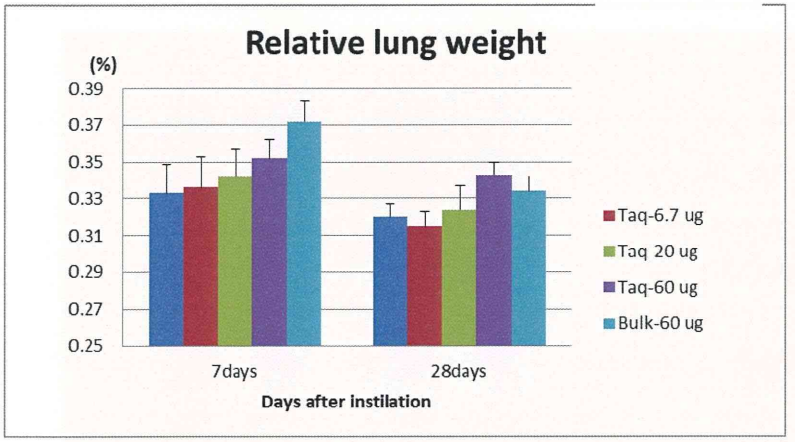


Control　T-CNT 6.7μg　T-CNT 20μg
T-CNT 60μg　Bulk-CNT 60μg

図 37　T-CNT 単回気管内投与ラットの肺比重量

C-1-（4）肺組織内侵入後の T-CNT の遠隔臓器への移行調査

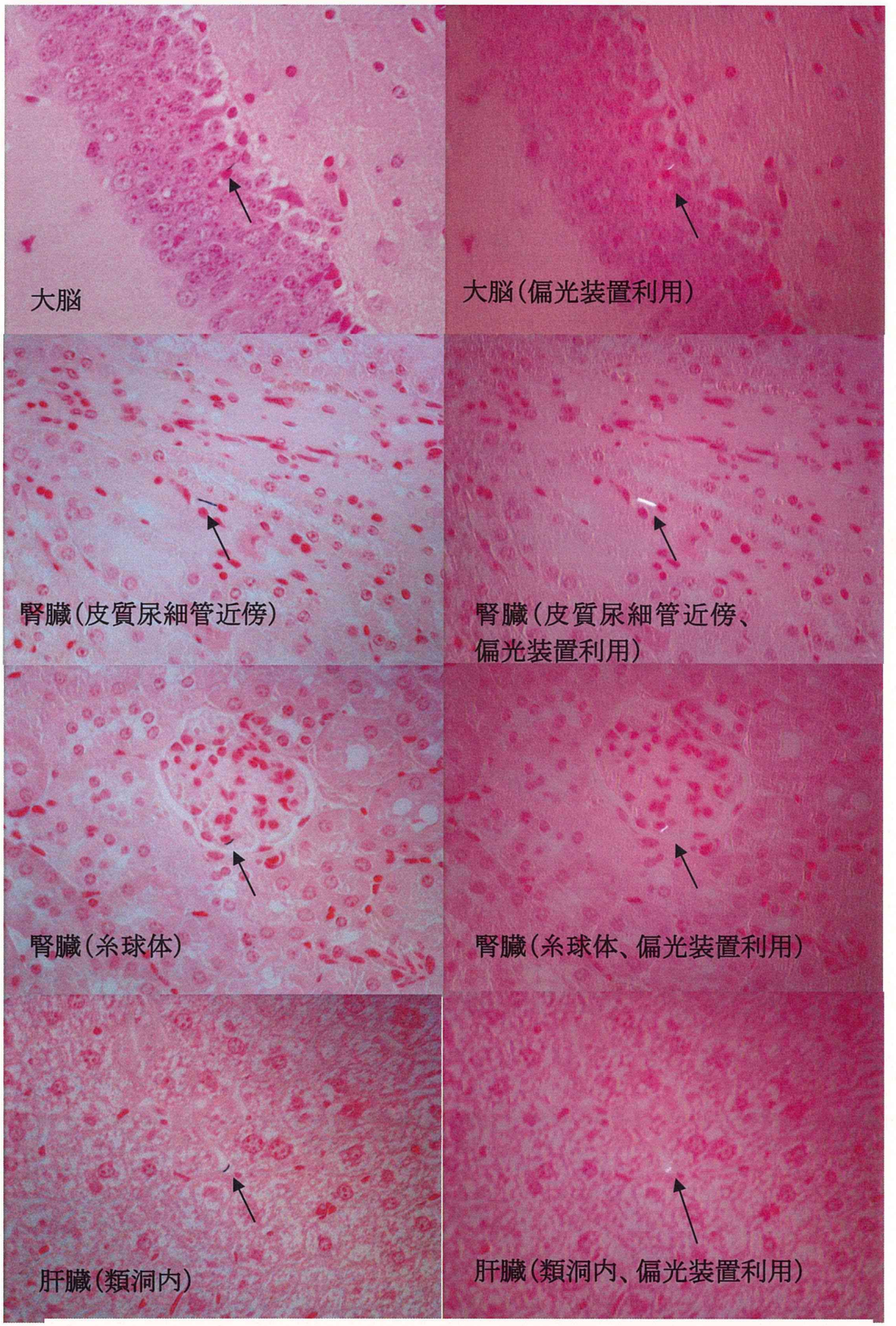
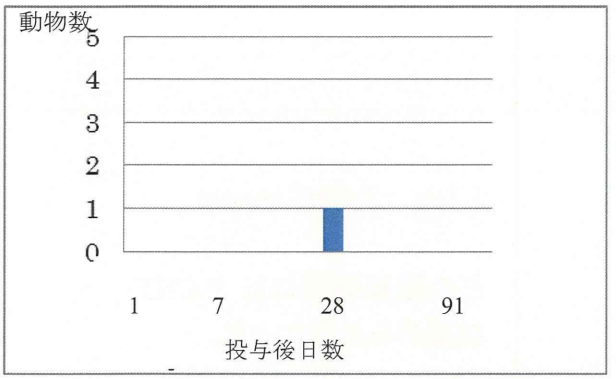
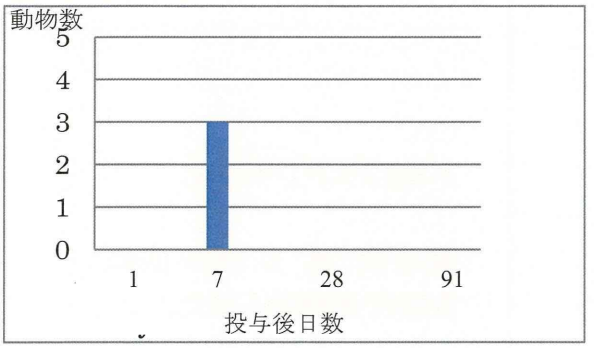


図 38 T-CNT10μg 気管内投与後 91 日のマウスの脳、腎臓及び肝臓で認められた T-CNT



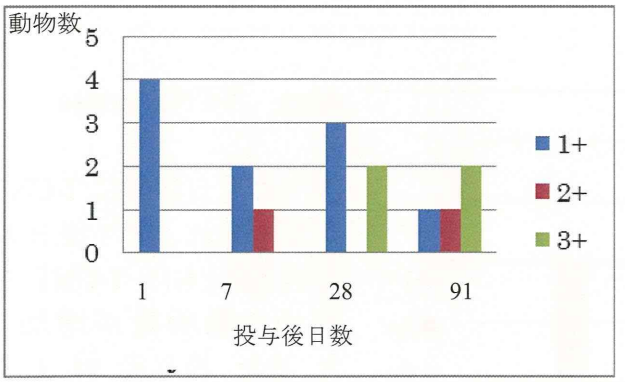
1.1μg　T-CNT /mouse

投与後 28 日に 1 匹に T-CNT が認められた。



3.3μg　T-CNT /mouse

投与後 7 日に 1 匹に T-CNT が認められた。

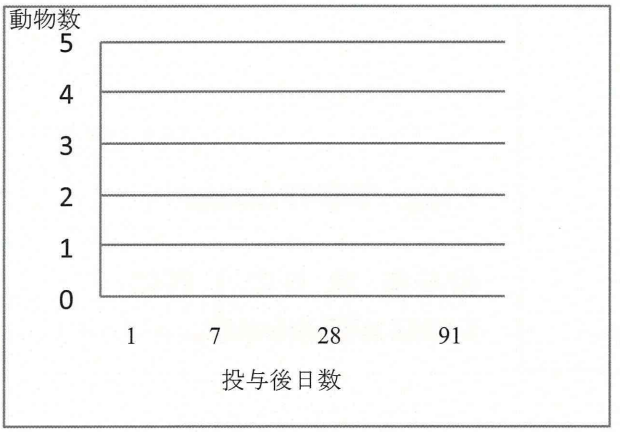


10μg　T-CNT/mouse

投与後 1 日から T-CNT が認められ、投与後日数の経過とともに沈着のグレードが増加した。

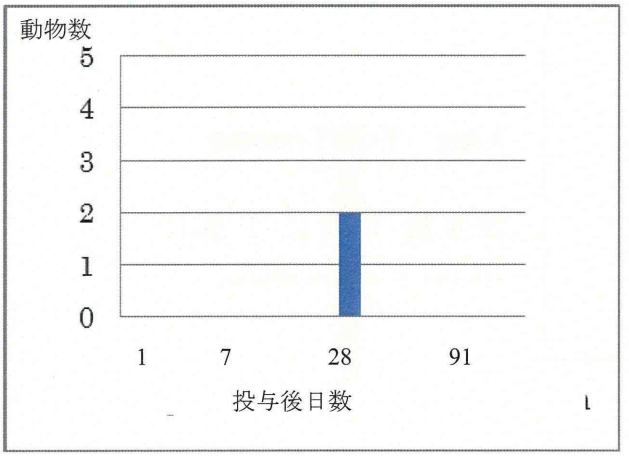
図 39 肺関連リンパ節への T-CNT の移行

1+: 1-10 Spots of T-CNT
2+: 11-20 Spots of T-CNT
3+: 21-50 Spots of T-CNT



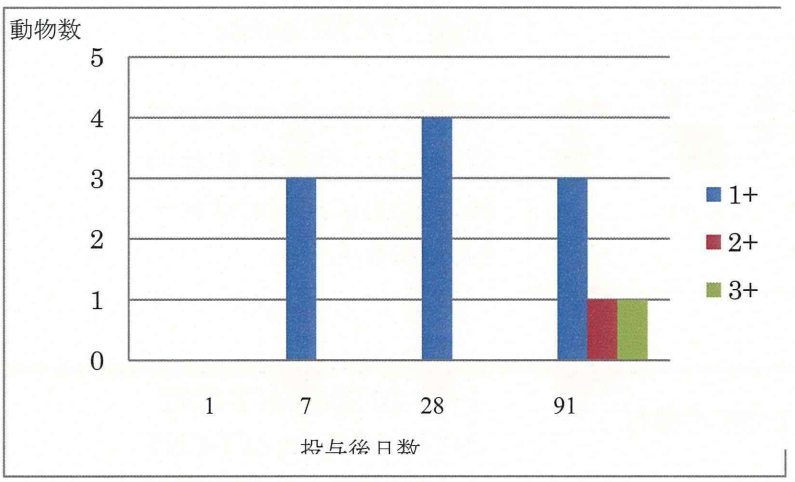
1.1μg　T-CNT/mouse

どの観察時期にも T-CNT は認められなかった。



3.3μg T-CNT/mouse

投与後 28 日で 2 匹に T-CNT が認められた。

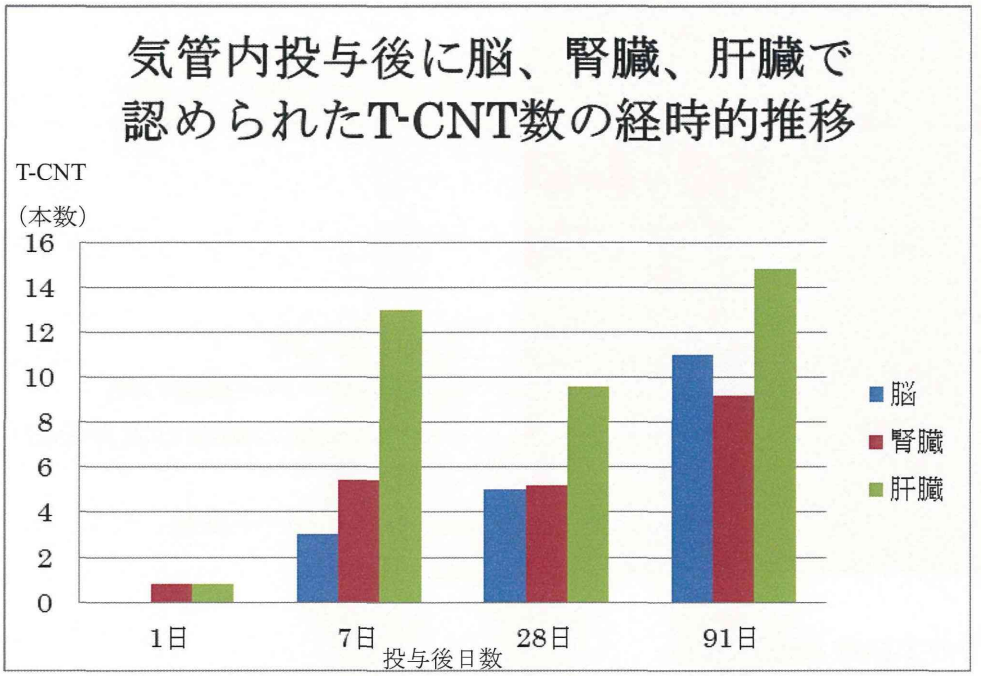


10μg　T-CNT/mouse

投与後 7 日以降に T-CNT が認められ、投与後日数の経過とともに T-CNT を認めた動物数が増加した。また、投与後 91 日では沈着のグレードが増加した。

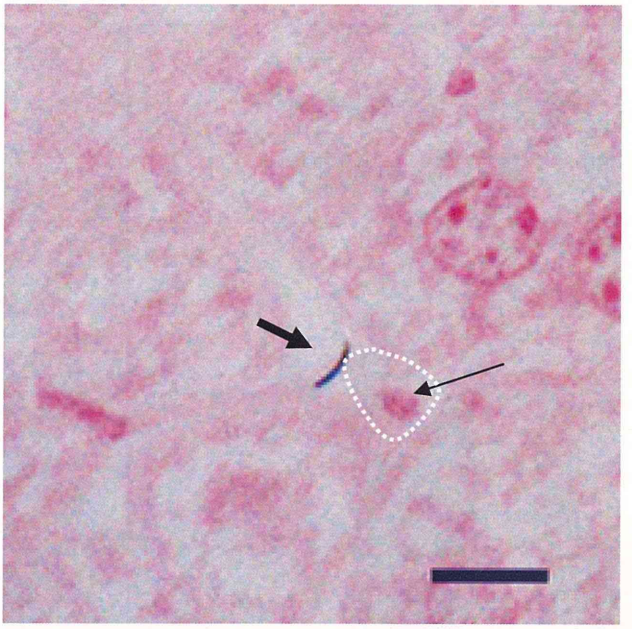
1+: 1-10 Spots of T-CNT
2+: 11-20 Spots of T-CNT
3+: 21-50 Spots of T-CNT

図 40 脾臓への T-CNT の移行



		T-CNT 数			
	投与後日数	1 日	7 日	28 日	91 日
脳	平均値	0	3	5	11
	標準偏差	0	1	3	8
腎臓	平均値	0.8	5.4	5.2	9.2
	標準偏差	0.84	3.21	2.17	4.97
肝臓	平均値	0.8	13	9.6	14.8
	標準偏差	0.84	8.06	7.02	10.57

図 41 気管内投与後に脳、腎臓、肝臓で認められた T-CNT 数の経時的推移



太矢印:T-CNT
細矢印:クーパー細胞の核
クッパー細胞の細胞質境界を白色点線で示した。
ケルンエヒトロート染色
Ber:10μm

図 42 肝蔵での T-CNT の沈着状況
T-CNT 10μg 気管内投与後 91 日
(図 11 の最下段左列の写真を拡大)

C-2.　電子顕微鏡を用いたT-CNTによる生体影響の検索

C-2-（1）　ナノマテリアルの生体影響検索に有効な TEM 検索方法の開発

H23-化学-一般-001 での技術開発が順調に進み、本研究分担「B-2-(3) 気管内投与した T-CNT の肺組織内侵入経路の解明を目的とした TEM 検索」は、その成果を利用して研究を進めた。

C-2-(2) MWCNT 原体（MWCNT-7）を気管内投与したラット肺のサンプルを用いた短期毒性病態の超微細形態学的検索

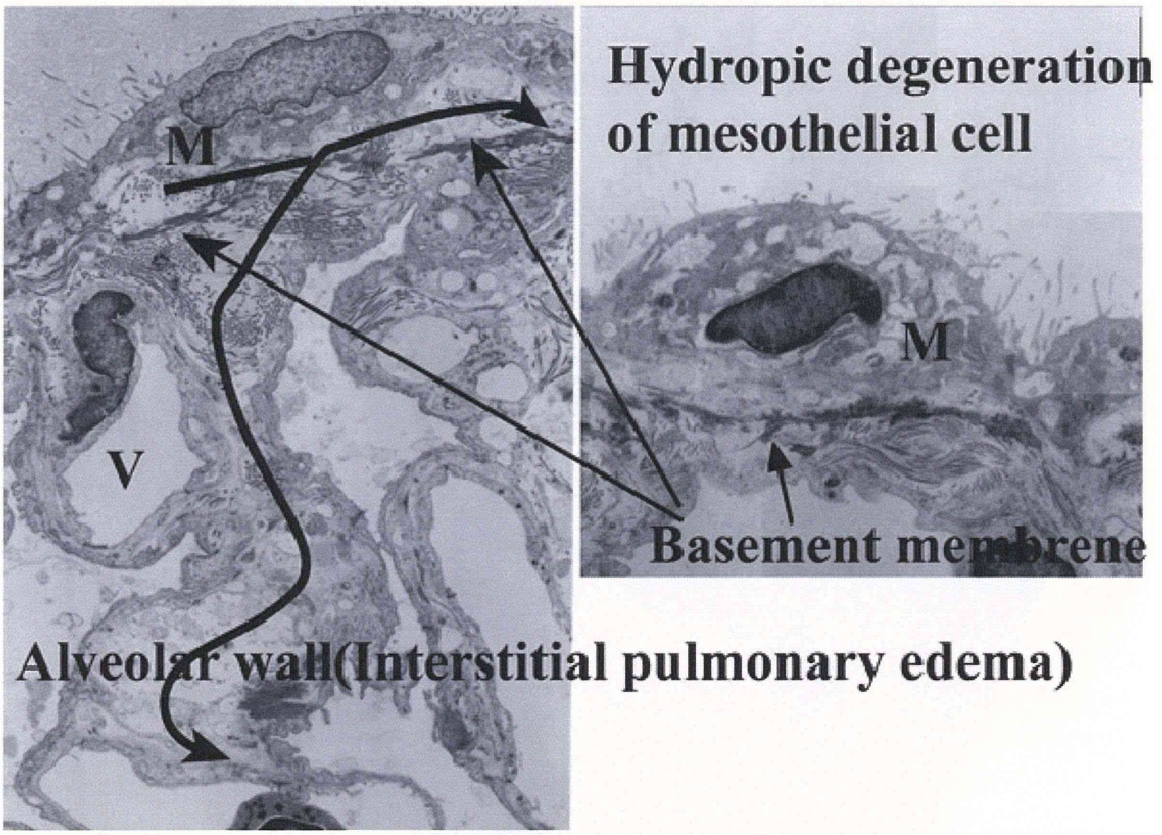
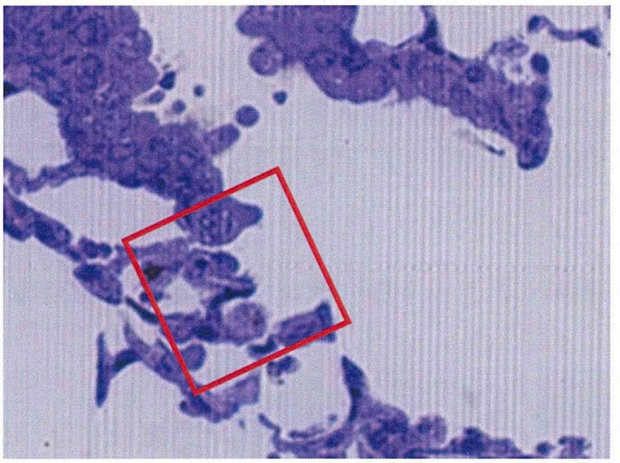


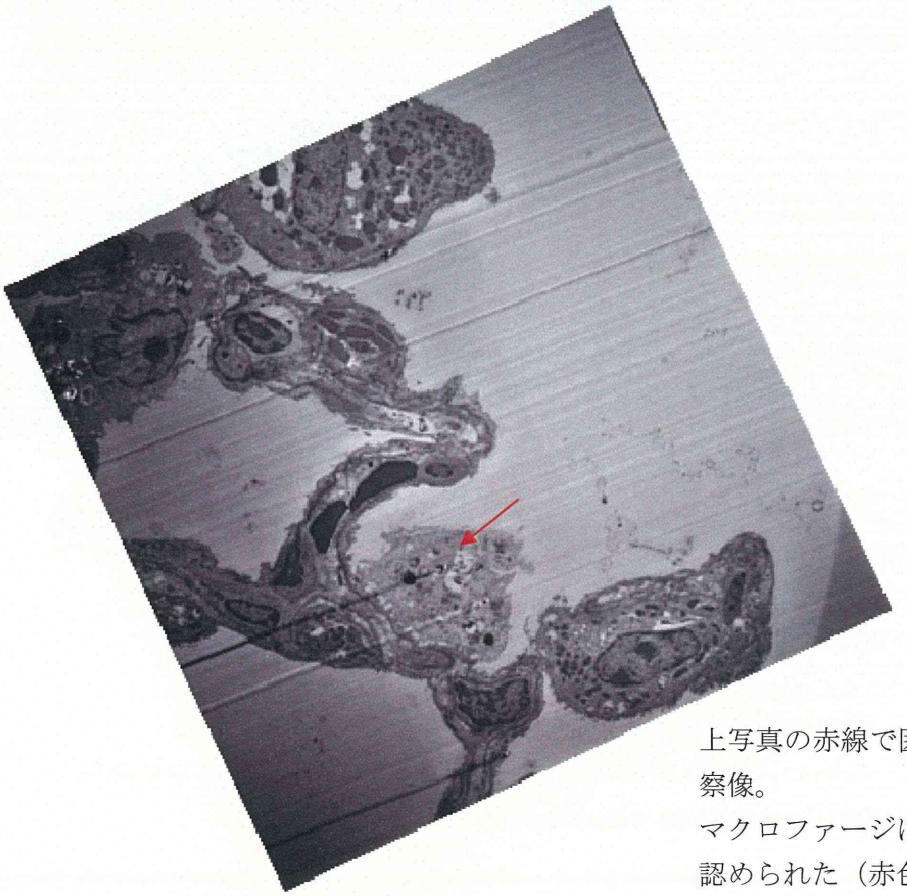
図 43　F344 雄ラットの肺の透過型電子顕微鏡写真

MWCNT（MWNT-7 原体）160μg/匹を 13 週齢で単回気管内投与し、投与後 28 日に解剖
双方向矢印で示した部位に間質の水腫を認める。

C-2-(3) 気管内投与したT-CNTの肺組織内侵入経路の解明を目的としたTEM検索

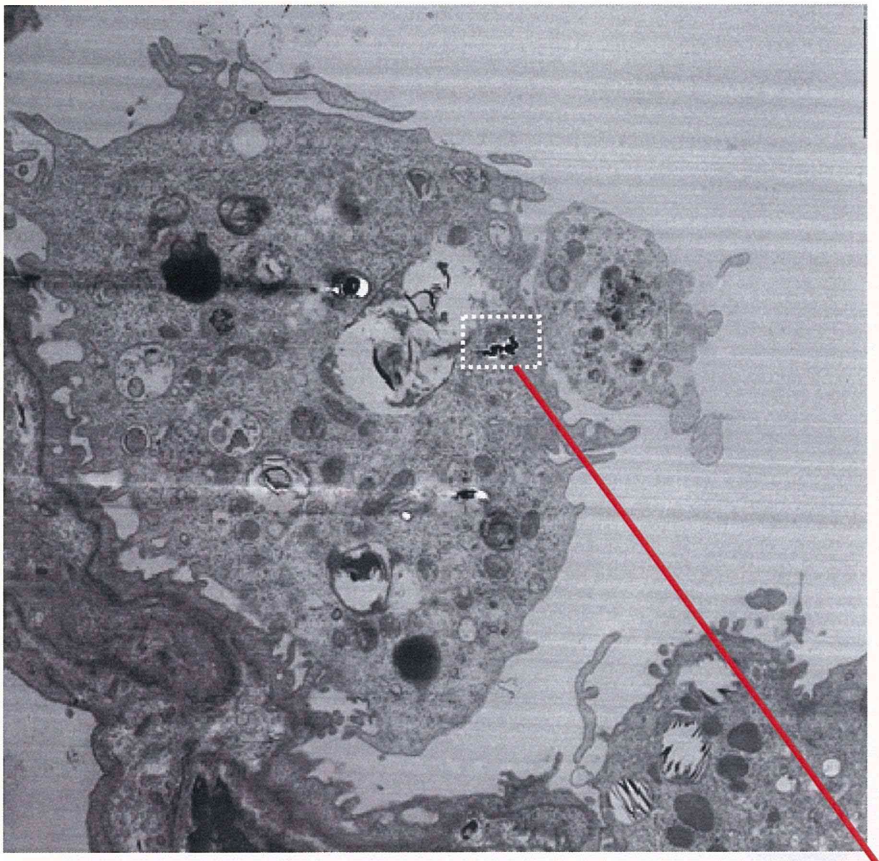


気道終末部と肺胞域との移行部

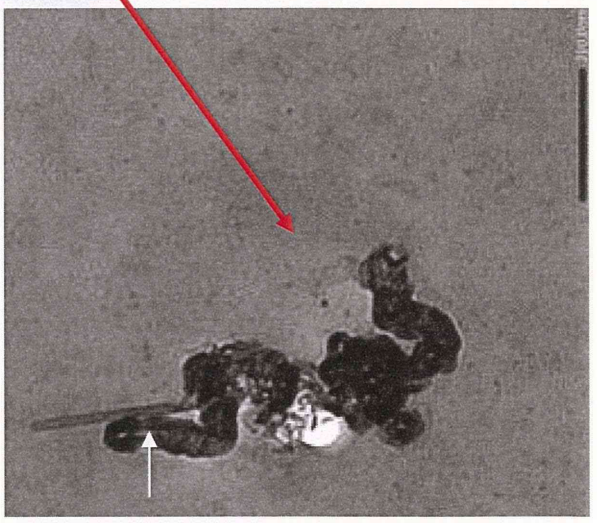


上写真の赤線で囲んだ枠内の TEM 観察像。
マクロファージに貪食されたT-CNTが認められた（赤色矢印）。

図 44 気道終末部と肺胞壁の電顕像
T-CNT 10μg 投与後 1 日の肺

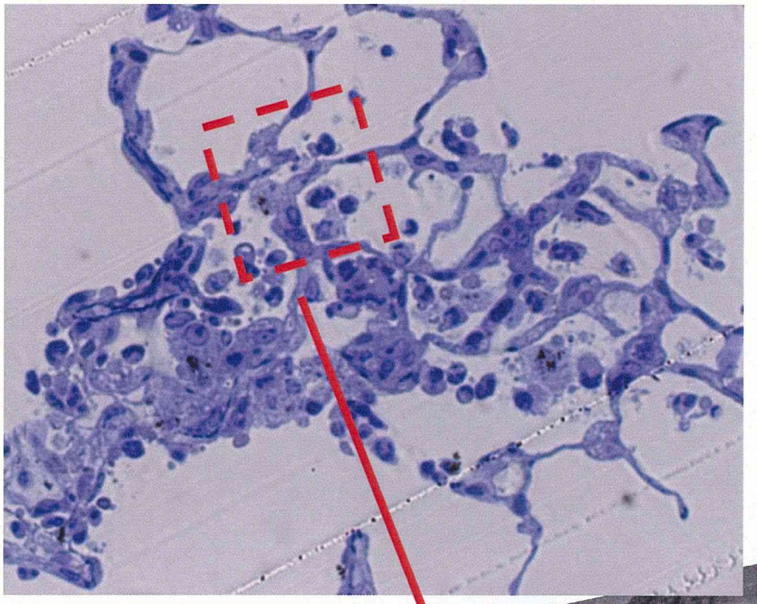


肺胞マクロファージの細胞質内に不定形の異物が認められた(白色点線で囲んだ枠内)。



拡大を上げて観察すると、不定形の異物は中空構造(白色矢印)を示すT-CNTであることが確認された。

図 45 マクロファージに貪食された T-CNT の電顕像
T-CNT 10μg 投与後 1 日の肺



赤破線で示した枠内を TEM で観察。
肺胞マクロファージに貪食された T-CNT（赤色矢印）が認められた。

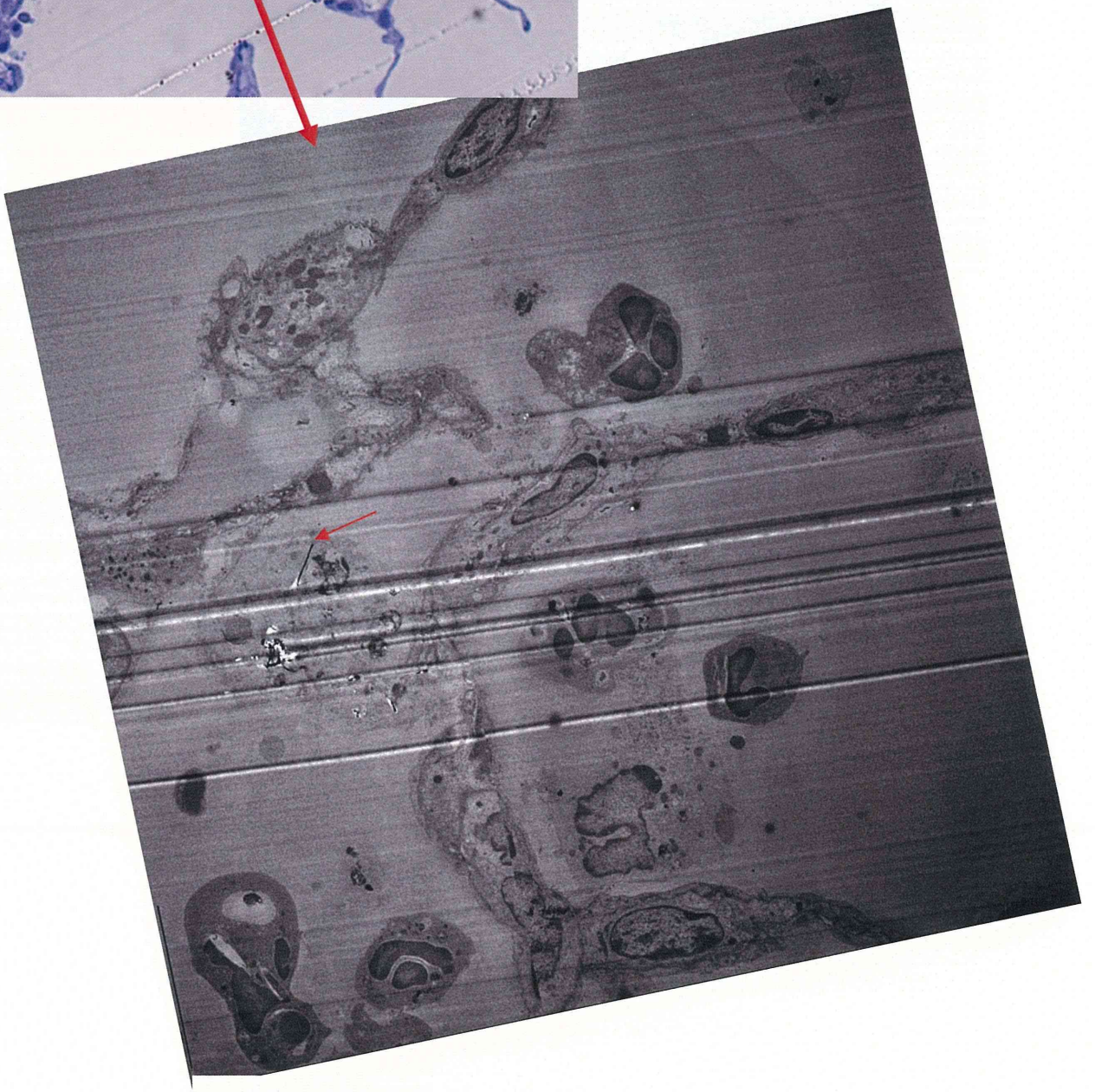
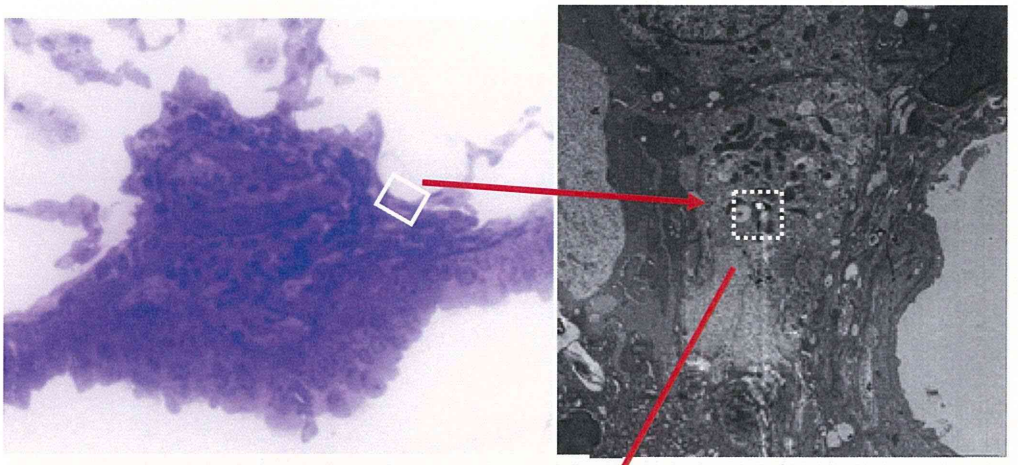
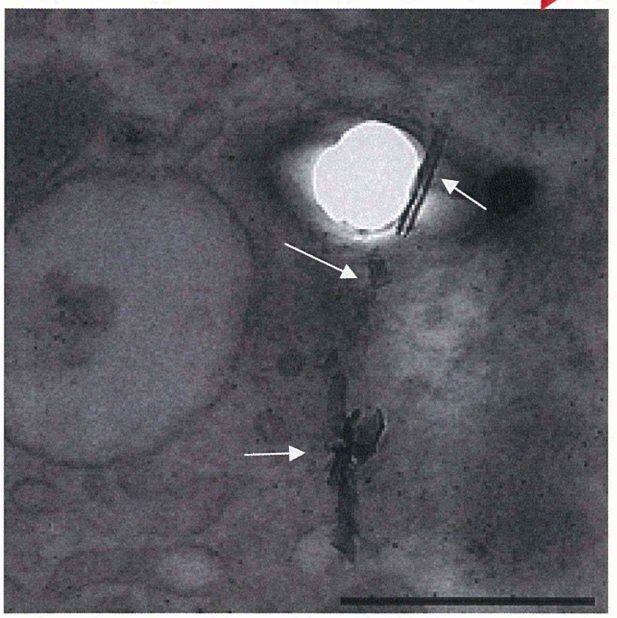


図 46 肺胞腔内：T-CNT 貪食マクロファージの集簇箇所の電顕像
T-CNT 10μg 投与後 1 日の肺



セミシン標本(トルイジンブルー染色)
光学顕微鏡で透過型電顕による検索部位を選(白枠で囲んだ部分)

透過型電子顕微鏡による観察像
マクロファージの細胞質内にT-CNTが存在(白点線で囲んだ部分)



右上の観察部位を拡大して観察。
T-CNT は外径 27.4 nm、内径 11.8nm の中空構造を持つ MWCNT であることが確認された。

白色矢印:T-CNT、
(3本の赤色矢印で示したT-CNTは連続していると思われる)
Bar: 500nm

図 47 細気管支の電顕像
T-CNT 10μg 投与後 3 日の肺

別添５

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yokohira M, Hashimoto N, Yamakawa K, Saoo K, Kuno T, Imaida K	Lack of promoting effects from physical pulmonary collapse in a female A/J mouse lung tumor initiated with 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK) with remarkable mesothelial cell reactions in the thoracic cavity by the polymer.	Exp. Toxicol. Pathol.	63	181 - 185	2011
Yokohira M, Nakano Y, Hashimoto N, Yamakawa K, Ninomiya F, KIshi S, Saoo K, Imaida K	Toxicity of nicotine by repeated intratracheal instillation to F344 rats.	J. Toxicol. Pathol.	25	257 - 263	2012
Nakada T, Kiyotani K, Iwano S, Uno T, Yokohira M, Yamakawa K, Fujieda M, Saito T, Yamazaki H, Imaida K, Kamataki T	Lung tumorigenesis promoted by anti-apoptotic effects of cotinine, a nicotine metabolite through activation of PI3K/Akt pathway.	J. Toxicol. Sci.	37	555-563	2012
Yokohira M, Kishi S, Yamakawa K, Nakano Y, Ninomiya F, Kinouch S, Tanizawa J, Saoo K, Imaida K	Napsin A is possibly useful marker to predict the tumorigenic potential of lung bronchiolo-alveolar hyperplasia in F344 rats.	Toxicol. Pathol.			2013 in press

	Exp.				
Ninomiya F, Yokohira M, Kishi S, Nakano Y, Yamakawa K, Inoue T, Kuno T, Imaida K	Gender-dependent effects of gonadectomy on lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female and male A/J mice.	Oncol. Rep.	30	2632 - 2638	2013
Yokohira M, Nakano Y, Yamakawa K, Kishi S, Ninomiya F, Saoo K, Imaida K	Strain differences in pleural mesothelial cell reactions induced by potassium octatitanate fibers (TISMO) infused directly into the thoracic cavity.	Exp. Toxicol. Pathol.	65 (6)	925 - 932	2013
Taquahashi Y, Ogawa Y, Takagi A, Tsuji M, Morita K, Kanno J	An Improved dispersion method of multi-wall carbon nanotube for inhalation toxicity studies of experimental animals.	J. Toxicol. Sci.	38 (4)	619 - 628	2013
Takagi A, Hirose A, Futakuchi M, Tsuda H, Kanno J.	Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice.	Cancer Sci.	103	1440 - 1444	2012
Hirose A, Takagi A, Nishimura T, Tsuda H, Sakamoto Y, Ogata A, Nakae D, Hino O, Kanno J.	Importance of researches on chronic effects by manufactured nanomaterials.	Yakugaku Zasshi	131 (2)	195 - 201	2011

Nagano K, Gotoh K, Kasai T, Aiso S, Nishizawa T, Ohnishi M, Ikawa N, Eitaki Y, Yamada K, Arito H, Fukushima S.	Two- and 13-week Inhalation Toxicities of Indium-Tin Oxide and Indium Oxide in Rats.	J. Occup. Health	53	51 - 63	2011
Aiso S, Kubota H, Umeda Y, Kasai T, Takaya M, Yamazaki K, Nagano K, Sasaki T, Koda S, Fukushima S.	Translocation of Intratracheally Instilled Multi- wall Carbon Nanotubes to Lung-Associated Lymph Nodes in Rats.	Indust. Health	49	215 - 220	2011
Kano H, Suzuki M, Senoh H, Yamazaki K, Aiso S, Matsumoto M, Nagano K, Fukushima S.	2,4-Dichloro-1-nitrobenzene exert carcinogenicities in both rats and mice by two years feeding.	Arch. Toxicol.	86	1763 - 1772	2012
Umeda Y, Kasai T, Saito M, Kondo H, Toya T, Aiso S, Okuda H, Nishizawa T, Fukushima S.	Two-week Toxicity of Multi-walled Carbon nanotubes by wholebody Inhalation Exposure in Rats.	J. Toxicol. Pathol.	26	131 - 140	2013
Saito A, Sasaki T, Kasai T, Katagiri T, Nishizawa T, Noguchi T, Aiso S, Nagano K, Fukushima S.	Hepatotumorigenicity of ethyl tertiary-butyl ether with 2-year inhalation exposure in F344 rats.	Arch. Toxicol.	87	905 - 914	2013

別添６

Ⅳ．研究成果の刊行物・別刷

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Lack of promoting effects from physical pulmonary collapse in a female A/J mouse lung tumor initiated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) with remarkable mesothelial cell reactions in the thoracic cavity by the polymer

Masanao Yokohira [a], Nozomi Hashimoto [a,b], Keiko Yamakawa [a], Kousuke Saoo [a], Toshiya Kuno [a], Katsumi Imaida [a,*]

[a] Onco-Pathology, Department of Pathology and Host-Defence, Faculty of Medicine, Kagawa University, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
[b] Gastrointestinal Surgery, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan

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ABSTRACT

Experimental identification of potential chemopreventive or tumor promotive agents in the lung is important. Establishment of short-term bioassay models is therefore a high priority. In an attempt to induce strong promotion effects, in Experiment 1, left thoracotomy was performed on A/J mice at week 3 after initiation with 4-(methylnitrosamno)-1-(3-pyridyl)-1-butanone (NNK) (2 mg/0.1 ml saline/mouse i.p.) at weeks 0 and 1. In Experiment 2, at week 3, 0.2 ml of polymer gel was infused directly into the left cavity of the thorax with thoracotomy to occupy certain thoracic cavity volume and to examine the influence of physical pulmonary collapse. The experiments were terminated after 8, 10, 12 and 16 weeks in Experiment 1, and 12 weeks in Experiment 2 but no clear promotion effects in either experiment or pulmonary collapse due to infused polymer were apparent. However, a pronounced mesothelial cell reaction to the infused polymer was evident on the left lung surfaces and parietal pleura in Experiment 2. In conclusion, the present experiments did not demonstrate any clear lung tumor promotion effects of thoracotomy or physical left lung collapse. It remains possible, however, that alternative approaches might have greater efficacy and these need more consideration. In addition, mesothelial cells reaction was observed with the infused polymer.

Introduction

In smokers, the risk of lung cancer development remains elevated even after giving up the habit and environmental tobacco smoke from others continues to be a problem (Mitchell and Sanders, 2002). Therefore, identification of potential chemopreventive or tumor promotive agents is important. Previously, we demonstrated that 8-methoxypsoralen (8-MOP) treatment during the initiation phase strongly inhibits lung tumorigenesis induced by a single intraperitoneal injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice (Takeuchi et al., 2003). NNK is a tobacco-specific *N*-nitrosamine which conceivably plays an important role in tobacco-related human lung cancer, given its strong potential to induce lung tumorigenesis in rodents (Belinsky et al., 1992). CYP2A6 is involved in the mutagenic activation of promutagens such as tobacco-specific *N*-nitrosamines (Kushida et al., 2000) and is also responsible for metabolism of 70–80% of nicotine to the inactive metabolite cotinine in humans (Messina et al., 1997). 8-MOP is reported to inhibit CYP2A6 (Draper et al., 1997; Ono et al., 1996) and we earlier demonstrated that 3 days intake of 100 ppm 8-MOP strongly reduced induction of lung tumors (Takeuchi et al., 2006).

We have focused on establishing a short-term bioassay model for identification of chemopreventive agents acting during the initiation phase of lung carcinogenesis (Yokohira et al., 2008). Examination of the time course of NNK-induced lung tumor development to determine the most appropriate shortest period to assess effects of test agents, with 8-MOP as a typical example showed that two treatments with NNK and 12 weeks duration are effective for detection of lung cancer chemoprevention (Yokohira et al., 2008).

This present study was conducted in an attempt to reduce the experimental period needed by incorporating procedures with

* Corresponding author at: Onco-Pathology, Department of Pathology and Host-Defence, Faculty of Medicine, Kagawa University, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa, 761-0793 Japan. Tel.: +81 87 891 2111; fax, +81 87 891 2112.
E-mail address: imaida@med.kagawa-u.ac.jp (K. Imaida).

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strong tumor promotion effects. Left lobe pneumonectomy is reported to activate cell proliferation and to enhance lung tumorigenesis induced by 3-methylcholanthrene (Brown et al., 1999) and was therefore chosen for assessment. However, since pneumonectomy is a complex surgical procedure we performed thoracotomy in its place in Experiment 1. This is known to be induced hepatocyte growth factor (HGF), which stimulates proliferation of respiratory epithelial cells after pneumonectomy or thoracotomy (Sakamaki et al., 2002). In Experiment 2, a polymer was infused directly into the left cavity of the thorax with thoracotomy to occupy certain thoracic cavity volume to examine the effects of physical pulmonary collapse on lung tumorigenesis.

Methods

Chemicals

NNK was purchased from Toronto Research Chemicals (Toronto, Canada) and the polymer, with high molecule (FD-1000), sodium salt cross-linkage in the acrylic acid polymerization, was from Japan Tanner Corporation (Osaka, Japan). This polymer material absorbs moisture and brings about lung collapse by occupying the thoracic cavity through expansion. The polymer is a white powder form with 10% moisture content, pH 6–8, 150–300 mm average particle size and 60–75 g/g 0.9% NaCl water extraction efficiency.

Animals

Female A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Division of Animal Experiment, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. The protocols of the experiments were approved by the Animal Care and Use Committee for Kagawa University. The animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12 h light/dark cycle) and temperature (24 ± 2 °C). The experiments were started after a 2-week acclimation period.

Experimental design and tissue preparation

Experiment 1: A total of 65 mice at 7 weeks of age were divided into 2 groups of 31 (Group 1) and 34 (Group 2) mice pretreated with NNK (2 mg/0.1 ml saline/mouse i.p.) at weeks 0 and 1. At week 3, 34 mice of Group 2 underwent a left thoracotomy. The procedure was as follows. Each mouse was given i.p. of 0.2 ml pentobarbital sodium (Nembutal, Dainippon Sumitomo Pharma Co., Ltd.., Osaka, Japan) with 10 times dilution (0.06–0.1 ml/10 g body weight). Under deep anesthesia, a skin incision (about 7 mm) was performed on the left axilla. After confirmation of the location of the thoracic wall, thoracotomy was completed with the incision (about 5 mm) between ribs. Left lung was observed directly through this opened hole and also confirmed its atelectasis. The skin was clipped to close the thorax. The experiment was terminated after 8, 10, 12 and 16 weeks when 7, 8 and 9 mice of each group were sacrificed, under deep anesthesia.

Experiment 2: A total of 30 mice at 7 weeks of age were divided into 2 groups of 16 (Group 1) and 21 (Group 2) mice pretreated with NNK (2 mg/0.1 ml saline/mouse i.p.) at weeks 0 and 1. At week 3, 59 mice of Group 2 were given a left thoracotomy (the procedure was almost the same as Experiment 1) and infused with 0.2 ml polymer gel into the left cavity of the thorax. The polymer gel was made up with 1 g polymer powder and 30 ml saline. The experiment was terminated after 12 weeks and all mice of each group were sacrificed under deep anesthesia.

Table 1
Effects of thoracotomy with NNK on A/J mice.

Weeks	8		10		12		16	
Groups	1[a]	2[a]	1	2	1	2	1	2
Number of mice	8	8	8	8	8	9	8	9
Body weight (g)	22.23 ± 1.22	20.70 ± 1.47*	22.65 ± 1.28	21.11 ± 1.14*	22.72 ± 2.53	21.88 ± 1.59	23.48 ± 1.51	22.06 ± 2.15
Lung weight								
Right								
Absolute (mg)	90.4 ± 10.4	82.7 ± 11.8	88.9 ± 7.2	81.1 ± 9.9	86.8 ± 9.1	83.3 ± 11.4	97.5 ± 11.5	10.1 ± 7.4
Relative (%)	0.41 ± 0.03	0.40 ± 0.05	0.39 ± 0.04	0.39 ± 0.05	0.38 ± 0.05	0.38 ± 0.04	0.41 ± 0.03	0.46 ± 0.02
Left								
Absolute (mg)	47.6 ± 2.3	37.4 ± 3.3*	44.8 ± 1.4	36.4 ± 5.4*	44.0 ± 4.0	41.9 ± 7.7	52.1 ± 8.4	45.8 ± 6.2
Relative (%)	0.21 ± 0.02	0.18 ± 0.01*	0.20 ± 0.02	0.17 ± 0.03	0.19 ± 0.02	0.19 ± 0.03	0.22 ± 0.04	0.21 ± 0.03
Macroscopic nodules								
Left lung								
Incidence	37.5% (3/8)	25.0% (2/8)	0.0% (0/8)	25.0% (2/8)	75.0% (6/8)	88.9% (8/9)	100% (8/8)	100% (9/9)
Multiplicity	0.9 ± 1.7	0.4 ± 0.7	0.0 ± 0.0	0.3 ± 0.5	1.5 ± 1.4	2.6 ± 2.0	5.1 ± 3.8	3.3 ± 1.6
Right lung								
Incidence	12.5% (1/8)	50.0% (4/8)	50.0% (4/8)	87.5% (7/8)	100% (8/8)	88.9% (8/9)	100% (8/8)	100% (9/9)
Multiplicity	0.1 ± 0.4	0.5 ± 0.5	1.1 ± 1.7	1.9 ± 1.5	3.8 ± 2.3	3.1 ± 2.0	7.9 ± 5.4	4.9 ± 3.4
Bilateral lungs								
Incidence	50.0% (4/8)	50.0% (4/8)	50.0% (4/8)	87.5% (7/8)	100% (8/8)	100% (9/9)	100% (8/8)	100% (9/9)
Multiplicity	1.0 ± 1.7	0.9 ± 1.1	1.1 ± 1.7	2.1 ± 1.7	5.3 ± 2.0	5.7 ± 3.1	13.0 ± 8.8	8.2 ± 4.7
Histopathological tumors								
Bilateral lungs								
Hyperplasia	0.0 ± 0.0	0.4 ± 0.7	0.4 ± 0.7	0.5 ± 0.8	1.8 ± 1.0	1.7 ± 1.6	1.6 ± 1.4	1.2 ± 1.5
Adenoma	0.3 ± 0.7	0.1 ± 0.4	1.0 ± 0.8	0.8 ± 1.0	4.1 ± 1.6	3.3 ± 2.5	6.9 ± 3.2	4.9 ± 2.7
Total	0.3 ± 0.7	0.9 ± 0.9	1.4 ± 1.3	1.3 ± 1.3	5.9 ± 1.6	5.0 ± 3.5	8.5 ± 3.9	6.1 ± 3.7

[a] Group 1, NNK; Group 2, NNK+thoracotomy.
* $P < 0.05$ vs the NNK control group.

Experiments 1 *and* 2: At autopsy, the lungs were excised and weighed and then infused with 10% neutral buffered formalin, and carefully inspected grossly. After fixation, all macroscopically detected lung nodules were counted under a stereomicroscope, and each lung lobe was examined histopathologically.

Immunohistochemical analysis

In Experiment 2, lungs were immunostained for calretinin and anti-human mesothelial cell (HMBE-1) by the avidin–biotin complex (ABC) method, all staining processes from deparaffinization to counterstaining with hematoxylin being performed automatically using the Ventana Discovery™ staining system (Ventana Medical Systems, AZ, USA). Anti-mouse Calretinin monoclonal antibody, clone 5A5, purchased from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK) and anti-mouse HBME-1 monoclonal antibody, Lot no. 108, purchased from DAKO corporation (CA, USA) were used at 1:100 and 1:50 dilutions.

Statistical analysis

The body and lung weights, and multiplicity of lung proliferative lesions were analyzed by the Turkey-krummer test (multicomparison test). The incidences of lung proliferative lesions were analyzed by the Fisher's exact probability test and data for multiplicity by the Student's *t*-test.

Results

Experiment 1: Body and lung weight data are shown in Table 1. Body weights of the NNK alone group at 8 and 10 weeks were significantly greater than in the NNK+thoracotomy group. Left lung weights of the NNK+thoracotomy group at 8 and 10 weeks were significantly decreased as compared with the NNK group and adhesion of the left lung to the visceral pleura was observed. Lung whitish nodules were detected in all groups macroscopically. Incidences and multiplicities of hyperplasias and adenomas, diagnosed according to the criteria of 'International Classification of Rodent Tumors: The Mouse' (Dungworth et al., 2001) are also summarized in Table 1. The lung lesions in each group increased with time, but with no significant inter-group differences in either incidence or multiplicity.

Experiment 2: Body and lung weights in 12 weeks are shown in Table 2. Though body weights demonstrated no significant inter-group differences, the left lung weights of the NNK+polymer group were significantly decreased as compared with the NNK group. Fig. 1 shows macroscopical findings of the NNK+polymer group at autopsy at 12 weeks. The infused polymer formed a discrete mass in the cavity of the chest and deflected the heart slightly to the right. This remaining total mass volume in the left thorax at the sacrifice day on week 8 was approximately 10–40% of the primary volume, 0.2 ml, in week 3. Some adhesion with visceral and parietal pleura was evident. Lung whitish nodules were detected in each group macroscopically (Fig. 2). And data for macroscopic lung nodules and microscopic hyperplasias and adenomas are also summarized in Table 2. The multiplicity of macroscopic left lung nodules in the NNK+polymer group was significantly increased as compared with the NNK group, but there were no other significant inter-group differences in the incidences and multiplicities of macroscopic lung nodules and microscopic lung hyperplasias and adenomas.

Interestingly, reaction with the infused polymer was observed on the surface of the left lung histopathologically (Fig. 3A). Mesothelial cells immunohistochemically stained positive for

Table 2
Effect of the polymer with NNK on A/J mice.

Groups	1[a]	2[a]
Number of mice	16	21
Body Weight	22.8 ± 1.5	21.3 ± 1.6
Lung weight		
Right		
Absolute (mg)	84.2 ± 6.4	80.0 ± 10.7
Relative (%)	0.37 ± 0.03	0.38 ± 0.05
Left		
Absolute (mg)	42.2 ± 4.7	38.4 ± 5.8
Relative (%)	0.19 ± 0.02	0.18 ± 0.02
Macroscopic nodules		
Left lung		
Multiplicity	1.88 ± 1.59	3.39 ± 1.79*
Right lung		
Multiplicity	4.81 ± 2.20	5.72 ± 3.14
Bilateral lungs		
Multiplicity	6.69 ± 2.63	9.11 ± 4.14
Histopathological tumors		
Bilateral lungs		
Hyperplasia	1.13 ± 1.02	1.39 ± 0.78
Adenoma	3.94 ± 1.24	4.61 ± 2.57
Total	5.06 ± 1.84	6.00 ± 2.68

[a] Group 1, NNK; Group 2, NNK+polymer.
* $P < 0.05$ vs the NNK control group.

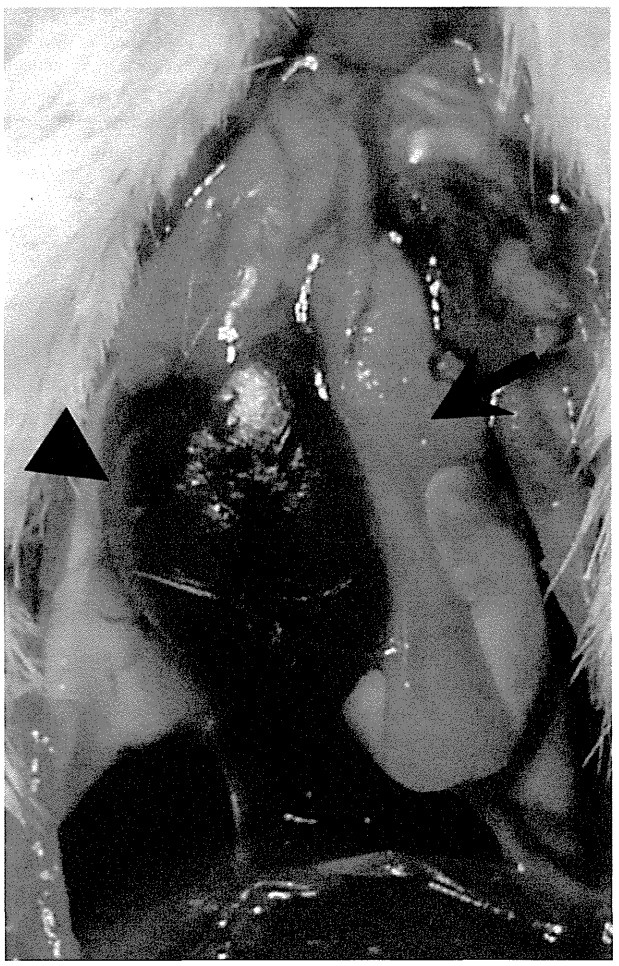

Fig. 1. Macroscopic findings for the NNK+polymer group at 12 weeks. The infused polymer forms a discrete mass in the cavity of the chest (arrow) and is deflecting the heart slightly to the right (arrow head).

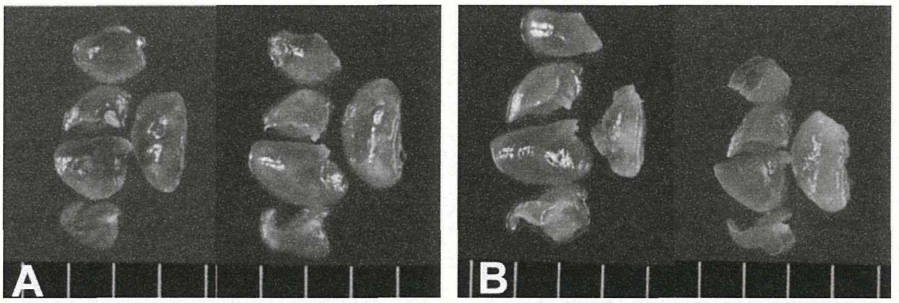

Fig. 2. Macroscopic findings of the lungs for the NNK group (A) and the NNK+polymer group (B) at 12 weeks. Note part of the left lung adhering to the visceral pleura in the latter. Lung whitish nodules were detected in each group.

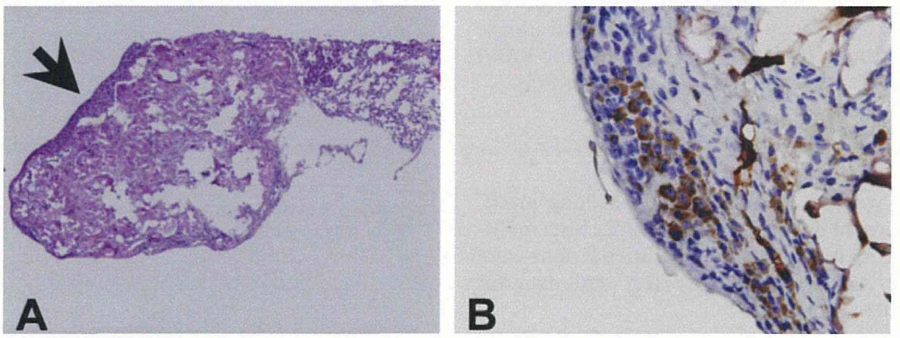

Fig. 3. Histopathological findings for the left lung in the NNK+polymer treated group at 12 weeks. The surface of the left lung demonstrates a reaction to the polymer and accumulation of mesothelial cells is evident (A: arrow). Note positive immunohistochemical staining of calretin in (B).

calretinin appeared to have accumulated around the polymer (Fig. 3B). An assessment of HMBE-1 antibody binding was also performed but it was difficult to detect positive cells because of strong background staining (data not shown).

Discussion

The present study demonstrated no promotion effects by thoracotomy or pulmonary collapse with foreign material on NNK induction of lung tumors. Compensatory lung growth after pneumonectomy was reported to occur in adult male foxhounds (Hsia et al., 1994) and rabbits (Cagle et al., 1988) and to enhance lung tumorigenesis induced by 3-methylcholanthrene in male BALB/cByJ mice (Brown et al., 1999). Sakamaki et al. (2002) reported that increase in HGF stimulates proliferation of respiratory epithelial cells during compensatory lung growth in female Institute of Cancer Research (ICR) mice. In this report, HGF level in the right lung of pneumonectomized mice increased on Day 1, although it thereafter increased to higher levels on Day 10. The HGF levels in the sham-operated mice were also transiently elevated to a level similar to that seen in pneumonectomized mice on Day 1, and then decreased to almost a normal level on Day 10. From this report, thoracotomy may promote initiated lung tumor by temporal HGF inducing. The reason for the lack of any promoting effects in our Experiment 1 is unclear although the fact that the thoracotomy was only performed once as an acute procedure may be important. For more continuous promoting effects in the lung we examined lung collapse by foreign material infused into the left cavity of thorax in Experiment 2. However, the results were again equivocal, despite lesions in the NNK+polymer group showing a tendency for increase. Though pneumonectomy might promote lung tumor induced by NNK, it is not an appropriate procedure to employ for bioassay models in which enough numbers of animals should be treated. With our approach the infused polymer appeared to induce a foreign-body reaction and seemed to be decreased in amount after 12 weeks. It is possible that more frequent infusions could exert stronger promoting effects.

However, there were interesting histopathological findings in Experiment 2 concerning the reaction of mesothelial cells, immunohistochemically positive for calretinin, with the infused polymer on the surface of the left lung. Calretinin is a calcium-binding protein of 29 kD. It is a member of the large family of EF-hand proteins, to which the S-100 protein also belongs. In a survey of the distribution of calretinin immunoreactivity in non-neural tissues, strong and consistent immunoreactivity of normal and reactive mesothelial cells were reported and the report also concluded calretinin is a useful marker for the positive identification of malignant mesotheliomas (Doglioni et al., 1996). In present experiment, though the observed mesothelial cells were not sufficiently atypical to be given a diagnosis of malignant mesothelioma, these findings should be confirmed whether they indicate the possibility of pleural mesothelioma or not by future long-term experiment. Epidemiologically, analyses using an age-cohort model in Japan showed that there will be about 100,000 deaths due to pleural mesothelioma in the next 40 years from 2006 (Murayama et al., 2006). Using 1973–2000 mesothelioma incidence data in the United States, the total number of male mesothelioma cases in 2003–2054 would be approximately 71,000 (Price and Ware, 2004). There are some reports of peritoneal mesothelioma induced by 5 chemicals or some fibers in wild rats (Crosby et al., 2000; Kamstrup et al., 2002; Kim et al., 2006; Krajnow and Lao, 2000) or pleural mesotheliomas in genetically modified animals, like the p53 knock out mouse (Jongsma et al., 2008). Lardinois et al. (2006) also reported efficacy of intrapleural application of cisplatin in an immune-competent rat model with malignant pleural mesothelioma inoculated with mesothelioma cells. However, to our knowledge there has been no report of pleural mesothelioma in experimental animals using the procedure of directly infusing material into the thoracic cavity.