Residual metals in carbon nanotubes suppress the proliferation of neural stem cells

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ABSTRACT — Carbon nanotubes (CNTs) are used in many fields; however, little is known about the effects of CNTs on the central nervous system (CNS). In this study, we found that extracts of sonicated CNTs suppressed the proliferation of neural stem cells (NSCs). Single-walled nanotubes (SWCNTs) and multi-walled CNTs (MWCNTs) were suspended in PBS (1 mg/mL) and sonicated for 5 hr using a water bath sonicator. Supernatants from both types of CNTs suppressed NSC proliferation. The effects weakened in a dilution-ratio-dependent manner and strengthened in a sonication time-dependent manner. Metal concentrations extracted from SCNTs and MCNTs after 5-hr of sonication were determined using inductively coupled plasma mass spectrometry. Mn, Rb, Cs, Tl, and Fe were detected in the SWCNT supernatant, and Mn, Cs, W, and Tl were detected in the MWCNT supernatant. The concentration of Mn, Rb, and Fe eluted from the SWCNTs and Rb eluted from MWCNTs following sonication were sufficient to suppress NSC proliferation alone. N-acetyl cysteine (NAC) and ascorbic acid (AA) reversed the effects of Mn and Fe and restored NSC proliferation. The effects of Rb and Tl were not affected by the antioxidants. Both antioxidants largely restored the suppression of NSC proliferation induced by the SWCNT and MWCNT supernatants. These results suggest that metals extracted from CNTs via a strong vibration energy can suppress NSC proliferation through ROS production by the extracted metals.

Key words: Carbon nanotube, Neural stem cell, Metals, Proliferation

INTRODUCTION

CNTs are fiber-shaped nanomaterials that consist of graphite hexagonal-mesh planes (graphene sheet) in a single-layer (single-walled carbon nanotubes (SWCNTs)) or in multiple layers with nest accumulation (multi-walled carbon nanotubes (MWCNTs)). The structure of SWCNTs is a honeycomb carbon lattice rolled into a cylinder, and the basic morphology consists of a sheet of tangle SWCNT (with a diameter of approximately 2 nm) bundles with diameters tens of nanometers in length. The structure of MWCNTs consists of honeycomb carbon lattices rolled into a multi-layer tubular shape, and the basic morphol-
However, recent reports showed that nano-particles can cross the blood–brain barrier (BBB) and enter the brain (Sharma and Sharma, 2007). Furthermore, it has been suggested that the olfactory nerve pathway is a portal of entry into the CNS (Henriksson and Tjalve, 2000; Persson et al., 2003; Mistry et al., 2009; Balasubramanian et al., 2013). Recent reports showed that MWCNTs are toxic to neural cells (Belyanskaya et al., 2013).

Preparation of supernatants of sonicated CNT solutions

SWCNTs and MWCNTs were suspended in PBS (1 mg/mL) and sonicated for 10 min or 5 hr using a water bath-sonicator (Hitachi-Kokusai Electric Inc., Tokyo, Japan) at a frequency of 36 kHz and a watt density of 65 W/264 cm². The supernatants of sonicated CNT suspensions were diluted with culture medium 10- to 1,000-fold.

Rat neural stem cell (NSC) culture

Rat NSCs were cultured as previously described (Reynolds et al., 1992; Hamanoue et al., 2009) with slight modifications. Briefly, the telencephalon was dissected from embryonic day 16 (E16) rats of either sex in ice-cold DMEM/F12. The tissue was then minced and dispersed into single cells by pipetting. Cells were then cultured in DMEM/F12 containing B27 supplement (1/200), 20 ng/mL fibroblast growth factor 2 (FGF2) and 20 ng/mL epidermal growth factor (EGF) for 7 days. The primary neurospheres were incubated with TrypLE Select for 15 min and dissociated by pipetting. Single cells were seeded in 96-well plates for the proliferation assay.

Measurement of metal concentrations

CNTs were suspended in PBS (1 mg/mL) and sonicated for 5 hr using a water bath sonicator. The metal concentrations in the CNT supernatants were quantified using an inductively coupled plasma mass spectrometer (ICP-MS) (Agilent 7500ce ICP-MS, Agilent Technologies, Santa Clara, CA, USA) fitted with a collision/reaction cell in helium mode. We first detected metals at concentrations exceeding the detection limits using a semi-quantitative analysis. Next, we determined the concentration of the detected metals (i.e., Mn, Fe, Rh, Cs, W, and Tl) using a full quantitative analysis with calibration curves.

Treatment of NSCs with the supernatants of sonicated CNT suspensions, metals, and antioxidants

NSCs were treated with the supernatants of sonicated CNT suspensions, MnCl₂ (1-100 ppb), RbCl (1-100 ppb), TlCl₃ (0.1-10 ppb), FeCl₂ (100-10,000 ppb) or FeCl₃ (100-10,000 ppb) with or without 10 μM N-acetyl cysteine (NAC) or 10 μM ascorbic acid (AA) for 24 hr.

NSC proliferation assay

We quantified NSC proliferation according the instructions from the BrdU cell proliferation assay kit (Calbiochem, Hayward, CA, USA). The primary neurospheres were dissociated into single cells and seeded in 96-well plates at a density of 2 x 10⁴ cells/
well. BrdU was added to the medium during the treatment of NSCs. After incubation, the cells were fixed, and BrdU-immuno-labeling was performed. The fluorescence intensities were used as a marker of proliferation. The fluorescence was measured at an excitation wavelength of 320 nm and emission wavelength of 460 nm with a fluorescence microplate reader (Spectra Max Microplate reader, Molecular Devices, Sunnyvale, CA, USA).

**Data analysis and statistics**

All data are shown as the mean ± S.E.M. The statistical analysis was performed using Student’s *t*-test or an ANOVA followed by a Tukey’s test. Differences were considered to be significant at *p* < 0.05.

**RESULTS**

SWCNTs and MWCNTs were suspended in PBS (1 mg/mL) and sonicated for 5 hr using a water bath sonicator. The supernatants of the sonicated CNT suspensions were collected and diluted with culture medium 10- to 1,000-fold. We found that a 24-hr treatment with supernatants of SWCNT and MWCNT suppressed NSC proliferation in a dilution ratio-dependent manner (Fig. 1). The suppression of proliferation was stronger with the SWCNT supernatant when compared with the MWCNT supernatant. The effects of sonication time were also assessed. The suppressive effects of both supernatants disappeared when the sonication time was changed from 5 hr to 10 min (Fig. 2). These results suggest that the suppression of NSC proliferation is due to factors released from CNTs in a sonication time-dependent manner.

CNTs are manufactured using metallic catalysts (Ding et al., 2008; Yazyev and Pasquarello, 2008; Banhart, 2009; Tyagi et al., 2011). Thus, we speculated that residual metals extracted from CNTs during the 5-hr sonication may be responsible for the suppression of NSC proliferation. We therefore quantified the metal contents in the CNT supernatants. The metals in the SWCNT and MWCNT supernatants were first analyzed using ICP-MS in a semi-quantitative mode. Next, the concentrations of metals were determined using calibration curves (Table 1). We found that a 5-hr sonication induced the extraction of multiple metals from the CNTs. Mn, Rb, Cs, Ti, and Fe were detected in the SWCNT supernatant, whereas Mn, Cs, W, and Ti were detected in the MWCNT supernatant. Among these metals, the concentration of Fe in SWCNT supernatant was remarkably high (from N.D. to 7,110 ppb). The concentrations of these metals in PBS were largely negligible and did not change after a 5-hr sonication.

Next, we examined the direct effects of the metals at concentration ranges detected in the supernatants. Fig. 3 shows the metals that had a suppressive effect on NSC proliferation. 

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**Fig. 1.** Effects of the supernatants of sonicated CNT suspensions on the proliferation of rat NSCs. The supernatants of SWCNTs and MWCNTs suppressed NSC proliferation in a dilution ratio-dependent manner. *: *p* < 0.05, **: *p* < 0.01 vs. control group (N = 6), ANOVA followed by a Tukey’s test.

**Fig. 2.** Sonication time-dependence of CNT supernatant effects. The effects of SWCNT and MWCNT supernatants disappeared with a sonication time of 10 min. However, a 5-hr sonication time produced a significant suppression of NSC proliferation. *: *p* < 0.05 vs. control group (N = 6), ANOVA followed by a Tukey’s test.
proliferation (Fig. 3). Mn\(^{2+}\), Rb\(^{+}\), Tl\(^{3+}\), Fe\(^{2+}\), and Fe\(^{3+}\) suppressed the proliferation of NSCs in a concentration-dependent manner. These results indicate that Mn, Rb, and Fe were present in the SWCNT supernatant at a concentration high enough to suppress NSC proliferation. This effect was induced by the Rb in the MWCNT supernatant. Some metals are known to produce reactive oxygen species (Ding et al., 2008) that can result in oxidative stress on lipids, DNA and proteins (Henriksson and Tjalve, 2000; Choi et al., 2007; Alekseenko et al., 2008; Kim et al., 2011; Latronico et al., 2013; Roth and Eichhorn, 2013; Sripetchwandee et al., 2013). Thus, we examined the involvement of ROS in the suppression of NSC proliferation. N-acetyl cysteine (NAC) (10 \(\mu\)M) and ascorbic acid (AA) (10 \(\mu\)M) are typical antioxidants that can significantly restore the suppression of the NSC proliferation caused by Mn\(^{2+}\), Fe\(^{2+}\), and Fe\(^{3+}\) (Fig. 4A). The effect of Rb and Tl were not affected by NAC or AA (data not shown). These results suggest that ROS is involved in the suppressive effects produced by Mn and Fe. We also examined whether ROS played a role in the suppression of NSC proliferation by the CNT supernatants (Fig. 4B). Both NAC and AA markedly restored the decrease in NSC proliferation caused by the SWCNT and MWCNT supernatants. We confirmed that both of these antioxidants alone did not affect NSC proliferation (data not shown). Taken together, these results suggest that the suppressive effects of the sonicated extract of CNTs were mainly caused by

### Table 1. Metals eluted from CNTs by sonication for 5 hr.

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<th>Concentrations of metals (ppb)</th>
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<tr>
<td>PBS</td>
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<td>SWCNT</td>
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<td>Mn</td>
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The metal concentrations in the supernatant of SWCNT and MWCNT were quantified using ICP-MS in a semi-quantitative mode followed by a full quantitative mode. Mn, Rb, Cs, W, Tl, and Fe were detected in the SWCNT supernatant. Mn, Rb, Cs, W, Tl, and Fe were detected in the MWCNT supernatant. The concentration of Fe in the SWCNT supernatant was remarkably high (7,110 ppb).

![Fig. 3](image-url) The direct effect of metals in CNT supernatants. Mn\(^{2+}\), Rb\(^{+}\), Tl\(^{3+}\), Fe\(^{2+}\), and Fe\(^{3+}\) suppressed NSC proliferation in a concentration-dependent manner. *: \(p < 0.05\), **: \(p < 0.01\) vs. control group (N = 12), ANOVA followed by a Tukey’s test.
We found that the supernatants of sonicated CNT suspensions suppress NSC proliferation. We also determined that these effects were largely mediated by ROS production from residual metals. To demonstrate the involvement of ROS, we used the two antioxidants NAC and AA. NAC exerts its protective by increasing glutathione levels (Yim et al., 1994; Arfsten et al., 2007; Li et al., 2009), directly scavenging ROS, and activating ERK1/2 (Zhang et al., 2011). AA is a powerful water-soluble antioxidant that acts by scavenging ROS and reactive nitrogen species (Carr and Frei, 1999; Kojo, 2004). The concentrations of NAC and AA used in this study were at a level shown to suppress the effects of ROS in previous studies (Carr and Frei, 1999; De la Fuente and Victor, 2001; Nakajima et al., 2009). Proliferative NSCs have a high endogenous ROS lev-

**Fig. 4.** Antioxidants attenuated the reduction in NSC proliferation caused by metals and CNT supernatants. The suppression of the NSC proliferation caused by Mn⁴⁺, Fe⁴⁺, Fe⁵⁺ (A) and the supernatants of CNTs (B) was significantly restored by NAC (10 μM) and AA (10 μM). *: p < 0.05, **: p < 0.01 vs. control group, #: p < 0.05, ##: p < 0.01 vs. metal or CNT-supernatant-treated groups (N = 7), ANOVA followed by a Tukey’s test.
el (Le Belle et al., 2011), and redox balance is important to regulate NSC/neural progenitor cell (NPC)-self-renewal and differentiation (Smith et al., 2000; Li et al., 2007; Hou et al., 2012; Topchyi et al., 2013). For example, mitochondrial superoxide negatively regulates NPC-self-renewal in the developmental cerebral cortex (Hou et al., 2012). High levels of ROS inhibit O-2A progenitor proliferation (Smith et al., 2000; Li et al., 2007). In other cases, NADPH oxidase (Nox) 4-generated superoxide drives mouse NSC proliferation (Topchyi et al., 2013). Ketamine-induced ROS enhanced the proliferation of NSCs derived from human embryonic stem cells (Bai et al., 2013). The effect of ROS on NSC/NPC proliferation may change depending on the subcellular localization of the ROS generation and the timing of the ROS generation.

The suppression of NSC proliferation by the supernatants of both CNTs were virtually restored by the antioxidants, suggesting that the effects of CNT-supernatants were mediated through ROS stress. After a 5-hr sonication, multiple metals were detected in the SWCNT and MWCNT supernatants using ICP-MS. Mn, Rb, Cs, Tl, and Fe were detected in the SWCNT supernatant, and Mn, Cs, W, and Tl were detected in the MWCNT supernatant. Out of these SWCNT metals, the effects of Mn and Fe were reversed by antioxidants, suggesting that Mn and Fe play the main role in the suppression of NSC proliferation by CNT supernatants. In the MWCNT supernatant, the concentrations of Mn and Fe were insufficient to suppress NSC proliferation. Thus, a combination of ROS produced by multiple metals might produce synergistic suppressive effects.

Fe is essential for biological processes, but it is also known to be toxic in excess. Fe^{2+} overload into the cells and shuttling of Fe^{3+} to Fe^{2+} leads to cellular malfunctions due to ROS production (Halliwell and Gutteridge, 1992; Touati, 2000). Although Fe^{3+} has been largely considered as non-cytotoxic (Braun, 1997; Bruins-Audom, 2008; Banhart, 2009; Tyagi et al., 2011). Recent studies showed that metal impurities play a major role in CNT cytotoxicity (Liu et al., 2008; Kim et al., 2010). The residual metals can remain in the contact solvent or embed inside the CNTs (Pumera, 2007; Fubini et al., 2011; Aldieri et al., 2013). In our study, the content of Fe in SWCNT was remarkable. A SWCNT is a graphene sheet protected metal core/shell of nanoparticles (Pumera, 2007). This structure may have caused the higher levels of metal impurities when compared with MWCNTs. Our data suggest that the residual metallic catalysts are released by vibration energy with a sonication frequency of 36 kHz, watt density of 65 W/264 cm² and sonication time of 5 hr. Pumera et al. indicated that washing with concentrated nitric acid removed up to 88% (w/w) of metal catalyst nanoparticles (Pumera, 2007). For public health and the safer applications of CNTs in nano-medicine, it is preferable to decrease the amount of the metal impurities by improving the washing process.

Conflict of interest—The authors declare that there is no conflict of interest.

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