Ⅲ．研究成果の刊行に関する一覧表

書籍

著者名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

著者名	論文タイトル	発表誌名	巻号	ページ	出版年
Takagi A, Hirose A, Nishimura T, Fukumori N, Ogata A, Ohashi N, Kitajima S, Kanno J.	Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube.	Toxicol Sci.	33(1)	105-116	2008
Onishi, T., Fukamachi, K., Ohshima, Y., Jiegou, X., Ueda, S., Iigo, U, Takasuka, N., Naito, A., Fujita K., Matsuoka Y., Izumi K., and Tsuda, H.	Possible application of human c-Ha-ras proto-oncogene transgenic rats in a medium-term bioassay model for carcinogens.	Tox. Pathol.	35	436-443	2007
Tsuda, H., Iigo, M., Takasuka, N., Ueda, S., Ohshima Y., Fukamachi, K., Shirai, T., Hirano, S., Matsuda, E., and Wakabayashi, K.	Possible enhancing activity of diacylglycerol on 4-nitroquinoline 1-oxide induced carcinogenesis of the tongue in human c-Ha-*ras* proto-oncogene transgenic rats.	Food and Chemical Toxicology	45	1013 -1019	2007
Iigo, M,, Shimamura, M., Hirano, S., Tokutake, S., Alexander, D.B. and Tsuda, H. Tanaka, T., (Editor)	Cancer prevention and anti-metastatic effects by oral administration of procyanidins.	Cancer: Dosease Progression and Chemoprev ention.	ISBN :81-3 08-01 50-7	277-297	2007
Naiki-Ito, A., Asamoto, M., Jplaowadp, M., Talajasjo S., Uamashita, H., Tsuda, H., Ogawa, K. and Shirai, T.	Gpx2 Is an Overexpressed Gene in Rat Breast Cancers Induced by Three Different Chemical Carcinogens.	Cancer Res	67 (23)	11353 -11358	2007
Matsuoka, T., Hamaguchi, T., Fukamachi, K., Yoshida, M., Watanabe G., Taaya, K., Tsuda, H. and Tsubura, A.	Molecular analysis of rat mammary carcinogenesis: an approach from carcinogenesis research to cancer prevention.	Med Mol Morphol	40	185-190	2007
西村哲治	ナノ粒子の有害性評価とリスク対策	技術情報協会		33-42	2007

Burlinson, B., Tice, RR., Speit, G., Agurell, E., Brendler-Schwaab, SY., Collins, AR., Escobar, P., Honma, M., Kumaravel, TS., Nakajima, M., Sasaki, YF., Thybaud, V., Uno, Y., Vasquez, M., and Hartmann, A.	Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup	Mutat. Res.	627	31-35	2007
Moore, MM., Honma, M., Clements, J., Bolcsfoldi, J., Burlinson, B., Cifone, M., Clark, J., Clay, P., Doppalapudi, R., Fellows, M., Gollapudi, B., Hou, S., Jenkinson, J., Muster, W., Pant, K., Kidd, DA., Lorge, E., Lloyd, M., Myhr, B., O'Donovan, M., Riach, C., Stankowski, Jr. LF., Thakur, AK., and Van Goethem, F.	Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment.	Mutat. Res.	627	36-40	2007
Ku, WW., Bigger, A., Brambilla, G., Glatt, H., Gocke, E., Guzzie, PJ., Hakura, A., Honma, M., Martus, H-J., Obach, RS., and Roberts, R.	Strategy for genotoxicity testing-Metabolic considerations	Mutat. Res.	627	59-77	2007
Wang, J., Chen, T., Honma, M., Chen, L.,Moore, M.	3'-Azido-3'-deoxythymidine induces deletions in L5178Y mouse lymphoma cells.	Environ. Mol. Mutagen.	48	248-257	2007
Honma, M., Sakuraba, M., Koizumi, T., Takashima, Y., Sakamoto, H., and Hayashi, M.	Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells.	DNA Repair	6	781-788	2007
Luan Y, Suzuki T, Palanisamy R, Takashima Y, Sakamoto H, Sakuraba M, Koizumi T, Saito M, Matsufuji H, Yamagata K, Yamaguchi T, Hayashi M, Honma M.	Potassium bromate treatment predominantly causes large deletions, but not GC>TA transversion in human cells.	Mutat. Res.	619	113-123	2007
Newwirth, E., Honma, M., and Grosovsky, A.	Interchromosomal crossover in human cell is associated with long gene conversion tracts.	Mol. Cell. Biol.	27	5261 -5274	2007

Yatagai, F, Umebayashi, Y., Suzuki, M., Abe, T., Suzuki, H., Shimazu, T., Ishioka, N., Iwaki, M, and Honma, M.	Influence of low-dose and low-dose-rate ionizing radiation on mutation induction in human cells.	Advan. Space Res.	40	470-473	2007
Yatagai, F., Umebayashi, Y., Honma, M., Sugasawa, K., Takayama, Y., and Hanaoka, F.	Mutagenic radioadaptation in a human lymphoblastoid cell line.	Mutat. Res.	638	48-55	2008
Sato K, Akaishi T, Matsuki N, Ohno Y, Nakazawa K	β-Estradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells.	Brain Res.	1150	108-120	2007
Sato K, Saito Y, Oka J, Ohwada T, Nakazawa K	Effects of tamoxifen on L-glu transporters of astrocytes.				(Submitted)
Usami, M., Mitsunaga. K. and Nakazawa, K.	Two-dimensional electrophoresis of protein from cultured postimplantation rat embryos for developmental toxicity studies	Toxicol. In Vitro	21	521-526	2007
Nakajima, M., Takahashi, H., Nakazawa K.and Usami, M.	Fetal cartilage malformation by intravenous administration of indium trichloride to pregnant rats	Reproduct. Toxicol.	24	409-413	2007
Usami, M., Mitsunaga, K. and Nakazawa, K.	Comparative proteome analysis of the embryo proper and yolk sac membrane of day 11.5 cultured rat embryos	Birth Defects Res.	B 80	383-395	2007

Ⅳ．研究成果の刊行物・別冊

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.33, No.1, 105-116, 2008

Original Article

Induction of mesothelioma in p53+/– mouse by intraperitoneal application of multi-wall carbon nanotube

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(Received November 20, 2007; Accepted December 9, 2007)

ABSTRACT — Nanomaterials of carbon origin tend to form various shapes of particles in micrometer dimensions. Among them, multi-wall carbon nanotubes (MWCNT) form fibrous or rod-shaped particles of length around 10 to 20 micrometers with an aspect ratio of more than three. Fibrous particles of this dimension including asbestos and some man-made fibers are reported to be carcinogenic, typically inducing mesothelioma. Here we report that MWCNT induces mesothelioma along with a positive control, crocidolite (blue asbestos), when administered intraperitoneally to p53 heterozygous mice that have been reported to be sensitive to asbestos. Our results point out the possibility that carbon-made fibrous or rod-shaped micrometer particles may share the carcinogenic mechanisms postulated for asbestos. To maintain sound activity of industrialization of nanomaterials, it would be prudent to implement strategies to keep good control of exposure to fibrous or rod-shaped carbon materials both in the workplace and in the future market until the biological/ carcinogenic properties, especially of their long-term biodurability, are fully assessed.

Key words: Multi-wall carbon nanotube (MWCNT); Asbestos; Fullerene; Mesothelioma; P53 heterozygous mouse; Micrometer particles

INTRODUCTION

A rapid increase in the usage of nanomaterials in consumer products and medical applications in the near future underlines the importance of understanding its potential toxicity to people and the environment (Lam *et al.*, 2006; Donaldson *et al.*, 2006). Among them, carbon nanotubes and fullerenes have been one of the most extensively researched and developed nanoparticles. Carbon nanoparticles tend to aggregate into micrometer particles due to their cohesive characteristics (Lam *et al.*, 2006; Luo *et al.* 2004). And they are considered to be very stable in the organism. These two elements lead us toxicologists to consider a concern of the chronic toxicity of micrometer-sized particles before any consideration is made for their pure nanometer-sized properties in our body. Once inside the body, the long-lasting scavenging and inflammatory activities towards the non-degradable micrometer-sized particles would lead to the continuous oxidative stress at their deposit sites, which eventually lead to tissue destruction and, on some occasion, carcinogenesis (Coussens and Werb, 2002). Additional concern is given to the fibrous or

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rod-shaped particles of micrometer length that share the dimension of asbestos reported to be carcinogenic to humans and experimental animals (Hei *et al.*, 2006; WHO,1986, 1998). Another factor reported to relate with carcinogenic potency of asbestos is the iron (Fe) content. The most potent asbestos (crocidolite or blue asbestos) contains the highest amount of Fe (WHO, 1986). It is explained that Fenton reaction would accelerate the generation of oxygen radical species that lead to carcinogenesis (Jiang *et al.*, 2006; Gulumian and Wyk, 1987).

MWCNTs form micrometer-sized particles of fiber or rod-shape. The diameter ranges from 0.01 to 0.2 micrometer (Hou *et al.*, 2003) and lengths may reach tens of micrometers that correspond to the size and shape of asbestos. Additionally, some CNTs are reported to contain a considerable amount of Fe due to its manufacturing process (Lam *et al.*, 2006). Deduced from those factors, we hypothesized that MWCNT might have carcinogenic potency similar to asbestos when administered to organisms via the same route of exposure. Here, we adopted a short-term bioassay, i.e., the p53 heterozygous mouse intraperitoneal exposure model reported to be sensitive to asbestos and develop mesotheliomas fast (Marsella *et al.*, 1997; Vaslet *et al.*, 2002). This mouse model has been reported to be sensitive not only to genotoxic carcinogens (Pritchard *et al.*, 2003) but also to reactive oxygen species (ROS)-related carcinogenesis (Tazawa *et al.*, 2007) and therefore fits with the postulated carcinogenesis mechanisms of asbestos and asbestos-like particles (Marsella *et al.*, 1997; Vaslet *et al.*, 2002).

MATERIALS AND METHODS

Experimental animals

The p53-heterozygos (p53(+/−)) mice were generously given by Dr. S. Aizawa (Tsukada *et al.*,1993). This p53 (+/−) mice were bred with normal wild-type C57BL/6 females (SLC, Shizuoka, Japan). After more than 20 generations of backcrossing, seventy-six male p53(+/−) mice of an age of 9 to 11 weeks were used in this experiment (nineteen per group). All mice were housed individually under specific pathogen-free conditions, with a 12 hr light-dark cycle at the animal facility of NIHS. They were given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., ltd.) *ad libitum*. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, Japan.

Histology

For evaluation of carcinogenicity, visceral organs including liver, kidney, spleen, lung, digestive tract and macroscopic tumors (*en bloc* in case of severe peritoneal adhesion) were fixed in 10% neutral buffered formalin. After conventional processing, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and

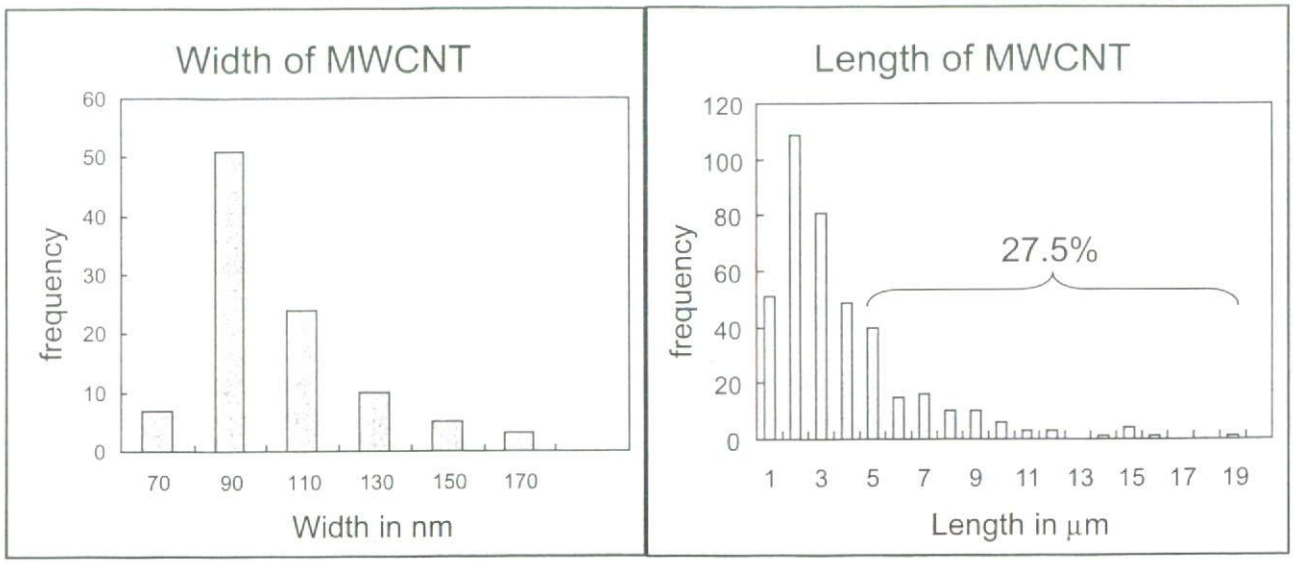

Fig. 1. Width and length distribution of MWCNT:
Width and length distribution of MWCNT (MITSUI MWCNT-7, Lot NO. 060125-01k) was measured at Tokyo Metropolitan Institute of Public Health. The average width was about 100 nm, and 27.5% of the particles were longer than 5 micrometer.

examined histopathologically under a light microscope.

Materials

Multi-wall carbon nanotube (MITSUI MWCNT-7, Lot NO. 060125-01k), UICC-grade Crocidolite (NIHS material stock), and fullerene (C_{60}, Nanom purple, Frontier Carbon Corporation, Tokyo, Japan) were used in this study.

The number of particles per weight and size distribution of MWCNT was determined as follows: 1.03 mg of MWCNT was suspended in 5 ml of 5%Triton X-100 (Qbiogene, CA, USA) and sonicated for 30 min, immediately diluted x100 by 5%TX-100, and then an aliquot of 5 microliters was mounted on a glass plate. The plate was heated up to 480 °C for 20 min by an electric oven, metallized by platinum and palladium, and subjected to scanning electron microscope observation. All visual fields were photographed. Number and length of the particles were measured on the enlarged photo prints. As a result, one gram of MWCNT corresponded to 3.55×10^{11} particles. The length and width distribution is shown in Fig. 1. The number of particles per weight of the UICC Crocidolite was reported as 2.93×10^{12} fibers/g (Moalli *et al.*, 1987). The contents of elements in the MWCNT were determined by collision type inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies, Inc. Santa Clara, CA, USA) and combustion ion chromatography (DX-120, Dionex Corporation, Sunnyvale, CA, USA). The average content of Fe was about 3,500 ppm (0.35%) by a microwave-assisted dissolution procedure with a mix-

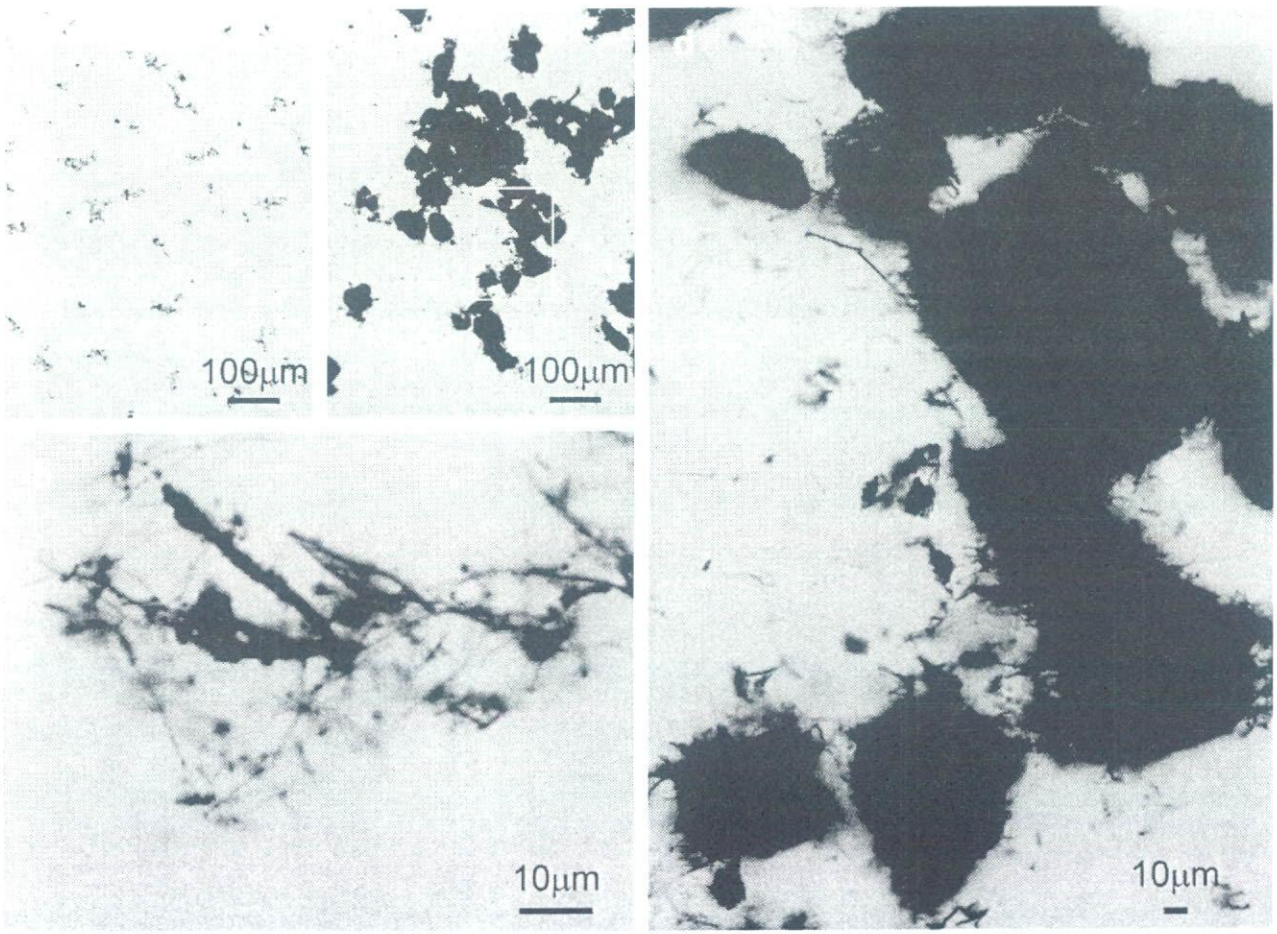

Fig. 2. Light microscopic view of administered MWCNT:
Light microscopic view of sonicated MWCNT sample suspension mounted on slide glasses. a) Well-dispersed area of the preparation. b) Close-up view of the boxed area in a). Fine fiber or rod-shaped particles longer than 10 micrometers are seen. c) Aggregated MWCNT. d) Close-up view of the boxed area in c) Aggregates are 50 to 200 micrometers in dimensions.

ture of nitric acid and perchloric acid. Sulfur content was about 470 ppm. Chlorine was 20 ppm and fluorine and bromine were below detection levels (5 and 40 ppm, respectively).

Preparation of particle suspension

MWCNT, crocidolite and fullerene were suspended at a concentration of 3 mg/ml to 0.5% methyl cellulose (Shin-Etsu Chemical Co., Ltd.) solution and autoclaved (121 °C, 15 min). After addition of Tween 80 (Tokyo Chemical Industry Co., Ltd.; final 1.0% conc.), the solutions were subjected to sonication by ultrasonic homogenizer (VP30s, TAITEC Co. Japan) (cf. Fig. 2).

Treatment of mice

Nineteen male p53 (+/–) mice at the age of 9 to 11 weeks were given single i.p. injection of 1×10^9 of MWCNT particles (corresponding to 3 mg/head) in 1 ml suspension. The number of the particles was set to a moderate value of the reported ranges (Roller *et al.*, 1997) which corresponds to the maximum value recommended by the draft guideline for man-made mineral fibers (Bernstein and Riego Sintes, 1999). Another 19 mice were given single i.p. injection of 3 mg/head suspension (1 ml) of fullerene, and as a positive control of this carcinogenesis study, another 19 mice were given 1×10^{10} of crocidolite in 1 ml of suspension (corresponds to 3 mg/head) at the first day of experiment. Vehicle solution (1 ml) was given to 19 mice as negative controls. Satellite groups consisting

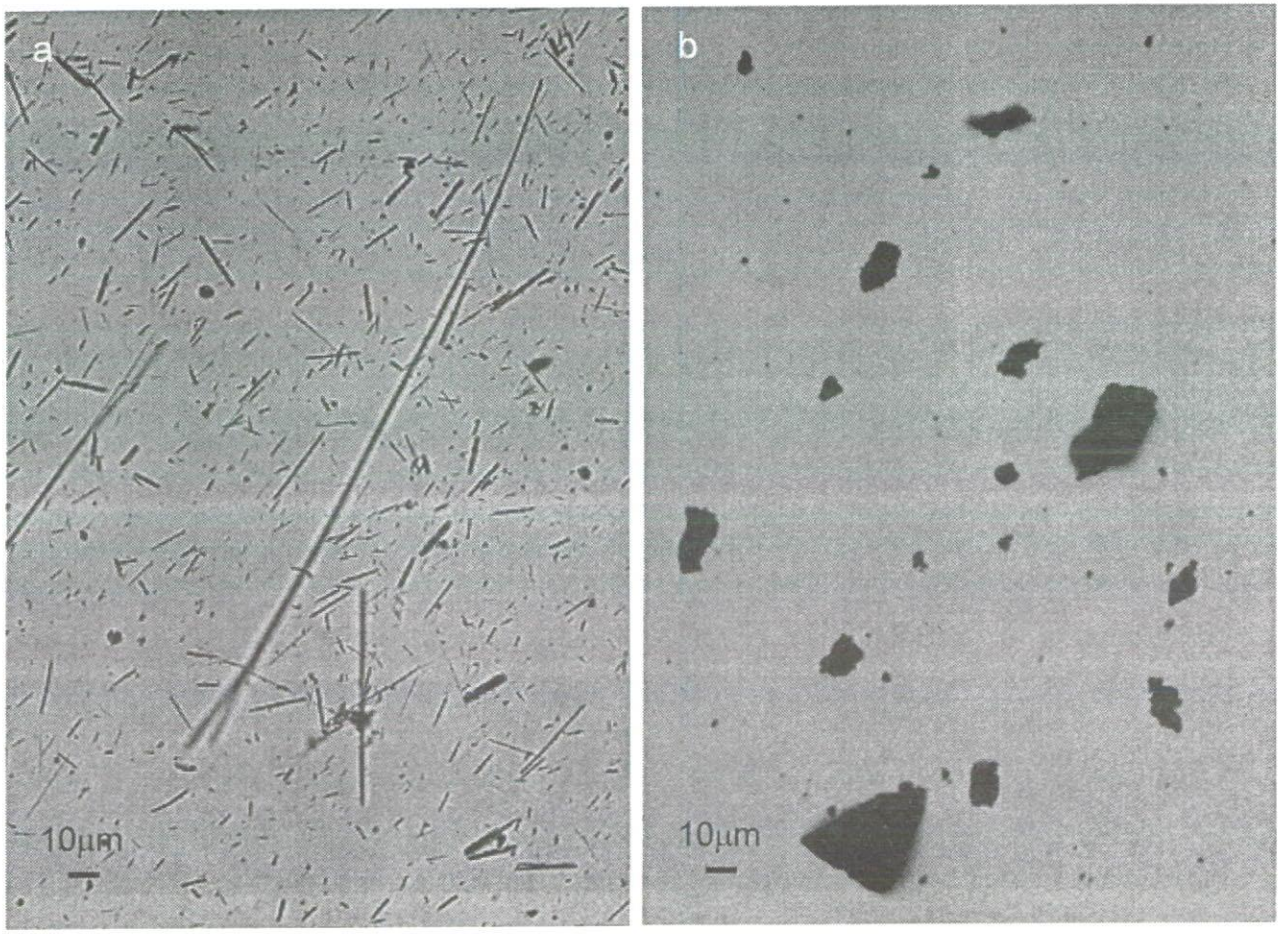

Fig. 3. Light microscopic views of administered crocidolite and fullerene:
Light microscopic views of administered crocidolite and fullerene. a) Crocidolite sample consisting of various lengths of rod-shaped particles. b) Fullerene sample consisted of sand grain-like particles of sizes ranging up to 50 micrometers.

Mesothelioma by MWCNT in p53 +/− mouse.

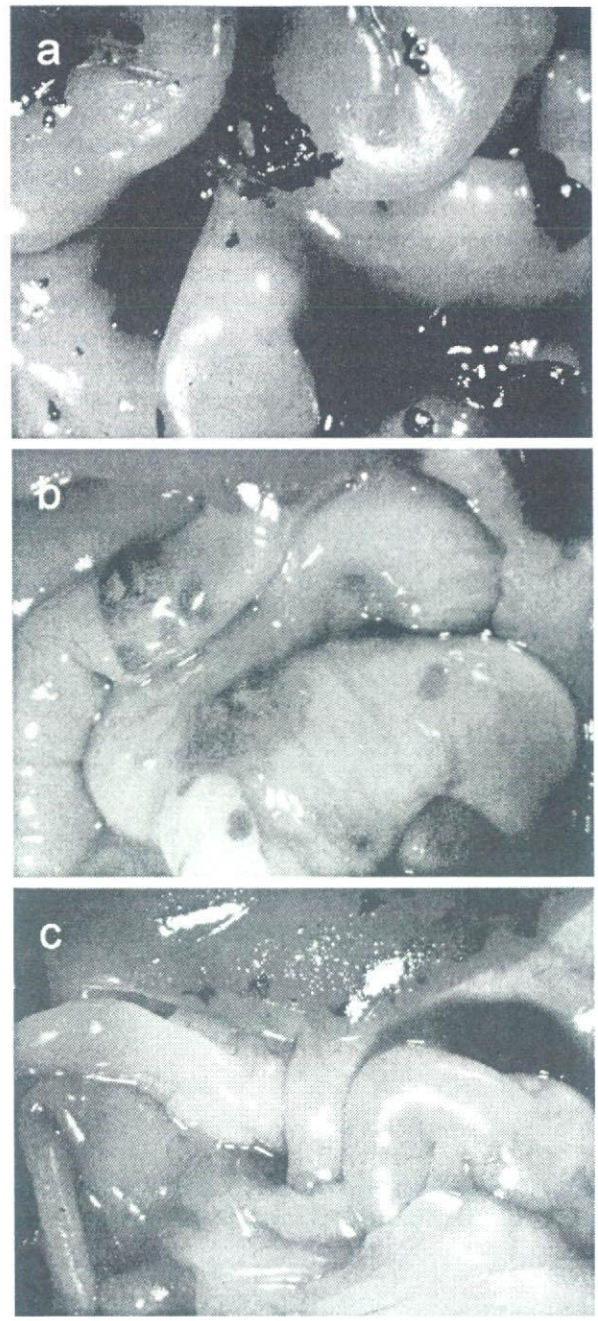

Fig. 4. Early peritoneal responses to MWCNT, crocidolite, and fullerene (10 days after i.p. injection):
Early findings of peritoneal cavity 10 days after i.p. administration of a) MWCNT inducing slight fibrinous deposit, adhesion, ascites retention, and edematous and hypotonic intestinal loops, b) crocidolite inducing slightly edematous intestinal loops, and c) fullerene with no obvious change except for black patchy deposits on the serosal surface.

of 6 wild-type C57BL/6 male mice each were similarly treated and sacrificed at day 10 for the observation of early peritoneal responses.

RESULTS

Although rigorously agitated prior to i.p. injection, the MWCNT sample contained aggregates among dispersed rod-shaped or fibrous particles (Fig. 2). Crocidolite sample was made of evenly dispersed rod-shaped or fibrous particles (Fig. 3a). Fullerene was in polygonal particles of micrometer size (Fig. 3b).

At day 10, the satellite groups were monitored for early responses (Fig.4). MWCNT mice showed slight fibrinous adhesion with a trace amount of ascites with scattered black spots of MWCNT aggregates. The intestine loops were edematous and hypotonic. Crocidolite mice showed similar responses but to a lesser extent, and there were no overt peritoneal adhesions. Bluish green spots of crocidolite aggregates were seen on the peritoneal surface. The Fullerene group showed minimal changes except for the black spots of aggregates on the serosal surfaces.

The vehicle control mice showed no overt change in peritoneal cavity.

The mice of main groups were monitored until one of the groups reached 100% mortality. The highest lethality

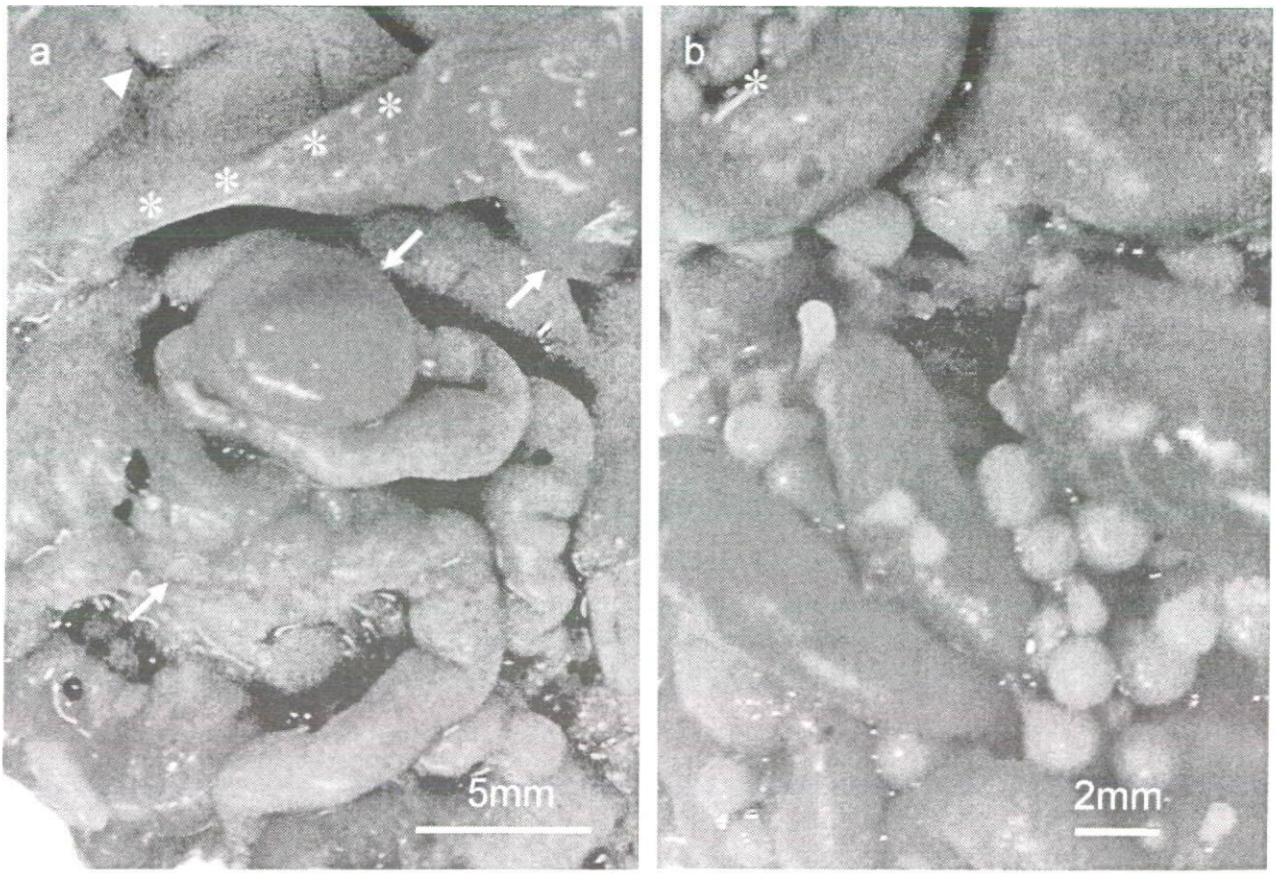

Fig. 5. Macroscopic view of abdominal viscera of MWCNT-treated and crocidolite-treated mouse:
Macroscopic view of the abdominal viscera excised *en bloc* of a) MWCNT-treated mouse that died at day 147, and b) crocidolite-treated mouse moribund on day 172 due to ileus. a) Fibrous adhesions of the visceral organs and multiple peritoneal tumor formation (arrows) are seen. Asterisks indicate the ventral cut end of diaphragma. One tumor penetrates the diaphragma and protrudes into pleural cavity (arrow head). Black spots are the aggregates of MWCNT. b) Multiple nodules up to 2 mm in diameter are induced on the serosal surface including liver (asterisk). Bluish green spots are the aggregates of crocidolite. Histology of the nodules is shown in Fig. 7a.

was seen in the MWCNT group followed by the Crocidolite group, and the study was terminated at week 25 (day 180) and all mice of the Control and the Fullerene groups and 6 of the Crocidolite group were subject to autopsy. MWCNT-treated mice revealed moderate to severe fibrous peritoneal adhesion with slight ascites, fibrous peritoneal thickening with occasional black-colored depositions and a high incidence of macroscopic peritoneal tumors up to 2.7 × 1.5 cm in size (Fig. 5a). Similar findings but to a lesser extent with bluish green deposits were seen in asbestos-treated mice. In some cases, small polyp-like nodules were seen over the serosal surface (Fig. 5b). The Fullerene group showed no peritoneal adhesion, fibrous thickening nor tumor induction. Only small black plaques were scattered on the serosal surface.

Histologically, peritoneal adhesion and fibrous thickening of the MWCNT group mice was due to the formation of fibrous scars and foreign body granulomas against the MWCNT with phagocytic cells including multinucleated giant cells. Adjacent to those fibrogranulomatous lesions, a spectrum of peritoneal mesothelial lesions was seen, from nodular mesotheliomatous pile-ups of atypical mesothelial cells (Fig. 6), typical epithelial mesotheliomas with occasional hobnail appearance and mild to moderate fibrovascular stem formation (Fig. 7a), to large tumors measuring up to 2.7 × 1.5 cm in size composed of anaplasic cells with high mitotic rate and occasional central necrosis compatible with the diagnosis of high-grade malignant mesothelioma (Fig. 7b). Large tumors are invasive to the abdominal wall, diaphragma, liver parenchyma, and pancreas, and in some cases involving the thoracic cavity. No distant metastasis was observed so far as exam-

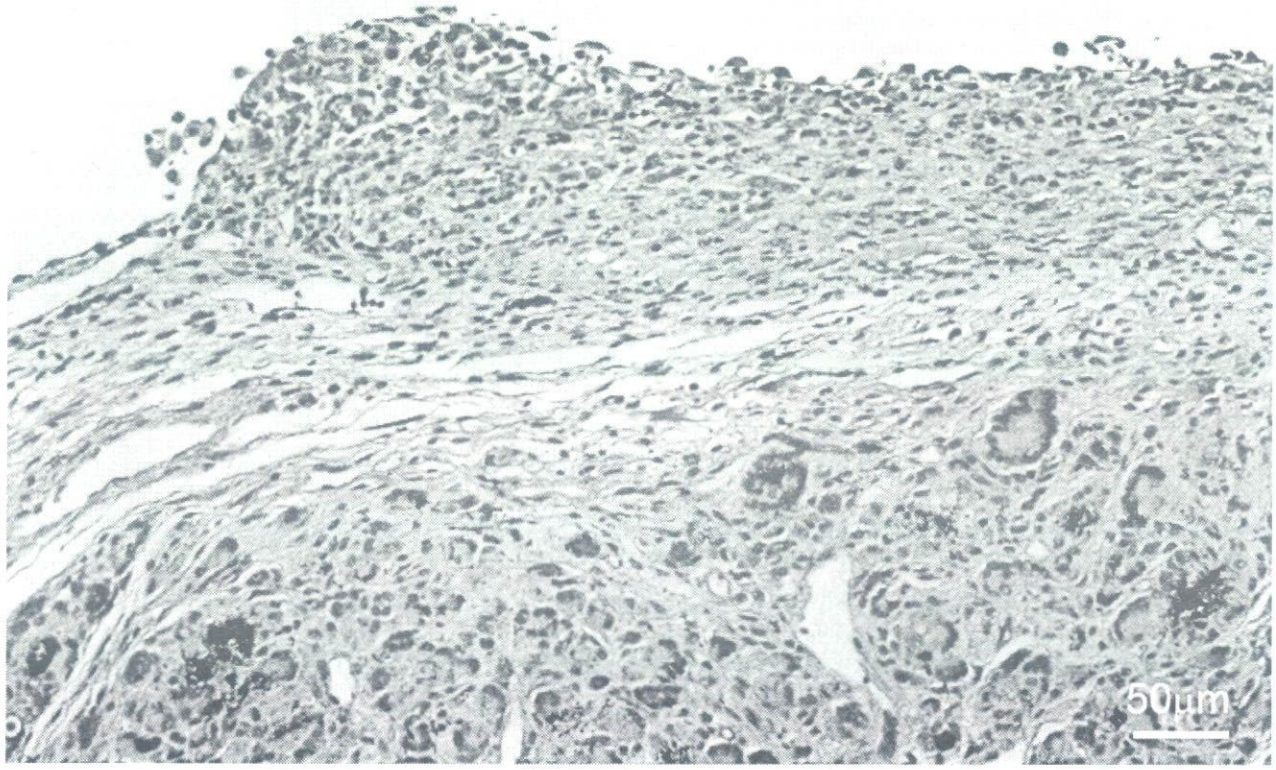

Fig. 6. Mesothelial response in MWCNT-treated mice:
Fibrous thickening of the peritoneum and foreign body granulomas against the MWCNT with phagocytic cells including multinucleated giant cells are formed in the MWCNT-treated mouse. Mesothelial lesions were found in the vicinity of fibrosis and granulomas. Microscopic mesotheliomatous plaques on the fibrotic peritoneum above a granuloma (MWCNT-treated mouse moribund on day 144 due to multiple mesotheliomas with severe peritoneal adhesion).

ined.

Cumulative mortality rate by mesothelioma is shown in Fig. 8. Mice with large/invasive mesotheliomas considered as cause of death are plotted by Kaplan-Meier method. Second major cause of death was constriction ileus due to severe peritoneal adhesion. Among those moribund/dead or terminated at week 25, there were 3 mice with incidental mesotheliomas in the MWCNT group (cause of death: all three by ileus) and 6 incidental mesotheliomas in the Crocidolite group (cause of death: three by ileus and three terminated at week 25). The overall incidence of mesothelioma after the first incidental case found in the MWCNT group at day 84 were 14/16 (87.5%, 11 found as cause of death, 3 as incidental) in MWCNT and 14/18 (77.8%, 8 found as cause of death, 6 as incidental including 3 terminated at week 25) in the Crocidolite group. Neither tumor induction nor interim death was observed in the Control and the Fullerene groups except for one moriband mouse by chronic pyelonephritis at day 152.

In large fibrous scars/granulation, aggregates similar to those shown in Fig. 2c and 2d were found embedded. Dispersed fibers of MWCNT and crocidolite were found extracellular in the fibrotic lesions or phagocytized by the phagocytic cells. Such fiber-laden cells were found not only in the peritoneal lesions but also in the liver within the hepatic sinusoids or along with the fibrous septum between the hepatic lobes, and in the mesenteric lymph nodes (Fig. 9).

In the Fullerene group, peritoneal lesion was minimal. Only small brownish black plaques were seen on the serosal surface. Histologically, the plaques contained polygonal clefts and lacunae surrounded by a thin layer of foamy cells and separated by thin fibrous septa (Fig.10). The clefts/lacunae corresponded to the injected fullerene aggregates in size and shape. Since fullerene dissolves well in organic solvents, especially in xylene, the embedded particles were washed away during histology preparation, leaving clefts behind. It is noted that the edge of the clefts are tinted brown, indicating possible biodegradation of the surface of the fullerene particles by the phagocytic cells, blending proteins and/or other organic components so that the sub-micrometer fullerene grains become resis-

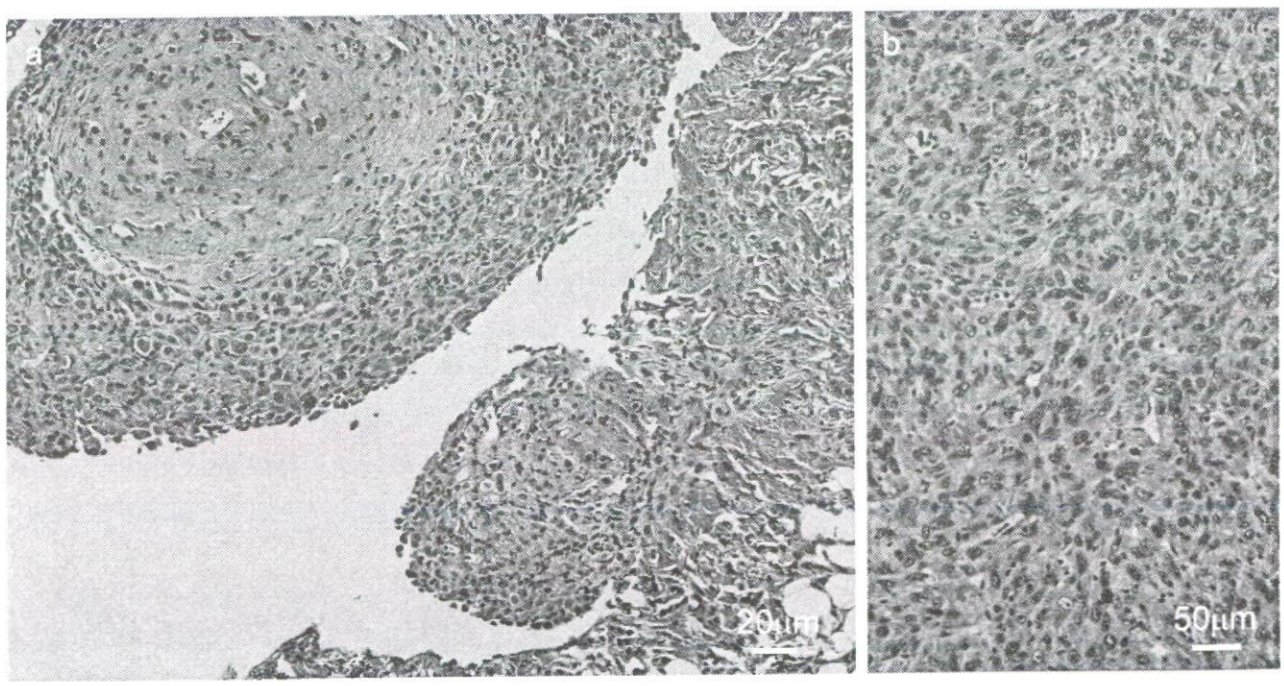

Fig. 7. Mesotheliomas in the Crocidolite group:
a) Typical mesothelioma nodules with fibrous stem induced in crocidolite-treated mouse (moribund on day 172 with multiple mesotheliomatous nodules with hemorrhagic ascites and peritoneal adhesion). b) Undifferentiated form of mesothelioma (so-called high-grade malignant mesothelioma) found as an invasive tumor of 1×1 cm in size (moribund case on day 170 with multiple invasive mesotheliomas up to 1×1.5 cm in size, severe peritoneal fibrosis and jaundice).

Mesothelioma by MWCNT in p53 +/− mouse.

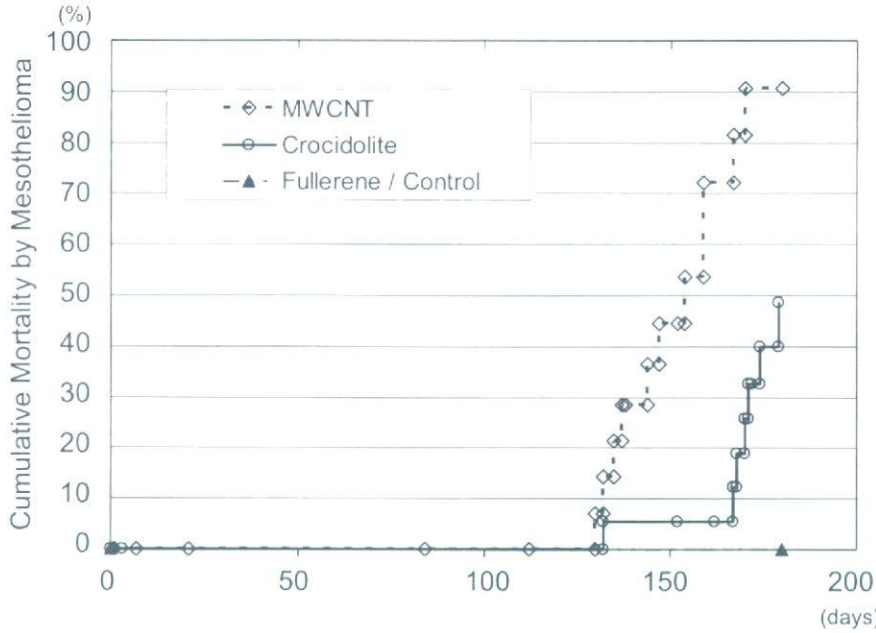

Fig. 8. Cumulative mortality of MWCNT and crocidolite treated mice by mesothelioma: Mice with large/invasive mesotheliomas considered as cause of death are plotted by Kaplan-Meier method. Second major cause of death was constriction ileus due to severe peritoneal adhesion. Among those moribund/dead or terminated at week 25 (day 180), there were 3 mice with incidental mesotheliomas in the MWCNT group and 6 incidental mesotheliomas in the Crocidolite group. No tumor induction was observed in the Fullerene and the Control groups.

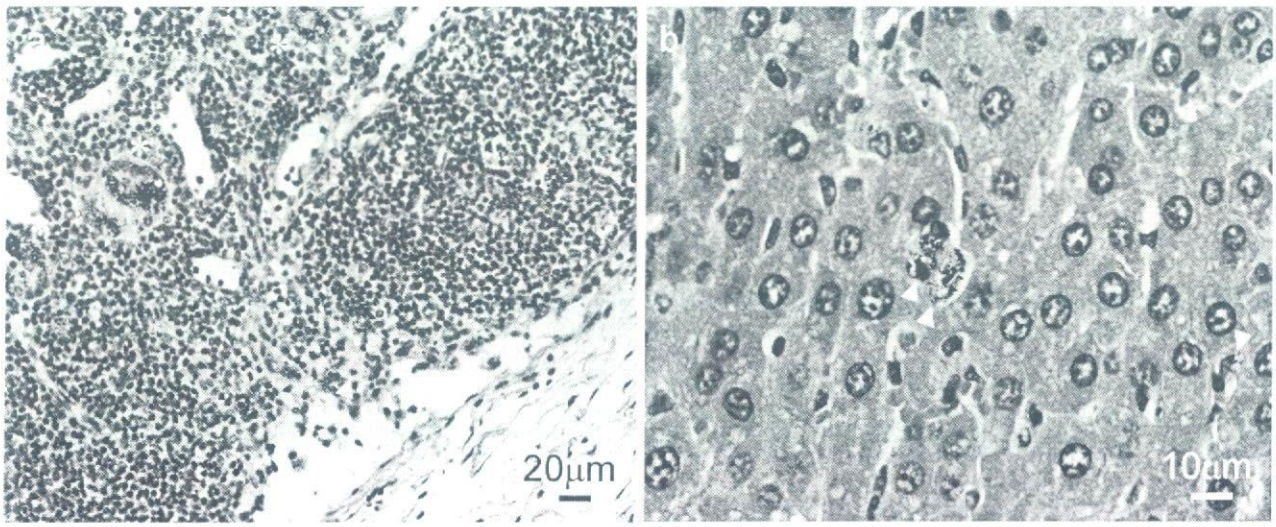

Fig. 9. Extraperitoneal migration of shorter fibers:
Phagocytized shorter fibers are found in hepatic sinusoids and local lymph nodes. a) Multinuclear giant cells (asterisks) and mononuclear phagocytic cells (arrow heads) with black fibers are seen in mesenteric lymph nodes (MWCNT-treated moribund mouse on day 159 with mesotheliomas and fibrous adhesion). b) MWCNT-laden phagocytic cells in hepatic sinusoids (arrow heads)(MWCNT-treated mouse found dead on day 84 with multiple mesotheliomas up to 0.7×0.7 cm in size, severe peritoneal fibrosis and pleural effusion).

tant to the solvents.

In summary, intraperitoneally administered MWCNT has induced mesothelioma in the p53(+/−) mice carcinogenesis model, probably due to its resemblance to asbestos in size and shape, and biopersistency.

DISCUSSION

The foreign body carcinogenesis is a category among various mechanisms of carcinogenesis. It has been postulated that ROS and/or RNS generated locally by the inflammatory reactions against non-digestive, long-lasting foreign bodies induces carcinogenic response (Tazawa *et al.*,2007). And one particular shape and size to enhance

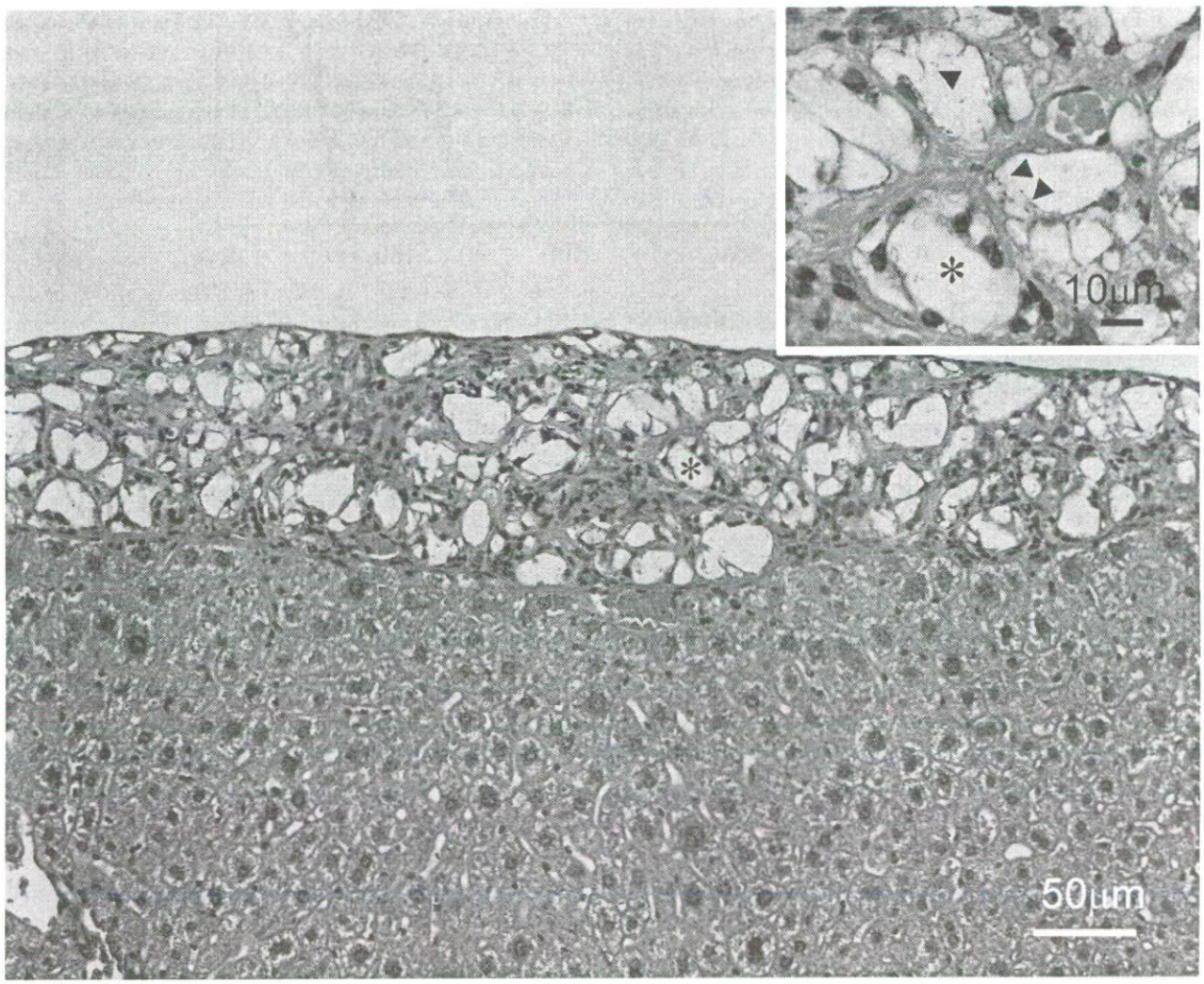

Fig. 10. Fullerene deposits:
Serosa of fullerene-treated mice showed minimum response within this 25-week period. Only black spots were occasionally seen on the surface. Histologically, the spots were made of polygonal slits surrounded by foamy cells and fibrous septa forming a compact fibrous scar. There were no signs of mesothelial response by this treatment. Since fullerene dissolves well to organic solvents especially xylene, the embedded particles were washed away leaving clefts behind. It is noted that the edges of the clefts are tinted brown, indicating possible biodegradation of the surface of the fullerene particles by the phagocytic cells, blending proteins and/or other organic components so that the sub-micrometer fullerene grains become resistant to the solvents (arrow head in inset).

this potency has been extensively studied on asbestos and man-made fibers (WHO, 1986, 1998). To study the asbestos-type carcinogenesis, the intraperitoneal route has been adopted in parallel to inhalation or transtracheal route of lung exposure. There has been some debate on whether rodent models are equivalent to the inhalation exposure to humans (Pott *et al.*,1994). Current understanding would be that the intraperitoneal model has considerable value on hazard identification in this regard (WHO, 1998, 2002). On the other hand, the p53 (+/–) mice, in general, have been suggested to be a good model to predict carcinogen, especially of a genotoxic nature (Pritchard *et al.*,2003). Relatively recently, this model has been reported to be sensitive to oxidative stress-mediated carcinogenesis such as foreign body carcinogenesis, producing a tumor with shorter latency periods than in wild-type mice (Tazawa *et al.*,2007). When asbestos was applied intraperitoneally to this model, mesotheliomas were induced with short latency as well (Marsella *et al.*,1997; Vaslet *et al.*,2002). Here, although the genotoxic effect of MWCNT is unclear, our results suggested that intraperitoneal administration of MWCNT possesses carcinogenic potential in p53(+/–) mice presumably depending on its size/shape and persistency in the organism.

Prediction of the mesotheliomagenic potential of MWCNT in humans cannot be completed by this p53+/– mouse model study. For example, glass fiber of a same shape and size to asbestos tends to fail to induce mesothelioma in humans because of its relatively faster disappearance from the deposition sites (Lippmann, 1990). Biodurability of MWCNT has to be rigorously tested before making any strong regulatory action. Likewise, Fe content of the material may be an important aspect to its carcinogenicity although our MWCNT contained lower Fe than crocidolite (WHO, 1986).

As shown in Fig.1, MWCNT studied here consists of rods and fibers of various size. In general, a bulk of a nanomaterial may contain a wide spectrum of particles at least in their size, from tens of micrometer down to true nanometer ranges. As suggested in this study by fullerene, micrometer-sized particles may become much smaller by biological activities, such as foreign body digestion activities of phagocytic cells. And yet, it is important to limit the significance of this study to the monitoring of biological activity of a compartment of the MWCNT longer than 5 micrometer. There is no information that this study method would be sensitive to pure nanometer-sized particles within this timeframe, i.e. 25 weeks. Again, this study is considered sufficient for detection of mesotheliomagenesis only by rod-shaped micrometer-sized particles. The biological effects of pure nanometer-sized CNTs and fullerene are not assessed in this study, and therefore, this remains open to further research.

The safety assessment for the new materials such as nanoparticles poses a new paradigm. The key to it is that the full-scale exposure to the public has not yet started. Therefore, there is a good chance that the information from hazard identification studies can directly be fed back to the product development plans so that harmful exposure can be prevented before it happens. In this way, manufacturers can produce safer products without risking themselves and the consumers by waiting for the full chronic toxicology studies including carcinogenicity studies to be finished after their initial (less safe) products are widely marketed.

ACKNOWLEDGMENTS

The authors thank Mr. Masaki Tsuji for technical support. This study was supported by Health Sciences Research Grants H17-kagaku-012 and H18-kagaku-ippan-007 from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

Bernstein, D.M. and Riego Sintes, J.M. (1999): Methods for the determination of the hazardous properties for human health of man made mineral fibres (MMMF). In: European Commission Joint Research Centre. Institute for Health and Consumer Protection, Unit: Toxicology and Chemical Substances. European Chemicals Bureau. pp. 44-45.

Coussens, L.M. and Werb, Z. (2002): Inflammation and cancer. Nature, **420**, 860-867.

Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G. and Alexander, A. (2006): Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol. Sci., **92**, 5-22.

Gulumian, M. and van Wyk, J.A. (1987): Hydroxyl radical production in the presence of fibres by a Fenton-type reaction. Chem. Biol. Interact., **62**, 89-97.

Hei, T.K., Xu, A., Huang, S.X. and Zhao, Y. (2006): Mechanism of fiber carcinogenesis: From reactive radical species to silencing of the beta igH3 gene. Inhal. Toxicol., **18**, 985-990.

Hou, P.-X., Xu, S.-T., Ying, Z., Yang, Q.-H., Liu, C. and Cheng, H.-M. (2003): Hydrogen adsorption/desorption behavior of multi-walled carbon nanotubes with different diameters. Carbon, **41**, 2471-2476.

Jiang, L., Zhong, Y., Akatsuka, S., Liu, Y.T., Dutta, K.K., Lee, W.H., Onuki, J., Masumura, K., Nohmi, T. and Toyokuni, S. (2006): Deletion and single nucleotide substitution at G:C in the kidney of gpt delta transgenic mice after ferric nitrilotriacetate treatment. Cancer Sci., **97**, 1159-1167.

Lam, C.W., James, J.T., McCluskey, R., Arepalli, S. and Hunter, R.L. (2006): A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Crit. Rev. Toxicol., **36**, 189-217.

Lippmann, M. (1990): Effects of fiber characteristics on lung deposition, retention, and disease. Environ. Health Perspect., **88**, 311-317.

Luo, J., Peng, L.-M., Xue, Z.Q. and Wu, J.L. (2004): Positive electron affinity of fullerenes: Its effect and origin. J. Chem. Phys., **120**, 7998-8001.

Marsella, J.M., Liu, B.L., Vaslet, C.A. and Kane, A.B. (1997): Susceptibility of p53-deficient mice to induction of mesothelioma by crocidolite asbestos fibers. Environ. Health Perspect., **105** (Suppl. 5), 1069-1072.

Moalli, P.A., MacDonald, J.L., Goodglick, L.A. and Kane, A.B. (1987): Acute injury and regeneration of the mesothelium in response to asbestos fibers. Am. J. Pathol., **128**, 426-445.

Pott, F., Roller, M., Kamino, K. and Bellmann, B. (1994): Significance of durability of mineral fibers for their toxicity and carcinogenic potency in the abdominal cavity of rats in comparison with the low sensitivity of inhalation studies. Environ. Health Perspect., **102** (Suppl. 5), 145-150.

Pritchard, J.B., French, J.E., Davis, B.J. and Haseman, J.K. (2003): The role of transgenic mouse models in carcinogen identification. Environ. Health Perspect., **111**, 444-454.

Roller, M., Pott, F., Kamino, K., Althoff, G.H. and Bellmann, B. (1997): Dose-response relationship of fibrous dusts in intraperitoneal studies. Environ. Health Perspect., **105** (Suppl. 5), 1253-1256.

Tazawa, H., Tatemichi, M., Sawa, T., Gilibert, I., Ma, N., Hiraku, Y., Donehower, L.A., Ohgaki, H., Kawanishi, S. and Ohshima, H. (2007): Oxidative and nitrative stress caused by subcutaneous implantation of a foreign body accelerates sarcoma development in Trp53+/– mice. Carcinogenesis, **28**, 191-198.

Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S-I., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., Matsuo, I., Ikawa, Y. and Aizawa, S-I. (1993): Enhanced proliferative potential in culture of cells from p53-deficeint mice. Oncogene, **8**, 3313-3322.

Vaslet, C.A., Messier, N.J. and Kane, A.B. (2002): Accelerated progression of asbestos-induced mesotheliomas in heterozygous p53+/– mice. Toxicol. Sci., **68**, 331-338.

World Health Organization (1986): Environmental Health Criteria, **53**. Asbestos and Other Natural Mineral Fibres. World Health Organization, Geneva.

World Health Organization (1998): Environmental Health Criteria, **203**. Chrysotile Asbestos. World Health Organization, Geneva.

World Health Organization (2002): Man-made vitreous fibres. IARC monographs on the evaluation of carcinogenic risk to humans., Vol., **81**, IARC Lyon.

Possible application of human c-Ha-ras proto-oncogene transgenic rats in a medium-term bioassay model for carcinogens

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Running title: Hras rat medium-term carcinogen bioassay

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Keywords. Transgenic rat; Hras; mammary tumors; medium-term assay

The abbreviations used are: MNU, *N*-methyl-*N*-nitrosourea; DMBA, dimethylbenzoanthracene; 3-MC, 3-methylcholanthrene; B[a]P, benzo[a]pyrene; IQ, 2-amino-3-methylimidazo[4, 5-*f*]quinoline; MeIQx, 2-amino-3, 8-dimethylimidazo[4, 5-*f*]quinoxaline; NNK, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone; DEN, diethylnitrosamine; AOM, azoxymethane; DMA, dimethylarsinic acid; PCR, polymerase reaction chain; RFLP, restriction fragment length polymorphisms

Abstract

With the aim of developing a medium-term assay for screening of environmental carcinogens, we exposed mammary carcinogen sensitive human c-Ha-ras proto-oncogene transgenic (Hras128) rats to various carcinogens, including compounds which do not normally induce mammary tumors. Seven-week-old Hras128 rats and wild type littermates received three oral administrations of 3-methylcholanthrene (3-MC), benzo[a]pyrene (B[a]P), anthracene or pyrene (200 mg/kg), 2-amino-3-methylimidazo[*4,5-f*]quinoline (IQ) or 2-amino-3,8-dimethylimidazo[*4,5-f*]quinoxaline (MeIQx)(80 mg/kg), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or dimethylarsinic acid (DMA)(100 mg/kg), two oral administrations of diethylnitrosamine (DEN)(100 mg/kg), or one oral administration of azoxymethane (AOM)(50 mg/kg) and were killed at week 12 (females) (at week 10 for the 3-MC group) or week 20 (males). Female Hras128 rats receiving NNK, DEN, or DMA showed a significant increase in mammary tumor incidence and/or multiplicity compared to the respective values with olive oil or deionized distilled water (DDW) vehicles. In male Hras128 rats, significant increase in mammary tumors was also observed in groups administered 3-MC, B[a]P, anthracene, IQ, and NNK. Mutations of transgenes were observed in codons 12 and/or 61 in the induced tumors by PCR-RFLP except in the DEN group in female and in the MeIQx group in male Hras128 rats. Thus various carcinogens, not necessarily limited to those normally targeting the breast, were found to induce mammary carcinomas in Hras128 rats, especially in females, pointing to potential use for medium-term screening.

Introduction

We have generated human c-Ha-ras proto-oncogene transgenic (Hras128) rats which are highly sensitive to mammary carcinogens, rapidly developing carcinomas after exposure to *N*-methyl-*N*-nitrosourea (MNU), dimethylbenzo[a]anthracene (DMBA), or PhIP (Asamoto et al., 2000; Tsuda et al., 2001). Furthermore, the Hras128 rats are also highly susceptible to induction of lesions in the esophagus, bladder, skin and tongue (Asamoto et al., 2002; Ota et al., 2000; Park et al., 2004; Suzuki et al., 2005).

Incidence of spontaneous tumors in the mammary gland of Hras128 rats was 52.8% at 40 weeks and slightly increased as compared for female Sprarue-Dawley wild type rats (Tsuda et al., 2005). Taking advantage of these characteristics, we have focused on whether our transgenic animals might have advantages for use in short- or medium-term assay systems for screening environmental carcinogens. One problem is that carcinogens generally have specific organotropic actions as initiating agents (Tsuda et al., 1999). One way to overcome this is to use multi-organ carcinogenesis models (Imaida and Fukushima, 1996; Ito et al., 1988) in which animals are first treated with various carcinogens initiating carcinogenesis in the major organs and then assaying promotion or other modulation effects. However, the established protocols require upwards of 30 weeks until tumors or preneoplastic lesions are induced. As a single organ model, the Ito approach in the liver has many advantages in terms of cost and duration, at 8 weeks, but requires partial hepatectomy to enhance carcinogenesis (Ito et al., 1989; Tsuda et al., 1980). While transgenic (rasH2) mice bearing a human c-H-ras proto-oncogene have attracted interest for testing purposes (Ando et al., 1992; Yamamoto et al., 1996), the assay takes 26 weeks and cannot be said to be short-term. Our Hras128 rats develop tumors within 8 weeks.

For validation in the present study, a number of known carcinogens were selected. These were genotoxic agents as the results could not be applied directly to non-genotoxic agents. The polycyclic aromatic hydrocarbons 3-methylcholanthrene (3-MC) and benzo[a]pyrene (B[a]P) and their parent nuclear substances anthracene and pyrene are included in exhaust gas and tobacco smoke. 3-MC and B[a]P in particular are known to be causative agents for lung cancer in humans and mammary cancers in rats (Bolasny et al., 1963; Gingell et al., 1981). The heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3, 8-dimethylimidazo[4, 5-*f*]quinoxaline (MeIQx) are contained in broiled meat and fish (Sugimura, 1985) and 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK) is found in tobacco smoke (Brown et al., 1999). Diethylnitrosamine (DEN) is an N-nitroso compound commonly used in liver cancer experiments (Ito et al., 1989), while azoxymethane (AOM) specifically induces aberrant crypt foci and tumors in the colon (Thorup et al., 1995). Dimethylarsinic acid (DMA) is an arsenic compound present in the environment (Braman and Foreback, 1973) which is known to cause skin, lung and urinary bladder cancers (Chen et al., 1988; Cohen et al., 2001).