21.03% of the total inertia) opposed the Arc-PMWCNT, Arc-A-MWCNT and Arc-HA-MWCNTs (high intensity ratings in “Diameter”, “Length”, “Raman shift (D)”, EPR intensity”, “EC$_{50}$” and “Oxidation temperature”) to CVD-PMWCNTs and CVD-H-MWCNTs (high intensity ratings in “Iron”, “Aluminum”, “TGA metal catalyst” and “surface area”).

In PCA using data of CVD-based-MWCNTs, the factor “Defect (I$_D$/I$_G$)”, which projected to the side opposite the factor “EC$_{50}$”, had the greatest relevance with cellular toxicity of CVD-based-MWCNTs (Figure 2-10B). In addition, surface charge, which stretched in the same direction with “EC$_{50}$”, is also positively correlated to cellular toxicity because it has negative value (Figure 2-10B). Surface charge becomes larger,
which means the value of surface charge get close to positive value, the EC$_{50}$ increases. Consequently, it means that surface charge becomes larger, the cellular toxicity decreases. On the other hand, “Iron” content and “length” was negatively correlated to cellular toxicity. In case of arc-discharge-based MWCNTs, the factors “Surface area”, “EPR peak intensity” and “Defect (I$_D$/I$_G$)”, which projected to the side opposite the factor “EC$_{50}$”, had great relevance (Figure 3-11B).
Figure 3-9 | Correlation of the factors of 8 different MWCNTs using PCA. (A) Individual factor map showed different clusters of MWCNTs. (B) Variable factor map showing correlations between physicochemical factors and factor “EC_{50}”. The factor “Raman shift (G)”, which projected to the side opposite the factor “EC_{50}” had the greatest relevance with cellular toxicity of MWCNTs because the EC_{50} and toxicity is inversely related.
Figure 3-10 | Correlation of the factors of 4 different CVD-based MWCNTs using PCA. (A) Individual factor map showed different clusters of CVD-based MWCNTs. (B) Variable factor map showing correlations between physicochemical factors and factor “EC$_{50}$”. The factor “Defect (I$_D$/I$_G$)”, which projected to the side opposite the factor “EC$_{50}$”, had the greatest relevance with cellular toxicity of CVD-based-MWCNTs.
**Figure 3-11 | Correlation of the factors of 4 different arc-discharge-based MWCNTs using PCA.**

**(A)** Individual factor map showed different clusters of CVD-based MWCNTs. **(B)** Variable factor map showing correlations between physicochemical factors and factor “EC$_{50}$”. The factors “Surface area”, “EPR peak intensity” and “Defect ($I_D/I_G$)”, which projected to the side opposite the factor “EC$_{50}$”, had great relevance with cellular toxicity of arc-discharge-based-MWCNTs.
3.4. DISCUSSION

This study was aimed at describing which physicochemical properties were involved in determining the cellular toxicity of MWCNTs. Generally, the toxicity of certain nanoparticles such as CNTs is known to be related to factors such as surface area, length, degree of agglomeration, water solubility, metal catalyst remnants, and surface charge (Donaldson et al., 2006). However, studies seeking mainly to identify physicochemical determinants that have dominant roles in toxicity according to synthetic methods and post-acid and -thermal treatment have not provided detailed descriptions of these factors (Aillon et al., 2009). In fact, studying the toxicity of MWCNTs is difficult in terms of restricting the particle conditions because the lengths of MWCNTs are difficult to control during synthesis, and prepared MWCNTs tend to be contaminated with impurities such as metal catalyst particles, amorphous carbon broadness in the diameter, and chirality distribution of tubes (Peng and Wong, 2009). Therefore, we used eight types of MWCNTs showing different characteristics. They were prepared from two different synthetic methods and two post-treatment steps to compare cellular toxicity (Figure 3-1 and Figure 3-2). By this simple comparison, we observed which physicochemical properties had the greatest impact on cellular toxicity and which characterization methods were useful to predict the result at the same time.

Physicochemical changes in thermal-treated MWCNTs vary according to temperature (Behler et al., 2006). Raising the temperature up to 1800–2000°C increased the crystallinity of MWCNTs by annealing them; however, raising the
temperature up to 500°C has the opposite effect by creating defective sites on the side walls (Behler et al., 2006). We applied the latter method; therefore, the effects of thermal treatment were expected to decrease amorphous carbon and the creation of binding sites by breaking C–C bonds. This phenomenon was apparent in Arc-H-MWCNTs but not in CVD-H-MWCNTs and was well characterized by the Raman G-peak shift (Figure 3-3A, Figure 3-3B, and Table 3-1). In PCA analysis (Figure 3-10B), broken C–C bonds represented as this Raman G-peak shift revealed the major determinant, and subsequent particle characterization results did not have any correlation with the EC$_{50}$ of MWCNTs. The broken C–C bond may attack important molecules in the cell since π-orbital misalignment between adjacent carbon atoms has an influence on increment of overall reactivity (Hamon et al., 2001; Niyogi et al., 2002).

Through acid treatment, MWCNTs are decapped by carboxylated groups, and sidewall defects are functionalized to form functional groups, including hydroxyl groups. Consequently, the physicochemical characteristics of PMWCNTs are changed (Peng and Wong, 2009; Vaisman et al., 2006; Yan et al., 2011). First, A-MWCNTs showed enhanced dispersion in water or organic medium because of the introduction of functional groups to the defects (Figure 3-3B and Table 3-1). Second, A-MWCNTs were shorter in length (Figure 3-6A). Third, A-MWCNTs exhibited negative charge shifts of zeta potential (Table 3-4) as the ionization reaction of carboxylic acids in water was sufficiently effective (Vaisman et al., 2006). Fourth, metal catalysts were removed (Table 3-1 and 3-2). Fifth, TMWCNTs exhibited lesser surface area than
PMWCNTs (Figure 3-7); this was caused by rebundling of the tube walls (Chakraborty et al., 2006). These features were observed in CVD-A-MWCNTs, but not in Arc-A-MWCNTs. Since Arc-PMWCNTs originally showed high crystallinity and had few defects (Tessonner et al., 2009), acid oxidation through defective walls may occur, albeit in a limited fashion. Consequently, Arc-A-MWCNTs did not show significant differences in cell viability compared to Arc-PMWCNTs (Figure 3-8C). Generally, among several physicochemical characteristics, length and surface area are generally believed to be positively correlated with toxicity (Liu et al., 2012). Metal remnants and negative surface charge are also known to enhance the toxicity of reactive oxygen species (Donaldson et al., 2006). However, in correlation analysis with “EC$_{50}$” and physicochemical factors using data all of 8 MWCNTs, the factor “Raman shift (G)”, which projected to the side opposite the factor “EC$_{50}$” had the greatest relevance because the EC$_{50}$ and toxicity is inversely related. In PCA using the data only of CVD-based-MWCNTs, the factor “Defect (I$_D$/I$_G$)”, which projected to the side opposite the factor “EC$_{50}$”, and “Surface charge” which stretched in the same direction with “EC$_{50}$”, had the greatest relevance with cellular toxicity of CVD-based-MWCNTs (Figure 2-10B). It is interesting that, “Iron” and “Length” are negatively correlated to cellular toxicity in the analysis of CVD-based MWCNTs. Because this study is cell viability based on 48 h treatment, this result cannot explain long-term tumorigenic study of CVD-based MWCNTs. However, it may be possible that these factors have long-term effect on tumorigenicity of CVD-based MWCNTs which was studied in Chapter II. Future study is needed for this. In case of arc-discharge-based MWCNTs, the factors
“Surface area”, “EPR peak intensity” and “Defect (I_D/I_G)”, which projected to the side opposite the factor “EC_{50}”, had great relevance (Figure 3-11B).

From this simple comparative study, we identified some important determinants of the physicochemical characteristics of MWCNTs according to synthetic method and post-treatments. In both Arc- and CVD-MWCNTs, creation of binding sites on the tube walls by breaking the C–C bonds after thermal oxidation or introducing functional groups by acid treatment played a pivotal role in increased toxicity. This broken C–C bond feature was most clearly demonstrated by the “Raman shift (G)”, which is related to generation of D’-peak at 1615 cm\(^{-1}\), a double resonance Raman feature (Behler et al., 2006; Lehman et al., 2011).

These results indicated that among the factors known to be related to cellular toxicity, such as length, diameter, surface charge, metal catalyst remnants, and increased reactivity, increased reactivity by C–C bond breakage which could be identified by changes in the Raman peak could be widely used for predicting the toxicity of MWCNTs, regardless of the synthesis method and post–treatment conditions. Further studies to identify correlations between cellular and long-term systemic toxicities of these different MWCNTs may be required for the prediction of the safety of MWCNTs.
GENERAL CONCLUSION

The results of this study indicated that the mechanisms of toxicity or physicochemical toxicity determinants were different according to the synthesis method or post-treatments used to prepare MWCNTs, and these differences should be considered for predicting systemic or cellular toxicities. At the systemic level, the inflammogenicity resulting from interactions with various types of cells in the lung and the particle clearance rate from the lung by alveolar macrophages were proposed to be the major determinants of toxicity, in addition to metal catalyst remnants, which can accelerate the Fenton reaction and lysosomal degradation processes in the lung during tumorigenesis. At the cellular level, any other factors that known to induce toxicity, i.e., defects caused by adding functional groups or C–C bond breakage on the surface of MWCNTs, played a pivotal role in cellular toxicity, and these factors could be clearly described and predicted by the Raman peak $I_D/I_G$ ratio and G-peak shift. Anticipating the toxicity of MWCNTs is important because prepared MWCNTs are not homogeneous and because it is not feasible to test every newly synthesized MWCNTs. By uncovering the contribution of physicochemical determinants to the toxic response, the results and methods of this study can be applied for the benign design of MWCNTs and for predicting toxicity of newly synthesized MWCNTs.
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국문 논문 초록

다중벽탄소나노튜브의 마우스 폐에서의 독성 기전
(지도 교수: 조 명 행)

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다중벽탄소나노튜브는 높은 탄성과 인장강도를 가져 보강물질로서 큰 관심을 받고 있으나, 석면과 유사한 큰 종횡비로 인하여 인체에 독성을 유발 할 수도 있다는 가능성이 제기되었다. 다중벽탄소나노튜브는 다른 나노 입자들과는 달리 입자의 물리화학적 특성에 대한 조건 조절이 힘들기 때문에, 독성학자들이 입자의 독성 원 인에 대한 연구를 하기에 어려움이 있다. 이에 본 연구에서는, 다중벽탄소나노튜브에 물리화학적 특성 변화를 주고, 이렇게 준비된 다양한 다중벽탄소나노튜브의 독 성 결과를 비교함으로써, 입자 특성과 독성의 상관 관계를 규명해 보고자 하였다.

먼저, 화학증착법으로 합성한 그대로의 다중벽탄소나노튜브(PMWCNT)와 산처리를 통해 친수성 기능기를 도입한 다중벽탄소나노튜브(TMWCNT)의 물질 특성을 확인하고, 마우스 폐에서의 염증 반응, 청소율, 발암성의 차이를 비교하였다. 마우스 폐로, 대조군은 멸균 식염수, 저농도군은 0.01 mg/kg, 고농도군은 0.1 mg/kg으로 기관지 점적 방법으로 물질을 투여 하고, 최장 일년간 관찰 하였다. 실험 결과, 합성 상태 그대로의 다중벽탄소나노튜브는 산처리 한 다중벽탄소나노튜브 보다, 더 심한 급성 염증반응을 유발하며, 폐에서의 청소율이 더 느린 것으로 나타났다. 세포 증생(hyperplasia)과 분열상(mitotic figure), 세포질 부등 (anisocytosis), 핵
부등 (anisokaryosis)과 같은 가역적 전단병변인 이형 변화 (dysplastic change)는 두 입자 모두에서 육아종성 염증이 대부분 사라진 4개월 후에도 관찰되었으나, 발암성 비교 실험 결과, 합성 그대로의 소수성 다중벽탄소나노튜브를 투여한 군에서만 폐암의 발생을 관찰 할 수 있었다. 이러한 암 발생은 cathepsin-D와 Bcl-2의 발현이 증가된 것과 관련되어 있었으며, 암 발생 그룹에서는 혈관신생인자 (VEGF)와 증식세포핵항원 (PCNA) 또한 증가되어 있었다.

다중벽탄소나노튜브의 물리화학적 특성과 세포 독성과의 상관 관계를 자세히 알 아보기 위하여, 다른 생산 방법, 후처리 방법에 의해 준비된 여덟 종류의 다중벽탄소나노튜브를 이용하여 세포 독성과 높은 상관관계를 갖는 물리화학적 결정인자를 확인 하였다. 결과, 여러 독성 유발 인자를 가운데, 600°C에서의 열처리 혹은 산처리에 의해 발생되는 탄소-탄소 연결의 끊어짐으로 인한 파이 오비탈의 불안정화, 그로 인한 표면 반응성의 증가가 가장 큰 세포 독성의 원인으로 작용함을 확인 할 수 있었다. 이는 기존에 알려진 라만 스펙트럼의 I_D/I_G 외에도 탄소-탄소 연결의 끊어짐을 나타내 주는 G peak 중심의 이동 정도가 다중벽탄소나노입자의 독성을 결정짓는 데 매우 중요한 예측인자로 사용 될 수 있음을 보여준다. 또한 적성형의 아크방전으로 합성된 입자와 달리, 화학증착방식으로 합성된 끝이지 못한 입자는 그 형태를 나타내는 다중벽탄소나노입자의 경우는 길이와 질의 농도가 오히려 세포 독성과 음의 상관관계를 나타낼 수 있었다. 이는 길이가 길수록 오히려 세포의 ATP 생성량을 증가시키며 세포의 증상을 증가시킨다는 것을 의미하며, 이는 앞선 발암성 연구와 일맥 상통한다.

본 연구는 다중벽탄소나노튜브가 그 특성과 종류에 따라 독성의 기전이 다름을 보여준다. 본 연구에서 얻어진 다중벽탄소나노튜브의 물리화학적 특성에 따른 생체에서의 반응 차이와 세포 독성과 물리화학적 특성의 상관관계에 대한 분석은, 다양한 방법으로 합성된 다중벽탄소나노튜브의 독성을 예측하는데 활용되어, 산업현장에서의 안전한 다중벽탄소나노튜브의 사용에 적용 될 수 있을 것이라 생각한다.

주요어: 다중벽탄소나노튜브, 기관지점적, 육아종성 염증, 구별되는 독성 작용, 폐암, 물리화학적 특성, 후처리, 아크방전, 화학증기증착
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