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G. 知的財産権の出願・登録状況 (予定を含む)

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特許出願人: 公立大学法人名古屋市立大学

2. 実用新案登録

なし

3. その他

なし

Ⅱ. 研究成果の刊行に関する一覧表

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Ⅲ. 研究成果の刊行物・別冊

Original Article

Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats

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ABSTRACT — The present study assessed a carcinogenic hazard of multi-wall carbon nanotube (MWCNT) in intact (not genetically modified) rodents. MWCNT (1 mg/kg body weight, 7 animals), crocidolite (2 mg/kg body weight, 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) was administered to male Fischer 344 rats (12 weeks old) by a single intrascrotal injection. Rats were autopsied immediately after death, when becoming moribund or at the end of the maximal observation period scheduled to be 52 weeks. After 37-40 weeks, however, 6 MWCNT-treated animals died or became moribund due to intraperitoneally disseminated mesothelioma (6/7, 85.7%) with bloody ascites. Peritoneal mesothelium was generally hypertrophic, and numerous nodular or papillary lesions of mesothelioma and mesothelial hyperplasia were developed. While mesothelioid cells were predominant in relatively early stage tumors, advanced stage mesotheliomas were constituted by 2 portions occupied by mesothelioid cells on the surface and spindle-shaped sarcomatous cells in the depth. In the latter, the histological transition was apparently observed between these 2 portions. Mesotheliomas were invasive to adjacent organs and tissues, and frequently metastasized into the pleura. Only 1 rat survived for 52 weeks in the MWCNT-treated group, and similar findings except mesothelioma were observed. All 10 crocidolite-treated and 5 vehicle-treated rats survived for 52 weeks without any particular changes except deposition of asbestos in the former case. It is thus indicated that MWCNT possesses carcinogenicity causing mesothelioma at a high rate in intact male rats under the present experimental conditions. The present data identifies a carcinogenic hazard of MWCNT and will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of this and similar upcoming materials.

Key words: Multi-wall carbon nanotube, Mesothelioma, Nanomaterial, Carcinogenicity, Hazard identification, Rat

INTRODUCTION

Hazardous and risky substances present in the human environment must be avoided or strictly controlled. For

this purpose, the risk assessment process is critical and should be conducted using established protocols based on scientific evidence. In recent years, progress of research and development in the industrial field has been con-

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tinuously introducing new materials into our society to make our life more comfortable and convenient than ever. Potential human and environmental risks of these newcomers, however, should be carefully clarified at as an early stage as possible during its development and prevalence in order to protect benefits of all stakeholders, including not only health profits of general consumers and people participating in the manufacturing, but also business interests of manufacturers.

Nanomaterials, provisionally defined as substances of which sizes are smaller than 100 nm at least in 1 dimension, have attracted much attention and being enthusiastically developed and supplied because of its promising future with a wide variety of potential application. It should be noted, however, that their outstanding reactivity due to the enormous surface area achieved by the infinitesimal size serves as their best merit at one side but also their worst demerit at the other side, especially when they come into the human and other living creatures' bodies (Medina *et al.*, 2007). Possible human and environmental risks of nanomaterials should thus be urgently but carefully elucidated, and efforts have globally started to be made on this issue (Donaldson *et al.*, 2006; Lam *et al.*, 2006; Singh and Nalwa, 2007).

Carbon nanotube, a new form of technological crystalline carbon, is one of the most anticipated nanomaterials because of its unique properties that are suitable for a variety of industrial products such as high-strength materials, electronics and biomedical apparatuses (Martin and Kohli, 2003; Scott, 2005). Regarding potential biological risks of carbon nanotube, only limited information is currently available, especially for its chronic effects. Nevertheless, cohesive and thus easily agglomerating nature and estimated long stability in the body are considered as possible factors for toxic influence (Lam *et al.*, 2006; Luo *et al.*, 2004; Takagi *et al.*, 2008a). There is another concern that carbon nanotube might cause a tragedy similar to that caused by asbestos, because of their similarities such as a fibrous/rod-shaped structure with a superbly high aspect ratio and a contamination of iron, and of the fact that carbon nanotube may be used as an asbestos substitute (Donaldson *et al.*, 2006; Maynard *et al.*, 2004; Lam *et al.*, 2006; Singh and Nalwa, 2007).

In 2008, our colleagues of the National Institute of Health Sciences of Japan have reported that multi-wall carbon nanotube (MWCNT) induces peritoneal mesotheliomas at an incidence (14/16, 87.5%) similar to the case of crocidolite (14/18, 77.8%), within 25 weeks after a single intraperitoneal administration to male mice heterozygously deficient in the *p53* gene, in which some of us participated (Takagi *et al.*, 2008a). While the study clear-

ly indicates a potential carcinogenic hazard of MWCNT, it is then absolutely necessary to perform the hazard identification using intact (not genetically modified) animals before forwarding steps/stages of the risk assessment process. In this context, we planned and conducted the present small-sized study simply aiming at an identification of a carcinogenic hazard of MWCNT in intact rodents, before starting a more detailed dose-dependent study that is now being executed in our laboratories.

MATERIALS AND METHODS

Ethical consideration of the experiments

An experimental protocol was approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to its execution and monitored at every step during the experimentation for its scientific and ethical appropriateness, including concern for animal welfare, with strict obedience to the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and other similar laws, guidelines, rules and *etc.* provided domestically and internationally.

Animals

A total of 22 male Fischer 344 DuCrjCrj rats were purchased at their age of 4 weeks old from Charles River Inc. (Kanagawa, Japan). Rats were housed individually in stainless-steel cages (220 x 200 x 160, in millimeter) with wire-netting fronts and floors. The cages were suspended from belt-type racks with an automatic water-supply system providing tap water. The animal room was air-conditioned as 24-25°C, 50-60% relative humidity, and 10 times ventilation per hour using air drawn into the animal room by passing through a filter at the efficiency of 99.9% (HEPA filter). Fluorescent lighting was controlled to give a 12-hr light (6:00-18:00)/dark cycle. After an 8-week acclimation, rats were used for experimentation at their age of 12 weeks old, when the average body weight was 235 g. Animals were given tap water and a CE-2 pellet diet (Clea Japan Inc., Tokyo, Japan) *ad libitum*, critically monitored to detect any clinical signs and deaths, and weighed weekly throughout the acclimation and experimental periods.

Test chemicals

The presently utilized test chemicals, MWCNT (MITSUI MWCNT-7; lot number, 060125-01k) and UICC-

grade crocidolite (stocked at the Tokyo Metropolitan Institute of Public Health) were exactly identical to those used in the *p53* gene-deficient mice study of Takagi *et al.* (2008a). To examine the property of MWCNT, therefore, the same methods were utilized as described by Takagi *et al.* (2008a) such that particle number per unit weight as well as width and length distribution were measured scanning electron microscopically using a 5% Triton X-100 (Qbiogene, CA, USA) suspension (1.03 mg/5 ml), while contents of elements such as iron, sulfur, chlorine, fluorine and bromine were determined by a collision type inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies, Inc., Santa Clara, CA, USA) and a combustion ion chromatography (AQF-100, DX-120, Dia Instrument Co., Ltd, Kanagawa, Japan).

MWCNT and crocidolite were administered to rats as suspensions in 2% carboxymethyl cellulose (CMC) (Kanto Chemical Co., Inc., Tokyo, Japan) solution at concentrations of 0.5 and 1.0 mg/ml for MWCNT and crocidolite, respectively. These suspensions as well as a vehicle 2% CMC solution were sterilized by an autoclave at 120°C for 20 min and vigorously mixed by hand-shaking immediately prior to the administration. States of MWCNT and crocidolite in the administering suspensions in 2% CMC were assessed light microscopically, and in addition a state of MWCNT in a water suspension was separately assessed using a transmission electron microscope (TEM), because CMC cannot be used as a medium for the ultrastructural assessment.

Animal treatments

Rats at an average body weight of 235 g were administered vehicle (5 animals), crocidolite (10 animals, 2.0 mg/kg body weight corresponding to 0.47 mg/rat) or MWCNT (7 animals, 1.0 mg/kg body weight corresponding to 0.24 mg/rat) by a single intrascrotal injection for which the anterior skin of the scrotum was surgically incised 2-3 mm in length under the anesthesia with pentobarbital (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and suspensions of test chemicals or a vehicle solution were administered into the scrotal cavity at a volume of 2 ml/kg body weight. The doses of test chemicals were decided as described below. Rats were then maintained under critical monitoring for the maximal observation period scheduled to be 52 weeks. When rats died or became moribund in the middle of such a period, they were immediately autopsied. At the end of week 52, all surviving animals were sacrificed under light ether anesthesia by exsanguination.

Pathological assessment

At autopsy, rats were macroscopically examined throughout the body including celomic cavities. All major organs and tissues, especially tumor tissues of the inner-surface of such cavities and organs suspected to be involved by tumor, were taken, fixed in 10% neutrally-buffered formalin, embedded in paraffin and processed by routine hematoxylin and eosin staining for the histological examination.

Statistical analysis

Statistical significance of intergroup difference for the tumor incidence was assessed using Fisher's exact test, and *p*-values less than 0.05 were considered significant.

RESULTS

Property of test chemicals, their state in suspensions and decision of their administrating doses

As aforementioned, the test chemicals used in the present study were exactly identical to those used in the *p53* gene deficient mice study of Takagi *et al.* (2008a), and these 2 studies utilized the same methods to determine their property. As expected, therefore, property of test chemicals of the present study was virtually the same as that described by Takagi *et al.* (2008a). Number of particles per unit weight of MWCNT was 3.55×10^9 particles/mg, while that of crocidolite was 2.93×10^9 particles/mg (Moalli *et al.*, 1987). Width of MWCNT particles formed Gaussian distribution with a peak at 90 nm, and 82% of particles belonged in a range of 70-110 nm (Fig. 1a). In the case of crocidolite, a peak of width distribution located between 110-200 nm, and 81.3% of particles belonged in a range of 30-400 nm (Moalli *et al.*, 1987). Length of MWCNT particles also formed Gaussian distribution with a peak at 2 μ m, and 72.5% of particles belonged in a range of 1-4 μ m (Fig. 1b). In the case of crocidolite, a peak of length distribution located between 1.1-2.0 μ m, and 91.5% of particles belonged in a range of 0.1-5 μ m (Moalli *et al.*, 1987). Within a certain weight of MWCNT, therefore, substantially fewer and thinner particles were present when comparing with crocidolite, while length distribution was similar for both test chemicals.

The contents of iron, sulfur and chlorine of MWCNT were 3,500, 470 and 20 ppm, respectively, whereas fluorine and bromine (the respective detection limits being 5 and 40 ppm) could not be detected. The iron content of MWCNT was thus only 1.2-1.3 hundredths of that of crocidolite, estimated to be approximately 26-29% (Roller *et al.*, 1996) or 27% (Matsuoka *et al.*, 2003; Poser *et al.*,

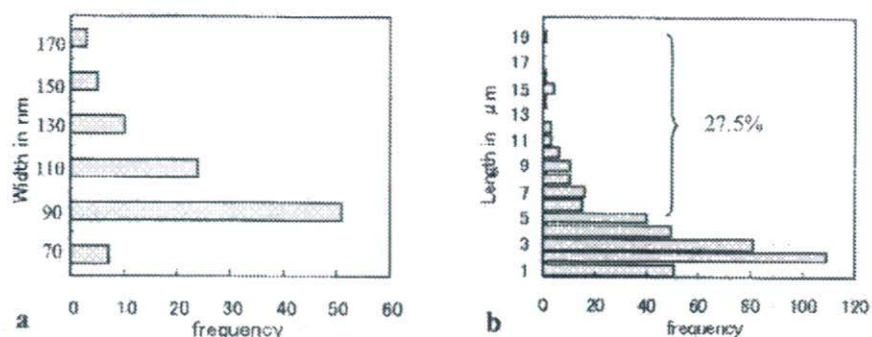


Fig. 1. Size distribution, (a) width and (b) length, of MWCNT used in the present study.

2003).

In a water suspension, the state of MWCNT was demonstrated by TEM as fine fibrous particles in occasional association with agglomerates (Fig. 2a). At higher magnifications, MWCNT particles appeared as multi-layered hollow fibers with round ends and highly electron-dense areas scattering outside of the fiber walls (Figs. 2b-d). In a CMC suspension, the state of MWCNT was light microscopically demonstrated as the coexistence of agglomerates and dispersed as multi-sized rod-shaped or fibrous particles (Fig. 2e), well in accordance with the above TEM image and suggesting the coexistence of minute fibrous particles that could not be seen under a light microscope. In contrast, crocidolite was dispersed in CMC as rod or needle-shaped particles (Fig. 2f).

Taking above-mentioned property and state in suspensions into consideration, the dose of MWCNT was decided to be 0.24 mg/animal (1 mg/kg body weight), corresponding to 0.85×10^5 particles/animal (3.62×10^6 particles/kg body weight) to be approximately 1/10 of a moderate value of the reported ranges (Roller *et al.*, 1997) corresponding to the maximum value recommended by the draft guideline for man-made mineral fibers (Bernstein and Riego Sintes, 1999). The dose of crocidolite was then decided to be 0.47 mg/animal (2 mg/kg body weight), corresponding to 13.77×10^6 particles/animal (58.60×10^8 particles/kg body weight), because we planned to make the doses of test chemicals in an equivalent range at least as a weight basis under consideration of the fact that the dose of crocidolite needs to be high enough to cause carcinogenicity (Adachi *et al.*, 2001; Cullen *et al.*, 2002; Davis, 1976; Mackay *et al.*, 1987; Vasilieva *et al.*, 1998; Wagner *et al.*, 1984; Whitaker *et al.*, 1984).

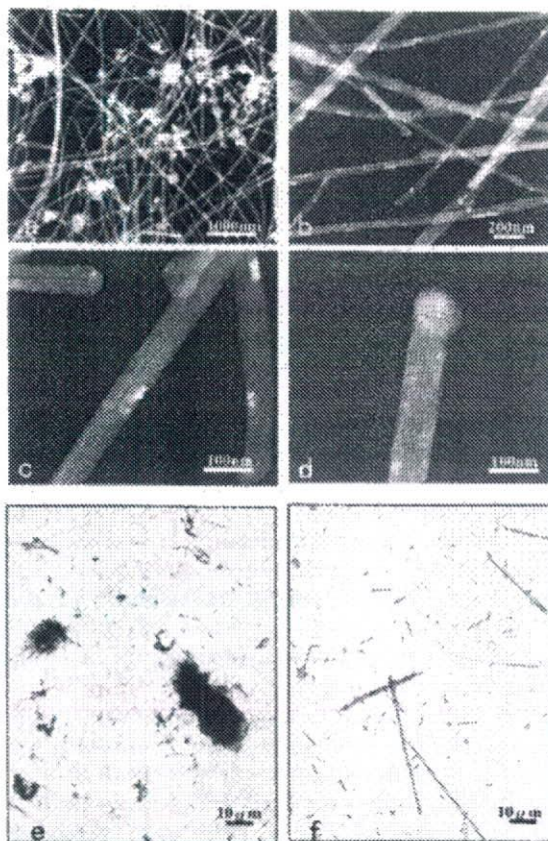


Fig. 2. Representative TEM image of a water suspension of MWCNT (a, x 10000; b, x 30000; c, x 50000; d, x 100000), and light microscopic appearance of 2% CMC suspensions of (e) MWCNT and (f) crocidolite used for the administration.

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Animal experiment**General findings**

The timings of autopsy are summarized in Table 1. All vehicle- and crocidolite-treated rats healthily survived throughout the 52-week maximal observation period. In contrast, 4 out of 7 MWCNT-treated rats died during weeks 37-40, 2 other rats became moribund at the ends of week 40 and week 50, and only 1 rat healthily survived until the end of week 52. In dead and moribund rats treated with MWCNT, severe anemia and enlargement of abdomen due to accumulation of ascites were common-

ly observed, and their body weights were decreased, or in some cases conversely increased due to marked ascites, for several weeks before autopsied. It is thus meaningless to compare group values of body weight between MWCNT-treated rats and other rats, whereas body weight values or a growth trend of crocidolite-treated animals were not different from those of vehicle-treated, control animals.

Macroscopic findings

The macroscopic findings observed in rats are summarized as an individual animal basis in Table 2. There were

Table 1. Summary of timing of autopsy

Treatment	Total number of rats	Timing of autopsy (weeks after commencement)							
		26	27	30	37	39	40	50	52
Vehicle	5								5 (S)*
Crocidolite	10								10 (S)
MWCNT	7				1 (D)	1 (D)	2 (D), 1 (M)	1 (M)	1 (S)

*Number of rats autopsied with the autopsy status specified in the parenthesis: S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

Table 2. Macroscopic findings as an individual animal basis

Treatment	Animal number	Autopsy status*	Timing of autopsy (weeks after commencement)	Findings**			
				Hemorrhagic ascites	Adhesion	Tumor nodule	
						Peritoneal cavity	Thoracic cavity
Vehicle	V1-V5	all S	all 52	-	-	-	-
Crocidolite	C1-C10	all S	all 52	-	-	-	-
MWCNT	M1	M	50	++	+++	+++	-
	M2	D	37	++	+++	+++	+
	M3	D	40	++	++	++	+
	M4	D	39	+++	+++	+++	+
	M5	S	52	-	++	+	-
	M6	M	40	+	++	++	-
	M7	D	40	+++	+++	+++	+

*S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

**Presented as grade determined under the following criteria.

Hemorrhagic ascite (volume): +, < 20 ml; ++, 20-50 ml; +++, > 50 ml.

Adhesion (affected area): +, < 5%; ++, 25-75%; +++, > 75%.

Tumor nodules: +, < 2 mm in diameter, focal; ++, 2-10 mm in diameter, diffuse; +++, 2-10 mm in diameter, diffuse and the presence of large tumor mass involving in the diaphragm, liver, stomach, pancreas and spleen.

no apparent macroscopic findings observed in vehicle-treated, control rats. In crocidolite-treated rats, scattered bluish-green spots were observed on the serosal surface.

Representative macroscopic appearances of MWCNT-treated rats upon autopsy are demonstrated in Fig. 3. Hemorrhagic ascites at an amount of 5-75 ml was present in the abdominal cavity of 4 dead and 2 moribund rats in association with severe fibrous adhesion of organs/tissues and the peritoneum, especially among the diaphragm, liver, stomach, pancreas, spleen and omentum. The liver was strongly deformed, resulting in difficulty to identify lobular segmentation. In such animals, whitish nodules with varied sizes and polypoid or papillary shapes were disseminated throughout the peritoneal wall including that of the scrotal cavity and occupied visceral peritoneum of

organs/tissues. While the majority of such nodules were small (up to 2 mm in diameter), large tumors (5-20 mm in diameter) were occasionally observed, mostly around the diaphragm and involving the liver, stomach, pancreas, spleen and their surrounding stroma. Abdominal adipose tissues were largely replaced by tumor nodules. In the thoracic cavity, tumor nodules were observed only on the surface of the diaphragm with the exceptions of metastatic lesions detected on the peri- and epicardium detected of 4 dead animals. In the MWCNT-treated rat surviving at the end of week 52, moderate fibrous adhesions and small tumor nodules were observed on the parietal and visceral peritoneum including the wall of the scrotal cavity, but ascites were not present. In addition, small black-colored spots scattered on the peritoneum of all MWCNT-treated rats.

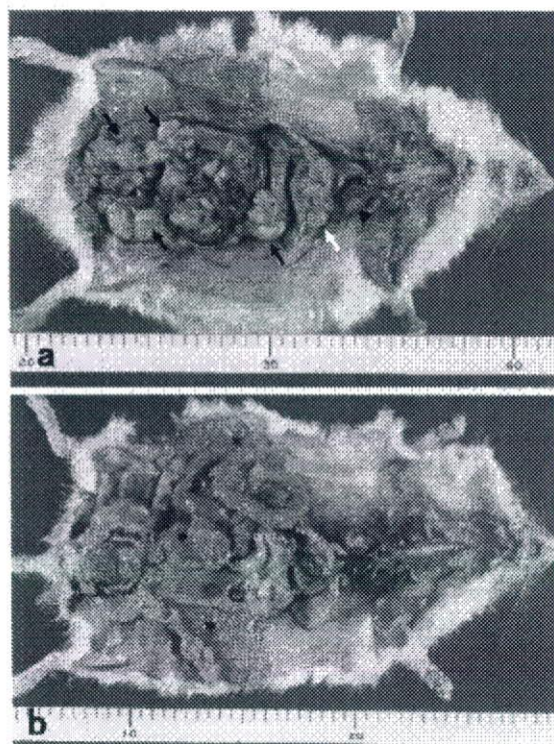


Fig. 3. Representative macroscopic appearances of rats treated with MWCNT. (a) Multiple tumor nodules were observed on the peritoneum (black arrows) and the epicardium (arrowhead), and a large tumor mass involved in diaphragm and liver (white arrow). Visceral organs were severely adhered. (b) Multiple small nodules spread on visceral and parietal peritoneum (asterisk). Black deposits of MWCNT were observed on various sites of the peritoneum.

Histological findings regarding mesothelial proliferating lesions

Histological findings for mesothelial proliferative lesions observed in rats are summarized as an individual animal basis in Table 3. While no mesothelial abnormality was found in vehicle- or crocidolite-treated rats, mesothelial hyperplasias and mesotheliomas were observed in 7 and 6 out of 7 MWCNT-treated rats, respectively. The overall incidence of mesothelioma in MWCNT-treated rats was thus calculated to be 86%, and this value was significantly higher than those of vehicle- or crocidolite-treated rats (both 0%) ($p < 0.05$).

In the peritoneum of MWCNT-treated rats, mesothelial cells were generally hypertrophic, and mesothelial hyperplasias and mesotheliomas were frequently observed. Small-sized polypoid or papillary mesotheliomas were early-stage tumors (representative histology demonstrating in Fig. 4) that grew up toward the celomic cavity and consisted of enlarged, pleomorphic mesothelioid tumor cells having nuclei with prominent nuclear membrane and nucleoli, and basophilic cytoplasm. In the central region of such tumors, necrotic tissue and/or a fibrous matrix were often present. In contrast, massive mesotheliomas were advanced-stage tumors (representative histology demonstrating in Fig. 5) that invaded into adjacent organs/tissues and destructed their architecture. These tumors were composed of 2 morphologically different portions; *id est*, the superficial layer consisting of mesothelioid tumor cells and the deep layer consisting of spindle-shaped sarcomatoid cells; and the histological transition between these portions was apparently observed. Furthermore in these large tumors, osteoid and carcifying osteoid changes were sometimes observed as a secondary, reactive phenomenon. These histological characteristics of mesotheli-

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Table 3. Histological findings for mesothelial proliferating lesions as an individual animal basis

Treatment	Animal number	Autopsy status*	Timing of autopsy (week after commencement)	Mesothelial hyperplasia	Mesothelioma**		
					Development	Invasion	Osteoid change
Vehicle	V1-V5	all S	all 52	-	-	-	-
Crocidolite	C1-C10	all S	all 52	-	-	-	-
MWCNT	M1	M	50	+	+	+	+
	M2	D	37	+	+	+	-
	M3	D	40	+	+	+	+
	M4	D	39	+	+	+	+
	M5	S	52	+	-	-	-
	M6	M	40	+	+	+	-
	M7	D	40	+	+	+	+

*S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

**The overall incidence of mesothelioma in rats treated with MWCNT was 6 out of 7 (86%), significantly higher than those in rats treated with vehicle (0 out of 5, 0%) or crocidolite (0 out of 10, 0%) ($p < 0.05$).

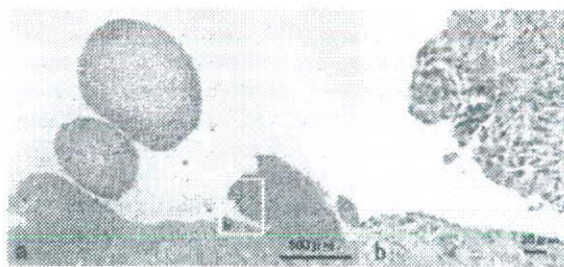


Fig. 4. Representative histology of relatively early-stage mesotheliomas observed in rats treated with MWCNT. (a) Mesotheliomas arose from the peritoneal surface with necrotic tissue and/or a fibrous matrix in its central region of nodules. (b) Under high magnification, round and basophilic mesothelioid tumor cells proliferated in the peripheral region of polypoid or papillary mesothelioma nodules.

oma were identically observed in the thoracic metastatic lesions without any continuity from the mesothelial neoplastic or non-neoplastic changes seen in the peritoneal cavity and the thoracic-side of the diaphragm.

Histological findings regarding granulomatous lesions in the celom

Granulomas were found in the celom of both crocidolite- and MWCNT-treated, but not vehicle-treated, animals (Fig. 6).

In the MWCNT-treated rats, granulomas scattered in the submesothelial layer of the fibrously thickened parietal and visceral peritoneum and were relatively large in the scrotal cavity. Such granulomas were with high cellularity including macrophages and multinucleated giant cell, and contained MWCNT agglomerates and non-agglomerated particles (Fig. 6a), indicating them to be active. The distribution patterns of these granulomas and mesotheliomas were totally independent.

In the crocidolite-treated rats, granulomas were distributed similarly to the MWCNT-treated rats. The cellularity was, however, much lesser, and crocidolite was found as fine fiber-shaped particles within a rich collagenous matrix (Fig. 6b), indicating them to be almost inactive.

Histological findings regarding intraorgan distribution of test chemicals

Fibrously shaped MWCNT particles were found also within organs, for instance in the cytoplasm of portal macrophages (Fig. 7a) and Kupffer cells (Fig. 7b) of the liver, as well as macrophages and multinuclear giant cells of the mesenteric lymph nodes (Fig. 7c). Similar phenomena were found in crocidolite-treated rats infrequently and with lesser amount of particles (data not shown).

Other histological findings

Several other lesions were histologically found in rats, but their incidences, multiplicities and severity were in a