

Basic study of intratracheal instillation study of nanomaterials for the estimation of the hazards of nanomaterials

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Abstract: In order to examine the usefulness of intratracheal instillation of nanoparticles for the screening of the harmful effects of nanoparticles, we performed intratracheal instillation studies of nanomaterials on rats using different delivery devices and postures as a basic study. Multiwall carbon nanotubes (MWCNTs) with a geometric mean length and secondary diameter of 2.16 μm and 752 nm, respectively, were used as the nanomaterials. Male F344 rats were intratracheally exposed to 0.04 or 0.2 mg/rat of MWCNT, were dissected at 1 d and 3 d, and cell analyses of the bronchoalveolar lavage fluid (BALF) were analyzed. Two delivery devices were used for the intratracheal instillation of the MWCNTs: a gavage needle and a microsyringe aerolizer. Both induced neutrophil influx in the lung at 1 and 3 d, and there were no significant differences in neutrophil inflammation between the two delivery devices. The main distribution of pulmonary inflammation by both delivery devices was in the centrilobular spaces in the lung. Two postures were used: an angle of approximately 45 degrees and a standing posture on a board, both of which also induced pulmonary influx in BALF and pulmonary inflammation mainly in the centrilobular spaces, with no large difference in pulmonary inflammation between the two postures. Taken together, the differences in the delivery devices and postures of the rats in the intratracheal instillation did not affect the acute pulmonary toxicity of the nanomaterials.

Key words: Nanomaterial, Intratracheal instillation, Inflammation, Harmful effect, Administration

Introduction

Various manufactured nanomaterials have been produced with the increasing development of nanotechnology, but the pulmonary toxicity of these manufactured nanomaterials is not fully understood. Although the most reliable studies for exploring the pulmonary toxicity of respirable

chemicals are inhalation studies, due to their having the most realistic exposure for real-life humans¹⁾, it is impossible for the pulmonary toxicity of all nanomaterials to be examined this way because of the high cost, the need for large facilities, and securing technical expertise for maintaining stable exposure and keeping a desirable dispersion of nanomaterials^{2, 3)}. Intratracheal instillation studies, on the other hand, are useful for examining the dosage dependence of nanomaterials and the clarification of the mechanism involved in exposure to nanomaterials^{4–7)}, but the findings of pulmonary toxicity of nanomaterials are lim-

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ited. Intratracheal instillation studies have garnered attention in the research of the pulmonary toxicity of nanomaterials because of their relatively inexpensive cost and their requirement of relatively simple equipment⁸).

Comparing inhalation and intratracheal instillation studies for the pulmonary response induced by nanomaterials, acute pulmonary inflammation due to a bolus effect is observed following intratracheal instillation^{3, 4}), unlike inhalation studies. We conducted inhalation and intratracheal instillation studies of nanomaterials with high and low toxicity, and found that the pattern of pulmonary ranking of nanomaterials is the same when pulmonary inflammation in not only the acute phase but also in the chronic phase are examined^{3, 9, 10}). There are some other studies in which the ranking of pulmonary inflammation by nanomaterials is the same in inhalation studies and intratracheal instillation studies^{4, 8, 11}), suggesting that intratracheal instillation studies may be useful for ranking the harmful effects of nanomaterials.

There are not yet enough studies about the methodology of intratracheal instillation. Although there is a report that the results of an interlaboratory evaluation of rodent pulmonary responses to intratracheally exposed nanomaterials were similar, a detailed methodology of the intratracheal instillation studies was not shown¹²). Even if there are different results between laboratories, it cannot be denied that the differences might be related to a difference in the basic techniques of intratracheal instillation. Therefore, in order to examine whether or not pulmonary toxicity following intratracheal instillation of nanomaterials is a reflection of the technique used, we examined differences in pulmonary inflammation resulting from different delivery devices and different positions of animals in intratracheal instillations.

Materials and Methods

Preparation of samples of multiwall carbon nanotube (MWCNT) suspensions

The tested carbon nanotubes (CNTs) were MWCNTs (Mitsui-7) purchased from Mitsui & Co., Ltd., the physico-chemical properties of which are shown in Table 1. A 50 ml bovine serum albumin (BSA)(08587-42, Nacalai Tesque, Inc) aqueous solution of 10 mg/ml was added to the MWCNT of 50 mg sampled in a glass vial of 100 ml. The vial was placed in an ultrasonic bath (5510-MT, BRANSON) and sonication at 70 W was applied to the suspension for 1.5 h. The suspension of dispersed MWCNT was filtered through a cell strainer with a pore size of 40 μm in order to remove the coarse agglomerates. The con-

Table 1. Physico-chemical properties of MWCNTs

| Physico-chemical Properties | Value |
|------------------------------|----------------------------|
| Maker | Mitsui & Co., Ltd |
| Sample | Mitsui-7 |
| Geometric mean diameter (SD) | 52 nm (1.5) |
| Geometric mean length (SD) | 2.16 μm (4.2) |
| Specific surface area (BET) | 23.0 m^2/g |
| Purity | More than 99.6 % |
| Shape | Fibrous |
| Ratio of GD* | 13 \pm 3 |
| Secondary diameter(DLS) | 752 nm |
| Solubility | Low |

*Intensity ratio of G-band to D-band

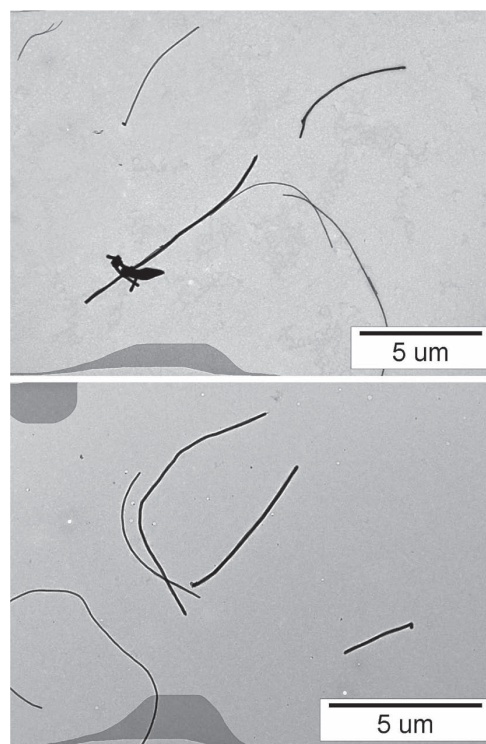


Fig. 1. TEM photograph of MWCNTs dispersed by sonication using an ultrasonic bath. The MWCNTs in the suspension were well-dispersed.

centration of the MWCNT in the filtered suspension was prepared to 0.1 or 0.5 mg/ml, and the suspensions were used as stock suspensions for the intratracheal instillation tests. The mean size of the secondary particles was characterized by the dynamic light scattering method (Zetasizer Nano ZS, Malvern) and the cumulant mean was 752 nm. The state of dispersing in the suspension was observed by TEM (JEM-1010, JEOL). The CNTs were well dispersed, as shown in Fig. 1. Measuring the distribution of the length of the dispersed CNTs the TEM photos, the geometric

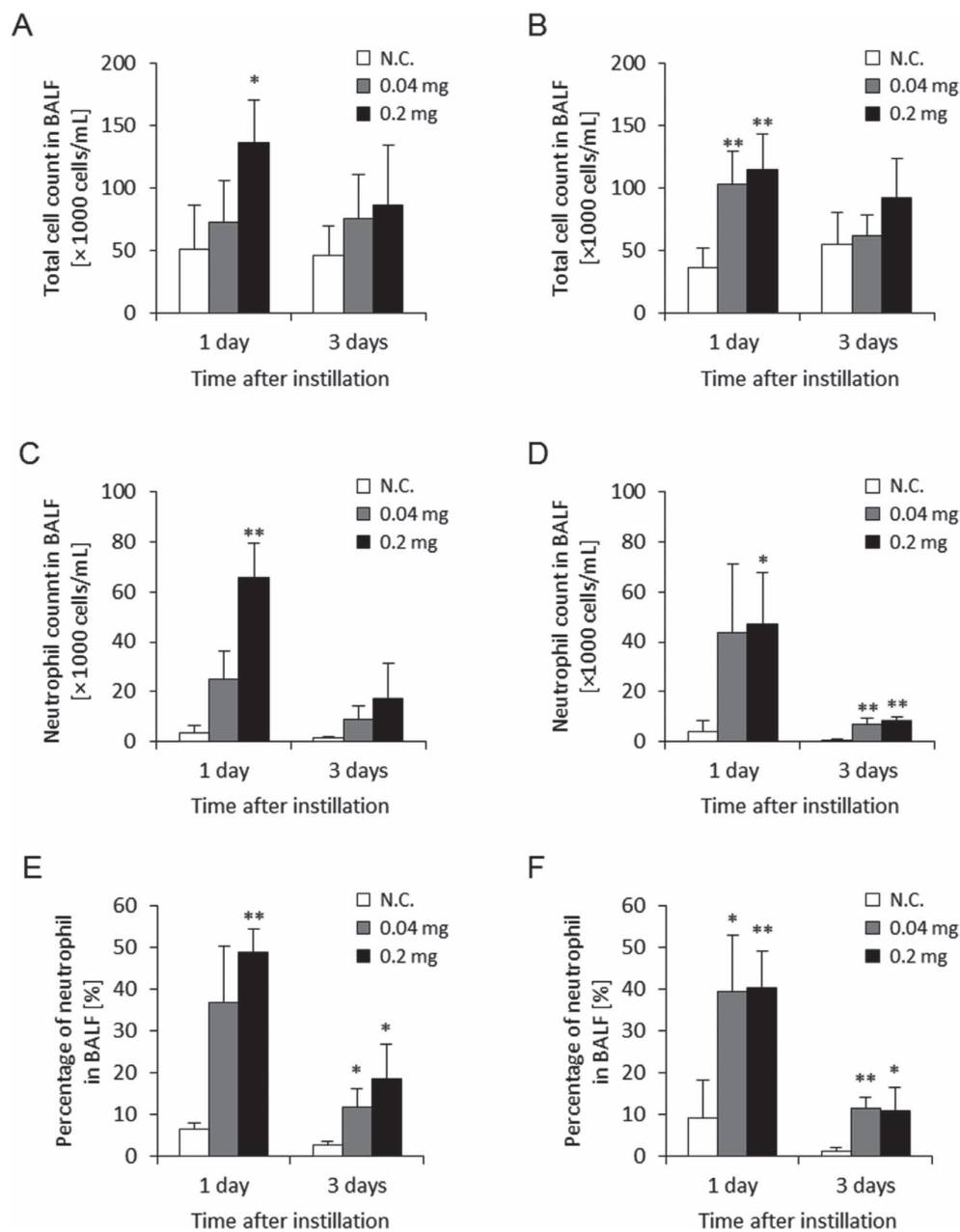


Fig. 2. Cell analysis of bronchoalveolar lavage fluid (BALF) with two delivery devices. (A) Total cell count in BALF using gavage needle, (B) Total cell count in BALF using microspray aerosolizer, (C) Neutrophil count in BALF using gavage needle, (D) Neutrophil count in BALF using microspray aerosolizer, (E) Neutrophil percentage in BALF using gavage needle, (F) Neutrophil percentage in BALF using microspray aerosolizer. No difference between delivery devices was observed in neutrophil influx in the lung. (N.C.; Negative control, * $p < 0.05$, ** $p < 0.01$)

mean and the geometric SD were $2.16 \mu\text{m}$ and 4.2, respectively. The geometric mean diameter and the geometric SD were 52 nm and 1.5, respectively.

Animals

Male Fischer 344 rats (10 wk old) were purchased from

Charles River Laboratories International, Inc. (Japan). The animals were kept in the Laboratory Animal Research Center of the University of Occupational and Environmental Health for two wk with access to free-feeding of commercial diet and water. All procedures and animal handling were done in accordance with the guidelines described 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.

Intratracheal instillation of nanomaterial

The MWCNTs were suspended in 0.4 ml distilled water, and 0.04 mg (0.16 mg/kg) or 0.2 mg (0.8 mg/kg) of MWCNTs was administered to rats (12 wk old) by a single intratracheal instillation. Negative control groups received distilled water including 10% BSA. Three methods of intratracheal instillation were used after anesthetization by isoflurane inhalation: 1) Rats maintained a standing posture on a board and were exposed to MWCNTs by a gavage needle inserted into the tracheal lumen; 2) Rats were kept in a supine posture angled approximately 45 degrees to a board, and were exposed to MWCNTs by a microspray aerosolizer inserted into the tracheal lumen; and 3) Rats maintained a standing posture on a board and were exposed to MWCNTs by a microspray aerosolizer inserted into the tracheal lumen.

Animals (5 rats in each group) were dissected at 1 d and 3 d after the instillation.

There were 5 rats each in the control, low dose, and high dose groups at each time course. The right lungs were inflated with total 20 ml physiological saline under a pressure of 20 cm water, and BALF was collected and divided into two to three times. Between 15 and 18 ml of BALF was collected in collection tubes by free fall. The histopathological evaluation was performed with the left lung inflated and fixed by 10% formalin solution at 25 cm H₂O pressure.

Analysis of inflammatory cells in BALF with cytopsin

From 10 to 13 ml of BALF from the first subgroups was centrifuged at 400 g at 4°C for 15 min. The supernatant was transferred to a new tube and used for measuring the cytokines in the BALF. The pellets were washed by suspension with polymorphonuclear leukocyte (PMN) Buffer (137.9 mM NaCl, 2.7 mM KCl, 8.2 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 5.6 mM C₆H₁₂O₆) and centrifuged at 400 g at 4°C for 15 min. After the supernatant was removed, the pellets were resuspended with 1 ml of PMN Buffer. The cell number in the BALF was counted by Celltac (Nihon Kohden Corp., Tokyo, Japan), and cells were splashed on a slide glass using cytopsin. After the cells were fixed and stained with Diff-Quik (SYSMEX Corp., Hyogo, Japan), the number of neutrophils were counted by microscopic observation.

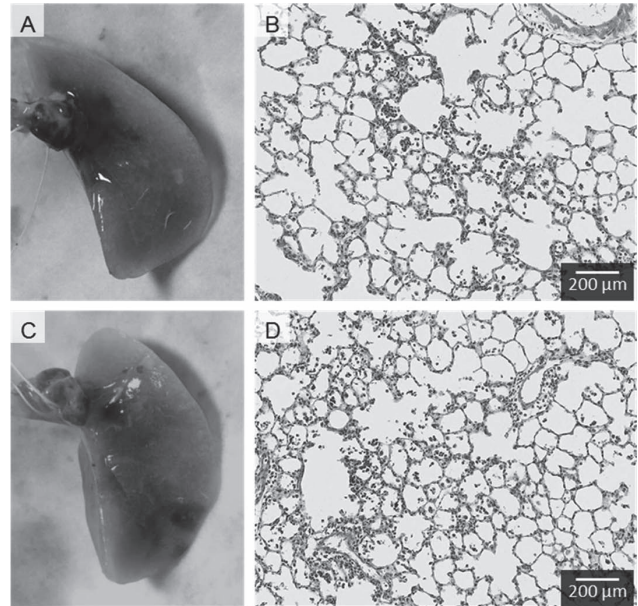


Fig. 3. Representative photomicrographs of H&E-stained lung tissue in rat. (A) Macroscopic finding of lung tissue following intratracheal instillation of MWCNT using gavage needle in posture angled approximately 45 degrees, (B) Lung tissue following intratracheal instillation of MWCNT using gavage needle, (C) Macroscopic finding of lung tissue following intratracheal instillation of MWCNT using microspray aerosolizer in posture angled approximately 45 degrees, (D) Lung tissue following intratracheal instillation of MWCNT using microspray aerosolizer. Distribution of infiltration of inflammatory cells in the lung were mainly in the centrilobular lesions with both gavage needle and microspray aerosolizer.

Parameter in BALF

The concentration of Rat cytokine-induced neutrophil chemoattractant (CINC) -1 in the BALF was measured by ELISA kits, #RCN100 (R&D Systems, Minneapolis, MN). The concentration of total protein was measured using Pierce TM 660 nm Protein Assay Reagent (22660, Thermo Fisher Scientific K.K. Yokohama, Japan) by the colorimetric determination method. The concentration of rat albumin was measured by Albumin Rat ELISA Quantitation Set (E110-125) and ELISA Starter Accessory Package Kit I (E101) (Bethyl Laboratories, Inc. Montgomery, TX).

Histopathology

The lung tissue, which was inflated and fixed with a 10% formalin solution under a pressure of 25 cm water, was embedded in paraffin, and 4 μm-thick sections were cut from the lobe, then stained with hematoxylin and eosin.

Statistical analysis

Analysis of variance (ANOVA) and Dunnett's test were

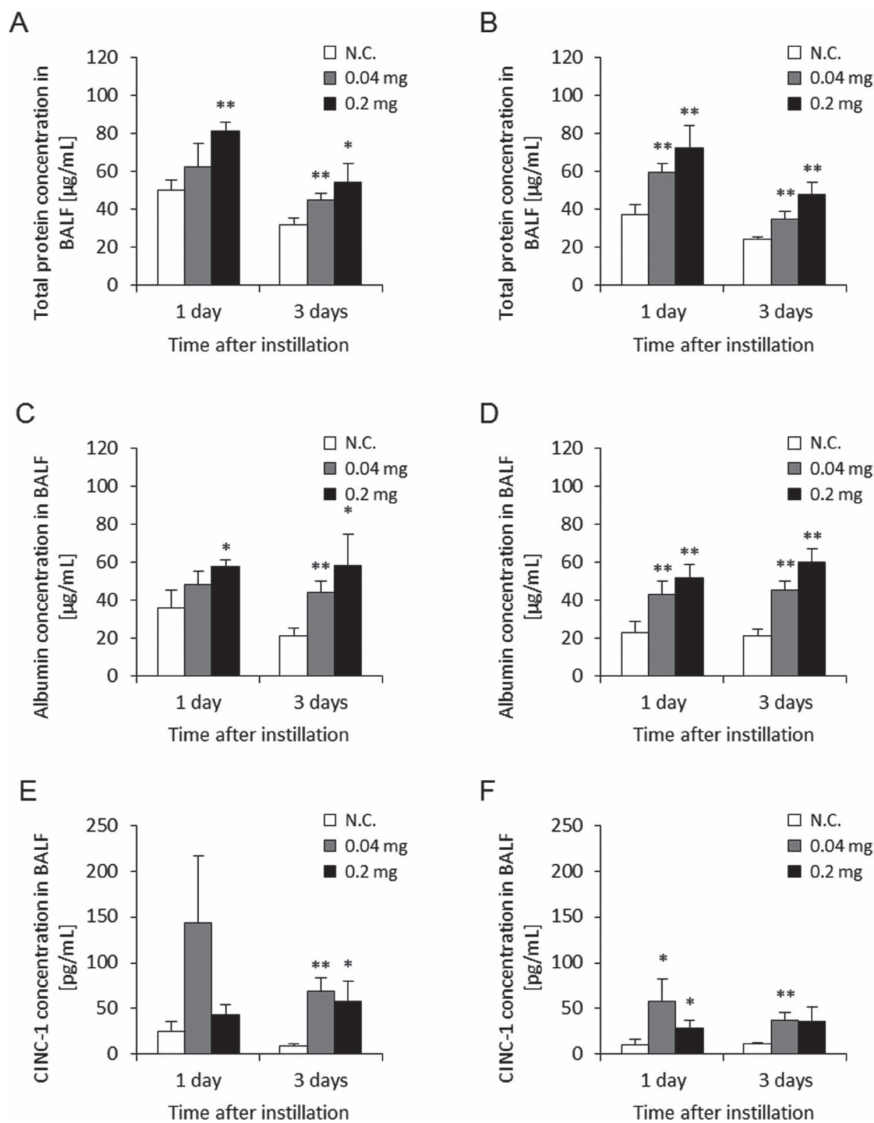


Fig. 4. Concentration of parameter in BALF with two delivery devices. (A) Concentration of albumin in BALF using gavage needle, (B) Concentration of albumin in BALF using microspray aerosolizer, (C) Concentration of protein in BALF using gavage needle, (D) Concentration of protein in BALF using microspray aerosolizer, (E) Concentration of CINC-1 in BALF using gavage needle, (F) Concentration of CINC-1 in BALF using microspray aerosolizer. No difference was observed between delivery devices for these factors. (N.C.; Negative control, * $p < 0.05$, ** $p < 0.01$)

applied where appropriate to determine individual differences using a computer statistical package (SPSS, SPSS Inc., Chicago, IL, U.S.A.).

Results

1) Comparison of pulmonary inflammation and delivery devices

Figure 2 shows the total cell count, and the neutrophil count and percentage of neutrophils in BALF in the two

approaches of gavage needle and microsyringe aerosolizer. These three parameters in BALF using both approaches increased somewhat in the 0.04 mg and 0.2 mg/rat at 1 d and 3 d postexposure. There were no persistent differences in these data between the two approaches during the observation periods. The level of these parameters at 1 d was higher than that at 3 d, but considering that MWCNTs have a high biopersistence, these phenomenon may be due to the addition of the bolus shot.

Both approaches show infiltration of inflammatory cells

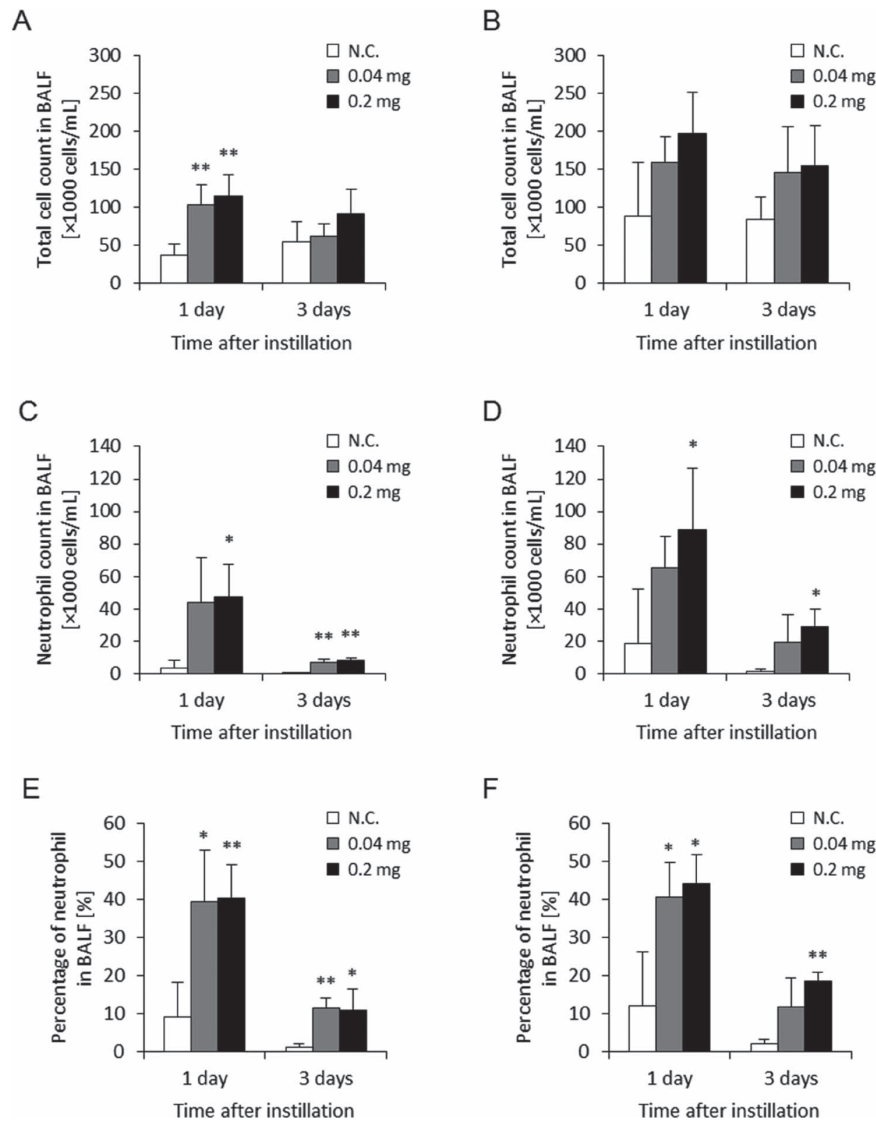


Fig. 5. Cell analysis in bronchoalveolar lavage fluid (BALF) in intratracheal instillation using microspray aerosolizer with two types of posture. (A) Total cell count in BALF in posture angled approximately 45 degrees, (B) Total cell count in BALF in vertical posture, (C) Neutrophil count in BALF in posture angled approximately 45 degrees, (D) Neutrophil count in BALF in vertical posture, (E) Neutrophil percentage in BALF in angled posture, (F) Neutrophil percentage in BALF in vertical posture. No difference was observed in neutrophil influx in the lung in the two types of posture. (N.C.; Negative control, * $p < 0.05$, ** $p < 0.01$)

such as macrophages and neutrophils in the centrilobular spaces, which are neighboring alveolus lesions around the peripheral respiratory tract (Fig. 3). No pathological features such as severe fibrosis, emphysematous changes, or granulomatous changes were observed.

Figure 4 shows the albumin, protein, and CINC-1 concentrations in BALF by the two approaches. Albumin, protein, and CINC-1 are makers of alveolar-capillary permeability, lung injury and chemokine for neutrophil. Albumin and protein in the BALF was dose-dependently higher in

the groups exposed to MWCNT at 1 d and 3 d post-exposure in both approaches. The concentration of CINC-1 in the BALF was higher in the 0.04 mg and 0.2 mg MWCNT-exposed groups during the observation period in both approaches. There were no persistent differences in these data between the two approaches during the observation periods.

2) Comparison of posture and pulmonary inflammation

Figure 5 shows the cell analysis in BALF in the pos-

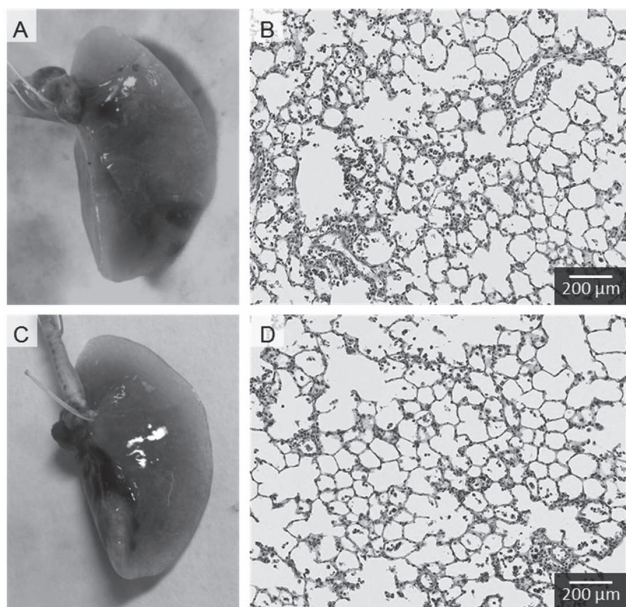


Fig. 6. Representative photomicrographs of H&E-stained lung tissue in rat. (A) Macroscopic finding of lung tissue following intratracheal instillation of MWCNT in posture angled approximately 45 degrees. (B) Lung tissue following intratracheal instillation of MWCNT in posture angled approximately 45 degrees. (C) Macroscopic finding of lung tissue following intratracheal instillation of MWCNT in vertical posture. (D) Lung tissue following intratracheal instillation of MWCNT in vertical posture. Distribution of infiltration of inflammatory cells in the lung were mainly in centrilobular lesions in both of 2 types of posture.

ture angled approximately 45 degrees and in the standing posture using the microsyringe aerosolizer. The three parameters in the BALF were higher in the two posture approaches in the 0.04 mg and 0.2 mg/rat at 1 d and 3 d post-exposure, but not significantly. There were no persistent differences in these data between the two postures during the observation periods.

Figure 6 shows pathological features in the two posture approaches. Both postures show infiltration of inflammatory cells in the centrilobular spaces in the lung. No pathological features such as severe fibrosis, emphysematous changes, or granulomatous changes were observed. The photograph shows that in both approaches the distribution shifted to the distal sites and close to the pleura, although the distribution shifted more into the lower area than the other areas.

Figure 7 shows the albumin, protein, and CINC-1 concentrations in the BALF in both approaches. The concentration of albumin and protein in the BALF in the MWCNT-exposed groups at 1 d and 3 d post-exposure was dose-dependently higher in both posture approaches, although not significantly. A higher concentration of

CINC-1 in the BALF was observed in the 0.04 mg and 0.2 mg MWCNT-exposed groups during the observation period in both posture approaches. There were no persistent differences in these data between the two postures during the observation periods.

Discussion

We conducted an intratracheal instillation study of MWCNTs using different delivery devices and postures and examined pulmonary inflammation in order to examine whether intratracheal instillation can be useful for the screening estimation of the pulmonary toxicity of nanomaterials.

There was no difference between the delivery devices, a gavage needle and a microsyringe aerosolizer, in the neutrophil influx in BALF induced by the MWCNTs. MWCNTs have a potential for high inflammation, and there are many reports^{13–15} that exposure to MWCNTs induced neutrophil inflammation in not only inhalation but also in intratracheal instillation studies, and that inhalation of MWCNTs induced persistent pulmonary inflammation and fibrosis^{14, 16, 17}. It has also been reported that intratracheal instillation of 1.5 mg/kg of short MWCNTs induced neutrophil inflammation in rat lung in the acute phase¹⁸. We determined 0.2 mg/rat as the maximum dose of MWCNTs because this is the maximum dose at which well-dispersed MWCNTs are kept in suspension. Silva *et al.*¹⁵ conducted an intratracheal instillation study at a dose of 0.2 mg of three types of MWCNTs (original, purified, carboxylic acid functionalized), and, according to the figures in their paper, the value of the PMNs in the BALF was approximately the same as those in the present study.

Considering that MWCNTs demonstrate inflammogenicity and fibrogenicity in animal studies^{2, 16–18}, it was expected that the PMN influx in the lung in both of the approaches in the present study would persist after 3 d post-exposure, but a decrease of PMN influx was observed. Biphasic pulmonary inflammation, acute and chronic inflammation, was observed in the lung following intratracheal instillation of MWCNTs in our previous study¹³, and, although titanium dioxide is not a fibrous material, Kobayashi *et al.*⁷ showed that the difference between a gavage needle and a microsyringe aerolyzer did not yield a difference in lung inflammation and injury following intratracheal instillation of titanium dioxide nanoparticles. Taken together, the two delivery devices might not cause a difference in pulmonary inflammation, but attention should be paid to the obstruction of the suspension in the micro-

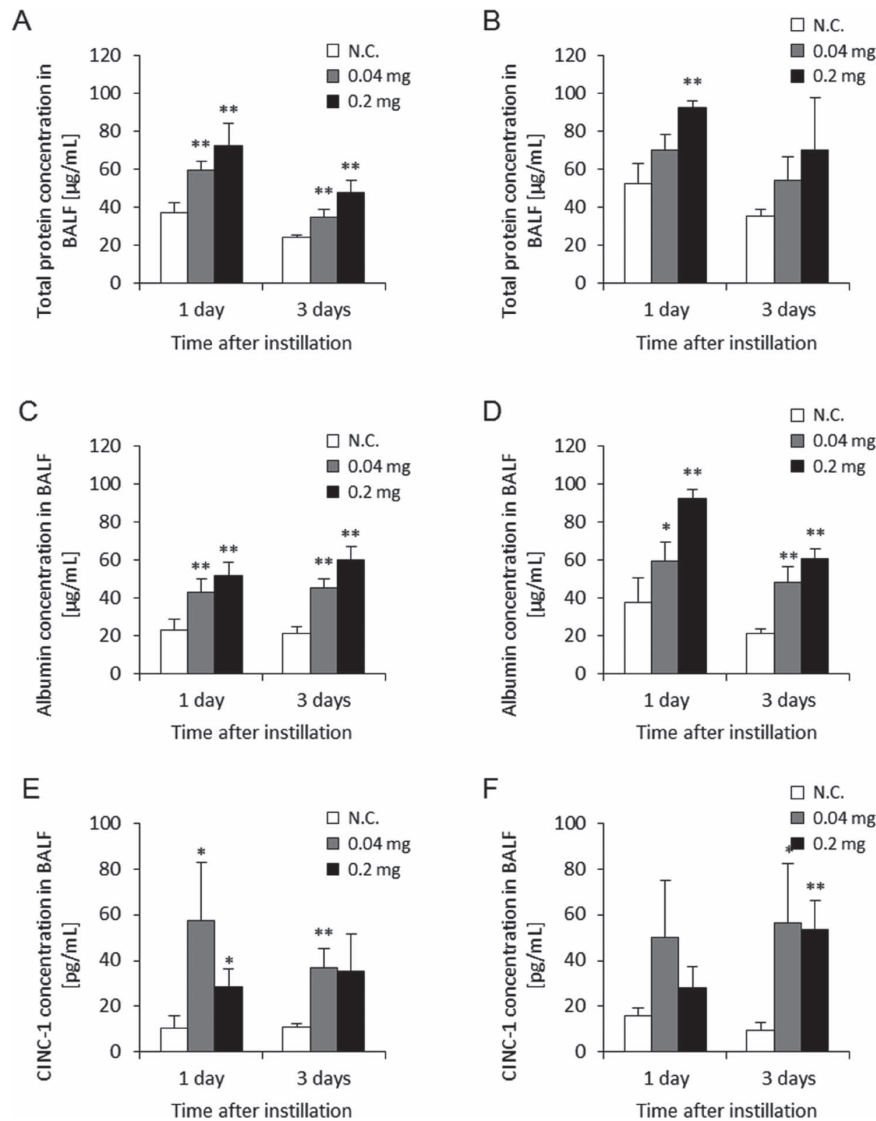


Fig. 7. Concentration of parameters in BALF in intratracheal instillation using microspray aerosolizer with two types of posture. (A) Concentration of albumin in BALF in posture angled approximately 45 degrees. (B) Concentration of albumin in BALF in vertical posture. (C) Concentration of protein in BALF in posture angled approximately 45 degrees. (D) Concentration of protein in BALF in vertical posture. (E) Concentration of CINC-1 in BALF in posture angled approximately 45 degrees. (F) Concentration of CINC-1 in BALF in vertical posture. No difference was observed in these factors between the two types of posture. (N.C.; Negative control, * $p < 0.05$, ** $p < 0.01$)

spray aerosolizer depending on the physicochemical properties of nanomaterials, such as agglomeration (data not shown).

Although it is an indirect sign of distribution of MWCNTs, we think that the distribution of pathological lesions in the lung was that of MWCNTs. The main distribution of inflammation in the lung induced by MWCNTs with both the gavage needle and the microspray aerosolizer was in the centrilobular spaces, which are the neigh-

boring alveolus lesions around the peripheral respiratory tract, and the distribution patterns in both approaches was fundamentally similar. In inhalation exposure studies of respirable chemicals, the main patterns of distribution of pulmonary inflammation are a centrilobular pattern^{19, 20}. In a study by Bermudez *et al.*²⁰, the macrophage accumulation and aggregation in the centrilobular and subpleural spaces in the rodent's lung was induced by inhaled titanium dioxide nanoparticles, but, compared with intratra-

cheal instillation, some inflammatory lesions following inhalation exposure were present deep in the alveolar wall and the subpleural space. There are no studies on the effect of inhaled nanoparticles on human lung, but there are some studies of exposure to welding fumes that include metal oxide nanoparticles^{21,22}). The distribution of opacity in the computed tomography image in patients with welding lung was compatible with that of centrilobular nodules, meaning that the deposition of nanoparticles from the welding fumes may be located in the centrilobular area^{21,22}).

Although there was little difference in pathological distributions between the gavage needle and the microspray aerosolizer, the distribution of inflammation in the gavage needle type shifted slightly to the proximal site, and no difference was observed between lobes in the distribution of inflammation. With the microspray aerosolizer, on the other hand, the distribution shifted to the distal sites and close to the pleura, although the distribution of inflammatory changes shifted more into the lower lobes than the other lobes. We speculated that the strong pressure of the microspray aerosolizer may have induced the lobular difference in inflammatory changes. Fujita *et al.*¹⁸⁾ reported that MWCNTs following intratracheal instillation by a microspray aerosolizer penetrated into the pleura, and Xu *et al.*²³⁾ reported that MWCNTs were observed in the pleural cavity in the acute phase following intratracheal instillation of MWCNTs with a microspray aerosolizer.

Intratracheal instillation of MWCNTs in the posture angled approximately 45 degrees and in the standing posture induced neutrophil influx in BALF, but the difference in posture did not change the degree of neutrophil influx. The main distribution of inflammation in the lung induced by intratracheal instillation of MWCNTs in the 45 degree angled posture and in the standing posture was in the centrilobular spaces, but there was no difference in pulmonary inflammation in the rat lung in the pathological findings. Taken together, a difference of posture at this level can be ignored when we estimate the pulmonary toxicity of nanomaterials. Hasegawa-Baba *et al.*²⁴⁾ reported that the distribution of india ink in lung in the 45 degree angled posture were more dispersible than that in the standing posture in intratracheal instillation studies. There are a different tendency of distribution of materials between our and their studies, suggesting that the difference in viscosity liquid between india ink and MWCNT suspensions may connect with difference of distribution in the lung.

There may be difference between lobes in the amount of deposited nanomaterials using either posture for the intratracheal instillation of nanomaterials. Costa *et al.*²⁵⁾

performed an intratracheal instillation of oil combustion particles and found greater amounts of deposition in the inferior lobe than in the superior lobe. Although we did not measure the deposition of MWCNTs in the present study, we can speculate this tendency for downward deposition from the photography of the lung after using the microspray aerosolizer. Costa *et al.*²⁵⁾ also performed an inhalation study of oil combustion particles and found a predominance of downward deposition. These results indicate that the difference in posture can be ignored.

In summary, we performed an intratracheal instillation study of MWCNTs for rat using different cannulas and postures as a basic study. The difference in delivery devices (gavage needle and microsprayer aerosolizer) did not result in a change of level and distribution of pulmonary inflammation in rat lung, and neither did the difference in posture. Taken together, intratracheal instillation studies can assess the acute pulmonary toxicity of nanomaterials within this condition.

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