Letter

Sub-toxic concentrations of nano-ZnO and nano-TiO₂ suppress neurite outgrowth in differentiated PC12 cells

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ABSTRACT — Nanomaterials have been extensively used in our daily life, and may also induce health effects and toxicity. Nanomaterials can translocate from the outside to internal organs, including the brain. For example, both nano-ZnO and nano-TiO₂ translocate into the brain via the olfactory pathway in rodents, possibly leading to toxic effects on the brain. Although the effects of nano-ZnO and nano-TiO₂ on neuronal viability or neuronal excitability have been studied, no work has focused on how these nanomaterials affect neuronal differentiation and development. In this study, we investigated the effects of nano-ZnO and nano-TiO₂ on neurite outgrowth of PC12 cells, a useful model system for neuronal differentiation. Surprisingly, the number, length, and branching of differentiated PC12 neurites were significantly suppressed by the 7-day exposure to nano-ZnO (in the range of 1.0×10^{-4} to 1.0×10^{-1} $\mu\text{g/mL}$), at which the cell viability was not affected. The number and length were also significantly inhibited by the 7-day exposure to nano-TiO₂ (1.0×10^{-3} to 1.0 $\mu\text{g/mL}$), which did not have cytotoxic effects. These results demonstrate that the neurite outgrowth in differentiated PC12 cells was suppressed by sub-cytotoxic concentrations of nano-ZnO or nano-TiO₂.

Key words: Nano-ZnO, Nano-TiO₂, PC12, Neurite outgrowth

INTRODUCTION

Nanomaterials have been extensively used in areas such as energy, healthcare, environment, materials, and electronics, and study of their toxicity and health effects is needed. The small size of nanomaterials provides them with specific properties, such as high surface-to-volume ratio and high surface charge (Linse *et al.*, 2007). It has been reported that nanomaterials translocate from the interstitial region of the lung after respiratory exposure to other organs, such as the liver and kidney, through blood circulation (L'Ajou *et al.*, 2008; Nemmar *et al.*, 2002; Semmler *et al.*, 2004; Elder and Oberdörster, 2006).

In the case of the brain, the olfactory-brain translocation pathway is considered the main route for entry of nanomaterials into the brain (Oberdörster *et al.*, 2005). Nasal exposure of airborne nano-ZnO or nano-TiO₂ in rodents results in their translocations into the brain (Kao *et al.*, 2012b; Wang *et al.*, 2008), and several groups have reported the effects of these nanomaterials on neuron-

al viability or neuronal excitability *in vitro* (Gramowski *et al.*, 2010; Valdiglesias *et al.*, 2013a, 2013b; Liu *et al.*, 2010; Long *et al.*, 2007; Jeng and Swanson, 2006). However, it is still unclear whether these nanomaterials affect the differentiation and development of neurons.

Neurons are differentiating entities, and neurogenesis in the mammalian brain continues into adulthood. Differentiated neurons assemble into functional networks by developing axons and dendrites (collectively called neurites) that can synapse with other neurons (Van Ooyen *et al.*, 1995). The morphologies of neurons are modulated by a variety of conditions including trophic factors, electrical activity, synaptogenesis, functional maturation, and differentiation of neurons (Thoenen, 1991; Van Ooyen *et al.*, 1995; Fields and Nelson, 1992). The neuronal morphology is also affected by the cell viability, as shown in neurodegenerative diseases (Klement *et al.*, 1998). Therefore, morphological changes *in vitro* can be sensitive markers for detecting neurotoxins. As a model of neuronal differentiation in both neurobiological and neurotoxicological

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studies, the PC12 cell line has been widely used. PC12 cells respond to nerve growth factor (NGF) exposure with dramatic morphological changes. NGF-treated, differentiated PC12 cells cease proliferation, extend neurites, and become electrically excitable (Das *et al.*, 2004).

In this study, we re-evaluated the cytotoxic effects of nano-ZnO or nano-TiO₂ and then investigated effects of these nanomaterials on neurite outgrowth in differentiated PC12 cells. We observed that the toxic concentration 50 (TC₅₀) after 4 days of exposure of PC12 cells to nano-ZnO was about 10 µg/mL, and that nano-TiO₂ did not affect the viability. We also found that the neurite outgrowth of differentiated PC12 cells was suppressed by a sub-toxic concentration of nano-ZnO or nano-TiO₂.

MATERIALS AND METHODS

Nanomaterials

Nano-ZnO was purchased from Sigma-Aldrich (St. Louis, MO, USA; 721077, average primary particle size ≤ 40 nm), and nano-TiO₂ was obtained as a gift from CIK NanoTek (Tokyo, Japan; average primary particle size ≤ 36 nm, 80% anatase type).

Measurement of average hydrodynamic size of the nanomaterials

Nano-ZnO or TiO₂ were diluted to 100 µg/mL in the differentiation medium: RPMI1640 (Gibco, Grand Island, NY, USA) medium containing 1% heat-inactivated horse serum (Gibco), 0.5% heat-inactivated fetal bovine serum (Gibco), a mixture of a penicillin-streptomycin solution (100 unit/mL and 100 µg/mL, respectively; Gibco), and NGF (50 ng/mL, mouse NGF 2.5S, Alamone labs, Jerusalem, Israel). The suspensions of nanomaterials were sonicated for 10 min to prevent aggregation, and then the average hydrodynamic sizes were measured by dynamic light scattering measurement using ELSZ-2NP (Otsuka Electronics, Osaka, Japan).

Culture of PC12 cells and application of nanomaterials

PC12 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown on BioCoat collagen I plates (Becton Dickinson, Franklin Lakes, NJ, USA) with the growth culture medium (RPMI1640 medium containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and a mixture of the penicillin-streptomycin solution). The cells were maintained in a CO₂ incubator at 37°C (Forma 310, ThermoFisher Scientific, Waltham, MA, USA) with a

water-saturated, 5% CO₂ atmosphere. PC12 cells were passaged at a 1:3 ratio when the cells reached 80-90% confluence. One day before the application of test nanomaterials, PC12 cells were plated onto collagen I-coated, 96-well plates (ThermoFisher Scientific) at a density of 2,500 cells/well with the differentiation medium (200 µL). When the nanomaterials were applied to the wells, the suspension of the nanomaterials was prepared fresh before use with the differentiation medium and dispersed for 10 min using a sonicator. The multi-well plates were incubated further for several days (maximum 7 days) in the same incubator. Triton-X (0.1% v/v) was used as a positive control reagent to induce complete cell death. Phase-contrast images were acquired using an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan), a phase contrast 20 × objective lens (Nikon), and a digital camera (DN100, Nikon).

Lactate dehydrogenase (LDH) assay

PC12 cells were exposed to the nanomaterials for 4 days for the assay. The supernatants of the culture medium (100 µL) were collected from each well and transferred to new 96-well plates. An LDH assay was performed according to the manufacturer's instructions (LDH-Cytotoxic Test, Wako Pure Chemical Industries, Osaka, Japan). The absorbance at 560 nm of the sample was measured using a plate reader (iMark, Bio-Rad, Hercules, CA, USA). The percentage of cytotoxicity was calculated as follows: $(S - N)/(P - N) \times 100$, where S is the absorbance of the samples, N is of the negative control (mean of wells without nanomaterials), and P is of the positive control (mean of wells treated with 0.1% v/v of Triton-X).

Thiazolyl blue tetrazolium bromide (MTT) assay

Supernatants of the culture medium (100 µL) were removed from each well and then the plates were used for the assay. Thiazolyl blue tetrazolium bromide (MTT, M2128, Sigma-Aldrich) was dissolved to a concentration of 5 mg/mL in PBS (–) and then sterilized using 0.22 µm pore filter membranes (= MTT solution). The solution (10 µL) was added to each well, and the plates were incubated further for 3 hr. The medium was then removed and the formazan produced in the cells was dissolved by adding isopropanol containing 0.04 M HCl (200 µL). The absorbance at 570 nm was measured, and the percentage cell viability was calculated as follow: $(S - B)/(N - B) \times 100$, where S is the absorbance of the sample, and B is the mean of the absorbance of the blank well without cells.

Measurement of neurite outgrowth

PC12 cells treated with nanomaterials were fixed after 8 days *in vitro* (DIV) in 4% (w/v) formaldehyde in PBS(–) (pH 7.4) for 30 min at room temperature (RT). The neurites were visualized using a combination of the following antibodies: mouse anti-beta III tubulin antibody (1:1000 dilution, 2 hr at RT, MAB5564, Merck Millipore, Darmstadt, Germany) and goat anti-mouse AlexaFluor 488-conjugated antibody (1:500 dilution, 2 hr at RT, A-1100, ThermoFisher Scientific). These antibodies were diluted in PBS (–) containing 0.3% Triton X-100, 0.12% λ -carrageenan, 1% goat serum, and 0.02% sodium azide (Irie *et al.*, 2014). The nuclei were labeled using Hoechst 33342 (1 μ g/mL, 30 min at RT, Dojindo, Kumamoto, Japan). The acquisition of the fluorescent images was performed with a high-content screening platform (Cellomics CellInsight, ThermoFisher Scientific) using a 10 $\times$ /0.3 numerical aperture objective lens (UPlanFL N, Olympus, Tokyo, Japan). Fifteen view fields, corresponding to 5.3 μ m², were imaged for each well. The measurement of neurite parameters was performed using the Cellomics Neuronal Profiling V4 BioApplication installed in the Cellomics CellInsight. Data from 15 view fields were then averaged.

Data analysis

The TC₅₀, the concentration of the test nanomaterials that causes 50% cell damage or decline in cell viability, was calculated using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was tested using one-way ANOVA with a post-hoc Dunnett's test (significance, $p < 0.05$), and data are presented as the means $\pm$ standard error of the mean unless otherwise stated. Numbers in parentheses in figures and n in the text indicate the number of experiments.

RESULTS

Characterization of the nano-ZnO and nano-TiO₂

As nanomaterials often form agglomerates, average hydrodynamic sizes of nano-ZnO or nano-TiO₂ dispersed in the differentiation medium were measured by dynamic light scattering (Dhawan and Sharma, 2010). The average hydrodynamic sizes of nano-ZnO and TiO₂ were 176 $\pm$ 21 nm ($n = 3$) and 321 $\pm$ 33 nm ($n = 3$), respectively. These values are comparable to those reported previously (Liu *et al.*, 2010; Wu *et al.*, 2010).

Effects of nano-ZnO and nano-TiO₂ on the viability of PC12 cells

To determine the cytotoxicity of nano-ZnO and nano-

TiO₂, PC12 cells were seeded onto collagen-I-coated plates in the differentiation medium and cultured for one day. Subsequently, the cells were exposed to the nanomaterials for 4 days in a CO₂ incubator, and the toxicity was then examined using LDH or MTT assays. Figure 1Ai - 1Aiii shows that the cytotoxicity of nano-ZnO was concentration-dependent. The phase-contrast images showed that PC12 cells exposed to lower concentrations of nano-ZnO (0.1 μ g/mL, Fig. 1Aii) had no change in morphology, but that those exposed to higher concentrations (10 μ g/mL, Fig. 1Aiii) exhibited obvious changes in their shapes: cells were aggregated and had rounder cell bodies with fewer neurites. The nano-ZnO cytotoxicity and cell viability are shown in Fig. 1B. The TC₅₀ of nano-ZnO determined by LDH and MTT assays were 9.9 and 9.1 μ g/mL, respectively. On the other hand, Fig. 1Aiv shows that a high concentration of nano-TiO₂ (100 μ g/mL) did not affect PC12 cells. LDH and MTT assays revealed that nano-TiO₂ did not damage PC12 cells nor affect the viability even at the highest concentration (Fig. 1Aiv, and 100 μ g/mL in Fig. 1Ci and Cii, $n = 5$ each). These results demonstrate that nano-ZnO has cytotoxic effects in a concentration-dependent manner on PC12 cells whereas nano-TiO₂ does not.

Time course of nano-ZnO-induced cytotoxicity in PC12 cells

To determine how different nanomaterial exposure periods affect the viability of PC12 cells, we measured the time course of the cell viability using an MTT assay. The time courses of percentage cell viabilities in nanomaterials are shown in Fig. 2. The highest concentration of nano-ZnO (100 μ g/mL) decreased the cell viability by more than 50% within 2 hr after application, and this decline continued thereafter (black circles in Fig. 2A; 2 hr, 40.3 $\pm$ 3.5%, $n = 6$, $p < 0.001$; 5 hr, 34.0 $\pm$ 3.2%, $n = 6$, $p < 0.001$; 2 days, 2.2 $\pm$ 0.4%, $n = 6$, $p < 0.001$; 4 days, 4.5 $\pm$ 0.8%, $n = 6$, $p < 0.001$; 7 days, 3.7 $\pm$ 2.1%, $n = 6$, $p < 0.001$). At higher concentrations of nano-ZnO (1, 10, and 100 μ g/mL), the declines in viability exhibited clear time-dependency (gray triangles, white squares, and black circles in Fig. 2A). On the other hand, lower concentrations of nano-ZnO (0.01 and 0.1 μ g/mL) did not affect the cell viability even 7 days after the application (0.01 μ g/mL, black square at 7 days in Fig. 2A, 104.7 $\pm$ 6.3%, $n = 6$; 0.1 μ g/mL, white circle at 7 days in Fig. 2A, 104.0 $\pm$ 6.4%, $n = 6$). A similar experiment was performed with nano-TiO₂ (Fig. 2B), but nano-TiO₂ did not affect the cell viability at any of the tested concentrations (in the range of 0.01 to 100 μ g/mL). These results demonstrate that a low concentration of nano-ZnO (0.01

Fig. 1. Concentration-dependent cytotoxicity of nano-ZnO and the absence of nano-TiO₂ toxicity. (A) Phase-contrast images of PC12 cells exposed to nano-ZnO or nano-TiO₂ for 4 days. The pictures were taken at 5 days *in vitro* (DIV). (Ai) negative control without nanomaterial, (Aii) 0.1 µg/mL of nano-ZnO, (Aiii) 10 µg/mL of nano-ZnO, and (Aiv) 100 µg/mL of nano-TiO₂. Note that the morphology of the cells in (Aiii) is clearly affected: cells are aggregating and show fewer neurites and rounder cell bodies. Apparent changes in the morphology are not seen in (Aii) compared with (Ai). At high concentrations of nano-TiO₂ (100 µg/mL), the cells do not show obvious morphological changes (Aiv). (B) Concentration-dependent cytotoxicity of nano-ZnO measured by the LDH assay (Bi) or MTT assay (Bii). The data were fit to the following equations: $Y = 100/[1 + 10^{(\log TC_{50} - X)}]$ for (Bi) or $Y = 100/[1 + 10^{(X - \log TC_{50})}]$ for (Bii), where TC_{50} is the abbreviation for the 50% toxicity concentration. Each data point in (B) was averaged from 8 wells. Here and in the following figures, numbers in parentheses in figures indicate the number of experiments, and error bars represent the standard error of the means. (C) Absence of cytotoxicity in PC12 cells exposed to nano-TiO₂. The toxicity was measured by the LDH (Ci) and MTT assay (Cii). Here and following figures, the statistical significance was tested using one-way ANOVA with a post-hoc Dunnett's test unless otherwise stated. n.s., not significant.

and 0.1 µg/mL) and nano-TiO₂ has no influence on the survival rate of PC12 cells.

Effect of sub-toxic concentrations of nano-ZnO and TiO₂ on neurite outgrowth

In the brain, differentiated neurons are connected to each other by developing neurites to form functional networks (Van Ooyen *et al.*, 1995). This indicates that proper growth of neurites and their connections are crucial for brain functions. To examine whether neurite outgrowth can be affected by the nanomaterials, we quantified several properties of PC12 neurites using immunohistochemical staining and a high-content screening platform (Fig. 3). Nano-ZnO exhibited cytotoxicity in the range of 1 µg/mL and greater when PC12 cells were exposed for 7 days (Fig. 2A). To examine the effects of a lower concentration of nano-ZnO on neurite outgrowth, nano-ZnO

concentrations of 0.1 µg/mL or lower were used. When PC12 cells were exposed to higher concentrations of nano-TiO₂ (10 and 100 µg/mL), neurite analysis could not be performed because the cells were covered with the precipitated nano-TiO₂. Therefore, concentrations of 1 µg/mL or lower of nano-TiO₂ were adopted.

In the absence of the nanomaterials, NGF-treated PC12 cells showed well-developed neurites at DIV8 (Fig. 3Aii', neurites highlighted with magenta color). Surprisingly, PC12 cells differentiated in the presence of 0.1 µg/mL of nano-ZnO (Fig. 3Bii') exhibited shorter neurites than those in the control group (Fig. 3Aii'). The graphs in Fig. 3D summarize the effects of nano-ZnO on the normalized total number of neurites per cell (Di), normalized total length of neurites per cell (Dii), and normalized number of neurite branch points per neurite (Diii). These graphs demonstrate that the length and branching of neurites in

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Fig. 2. Time course of cell viabilities in the presence of nanomaterials. Time course of cell viability percentage in the presence of nano-ZnO (A) or nano-TiO₂ (B). The viabilities were measured using MTT assay. Note: x-axis is a logarithmic scale. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. In (B), no data point was significantly different compared with control.

PC12 cells were clearly impaired at the lower concentrations of nano-ZnO, which did not affect the viability. The normalized number of neurites per cell, total length of neurites per cell, and number of branch points per neurite were significantly inhibited by nano-ZnO in the range of 1.0×10^{-4} to 1.0×10^{-1} $\mu\text{g/mL}$. These results are in contrast to those shown in Fig. 2A. A similar experiment was performed with nano-TiO₂. In contrast to the viability of cells exposed to nano-TiO₂, the length and branching of neurites were unexpectedly suppressed (Fig. 3Cii'). The normalized number of neurites per cell and total length

of neurites per cell were significantly inhibited by nano-TiO₂ in the range of 1.0×10^{-3} to $1.0 \mu\text{g/mL}$. The number of branch points per neurite was also reduced significantly at 1.0×10^{-3} and 1.0×10^{-2} $\mu\text{g/mL}$. These results indicate that neurite outgrowth in differentiated PC12 cells was impaired by the sub-toxic concentrations of nano-ZnO and nano-TiO₂.

DISCUSSION

Cytotoxic effect of nano-ZnO and absence of nano-TiO₂ toxicity

We demonstrate that nano-ZnO, but not nano-TiO₂, had a cytotoxic effect on differentiated PC12 cells in a concentration-dependent manner (Figs. 1 and 2). Our results using nano-ZnO are consistent with previous reports describing the toxicity of nano-ZnO to mouse neuro-2A neuroblastoma cells, human-derived SH-SY5 neuronal cells, or PC12 cells (Jeng and Swanson, 2006; Valdiglesias *et al.*, 2013a; Kao *et al.*, 2012b), although the toxic nano-ZnO concentrations reported were slightly different from those determined in this study. Nano-ZnO-induced cytotoxicity would be caused by Zn²⁺ ions released from nano-ZnO (Xu *et al.*, 2013). The Zn²⁺ concentration is elevated in both the cytosol and mitochondria, and the intracellular Zn²⁺ ions also can induce oxidative stress and/or elevated mitochondrial Zn²⁺, leading to mitochondrial dysfunction, caspase activation, and cell apoptosis (Fukui *et al.*, 2012; Kao *et al.*, 2012a). In contrast to the results observed with nano-ZnO, nano-TiO₂ did not exhibit cytotoxicity to PC12 cells. This observation agrees with that of Valdiglesias *et al.* (2013b), showing that neither anatase nor anatase plus rutile-type nano-TiO₂ reduce the viability of SH-SY5Y neuronal cells despite the visible nanoparticle uptake. Our results are also similar to those of Wu *et al.* (2010), demonstrating that nano-TiO₂ has a negligible effect on the viability of PC12 cells [$\sim 90\%$ viability in $100 \mu\text{g/mL}$ of anatase or rutile-type nano-TiO₂ after 24-hr incubation]. On the other hand, Liu *et al.* (2010) reported a different result: nano-TiO₂ reduces the viability of PC12 cells by $\sim 50\%$ in largely anatase-type nano-TiO₂ ($100 \mu\text{g/mL}$). The reason for the inconsistency is not clear but could be due to the differences in experimental materials and procedures.

Inhibition of neurite outgrowth by a low concentration of nano-ZnO and nano-TiO₂

In Fig. 3, we showed that sub-toxic concentrations of nano-ZnO or nano-TiO₂ significantly decreased the length and branching of neurites. To the best of our knowledge, this is the first report showing that a non-toxic level of

Fig. 3. Inhibition of neurite outgrowth by nano-ZnO and nano-TiO₂. (A-C) Fluorescent images of PC12 cells labelled with Hoechst 33342 (nuclei, Ai, Bi, and Ci) and anti-beta III tubulin antibody (cell bodies and neurites, Aii, Bii, and Cii). In Aii', Bii', and Cii', detected neurites are highlighted with magenta color. These images were acquired at 8 DIV. (D and E) Summary of neurite growth in the presence of nano-ZnO (D) or nano-TiO₂ (E). (Di and Ei) Normalized number of neurites per cell. (Dii and Eii) Normalized total length of neurites per cell. (Diii and Eiii) Normalized number of branch points per neurite. ****p* < 0.001, ***p* < 0.01, and **p* < 0.05.

nanomaterials can cause neurite maldevelopment. Neurite outgrowth is essential for the formation of neuronal networks, and the blockade of neurite outgrowth disturbs the normal development of the brain (Levi-Montalcini and Cohen, 1960; Cohen, 1960). Our data indicate the possibility that the nanomaterials we tested might affect the formation of the brain in humans by interrupting neurite development.

In PC12 cells, the signaling pathway underlying NGF-induced neurite outgrowth in cells has been clarified: NGF binds to tropomyosin receptor kinase A (TrkA) and leads to dimerization and autophosphorylation of TrkA. This triggers a series of signaling events consisting of PI3-kinase, Vav2/3, and Rac1/Cdc42, leading to control of the organization of the F-actin cytoskeleton and the promotion of axon extensions (Aoki *et al.*, 2005; Skaper, 2008). Our results imply that this signaling cascade could be impaired by nano-ZnO and TiO₂, and further studies are needed to clarify the mechanisms underlying the nanomaterial-induced inhibition of neurite outgrowth.

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

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