Long-term retention of pristine multi-walled carbon nanotubes in rat lungs after intratracheal instillation

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ABSTRACT: As a result of the growing potential industrial and medical applications of multi-walled carbon nanotubes (MWCNTs), people working in or residing near facilities that manufacture them may be exposed to airborne MWCNTs in the future. Because of concerns regarding their toxicity, quantitative data on the long-term clearance of pristine MWCNTs from the lungs are required. We administered pristine MWCNTs well dispersed in 0.5 mg ml⁻¹ Triton-X solution to rats at doses of 0.20 or 0.55 mg via intratracheal instillation and investigated clearance over a 12-month observation period. The pristine MWCNTs pulmonary burden was determined 1, 3, 7, 28, 91, 175 and 364 days after instillation using a method involving combustion oxidation and infrared analysis, combined with acid digestion and heat pretreatment. As 0.15- and 0.38-mg MWCNTs were detected 1 day after administration of 0.20 and 0.55 mg MWCNTs, respectively, approximately 30% of administrated MWCNTs may have been cleared by bronchial ciliary motion within 24 h of administration. After that, the pulmonary MWCNT burden did not decrease significantly over time for up to 364 days after instillation, suggesting that MWCNTs were not readily cleared from the lung. Transmission electron microscopy (TEM) showed that alveolar macrophages internalized the MWCNTs and retained in the lung for at least 364 days after instillation. MWCNTs were not detected in the liver or brain within the 364-day study period (<0.04 mg per liver, <0.006 mg per brain). Copyright © 2015 The Authors Journal of Applied Toxicology Published by John Wiley & Sons Ltd.

Introduction

Multi-walled carbon nanotubes (MWCNTs) possess unique physicochemical properties, including rigidity, a large surface area and electrical conductivity or semiconductivity depending on chirality. They are candidate molecules for many industrial (Javey et al., 2003) and medical (Allen and Cullis, 2004; Kam et al., 2005) nanotechnology applications, such as energy conversion and drug delivery. In the future, people working in or residing near manufacturing facilities may be exposed to airborne MWCNTs.

Recently, concern regarding the toxicity of MWCNTs has increased, and this issue has been the focus in many studies (Morimoto 2005). MWCNTs reportedly induced pulmonary symptoms, such as granulomatous inflammation and fibrotic responses, after inhalation exposure or intratracheal instillation in some studies (Muller et al., 2005, Li et al., 2007; Ma-Hock et al., 2009, Pauluhn, 2010), while no toxicity was reported by other studies (Mitchell et al., 2007, Muller et al., 2009, Kobayashi et al., 2010, Morimoto et al., 2012). These differences in toxicity were proposed to be as a result of the physicochemical characteristics of MWCNTs, such as length and diameter, impurities, components of their surface structure such as functional groups and dispersants (Donaldson et al., 2010; Nagai et al., 2011; Nakanishi, 2011).

Biopersistence is one of the most important factors associated with toxicity. If nanoparticles are retained in the lungs for a long period, chronic oxidative stress-induced damage could occur. According to the International Agency for Research on Cancer (IARC, 2002), lung fibrosis and thoracic tumors were often observed when fiber clearance from the lung is slow, and not when it is fast.
Therefore, information on the clearance of MWCNTs is required in addition to information on their pulmonary toxicity.

Several studies have been conducted to estimate the biopersistence and biodistribution of carbon nanotubes (CNTs). However, few studies have directly investigated the clearance and kinetics of pristine MWCNTs because these analyses are problematic. Catalytic cobalt analysis has shown that 90% of tangled MWCNTs found in the lung 1 day after inhalation exposure was still present 6 months later (Pauluhn, 2010). After intratracheal instillation of 14C-taurine-MWCNTs, 14C analysis showed that 78% was detected in the lungs and this had reduced to 28% by day 28 after instillation (Deng et al., 2007). After inhalation exposure to MWCNTs, a study using hybrid markers and high-performance liquid chromatography analysis found that lung retention did not vary between 1 and 56 days after exposure (Ohnishi et al., 2013). Ten to 20% of administered 14C-labeled MWCNTs were retained in lungs between 3 and 12 months after administration (Czarny et al., 2014). Based on these previous studies, lung clearance of MWCNTs was considered to occur very slowly. However, the clearance and translocation of MWCNTs could be vary with a different physiological property of MWCNTs; e.g., diameter, length, being tagged or not, and the dispersibility in solution for administration and in the organ after administration. Catalytic metal ions could separate from the CNTs and independently translocate to other organs within the body (Huang et al., 2010). As molecular tagging can alter physicochemical and biological properties (Liu et al., 2008; Marquis et al., 2009; Tamura et al., 2011), the kinetics and toxicity of tagged CNTs may differ from those of pristine CNTs (Leeuw et al., 2007). In the 14C-labeling determination, there are no data whether 14C-labeling could be homogeneously synthesized and/or the isotopic fractionation could not have occurred between exposure sample and a retained sample in the organ. In addition, the clearance could be accelerated or delayed for well-dispersed particles, as the degree of agglomeration affected the cytotoxicity of the carbon nanotube (Wick et al., 2007).

Therefore, the long-term clearance data for well-dispersed pristine MWCNTs are required after the detailed identification of the characteristics in order to elucidate their biopersistence as well as the translocation data for MWCNTs from the lungs to other organs, such as the liver and brain. In the present study, we administered the MWCNTs intratracheally to rats and determined lung, liver, and brain MWCNTs levels for 12 months. To achieve this, we used a recently developed method (Tamura et al., 2011) involving non-dispersive infrared (NDIR) analysis of the CO2 generated from the decomposition of pristine MWCNTs that was separated from tissues by acid-digestion and heat pretreatment. Pulmonary pristine MWCNTs were also observed over time using optical microscopy and transmission electron microscopy (TEM). The pulmonary clearance of pristine MWCNTs and their translocation to extrapulmonary organs were evaluated using these quantitative and qualitative data.

Materials and methods

Preparation of MWCNTs suspension

To prepare a stable suspension, MWCNTs, which were synthesized using a floating catalyst method (Nikkiso Co. Ltd., Japan), were sonicated (180W) for 30 min in an aqueous solution of 0.5 mg ml−1 polyoxyethylene octyl phenyl ether ( Triton-X, Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) using an ultrasonic bath (5510-MT; Branson Ultrasonics Co., Danbury, CT, USA) according to previously described methods (Kobayashi et al., 2010; Chen et al., 2011; Morimoto et al., 2012).

The total surface area of raw MWCNTs was measured using the Brunauer–Emmett–Teller (BET) N2 gas adsorption method, via surface area and pore size analyzers (Autosorb-1-C; Quantachrome Instruments, Boynton Beach, FL, USA). Metal impurities in the raw MWCNTs were determined using inductively coupled plasma-mass spectrometry (ICP–MS) (Agilent 7500a; Agilent Technologies, Inc., Santa Clara, CA, USA) after microwave-assisted acid digestion of the MWCNTs with HNO3, at 240 °C. The size of the MWCNTs in the suspension was evaluated by TEM (JEM-1010; JEOL Ltd, Tokyo, Japan).

Intratracheal instillation

MWCNTs were suspended in 0.4 ml of 0.5 mg ml−1 Triton-X aqueous solution at concentrations of 0.50 and 1.38 mg ml−1. They were intratracheally instilled into male Wistar rats, at a dose of 0.20 or 0.55 mg per rat using tip cutting feeding needle for rat (Cat No. 7204; Fuchigami Kikai Co., Kyoto, Japan). The rats were 8 weeks old with a mean body weight of 196 g (range 186–207) and had been anesthetized by inhalation of sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan). An aqueous solution of 0.5 mg ml−1 Triton-X was intratracheally instilled into the control rats. In each of the above 3 groups, 11 rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku Corporation, Japan) on days 1, 3, 7, 28, 91, 175, and 364 after instillation, and dissection and gross autopsy were performed. Five rats in each group were used for the MWCNTs analysis, 5 rats in each group were used for the optical microscope observations, and 1 rat was used for TEM observation.

All animal handling procedures were conducted in accordance with the guidelines in the Japanese Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan, or by the Institutional Animal Care and Use Committee, National Institute of Advanced Industrial Science and Technology.

Analysis

After drawing blood from the abdominal aorta, the left and right lungs, livers and brains of five rats in each group were dissected, separated and weighed. They were then cut into small pieces with scissors, homogenized in saline (3 ml for the left lung, right lung and brain; 25 ml for the liver) with an electric homogenizer (Polytron RT3100; Kinematica AG, Luzern, Switzerland), and stored at −20 °C prior to analysis.

Quantification of MWCNTs in the lungs, liver and brain was performed by acid digestion, muffle ashing, in-situ preheating and combustive oxidation-NDIR measurement, as described by Tamura et al. (2011). Three milliliters of 60% HNO3 was added to approximately 0.1 g of homogenized tissue, and the mixture was digested using a hot plate heat at 120 °C for 8 h. After cooling, the acid-digested samples were filtered with a quartz membrane filter (QR-100; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) which had been preheated at 900 °C for 15 min in a muffle furnace (FP42; Yamato Kagaku Co. Ltd., Tokyo, Japan). The filter containing the MWCNTs was washed with 5 ml of pure water five times and then heated at 400 °C for 15 min. After heating, the MWCNTs on the filter were quantified using a combustive oxidation chamber (SSM-5000A; Shimadzu, Japan) with an NDIR detector (TOC-V CPH, Shimadzu) at a temperature of 900 °C in the
presence of oxygen gas (purity, > 99.9%). A calibration curve for carbon content was generated using glucose (Wako Pure Chemical Industries Co. Ltd).

The individual variation of left lung MWCNTs burden was much larger than that observed in total lung tissue (Supporting Information Table 1), possibly reflecting differences in MWCNTs deposition in the right and left bronchi during instillation. This left lung variability was caused by the large individual variation in the initial amounts deposited in each lung. We, therefore, analyzed the total lung burden, because these data were not influenced by variability in distribution between the lungs.

**Quality assurance and quality control for the determination of MWCNTs**

The detection limit and the precision of analysis for pulmonary MWCNTs in the present study were checked in the previous study (Tamura et al., 2011). To check the validity of this method, the lung burdens of male Wistar rats (aged 8 weeks, mean body weight 272 g, range 245–299 g) were determined after a 28-day inhalation whole-body MWCNTs exposure (0.37 ± 0.18 mg m⁻³), and compared with our previously reported results for the same inhalation exposure using different methods (Oyabu et al., 2011).

**Optical microscope observation and TEM observation**

The lungs and lymph nodes of five rats from each group were fixed with 10% formalin, embedded with paraffin, sectioned, and stained with hematoxylin and eosin for optical microscope observation. For TEM, lung tissue was fixed using glutaraldehyde and osmium tetroxide solution, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin microtome sections were cut using a diamond knife. Some sections of the specimen were stained using 2% uranyl acetate solution and 0.5% lead citrate solution at room temperature. Conventional TEM observation was performed by H-7000 (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

**Results**

**Characteristics of MWCNTs**

The surface area of the MWCNTs was 69 ± 37 m² g⁻¹, as determined by the N₂ gas adsorption method. With regard to metal impurities, the levels of Li, Al, Ca, Fe and Cd in the MWCNTs were 0.5, 80, 176, 53 and 16 µg g⁻¹ respectively, as determined by ICP-MS.

The geometric mean diameter of the MWCNTs in the suspension used for the intratracheal instillation test was 48 nm [geometric standard deviation (GSD), 1.1] and the geometric mean length was 2.5 µm (GSD, 2.4), as determined by TEM (Fig. 1). The size of the MWCNTs in the suspension did not change from the raw MWCNT (geometric mean: 44 nm; GSD 1.3).

**Quality assurance and quality control for the determination of MWCNTs**

The detection limit of the determination method used in the present study, defined as a signal-noise ratio of 3, was 0.0003 mg of MWCNTs, and 0.003 mg per lung, 0.04 mg per liver and 0.006 mg per brain. The precision of analysis (repeatability) for pulmonary MWCNTs determination was 5.6% and the efficiency of recovery was > 95% (Tamura et al., 2011).

**Determination of MWCNTs burden in the lung**

The ratio of the MWCNTs burden in the right lung to the total MWCNTs lung burden 1 day after the instillation was 0.80 and 0.91 for the 0.20 mg and 0.55 mg doses, respectively. These ratios were not identical to the ratios of the weight of the right lung to the total lung, which were 0.67 and 0.70 for the 0.20 mg and 0.55 mg doses, respectively (Supplementary Information Table 1). At 3 days to 12 months after the instillation, the MWCNT burdens in the right lung were also much greater than the corresponding burdens in the left lung (ratio of burden in the right lung to the total were 0.88 and 0.90 for the 0.20 mg and 0.55 mg doses, respectively).

The individual variation of MWCNTs burden in the lung was evaluated using the relative standard deviations (RSDs) of the measured amounts. The left lung RSD values after administration of 0.20 and 0.55 mg MWCNTs were 49%–110% and 43%–130% for the entire period examined in this study, respectively. The equivalent values for total lung were 14%–30% and 20%–34%, respectively. Therefore, the burden values for total lung were used for the following data analysis.

The total lung burdens after intratracheal MWCNTs instillation are shown in Fig. 2. On days 1, 3, 7, 28, 91, 175 and 364 after the instillation of 0.20 mg MWCNTs, the mean ± standard deviation (SD) of total lung MWCNTs burdens were 0.15 ± 0.024, 0.13 ± 0.030, 0.15 ± 0.055, 0.15 ± 0.045, 0.12 ± 0.017, 0.14 ± 0.018, and 0.15 ± 0.028 mg per lung respectively (N = 5). For the 0.55 mg dose, the corresponding pulmonary MWCNTs burdens were 0.38 ± 0.11, 0.30 ± 0.06, 0.30 ± 0.084, 0.28 ± 0.073, 0.37 ± 0.086, 0.28 ± 0.082 and 0.30 ± 0.10 mg per lung (N = 5). As 0.15 and 0.38 mg MWCNTs...
were detected 1 day after administration of 0.20 and 0.55 mg MWCNTs, respectively, approximately 30% of administrated MWCNTs may have been cleared by bronchial ciliary motion within 24 h of administration. MWCNTs were not detected in the lungs of control rats (<0.003 mg per lung).

Observation using optical microscopy and TEM

Using optical microscopy, MWCNTs-laden macrophages were observed in the lung interstitium and alveoli throughout the observation period in both dosage groups (Fig. 3). On day 1 after instillation, many of the MWCNTs aggregates observed were within macrophages, with only a small number of extracellular aggregates (Fig. 3a). Three days after instillation, most of the MWCNTs aggregates were observed in macrophages (Fig. 3b). From days 7–90 after instillation, several MWCNTs-laden macrophages accumulated in the lung (Fig. 3c). From days 90–364 after instillation, most of the MWCNTs-laden macrophages were internalized in the alveolar interstitium and formed granuloma (Fig. 3d–f). TEM images of the alveolar macrophages in the lungs of rats

Figure 2. Pulmonary multi-walled carbon nanotubes (MWCNTs) burden by time elapsed after single intratracheal instillation at the doses of 0.20 and 0.55 mg per rat.

Figure 3. Multi-walled carbon nanotubes (MWCNTs)-laden macrophages in the interstitium and alveolus at (a) day 1, (b) day 3, (c) day 7, (d) day 28, (e) day 91, (f) day 175, and (g) and (h) day 364 after instillation in rats that were administered a high dose (0.55 mg per rat).
obtained on days 7 and 364 after the instillation of 0.20 mg MWCNTs are shown in Fig. 4. While MWCNTs were observed in phagolysosomes in alveolar macrophages, they were not found in the nuclei or other intracellular organelles. The internal multilayered structure of the MWCNTs was maintained.

MWCNT translocation to extrapulmonary organs
MWCNTs were not detected in the liver or the brain after intratracheal instillation (<0.04 mg per liver, <0.006 mg per brain). However, optical microscopy revealed the presence of MWCNTs in the peribronchial lymph nodes (Fig. 5).

Discussion
Well-dispersed MWCNTs were used in the present study. The biological behaviors of MWCNTs, such as toxicity and biodistribution, could depend on their aggregation state. In contrast to aggregated MWCNTs, dispersed MWCNTs can penetrate mesothelial cells and further induce cell injury (Nagai et al., 2011). Smaller nanoparticles showed more widespread organ distribution (De Jong et al., 2008). Exposure to small aggregates of TiO₂ nanoparticles produced increased oxidative stress effects and cytotoxicity than exposure to large aggregates of these nanoparticles (Noël et al., 2013).

Therefore, well-dispersed MWCNTs might be more toxic and show more widespread organ distribution than aggregated MWCNTs, although published data have not indicated that dispersion affected the toxicity of TiO₂ nanoparticles (Kobayashi et al., 2009). However, there are a few previous studies that have investigated the biodistribution of well-dispersed MWCNTs for observation periods of over 6 months (Mercer et al., 2013; Czarny et al., 2014). In the present study, TEM identified no large MWCNT aggregates and only a few fibers aggregate in the instilled suspension (Fig. 1). The present study directly determined the lung retention of pristine MWCNTs after intratracheal instillation. Most of the previous studies analyzed the organ distributions of MWCNTs indirectly, for example by analyzing catalytic metal (Pauluhn 2010) or functionalized MWCNTs (Deng et al. 2007, Liu et al. 2007). Pauluhn (2010) estimated pulmonary MWCNT burdens in rats for 26 weeks after a 13-week inhalation exposure by analyzing the catalytic Co. Catalytic metal can distribute throughout the body (Huang et al., 2010). In a study of the biodistribution of ⁹⁹ᵐTc-radiolabeled carbon nanoparticles, ⁹⁹ᵐTc or its oxides could enter the blood circulation independently of the nanoparticle (Mills et al., 2006). Deng et al. (2007) estimated the pulmonary ¹⁴C-taurine-functionalized MWCNT burdens in mice after intravenous injection, intratracheal instillation and stomach intubation for 12 h to 90 days after administration. Functionalization could change the biodistribution of...
inhaled particles depending on the type of functional group. Functionalized MWCNTs were phagocytosed more effectively than pristine MWCNTs in a study (Fraczek-Szczypta et al., 2011), although functionalized MWCNT and pristine MWCNTs showed no differences in clearance in a study (Silva et al., 2014). Therefore, analysis of a catalytic metal or functionalized MWCNTs may produce distribution data that cannot be extrapolated to pristine MWCNTs. Although the translocation of MWCNT after aspiration in mice using 14C-labeled MWCNTs, it is difficult to apply the determination of many kinds of MWCNTs owing to the synthesizing cost. Our direct determination of pristine MWCNTs was more suitable to determine the lung clearance and biodistribution of pristine MWCNTs. We also determined the burden of the total lung to accurately evaluate the pulmonary clearance but not one side lung. MWCNT burdens were actually higher in the right lung than in the left lung in the present...
study. In humans, aspiration pneumonitis tends to occur in the right lung (Kouno et al., 2006), indicating that this lung could be exposed more to ingested substances because the main right bronchus is shorter, thicker and less angled than the main left bronchus (Kouno et al., 2006). This difference in the right and left bronchi is due to the position of the heart on the left side of the body (Kouno et al., 2006). As the rat heart apex is also placed slightly to the left (Hiraiwa et al. 1960), there could be a difference between rat right and left bronchi. Therefore, we considered it is more accurate to determine the total lung burden when evaluating pulmonary clearance.

In the present study, approximately 70% of the MWCNTs were detected in lung tissue 1 day after the intratracheal instillation. Particles trapped on the trachea and bronchioles can be cleared by ciliary motion within 1 day because bronchial ciliary motion rates were 7.5–13.6 mm min⁻¹ (Lightowler and Williams 1969) and the length of trachea and bronchioles (until terminal bronchioles) were 5.1 mm in the rat (Yeh et al., 1979). Therefore, it can be presumed that 30% of the instilled MWCNTs might be trapped on the trachea and bronchioles and might be cleared by ciliary motion.

On day 1 after instillation, only a small number of extracellular aggregates of MWCNTs were observed although aggregates were not observed in suspension. One possible reason is that macrophage gathered them in a day. The other possible reason is that a part of dispersed MWCNTs aggregated when the suspension mixed with bio-surfactant in the alveolar region.

In the present study, MWCNTs were rarely cleared from the lung within 1 year after intratracheal instillation. Previous inhalation exposure tests using MWCNTs have also low clearance rates. After inhalation exposure to tangled MWCNTs (6.0 mg m⁻³), pulmonary retention was 26 weeks was >90% of that observed 1 day after exposure [0.1 mg m⁻³, half-time 5 month; 0.4–6.0 mg m⁻³, half-time 10 to > 13 months (Pauluhn, 2010)]. After inhalation exposure and intratracheal instillation to original and functionalized MWCNTs, no clearance was observed within 21 days (Silva et al., 2014). After inhalation exposure, 25% and 35% cleared at 168 and 336 days post-exposure compared with the burden at 1 day post-exposure in rats (Mercer et al., 2013). In contrast, spherical C60 nanoparticles were cleared from the lungs much faster than MWCNTs [half-life < 1 month (Shinohara et al., 2010)] although C60 and CNTs are both carbon allotropes, and both have a graphene structure. For other poorly soluble fibers, the pulmonary clearance was similarly slow to that observed in the present study [crocidolite and amosite, half-life > 13 months (Hesterberg and Hart, 2000); silicon carbide whisker, half-life 16 months (Akiyama et al., 2007)]. These reports comprehensively indicate that water-insoluble fibers such as MWCNTs showed slow clearance from the lung.

Another factor in elucidating the slow clearance, that is, the length of MWCNT, the pulmonary macrophage cytotoxicity, overload of instillation amount of MWCNTs, and dispersion of the CNTs, was examined.

We considered the possibility estimated that the MWCNTs length could be the cause of their cytotoxicity to macrophages. Previous studies indicated that longer fibers could not be fully phagocytosed by macrophages, and induced cytotoxicity [>15 μm in length (Searl et al., 1999; Poland et al., 2008)]. However, the MWCNTs used in the present study were much shorter (average length = 2.4 μm; 96%, < 0.5–10 μm 96%; 2.8%, 10–15 μm; 1.1%, > 15 μm) than those shown to block phagocytosis. In addition, our TEM observations did not observe any MWCNTs piercing macrophages. Therefore, these findings indicated that the length of the MWCNTs was not the cause of their lung retention in the present study.

The macrophage cytotoxicity of MWCNTs could also cause their slow clearance. There have been many studies showing that MWCNTs are cytotoxic to macrophages (Hirano et al., 2008; Cheng et al., 2009; Boncel et al., 2011; Chen et al., 2011; Zhang et al., 2012; Luo et al., 2012). The viability of mature human macrophages decreased after their exposure to, and uptake of MWCNTs (Cheng et al., 2009; Boncel et al., 2011; Zhang et al., 2012). MWCNTs have been reported to disrupt the macrophage plasma membrane integrity (Hirano et al., 2008), inhibit their migration (Hirano et al., 2010), increase markers of oxidative stress (Chen et al., 2011), and cause ultrastructural and morphological changes (Luo et al., 2012). In contrast, no significant macrophage toxicity was observed for C60 (Jia et al., 2005). Therefore, the MWCNT cytotoxicity might delay the macrophages’ pulmonary clearance.

Lung overload could be also the cause of delay MWCNT clearance from the lung. As overload occurred after administration of the exceeding threshold volume of MWCNTs (Pauluhn, 2010), we discuss the volume of MWCNTs employed in the present study. Assuming the density of MWCNT in the present study was a true density of 1.72 g cm⁻³ because the suspension was well dispersed, pulmonary MWCNTs was estimated to be only 0.12 μl g⁻¹ per lung, which is within the range of minimal lung overload for tangled MWCNTs [0.1–0.3 μl g⁻¹ per lung (0.1 mg m⁻³); Pauluhn, 2010]. In the present study, however, only 0.12 μl g⁻¹ per lung of MWCNTs induced a complete delay in clearance. In Pauluhn (2010), complete lung overload occurred at a much higher volume of tangled MWCNTs [1.2–12 μl g⁻¹ per lung (1.5–6.0 mg m⁻³)]. There are two possible explanations for this difference between Pauluhn (2010) and the present study. One possibility relates to the severity of cytotoxicity, as the toxicity of well-dispersed rigid MWCNTs could be more severe than that of tangled MWCNTs. In a previous in vitro test, greater cytokine induction was observed in macrophages after phagocytosis of long needle-like CNTs than after phagocytosis of long-tangled CNTs (Palomaki et al., 2011). The other possibility relates to the administration method resulting in different local doses in the lung, as the overload threshold could be higher for inhalation exposure than for intratracheal instillation. Brain et al. (1976) reported that intratracheal instillation resulted in less even lung distribution than inhalation exposure. Therefore, even if the total lung burden after intratracheal instillation and inhalation exposure is the same, the local burden per unit lung area at some points could be higher after intratracheal instillation than that after inhalation exposure. More research is required to study the relationship between cytotoxicity/overload and delayed clearance.

A recent study showed that MWCNT, which synthesized by a chemical vapor deposition process, were cleared by 80%–90% at 90–360 days post-aspiration in mice (Czarny et al., 2014). Their MWCNT size (3.9 μm of length and 40 nm of diameter) and dose per weight [1 mg kg⁻¹ per body weight (BW)] was similar to our study (2.5 μm of length and 48 nm of diameter; 1.0 and 2.8 mg kg⁻¹ per BW of dose). Therefore, the difference in pulmonary clearance between two studies might not be due to the size and dose.

In conclusion, MWCNTs were retained in the lung, even 1-year post-institution. However, these MWCNTs in pulmonary macrophages seemed to form stable granuloma and no tumors were observed for up to 1-year post-institution. Although the long-term retention observed in the present study might have the possibility of chronic MWCNT toxicity, our findings indicated that there was a low possibility of MWCNTs inducing severe chronic adverse effects on the lung.
Declaration of interest

This study was funded by a grant, ‘Evaluating risks associated with manufactured nanomaterials’ (no. P06041), from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. Intratracheal instillation tests were performed at the Kashima Laboratory, Mitsubishi Chemical Medience Corp, Kashima, Japan, and inhalation exposure tests for the validation of analysis were performed at the Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu, Japan. The authors report no conflicts of interest.

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Supporting information
Additional supporting information may be found in the online version of this article at the publisher’s web-site.