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Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats

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ABSTRACT

Biological responses of multi-wall carbon nanotubes (MWCNTs) were assessed after a single intratracheal instillation in rats. The diameter and median length of the MWCNTs used in this study were approximately 60 nm and 1.5 μm, respectively. Groups of male Sprague–Dawley rats were intratracheally instilled with 0.04, 0.2, or 1 mg/kg of the individually dispersed MWCNT suspension. After instillation, the bronchoalveolar lavage fluid was assessed for inflammatory cells and markers, and the lung, liver, kidney, spleen, and cerebrum were histopathologically evaluated at 3-day, 1-week, 1-month, 3-month, and 6-month post-exposure. Transient pulmonary inflammatory responses were observed only in the lungs of the group of rats exposed to 1 mg/kg of MWCNTs. Morphology of the instilled MWCNTs in the lungs of rats was assessed using light microscopy and transmission electron microscopy (TEM). Light microscopy examination revealed that MWCNTs deposited in the lungs of the rats were typically phagocytosed by the alveolar macrophages and these macrophages were consequently accumulated in the alveoli until 6-month post-exposure. The 400 TEM images obtained showed that all MWCNTs were located in the alveolar macrophages or macrophages in the interstitial tissues, and MWCNTs were not located in the cells of the interstitial tissues. There was no evidence of chronic inflammation, such as angiogenesis or fibrosis, induced by MWCNT instillation. These results suggest that MWCNTs were being processed and cleared by alveolar macrophages.

1. Introduction

Carbon nanotubes (CNTs) are fiber-shaped substances that consist of graphite hexagonal-mesh planes (graphene sheet) present as a single-layer or as multi-layers with nest accumulation. Tubes with single-wall structures and multi-wall structures are called single-wall carbon nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs), respectively. CNTs are regarded as nanomaterials because their diameters are within the nanoscale range (1–100 nm). Currently, various applied studies are focusing on CNTs because of their excellent physical–chemical properties.

However, there is a growing concern regarding the hazards of CNTs. Many pulmonary toxicity studies (e.g., inhalation exposure studies, intratracheal instillation studies, and pharyngeal aspiration studies) have reported that multifocal granulomas or fibrotic responses were persistently observed in the lungs of rats and mice after SWCNT exposure (Warheit et al., 2004; Lam et al., 2004; Mangum et al., 2006; Chou et al., 2008; Miyawaki et al., 2008; Shvedova et al., 2005, 2007, 2008a,b). MWCNT pulmonary toxicity studies also reported similar pulmonary responses as SWCNT exposure. Granulomatous inflammation and fibrotic responses were reported in MWCNT inhalation exposure studies (Muller et al., 2005; Li et al., 2007; Ma-Hock et al., 2009; Pauluhn, 2010).

The major concerns of CNT toxicity are that those objects are nanoscaled, and more importantly, they are fiber-shaped (i.e., they may have asbestos-like properties). Takagi et al. (2008) reported that most p53+/− transgenic mice died owing mesothelioma up to 180 days after intraperitoneal injection of MWCNTs at a dose of 3 mg/mouse (approximately 100 mg/kg body weight). Poland et al. (2008) reported that inflammatory responses were observed in mice exposed to fibers longer than 15 μm, but not in those exposed to shorter fibers, at 1 and 7 days after intraperitoneal injection of MWCNTs, asbestos, or carbon black particles at 50 μg/mouse. In a more recent intraperitoneal injection study with MWCNTs, however, there was no significant increase in the incidence of mesothelioma at doses of 2 and 20 mg/rat, even 2 years after injec-

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tion, although the incidence of mesothelioma was significantly increased after administration of crocidolite (Muller et al., 2009).

In most CNT toxicity studies, CNT agglomerates were used as the test samples. However, some studies indicate that dispersed CNTs are more toxic than agglomerated CNTs when inhaled or instilled into the lungs of experimental animals. Muller et al. (2005) reported that MWCNT samples ground by a ball mill induced greater inflammation than non-ground bulk MWCNT samples after intratracheal instillation in rats. In their reports, the average length of the MWCNT samples was greatly decreased from 5.9 to 0.7 μm because of the ball mill grounding; but major characteristics such as the diameter or surface area did not change. Mercer et al. (2008) reported that after pharyngeal aspiration exposure of mice to dispersed SWCNTs (average particle size, 0.69 μm) and non-dispersed SWCNTs (average particle size, 15.2 μm), thickening of the alveolar walls was observed only in the group exposed to dispersed SWCNTs. Mercer et al. (2008) concluded that the dispersed SWCNTs were rapidly incorporated into the alveolar interstitium. Porter et al. (2010) suggested that the dispersed MWCNTs could reach the pleura after pharyngeal aspiration exposure in mice. These findings indicate that toxicity studies using agglomerated CNTs are inadequate to evaluate the hazards and risks of CNTs. However, there are few toxicity studies with dispersed CNTs. Further, there is little information regarding the behavior of MWCNTs after deposition in the lungs.

There are many potential applications of MWNCTs (e.g., in electrically conducting ceramics, anti-static clothing, and heat-exchange materials, etc.). To explore these applications, MWCNT dispersion is a key factor. Extensive research on MWCNT dispersion is underway in several organizations. Therefore, it is possible that exposures to dispersed MWCNTs might occur in the near future, necessitating the evaluation of the hazards of exposure to dispersed MWCNTs.

In this study, individually dispersed MWCNTs were intratracheally instilled in rats, and the biological responses (e.g., pulmonary inflammation) were assessed. Further, light microscopic and transmission electron microscopic examinations were performed to evaluate the behavior of MWCNTs in the lungs.

2. Materials and methods

2.1. Sample preparation

MWCNT samples (MWNT-7, Lot#T050831-01) were purchased from Mitsui & Co. Ltd. (Tokyo, Japan). MWNT-7 is a highly pure MWCNT sample, in which the carbon content is 99.79% (determined by fluorescence X-ray analysis). MWNT-7 has been used in many toxicity studies such as those by Takagi et al. (2008) and Poland et al. (2008). MWNT-7 is produced as a dry powder and the tubes do not aggregate together. To disperse MWCNTs in liquid for intratracheal instillation, MWCNTs (0.04, 0.2, or 1 mg/mL) and a maximum of 10 mg/mL of polyoxyethylene sorbitan monooleate (Tween 80, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to Milli-Q water (Millipore Corporation, Billerica, MA, USA) and then ultrasonicated using an ultrasonic bath (5510J-MT, Branson Ultrasonics Div. of Emerson Japan, Ltd., Kanagawa, Japan) for 90 min at 135 W and a frequency of 42 kHz. PBS (10 mM) was then added to the ultrasonicated MWCNT suspension. The above MWCNT suspensions were used for intratracheal instillation the day after their preparation.

Tween 80 (10 mg/mL) in PBS (10 mM) was used as the negative (vehicle) control. Min-U-Sil 5 crystalline silica particles (US Silica Co., Berkley Springs, WV, USA), which produce continuous pulmonary inflammation in the lungs of rats with 5 mg/kg of intratracheal instillation (Warheit et al., 2006, 2007a,b; Kobayashi et al., 2009), was used as the positive control and was prepared as described for the MWCNT suspension. The concentration of the crystalline silica particles was adjusted to 5 mg/mL for intratracheal instillation.

2.2. MWCNT characterization

For both the bulk MWCNT samples and MWCNT suspensions, the agglomeration state and fiber length were evaluated based on observation using a scanning electron microscope (SEM) (SM-5410, JEOL Ltd., Tokyo, Japan) and a transmission electron microscope (TEM) (TM-1010, JEOL Ltd., Tokyo, Japan).

The BET surface area was measured by the N_2-adsorption method using Autosorb (Quantachrome Instrument, Boynton Beach, FL, USA) at a pressure ranging from 10.3 to 31.4 kPa. Purity of the MWCNT samples was measured by thermogravimetric analysis (TGA) using an auto simultaneous TG/DTA instrument (DTG-60H, Shimadzu Corporation, Kyoto, Japan). Furthermore, presence of defects in the graphene structure of the bulk MWCNT samples and the MWCNT suspensions was evaluated by Raman spectroscopy analysis (Nicolet Almega XR micro-Raman system, Thermo Fisher Scientific Inc., Japan). The resonance Raman scattering spectra were measured in the frequency regions of 100–3000 cm^{-1} with excitation wavelength at 532 nm.

The MWCNT suspension was characterized within 1 week of sample preparation. This is because we had monitored the time-dependent changes in the agglomeration state of the MWCNTs in the suspension using laser diffraction (Helos, Sympatec, Clausthal-Zellerfeld, Germany), and no significant difference was noted in the equivalent diameters of MWCNTs within 1 week of sample preparation.

2.3. Experimental animals

In this study, 8-week-old male Crl:CD(SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used. The rats were kept in an animal facility and housed in positive-pressure air-conditioned units (21–24 °C, 42–64% relative humidity) with 12 h light and dark cycles. After a 7-day acclimation, the body weight of each rat was measured and assigned to the study. Their body weight was in the range of 288–336 g at intratracheal instillation.

2.4. General experimental design

Rats were anesthetized with ether, and 1 mL/kg body weight of MWCNTs, negative control, or the positive control (crystalline silica particle) suspension were instilled into the trachea using a 18G indwelling needle, corresponding to doses of 0.04, 0.2, or 1 mg/kg body weight of MWCNTs and 5 mg/kg body weight of crystalline silica particles. Following instillation, the viability and general condition of the rats were observed once a day until dissection. The body weight of each rat was measured before instillation and at 1-, 3-, 7-, 14-, 21-, 28-, 35-, 42-, 49-, 56-, 63-, 70-, 77-, 84-, and 91-day post-exposure. Measurements of the organ weight of the left lung, bronchoalveolar lavage fluid (BALF) examination from the right lung, and histopathological evaluation of the left lung, liver, kidney, spleen, and cerebrum were performed at 3-day, 1-week (7 days), 1-month (28 days), and 3-month (91 days) post-exposure. Five rats per group were evaluated at each time point.

Animal experiments were performed in 2009 at the Kashima Laboratory, Mitsubishi Chemical Medience Corp. (Tokyo, Japan) in accordance with the Law for Partial Amendments to the Law Concerning the Protection and Control of Animals (2005). This study was approved by the Institutional Animal Care and Use Committee of the Testing Facility and performed in accordance with the ethics criteria contained in the bylaws of the Committee of National Institute of Advanced Industrial Science and Technology.

2.5. Bronchoalveolar lavage

The rats were euthanized by administrating an intraperitoneal injection of pentobarbital sodium (Nembutal injectable, Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan) followed by exsanguination. The left bronchus was clamped with forceps, and the right bronchus was cannulated. Subsequently, 3 mL of heated (~37 °C) saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was instilled and aspirated to and from the lung to recover the first BALF fraction (approximately 2 mL). The supernatant was obtained by centrifuging the BALF at 300 g for 5 min and was used for the biochemical and cytokine measurements. Thereafter, 2 mL of saline solution was instilled and aspirated to and from the lung twice, and then additional BALF (approximately 4 mL) was obtained, centrifuged at 300 g for 5 min after addition to the precipitation obtained by centrifugation of the first BALF. The cell fraction was used to determine cell counts in the BALF.

2.6. Pulmonary cell counts

The cell fractions were suspended in saline with addition of BSA (0.1%) and EDTA-2K (0.05 mM) dissolved in PBS, and the number of total cells, neutrophils, macrophages, lymphocytes, and eosinophils were counted with an automatic erythrocyte analyzer (XT-2000iV, Sysmex Corporation, Hyogo, Japan).

2.7. Biochemical measurements

Lactate dehydrogenase (LDH) and total protein (TP) concentrations in the supernatant obtained by centrifugation of the BALF were measured with an automatic biochemical analyzer (TBA-200FR, Toshiba Medical Systems Corporation, Tochigi, Japan). Interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, granulocyte monocyte colony stimulating factor (GM-CSF), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α concentrations were measured using a Rat Cytokine 10-Plex A Panel kit and Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc., Tokyo, Japan).

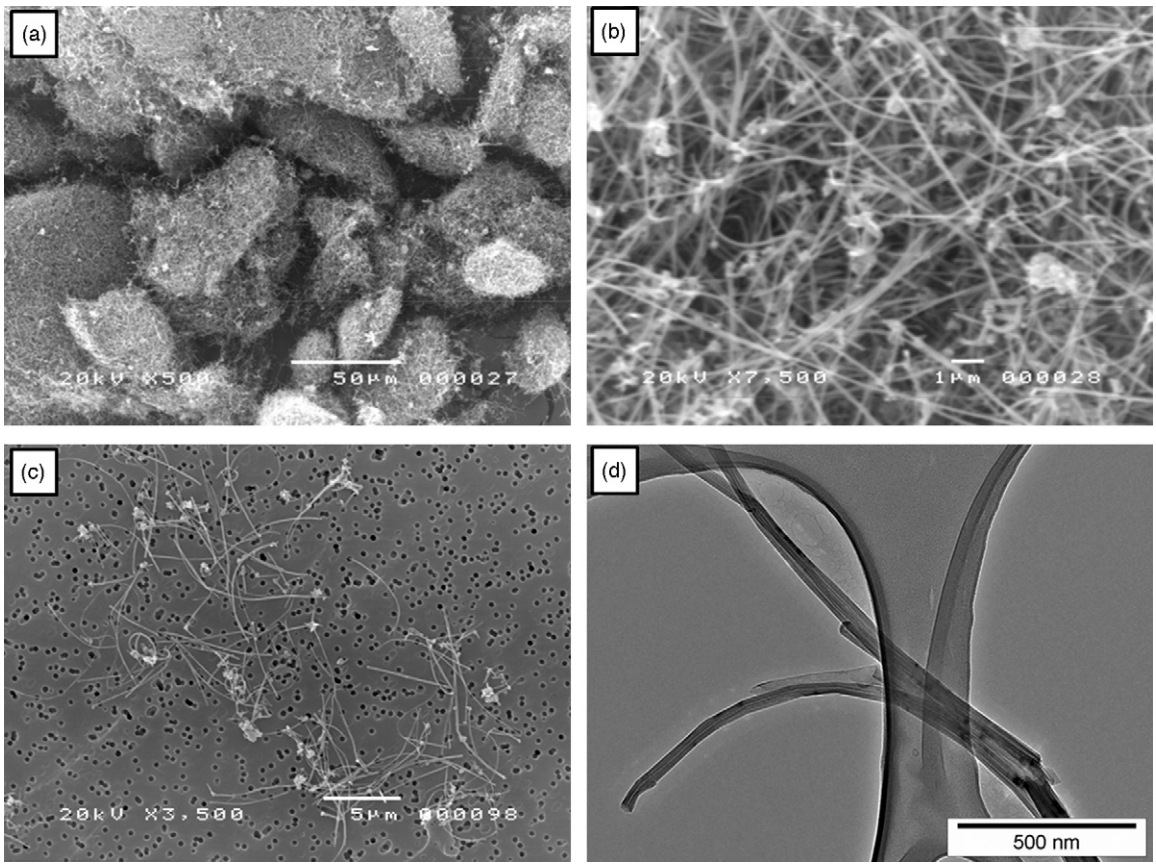

Fig. 1. SEM and TEM images of MWCNTs samples. SEM images of bulk MWCNTs (panels a and b). SEM (panel c) and TEM (panel d) images of MWCNTs dispersed by ultrasonication with an ultrasonic bath.

2.8. Histopathological evaluation

The trachea, left lung, liver, kidney, spleen, and cerebrum were fixed with 10% (v/v) neutral phosphate-buffered formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological evaluation under the light microscope.

2.9. Light and electron microscopic observation of MWCNTs in the lung

Morphology of the MWCNTs in the lung was observed with the light microscope. Sections of the right lung after lavage were fixed with glutaraldehyde and were resin-embedded to give ultrathin sections. Morphology of the individual tubes of instilled MWCNTs in the lung of rats was observed with TEM (JEM-100CX II, JEOL Ltd., Tokyo, Japan).

2.10. Statistical analyses

Statistical analyses of the body and lung weights, as well as the cell numbers and biochemical parameters in the BALF were conducted. Statistical significance was determined using multiple comparison tests between the negative control and MWCNT-exposed groups. First, the Bartlett's test was conducted. One-way layout analysis of variance was conducted when the variances were equal. Further, Dunnett's multiple comparison tests were conducted when the differences between the groups were significant. The Kruskal–Wallis test was used when the variances were not equal and Steel's multiple comparison tests were conducted when the differences were significant. Statistical significance was determined between the positive and negative control groups using intergroup comparison tests. First, the *F*-test was conducted; the Student's *t*-test was used when the variances were equal, and the Aspin–Welch *t*-test was used when the variances were not equal. Statistical significances were judged at the 0.05 probability level. SAS System version 6.12 (SAS Institute Japan Ltd., Tokyo, Japan) was used for all the statistical analyses.

3. Results

3.1. MWCNT characterization

SEM and TEM images of the bulk and dispersed MWCNT samples are shown in Fig. 1. In the bulk MWCNT samples, MWCNTs were in the form of agglomerates with sizes ranging from 50 to 100 μm, which are formed from tangled individual tubes with lengths of more than 10 μm (Fig. 1a). Objects other than fibrous shape materials (sizes less than 1 μm), which were considered to be carbon soot, were also observed in the bulk MWCNT samples (Fig. 1b). Their content was 7–8% by weight and catalysts were not detected (<0.1% by weight), based on results from the TG analysis. The BET surface area of the bulk MWCNTs was 23.0 m^2/g.

Most of the MWCNTs in the suspension were individually dispersed (Fig. 1c and d), which suggests that ultrasonication with an ultrasonic bath is effective for dispersing MWCNTs into the Tween 80 solution. Distribution of the MWCNT length in the 1 mg/mL of MWNT suspension, which is measured based on the SEM images, is shown in Fig. 2. The length of the all MWC-

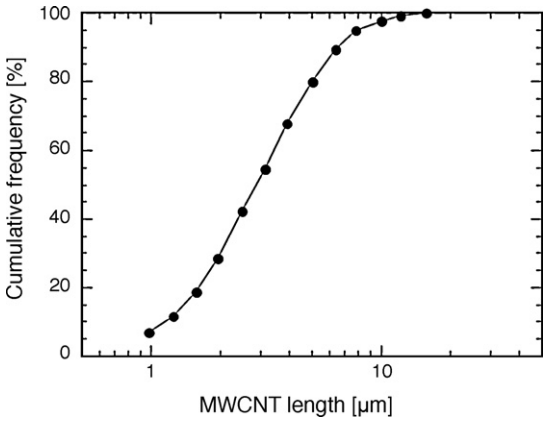

Fig. 2. Distribution of MWCNT length in a 1 mg/mL MWCNT suspension dispersed by ultrasonication in an ultrasonic bath.

NTs in the suspension was less than 20 μm, whereas longer tubes were present in the bulk sample. These results suggest that the MWCNTs were cut during ultrasonication. Generally ultrasonication processes can cause a degradation in sample quality by introducing defects in the graphene structure of MWCNT and producing carbon debris. In order to evaluate this degradation, an effective method is calculation of D/G ratio, the ratio of the intensities of disorder-induced mode (D-band) and graphene-induced mode (G-band) which are appeared in the Raman spectrum of MWCNT (Lee et al., 2008; Musumeci et al., 2008). The D/G ratio of the bulk MWCNT samples and the dispersed MWCNT suspension showed quite similar values of 0.091 and 0.085, respectively. This result implies that there are not significant degradation in sample quality after the ultrasonication process even the MWCNT fibers were cut into shorter segments.

3.2. Body and lung weights

Statistically significant differences in the body weights of experimental animals were not observed between any of the MWCNT or crystalline silica-exposed groups and the negative control group at any time point.

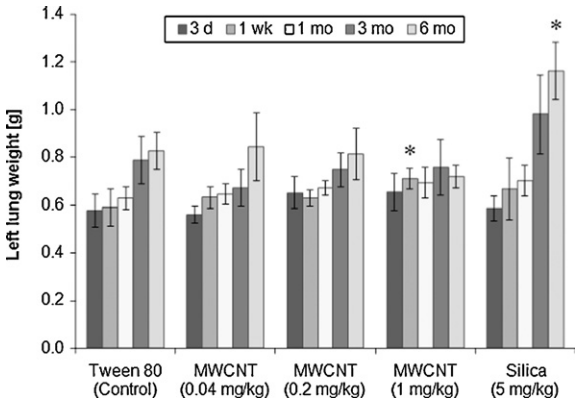

Fig. 3. Left lung weights of rats exposed to MWCNTs or crystalline silica particles and the corresponding controls at 3-day, 1-week, 1-month, 3-month, and 6-month post-instillation exposure. Values are represented as the mean ± SD. *Significant increase from control ($p < 0.05$).

Throughout the study period, no obvious increase in the lung weight was observed in any of the MWCNT-exposed groups when compared with the lung weight in the negative control group. In contrast, lung weight was significantly greater in the crystalline silica-exposed group (Fig. 3).

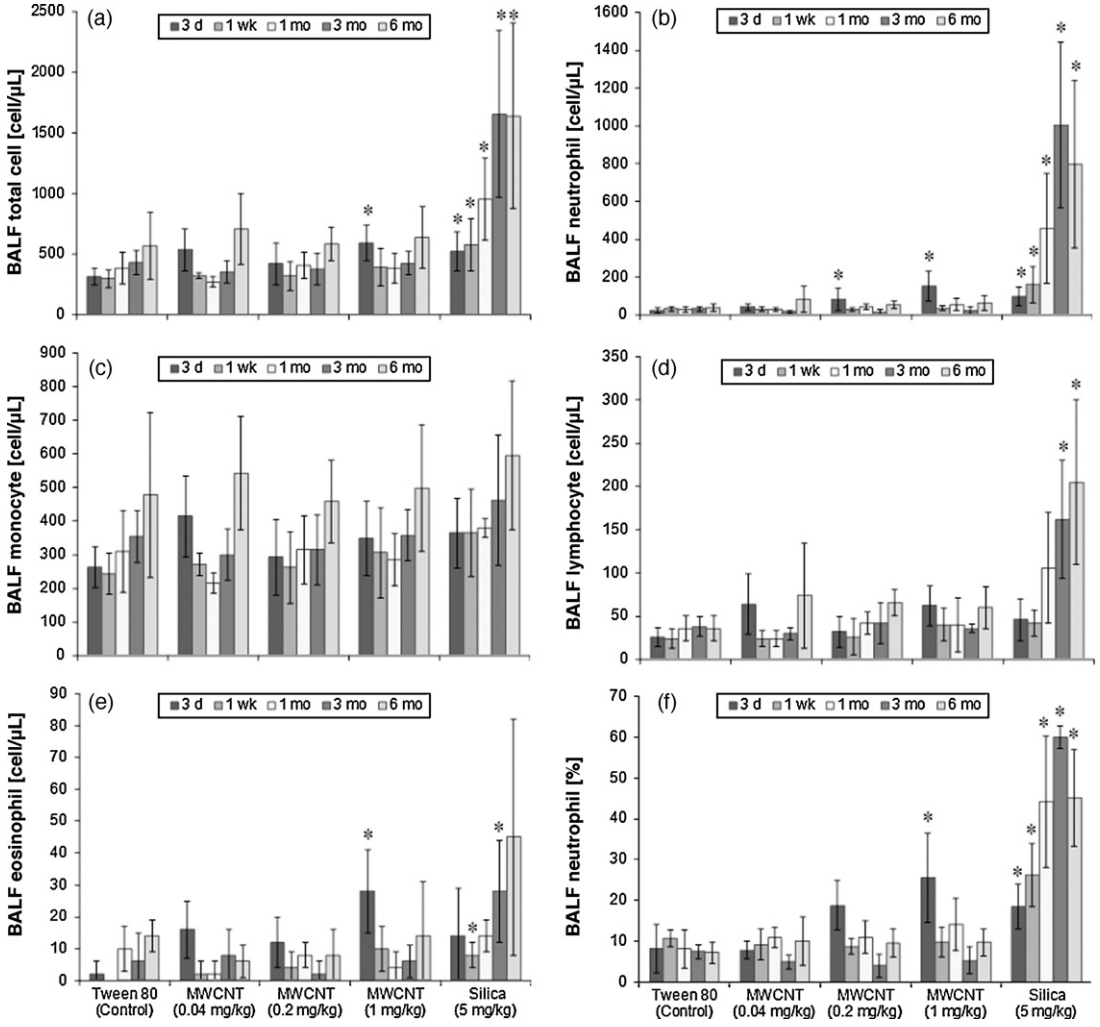

Fig. 4. Total number of BALF cells (a), neutrophils (b), macrophages (c), lymphocytes (d), and eosinophils (e), and the percentage of neutrophils (f) in rats exposed to MWCNTs, crystalline silica particles, or the corresponding controls at 3-day, 1-week, 1-month, 3-month, and 6-month post-instillation exposure. Values are represented as the mean ± SD. *Significant increase from control ($p < 0.05$).

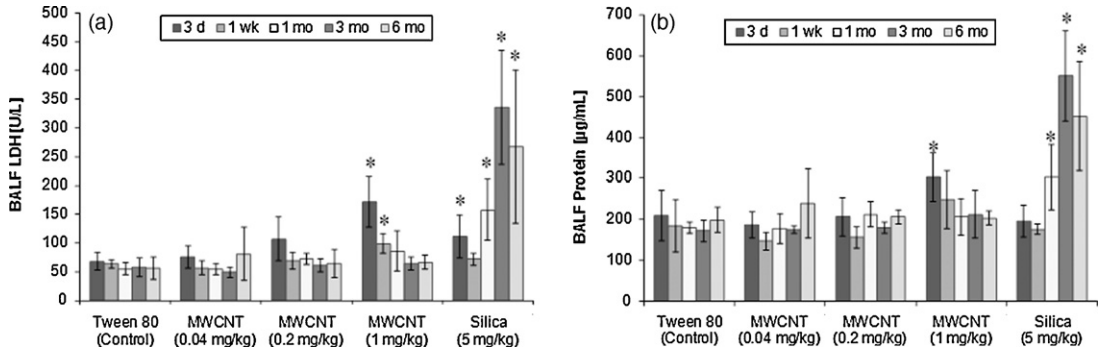

Fig. 5. BALF LDH (a) and protein (b) levels in rats exposed to MWCNTs, crystalline silica particles, or the corresponding controls at 3-day, 1-week, 1-month, 3-month, and 6-month post-instillation exposure. Values are represented as the mean ± SD. *Significant increase from control ($p < 0.05$).

3.3. Necropsy findings

In the negative control group and the group exposed to 0.04 mg/kg MWCNTs, abnormal findings were not observed at any of the time points. In the groups exposed to 0.2 and 1 mg/kg MWCNTs, brown or black spots were observed in the lung until 1- and 6-month post-exposure, respectively. These spots were considered to be the pigment of the agglomerated MWCNTs. In the crystalline silica-exposed group, significant changes were not observed until 1-month post-exposure, white spots were observed in the lung from 3- to 6-month post-exposure, and hypertrophy of the peribronchial lymph nodes and thymic lymph nodes was observed.

3.4. BALF results

3.4.1. Inflammatory cells

In the MWCNT-exposed groups, the number and percentage of BALF inflammatory cells were changed in a dose-dependent manner (Fig. 4). While no changes were observed in the group exposed to 0.04 mg/kg MWCNTs. BALF neutrophils were increased significantly only at 3-day post-exposure in the group exposed to 0.2 mg/kg MWCNTs. In the group exposed to 1.0 mg/kg MWCNTs, the total BALD cell numbers, neutrophils, eosinophils, and the percentage of neutrophils were increased significantly only at 3-day post-exposure. In the crystalline silica-exposed group, the total BALF cell numbers, neutrophils, lymphocytes, eosinophils, and

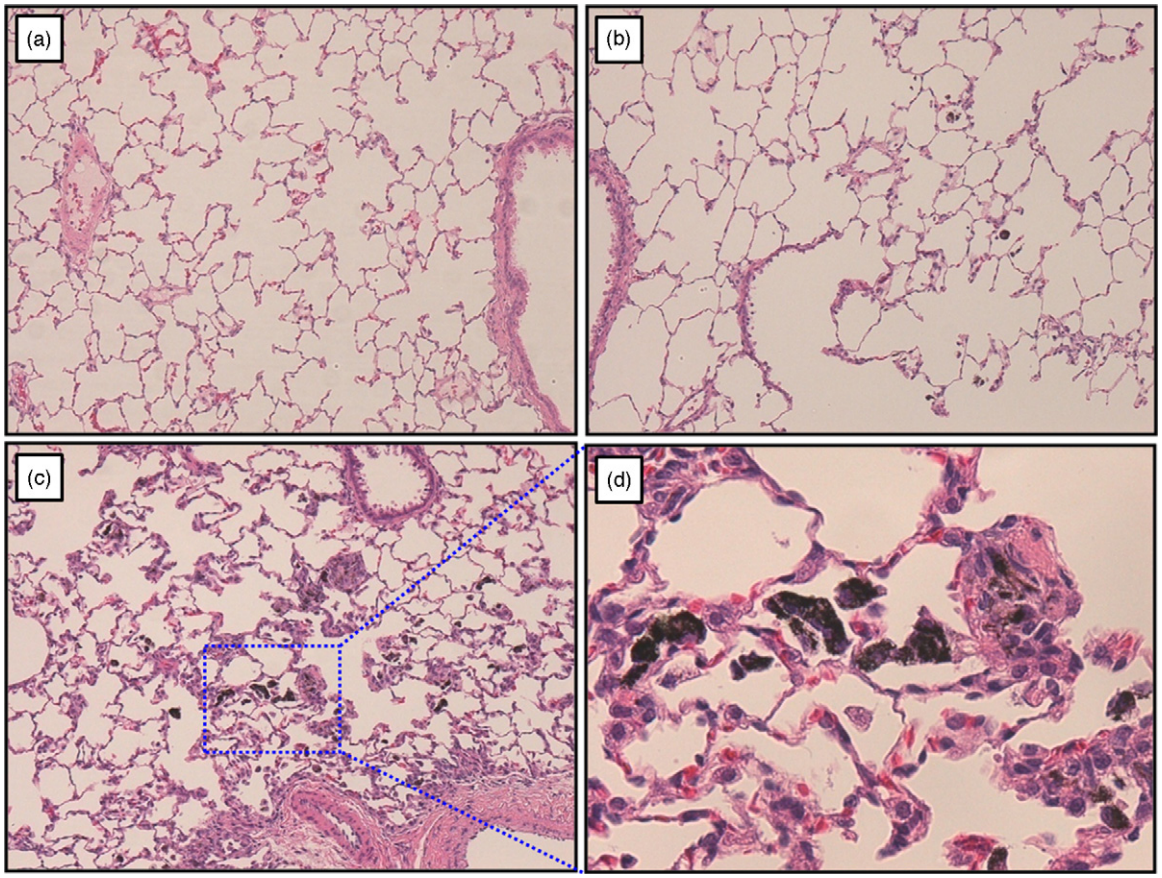

Fig. 6. Light micrographs of lung tissues of rats exposed to MWCNTs at 1-month post-instillation exposure (hematoxylin and eosin staining). No significant changes were observed in the group exposed to 0.04 mg/kg MWCNTs (panel a). Minimal macrophage accumulation and phagocytosed MWCNTs were observed in the alveoli of the group exposed to 0.2 mg/kg MWCNTs (panel b). MWCNT deposits and macrophage accumulation were observed in the alveoli of the group exposed to 1 mg/kg MWCNTs (panels c and d).

Table 1
Pulmonary histopathology severity scores[a] of rats exposed to MWCNTs or silica.

Findings	Timepoint	Control group	MWCNT-exposed group			Silica-exposed group
			0.04 mg/kg	0.2 mg/kg	1 mg/kg	
Administered substance accumulation in the alveoli	3 d	0	0	1	1	1
	1 wk	0	0	1	1.4	0.8
	1 mo	0	0	0.6	1	1
	3 mo	0	0	0.4	1	0
	6 mo	0	0	0.6	1	0
Administered substance accumulation in the interstitium	3 d	0	0	0	0.6	1
	1 wk	0	0	0	0.8	0
	1 mo	0	0	0.2	0.4	0
	3 mo	0	0	0.2	0.4	0
	6 mo	0	0	0	1.2	0
Inflammatory cell infiltration in the alveoli	3 d	0.2	0.2	0.8	0.8	0.6
	1 wk	0	0	0.4	0.4	0
	1 mo	0.4	0	0	0	0.2
	3 mo	0	0	0	0	0.6
	6 mo	0	0	0	0	1
Macrophage accumulation in the alveoli	3 d	0.6	0.2	1	1.4	1
	1 wk	0.2	0	1	1.4	0.8
	1 mo	0.2	0	0.8	1	1
	3 mo	0	0	0.4	1	1.8
	6 mo	0	0	0.6	1	2
Macrophage infiltration in the interstitium	3 d	0.4	0	0.6	1.2	1
	1 wk	0.2	0	0.2	1.2	0
	1 mo	0	0	0.2	0.4	0.6
	3 mo	0	0	0.2	0.4	1
	6 mo	0	0	0	1.2	0.3
Granuloma in the interstitium	3 d	0.4	0	0.6	0.4	0.2
	1 wk	0	0	0	0.4	0
	1 mo	0	0	0.2	0.4	0
	3 mo	0	0	0	0	0.2
	6 mo	0	0	0	0.4	0.8
Granuloma in the lymphoid tissue	3 d	0	0	0	0	0
	1 wk	0	0	0	0	0
	1 mo	0	0	0	0	0
	3 mo	0	0	0	0	0
	6 mo	0	0	0	0	1
Hypertrophy of the bronchial epithelium	3 d	0	0	1.4	1	0
	1 wk	0	0	0	0.2	0
	1 mo	0	0	0	0	0
	3 mo	0	0	0	0	0
	6 mo	0	0	0	0	0
Hypertrophy of the alveolar epithelium	3 d	0	0	0	0	0
	1 wk	0	0	0	0	0
	1 mo	0	0	0	0	0
	3 mo	0	0	0	0	0.8
	6 mo	0	0	0	0	1.8
Macrophage cytolysis in the alveoli	3 d	0	0	0	0	0
	1 wk	0	0	0	0	0
	1 mo	0	0	0	0	0.2
	3 mo	0	0	0	0	1.8
	6 mo	0	0	0	0	1.8
Foamy macrophage infiltration in the alveoli	3 d	0	0	0	0	0
	1 wk	0	0	0	0	0
	1 mo	0	0	0	0	0
	3 mo	0	0	0	0	1.4
	6 mo	0	0	0	0	1.3
Alveolar proteinosis	3 d	0	0	0	0	0
	1 wk	0	0	0	0	0
	1 mo	0	0	0	0	0
	3 mo	0	0	0	0	1.2
	6 mo	0	0	0	0	1.8

[a] Severity scores given to individual animals from a complete pathological examination are 0, not remarkable; 1, minimal; 2, slight/mild; 3, moderate; and 4, severe; based upon relative evaluation of lesions. Severity scores for each animal within a group were added, and an average score per animal was calculated, which is shown in the table.

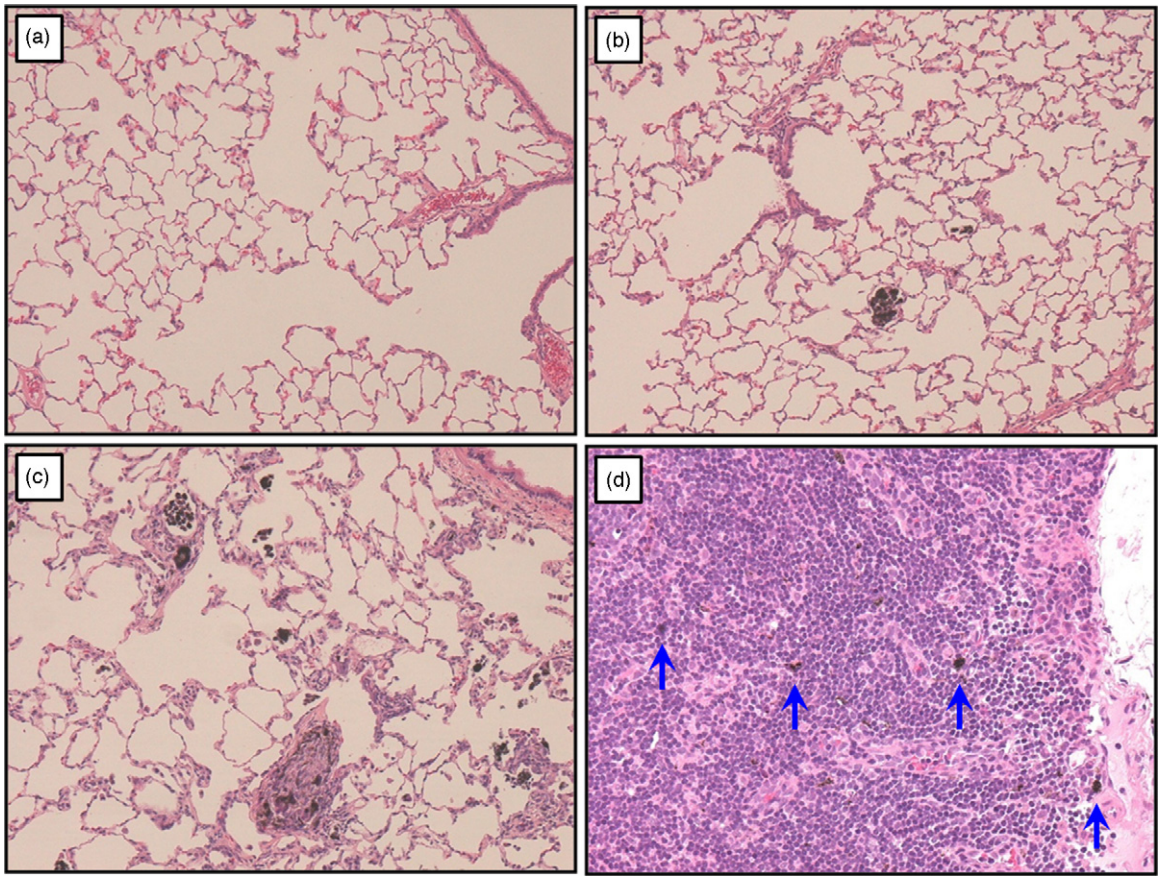

Fig. 7. Light micrographs of lung tissues of rats exposed to MWCNTs at 6-month post-instillation exposure (hematoxylin and eosin staining). No significant changes were observed in the group exposed to 0.04 mg/kg MWCNTs (panel a). Minimal macrophage accumulation and phagocytosed MWCNTs were observed in the alveoli of the group exposed to 0.2 mg/kg MWCNTs (panel b). MWCNT deposits and macrophage accumulation were observed in the alveoli and interstitium of the group exposed to 1 mg/kg MWCNTs (panel c). Minimal MWCNT depositions (arrows) were observed in the peribronchial lymph nodes in the group exposed to 1 mg/kg MWCNTs (panel d).

the percentage of neutrophils were significantly increased until 6-month post-exposure.

3.4.2. Biochemical measurements

For the MWCNT-exposed groups, LDH and TP levels in the BALF were significantly increased only in the group exposed to 1 mg/kg MWCNTs; however, the changes were transient and recovered after 1-week post-exposure (Fig. 5). BALF cytokine levels were not significantly changed at any time point (data not shown). In contrast, LDH and TP levels in the BALF were significantly increased until 6-month post-exposure in the crystalline silica-exposed group (Fig. 5), and significant changes in IL-1β and IL-2 levels were observed in this group (data not shown).

3.5. Histopathological evaluation

For all the groups, histopathological changes due to the instillation exposure of MWCNTs or crystalline silica were observed only in the lungs and lung-associated lymph nodes, and not in the other tissues (i.e., the liver, kidney, spleen, and cerebrum). Table 1 summarizes the histopathological findings of the rats examined in this study and their severity scores at each time point.

In the MWCNT-exposed groups, dose-dependent histopathological changes were observed. In the group exposed to 0.04 mg/kg MWCNTs, no significant changes were observed at any time points (Figs. 6 and 7Figs. 6a and 7a). In the group exposed to 0.2 mg/kg MWCNTs, minimal macrophage accumulation and phagocytosed MWCNTs were observed in the alveoli (Figs. 6b and 7b). In the group exposed to 1 mg/kg MWCNTs, deposition of the MWCNTs and macrophage accumulation, part of which were granulomatous, was observed in the alveoli and interstitium from 3-day to 1-month post-exposure (Fig. 6c and d). Most MWCNTs were phagocytosed by alveolar macrophages. Further, hypertrophy of the bronchial epithelium and inflammatory cell infiltrations were observed. From 3- to 6-month post-exposure, histopathological findings were qualitatively similar to those at 1-month post-exposure; although the severity of the changes was gradually weaker. At 6-month post-exposure, deposition of the MWCNTs and macrophage accumulation, part of which were granulomatous, was observed in the alveoli and interstitium in the group exposed to 1 mg/kg MWCNTs; however, the severity of these changes was minimal (Fig. 7c). In the group exposed to 1 mg/kg MWCNTs, minimal MWCNT depositions were observed in the peribronchial lymph nodes at 6-month post-exposure (Fig. 7d).

In the crystalline silica-exposed group, only minimal macrophage accumulation in the alveoli and interstitium was observed up to 1-week post-exposure. However, the severity of macrophage accumulation was increased after 1-month post-exposure, and, cytolysis of macrophages was observed, which was most severe at 6-month post-exposure. At 6-month post-exposure, in addition to the infiltration of inflammatory cells, foamy macrophages and cytolysis of macrophages were observed in the alveoli; pulmonary alveolar proteinosis was induced (Fig. 8a). Hypertrophy of the alveolar epithelium and granulomas was observed in the interstitium (Fig. 8b). Multifocal granulomas were also observed in the intrapulmonary lymph nodes (Fig. 8c), peribronchial lymph nodes (Fig. 8d), and thymic lymph nodes (data not shown).

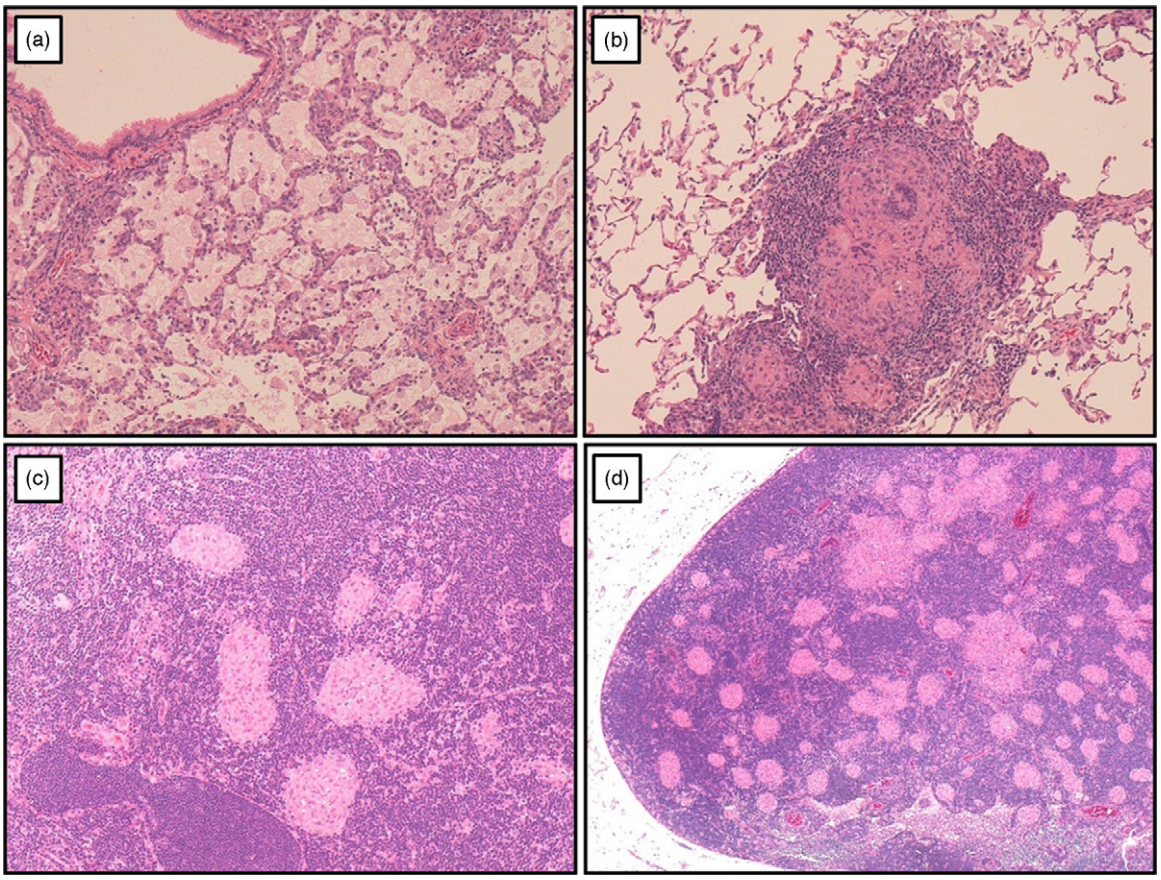

Fig. 8. Light micrographs of lung tissues of rats exposed to crystalline silica particles at 6-month post-instillation exposure (hematoxylin and eosin staining). Cytolysis of macrophages and foamy macrophage infiltrations were observed in the alveoli; pulmonary alveolar proteinosis was induced (panel a). Granuloma formations were observed in the interstitium (panel b). Multifocal granulomas were observed in the intrapulmonary lymph nodes (panel c) and peribronchial lymph nodes (panel d).

The results of the histopathological evaluations were consistent with that of BALF inflammatory cells and biochemical measurements.

3.6. Light and electron microscopic observation of MWCNTs in the lungs

On the basis of light microscopic examination, MWCNTs deposited in the lungs were phagocytosed by alveolar macrophages and were sequentially accumulated in the alveoli or interstitium until 6-month post-exposure (Fig. 9). Furthermore, the 400 TEM images, which displayed individual MWCNTs, showed that all the MWCNTs were presented in a similar form in the lungs. MWCNTs were located in the alveolar macrophages or in macrophages in the interstitial tissues, and individual MWCNTs were not present in the cells of the interstitial tissue (Fig. 10). The diameter and length of the 104 tubes in the TEM images were measured. The diameter of the MWCNTs in the lungs was almost the same as the instilled materials (approximately 60 nm). There were a wide range of MWCNT lengths in the lungs; the median length was approximately 1.5 μm (number basis), although some tubes were considerably longer, measuring up to 6 μm (Fig. 11).

4. Discussion

Biological responses due to the single instillation of MWCNTs were observed only in the lungs of rats, and not in the liver, kidney, spleen, or cerebrum (including the olfactory bulb) in all the groups. BALF inflammatory cells as well as LDH and TP levels were significantly increased in the group exposed to 1 mg/kg MWCNTs, but only at 3-day post-exposure. No significant changes in BALF inflammatory cells and markers were observed in the groups exposed to 0.04 and 0.2 mg/kg MWCNT at any time points. The severity of the lesions on histopathological examination of rat lungs was dose dependent although Warheit et al. (2004) and Mitchell et al. (2007) have reported non-dose-dependent pulmonary responses due to instillation of SWCNTs or inhalation of MWCNTs. Histopathological evaluation indicated deposition of the MWCNTs and macrophage infiltration, part of which were granulomatous, in the alveoli and interstitium in the group exposed to 1 mg/kg MWCNTs. On the basis of the light microscopic observations, MWCNTs were phagocytosed by macrophages. Hypertrophy of the bronchial epithelium and inflammatory cell infiltrations were also observed in this group. Some of the previous toxicity studies of MWCNT instillation or inhalation exposures in rats (Muller et al., 2005; Li et al., 2007; Ma-Hock et al., 2009; Pauluhn, 2010) have reported qualitatively similar pulmonary responses. However, our study revealed that pulmonary inflammatory responses were transient, and the histopathological changes were gradually weaker from 1-week to 6-month post-exposure. At 6-month post-exposure, significant changes were not observed in the group exposed to 0.2 mg/kg MWCNTs. In the group exposed to 1 mg/kg MWCNTs, deposition of the MWCNTs and macrophage accumulation, of which some of them were granulomatous, were observed in the alveoli and interstitium until 6-month post-exposure, although they were minimal changes. Studies have reported that pulmonary fibrosis is induced due to exposure to SWCNTs or MWCNTs (Muller et al., 2005; Shvedova et al., 2008a); however, pulmonary fibrosis was not observed in any of the groups in this study.

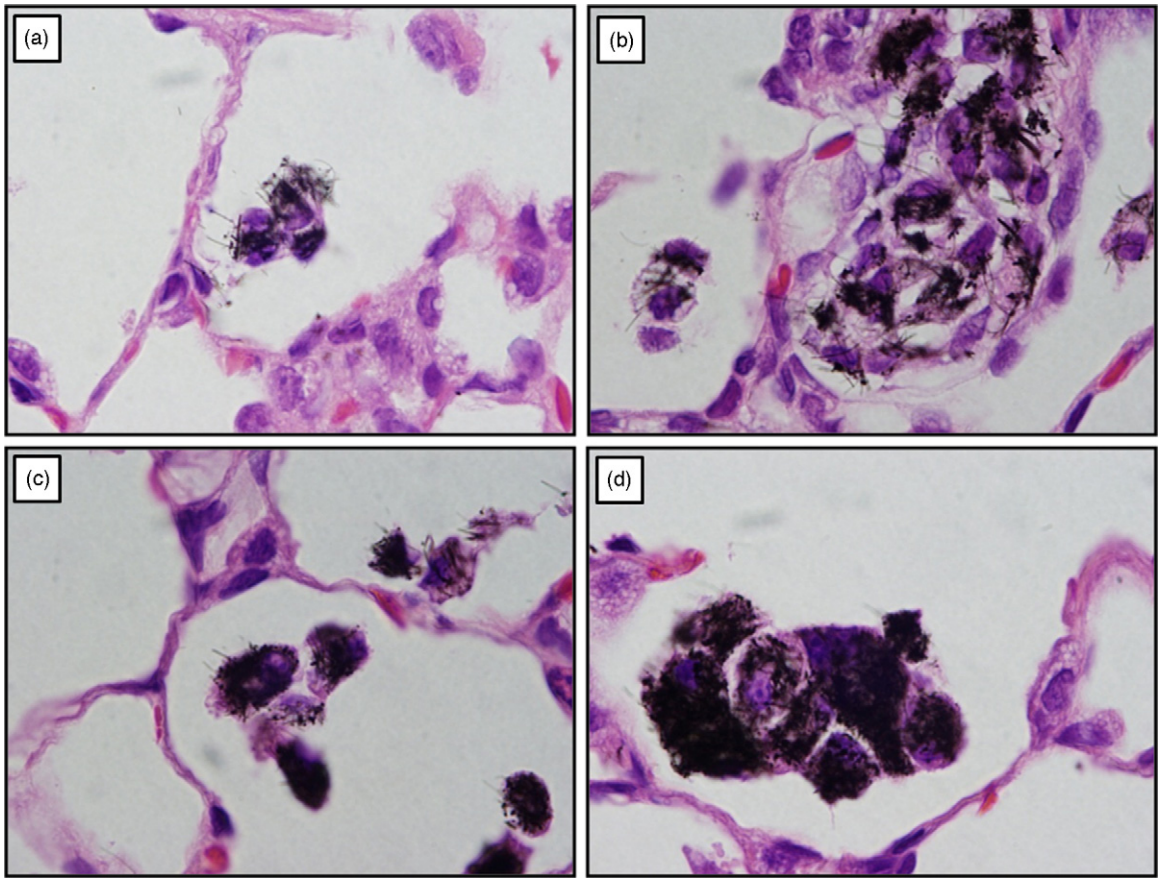

Fig. 9. Light micrographs of MWCNTs in the lungs of rats exposed to 1 mg/kg MWCNTs at 3-day (panel a), 1-week (panel b), 1-month (panel c), and 6-month (panel d) post-instillation exposure (hematoxylin and eosin staining, magnification: ×200). MWCNTs deposited in the lungs were typically phagocytosed by alveolar macrophages and were sequentially accumulated in the alveoli until 6-month post-instillation.

Light microscopy and TEM observations revealed that the MWCNTs deposited in the lungs were phagocytosed by alveolar macrophages and were sequentially accumulated in the alveoli. MWCNT translocation or penetration to the pleural was not observed. Furthermore, based on the 400 TEM images, it was shown that all the MWCNTs were located in the alveolar macrophages or phagocytosed by macrophages in the interstitial tissues, and individual MWCNTs were not presented in the cells of the interstitial tissue.

In contrast, inflammatory responses were observed in the lungs and lung-associated lymph nodes in the group exposed to 5 mg/kg crystalline silica, where BALF inflammatory cells, LDH, TP, IL-1β, and IL-2 levels were significantly increased after the instillation exposure, and these changes were the most severe at 6-month post-exposure. Furthermore, lung weights were significantly increased at 3- and 6-month post-exposure. Histopathological evaluation revealed that although short-term inflammatory responses were weak, the inflammatory responses were much stronger at 6-month post-exposure. Consequently, crystalline silica particles produced continuous inflammation with a 5 mg/kg dose of intratracheal instillation. These pulmonary responses were qualitatively and quantitatively different from the responses observed for MWCNTs instillation exposure.

The relationship of the dose of MWCNTs instilled into the lungs in this study and exposure levels of aerosolized MWCNTs to humans during the handling of CNTs in the work place is discussed below. The pulmonary deposition amount of MWCNTs in this study was considered to be almost 100% of the instilled dose of the MWCNTs (i.e., 0.04, 0.2, and 1.0 mg/kg). By measuring the BET surface area of the MWCNT samples, the doses can be expressed in terms of the CNT surface area dose, which are 0.0009, 0.1146, and 0.023 m^2/kg, for doses of 0.04, 0.2, and 1.0 mg/kg, respectively. Based on the density of the MWCNT samples reported by the manufacturer (2.1 g/cm^3) and assuming that the tube diameter and length are uniform (60 nm and 1.5 μm, respectively), and that all tubes are individually dispersed in the suspension, the doses can also be expressed in terms of tube numbers, which are 9.4×10^9, 4.7×10^{10}, and 2.4×10^{11} tubes/kg, for dosed of 0.04, 0.2, and 1.0 mg/kg, respectively.

On the other hand, there are only a few reports of atmospheric CNT concentrations measurements in the work place. Maynard et al. (2004) and Han et al. (2008) reported measured concentrations of SWCNTs and MWCNTs in the research facilities, respectively.

Maynard et al. (2004) reported that atmospheric concentrations of SWCNTs, which were estimated using an indicator of metal catalysts, were in range of 0.7–53 μg/m^3 during the collection and cleaning process, based on the investigation of SWCNT research facilities with laser-abrasion or the high pressure carbon monoxide (HiPco) method. Han et al. (2008) reported that the atmospheric mass concentration of total dust (including MWCNTs) was in range of 210–430 μg/m^3 during the blending process and in range of 37–190 μg/m^3 during the weighing and spraying process, based on the investigation of MWCNT research facilities with the thermal chemical vapor deposition (CVD) method. They also reported that the number concentration of MWCNTs was in range of $172.9–193.6 \times 10^6$ tubes/m^3 during these processes. Based on the atmospheric mass concentration or number concentration of CNTs reported in these studies, deposition amounts of MWCNTs to the lungs of humans working for 8 h/day and 5 days/week without any exposure protection can be calculated as

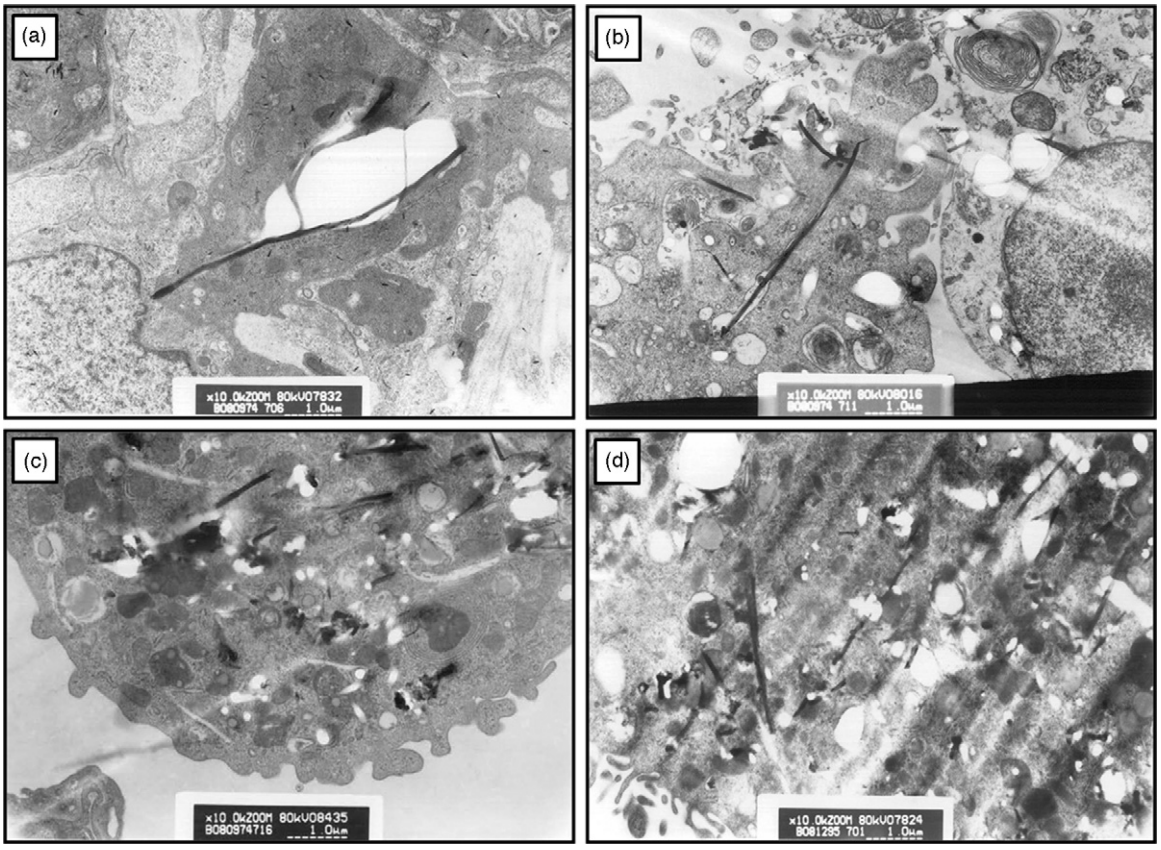

Fig. 10. TEM images of the lungs of rats exposed to 1 mg/kg of MWCNTs at 1-week (panel a), 1-month (panel b), 3-month (panel c), and 6-month (panel d) post-instillation exposure (magnification: ×10,000). MWCNTs observed in TEM images were located in the alveolar macrophages and not in the interstitial cells.

follows. Assuming that average daily exposure time is 8 h/day × 5 days/week × 60 min/h = 343 min/day, the deposition fraction of inhaled MWCNTs into the lungs is 0.1 (10%) based on the study of Miller (2000), the respiratory minute volume is 25 L/min, and body weight is 60 kg, then pulmonary deposition amounts of MWCNTs are calculated to be 0.01 and 6.2 μg/kg/day, based on the atmospheric concentration of 0.7 μg/m^3 (Maynard et al., 2004) and 434.5 μg/m^3 (Han et al., 2008), respectively. Therefore, instillation exposure of 1.0 mg/kg MWCNTs corresponds to pulmonary deposition amounts of 160–1300 days (i.e., several months to several years) in the working environment without any exposure protection when the maximum atmospheric concentration of MWCNTs is used in the calculation.

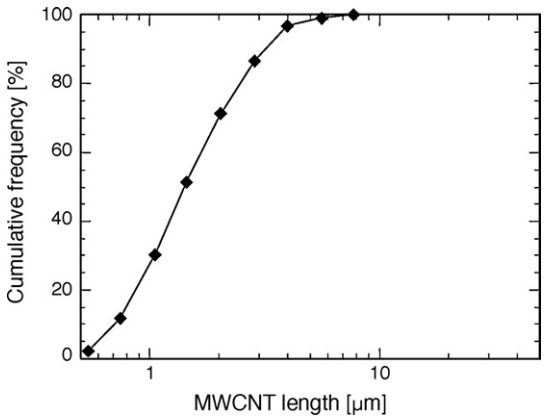

Fig. 11. Length of MWCNTs in the lungs of rats exposed to 1 mg/kg MWCNTs. The lengths of 104 MWCNTs observed by the TEM were measured. The median (minimum–maximum) length of the MWCNTs in the lungs was 1.5 (0.42–6.1) μm.

Based on the number concentration of CNTs, deposition of MWCNTs into the lungs per day per kg body weight were calculated to be 2.47–2.77 × 10^6 tubes/kg/day based on the atmospheric number concentration of 172.9–193.6 × 10^6 tubes/m^3 (Han et al., 2008). Therefore, 2.4 × 10^{11} tubes/kg (1.0 mg/kg) of instillation exposure of MWCNTs corresponds to a pulmonary deposition amount of 85,000–95,000 days, which is longer than the average human lifespan.

Collectively, our data indicated that the pulmonary inflammatory responses to MWCNT deposition in the lungs were dose dependent, and the responses were weak and transient under approximate pulmonary deposition amounts comparable to the work environment. Chronic inflammatory responses such as pulmonary fibrosis or angiogenesis were not observed. MWCNTs deposited in the lungs were mostly phagocytosed by alveolar macrophages and were sequentially accumulated in the alveoli, which suggests that MWCNTs were being processed and cleared by alveolar macrophages.

Competing interests

The authors declare that they have no competing interests.

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