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Translocation of Intratracheally Instilled Multiwall Carbon Nanotubes to Lung-Associated Lymph Nodes in Rats

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Abstract: In order to assess the extrapulmonary effects of multiwall carbon nanotubes (MWCNT), deposition of MWCNT and histopathologic changes in lung-associated lymph nodes (LALN) were examined in MWCNT-administered rats. At the age of 13 wk, male F344 rats were intratracheally instilled with MWCNT at a dose of 0 (vehicle), 40 or 160 μg/rat. The rats were sacrificed on Day 1, 7, 28 or 91 after instillation and light microscopic examinations were performed on LALN tissues. MWCNT was translocated to right and left posterior mediastinal lymph nodes and parathymic lymph nodes. Deposition of MWCNT was greater in the posterior mediastinal lymph node than in the parathymic lymph node, and the amount of MWCNT deposited in these two lymph nodes increased gradually and dose-dependently with time. MWCNT was phagocytosed by nodal macrophages, and some of the MWCNT-laden macrophages were aggregated. Transmission electron microscopic (TEM) observation confirmed the presence of MWCNT fibers with a characteristic multi-walled cylindrical structure.

Key words: Multiwall carbon nanotube, Lung-associated lymph node, Mediastinal lymph node, Macrophages, Granuloma

Rapid development of the carbon nanotube (CNT) industry and extensive industrial applications of CNTs in various sectors of industry have raised serious concerns over health risks of workers exposed to CNTs. Neither epidemiological nor medical case studies have been reported on health outcomes of CNT-exposed workers. Recent *in vivo* toxicity studies have shown induction of mesotheliomas after intraperitoneal injection of multiwall carbon nanotube (MWCNT) in *p*53 gene-deficient mice[1)] and asbestos-like pathogenicity after intraperitoneal injection of MWCNT in female mice[2)]. Asbestos fibers are known to cause mesotheliomas in exposed workers[3)] and animals[4)], and have been reported to migrate to the serosal tissues in insulation workers with long-term asbestos exposures[5)], and to translocate to the pleural cavity in rats administered chrysotile fibers by intratracheal instillation[6)]. We found in a previous study[7)] that intratracheal instillation of MWCNT in male rats increased the deposition of MWCNT dose- and time-dependently in the bronchus-associated lymphoid tissue (BALT) which is anatomically enclosed within the lung. However, whether or not BALT is involved in the pulmonary clearance of particles is still unclear[8)]. On the other hand, lung-associated lymph nodes (LALN) and their afferent and efferent lymphatic pathways have been described morphologically[8–10)], and are recognized as a structure important for lung defense and the sys-

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temic immune system[8]. The right and left posterior mediastinal lymph nodes and the parathymic lymph node have different lymphatic drainage pathways to clear particles from the lung[9, 10]. Recent rodent studies have addressed extrapulmonary effects of MWCNT on the systemic immune function[11, 12]. These extrapulmonary effects might be mediated in part through the translocation of MWCNT to the LALN[13], and finally to the general circulation, since pulmonary lymphatic ducts from the posterior mediastinal and parathymic lymph nodes drain particles into the blood stream at the subclavian vein[9, 10].

This short communication reports the time-course changes in the deposition of MWCNT and MWCNT-induced histopathologic changes in the right and left posterior mediastinal lymph nodes and parathymic lymph node of male rats that received intratracheal instillation of MWCNT.

Male F344/DuCrlCrlj rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan) at the age of 11 wk. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals[14], and the present study was approved by the ethics committee of the Japan Bioassay Research Center.

MWCNT was kindly supplied by MITSUI & Co. Ltd (MWNT-7, Lot No. 061220, Tokyo, Japan). The test substance was suspended in phosphate-buffered saline (PBS) containing 0.1% Tween 80 as a colloidal dispersant and subjected to ultrasonication for 20 min with an ultrasonic homogenizer (VP-30S, 20 kHz, 300 W, TAITEC Co., Ltd, Tokyo, Japan). Immediately before intratracheal instillation, the ultrasonicated suspension of MWCNT or the vehicle solution was subjected to additional ultrasonication for 30 s with a sonicator (US-2, As One Co., Ltd., Tokyo, Japan).

The suspension of MWCNT in the PBS-Tween 80 or the vehicle solution was intratracheally instilled in rats after inhalational anesthetization with isoflurane gas (Forane, Abbott Japan Co., Ltd., Tokyo, Japan). Experimental groups received MWCNT at a dose of 0 (vehicle), 40 or 160 μg/rat, as described in our previous study[7]. MWCNT- and vehicle-dosed rats were sacrificed on Day 1, 7, 28 or 91 following instillation.

Eight rats/group were sacrificed at each time point by exsanguination from the jugular vein under pentobarbital anesthesia, and the trachea was ligated. In preparation for light microscopic examination, organs and tissues were fixed by perfusion with physiological saline and subsequently 10% neutral buffered formalin. All posterior mediastinal and parathymic lymph nodes were removed together with the trachea and thymus, and embedded in paraffin. About 5 μm-thick slices of right and left posterior mediastinal and parathymic lymph nodes were sectioned, and stained with hematoxylin and eosin (H & E).

Deposition of MWCNT was semi-quantitatively evaluated for the extent of instilled MWCNT deposition in the LALN tissues. The extent of MWCNT deposition or the severity of histopathologic change was scored by microscopic observation of H & E-stained tissues, according to the following criteria. Score 1, termed "slight", indicates that slight MWCNT deposition or histopathologic change was observed in a limited part of the area. Score 2, termed "moderate", indicates that slight MWCNT deposition or histopathologic change was observed in a large part of the area or that moderate MWCNT deposition or histopathologic change was observed in a limited part of the area. Score 3, termed "marked", indicates that moderate MWCNT deposition or histopathologic change was observed in a large part of the area or that marked MWCNT deposition or histopathologic change was observed in a limited part of the area.

The right posterior mediastinal lymph node of a rat receiving MWCNT at a dose of 160 μg and sacrificed on Day 91 following instillation was examined by transmission electron microscopic (TEM) observation for MWCNT fibers (JEM-1400, JEOL, Tokyo, Japan). The lymph node was fixed with 0.074 M phosphate-buffer solution containing 2% paraformaldehyde and 1.0% glutaraldehyde and post-fixed with 1% osmium tetraoxide. Thereafter, the lymph node was dehydrated in graded ethanol, and embedded in epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate and used for the TEM observation.

Table 1 shows the deposition of MWCNT and the extent of MWCNT deposition in the right and left posterior mediastinal lymph nodes and in the parathymic lymph node as well as their MWCNT-induced histopathologic changes, i.e., aggregates of MWCNT-laden macrophages, on different days after instillation. Figure 1 also shows the time-course changes in the extent of MWCNT deposition in these lymph nodes expressed as the severity score averaged over the number of animals examined. No MWCNT fibers were found in the lymph nodes of 40 or 160 μg-dosed rats on Day 1. In the right and left posterior mediastinal lymph nodes, slight deposition of MWCNT fibers occurred on Day 7, and the incidence and severity of the MWCNT deposition increased dose- and time-dependently during the post-administration period after Day 7. In the parathymic lymph node, however, deposition of MWCNT fibers occurred only in three out of eight 160 μg-dosed rats on Day 91. Light-microscopic examination revealed

Table 1. Deposition of intratracheally instilled MWCNT and histopathologic changes in LALN at a dose of 0 (vehicle), 40 or 160 μg in the rats sacrificed on different days after instillation

Dose of MWCNT (/rat)	0 μg				40 μg				160 μg			
Days after instillation	1	7	28	91	1	7	28	91	1	7	28	91
Posterior mediastinal lymph node, right												
<No. of rats examined>	<6>	<2>	<8>	<8>	<3>	<4>	<8>	<7>	<5>	<4>	<7>	<8>
MWCNT deposition[1]	0	0	0	0	0	2	5	4	0	4	5	8
(Slight)						(2)	(5)	(2)		(4)	(3)	(0)
(Moderate)								(2)			(2)	(8)
Aggregate of MWCNT-laden macrophages	0	0	0	0	0	0	0	2	0	0	0	6
Posterior mediastinal lymph node, left												
<No. of rats examined>	<7>	<8>	<8>	<8>	<7>	<8>	<8>	<8>	<8>	<8>	<7>	<8>
MWCNT deposition	0	0	0	0	0	1	1	7	0	6	7	7
(Slight)						(1)	(1)	(4)		(6)	(1)	(2)
(Moderate)								(3)			(6)	(5)
Aggregate of MWCNT-laden macrophages	0	0	0	0	0	0	0	2	0	0	0	5
Parathymic lymph node												
<No. of rats examined>	<8>	<7>	<8>	<8>	<8>	<7>	<8>	<8>	<8>	<7>	<8>	<8>
MWCNT deposition	0	0	0	0	0	0	0	0	0	0	0	3
Slight												(1)
Moderate												(2)
Aggregate of MWCNT-laden macrophages	0	0	0	0	0	0	0	0	0	0	0	2

[1]MWCNT fibers were engulfed by macrophages.
Because of failure in sampling the right posterior mediastinal lymph node, the right side of several rats was not available for histopathological analysis.

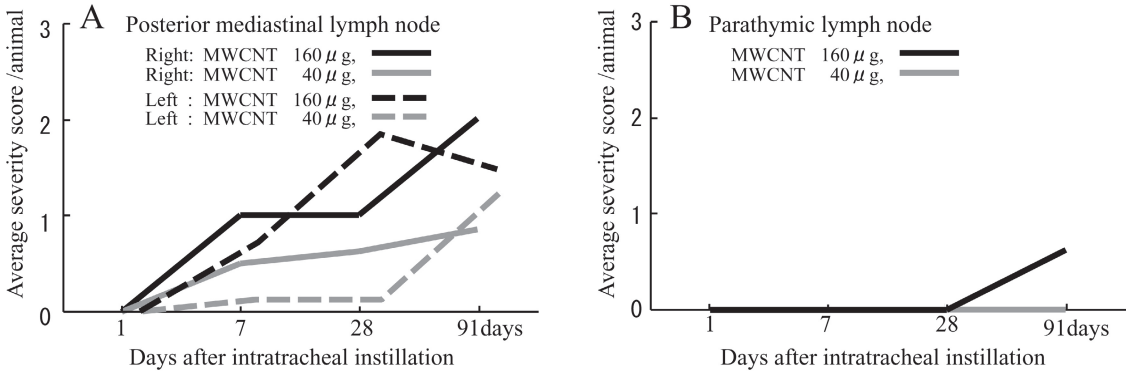

Fig. 1. Time-course changes in the extent of which MWCNT deposition in the right and left posterior mediastinal (A) and parathymic lymph (B) nodes of rats which received intratracheal instillation of MWCNT.

that macrophages were present in both the mediastinal and parathymic lymph nodes. Nodal macrophages were less abundant than alveolar macrophages in the alveolar space and wall, which we observed in the previous study[7]. As indicated by arrows in Fig. 2, dispersed MWCNT fibers engulfed by macrophages were observed in the lymph nodes, and some of the macrophages were filled with MWCNT fibers, the extent of which was scored as moderate. Small aggregates of several MWCNT-laden macrophages were focally formed on Day 91 in both the posterior mediastinal lymph nodes of 40 and 160 μg-dosed rats and in the parathymic lymph node of 160 μg-dosed rats, as indicated by the asterisks in Fig. 2. However, development of aggregated MWCNT-laden macrophages to granulomas and associated inflammation was not evident in any of the mediastinal or parathymic lymph nodes of the 40 or 160 μg-dosed rats throughout the 91-d postexposure period.

As shown in Fig. 3, TEM observation of the right

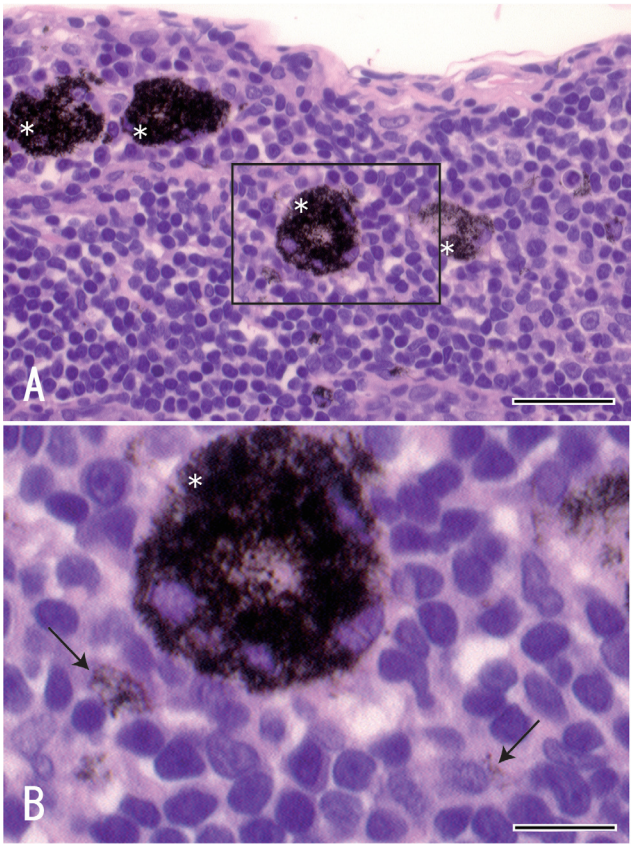

Fig. 2. Deposition of MWCNT in the right posterior mediastinal lymph node of a rat which received intratracheal instillation of MWCNT at a dose of 160 μg and was sacrificed on Day 91 after instillation.

In A, small aggregates of several MWCNT-laden macrophages are indicated by asterisks. H & E stain. Bar indicates 50 μm. In B, a magnified image of the enclosed area showen in A. Arrows indicate MWCNT fibers engulfed by macrophages. Bar indicates 17 μm.

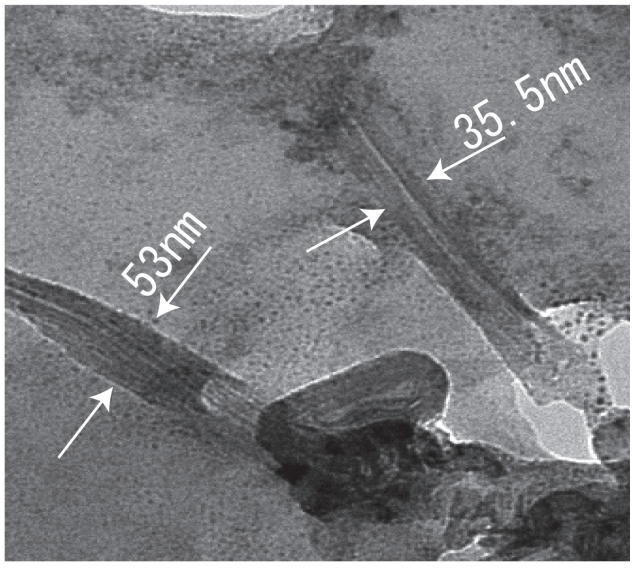

Fig. 3. A TEM image of MWCNT in the right posterior mediastinal lymph node of a rat which received intratracheal instillation of MWCNT at a dose of 160 μg and was sacrificed on Day 91 after instillation.

posterior mediastinal lymph node revealed that fibers having multi-layered cylindrical structures of 35.5 or 53 nm in width, which were characteristic of MWCNT morphology, were present in the cytoplasm of nodal macrophages.

In the present study, deposition of intratracheally instilled MWCNT in both the right and left posterior mediastinal lymph nodes of rats was found to increase gradually and dose-dependently during the 91-d postexposure period, and MWCNT fibers focally formed small aggregates of several MWCNT-laden macrophages only on Day 91 after instillation. Ma-Hock *et al.*[13] reported deposition of MWCNT in the mediastinal lymph node of rats exposed by inhalation to MWCNT aerosol. The translocation of MWCNT to the mediastinal lymph node found in the present study is comparable with the findings of Ma-Hock *et al.*[13] that 3-month inhalation exposure of rats to MWCNT aerosol at 0.1–2.5 mg/m^3 induced granulomatous inflammation and lymphoreticulocellular hyperplasia in the mediastinal lymph node as well as particle-laden macrophages. The principal difference in the character of the MWCNT-induced lesions in the mediastinal lymph node between the two studies is the slight formation of aggregated, MWCNT-laden macrophages in the present study, as compared with induction of granulomas and associated inflammation reported by Ma-Hock *et al*[13]. This difference might be due to the difference in amount of pulmonary deposition of MWCNT. Ma-Hock *et al.*[13] estimated that 46.8, 243 and 1,170 μg of MWCNT per lung were deposited in the lung after 90-d inhalation exposure to 0.1, 0.5 and 2.5 mg/m^3, respectively, while 40 and 160 μg per lung were intratracheally instilled in the present study. These values indicate that amount of MWCNT depositing in the pulmonary region was much greater in the study of Ma-Hock *et al.*[13] than in the present study. The small aggregates of several MWCNT-laden macrophages in the LALN were much smaller than the microgranulomas found in a previous study, which had diameters up to 150 μm in the alveolar wall and were composed of the MWCNT-laden alveolar macrophages[7]. It can be inferred, therefore, that small aggregations of MWCNT-laden macrophages progress to from microgranulomas and associated inflammation in the LALN when the dose level of MWCNT is increased.

TEM observation confirmed that in the cytoplasm of MWCNT-laden, nodal macrophages there were fibers

having multi-layered cylindrical structures of 35.5 or 53 nm in width, which were characteristic of MWCNT morphology[12, 15].

Increased deposition of MWCNT in the parathymic lymph node was seen in only three 160 μg-dosed rats on Day 91, together with slight or moderate formation of aggregated MWCNT-laden macrophages. The MWCNT deposition and aggregates of MWCNT-laden macrophages were much milder in the parathymic lymph node than in the posterior mediastinal lymph node. The present finding of MWCNT deposition in the LALN suggests that the right and left posterior mediastinal lymph nodes receive MWCNT from the alveolar space and interstitium through a lymphatic drainage pathway, while a small fraction of MWCNT is translocated to the parathymic lymph node through different drainage pathways. Morrow[10] argued that there are two different lymphatic pathways for pulmonary dust clearance, i.e., deep-set and pleural drainage pathways, and that the pleural lymphatics differ topographically from the deep-set in that the pleural pathway follows the surface of the lung segments and lobes to the hilar region. Therefore, it is likely that the intratracheally instilled MWCNT fibers depositing on the alveolar wall would migrate from the alveolar interstitium through the pleural lymphatic pathway into the posterior mediastinal lymph node and to a lesser extent into the parathymic lymph node, and finally into the blood stream at the subclavian vein[9, 10], thereby reaching the reticuloendothelial organs such as spleen and liver.

The LALN such as the posterior mediastinal lymph node, are recognized as a structure important to functioning of the systemic immune system[8]. Notably, Mitchell *et al.*[11] reported that the systemic immune function is suppressed by repeated inhalation exposure of male mice to MWCNT aerosol at 0.3–5 mg/m^3, and hypothesized that an extrapulmonary mechanism, transforming growth factor-β released from the MWCNT-laden alveolar macrophages in the lung, activates the cyclooxygenase pathway in the spleen, ultimately causing T-cell dysfunction and altered systemic immunity. However, Mitchell *et al.*[12] argued that the translocation of MWCNT from the lung to the spleen through circulation was unlikely, since no sign of foreign material was detected in the spleen following inhalation exposure. Further studies will be needed to examine whether or not MWCNT-induced nodal lesions varying from the small aggregates of several MWCNT-laden macrophages found in the present study to the reported formation of granulomas and associated inflammation found by Ma-Hock *et al.*[13], damages the posterior mediastinal lymph node to the extent that the systemic immune function is suppressed.

In conclusion, MWCNT, intratracheally instilled in male F344 rats, was found to migrate to the right and left posterior mediastinal lymph nodes and the parathymic lymph node. The deposition of MWCNT in these lymph nodes gradually dose-dependently increased during the postexposure period, and culminating in the formation of small aggregates of MWCNT-laden macrophages on Day 91, which possibly progress to form microgranulomas. TEM observation confirmed presence of MWCNT fibers in the nodal macrophage, which were characterized by a multi-walled cylindrical structure.

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