Original

Lung Carcinogenic Bioassay of CuO and TiO$_2$ Nanoparticles with Intratracheal Instillation Using F344 Male Rats

Masanao Yokohira[1], Nozomi Hashimoto[1], Keiko Yamakawa[1], Satoshi Suzuki[1], Kousuke Saoo[1], Toshiya Kuno[1], and Katsumi Imaida[1]

[1]Onco-Pathology, Department of Pathology and Host-Defense, Faculty of Medicine, Kagawa University, 1750–1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Abstract: Toxicity assessment of nanoparticles, now widespread in our environment, is an important issue. We have focused attention on the carcinogenic potential of copper oxide (CuO) and titanium dioxide (TiO$_2$). In experiment 1, a sequential pilot study, the effectiveness of a carcinogenic bioassay featuring intraperitoneal injection (i.p.) of 20 mg 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or 0.1 % *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) in drinking water for 2 weeks was examined. Based on the results, DHPN, as the lung carcinogen, and evaluation at week 30 were selected as the most appropriate for our purposes in Experiment 1. In experiment 2, the carcinogenic bioassay was used to assess the carcinogenic potentials of instilled nanoparticles of CuO and TiO$_2$. There were no significant intergroup differences in the lung neoplastic lesions induced by DHPN, although the neoplastic lesions induced by the nanoparticles in the CuO or TiO$_2$ intratracheal instillation (i.t.) groups, demonstrated a tendency to increase compared with the microparticles administration. At the very least, the carcinogenic bioassay with DHPN proved useful for assessment of the modifying effects of instilled particles, and further assessment of the carcinogenic potential of nanoparticles appears warranted. (J Toxicol Pathol 2009; **22**: 71–78)

Key words: nanoparticles, lung carcinogenicity, intratracheal instillation, NNK, DHPN

Introduction

The health risk posed by fine particles, especially nanoparticles in the air, has attracted much greater attention with the development of nanotechnology. The nanoparticle is a source of air pollution[1], but there are very few established evaluation methods for detection of lung carcinogenicity.

We have previously described a rat *in vivo* bioassay for detection of hazards due to fine particles by intratracheal instillation (i.t.)[2], which can be used for risk assessment, at an early stage, posed by materials inhaled into deep lung tissue. Sequential analysis of the effects of quartz (DQ-12, 4 mg/rat), a typical lung toxic agent[3], revealed days 1 and 28 after instillation to be most appropriate for detection of acute and subacute inflammatory changes, respectively. For further validation, the toxicity of fine particles from various materials (quartz, hydrotalcite, potassium octatitanate, palladium oxide and carbon black) was examined in our *in*

vivo bioassay[4]; 16 particle types, including nanoparticles were also examined[5]. With the same volume of particles, 2 mg/rat i.t., the nanoparticles of both CuO and NiO caused much more severe acute toxicity, such as acute death and severe inflammatory changes in neutrophil infiltration and edema, than micrometer-diameter particles of the same materials. These experiments confirmed the suitability of this bioassay for fine particle toxicity assessment. However, it cannot predict carcinogenicity.

We therefore aimed to establish a bioassay using the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as an initiating carcinogen. In the lungs of A/J female mice, the initial event in this model is reported to be formation of O^6-methylguanine-DNA, a major promutagenic adduct that leads to GC>AT transitional mispairing and subsequent activation of the K-*ras* proto-oncogene[6,7]. The bioassay model of NNK inducing lung tumor using A/J mice is used extensively, and we have previously reported the inhibitory effects of 8-methoxypsoralen, a potent human CYP2A6 inhibitor[8,9]. For carcinogenic assessment of fine particles by i.t., however, rats are more suitable because their tracheas are thick enough to allow instillation. There have been a number of reports of NNK-induced bioassay models using rats[10,11], and in the present study, experiment 1, a pilot study, was conducted to investigate the effectiveness of NNK for a carcinogenic

bioassay model using F344 rats. For comparison, *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), a carcinogen targeting the lung, nasal cavity, paranasal cavity, liver, thyroid gland, kidney, esophagus, colon and urinary bladder[12–14], was applied. Quartz was employed as a typical lung toxic agent to assess the influence of its inflammatory effects on lung tumor development.

In experiment 2, the bioassay model with DHPN assessed in experiment 1 was used to assess the carcinogenic potentials of nanoparticles of copper oxide (CuO) and titanium dioxide (TiO$_2$) in comparison with the same materials of micrometer order size.

Materials and Methods

Chemicals

NNK (Toronto Research Chemicals, Toronto, ON, Canada) and DHPN (Nacalai Tesque Inc., Kyoto, Japan) were employed for initiation. Quartz dust (DQ-12) was obtained from Deutsche Montan Technologie (GmbH, Germany) with a particle diameter of less than 7 μm. Titanium dioxide with a particle diameter of less than 5 μm (Rutile form, Lot. TCG4139) (TiO$_2$ micro) and titanium dioxide with a particle diameter of 80 nm (Lot. DPN0960) (TiO$_2$ nano) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Copper oxide with a particle diameter of less than 5 μm (CAS1317-38-0; CuO micro) and copper oxide with a particle diameter of 33 nm (CAS1317-38-0; CuO nano) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The particles were all suspended in saline (Otsuka Isotonic sodium chloride solution, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan).

Animal treatment

Male F344/DuCrlCrj rats (4 weeks of age) purchased from Japan Charles River (Kanagawa, Japan) were maintained in the Kagawa University Animal Facility according to the Institutional Regulations for Animal Experiments. The protocols of the experiments were approved by the Animal Care and Use Committee. The animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., 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.

Experiment 1: The experimental design is shown in Fig. 1. A total of 140, 6-week-old, male F344 rats were randomly separated into 7 groups of 20 rats each. At the start of the experiment, groups 2 and 3 were administered a single intraperitoneal injection (IP) of 20 mg NNK suspended in 1 ml saline, groups 4 and 5 received 3 IPs of 10 mg NNK suspended in 1 ml saline in weeks 0, 1 and 2 and groups 6 and 7 were administered 0.1% DHPN in drinking water for 2 weeks. In week 4, groups 1, 3, 5 and 7 were exposed by i.t. to 0.5 mg quartz suspended in saline (0.2 ml) using an
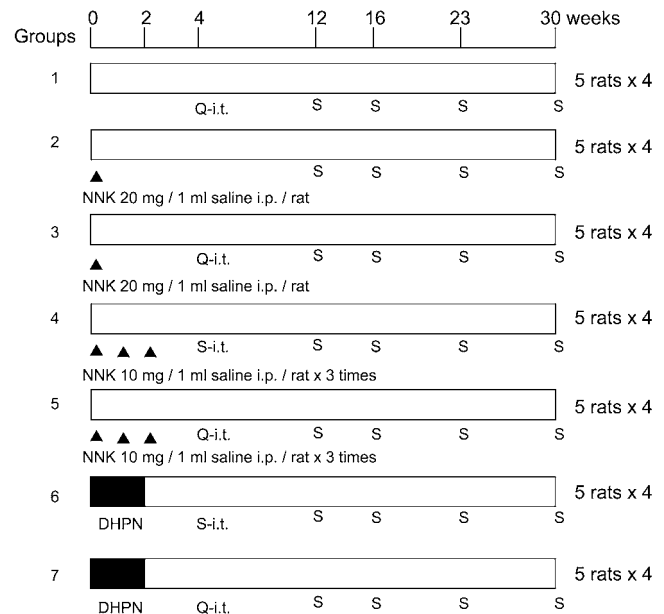

Fig. 1. Design of experiment 1. A total of 140, 6-week-old, male F344 rats were employed. Groups 2 and 3: a single IP of 20 mg NNK/1 ml saline. Groups 3 and 4: 3 × IP of 10 mg NNK/1 ml saline at weeks 0, 1 and 2. Groups 6 and 7: 0.1% DHPN in drinking water for 2 weeks. At week 4, groups 1, 3, 5 and 7 received i.t. of 0.5 mg quartz (DQ-12)/0.2 ml saline (Q-i.t.), and groups 4 and 6 received 0.2 ml of saline (S-i.t.). Subgroups of 5 rats each were sacrificed (S) at weeks 12, 16, 23 and 30 thereafter.
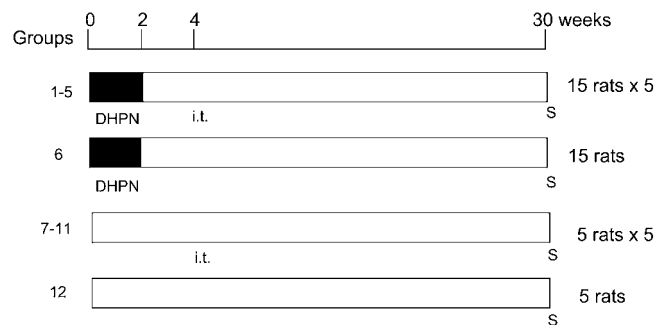

Fig. 2. Design of experiment 2. A total of 120 rats (6-week-old) were employed. Groups 1–6: 0.1% DHPN in drinking water for 2 weeks. Groups 1–5 and 7–11: i.t. of 0.5 mg/rat quartz (groups 1 and 7), CuO micro (groups 2 and 8), CuO nano (groups 3 and 9), TiO$_2$ micro (groups 4 and 10) and TiO$_2$ nano (groups 5 and 11) in week 4. Group 6 was a DHPN control group, and Group 12 was an untreated control group. All rats were sacrificed (S) at week 30.

aerosolizer (PennCentury Philadelphia, PA, USA), and groups 4 and 6 were exposed by i.t. to 0.2 ml saline as vehicle control groups. The test solutions were sonicated to maintain as much suspension as possible and 0.2 ml solutions were instilled into each rate with 1 ml of air so that the solution would be spread throughout in the lung. Subgroups of 5 rats each were sacrificed in weeks 12, 16, 23

Table 1. Number of Lesions in Lung Tissues Sections at Different Time Points (Experiment 1)

Groups	Treatment	No.*	Lesions	Weeks			
				12	16	23	30
Group 1	Saline i.p.		Hyperplasia	0.0	4.4 ± 1.1	0.0	0.4 ± 0.5
	+	20	Adenoma	0.0	0.0	0.0	0.0
	Quartz i.t.		Adenocarcinoma	0.0	0.0	0.0	0.0
Group 2	20 mg NNK i.p.		Hyperplasia	0.8 ± 1.3	2.0 ± 2.9	0.6 ± 0.9	0.0 ± 0.0
		20	Adenoma	0.0	0.0	0.0	0.0
			Adenocarcinoma	0.0	0.0	0.0	0.0
Group 3	20 mg NNK i.p.		Hyperplasia	0.2 ± 0.4	2.0 ± 1.0	1.2 ± 0.8	1.0 ± 0.7
	+	20	Adenoma	0.0	0.0	0.0	0.0
	Quartz i.t.		Adenocarcinoma	0.0	0.0	0.0	0.0
Group 4	10 mg NNK i.p. ×3		Hyperplasia	3.2 ± 2.6	3.2 ± 4.9	0.2 ± 0.4	0.8 ± 0.4
	+	20	Adenoma	0.0	0.0	0.0	0.0
	Saline i.t.		Adenocarcinoma	0.0	0.0	0.0	0.0
Group 5	10 mg NNK i.p. ×3		Hyperplasia	1.0 ± 0.7	3.2 ± 2.4	3.0 ± 1.6	0.8 ± 0.8
	+	20	Adenoma	0.0	0.0	0.0	0.0
	Quartz i.t.		Adenocarcinoma	0.0	0.0	0.0	0.0
Group 6	DHPN		Hyperplasia	7.6 ± 4.0	17.0 ± 7.1	23.8 ± 7.3	26.4 ± 4.4
	+	20	Adenoma	1.2 ± 1.3	1.6 ± 2.1	1.0 ± 0.7	1.8 ± 1.1
	Saline i.t.		Adenocarcinoma	0.0	0.0	0.0	0.4 ± 0.5
Group 7	DHPN		Hyperplasia	6.8 ± 2.2	14.3 ± 2.2	19.8 ± 4.0	17.4 ± 4.0
	+	20	Adenoma	0.8 ± 0.8	0.8 ± 0.5	0.8 ± 1.3	2.0 ± 1.4
	Quartz i.t.		Adenocarcinoma	0.0	0.0	0.0	0.4 ± 0.5

*Effective number of rats: 20 rats were employed in each group, and subgroups of 5 rats each were assessed in weeks 12, 16, 23 and 30.
There is no significant difference between group 2 and 3, 4 and 5 or 6 and 7.

and 30 thereafter.

Experiment 2: The experimental design is shown in Fig. 2. A total of 120 rats (6-week-old) were randomly separated into 6 groups of 15 animals and an additional 6 groups of 5 animals each. Groups 1-6 were administered 0.1% DHPN in drinking water for 2 weeks. Groups 1–5 and 7–11 were exposed by i.t. to 0.5 mg/rat quartz (groups 1 and 7), CuO micro (groups 2 and 8), CuO nano (groups 3 and 9), TiO$_2$ micro (groups 4 and 10) and TiO$_2$ nano (groups 5 and 11) in saline (0.2 ml) using an aerosolizer in week 4. Group 6 was a DHPN control group, and Group 12 was an untreated control group. All rats were sacrificed in week 30.

At autopsy, the lungs, liver and kidneys were removed. The lungs, including the trachea and heart, were weighed, infused with 10% phosphate buffered formalin and then rinsed in 10% phosphate buffered formalin. The weights of the lungs were calculated by subtraction of the weight of the remaining trachea and heart. The organs were immersed in 10% phosphate buffered formalin for a week, and slices were routinely processed for embedding in paraffin for histopathological examination of H and E stained sections. The histopathological specimens were routinely 2 slices of the left lobe and 1 slice each of the other lobes.

Histopathological analysis

Lung lesions were categorized as hyperplasia, adenoma and adenocarcinoma in accordance with the established criteria[15]. Incidences were assessed along with numbers of hyperplasias, adenomas and adenocarcinomas in experiment 1. In experiment 2, in addition to the numbers, the areas of adenomas and adenocarcinomas were assessed using an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice Corporation, Osaka, Japan)[16]. The numbers and areas in experiment 2 were indicated as: No. per 1 cm^2, which was calculated as (number of lesions / total area cm^2 of lung per a slide), and % Area, which was calculated as (total area of lesions / total area of lung per a slide) ×100.

Statistics

Body and organ weights and numbers and areas of the induced lung lesions were analyzed by the Tukey-Kramer post-hoc test. *P* values less than 0.05 were considered significant.

Results

Experiment 1

After intratracheal instillation, no symptoms of dyspnea were observed. The general conditions of the rats in all the groups did not change remarkably during the experimental period.

The average body weight of group 7 (DHPN and quartz i.t. group), 337.4 ± 19.6 (g), was greater than that of group 5

Table 2. Body and Organ Weights of the Rats (Experiment 2)

Groups	Treatment	Instilled particles	No. of rats	Body weight (g)	Lung Absolute (g)	Lung Relative (%)	Liver Absolute (g)	Liver Relative (%)
1	DHPN	Quartz	15	321.0 ± 14.2	1.43 ± 0.12	0.44 ± 0.07	7.88 ± 0.46	2.45 ± 0.07[a]
2	DHPN	CuO micro	15	321.0 ± 14.6	1.32 ± 0.11	0.41 ± 0.08	7.73 ± 0.44[a]	2.41 ± 0.08[a]
3	DHPN	CuO nano	15	334.5 ± 26.4	1.56 ± 0.43	0.47 ± 0.15	8.42 ± 0.71	2.52 ± 0.09
4	DHPN	TiO_2 micro	15	340.9 ± 12.9	1.43 ± 0.05	0.42 ± 0.02	8.70 ± 0.41	2.55 ± 0.09
5	DHPN	TiO_2 nano	15	334.2 ± 16.7	1.45 ± 0.13	0.43 ± 0.04	8.58 ± 0.46	2.57 ± 0.10
6	DHPN	Untreated	15	329.5 ± 22.2	1.44 ± 0.11	0.44 ± 0.03	8.45 ± 0.62	2.56 ± 0.08
7	Untreated	Quartz	5	323.4 ± 19.1	1.36 ± 0.09	0.42 ± 0.01	8.22 ± 0.64	2.54 ± 0.09
8	Untreated	CuO micro	5	325.7 ± 12.3	1.18 ± 0.12	0.36 ± 0.03	8.43 ± 0.61	2.59 ± 0.11
9	Untreated	CuO nano	5	349.0 ± 31.8	1.37 ± 0.07	0.39 ± 0.02	9.10 ± 1.07	2.60 ± 0.13
10	Untreated	TiO_2 micro	5	350.7 ± 19.3	1.36 ± 0.08	0.39 ± 0.02	9.15 ± 0.77	2.61 ± 0.08
11	Untreated	TiO_2 nano	5	334.4 ± 15.4	1.34 ± 0.08	0.40 ± 0.02	8.38 ± 0.48	2.51 ± 0.05
12	Untreated	Untreated	5	347.8 ± 15.5	1.37 ± 0.18	0.39 ± 0.04	9.17 ± 0.78	2.63 ± 0.14

[a] $P<0.05$ vs. DHPN control group (Group 6).

(10 mg NNK and quartz i.t. group), 297.9 ± 14.6, in week 23. There were no significant intergroup differences in body weight in the other groups. The absolute and relative weights of the lungs treated with quartz (groups 3, 5 and 7) were increased significantly compared with the control groups (groups 2, 4 and 6, respectively; data not shown).

In regard to macroscopic findings, white spots were seen on the surfaces of the lungs in the quartz i.t. groups. White nodules were observed in groups 6 and 7 (DHPN groups) in week 12, and these nodules grew sequentially thereafter. In the NNK and DHPN groups (groups 2–7), there were no differences in nodule appearance with or without quartz i.t.

In regard to histopathological changes in the lungs, hyperplastic lesions with mild inflammatory changes were observed in groups 2–5 (NNK i.p. groups) at week 12, but the hyperplastic lesions then decreased with time. After week 23, large granular lymphocytic lymphomas (LGL lymphomas) were found around the trachea were apparent in all rats treated with NNK i.p. (groups 2–5; Fig. 3). The data for the numbers of lung lesions in the various groups are summarized in Table 1. The NNK IP groups (groups 2–5) did not demonstrate obvious development of epithelial tumors in the lungs despite the inflammatory changes caused by quartz. In the DHPN groups (groups 6 and 7), lung tumors and many hyperplasias were observed that increased in number and size with time. In the quartz i.t. groups (groups 1, 3, 5 and 7), severe inflammatory changes with neutrophil infiltration, histiocyte infiltration, pulmonary edema and granuloma formation were observed at weeks 12, 16 and 23. At week 30, they were somewhat reduced. The treatment with quartz i.t. did not influence the lung tumor development in this experiment. The livers and kidneys did not demonstrate any significant histopathological alteration in any group.

Experiment 2

No symptoms of dyspnea were observed after i.t.

treatment, and the general conditions of the rats in all the groups did not change remarkably during the experimental period.

The final data for the body and organ weights in week 30 are shown in Table 2. The absolute and relative liver weights in group 2 (DHPN and CuO micro i.t.) were decreased significantly compared with group 6 (DHPN control group), while the absolute liver weights in group 1 (DHPN and quartz i.t.) were increased. The body, lung and kidney weights indicated no significant intergroup differences.

In regard to macroscopical findings, nodules in the lungs were observed in the DHPN groups 1–6, but there was no intergroup variation in number or area. In the DHPN untreated groups (groups 7–12), quartz i.t. (group 7) caused some white spots on the surface of the lungs, while CuO micro or nano i.t. (groups 8 and 9) induced mild nodulated changes. However, no lung lesions were found with TiO_2 micro or nano i.t. (groups 10 and 11).

In regard to histopathological assessment, all the DHPN groups (groups 1–6) contained hyperplasias, adenomas and adenocarcinomas in the lungs (Fig. 4). In the DHPN untreated groups (Fig. 5), quartz i.t. (group 7) and, to a lesser extent, CuO micro i.t. (group 8) caused granulomas. CuO nano i.t. (group 9) induced a milder inflammatory change. Both TiO_2 micro i.t. (group 10) and TiO_2 nano i.t. (group 11) were not associated with any appreciable inflammatory change. The data for histopathological lesions in the DHPN groups (groups 1–6) are summarized in Tables 3 (the number of lesions per cm^2 in a histopathological sections) and 4 (% areas). There were no significant intergroup differences in the numbers and areas of hyperplasias, adenomas and adenocarcinomas.

Discussion and Conclusions

In experiment 1 of the present study, DHPN was found to be more effective for induction of lung lesions than NNK,
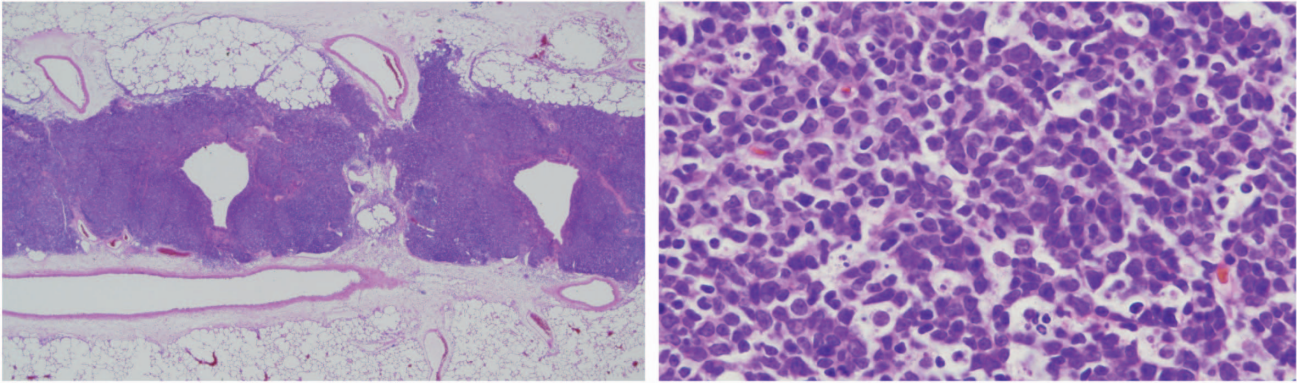
Fig. 3. Histopathological appearance of a large granular lymphocytic lymphoma (LGL lymphoma) in group 2 (20 mg NNK i.p. group) in experiment 1. A, magnification 12.5×; B, magnification 400×.
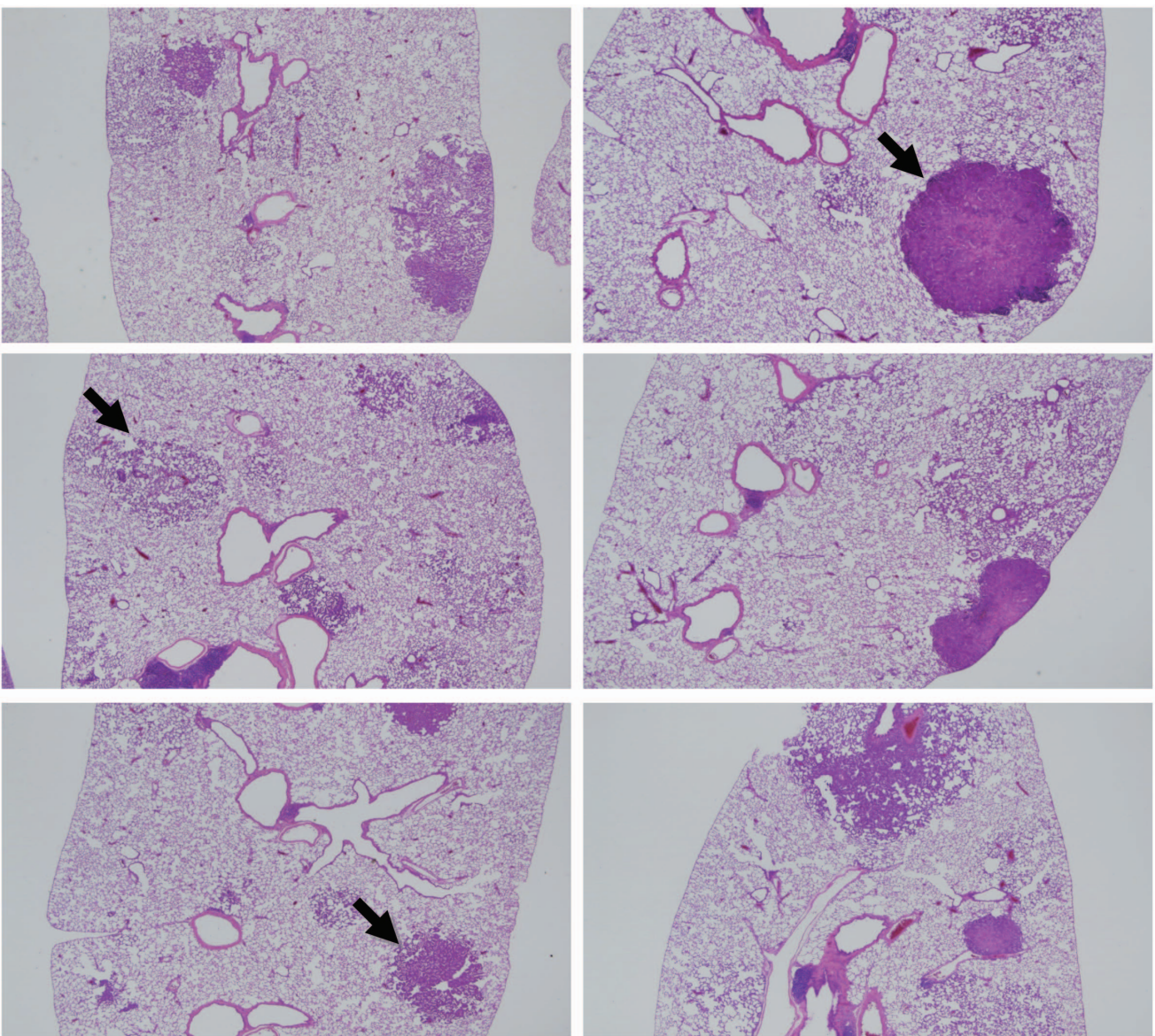

Fig. 4. Histopathological findings for the DHPN groups (groups 1–6) in experiment 2. A, group 1 (quartz); B, group 6 (DHPN control), adenocarcinoma indicated by the arrow; C, group 2 (CuO micro), hyperplasia indicated by the arrow; D, group 3 (CuO nano); E, group 4 (TiO$_2$ micro), adenoma indicated by the arrow; F, group 5 (TiO$_2$ nano). The magnification in A–F is 12.5×.
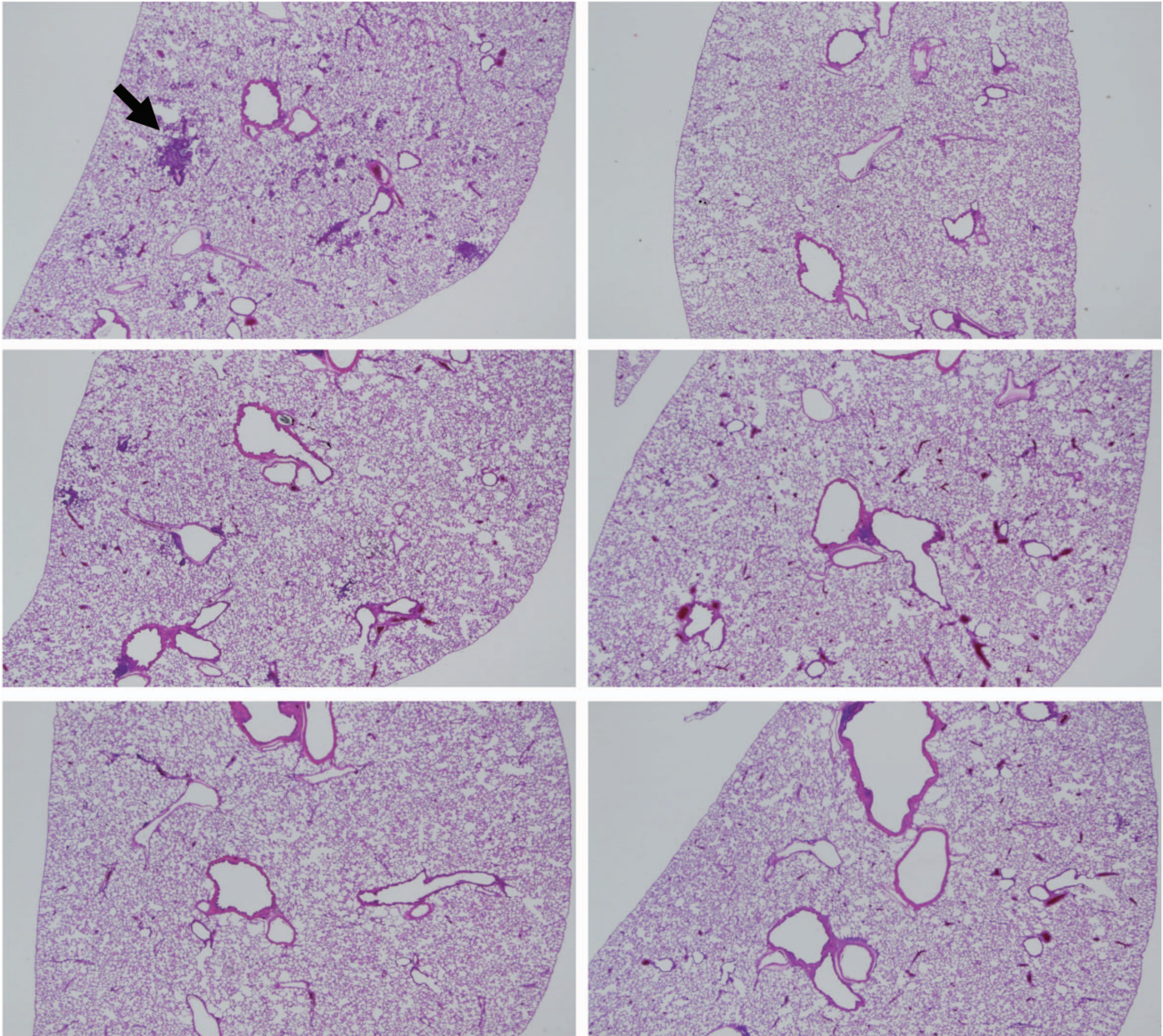
Fig. 5. Histopathological findings for the DHPN untreated groups (groups 7–12) in experiment 2. A, group 7 (quartz), granuloma indicated by the arrow; B, group 12 (untreated); C, group 8 (CuO micro); D, group 9 (CuO nano); E, group 10 (TiO$_2$ micro); F, group 11 (TiO$_2$ nano). The magnification is A–F is 12.5×.

the latter causing a 100% incidence of LGL lymphomas. The LGL lymphoma may be observed as a spontaneous neoplastic lesion in Fischer rats[15]; however Chung, *et al.* reported that 1.5 mg/kg body weight NNK administered by subcutaneous injection 3 times weekly for 21 weeks induces a 36% incidence in F344 rats[11]. In the DHPN groups (groups 6 and 7), lung hyperplasia, adenoma and adenocarcinoma increased sequentially, but quartz had no effect on their development. At an early stage of the experiment, severe inflammatory changes, especially neutrophil infiltration, were observed in the acute phase that became milder with time. Based on the results of experiment 1, DHPN, employed as a lung carcinogen, and evaluation at week 30 were concluded to be most appropriate for our purposes.

The results of experiment 2 showed that the numbers

and areas of neoplastic lesions induced by DHPN exhibited no significant intergroup differences in groups 1–6. However, in groups 3 and 5, the CuO or TiO$_2$ nanoparticle i.t. groups, the numbers and areas of neoplastic lesions tended to increase compared with the data for microparticles (groups 2 and 4). Copper undergoes redox-cycling reactions and produces reactive oxygen species[17], though it has an important role in the biological systems of various metalloenzymes[18,19]. Abe *et al.* reported about the carcinogenic risk of copper gluconate, a copper-containing compound, using a rat medium-term multi-organ carcinogenicity bioassay model[20]. Their report showed that copper gluconate admixed in a diet at a concentration of 300–6000 ppm might pose carcinogenic risks for the liver and forestomach at high dose levels. TiO$_2$ has been

Table 3. Number of Histopathological Lesions per cm^2 of Lung (Experiment 2)

Groups	Treatment	Instilled particles	No.	Hyperplasia	Adenoma	Adenoca.	Total	Adenoma+Adenoca.
1	DHPN	Quartz	15	14.2 ± 4.4	2.0 ± 1.9	0.3 ± 0.6	16.5 ± 4.5	2.3 ± 2.3
2	DHPN	CuO micro	15	15.8 ± 4.1	1.7 ± 1.1	0.3 ± 0.5	17.8 ± 4.8	2.0 ± 1.1
3	DHPN	CuO nano	15	16.0 ± 4.3	2.6 ± 1.5	0.2 ± 0.5	18.9 ± 4.8	2.8 ± 1.5
4	DHPN	TiO$_2$ micro	15	19.9 ± 5.6	2.5 ± 1.5	0.1 ± 0.3	22.5 ± 6.3	2.6 ± 1.5
5	DHPN	TiO$_2$ nano	15	20.0 ± 4.7	2.6 ± 1.4	0.3 ± 0.5	22.9 ± 5.4	2.9 ± 1.5
6	DHPN	Untreated	15	17.9 ± 2.8	2.0 ± 1.2	0.2 ± 0.6	20.1 ± 3.4	2.2 ± 1.1

There was no significant intergroup difference.

Table 4. Areas (%) of the Histopathological Lesions in the Lung Tissue Sections (Experiment 2)

Groups	Treatment	Instilled particles	No.	Adenoma	Adenoca.	Total
1	DHPN	Quartz	15	4.4 ± 6.1	2.0 ± 3.7	6.4 ± 8.0
2	DHPN	CuO micro	15	4.0 ± 4.7	1.7 ± 3.4	5.7 ± 4.9
3	DHPN	CuO nano	15	4.6 ± 3.9	3.9 ± 11.4	8.5 ± 11.2
4	DHPN	TiO$_2$ micro	15	5.3 ± 3.8	1.4 ± 3.0	6.7 ± 5.0
5	DHPN	TiO$_2$ nano	15	4.9 ± 2.4	6.0 ± 18.7	10.9 ± 18.4
6	DHPN	Untreated	15	3.4 ± 4.2	2.8 ± 7.5	6.3 ± 7.5

There was no significant intergroup difference.

classified as belonging to Group 2B (possibly carcinogenic to humans) in an International Agency for Research on Cancer (IARC) publication[21]. It has also been reported that TiO$_2$ has carcinogenic potential[22] and causes formation of DNA adducts[23]. On the other hand, it has also been reported that TiO$_2$ should not exert carcinogenic effects on the human lung based on an overview epidemiology and toxicology studies in mice, rats and hamsters[24].

In general, nanoparticles are likely to exert a greater impact because of the areas and characters of their surfaces[25]. The causes of the lack of clear effects in experiment 2 might be related to the low doses or frequencies of instillation used. Intratracheal administration of nanoparticles induces severe inflammatory changes in the acute phase in the rat lung at high doses[5], but given chronically, the nanoparticles may be eliminated easily and may induce only mild inflammatory changes. It is well known that nanoparticles spread from the primary target-organ to other organs. For example, in an oral administration study in mice, nanoparticles of titanium dioxide were found to be retained in many organs, such as the liver, spleen, kidneys and lung tissues, after uptake through the gastrointestinal tract[26]. This spreading ability to other organs of nanoparticles may indicate that the nanoparticle is cleared from the primary organ, like the lungs, earlier than microparticles. However there are no reports supporting this hypothesis, and further experimentation is needed to validate the evidence.

In conclusion, there were no significant intergroup differences in the neoplastic lesions induced by DHPN with nanoparticles of CuO or TiO$_2$ by i.t. despite a tendency for increase compared with the microparticle i.t. groups. However, this carcinogenic bioassay with DHPN appears useful for detection of the modified effects of instilled particles. Assessment of the harmful effects of nanoparticles is an important priority and should be pursued in more investigations, such as with intraperitoneal application of multiwall carbon nanotubes, which have been observed induce mesotheliomas in the p53+/- mouse[27].

References

1. Donaldson K and Seaton A. The Janus faces of nanoparticles. J Nanosci Nanotechnol. **7**: 4607–4611. 2007.
2. Yokohira M, Takeuchi H, Yamakawa K, Saoo K, Ikeda M, Matsuda Y, Zeng Y, Hosokawa K, Maeta H, and Imaida K. Establishment of a bioassay system for detection of lung toxicity due to fine particle instillation: Sequential histopathological changes with acute and subacute lung damage due to intratracheal instillation of quartz in F344 male rats. J Toxicol Pathol. **18**: 13–18. 2005.
3. Bruch J, Rehn B, Song W, Gono E, and Malkusch W. Toxicological investigations on silicon carbide. 2. In vitro cell tests and long term injection tests. Br J Ind Med. **50**: 807–813. 1993.
4. Yokohira M, Takeuchi H, Yamakawa K, Saoo K, Matsuda Y, Zeng Y, Hosokawa K, and Imaida K. Bioassay by intratracheal instillation for detection of lung toxicity due to

fine particles in F344 male rats. Exp Toxicol Pathol. **58**: 211–221. 2007.

5. Yokohira M, Kuno T, Yamakawa K, Hosokawa K, Matsuda Y, Hashimoto N, Suzuki S, Saoo K, and Imaida K. Lung toxicity of 16 fine particles on intratracheal instillation in a bioassay model using F344 male rats. Toxicol Pathol. **36**: 620–631. 2008.

6. Peterson LA and Hecht SS. O6-methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. Cancer Res. **51**: 5557–5564. 1991.

7. Ronai ZA, Gradia S, Peterson LA, and Hecht SS. G to A transitions and G to T transversions in codon 12 of the Ki-ras oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents. Carcinogenesis. **14**: 2419–2422. 1993.

8. Takeuchi H, Saoo K, Yokohira M, Ikeda M, Maeta H, Miyazaki M, Yamazaki H, Kamataki T, and Imaida K. Pretreatment with 8-methoxypsoralen, a potent human CYP2A6 inhibitor, strongly inhibits lung tumorigenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in female A/J mice. Cancer Res. **63**: 7581–7583. 2003.

9. Takeuchi H, Saoo K, Matsuda Y, Yokohira M, Yamakawa K, Zeng Y, Miyazaki M, Fujieda M, Kamataki T, and Imaida K. Dose dependent inhibitory effects of dietary 8-methoxypsoralen on NNK-induced lung tumorigenesis in female A/J mice. Cancer Lett. **234**: 232–238. 2006.

10. Huynh HT and Teel RW. Effects of intragastrically administered Pycnogenol on NNK metabolism in F344 rats. Anticancer Res. **19**: 2095–2099. 1999.

11. Chung FL, Kelloff G, Steele V, Pittman B, Zang E, Jiao D, Rigotty J, Choi CI, and Rivenson A. Chemopreventive efficacy of arylalkyl isothiocyanates and N-acetylcysteine for lung tumorigenesis in Fischer rats. Cancer Res. **56**: 772–778. 1996.

12. Kitamura Y, Umemura T, Kanki K, Ishii Y, Kuroiwa Y, Masegi T, Nishikawa A, and Hirose M. Lung as a new target in rats of 2-amino-3-methylimidazo[4,5-f]quinoline carcinogenesis: results of a two-stage model initiated with N-bis(2-hydroxypropyl)nitrosamine. Cancer Sci. **97**: 368–373. 2006.

13. Seike N, Wanibuchi H, Morimura K, Nishikawa T, Kishida H, Nakae D, Hirata K, and Fukushima S. Lack of promoting effect due to oral administration of dimethylarsinic acid on rat lung carcinogenesis initiated with N-bis(2-hydroxypropyl)nitrosamine. Cancer Lett. **175**: 113–119. 2002.

14. Konishi Y, Kondo H, Ikeda T, Kawabata A, Shoji Y, and Denda A. Effect of dose on the carcinogenic activity of orally administered N-bis(2-hydroxypropyl)nitrosamine in rats. Gann. **69**: 573–577. 1978.

15. Gary AB, Scot LE, Michel RE, Charles AM, and William

16. FM. Pathology of the Fischer Rat, Reference and Atlas. Academic Press, 350–357. 1990.

16. Watanabe T, Katsura Y, Yoshitake A, Masataki H, and Mori T. IPAP : Image processor for analytical pathology. J Toxicol Pathol. **7**: 353–361. 1994.

17. Valko M, Morris H, and Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. **12**: 1161–1208. 2005.

18. Prohaska JR, Geissler J, Brokate B, and Broderius M. Copper, zinc-superoxide dismutase protein but not mRNA is lower in copper-deficient mice and mice lacking the copper chaperone for superoxide dismutase. Exp Biol Med (Maywood). **228**: 959–966. 2003.

19. Wasser IM, de Vries S, Moenne-Loccoz P, Schroder I, and Karlin KD. Nitric oxide in biological denitrification: Fe/Cu metalloenzyme and metal complex NO(x) redox chemistry. Chem Rev. **102**: 1201–1234. 2002.

20. Abe M, Suzuki N, Yoshida M, Usuda K, Furukawa S, Juneja LR, Okubo T, and Nakae D. Possible carcinogenic risks of copper gluconate and their prevention by co-administered green tea catechins evaluated by a rat medium-term multi-organ carcinogenicity bioassay protocol. Food Chem Toxicol. **46**: 1760–1770. 2008.

21. Overall Evaluations of Carcinogenicity to Humans, Group 2B: Possibly carcinogenic to humans. IARC Monograph. **93**: 1–5. 2006.

22. Lee KP, Trochimowicz HJ, and Reinhardt CF. Pulmonary response of rats exposed to titanium dioxide (TiO_2) by inhalation for two years. Toxicol Appl Pharmacol. **79**: 179–192. 1985.

23. Gallagher J, Heinrich U, George M, Hendee L, Phillips DH, and Lewtas J. Formation of DNA adducts in rat lung following chronic inhalation of diesel emissions, carbon black and titanium dioxide particles. Carcinogenesis. **15**: 1291–1299. 1994.

24. Hext PM, Tomenson JA, and Thompson P. Titanium dioxide: inhalation toxicology and epidemiology. Ann Occup Hyg. **49**: 461–472. 2005.

25. Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, and Roberts SM. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol Sci. **90**: 296–303. 2006.

26. Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, and Chai Z. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett. **168**: 176–185. 2007.

27. Takagi A, Hirose A, Nishimura T, Fukumori N, Ogata A, Ohashi N, Kitajima S, and Kanno J. Induction of mesothelioma in p53+/– mouse by intraperitoneal application of multi-wall carbon nanotube. J Toxicol Sci. **33**: 105–116. 2008.