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Original Article

Phagocytosis-dependent and independent mechanisms underlie the microglial cell damage caused by carbon nanotube agglomerates

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ABSTRACT — Although carbon nanotubes (CNTs) are used in many fields, including energy, healthcare, environmental technology, materials, and electronics, the adverse effects of CNTs in the brain are poorly understood. In this study, we investigated the effects of CNTs on cultured microglia, as microglia are the first responders to foreign materials. We compared the effects of sonicated suspensions of 5 kinds of CNTs and their flow-through filtered with a 0.22 μm membrane filter on microglial viability. We found that sonicated suspensions caused microglial cell damage, but their flow-through did not. The number of microglial aggregates was well correlated with the extent of the damage. We also determined that the CNT agglomerates consisted of two groups: one was phagocytosed by microglia and caused microglial cell damage, and the other caused cell damage without phagocytosis. These results suggest that phagocytosis-dependent and independent mechanisms underlie the microglial cell damage caused by CNT agglomerates and it is important to conduct studies about the relationships between physical properties of nanomaterial-agglomerates and cell damage.

Key words: Carbon nanotube, Microglia, Phagocytosis, Cytotoxicity

INTRODUCTION

Carbon nanotubes (CNTs) are fiber-shaped nanomaterials that consist of graphite hexagonal-mesh planes (graphene sheets) in a single layer or in multiple layers with nest accumulation. The structure of single-walled carbon nanotubes (SWCNTs) is a honeycomb carbon lattice rolled into a cylinder, and the basic morphology consists of a sheet of tangled SWCNTs (each with a diameter of approximately 2 nm) bundles with diameters of tens of nanometers. The structures of multi-walled carbon nanotubes (MWCNTs) consist of honeycomb carbon lattices that are rolled into a multi-layer tubular shape, and the basic morphology is composed of particles of tangled MWCNTs with a diameter of approximately 30 nm. Although CNTs are used in many fields, including energy, healthcare, environmental technology, materials, and electronics, the adverse effects of CNTs in the brain are poorly understood. Thus far, most studies have focused on lung toxicity, as airborne CNT particles primarily enter through the respiratory tract (Jaurand *et al*., 2009; Pacurari *et al*., 2010). However, recent studies have suggested that the olfactory nerve pathway is a portal of entry to the central nervous system (CNS) for nanoparticles (Mistry *et al*., 2009; Balasubramanian *et al*., 2013). It was also reported that intranasal exposure to nanoparticles (Fe_2O_3) caused pathological alterations associated with microglial activation in the olfactory bulb, the hippocampus, and the striatum (Wang *et al*., 2011). Furthermore, it has been shown that nanoparticles cross the blood-brain barrier (BBB) and infiltrate the brain (Sharma and Sharma, 2007). We recently determined that residual metals extracted from CNTs possibly suppress the proliferation of neural stem cells via the production of reactive oxygen species (ROS) (Shigemoto-Mogami *et al*., 2014b).

Microglia are important cells to maintain CNS homeostasis (Shigemoto-Mogami *et al*., 2014a; Sato, 2015). In pathological conditions of the CNS, microglia are acti-

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vated and trigger the endogenous defense/immune system (Nakajima and Kohsaka, 1993; Kreutzberg, 1996; Graeber and Streit, 2010; Kettenmann *et al*., 2011; Fu *et al*., 2014), including phagocytosis (Lai and Todd, 2006; Cartier *et al*., 2014). Microglia thereby determine the severity of the pathology, as macrophages do so outside the brain. Therefore, in this study, we investigated the effects of CNTs on cultured microglia, as microglia are the first responders to foreign matters. We compared the effects of sonicated suspensions of 5 kinds of CNTs and found that the CNT agglomerates cause microglial cell damage. In addition, the CNT agglomerates consisted of 2 groups in view of mechanisms of their effects: one was phagocytosed by microglia and caused microglial cell damage, and the other caused cell damage not through phagocytosis.

MATERIALS AND METHODS

Materials

Single-wall CNT SW1859 (purity > 95%) (#1) and multi-wall CNT 04-12/10#1-(4) (purity: > 98%) were supplied by Nikkiso Co., Ltd. (Tokyo, Japan). Multi-wall CNT CNT-SD1 (purity > 95%) and multi-wall CNT SD2 (purity: > 95%) were supplied by Showa Denko Co., Ltd. (Tokyo, Japan). Multi-wall CNT (purity: > 95%) was supplied by Mitsui Chemicals, Inc. (Tokyo, Japan). The MTT cell growth assay kit was purchased from Merck Millipore (Darmstadt, Germany). Normal goat serum was purchased from Vector Laboratories (Burlingame, CA, USA). Anti-Iba1 antibody [019-9741] was purchased from Wako (Osaka, Japan). Anti-CD68 antibody [MCA341R] was purchased from AbD serotec (Oxford, UK). DAPI (4'6-diamidino-2-phenylindole), anti-mouse rabbit IgG conjugated to Alexa Fluors, and pHrodo Red succinimidyl ester (pHrodo) were purchased from Molecular Probes (Eugene, OR, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Life Technologies (Grand Island, NY, USA).

Preparation of sonicated CNT solution supernatants

CNTs #1, #2, #3, #4, and #5 were suspended in PBS (1 mg/mL) and sonicated 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 cm^2 for 30 min before being allowed to stand for 1 min. The supernatant of the sonicated CNT suspension was diluted with the culture medium described below by a factor of 10 to 1,000 and was then applied to the cultured microglia for 24 hr. When appropriate, the sonicated suspension was filtered with a 0.22 μm PVDF membrane filter, and the flow-through was diluted with culture medium by a factor of 10 to 1,000.

Microglial Cell Culture

Rat microglia were cultured as previously described (Nakajima *et al*., 1992; Shigemoto-Mogami *et al*., 2014a). Briefly, mixed glial cultures were prepared from the cerebral cortex of P1 Wistar rats and maintained for 12-23 days in DMEM containing 10% FBS. The microglia floating over the mixed glial cultures were collected and transferred to 96-well plates at 5.7 x 10^4 cells/cm^2 or to 8-well slide chambers at 1.5 x 10^5 cells/cm^2.

MTT assay

To examine microglial cytotoxicity, we used a MTT cell growth assay kit (Merck Millipore). The microglia were treated with various concentrations of CNT suspensions and cultured for 24 hr. The MTT reagent was added to each well, and the cultures were incubated at 37°C for 4 hr. The amount of MTT formazan was determined by measuring the absorbance at 570 nm (test wavelength) and 655 nm (reference wavelength) with iMark™ microplate reader (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry

After incubation with or without the various CNT suspensions for 24 hr, the microglia in the 8-well chambers were stained immunocytochemically. Cells were fixed with 4% paraformaldehyde (PFA) before 2-hr incubation in a blocking solution (3% normal goat serum, 0.3% Triton X-100 in PBS) at room temperature (RT). The cells were then incubated in a solution that included the primary antibodies (rabbit anti-Iba1 antibody [019-9741, Wako; 1:500] or mouse anti-rat CD68 antibody [MCA341R, AbD Serotec; 1:100]) for 16 hr at 4°C. The cells were washed and incubated in 0.1% Triton X-100 in PBS containing the secondary antibodies (anti-rabbit IgG conjugated with Alexa Fluor 488 or anti-mouse IgG conjugated with Alexa Fluor 594 [1:1000]) for 3 hr at RT. The cells were washed and then counterstained with DAPI [1:1000]. Fluorescent images were obtained using a Nikon A1R-A1 confocal microscope system (Nikon, Tokyo, Japan).

CNT phagocytosis analysis

To determine whether the CNTs were phagocytosed by the microglial cells, we used the pH-sensitive dye pHrodo. The sonicated CNTs were incubated with 200 μM pHrodo for 24 hr at RT in the dark. After washing, the pHrodo-labeled CNTs were suspended in PBS

(1 mg/mL), and the supernatants were diluted with culture medium by a factor of 10 to 1,000. The microglial cells were incubated with solutions containing various concentrations of labeled CNTs for 6 hr. The cells were then fixed with 4% PFA and immunostained using anti-Iba1 antibody. Images of the stained cells were obtained using a Nikon A1R-A1 confocal microscope system. The phagocytosis index was determined as the total pHrodo fluorescence intensity (FI) divided by the number of DAPI-positive cells in a 635 µm x 635 µm region according to the previous report (Gagen *et al*., 2013).

Data analysis and statistics

All data are shown as the mean ± S.E.M. Statistical analysis was performed using Tukey's test following ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS

CNTs #1, #2, #3, #4, and #5 were suspended in PBS at 1 mg/mL and sonicated in a water-bath sonicator for 30 min as in our previous report (Shigemoto-Mogami *et al*., 2014b). The visual appearance of these suspensions immediately after the sonication is shown in Fig. 1A-a1. After 1 min of incubation without agitation to allow settling, the supernatant of each sonicated CNT suspension was diluted with culture medium by a factor of 10 to 1,000 and was then applied to the cultured microglia. We found that all of the sonicated CNT suspensions damaged the microglia as a function of their concentrations (Fig. 1A-a2). The supernatant of #3 showed the strongest toxicity. To verify the size of the CNTs that was related to the toxicity, we filtered the sonicated CNT suspensions using 0.22 µm PVDF membrane filters. The flow-through contained no visible CNT clumps (Fig. 1B-b1) and the flow-through no longer caused microglial cell damage (Fig. 1B-b2). These results suggest that the size of CNT clumps that caused microglial cell damage is bigger than 0.22 µm.

Next, we examined the morphological changes in microglia that were caused by the treatment with unfiltered or filtered suspensions of sonicated CNTs. In the media that contained unfiltered CNTs, CNT agglomerates appeared after 24 hr. In all of the groups treated with the unfiltered CNT suspensions, a large number of microglial aggregates were observed (Fig. 2A-a, arrows). The photographs in Fig. 2B-a are typical magnified images of microglial aggregates. In all fields of view that we observed, the microglial aggregates consisted of 3-10 microglia. Few microglial aggregates were observed in the PBS control group (Fig. 2A-a, PBS) or in the groups treated with 0.22 µm filtered suspensions (Fig. 2A-b). We then evaluated the relationship between microglial cell damage and the number of microglial aggregates. We counted the number of microglial aggregates that consisted of more than 3 microglia (Fig. 2B-a, b). As shown in Fig. 2B-b, the number of microglial aggregates induced by the unfiltered CNT suspensions were significantly

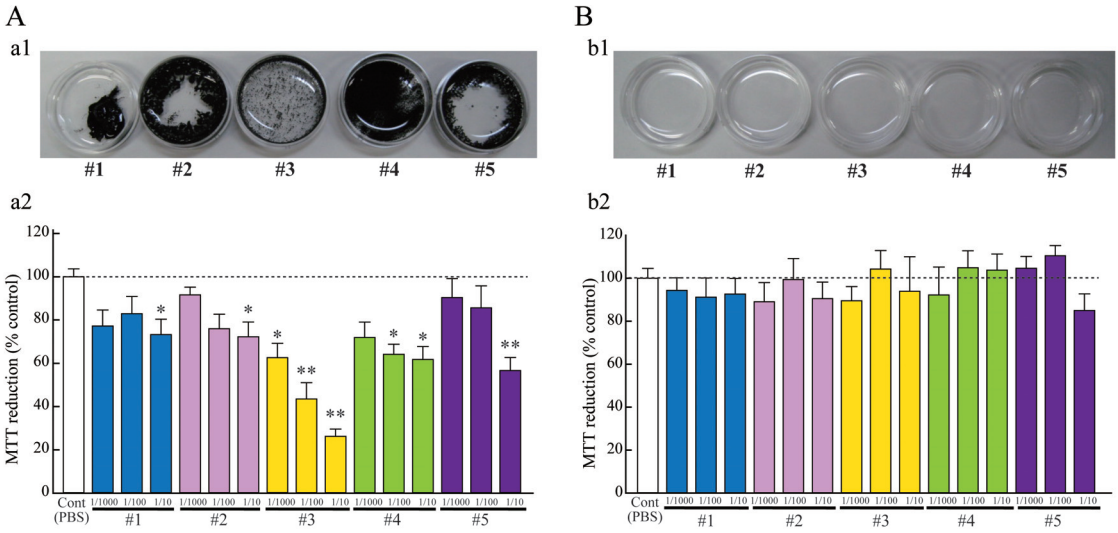

Fig. 1. CNT agglomerates damaged microglia. CNTs #1, #2, #3, #4, and #5 were suspended in PBS (at 1 mg/mL) and sonicated in a water bath sonicator. The appearances of the 1 mg/mL CNT suspensions are shown (A-a1). All of the sonicated CNT suspensions damaged microglia as a function of concentration (A-a2). When the CNT suspensions were filtered with a 0.22 µm filter, the sample contained few visible black clumps of CNTs (B-b1). The filtered CNT suspensions no longer caused microglial cell damage (B-b2). *, $p < 0.05$; **, $p < 0.01$ vs. control group (n = 10-14), Tukey's test following ANOVA.

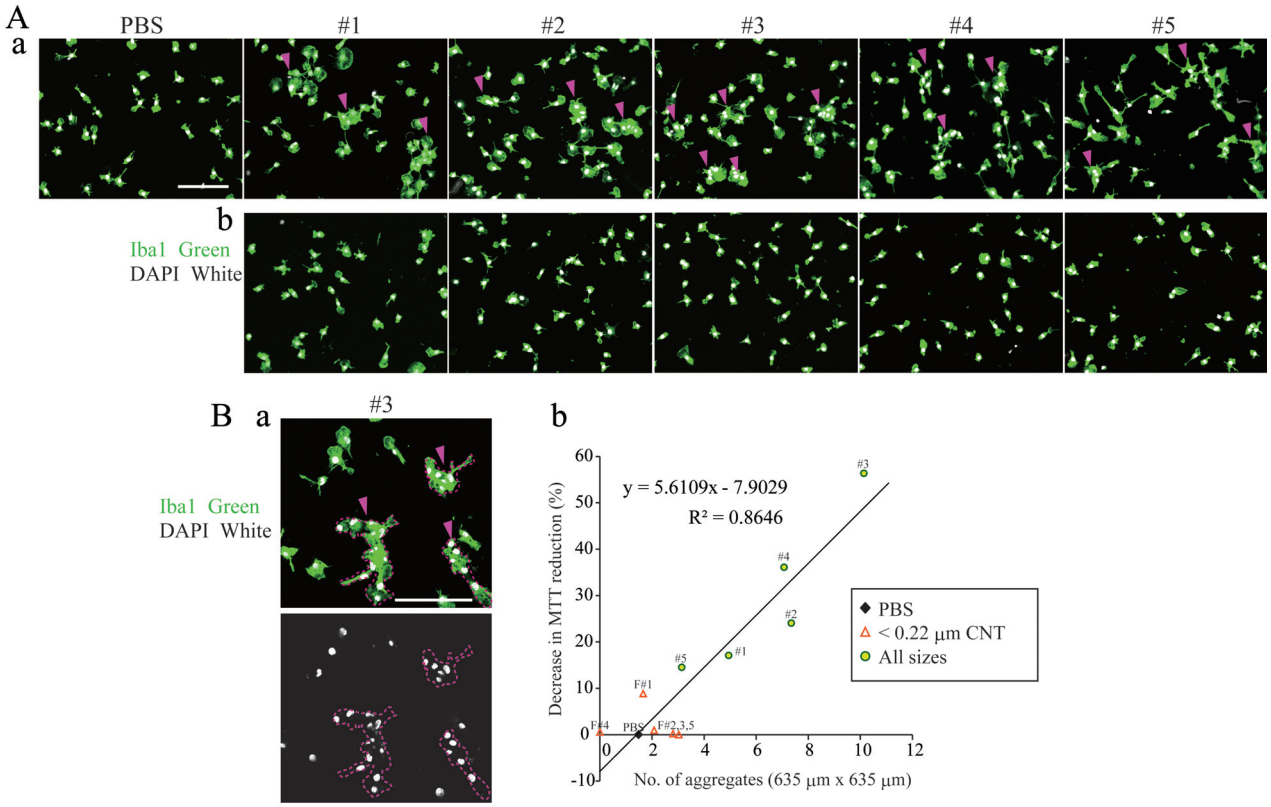

Fig. 2. The number of microglial aggregates correlated with microglial cell damage. Microglia were treated with CNT suspensions (A-a) or 0.22 μm-filtered CNT suspensions (A-b) for 24 hr and were immunostained with anti-Iba1 (green: microglia), followed by DAPI staining (white: nuclei). The aggregates of microglia (> 3 microglia) are indicated with pink arrows. Magnified images of typical microglial aggregates are shown (pink arrowheads in B-a). The number of microglial aggregates that were induced by the supernatants of the unfiltered CNT suspensions significantly correlated with microglial cell damage ('all sizes' in B-b).

correlated with microglial cell damage quantified with the decrease in MTT reduction ('all sizes' in Fig. 2B-b). The R^2 value for these plots was 0.8646. We confirmed that the numbers of microglial aggregates were low and the cell damage was minor in the groups treated with filtered suspensions ('< 0.22 μm CNT' in Fig. 2B-b). These results suggest that the number of microglial aggregates correlates with microglial cell damage.

Microglial aggregation and phagocytosis are simultaneous progressive phenomena. Thus, we next evaluated the relationship between microglial cell damage and phagocytosis of CNTs. To quantify direct phagocytosis of the CNTs by microglia, we measured the phagocytosis index using CNTs labeled with pHrodo, as in a previous report (Lööv *et al*., 2012). Because pHrodo is almost non-fluorescent at neutral pH but becomes fluorescent in acidic environments, it is suitable for visualizing early phagosomes containing labeled CNTs. We treated microglia with supernatants of unfiltered pHrodo-labeled CNT suspensions for 6 hr and immunostained for the microglial marker Iba1 (Fig. 3A). CNTs #3 and #4 exhibited strong pHrodo fluorescence and were followed by #5. Fig. 3B shows the phagocytosis index of CNTs #1-5. CNTs #3, #4, and #5 showed a significantly increased phagocytosis index, whereas the 0.22 μm filtered CNT suspensions rarely induced phagocytosis. When we evaluated the correlation between the phagocytosis index and microglial cell damage, the R^2 was 0.7715 (Fig. 3C-a). The correlation graph suggests that the CNT agglomerates could be categorized into 2 groups according to their phagocytosis indices. The first group showed a near-zero phagocytosis index (#1, #2), while the second group showed phagocytosis indices that correlated well with microglial cell damage (#3, #4, and #5). The correlation coefficient of CNTs #3-5 with cell damage reached an R^2 value of 0.9537 (Fig. 3C-b). These data suggest that CNTs #3, #4, and #5

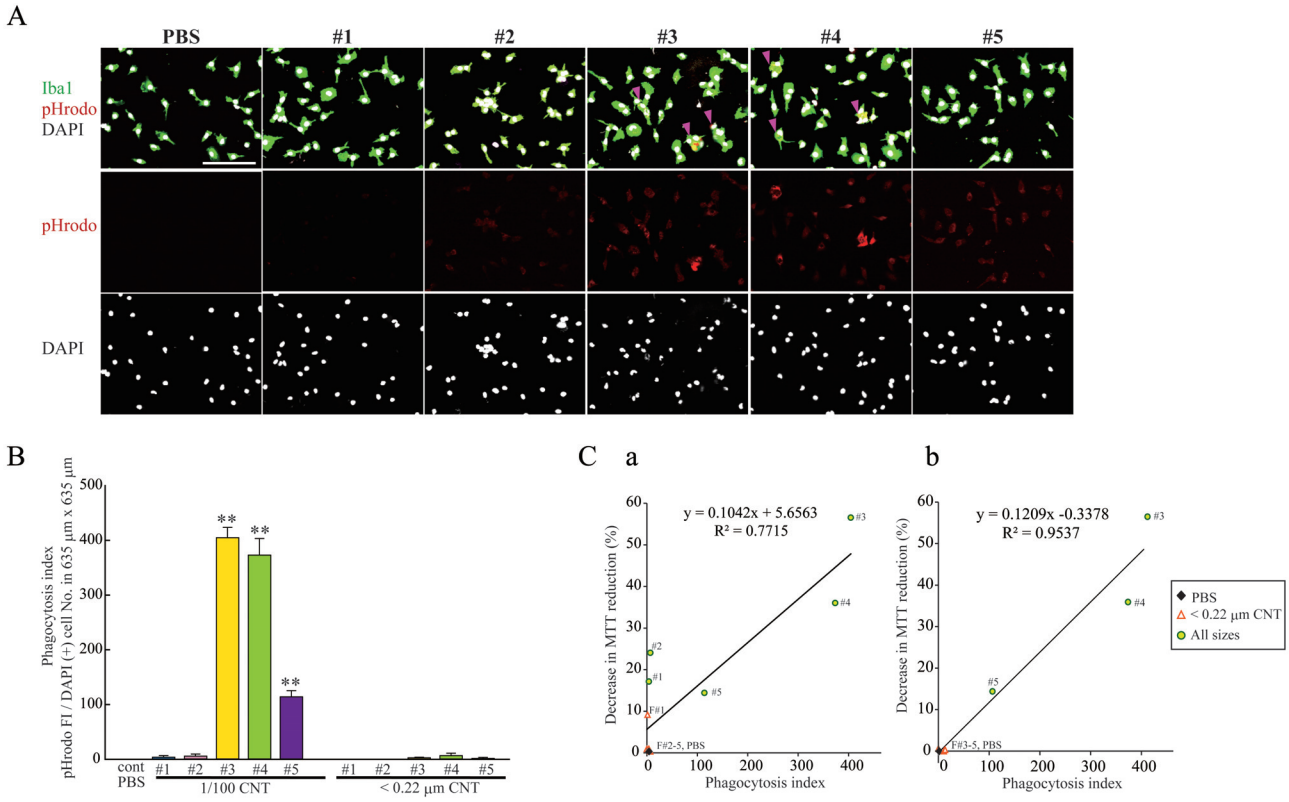

Fig. 3. CNTs #3-5 suspensions caused microglial cell damage via direct phagocytosis. Microglia were treated with pHrodo-labeled CNT suspensions or 0.22 μm-filtered pHrodo-labeled CNT suspensions and were then incubated for 6 hr. After incubation, the cells were immunostained for Iba1 followed by DAPI staining (A). CNTs #3-5 exhibited high phagocytosis indices (pHrodo FI/DAPI-positive cell number) (B). The correlations between the phagocytosis indices and microglial cell damage (C-a) and between the phagocytosis indices and microglial cell damage (C-b) are shown. The correlation coefficients for CNTs #3-5 with cell damage reached $R^2 = 0.9537$. **, $p < 0.01$ vs. control group (n = 4), Tukey's test following ANOVA.

agglomerates caused microglial cell damage via phagocytosis, and CNTs #1 and #2 agglomerates caused microglial cell damage independent of phagocytosis. As shown in Fig. 4A, CNT #3 exhibited a concentration-dependent increase in the phagocytosis index, whereas the phagocytosis index was 0 for the filtered CNT#3 suspension (column F in Fig. 4A). Typical images of microglia that have ingested CNT #3 are shown in Fig. 4B. All of the microglia that phagocytosed CNT #3 were positive for pHrodo (Fig. 4B-a). CD68 is a typical marker for phagocytic microglia. Nearly all of the microglia that had ingested CNT #3 agglomerates were also positive for CD68 (yellow arrows in Fig. 4B-b), confirming that the pHrodo-positive microglia were phagocytic microglia.

According to the data in Figs. 3 and 4, we suggest that CNT agglomerates with the size bigger than 0.22 μm cause microglial cell damage. In addition, the CNT agglomerates consisted of 2 groups in view of mechanisms of their effects: #3, #4, and #5 CNT agglomerates are directly phagocytosed by microglia and cause microglial cell damage, whereas #1 and #2 agglomerates cause microglial cell damage without phagocytosis. In both cases, the damaged microglial cell debris finally cause microglial aggregation, which well reflects the extent of microglial cell damage, as shown in Fig. 2.

DISCUSSIONS

The CNT agglomerates with the size bigger than 0.22 μm caused microglial cytotoxicity and the number of microglial aggregates is well correlated with the extent of cytotoxicity. Microglia are reported to migrate to the sites of cell death and aggregate to phagocytose cell debris (Nakajima and Kohsaka, 1993; Kreutzberg, 1996; Davalos *et al*., 2005; Hanisch and Kettenmann, 2007; Fu *et al*., 2014). Here, we determined

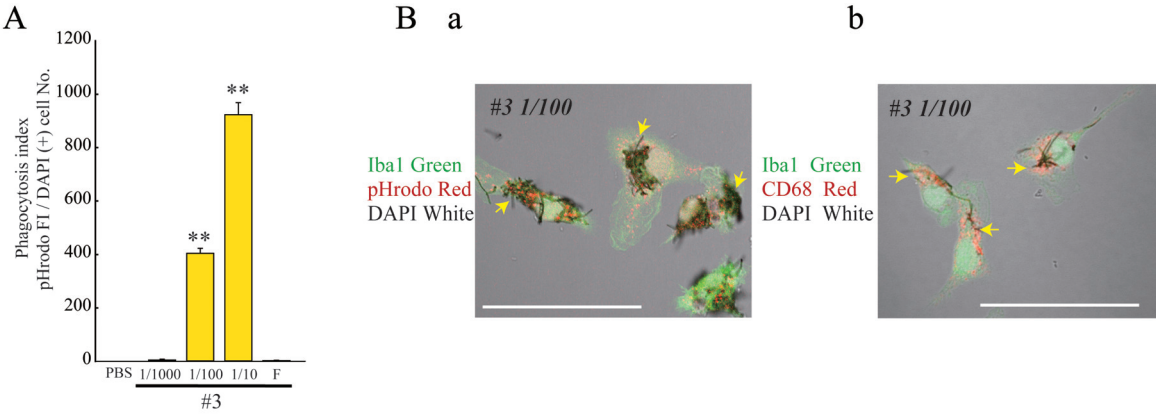

Fig. 4. A concentration-dependent increase in the phagocytosis index of #3 CNTagglomerates and typical images of phagocytosed #3 CNT agglomerates. The microglia phagocytosed the #3 CNTs as a function of concentration (A). In magnified images of microglia, black CNT agglomerates were observed in the CD68-positive microglia (yellow arrows in B-a) and in their pHrodo-positive lysosomes (B-b). **, $p < 0.01$ vs. control group ($n = 10$), Tukey's test following ANOVA.

that the number of microglial aggregates correlates well with microglial cell damage. We suggest that the number of microglial aggregates represent a good biomarker for microglial cell damage.

We also determined that the CNT agglomerates consisted of 2 groups in view of their mechanisms: one was phagocytosed by microglia and caused microglial cell damage, and the other caused cell damage without phagocytosis. Our results do not exclude the possible cytotoxicity of < 0.22 μm-sized CNTs, because it is possible that the concentrations of < 0.22 μm-sized CNTs in the filtered suspensions are too low to manifest detectable effects. In this paper, the internalization of CNT agglomerates is written as 'phagocytosis' collectively. Cells internalize larger particles by phagocytosis or macropinocytosis (> 1 μm), and smaller particles by endocytosis (60-120 nm) (May *et al*., 2000; Conner and Schmid, 2003). Phagocytosis and macropinocytosis are closely related processes (Swanson, 2008, 2014), however, the regulatory architecture of these processes are not fully understood (Bloomfield *et al*., 2015). These processes are triggered by the interaction between the particles and various kinds of receptors (Jang *et al*., 2009; Ramirez-Ortiz *et al*., 2013) As for CNTs, phagocytosis of SWCNTs by macrophages (Dong *et al*., 2013) and macropinocytosis of MWCNTs through macrophage receptor with collagenous structure (MARCO) (Hirano *et al*., 2012) have been reported, while the interaction of CNTs with scavenger receptors (ScRs), which are important for both phagocytosis and micropinocytosis, has also been reported (Wang *et al*., 2012). The surface modification, size, and shape of the CNTs or their agglomerates may affect their internalization pathways. In case of microglia, although phagocytosis of MWCNTs was reported (Boyles *et al*., 2015), microglia are equipped with the receptors and architectures for both phagocytosis (Brown and Neher, 2014) and macropinoytosis (Uesugi *et al*., 2012). Although the cytotoxicity of all of the CNT suspensions was disappeared by filtrating the suspensions with 0.22 μm filter, the CNT agglomerates which caused cytotoxicity by internalization in our experimental condition include those with a wide range of sizes and shapes. It is therefore highly possible that both phagocytosis and macropinocytosis were induced by the CNT agglomerates in this experimental condition.

As summarized above, 2 groups of CNT agglomerates were identified in this study: one in which the phagocytosis index was near zero (#1, #2), and an other in which the phagocytosis indices correlated well with microglial cell damage (#3, #4, and #5) . The high correlation coefficients of the phagocytosis indices with microglial cell damage suggest that phagocytosis of CNT agglomerates lead microglial cell damage. It has been reported that phagocytosis of nanomaterials such as asbestos and MCNTs causes macrophage cell death via the activation of inflammasomes (Goodglick and Kane, 1986; Boyles *et al*., 2015). Microglia also have a high capacity for phagocytic removal of foreign matter including pathogens and materials (Halle *et al*., 2008; Villegas *et al*., 2014). Foreign materials, such as asbestos, are recognized by Toll-like receptors (TLRs) and NOD-like receptors (NLRs) to elicit innate responses including

phagocytosis and the release of inflammatory cytokines (Li, 2013). This mechanism might also have caused the microglial cell death in this study. It has also been reported that nanoparticles themselves induce the generation of ROS and stimulate inflammatory factors in microglia in the absence of phagocytosis (Wang *et al*., 2011). We and other groups have reported that some CNTs cause cytotoxicity and genotoxicity (Pumera, 2007; Liu *et al*., 2008; Shigemoto-Mogami *et al*., 2014b) by ROS production via metal residues that were used as catalysts (Ding *et al*., 2008; Yazyev and Pasquarello, 2008; Banhart, 2009; Tyagi *et al*., 2011). The cytotoxicity of the CNT #1 and #2 agglomerates may have been caused via ROS production.

The severity of the cytotoxicity of these CNT agglomerates show an order of #1=#2<#5<#4<#3, suggesting that phagocytosis of the CNT agglomerates induced a more severe cytotoxicity. This is consistent with previous studies reporting that nanoparticle agglomerates induced substantial biological responses after phagocytosis. For example, nano-aerosols that are composed of large agglomerates (> 100 nm) are more likely to promote pulmonary clearance via phagocytosis and the acute inflammatory responses of macrophages (Noël *et al*., 2013). It remains to be elucidated what factors determine whether the CNT agglomerates are phagocytosed, and how the extent of the CNT agglomerate toxicity is determined. The length and fiber diameter of each CNT itself (#1: no information; #2: mean length of 3.5 μm, fiber diameter of 30 nm, #3: mean length of 4.51 μm, fiber diameter of 150 nm, #4: mean length of 3.0 μm, fiber diameter of 10-15 nm, #5: mean length of 3.3 μm, fiber diameter of 40-50 nm) seem not to correlate with the extent of toxicity of its agglomerates in this study. The biological responses to nanoparticles reportedly depend on the dimensions and concentrations of the nanoparticle agglomerates (Gosens *et al*., 2010; Noël *et al*., 2012, 2013). Furthermore, recent reports have suggested that it is not only the size but also the shape that determines the extent of the toxicity (Bhattacharjee *et al*., 2012). Long fibers, such as asbestos and CNTs, are known to be phagocytosed by lung macrophages and dendritic cells, which may result in IL-1β secretion and inflammatory cell death through NLRP3 inflammasomes (Goodglick and Kane, 1986; Dostert *et al*., 2008; Meunier *et al*., 2012; Kanno *et al*., 2015). It has also been verified that agglomerates over a particular length threshold induce 'frustrated phagocytosis' (Murphy *et al*., 2012; Schinwald and Donaldson, 2012). When phagocytes are unable to engulf targets that are larger than a particular length, frustrated phagocytosis occurs, thereby causing the release of toxic pro-inflammatory mediators. Because the phagocytosed #3, #4, and #5 CNT agglomerates in this study were also fibrous, the mechanisms underlying their effects may include frustrated phagocytosis. A detailed study on the correlation between the physical properties of CNT agglomerates (both their sizes and shapes) and the extent of the cytotoxicity is well warranted.

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

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