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

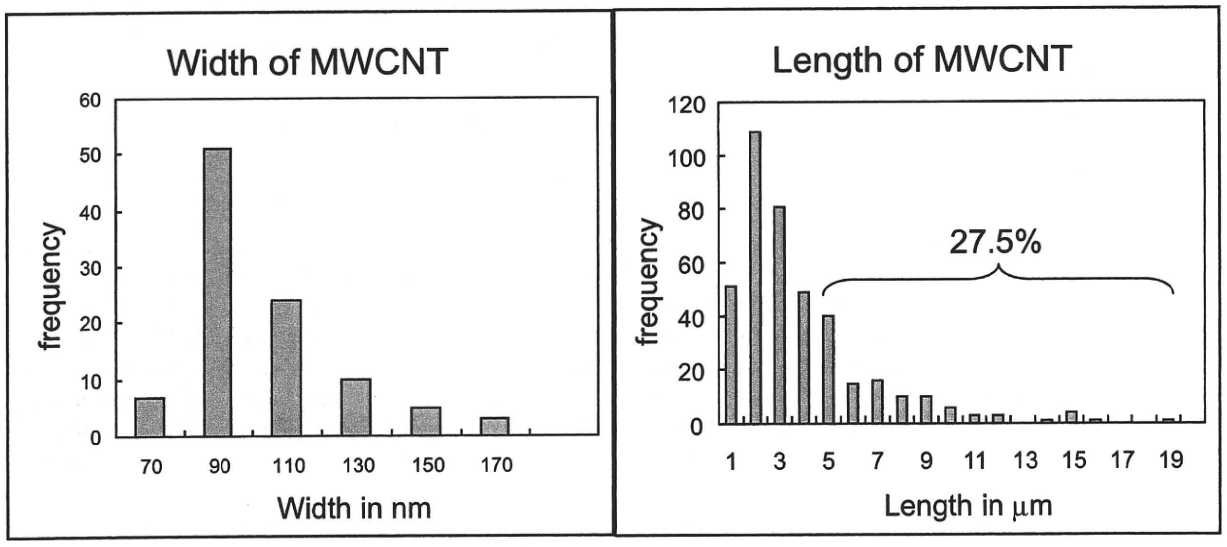

Fig. 1. Width and length distribution of MWCNT:
Width and length distribution of MWCNT (MITSUI MWCNT-7, Lot NO. 060125-01k) was measured at Tokyo Metropolitan Institute of Public Health. The average width was about 100 nm, and 27.5% of the particles were longer than 5 micrometer.

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-

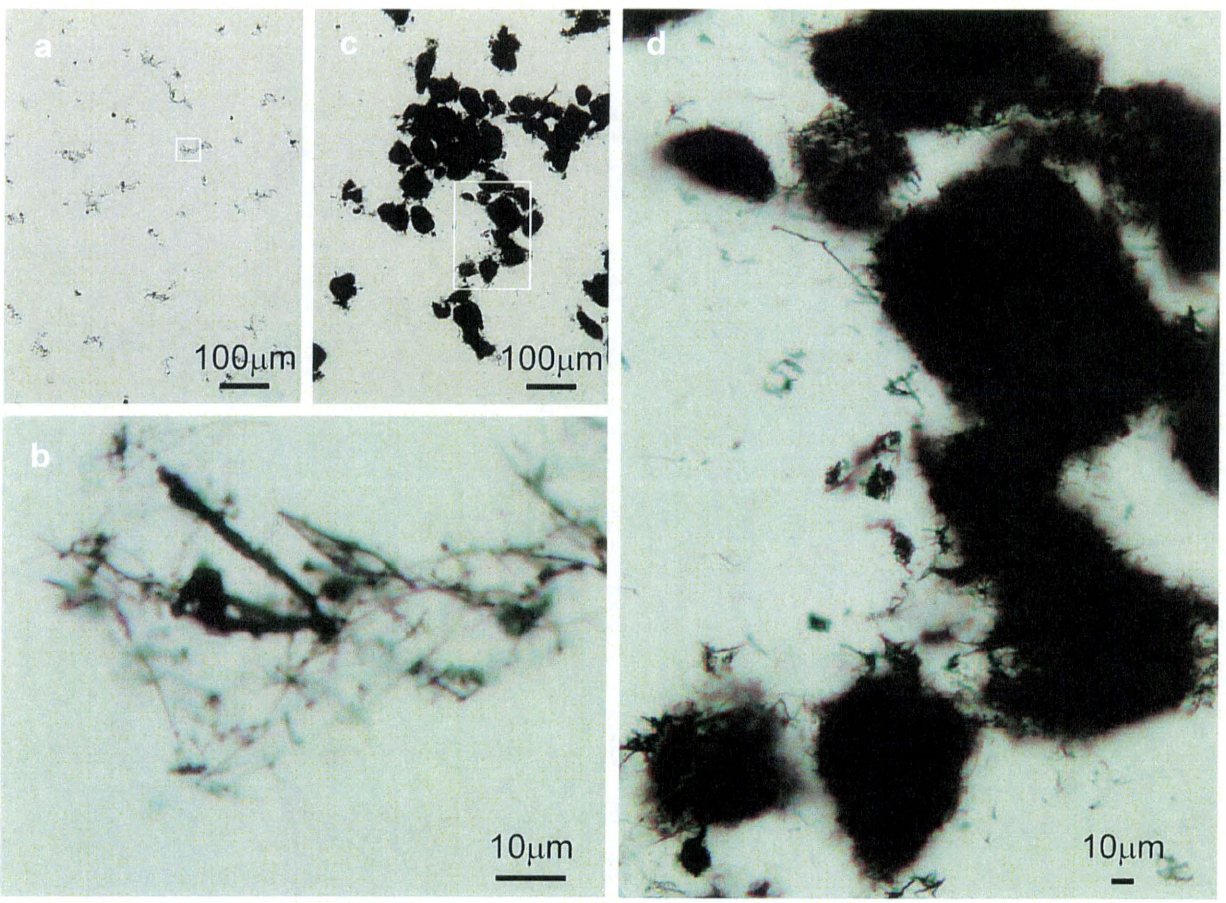

Fig. 2. Light microscopic view of administered MWCNT:
Light microscopic view of sonicated MWCNT sample suspension mounted on slide glasses. a) Well-dispersed area of the preparation. b) Close-up view of the boxed area in a). Fine fiber or rod-shaped particles longer than 10 micrometers are seen. c) Aggregated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

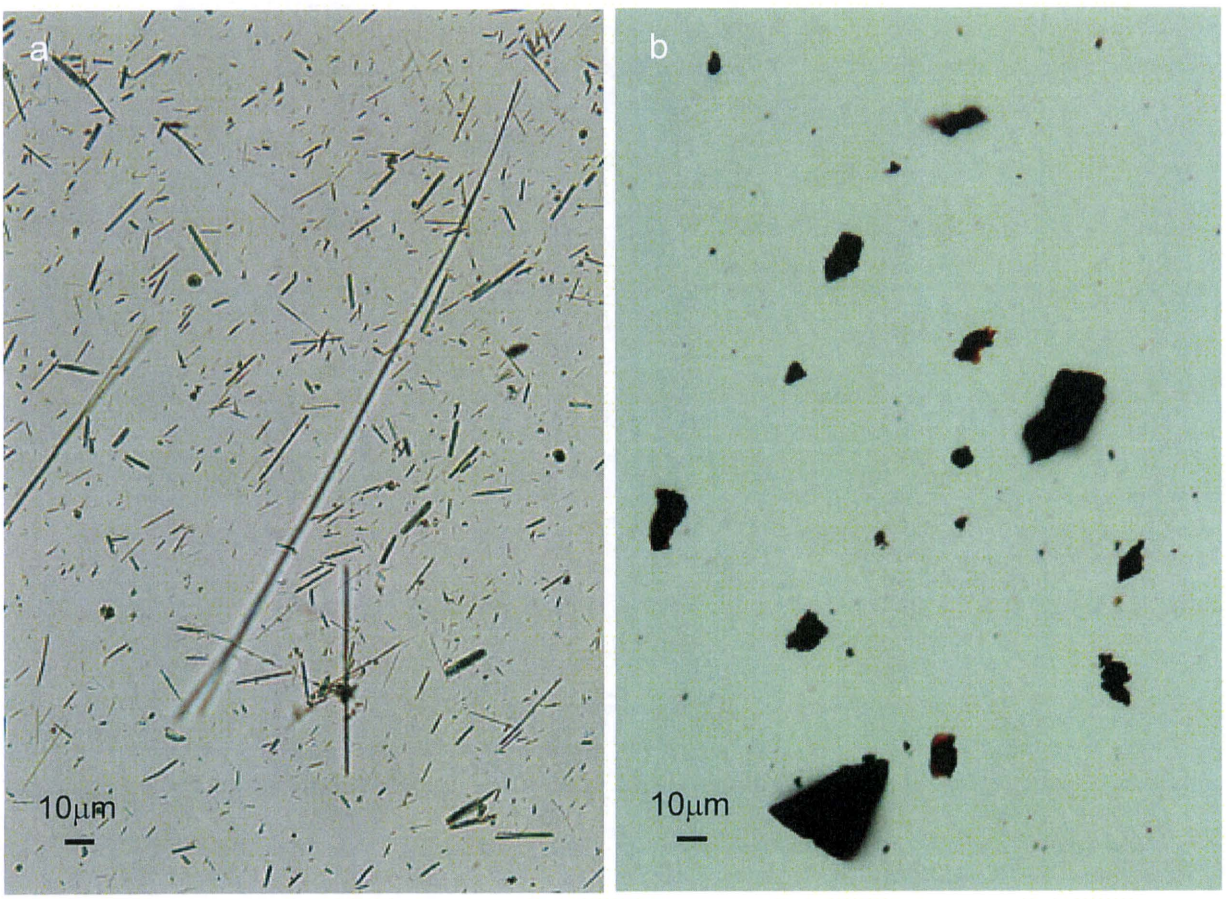

Fig. 3. Light microscopic views of administered crocidolite and fullerene:
Light microscopic views of administered crocidolite and fullerene. a) Crocidolite sample consisting of various lengths of rod-shaped particles. b) Fullerene sample consisted of sand grain-like particles of sizes ranging up to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

Mesothelioma by MWCNT in p53 +/– mouse.

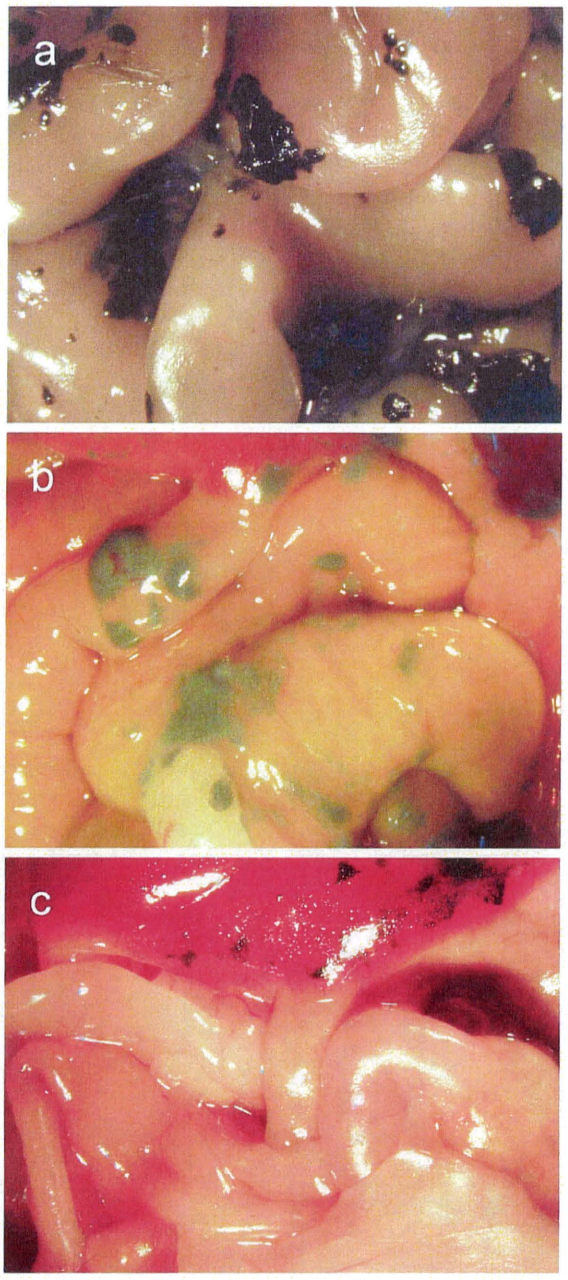

Fig. 4. Early peritoneal responses to MWCNT, crocidolite, and fullerene (10 days after i.p. injection):
Early findings of peritoneal cavity 10 days after i.p. administration of a) MWCNT inducing slight fibrinous deposit, adhesion, ascites retention, and edematous and hypotonic intestinal loops, b) crocidolite 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

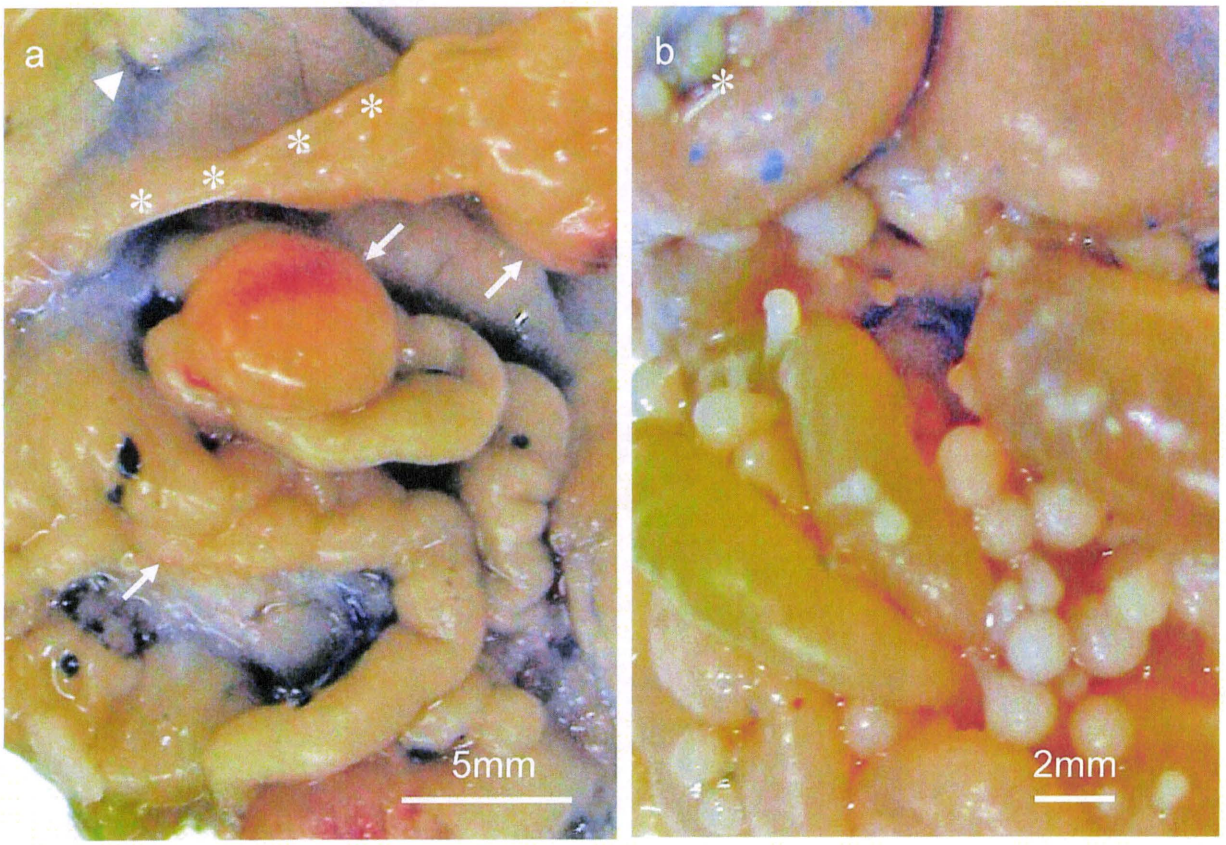

Fig. 5. Macroscopic view of abdominal viscera of MWCNT-treated and crocidolite-treated mouse:
Macroscopic view of the abdominal viscera excised *en bloc* of a) MWCNT-treated mouse that died at day 147, and b) crocidolite-treated mouse moribund on day 172 due to ileus. a) Fibrous adhesions of the visceral 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-

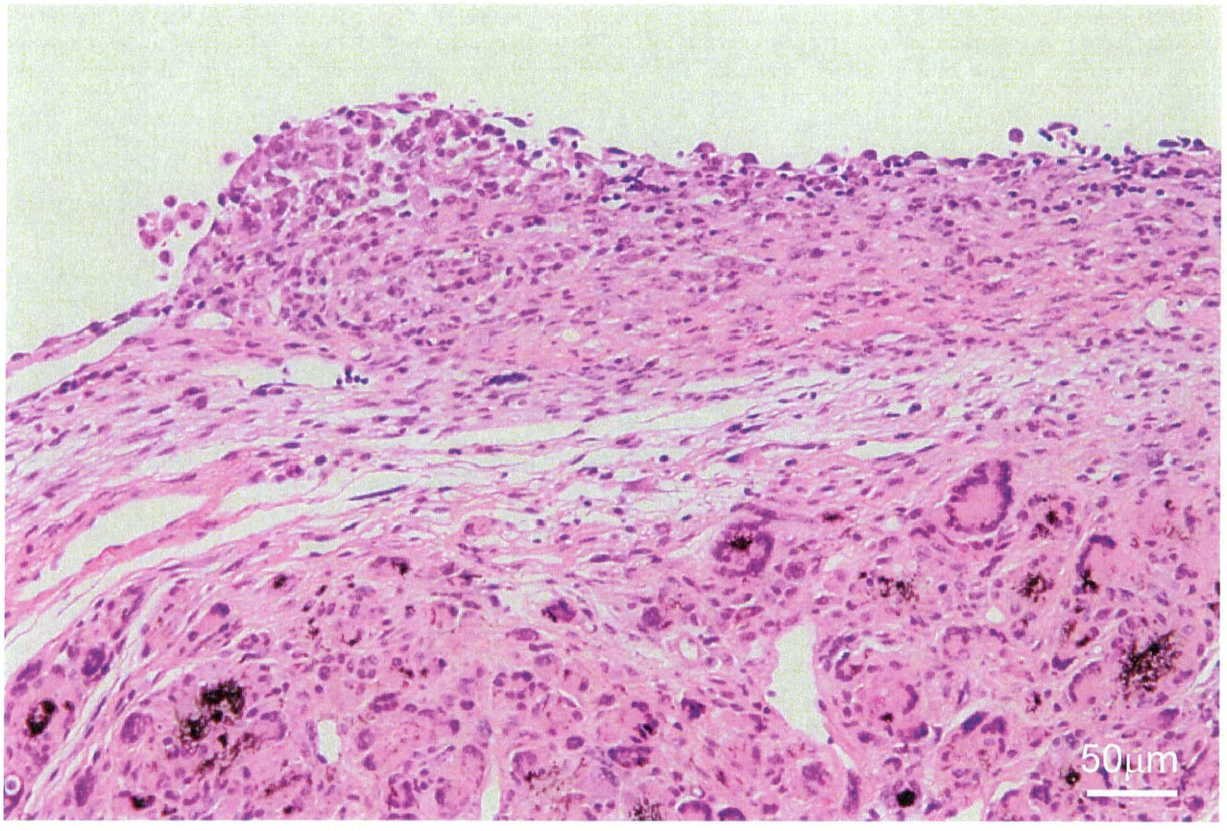

Fig. 6. Mesothelial response in MWCNT-treated mice:
Fibrous thickening of the peritoneum and foreign body granulomas against the MWCNT with phagocytic cells including multinucleated giant cells are formed in the MWCNT-treated mouse. Mesothelial lesions were found in the vicinity of fibrosis and granulomas. 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-

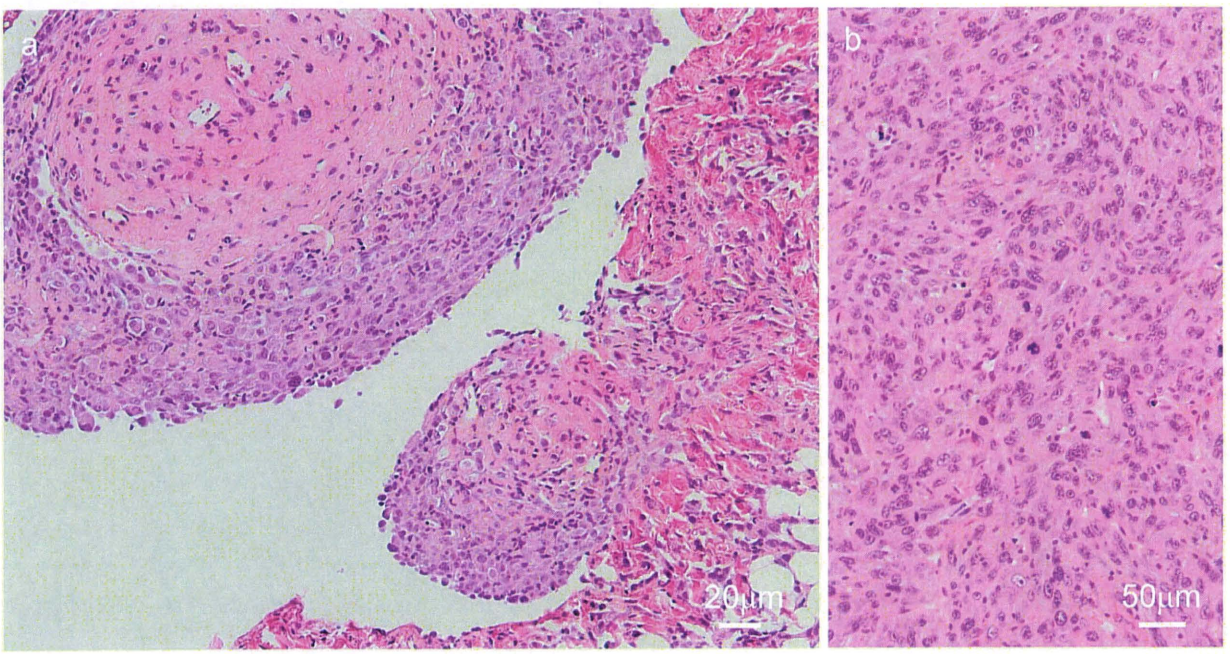

Fig. 7. Mesotheliomas in the Crocidolite group:
a) Typical mesothelioma nodules with fibrous stem induced in crocidolite-treated mouse (moribund on day 172 with multiple mesotheliomatous nodules with hemorrhagic ascites and peritoneal adhesion). b) Undifferentiated form of mesothelioma (so-called high-grade 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.

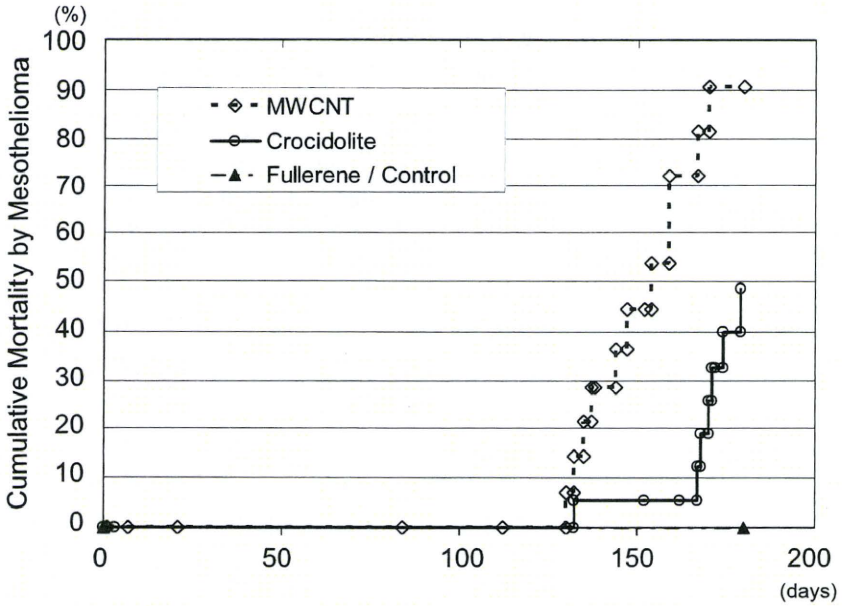

Fig. 8. Cumulative mortality of MWCNT and crocidolite treated mice by mesothelioma: Mice with large/invasive mesotheliomas considered as cause of death are plotted by Kaplan-Meier method. Second major cause of death was constriction ileus due to severe peritoneal adhesion. Among those moribund/dead or 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.

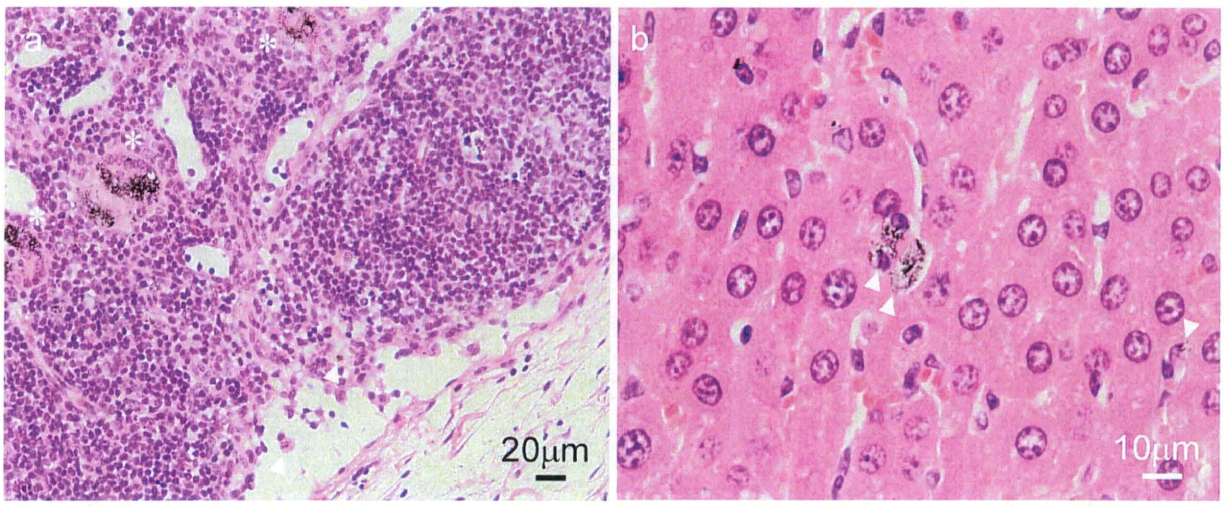

Fig. 9. Extraperitoneal migration of shorter fibers:
Phagocytized shorter fibers are found in hepatic sinusoids and local lymph nodes. a) Multinuclear giant cells (asterisks) and mononuclear phagocytic cells (arrow heads) with black fibers are seen in mesenteric lymph nodes (MWCNT-treated moribund mouse 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

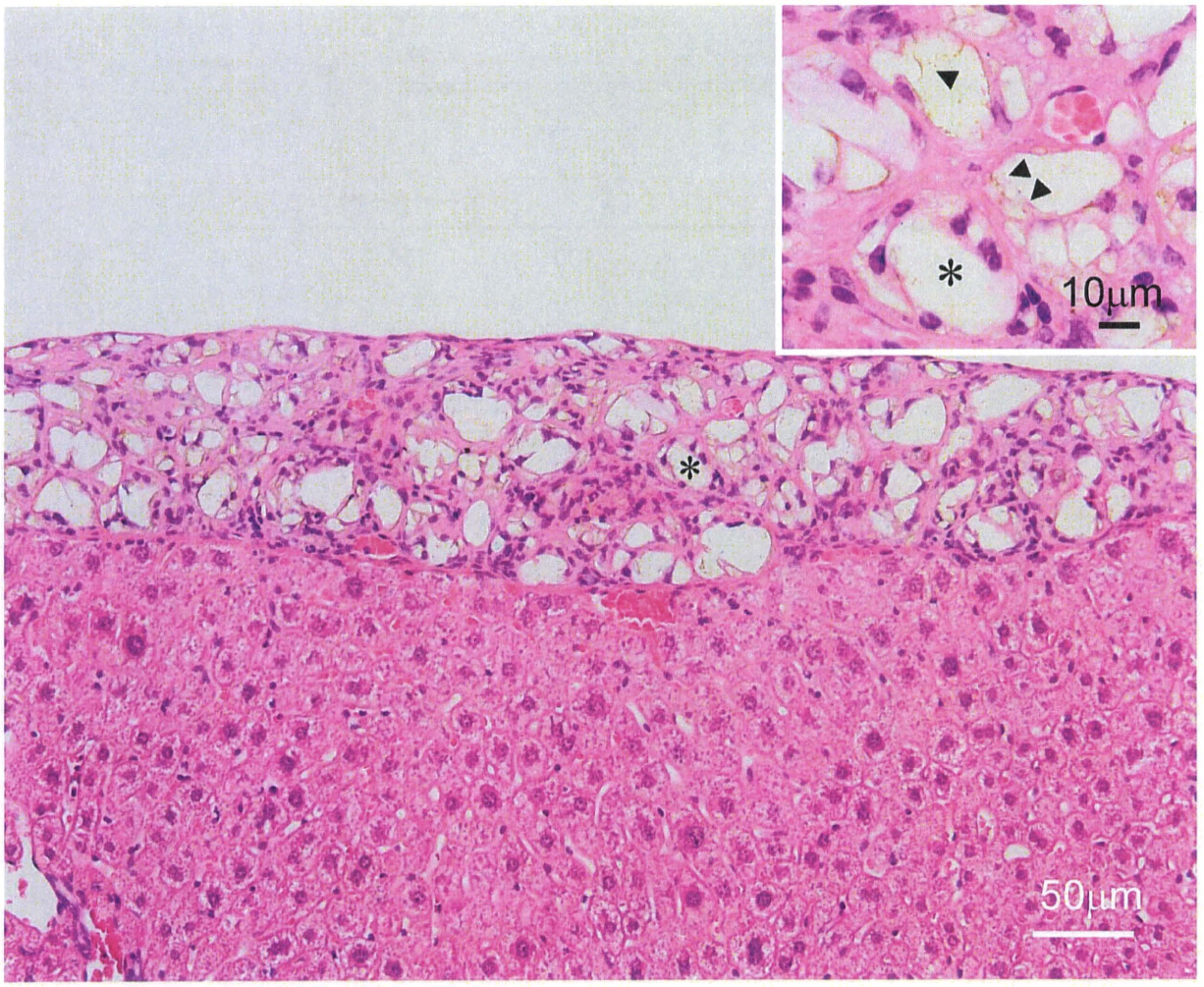

Fig. 10. Fullerene deposits:
Serosa of fullerene-treated mice showed minimum response within this 25-week period. Only black spots were occasionally seen on the surface. Histologically, the spots were made of polygonal slits surrounded by foamy cells and fibrous septa forming a compact fibrous scar. 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

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Thirteen-Week Inhalation Toxicity of 1,4-Dioxane in Rats

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Thirteen-week inhalation toxicity of 1,4-dioxane was examined by repeated inhalation exposure of male and female F344 rats to 0 (control), 100, 200, 400, 800, 1600, 3200, or 6400 ppm (v/v) 1,4-dioxane vapor for 6 h/day and 5 days/wk. All the 6400-ppm-exposed males and females died during the first week. Terminal body weight decreased, and relative weights of liver, kidney, and lung increased. AST increased in the 200 ppm- and 3200-ppm-exposed females, and ALT increased in the 3200-ppm-exposed males and females. Nuclear enlargement of nasal respiratory epithelial cells occurring in the 100-ppm-exposed males and females was the most sensitive, followed by the enlarged nuclei in the olfactory, tracheal, and bronchial epithelia. 1,4-Dioxane-induced liver lesions occurred at higher exposure concentrations than the nasal lesions did, and were characterized by single-cell necrosis and centrilobular swelling of hepatocytes in males and females. Glutathione *S*-transferase placental form (GST-P) positive liver foci were observed in the 1600-ppm-exposed females and 3200-ppm-exposed males and females, which are known as a preneoplastic lesion in rat hepatocarcinogenesis. Plasma levels of 1,4-dioxane increased linearly with an increase in the concentrations of exposure to 400 ppm and above. The enlarged nuclei in the nasal epithelia and the GST-P-positive liver foci were discussed in light of the possible development of nasal and hepatic tumors by long-term inhalation exposure to 1,4-dioxane. A lowest-observed-adverse-effect level (LOAEL) was determined at 100 ppm for the nasal endpoint in both male and female rats.

1,4-Dioxane is a highly flammable liquid, and has been widely used as a solvent for cellulose acetate, resins, oils, and waxes, and as a stabilizer in chlorinated solvents (Budavari et al., 1989; Lewis, 1993). The worldwide production volume of 1,4-dioxane was estimated to be 10000 tons in 1995 (EU Risk Assessment Report, 2002). The annual production of 1,4-dioxane in Japan was reported to amount to 4500 tons in 2004 (Chemical Daily, 2006). In the United States, release of 1,4-dioxane to atmosphere and surface water from manufacturing and processing facilities was estimated as 52 and 41 tons in 2004, respectively (Agency for Toxic Substances and Disease Registry [ATSDR], 2007). According to the Pollutant Release and Transfer Register Report from the Japan Ministry of the Environment (2007), 69 and 79 tons of 1,4-dioxane were released into atmosphere and public waters, respectively, from chemical industry in 2005. The National Institute for Occupational Safety and Health (NIOSH, 1977) reported that workplace air concentrations of 1,4-dioxane at 3 plants were 1.07, 0.9, and 6.5 ppm on average and the corresponding maximum values were 7.2, 2.0, and 23.6 ppm. Atmospheric concentrations of 1,4-dioxane at sampling points from various areas of Japan in 2000 were reported to range from 15 to 1200 ng/m^3 (0.3 ppb), while concentrations of 1,4-dioxane in public waters ranged from 0.08 to 160 μg/L (Japan Ministry of the Environment, 2002). Workers are at health risk when excessively exposed to 1,4-dioxane through inhalation and dermal contact in workplaces, while general populations are exposed to low levels of 1,4-dioxane in the urban ambient air through inhalation and in public waters through ingestion, inhalation, and dermal contact (DeRosa et al., 1996).

Excessive exposure of humans to 1,4-dioxane has been reported to cause deaths resulting from hepatic and renal failures in the acute and subacute phases (Johnstone, 1959; Rowe & Wolf, 1982). There have been no animal studies on subchronic and chronic inhalation toxicities of 1,4-dioxane, except for the

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Torkelson et al. study (1974) that found that repeated inhalation exposure of rats to 1,4-dioxane vapor at 111 ppm for 2 yr induced neither pathological changes nor tumors. The American Conference of Governmental Industrial Hygienists (ACGIH, 2001) and the German Research Foundation (DFG, 2003) established an occupational exposure limit (OEL) value of 20 ppm for 1,4-dioxane. The Japan Society for Occupational Health (JSOH, 1984) recommended an OEL value of 10 ppm for 1,4-dioxane, on the grounds that the OEL value is equivalent to one-tenth of the inhalation exposure level that did not induce any tumor in experimental animals. NIOSH (1977) recommended that occupational exposure to 1,4-dioxane be controlled so that employees are not exposed at airborne concentrations greater than 1 ppm, in the belief that 1,4-dioxane can be tumorigenic.

The present study was designed to better characterize the subchronic inhalation toxicity of 1,4-dioxane and to provide basic data on dose-response relationships for various endpoints for health risk assessment of humans exposed by inhalation to 1,4-dioxane vapor. In the present study, male and female F344 rats were exposed by inhalation to 1,4-dioxane vapor at 7 different concentrations or clean air for 6 h/day, 5 days/wk, for 13 wk. A potentially preneoplastic lesion of nuclear enlargement of epithelial cells in the nasal cavity and a preneoplastic lesion of glutathione *S*-transferase placental form (GST-P) positive liver foci were explored for detecting an early detectable stage participating in the possible development of nasal and hepatic tumors that would be induced after long-term inhalation exposure to 1,4-dioxane vapor. The OEL was discussed in light of the most sensitive nasal response to the inhaled 1,4-dioxane.

MATERIALS AND METHODS

The present study was conducted with reference to the Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemicals 413 (OECD, 1981). The present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1977).

Chemicals

1,4-Dioxane of reagent grade (greater than 99% pure) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The stock 1,4-dioxane was analyzed for purity and stability by gas chromatography–mass spectrometry (GC-MS) (Hitachi M-80B spectrometer, Hitachi, Ltd., Tokyo), gas chromatography (GC) (Hewlett Packard 5890, Agilent Technol., Santa Clara, CA), and infrared spectrometry (Hitachi 270-30, Hitachi, Ltd., Tokyo) before and after its use. Butylhydroxytoluene was detected in the 1,4-dioxane liquid by the GC-MS, and its concentration was quantitated as 1.3 ppm (w/w) by the GC. No gas chromatographic peak corresponding to butylhydroxytoluene was found in the air samples collected from the inhalation exposure chamber.

Animals

Four-week-old F344/DuCrj rats of both sexes were obtained from Charles River Japan, Inc. (Kanagawa, Japan). The animals were quarantined and acclimated for 2 wk, and then divided by stratified randomization into 8 body-weight-matched groups, each comprising 10 rats of both sexes. Inhalation exposure chambers were installed in the barrier system animal room where a temperature of 21 ± 2°C and a relative humidity of 60 ± 10% with 15–17 air changes/h were maintained. The animals were housed individually in stainless steel wire hanging cages (150 mm [W] × 216 mm [D] × 176 mm [H]) in the inhalation exposure chambers. The exposure chambers were maintained at a temperature of 20 to 24°C and a relative humidity of 30 to 70% with 12 air changes/h throughout the 13-wk exposure period. A 12-h light/dark cycle was automatically controlled. All the animals had free access to sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) and sterilized drinking water supplied by an automatic watering system.

Inhalation Exposure to 1,4-Dioxane Vapor

The animals were exposed to 1,4-dioxane vapor at a target concentration of 100, 200, 400, 800, 1600, 3200, or 6400 ppm for 6 h/day, 5 days/wk, for 13 wk. Groups of 10 rats of both sexes were exposed to clean air for 13 wk under the same conditions, and served as controls. Airflow containing 1,4-dioxane vapor at the target concentration was prepared by a vaporization technique. The saturated vapor–air mixture was generated by bubbling clean air through the 1,4-dioxane liquid in a temperature-regulated glass flask (30°C), and by cooling it through a thermostatted condenser at 20°C. The airflow containing the saturated vapor was diluted with clean air, and then warmed to 30°C in a thermostatted circulator that served to stabilize the vapor concentration by complete gasification of 1,4-dioxane. The flow rate of the vapor–air mixture was regulated with a flow meter, further diluted with humidity- and temperature-controlled clean air in a spiraling line mixer, and then supplied to an inhalation exposure chamber. Eight inhalation exposure chambers of 1060 L in volume were used in the present study. Each exposure chamber accommodated 20 individual cages for 10 males and 10 females. Chamber concentrations of 1,4-dioxane were monitored every 15 min with a gas chromatograph (GC-14B, Shimadzu Corp., Kyoto, Japan), equipped with a hydrogen flame ionization detector and a 1.5-m Shimadzu SBS-120 packed column operated at a column temperature of 90°C and with a gas injection volume of 2 ml. The concentrations were maintained constant at 100.2 ± 2.1 (mean ± SD), 200.7 ± 4.1, 403.9 ± 7.5, 799.8 ± 12.1, 1596.4 ± 27.2, 3198.4 ± 48.9 ppm throughout the 13-wk exposure period and 6409.5 ± 76.3 ppm for the first 1-wk surviving period. Accuracy and precision of the actual concentrations of 1,4-dioxane in the exposure chamber were kept by periodic injection of the certified standard 1,4-dioxane gas (Takachiho Co., Ltd., Tokyo) into the gas chromatograph for the calibration curve of 1,4-dioxane.

Clinical Observations and Analysis, and Pathological Examinations

The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured once per week. All animals underwent complete necropsy. The organs were removed, weighed, and examined for macroscopic lesions. For hematology and blood biochemistry, the surviving animals were bled from the abdominal aorta under diethyl ether anesthesia after overnight fasting at the end of the 13-wk exposure period. Hematological parameters were measured with an automatic blood cell analyzer (TECHNICON H-1, Technicon Instruments Corp., New York) and an automatic blood cell differential analyzer (Hitachi 8200, Hitachi, Ltd., Ibaraki, Japan). Blood biochemical parameters were measured with an automatic analyzer (Hitachi 705) and a flame photometer (Hitachi 750, Hitachi, Ltd., Ibaraki, Japan). Urinary parameters were measured in the last week of the 13-wk exposure period with Ames reagent strips (Multistix, Bayer Corp., New York). All the organs and tissues designated in the OECD test guideline (OECD, 1981) and the entire respiratory tract including the nasal cavity, pharynx, larynx, trachea, and bronchus were examined for histopathology. The tissues were fixed in 10% neutral buffered formalin, and embedded in paraffin. The nasal cavity was decalcified in a formic acid–formalin solution prior to trimming, and was transversely trimmed at three levels according to the procedure described in our previous paper (Nagano et al., 1997), i.e., at the level of the posterior edge of the upper incisor teeth (Level 1), at the incisive papilla (Level 2), and at the level of the anterior edge of the upper molar teeth (Level 3). Tissue sections of 5 μm in thickness were prepared, and stained with hematoxylin and eosin (H&E). Additionally, the livers of all the ten 800-, 1600-, and 3200-ppm-exposed rats of both sexes and ten control rats of both sexes were sectioned for further examination of the enzyme-altered liver foci by immunohistochemical staining with anti-GST-P (Sato et al., 1984; Tatematsu et al., 1985; Ito et al., 1988), using EnVision+ (EV+, Dako, Copenhagen, Denmark) of the two-layer dextran polymer visualization system (Vyberg & Nielsen, 1998). Polyclonal antibody of GST-P (Anti-GST-P) was obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Focal populations each having 50 or more hepatocytes homogeneously stained brown were defined as the GST-P-positive liver foci in the present study.

Determination of 1,4-Dioxane in Blood

Three rats of each sex per group except for the control were used to collect blood about 1 h after termination of day 3 exposure in wk 12 of the 13-wk exposure period. A 0.5-ml blood sample was collected from the tail vein into a heparinized tube and centrifuged at 3000 rpm for 5 min at 5°C. Then 100 μl plasma was diluted with 15 ml distilled water in a glass vial. 1,4-Dioxane was quantitatively determined with the headspace sampler gas chromatography–mass spectrometry (HS/GC-MS) (HS, Hewlett Packard 7694; GC-MS, Hewlett Packard 5989, Agilent Technol., Santa Clara, CA).

Statistics and Data Analysis

Body weight, food consumption, organ weight, and hematological and blood biochemical parameters were analyzed by Dunnett's test as described previously (Aiso et al., 2005). Histopathological findings and urinary parameters were analyzed by chi-square test. A two-sided analysis with p values of .05 and .01 was performed to determine statistical significance.

A no-observed-adverse-effect level (NOAEL) or a lowest-observed- adverse-effect level (LOAEL) was determined according to the WHO definition (International Programme on Chemical Safety [IPCS], 1994).

RESULTS

Survival, and Body Weights and Organ Weights

All the 6400-ppm-exposed rats of both sexes died during wk 1 of the 13-wk exposure period. The histopathological examination revealed that their deaths were primarily caused by renal failure, because all the 6400-ppm-exposed animals suffered from marked necrosis in the renal tubules. Moreover, lung congestion was observed in the 6400-ppm-exposed males and females (data not shown). All the other groups survived to the end of the 13-wk exposure period. In the surviving animals, no abnormal clinical sign was found in any 1,4-dioxane-dosed rats of either sex throughout the 13-wk exposure period. Terminal body weight significantly decreased in the 200-ppm- and 3200-ppm-exposed males and in the females exposed to 200 ppm, 800 ppm, and above (Table 1). Relative liver weight significantly increased in the males and females exposed to 800 ppm and above (Table 1). Relative kidney weight significantly increased in the 3200-ppm-exposed males and in the females exposed to 800 ppm and above. Relative lung weight increased in the males exposed to 200 ppm and 1600 ppm and above and in the females exposed to 200 ppm and above.

Hematology, Blood Biochemistry, and Urinalysis

Some erythrocyte parameters were increased in the 3200-ppm-exposed males and females with slight but statistical significance (Table 2). A slight but statistically significant increase was found in both aspartate aminotransferase (AST) in the 200-ppm- and 3200-ppm-exposed females and alanine aminotransferase (ALT) in the 3200-ppm-exposed males and females. The exposure to 3200 ppm decreased blood levels of glucose and triglyceride only in the males, but not in the females. Urinary protein slightly decreased in the males exposed to 3200 ppm (data not shown).

Histopathology

Repeated inhalation exposure to 1,4-dioxane vapor for 13 wk affected the upper and lower respiratory tracts and liver in both males and females and kidneys in females (Tables 3 and 4). The most sensitive lesion occurred in the nasal cavity,

TABLE 1

Terminal body weights and relative organ weights of the male and female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

Group (ppm)	Control	100	200	400	800	1600	3200
Male							
No. of animals examined	10	10	10	10	10	10	10
Body weight (g)	323 ± 14	323 ± 14	304 ± 11*	311 ± 19	317 ± 12	312 ± 14	301 ± 11**
Liver (%)	2.610 ± 0.069	2.697 ± 0.092	2.613 ± 0.084	2.666 ± 0.080	2.726 ± 0.082*	2.737 ± 0.077**	2.939 ± 0.101**
Kidneys (%)	0.589 ± 0.016	0.596 ± 0.021	0.612 ± 0.013	0.601 ± 0.020	0.610 ± 0.015	0.606 ± 0.021	0.647 ± 0.026**
Lungs (%)	0.310 ± 0.011	0.312 ± 0.007	0.325 ± 0.008*	0.320 ± 0.009	0.321 ± 0.011	0.333 ± 0.009**	0.346 ± 0.017**
Female							
No. of animals examined	10	10	10	10	10	10	10
Body weight (g)	187 ± 5	195 ± 8	174 ± 10**	180 ± 5	175 ± 6**	173 ± 8**	168 ± 4**
Liver (%)	2.353 ± 0.081	2.338 ± 0.092	2.395 ± 0.092	2.408 ± 0.066	2.513 ± 0.076**	2.630 ± 0.139**	2.828 ± 0.144**
Kidneys (%)	0.647 ± 0.014	0.631 ± 0.019	0.668 ± 0.012	0.662 ± 0.024	0.679 ± 0.018**	0.705 ± 0.028**	0.749 ± 0.024**
Lungs (%)	0.402 ± 0.013	0.402 ± 0.015	0.435 ± 0.018**	0.429 ± 0.029*	0.430 ± 0.013**	0.454 ± 0.018**	0.457 ± 0.016**

Note. Values are represented as mean ± SD. Significant difference indicated by $^{*}p \leq .05$ and $^{**}p \leq .01$ by Dunnett's test.
%: Relative organ weight.
Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

TABLE 2

Hematology and blood biochemistry of the male and female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

Group (ppm)	Control	100	200	400	800	1600	3200
Male							
No. of animals examined	10	10	10	10	10	10	10
Red blood cell ($10^6/\mu L$)	9.55 ± 0.17	9.53 ± 0.24	9.54 ± 0.18	9.59 ± 0.26	9.55 ± 0.18	9.58 ± 0.14	9.57 ± 0.37
Hemoglobin (g/dL)	16.0 ± 0.2	16.1 ± 0.4	15.9 ± 0.2	16.1 ± 0.3	16.0 ± 0.3	16.2 ± 0.3	16.4 ± 0.4*
Hematocrit (%)	46.2 ± 1.2	46.3 ± 1.3	46.3 ± 0.9	46.3 ± 1.4	46.3 ± 1.1	46.8 ± 0.9	47.3 ± 1.7
MCV (fL)	48.4 ± 0.7	48.6 ± 0.7	48.6 ± 0.4	48.3 ± 0.4	48.5 ± 0.6	48.9 ± 0.6	49.4 ± 0.5**
AST (IU/L)	73 ± 8	75 ± 14	73 ± 10	72 ± 5	72 ± 3	70 ± 4	73 ± 4
ALT (IU/L)	27 ± 3	27 ± 4	27 ± 4	28 ± 1	27 ± 2	27 ± 2	30 ± 2*
Glucose (mg/dL)	197 ± 17	206 ± 13	192 ± 9	190 ± 12	187 ± 15	184 ± 12	170 ± 11**
Triglyceride (mg/dL)	125 ± 17	148 ± 37	118 ± 33	131 ± 30	113 ± 27	106 ± 24	87 ± 22*
Female							
No. of animals examined	10	9[a]	9[a]	9[a]	10	9[a]	10
Red blood cell ($10^6/\mu L$)	8.77 ± 0.23	8.69 ± 0.21	8.73 ± 0.25	8.88 ± 0.21	8.68 ± 0.69	8.86 ± 0.16	9.15 ± 0.12**
Hemoglobin (g/dL)	16.2 ± 0.3	16.0 ± 0.3	16.3 ± 0.4	16.2 ± 0.4	16.2 ± 0.6	16.3 ± 0.2	16.6 ± 0.2*
Hematocrit (%)	46.0 ± 1.5	45.5 ± 1.2	45.8 ± 1.7	46.5 ± 1.5	45.4 ± 3.6	46.2 ± 0.7	47.5 ± 0.6*
MCV (fL)	52.5 ± 0.7	52.3 ± 0.7	52.4 ± 0.7	52.4 ± 0.8	52.3 ± 0.6	52.1 ± 0.5	52.0 ± 0.7
AST (IU/L)	64 ± 6	65 ± 3	74 ± 14*	69 ± 5	68 ± 6	70 ± 5	76 ± 5**
ALT (IU/L)	23 ± 3	21 ± 2	26 ± 10	25 ± 3	24 ± 4	25 ± 3	30 ± 3**
Glucose (mg/dL)	143 ± 18	144 ± 18	137 ± 9	140 ± 15	141 ± 15	139 ± 11	139 ± 18
Triglyceride (mg/dL)	45 ± 5	48 ± 6	42 ± 4	47 ± 8	42 ± 6	39 ± 7	42 ± 7

Note. Values are represented as mean ± SD. Significant difference indicated by $^{*}p \leq .05$ and $^{**}p \leq .01$ by Dunnett's test.
MCV: Mean corpuscular volume. AST : Aspartate aminotransferase. ALT: Alanine aminotransferase.
[a]The measurement could not be carried out for 1animal because of shortage of blood volume.
Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

TABLE 3
Incidences and severities of selected lesions in the male rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

	Male						
Group (ppm)	Control	100	200	400	800	1600	3200
No. of animals examined	10	10	10	10	10	10	10
Nasal cavity							
Nuclear enlargement: respiratory epithelium	0	7** (1+:7)	9** (1+:9)	7** (1+:7)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Nuclear enlargement: olfactory epithelium	0	0	5* (1+:5)	10** (1+:10)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Vacuolic change: olfactory epithelium	0	1 (1+:1)	3 (1+:3)	6* (1+:6)	10** (1+:10)	10** (1+:10)	9** (1+:9)
Trachea							
Nuclear enlargement: epithelium	0	0	0	0	1 (1+:1)	10** (1+:10)	10** (1+:10)
Bronchus							
Nuclear enlargement: bronchial epithelium	0	0	0	0	0	9** (1+:9)	10** (1+:10)
Vacuolic change: bronchial epithelium	0	0	0	0	4 (1+:4)	6* (1+:6)	6* (1+:6)
Liver							
Necrosis: single cells	0	0	0	0	0	1 (1+:1)	8** (1+:8)
Swelling: centrilobular	0	0	0	0	0	1	10** (1+:10)

Note. The number of the animals bearing the lesion in each exposed or control group are shown in the upper column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity ie., 1+: slight, 2+: moderate, 3+: marked, 4+: severe.

Significant difference indicated by* $p \leq .05$, ** $p \leq .01$ by chi square test.

Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

where the incidences and severities of nuclear enlargement in the respiratory epithelium were significantly increased dose-dependently in both males and females exposed to 100 ppm and above. The nuclear enlargement (Figure 1) was morphologically characterized by the appearance of the epithelial cells having round to oval or elongated nuclei that were at least four times as large in diameter as the normal nuclei of respiratory epithelial cells. The enlarged nuclei of respiratory epithelial cells were

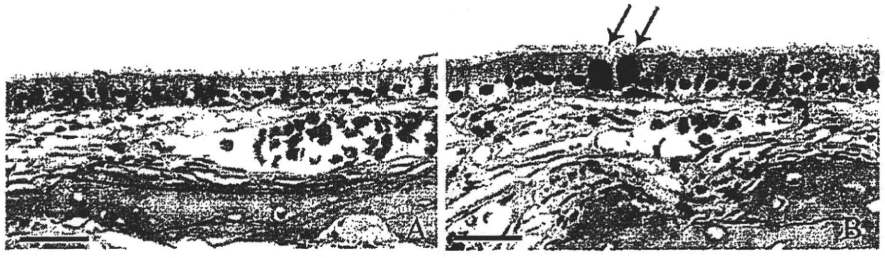

FIG. 1. (A) The normal respiratory epithelium in a male rat exposed to clean air for 13 wk. (B) Nuclear enlargement (arrows) in the respiratory epithelium at Level 2 of nasal cavity in a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. H&E stain. Bars indicate 50 μm.

TABLE 4

Incidences and severities of selected lesions in the female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

	Female						
Group (ppm)	Control	100	200	400	800	1600	3200
No. of animals examined	10	10	10	10	10	10	10
Nasal cavity							
Nuclear enlargement: respiratory epithelium	0	5* (1+:5)	9** (1+:9)	10** (1+:10)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Nuclear enlargement: olfactory epithelium	0	2 (1+:2)	6* (1+:6)	10** (1+:9, 2+:1)	10** (1+:10)	10** (1+:7, 2+:3)	10** (2+:10)
Vacuolic change: olfactory epithelium	0	1 (1+:1)	2 (1+:2)	3 (1+:3)	7** (1+:7)	9** (1+:9)	10** (1+:10)
Atrophy: olfactory epithelium	0	0	2 (1+:2)	3 (1+:3)	5* (1+:5)	5* (1+:5)	4 (1+:4)
Trachea							
Nuclear enlargement: epithelium	0	0	0	0	2 (1+:2)	7** (1+:7)	10** (1+:10)
Bronchus							
Nuclear enlargement: bronchial epithelium	0	0	0	0	0	0	10** (1+:10)
Vacuolic change: bronchial epithelium	0	0	0	1 (1+:1)	1 (1+:1)	3 (1+:3)	4 (1+:4)
Liver							
Necrosis: single cells	0	0	0	0	0	0	3 (1+:3)
Swelling: centrilobular	0	0	0	0	0	1 (1+:1)	8** (1+:8)
Kidney							
Hydropic change: proximal tubule	0	0	0	0	0	0	6* (1+:6)

Note. The number of the animals bearing the lesion in each exposed or control group are shown in the upper column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity ie., 1+: slight, 2+: moderate, 3+: marked, 4+: severe.

Significant difference indicated by $^{*}p \leq .05$, $^{**}p \leq .01$ by chi square test.

Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

localized at Level 1 in the males exposed to 100 and 200 ppm and in the females exposed to 100 ppm, and at Levels 1 and 2 in the females exposed to 200 ppm, while the respiratory epithelial cells having the enlarged nuclei were extended over the entire respiratory region at Level 1 through 3 in both males and females exposed to 400 ppm and above. The enlarged nuclei of the sustentacular cells in the olfactory epithelium (Figure 2) were significantly increased in both males and females exposed to 200 ppm and above, and were distributed over almost the entire area of the olfactory region at Levels 2 and 3. The incidences and severities of nuclear enlargement in the 1,4-dioxane-exposed males and females tended to decrease along the passage of inspiratory airflow through the upper and lower respiratory tracts. The incidence of degenerative change in the olfactory sensory cells observed as the vacuolic change (Figure 2) was significantly increased in the males exposed to 400 ppm and above, while the females exposed to 800 ppm and above exhibited olfactory epithelial atrophy characterized by a decreased number of the olfactory sensory cells and vacuolic change. Vacuolic change in the bronchial epithelium also occurred with statistical significance in the males exposed to 1600 ppm and above.

The 1,4-dioxane-induced liver lesions were characterized by significant increases in the incidences of both single-cell necrosis and centrilobular swelling of hepatocytes in the 3200-ppm-exposed males, and in the incidence of centrilobular swelling of hepatocytes in the 3200-ppm-exposed females. In addition, the GST-P-positive liver foci (Figure 3) were observed in three 3200-ppm-exposed males, two 3200-ppm-exposed females, and

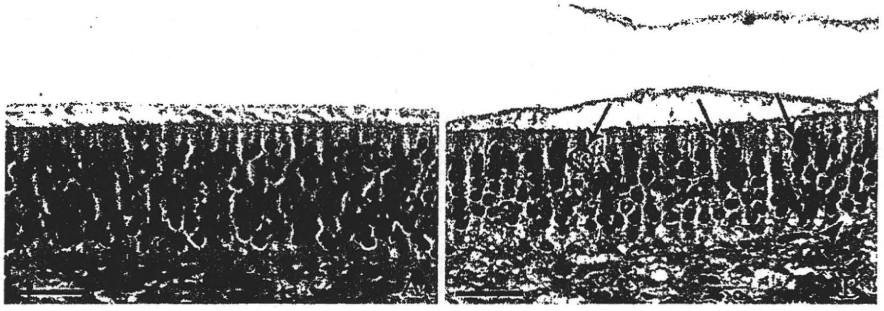

FIG. 2. (A) The normal olfactory epithelium in a male rat exposed to clean air for 13 wk. (B) Nuclear enlargement (arrows) of sustentacular cells and vacuolic change (arrowhead) of the olfactory sensory cells in the olfactory epithelium at Level 3 of nasal cavity in a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. H&E stain. Bars indicate 50 μm.

four 1600-ppm-exposed females out of each ten 1,4-dioxane-exposed rats of either sex, whereas the GST-P-positive foci could not be found in any of the 800- and 1600-ppm-exposed males and 800-ppm-exposed females and control groups of both sexes. The GST-P-positive hepatocytes exhibited morphologically focal and clonal proliferation.

The incidence of hydropic change in the renal proximal tubules was significantly increased in the 3200-ppm-exposed females.

Blood Levels of 1,4-Dioxane

Plasma levels of 1,4-dioxane were detected in the concentrations of exposure to 400 ppm and above, and increased linearly with the increase in the exposure concentration. The mean plasma levels of 1,4-dioxane in the 400-, 800-, 1600-, and 3200-ppm-exposed rats were 48, 152, 319, and 730 μg/ml for males, and 80, 209, 468, and 1054 μg/ml for females, respectively. Plasma levels of 1,4-dioxane were higher in females than in males. Figure 4 shows a highly linear relationship for each sex between the plasma levels of 1,4-dioxane and concentrations of inhalation exposure to 1,4-dioxane at 400 ppm and above, as indicated by the adjusted coefficient R^2 values greater than .9.

DISCUSSION

In this study, repeated inhalation exposure to 1,4-dioxane vapor for 13 wk was found to affect the upper and lower respiratory tracts and liver in rats of both sexes and kidneys in female rats. Nuclear enlargement of epithelial cells in the nasal respiratory epithelium was the most sensitive lesion occurring in the 100-ppm-exposed males and females. The incidence and severity of enlarged nuclei of epithelial cells decreased along the passage of 1,4-dioxane-containing inspiratory flow through the upper and lower respiratory tracts, suggesting that the nuclear enlargement tended to decrease with the presumably gradual decrease in the amount of 1,4-dioxane absorbed in the mucous layer of the respiratory, olfactory, tracheal, and bronchial epithelia. Notably, oral administration of 1,4-dioxane in drinking water for 13 wk was also found to induce nuclear enlargement in the nasal epithelia

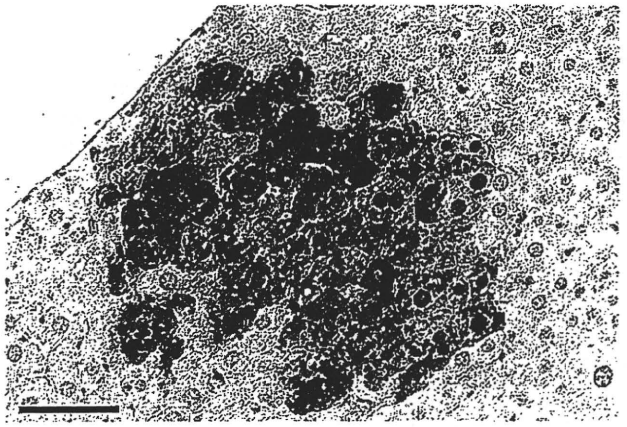

FIG. 3. A GST-P-positive focus in the liver of a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. Immunochemical staining with GST-P antibody. Bar indicates 100 μm.

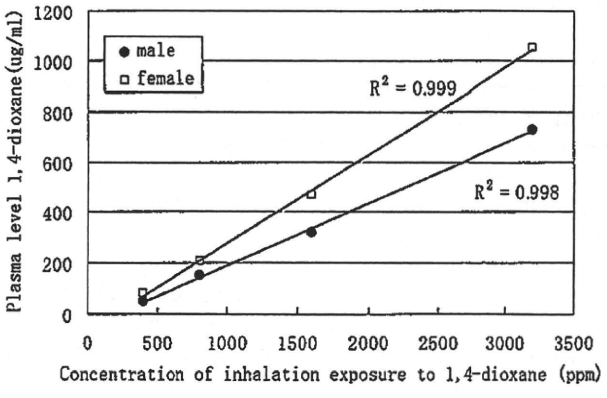

FIG. 4. Plasma levels of 1,4-dioxane as a function of the concentrations of exposure to 1,4-dioxane vapor. Filled circles and open squares represent the mean values of male and female rats, respectively.

of rats and mice of both sexes (Kano et al., 2008). However, there is a clear difference in the location of the 1,4-dioxane-induced enlarged nuclei of the nasal epithelial cells between the inhalation exposure and the oral administration. The respiratory epithelial area having the enlarged nuclei was expanded from the anterior portion (Level 1) to the entire region (Levels 1, 2, and 3) with an increase in the concentrations of inhalation exposure to 1,4-dioxane from 100 ppm to 3200 ppm. On the other hand, the oral administration of 1,4-dioxane-formulated drinking water uniformly produced the nuclear enlargement over the entire region (Levels 1, 2, and 3) of the respiratory epithelium without any anterior–posterior gradient along the nasal passage. This difference in the route of exposure can be accounted for in terms of a first-pass effect such that the inhaled 1,4-dioxane comes into first contact with the anterior portion of the respiratory epithelium, while the orally administered 1,4-dioxane is conveyed to the respiratory epithelial cells through the nasal blood flow after its first entrance in the gastrointestinal system including the liver. The nuclear enlargement was reported to occur as an early histopathological change in the upper respiratory tract of the rat simultaneously exposed to sulfur dioxide and intraperitoneally injected with several *N*-nitrosamines that are known to induce nasal tumors in rats (Fowlie et al., 1990). Grant and Grasso (1978) showed a good correlation between in vivo carcinogenicity and the extent of nuclear enlargement in HeLa cells in vitro. It can be inferred, therefore, that the nuclear enlargement of nasal epithelial cells found in the present study represents an early detectable and potentially preneoplastic lesion participating in the possible development of nasal tumors that would be induced by 2-yr inhalation exposure of rats to 1,4-dioxane vapor.

A significant increase in the degenerative change was evident in the olfactory epithelium of the males exposed to 400 ppm and above and of the females exposed to 800 ppm and above. The degenerative change was characterized by a vacuolic change and a decreased number of olfactory sensory cells, suggestive of possible impairment of olfactory sensation. Therefore, the olfactory epithelial degeneration is thought to represent a biologically significant adverse effect allowing extrapolation of animal toxicity data to humans, since the degenerative lesion of nasal mucosa might cause attenuation or loss of the olfactory acuity in humans (Tvedt et al., 1991; Hirsch & Zavala, 1999).

Another quantitative difference in the 1,4-dioxane-induced nasal and hepatic lesions between the present inhalation study and the oral administration study (Kano et al., 2008) can be recognized in light of the first-pass effect on the target organs. In the oral administration study, the nasal cavity lesions were induced at the drinking-water concentration of 1600 ppm, which corresponded to the estimated daily doses of 126 and 185 mg/kg in male and female rats, respectively. In the present inhalation study, however, the nasal cavity lesions were observed at the lowest inhalation exposure concentration of 100 ppmb (v/v), which corresponded to the estimated 1,4-dioxane uptake of 73 mg/kg/day, assuming that the minute volume is 561 ml/min/kg body weight for rats (Mauderly et al., 1979) and that the uptake ratio of 1,4-dioxane by the upper and lower respiratory tracts is 100%. Evidently, the 1,4-dioxane-induced nasal cavity lesions appeared at lower uptake levels by the inhalation exposure than by the oral administration. On the other hand, the oral administration of 1,4-dioxane induced centrilobular swelling of hepatocytes at the estimated daily uptake of 126 mg/kg in male rats and 756 mg/kg in female rats (Kano et al., 2008). The inhalation exposure to 1,4-dioxane induced centrilobular swelling of hepatocytes only at the highest exposure concentration of 3200 ppm (v/v), which corresponded to the estimated 1,4-dioxane uptake of 2336 mg/kg/day. Thus, the 1,4-dioxane-induced hepatic lesions appeared at lower uptake levels by oral administration than by inhalation exposure. These differences between oral administration and inhalation exposure can also be accounted for in the same terms of the first-pass effect on the gastrointestinal tract, including the liver, causing higher hepatic levels of 1,4-dioxane by oral administration than by inhalation exposure.

The hepatic lesions that occurred at higher concentrations of inhalation exposure to 1,4-dioxane than the nasal lesions did were characterized by both single-cell necrosis and hepatocellular swelling in the male rats and hepatocellular swelling in the females, in addition to GST-P-positive liver foci. The GST-P-positive hepatocytes appeared to be focally and clonally proliferating. It was reported, however, that a broad range of in vitro and in vivo genotoxicity assays for 1,4-dioxane produced negative results (IARC, 1999; Morita & Hayashi, 1998), and that no tumor was induced by 2-yr inhalation exposure of rats to 1,4-dioxane vapor at a level of 111 ppm (Torkelson et al., 1974). GST-P is known as a good specific marker enzyme for detecting an early histogenetic stage participating in the development of rat hepatocellular tumors by chemical carcinogens (Sato et al., 1984; Tatematsu et al., 1985; Ito et al., 1988). Therefore, a portion but not all of the GST-P-positive hepatocellular foci occurring after 13-wk inhalation exposure to 1,4-dioxane is considered to undergo further focal and clonal proliferation and progress finally to hepatocellular tumors that would be induced after 2-yr inhalation exposure to 1,4-dioxane at appropriately selected exposure concentrations in due consideration of the criterion of maximum tolerated dose (MTD) (Sontag et al., 1976; Bannasch et al., 1986). Indeed, the MTD for the 2-yr bioassay study for rat carcinogenicity of 1,4-dioxane is estimated to be much higher than the exposure concentration of 111 ppm used by Torkelson et al. (1974).

Inhalation exposure to 1,4-dioxane was found to severely affect the kidneys at high exposure concentrations, because the cause of deaths in the 6400-ppm-exposed rats of both sexes was attributed primarily to the renal failure resulting from marked necrosis in the renal tubules. The renal lesions of surviving animals were characterized by hydropic change in the proximal tubules occurring only in the 3200-ppm-exposed females but not in the males and by significantly increased relative kidney weight occurring in the 3200-ppm-exposed males and in the

females exposed to 800 ppm and above. Thus, higher susceptibility of females to the renal toxicity of 1,4-dioxane than that of males is suggested. This gender difference might be causally related to the following two factors. First, it was found in the present study that the mean plasma levels of 1,4-dioxane were higher in female rats than in males. Second, it is known that the level of CYP2E1 in the rat is greater in females than in males (Parkinson, 2001). Since Nannelli et al. (2005) demonstrated the induction of renal CYP2E1 enzymes in the rat receiving acute and chronic oral administration of 1,4-dioxane, higher susceptibility of females to the renal toxicity might be related to either the increased kidney levels of 1,4-dioxane expected from the increased plasma level of 1,4-dioxane in females over males or the enhanced production of its toxic metabolites by the renal CYP2E1 induction in females. However, the exact role of 1,4-dioxane or its metabolites involved in the mechanism of renal toxicity still remains unsolved (Woo et al., 1978; Hecht & Young, 1981; Nannelli et al., 2005).

A highly linear increase in plasma levels of 1,4-dioxane with an increase in the concentrations of inhalation exposure to 1,4-dioxane at up to 3200 ppm can be taken to indicate that the metabolic capacity is not saturated up to plasma levels of 1,4-dioxane at 730 μg/ml for male rats and 1,054 μg/ml for female rats. This result appears to be in sharp contrast to the Young et al. finding (1978) that single oral doses of 1,4-dioxane resulting in plasma levels above 100 μg/ml are removed from the body more slowly due to the saturation of metabolism. It was reported that 1,4-dioxane is metabolized by cytochrome P-450 (P450) enzymes and excreted primarily into urine as 2-hydroxyethoxyacetic acid or 1,4-dioxane-2-one (Braun & Young, 1977; Young et al., 1978; Woo et al., 1978; Nannelli et al., 2005). The 1,4-dioxane metabolism was reported to be enhanced by the induction of hepatic P450 enzymes in the rat pretreated with phenobarbital (Woo et al., 1978) and in the rat receiving repeated oral administration of 1,4-dioxane (Young et al., 1978; Nannelli et al., 2005). Nannelli et al. (2005) also reported that P450 2E1 enzymes in the nasal cavity and kidney are induced by repeated oral administration of 1,4-dioxane in drinking water. Therefore, lack of the metabolic saturation of 1,4-dioxane found in the present study might be attributed to the enhanced metabolism by the possible induction of P450 enzymes including CYP2E1 by 13-wk repeated inhalation exposure to 1,4-dioxane at up to 3200 ppm.

In the present inhalation study, a LOAEL value can be determined at 100 ppm for the nasal endpoint, because of the significantly increased incidence of nuclear enlargement in the nasal respiratory epithelium of both male and female rats exposed to the lowest exposure concentration of 100 ppm 1,4-dioxane. This LOAEL value is thought to be lower than the reported NOAEL of 400 mg 1,4-dioxane/m^3 (111 ppm) (EU Risk Assessment Report, 2002), which was based on Torkelson et al.'s report (1974). In the previous study of 13-wk oral administration of 1,4-dioxane (Kano et al., 2008), the most sensitive sign was both the nuclear enlargement in the nasal respiratory epithelium and the centrilobular swelling of hepatocytes. A NOAEL value was determined at a drinking-water concentration of 640 mg 1,4-dioxane/kg water for those 2 endpoints, which corresponded to the estimated 1,4-dioxane uptake of 52 and 83 mg/kg/day in male and female rats, respectively. The LOAEL value of 100 ppm (v/v) obtained by the daily 6-h inhalation exposure of F344 rats to 1,4-dioxane vapor for 13 wk corresponded to the estimated 1,4-dioxane uptake of 73 mg/kg body weight/day. Therefore, taking into consideration the difference in the definition between LOAEL and NOAEL (IPCS, 1994), the presumed NOAEL value expected from the LOAEL of 100 ppm obtained by the 13-wk inhalation exposure to 1,4-dioxane vapor is lower than the NOAEL obtained by the 13-wk oral administration. The present finding that the presumed NOAEL for the nasal response to the inhaled 1,4-dioxane is more sensitive than that for the same endpoint to the ingested 1,4-dioxane can also be attributable to the first-pass effect discussed earlier for the nasal cavity lesion, and therefore may implicate the importance of the nasal response as an endpoint that can be used for risk assessment of humans exposed to hazardous chemicals by inhalation.

Recommendation of the OEL value of 20 ppm for 1,4-dioxane by the ACGIH (2001) was based on both animal and human studies. For the animal basis of toxicity, the ACGIH used the NOAEL of 111 ppm, judging by the Torkelson et al. (1974) result that neither pathological changes nor tumors were induced on gross and microscopic examinations of organs and tissues in male and female Wistar rats exposed by inhalation to 1,4-dioxane vapor at 111 ppm for 7 h/day, 5 days/wk, for 104 wk, although the nasal cavity was not listed in the organs subjected to the microscopic examination in their report. The nasal-cavity lesions found in the 1,4-dioxane-inhaled rat are relevant to possible adverse health effects arising from workers' inhalation exposure to 1,4-dioxane, and the route of exposure and the daily and weekly exposure regimens employed in the present study are closely simulated for human exposure to 1,4-dioxane in workplaces. The presumed NOAEL value of 1,4-dioxane for the nasal endpoint is estimated to be lower than the LOAEL of 100 ppm found in the present inhalation study. Assuming an uncertainty factor of 10 for extrapolation of rodent data to humans (Barns, 1988), one-tenth of the presumed NOAEL value for 1,4-dioxane falls below the current OEL value of 20 ppm, suggesting the need to reconsider the OEL for 1,4-dioxane.

In conclusion, the present study demonstrated toxic effects in the upper and lower respiratory tract, liver, and kidneys in the rats exposed by inhalation to 1,4-dioxane vapor. Nuclear enlargement of the respiratory epithelial cells occurring at 100 ppm was the most sensitive lesion. The enlarged nuclei in the nasal epithelia might represent an early recognizable stage suggestive of the possible development of nasal tumors. The hepatic lesions that occurred in higher concentrations than did the nasal-cavity lesions were characterized by single-cell necrosis and centrilobular swelling of hepatocytes, in addition to the preneoplastic GST-P-positive liver foci. These results might predict that 2-yr