was a more pronounced change than that with MWCNTs (Fig. 4). In addition, mRNA levels of *IL-5* were 5, 11, 1, and 1 times higher (Fig. 4), and those of *MCP-1* were 14, 9, 2, and 4 times higher (Fig. 5) at the end of 2, 4, 10, and 20 weeks, respectively; however, these changes were faint and transient.

Effects on the peripheral blood cells

MWCNT treatment increased the total number of leukocytes, granulocytes, and monocytes in the peripheral blood as early as 1 week after its administration, and these high levels were maintained up to the end of week 20 (Figs. 6a, 6b and 6c). The number of total lymphocytes was also increased, but only at the end of week 20. B and T cells were increased, although not significantly, within the 20-week experimental period in the MWCNT-treated mice (Figs. 6d, 6e and 6f). In the crocidolite treatment mice, the numbers of leukocytes, granulocytes, and monocytes exhibited a statistically significant, although minimal, transient increase at the end of week 1 (Figs. 6a, 6b and 6c). CB and crocidolite treatment increased the numbers of lymphocytes, B, and T cells at the end of day 2 and 1 week, but not significantly, and then decreased (Figs. 6d, 6e and 6f).

Expression of leukocyte adhesion molecules on the peripheral blood cells

MWCNT treatment induced overexpression of CD49d and CD54, but not CD11b, on granulocytes as early as 2 and 1 weeks, respectively, after its administration, and these high levels were maintained up to the end of week 20 (Fig. 7a). The expression of adhesion molecules was not altered on monocytes, with the exception that a statistically significant, although minimal, transient overexpression was observed for CD49d at the end of week 4 (Fig. 7b). CB and crocidolite did not induce overexpression of any of the leukocyte adhesion molecules on the peripheral blood cells, and in fact their expression was transiently decreased in some cases (Fig. 7).

OVA-specific immunoglobulins in serum

Figure 8 summarizes the results for the serum con-

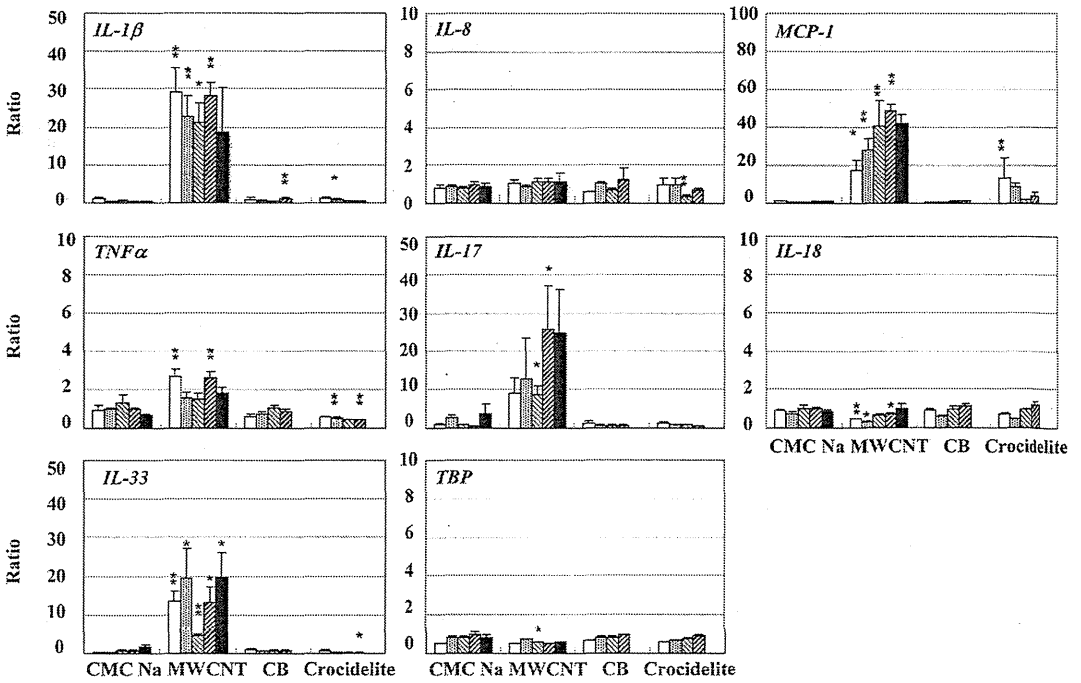

Fig. 5. mRNA expression of inflammatory cytokine genes in peritoneal cells obtained from mice in the first animal experiment. For each group of mice exposed to a test chemical or vehicle, columns from the left to the right are average values (n = 3-6) at 2, 4, 10, 20, and 34 weeks after exposure. These determinations were not made at the end of week 34 for the CB- and crocidolite-treated groups. (*$p < 0.05$, **$p < 0.01$).

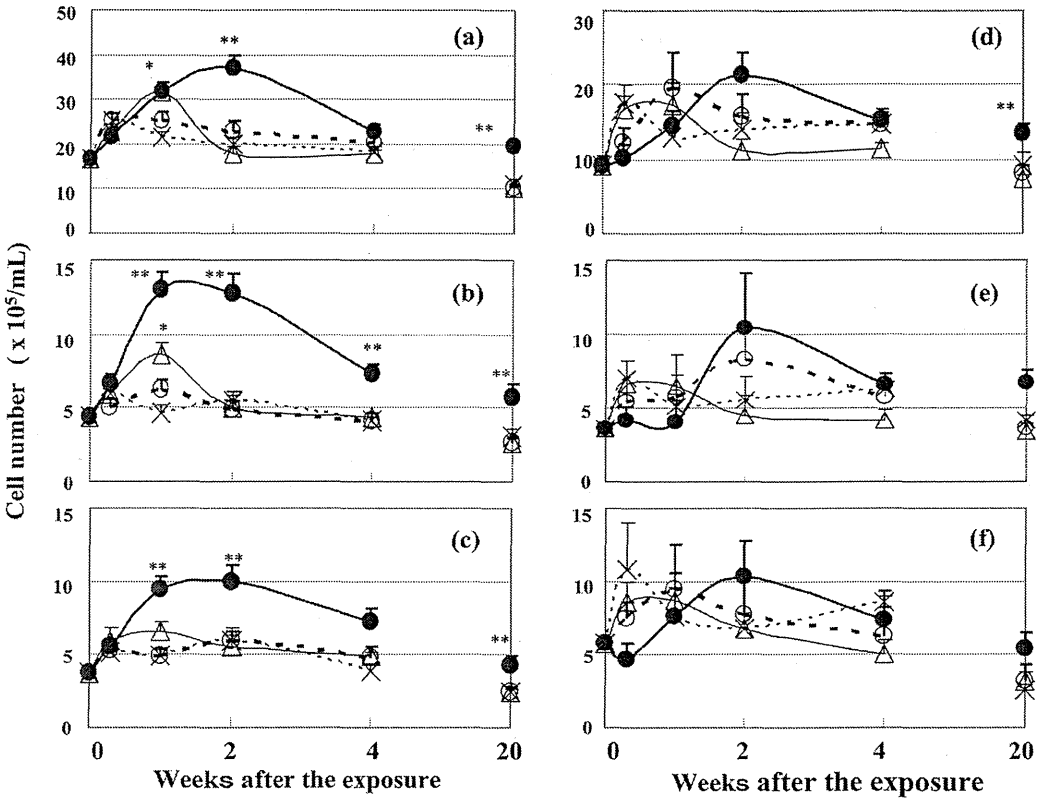

Fig. 6. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the numbers of (a) leukocytes, (b) granulocytes, (c) monocytes, (d) lymphocytes, (e) B cells, and (f) T cells after exposure to CMC Na (open circles), MWCNTs (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± standard deviations (n = 4). Asterisks indicate that results are significantly different from those of controls (*$p < 0.05$, **$p < 0.01$).

centrations of OVA-specific IgM (Fig. 8a) and IgG_1 (Fig. 8b). For mice treated with MWCNTs, CB, crocidolite, and CMC Na, the relative amounts (arbitrary units; AU) of OVA-specific IgM were, 1.33 ± 0.20, 1.07 ± 0.20, 1.07 ± 0.15, and 0.79 ± 0.12 AU, respectively, while those for OVA-specific IgG_1 were, 3.68 ± 0.57, 2.49 ± 0.29, 2.13 ± 0.32, and 2.28 ± 0.35 AU, respectively. Thus, MWCNT and not CB or crocidolite, significantly enhanced the production of OVA-specific immunoglobulins in mice.

DISCUSSION

The present study clearly shows that MWCNTs stimulated immune and inflammatory responses in mice and these effects sustained until the mice died. It has been previously shown in other animal models that a single intraperitoneal administration of MWCNT caused severe inflammation throughout the abdominal cavity and mesothelioma. Male Fisher 344 rats died at 37-52 weeks after administration (Sakamoto *et al.*, 2009) and and male C57BL/6-originated mice heterozygously deficient in the *p53* gene died within 25 weeks of administration (Takagi *et al.*, 2008).

The toxicity caused by MWCNTs in the present study did not involve tumor formation, but did induce severe inflammation, and 2 of 6 mice had died by the end of 32 weeks. The differences in the magnitudes of MWCNT toxicity between the present and previous studies was apparently because of differences in species, strains, and/or genders. To extrapolate the animal toxicity data to information important for human health concerns, further investigations are required. The most aggressive morphological change we observed was the infiltration of macrophages, eosinophils, plasma cells, and immature myeloid cells into the fibrously thickened visceral peritoneum of the liver with occasional granulation, and severe fibrous adhesions to the internal organs.

Light microscopic examination revealed that MWCNT

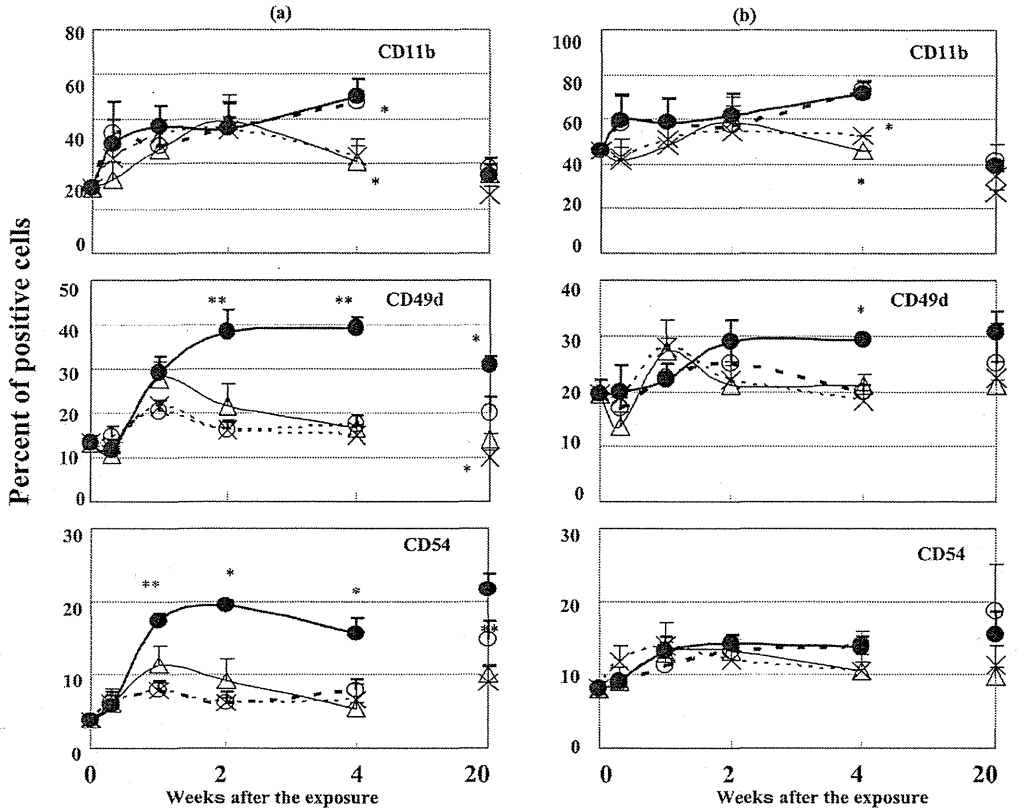

Fig. 7. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the expression of adhesion molecules on the surfaces of (a) granulocytes and (b) monocytes after exposure to CMC Na (open circles), MWCNT (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± S.D. (n = 4). Asterisks indicate that values are significantly different from those of controls ($*p < 0.05$).

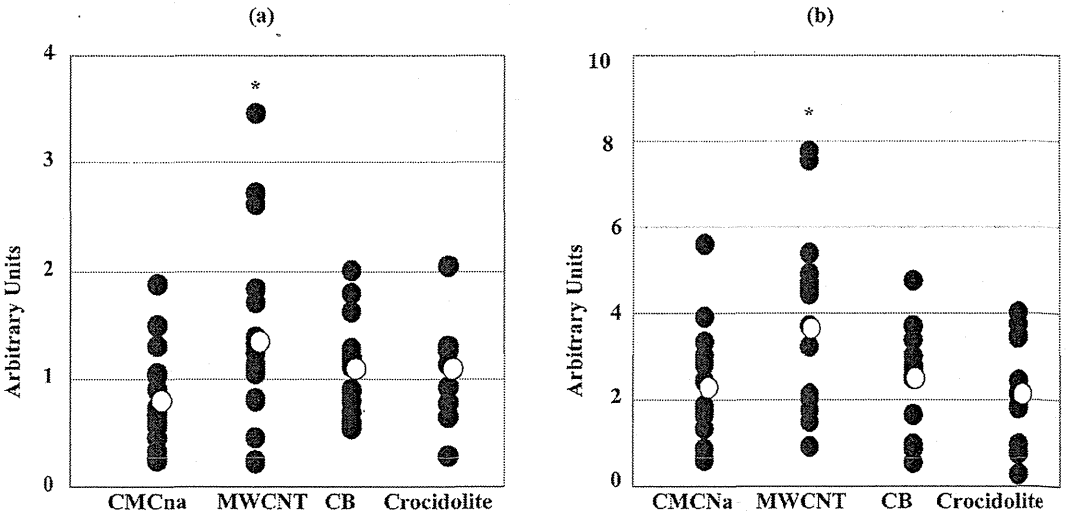

Fig. 8. Production of OVA-specific antibodies by mice in the third animal experiment. Serum concentrations of OVA-specific (a) IgM and (b) IgG_1 Open circles are average values, and closed circles are individual values (n = 10-19 for IgM, and n = 15 for IgG_1). Asterisks indicate that values are significantly different from those of controls ($*p < 0.05$).

were present in macrophages in these lesions. Frustrated phagocytosis has frequently been postulated to be involved in the mechanisms by which MWCNTs causes toxicity, including inflammation and carcinogenesis (Poland *et al.*, 2008). Thus, these lesions may have some fundamental biological importance. The sustained overexpression of cytokine mRNA in peritoneal cells may suggest one possibility, although no MWCNTs were observed in the peritoneal cells by light microscopy. An *in vitro* time lapse experiment revealed that dead cells with MWCNTs had been re-engulfed by other macrophages (data not shown), suggesting a cycle of sustained inflammation. MWCNTs either could not be limited by granuloma formation or subvisible MWCNTs may remain in the cavity. Thus, macrophages may continue their phagocytic activity, and as a result inflammatory cytokines/chemokines would be continuously produced.

Our results indicated that MWCNTs caused a systemic inflammation that was sustained for at least 20-34 weeks after a single intraperitoneal administration, because the numbers of leukocytes, granulocytes, and monocytes in the peripheral blood were increased from 1 to 20 weeks after MWCNT administration. During a similar period, CD49d and CD54 were overexpressed on granulocytes, which may have been involved in their infiltration into the inflammatory sites past vascular endothelial cells. Crocidolite increased the numbers of leukocytes, granulocytes, and monocytes at 1 week, but returned to the basal level after 2 weeks, and the effects were weaker than MWCNTs. The number of leukocytes was slightly increased at 2 days and 1 week after CMCNa exposure, and mRNA level of *IFNγ* was increased at 2 and 4 weeks after CMCNa exposure, but decreased thereafter. However the effect of CMCNa has not been known, there is a possibility CMCNa acts as a xenobiotic although the effect is little. Furthermore, the number of peripheral lymphocytes was also increased at 20 weeks after MWCNT administration, and this corresponded to the enhanced T cell-dependent production of OVA-specific antibodies, as indicated by their increased serum concentrations. The overexpressed mRNA of Th2 cytokine genes seen in peritoneal cells suggested that these cytokines have been involved in this enhanced antibody production. Although the underlying mechanisms need to be clarified, MWCNTs may promote these immune responses by acting as an adjuvant (Inoue *et al.*, 2009; Nygaard *et al.*, 2009).

The present study demonstrated the overexpression of mRNA for various cytokines/chemokines in peritoneal cells after a single intraperitoneal administration of MWCNTs. To the best of our knowledge, this is the first report of results obtained for peritoneal cells with regard to changes in cytokine/chemokine mRNA expression after MWCNT exposure *in vivo*. Previous reports focused primarily on short-term effects of MWCNT exposure. Mitchell *et al.* (2007) reported that *IL-10* levels increased in spleen homogenates after 14 consecutive days of whole body inhalation exposure for male C54BL/6 mice. Park *et al.* (2009) found that the protein levels of proinflammatory cytokines were increased both in the BAL fluid and in the peripheral blood, in which Th2 cytokines were increased to a greater extent than Th1 cytokines, in mice given intratracheal administrations of MWCNTs. In these reports, the levels of cytokines reached a peak at day 1 after the exposure and remained high at day 14.

In a study by Ryman-Rasmussen *et al.* (2009b) intratracheally administered MWCNTs potentiated the development of airway fibrosis in mice with allergic asthma induced by OVA sensitization, in which the levels of *IL-13* and *IL-5* increased at day 1, but returned to normal levels at day 14 when airway fibrosis became significant. Inoue *et al.* (2009) used MWCNT instillation for 6 weeks. At 24 hours after the final treatment, they observed significant exacerbation of murine allergic airway inflammation and high levels of Th1 and Th2 cytokine proteins. In the present study, the time-courses of changes in mRNA levels corresponded to functional groups of cytokines/chemokines.

mRNA overexpression of some pro-inflammatory cytokine genes, *IL-1β and IL-33*, occurred within 2 weeks and remained elevated up to the end of week 34. These are known to induce Th2 cytokines (Schmitz *et al.*, 2005; Amatucci *et al.*, 2007; Komai-Koma *et al.*, 2007; Kondo *et al.*, 2008). Therefore, mRNA of Th2 cytokine genes, *IL-4*, *IL-5* and *IL-13*, were also overexpressed within 2 weeks and remained elevated up to week 20. Among these, mRNA level for *IL-5* was still high, but levels for *IL-4* and *IL-13* decreased at the end of week 34.

mRNA level of a Th17 cytokine gene, *IL-17*, was also increased within 2 weeks, it was increased significantly after 10 to 20 weeks, and was still high, although not significantly, at the end of week 34. mRNA of Th1 cytokine genes, *IL-2* and *IFNγ*, were also overexpressed; however, this occurred at 20-34 weeks (*IL2*) and 34 weeks (*IFNγ*) after MWCNT exposure. While the details of the underlying mechanisms need to be clarified, the differential, time-dependent overexpression of Th2 and Th17 cytokine genes at first followed by Th1 cytokine genes may provide for some optimum balance between these inflammatory mediators for the sustained stimulating effects of MWCNTs on immune and inflammatory responses.

The present study indicated a rapid, drastic and sus-

tained increase in mRNA levels of Th2 cytokine genes, *IL-4*, *IL-5*, and *IL-13*, in peritoneal cells after exposure to MWCNT. In asthmatic and atopic patients, Th2 cytokines are induced and enhance inflammation and fibrosis (Schmid-Grendelmeier *et al.*, 2002; Izuhara 2003; Doherty and Broide 2007; Choi *et al.*, 2008). Thus, the overexpression of the Th2 cytokine genes in the present study may indicate their critical roles in MWCNT-induced inflammatory changes.

Among these overexpressed Th2 cytokines, mRNA level of *IL-5* increased most strikingly; it was 100 times higher than the control level at the end of week 2 and 50 times higher even at the end of week 34. Administration of an anti-*IL-5* antibody to mild atopic asthmatic patients reduced the numbers of airway eosinophils and fibrosis (Flood-Page *et al.*, 2003), and that *IL-5* deficient mice have significantly less peribronchial fibrosis (total collagen content) and significantly less peribronchial smooth muscle (thickness of peribronchial smooth muscle layer, α-smooth muscle actin immunostaining; Cho *et al.*, 2004). Thus, *IL-5* may be biologically important in the inflammatory reactions related to immune system disturbances. In these reports, *IL-5* and *TGFβ* were involved in the infiltration of eosinophils. Although the mRNA level of *TGFβ* was not altered in the present study, *IL-5* overexpresion may have caused the eosinophil infiltration into inflammatory sites.

It has recently been shown that *IL-1β, IL-18*, and *IL-33* are produced by the innate immune system, followed by the subsequent induction of Th2 cytokines (Schmid-Grendelmeier *et al.*, 2002; Izuhara, 2003; Doherty and Broide, 2007; Choi, *et al.*, 2008; Kroeger, *et al.*, 2009). Microbial pathogens, dead cells, and foreign bodies, such as asbestos or silica, can impose stress on phagocytes, which then develop inflammasomes.

The inflammasome is a multi-protein complex that is activated by ligand-induced intermediates, such as reactive oxygen species (ROS), K^+ efflux, or by lysosome destabilization (Dostert *et al.*, 2008; Petrilli *et al.*, 2007; Hornung *et al.*, 2008), and then by cysteine protease caspase-1. (Martinon *et al.*, 2002). Caspase-1 can initiate an apoptotic pathway and, at the same time, cleave cytokine precursors, such as pro-*IL-1β* and pro-*IL-18*, to form their mature forms (Dostert *et al.*, 2008; Yazdi *et al.*, 2010).

IL-33 is another member of the *IL-1* family that is produced by endothelial cells, epithelial cells (Moussion *et al.*, 2008), and myeloid cells (Schmitz *et al.*, 2005; Nile *et al.*, 2010). *IL-33* is processed by caspases in a manner similar to *IL-1β* and *IL-18* during apoptosis, although its cleavage product is not biologically active, and the full active form of *IL-33* must be released from damaged or necrotic cells. *IL-1β, IL-18*, and *IL-33* have been shown to activate Th2 cells, eosinophils, basophils, and mast cells to produce Th2 cytokines (Chow *et al.*, 2010; Komai-Koma *et al.*, 2007; Kondo *et al.*, 2008; Schmitz *et al.*, 2005), which induce inflammatory, allergic, and fibrotic changes (Finkelman *et al.*, 1999; Choi *et al.*, 2008; Doherty and Broide 2007).

In the present study, the mRNAs of *IL-1β* and *IL-33*, but not *IL-18*, were shown to be overexpressed, which suggests the involvement of the innate immune system in MWCNT-induced inflammatory changes. This may also be supported by the observation of the mRNA overexpression of the TLR adapter protein gene *MyD88*. TLR-related signals can also activate caspase-1 and may be a minor pathway in MWCNT-induced innate immune responses, because the magnitude of the overexpression of *MyD88* was small, although significantly increased.

The present results indicate that MWCNTs exert stronger effects than CB or crocidolite in female ICR mice. The latter two did not cause any particular pathological changes, and there were no apparent increases in leukocyte numbers, increased expression of leukocyte adhesion molecules on the peripheral blood cells, or enhanced production of OVA-specific antibodies. In addition, CB did not induce overexpression of any cytokine mRNAs in peritoneal cells, even though phagocytic activity may have been involved for up to 20 weeks. In fact, previous reports described no adverse effects of CB (Tabet *et al.*,2009; Teeguarden *et al.*, 2011). Crocidolite caused a sustained overexpression of *IL-6* mRNA in peritoneal cells, but *IL-6* has been reported not to stimulate or injure vascular vessel permeability (Manhiani *et al.*, 2007; McClintock *et al.*, 2005).

In conclusion, under the present experimental conditions, MWCNTs exhibited sustained stimulating effects on immune and inflammatory responses, unlike the other mineral fibers with structural similarities.

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Original Article

Teratogenicity of multi-wall carbon nanotube (MWCNT) in ICR mice

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ABSTRACT — A possible teratogenicity of multi-wall carbon nanotube (MWCNT) was assessed using ICR mice. MWCNTs were suspended in 2% carboxymethyl cellulose and given intraperitoneally or intratracheally to pregnant ICR mice on day 9 of the gestation. All fetuses were removed from the uterus on day 18 of the gestation, and were examined for external and skeletal anomalies. In the intraperitoneal study, various types of malformation were observed in all MWCNT-treated groups (2, 3, 4 and 5 mg/kg body weight, intraperitoneal). In contrast, such malformations were observed in groups given 4 or 5 mg/kg body weight, but not in that treated with 3 mg/kg in the intratracheal study. In either study, the number of litters having fetuses with external malformation and that of litters having fetuses with skeletal malformations were both increased in proportion to the doses of MWCNT. The present results are the first to report that MWCNT possesses the teratogenicity at least under the present experimental conditions. Mechanism(s) to result such malformations is yet unclear and further experiment is necessary.

Key words: Multi-wall carbon nanotube, Nanomaterial, Teratogenicity, Hazard identification, Mice

INTRODUCTION

Carbon nanotube is a new form of the technological crystalline carbon and one of the most anticipated nanomaterials, because of its unique properties suitable for a variety of industrial products such as high strength materials, electronics and biomedical apparatuses (Martin and Kohli, 2003; Scott, 2005). On the other hand, potential hazards and/or risk for humans of carbon nanotube has been concerned, and large efforts have been internationally being made to investigate and evaluate them (Lam *et al.*, 2006; Pacurari *et al.*, 2010; Hubbs *et al.*, 2011). Among those, a possible carcinogenicity has been concerned most, assuming the structural similarity between carbon nanotubes and asbestos. Takagi *et al.* (2008) have first reported that multi-wall carbon nanotube (MWCNT) induces mesotheliomas, when intraperitoneally administered to male p53 gene deficient mice. Shortly afterwards, Sakamoto *et al.* (2009) have demonstrated that the carcinogenicity of MWCNT is a universal event and not specific to mice or genetically modified animals, by showing the mesothelioma development in male intact (not genetically modified) rats, intrascrotally administered the same MWCNT. Since then, carcinogenicity of MWCNT has enthusiastically been being studied but the mechanism(s) of such carcinogenicity is yet not clearly understood. Because the damage to DNA, directly or indirectly, by MWCNT is to be evaluated by prenatal stage, a possible teratogenicity must be another big issue for the risk assessment of MWCNT. To the best of our knowledge there have been no reports dealing with this issue in the literature. In this content, the present study was conducted to assess a possible teratogenicity of MWCNT.

MATERIALS AND METHODS

Ethical consideration of the experiments

An experimental protocol was approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to its execution and monitored at every step during the experimentation for its scientific and

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ethical appropriateness, including concern for animal welfare, with strict obedience to the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and other similar laws, guidelines, rules and *et cetera* provided domestically and internationally.

Animals

Specific pathogen free Crlj:CD1(ICR) mice, 5 weeks old, were purchased from Charles River Japan Inc., Kanagawa, Japan and were sufficiently acclimatized before use. Mice were housed individually in plastic cage (180 x 305 x 110mm^3) with cedar chip bedding and free access to the standard diet CE2 (Nihon Clea, Inc., Tokyo, Japan) and water. The animal room was maintained at 23-25°C with a relative humidity of 50-60%, with 10 ventilation per hour (drawing fresh air through an HEPA-filter, 0.3 μm, 99.9% efficiency) and on a 12 hr light/dark cycle. At 8 to 13 weeks old, a nulliparous female was housed overnight with a male and the next morning the female was checked for the presence of a vaginal plug. The day when vaginal-plug formation was observed was regarded as day 0 of the gestation.

Test chemicals

The presently utilized test chemicals, MWCNT (MITSUI MWCNT-7; lot number, 060125-01k) was exactly identical to those used in the carcinogenicity studies in *p53* gene deficient mice (Takagi *et al.*, 2008) and in intact rats (Sakamoto *et al.*, 2009). MWCNT was suspended in 2% carboxymethyl cellulose sodium (CMCNa; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) solution at concentrations of 0.2, 0.3, 0.4 or 0.5 mg/ml for the intraperitoneal study to achieve a uniform administration volume of 10 ml/kg body weight. In the case of the intratracheal study, 3, 4 and 5 mg/ml suspensions were prepared to achieve a uniform administration volume of 1 ml/kg. The control (0 mg/kg body weight) animals were received 2% CMCNa solution, intraperitoneally or intratracheally, respectively. These suspensions as well as a vehicle 2% CMCNa solution were sterilized by an autoclave at 120°C for 20 min and vigorously mixed by hand shaking immediately prior to the administration.

Animal treatment and assessments

Two independent experiments were performed. In experiment 1, pregnant female mice were given a single intraperitoneal administration of MWCNT at dosages of 2, 3, 4 or 5 mg/kg body weight on day 9 of the gestation. On the other hand, in experiment 2, mice were given a single intratracheal spray administration of 3, 4 or 5 mg/kg body weight using intratracheal aerosolizer (MicroSprayer Model IA-1B; Penn-Century, Inc., Philadelphia, PA, USA) on day 9 of the gestation.

In either experiment, body weights of mated females were measured daily, and clinical observations were recorded. All mice were killed on day 18 of the gestation under light ether anesthesia. The liver, lung, spleen, heart, kidney, thymus and tracheobronchial lymph node of each dam were removed and weighed. Peripheral blood was examined for the leukocyte counting by Sysmex KX-21NV. Blood films were made, stained by May/Grünwald;Giemsa and counted for the subtypes of leucocytes under the light microscopy.

The uterus was opened to examine for early and late fetal deaths, and to record the position of dead and live fetuses. The numbers of implantation sites and corpora lutea in the ovaries were also counted. Each live fetus was weighed and examined for external anomalies. Fetuses were fixed in 95% ethanol and stained with Alizarin Red S (Dawson, 1926) to examine skeletal anomalies.

Statistical analysis

Scheffe's multiple comparison was applied for the organ weights of dams, maternal body weights, number of implantations and live fetuses, and fetal body weights. The incidence of pregnant females and of litters with malformed fetuses, and the number of malformed fetuses were analyzed using the Chi square test. The rank sum test was used for data on the resorption and the percent incidence of malformations (Nishimura, 1976). The trend test (cumulative X2 test) was performed to evaluate the significance of the development of malformations by the administered doses of MWCNT.

RESULTS

Experiment 1, the intraperitoneal study

The pregnant status is summarized in Table 1. No animals died after the MWCNT administration. While most of the mated mice were gestated regardless to treatments, 1, 1, 6 and 6 pregnant mice, which were dosed 2, 3, 4 and 5 mg/kg body weight of MWCNT respectively, did not have any living fetuses on 18 day of the gestation. The statistical significances of this change were obtained in the 4- and 5-mg/kg groups. Similarly, the rates of early resorption of fetuses were significantly increased, with the number of live fetuses per litter being decreased, in these groups. In addition, the body weights of live fetuses were significantly lower in the 2-, 3- and 4-mg/kg groups,

Table 1. Experiment 1; pregnant status

Reproductive parameters	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
Female mated[1)]	11	12	12	15	10
Female died[2)]	0	0	0	0	0
Female gestated[3)]	10	8	9	13	9
Female with >1 live fetus	10	7	8	7*	3**
Corpora lutea/litter[#]	15.8 ± 1.9	15.6 ± 1.6	16.0 ± 4.1	15.4 ± 1.8	14.4 ± 2.2
Implantations/litter[#]	14.5 ± 2.5	14.4 ± 1.5	12.3 ± 2.7	14.0 ± 2.1	12.7 ± 3.8
Resorption of fetuses(%)[4)#]					
Early	11.0 ± 13.5	35.3 ± 34.9	41.7 ± 34.8	67.1 ± 38.8**	81.7 ± 28.2***
Late	1.7 ± 3.7	2.4 ± 3.4	0	1.6 ± 3.1	0.9 ± 2.6
Live fetus /litter[#]	12.6 ± 2.6	9.5 ± 5.1	7.3 ± 4.1	4.8 ± 5.8**	1.4 ± 3.3***
Body wt of live fetus (g) [#]					
Male	1.48 ± 0.10	1.29 ± 0.08*	1.28 ± 0.10**	1.31 ± 0.08*	1.42 ± 0.12
Female	1.43 ± 0.13	1.23 ± 0.09*	1.24 ± 0.12*	1.21 ± 0.11*	1.33 ± 0.02

[1)] Number of animals with vaginal plug. [2)] Number of animals died before the scheduled sacrifice on day 18 of the gestation. [3)] Number of animals with implantation sites. [4)] 'Early' was defined as a case showing the implanted sites and amorphous mass, while 'Late' was defined as a case showing the head and limbs. [#] Values are the means ± S.D. The percent resorption and foetal body weight were obtained by averaging the value for each litter. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

but not in the 5-mg/kg group, than in the control group.

Figure 1 illustrates changes of the maternal body weight, of which increment was retarded by MWCNT with a dose-dependent tendency. The body and organ weights and leucocyte typing and counting data of dams are summarized in Table 2. The final body weights were significantly decreased in the 4- and 5-mg/kg groups. The liver weight tended decreased in the dose groups but changes were not statistically significant. The weight of the spleen was significantly increased in the dose groups but no other adverse effect was evident. The numbers of total white blood cells, neutrophils, eosinophils and monocytes, lymphocytes as well but lesser degree, all tended increased in all MWCNT-treated groups. The statistical significances of these changes were obtained in the 3-mg/kg group for the total white blood cells and in 2- 3- and 4-mg/kg groups for the neutro- and eosinophils.

The incidences of malformations were summarized in Table 3. Various types of external and skeletal malformations, such as reduction deformity of limb, short or absent tail, cleft palate, fusion of vertebrae, hypophalangia and hyperphalangia, were observed not in the control group but in all MWCNT-treated groups. Whereas respective incidences of such malformations were a few, the ratio of litters with malformed fetuses, the percent incidence of malformations and the ratio of malformed fetuses were all increased in all MWCNT-treated groups, most of them being with the statistical significance. The trend test evaluated that the development of skeletal malformations by the administered doses of MWCNT was significant ($p < 0.05$).

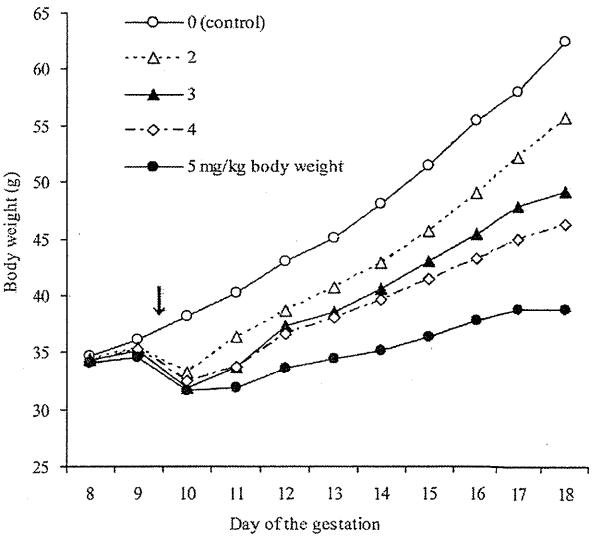

Fig. 1. Experiment 1; changes of the maternal body weights. The arrow represents the timing of the intraperitoneal administration of MWCNT.

Table 2. Experiment 1; body and organ weights, and leucocyte typing and counting of dams

Items	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
Number of dam	10	8	9	13	9
Body weight on day 9 of the gestation	36.1 ± 1.3	35.3 ± 1.9	35.2 ± 2.9	35.2 ± 2.6	34.6 ± 2.4
on day 18 of the gestation	62.4 ± 2.8	55.7 ± 12.0	49.2 ± 7.9	46.3 ± 11.9*	38.8 ± 9.4***
Organ weight					
Liver (g)	3.11 ± 0.40	3.17 ± 0.53	2.99 ± 0.49	2.80 ± 0.52	2.42 ± 0.64
Kidney (mg)	478 ± 133	503 ± 52	447 ± 48	472 ± 55	452 ± 55
Heart (mg)	179 ± 17	180 ± 23	167 ± 18	165 ± 16	157 ± 22
Lung (mg)	189 ± 8	181 ± 3	176 ± 20	188 ± 15	202 ± 20
Spleen (mg)	145 ± 40	297 ± 88*	323 ± 86**	333 ± 99**	372 ± 91***
Thymus (mg)	26.6 ± 12.9	22.6 ± 5.4	17.2 ± 9.2	25.3 ± 7.4	37.8 ± 15.0
Tracheobronchial lymph node (mg)	7.4 ± 8.3	7.3 ± 3.5	8.8 ± 4.5	6.2 ± 4.5	14.6 ± 5.1
Leucocyte count (10^2/μl)					
Total	47 ± 19	115 ± 34	124 ± 48*	109 ± 68	82 ± 38
Lymphocyte	28.9 ± 11.7	38.8 ± 12.0	33.6 ± 15.1	33.0 ± 35.7	23.9 ± 11.4
Neutrophil	15.0 ± 7.0	54.6 ± 19.2*	66.1 ± 23.0**	53.6 ± 37.0*	46.5 ± 23.9
Eosinophil	0.9 ± 0.5	17.5 ± 14.1**	16.1 ± 8.9**	15.5 ± 8.1**	6.7 ± 5.0
Monocyte	1.9 ± 1.7	4.1 ± 3.0	8.2 ± 7.8	7.0 ± 4.2	5.5 ± 4.0

Values are the mean ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

Table 3. Experiment 1; incidences of malformations

Items	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
External malformation					
Numbers of litters with malformed fetuses/examined (percentages in the parentheses)					
	0/10(0)	2/7(28.6)	2/8(25.0)	3/7(42.9)*	1/3(33.3)
Percent incidence of malformations#	0	9.2 ± 18.8	3.6 ± 6.8	4.6 ± 6.5	6.7 ± 11.5
Numbers of malformed fetuses/examined	0/126	3/76*	3/66*	3/63*	2/13***
Numbers of fetuses with					
short or absent tail	0	2	1	1	0
cleft palate	0	0	0	1	0
reduction deformity of limb	0	2	3	1	2
Skeletal malformation					
Numbers of litters with malformed fetuses/examined (percentages in the parentheses)					
	0/10(0)	4/7(57.1)**	3/8(37.5)*	3/7(42.9)*	2/3(66.7)**
Percent incidence of malformations#	0.0 ± 0.0	14.4 ± 18.1	11.1 ± 21.7	11.9 ± 19.2	40.0 ± 52.9
Numbers of malformed fetuses/examined	0/126	9/76***	7/66***	7/63***	5/13***
Numbers of fetuses with					
fusion of ribs	0	3	1	2	0
fusion of vertebral bodies and arches	0	6	7	0	3
hypophalangia	0	2	2	3	2
hyperphalangia	0	0	0	2	0

#Calculated by averaging the percentage in each litter (*i.e.* numbers of malformations/fetuses) and shown as the means ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

Experiment 2, the intratracheal study

The pregnant status is summarized in Table 4. No animals died after the MWCNT administration. Most of the treated mated mice were gestated, and all of them had living fetuses. The rates of early as well as late resorption of fetuses were increased in the 4- and 5-mg/kg groups, respectively, but these changes were not statistically significant because of a large dispersion. The numbers of live fetuses per litter in MWCNT-treated groups were well maintained, although slight decreases were seen in the 4- and 5-mg/kg groups. In contrast, the body weight of live fetuses was significantly lower in the 5-mg/kg group.

Figure 2 illustrates changes of the maternal body weights, of which increment was retarded in the 5-mg/kg group. The body and organ weights, and leucocyte typing and counting data of dam are summarized in Table 5. The final body weight was significantly decreased in the 5-mg/kg group. The weight of the lung and tracheobronchial lymph nodes tended increased in a dose-dependent tendency, and the statistical significance was achieved for the lung in the 5-mg/kg group. Lungs of dosed groups looked blackened. The numbers of total white blood cells tended increased in a dose-dependent tendency, and the statistical significance was achieved in the 4- and 5-mg/kg group, but the magnitude of this change was not so high. The numbers of all types of white blood cell looked increased in some MWCNT-treated group, but the changes were modest and lacked statistical significances.

The incidences of malformations were summarized in Table 6. Various types of external and skeletal malformations, as seen in experiment 1, were observed not in the control group and scarcely in the 3-mg/kg group. In the 4- and 5-mg/kg groups, however, such malformations occurred frequently and significantly. Typical features of the reduction deformity of limb and the fusion of vertebrae and ribs are demonstrated in Figs. 3 and 4, respectively. The ratio of litter with malformed fetuses, the percent incidence of malformations and the ratio of malformed fetuses were all increased in 4- and 5-mg/kg group, most of them being with the statistical significance.

DISCUSSION

The present results clearly elicit that MWCNT is teratogenic in mice, at least under the present experimental conditions. This is the first report demonstrate the teratogenicity of this nanomaterials. Also, there is no report on teratogenicity of other exogenous fibers such as single wall nanotubes, asbestos and glass fibers. It is sometimes difficult to judge teratogenicities of chemicals, especially when the maternal toxicity is present. Because the maternal toxicity was in fact observed in some MWCNT-treated groups of the present study, one might consider the malformation of the fetuses only reflected and thus did not necessarily indicate the "true" teratogenicity of MWC-

Table 4. Experiment 2; pregnant status

Reproductive parameter	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
Female mated[1)]	11	12	16	6
Female died[2)]	0	0	0	0
Female gestated[3)]	10	10	15	5
Female with >1 live fetus	10	10	15	5
Corpora lutea/litter	14.6 ± 1.5	16.0 ± 1.8	15.1 ± 1.8	15.8 ± 2.3
Implantations/litter	12.8 ± 1.6	14.8 ± 2.2	13.8 ± 2.7	11.8 ± 2.9
Resorption of fetuses(%) [4)#]				
Early	9.8 ± 13.4	8.8 ± 8.4	21.0 ± 29.8	20.0 ± 17.7
Late	2.0 ± 4.6	0.6 ± 1.8	0.8 ± 2.2	6.3 ± 10.1
Live fetus/litter[#]	11.3 ± 2.1	13.3 ± 1.5	10.5 ± 4.4	8.8 ± 2.9
Body weight of live fetus (g) [#]				
Male	1.41 ± 0.14	1.36 ± 0.12	1.23 ± 0.19	1.07 ± 0.20*
Female	1.35 ± 0.13	1.31 ± 0.11	1.19 ± 0.19	1.06 ± 0.18*

[1)] Number of animals with vaginal plug. [2)] Number of animals died before the scheduled sacrifice on day 18 of the gestation. [3)] Number of animals with implantation sites. [4)] 'Early' was defined as a case showing the implanted sites and amorphous mass, while 'Late' was defined as a case showing the head and limbs. [#] Values are the means ± S.D. The percent resorption and fetal body weight were obtained by averaging the value for each litter. Asterisks represent that the values are significantly different from the control value (* indicating $p < 0.05$).

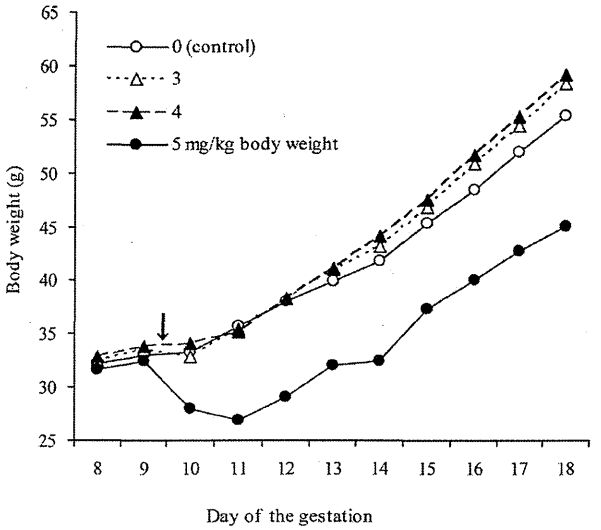

Fig. 2. Experiment 2; changes of the maternal body weights. The arrow represents the timing of the intratracheal administration of MWCNT.

Fig. 3. Experiment 2; an 18-day-old fetus, showing the reduction deformity of the limb, from a dam intratracheally administered MWCNT at a dose of 4 mg/kg body weight on day 9 of the gestation.

NT. Malformations were, however, induced even in the 4-mg/kg group of the intratracheal study, in which MWCNT did not apparently cause the maternal toxicity. In addition, the malformations induced by the MWCNT administration belonged to a reduction type, such as the reduction

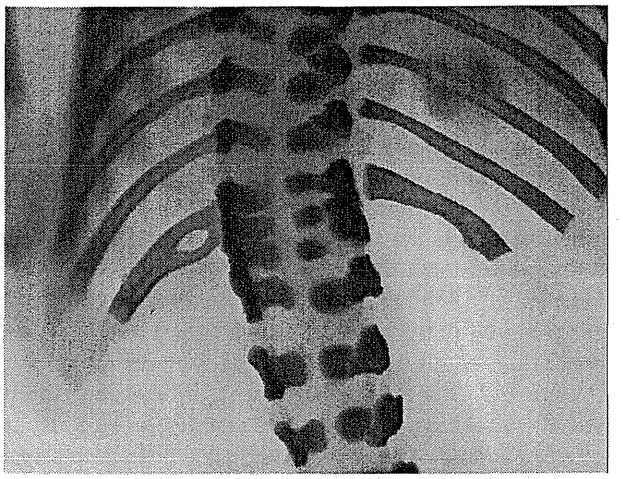

Fig. 4. Experiment 2; an 18-day-old fetus, showing the fusion of vertebrae and ribs, from a dam intratracheally administered MWCNT at a dose of 4 mg/kg body weight on day 9 of the gestation.

deformity of limbs and the short or absent tail. The malformations in this type have not been found among about 7,000 fetuses of ICR mice historically examined so far in our laboratory (Ogata *et al.*, 1984, 1987, 1989 and 1999). Also in other laboratories, the spontaneous incidence of the reduction deformity of limb of ICR mice is usually very low. For instance, the incidence of amelia and oligoductilia has both been reported to be 0.02% among 5,000 fetuses in another laboratory and no deformities have observed among 4,335 fetuses in another laboratory (Kameyama *et al.*, 1980). The malformations observed in this study are uncommon in merely by the maternal toxicity. It is thus safe to say that the teratogenicity of MWCNT demonstrated in the present study is true with a biological significance.

The reasons why we at first conducted the intraperitoneal study and used very high doses were to avoid missing a teratogenicity of MWCNT, if it is present, under the experimental condition as sensitive as possible form the point of the hazard identification. This is the same strategy that was adopted in the studies identifying the carcinogenic hazard of MWCNT (Sakamoto *et al.*, 2009; Takagi *et al.*, 2008). The relatively severe maternal toxicity in the high doses is thus rather expected. Nevertheless, the present intraperitoneal study can demonstrate the teratogenicity of MWCNT as stated above, witch then led us to confirm this hazard using a more human-relevant exposure route. In the intratracheal study, MWCNT was administered into the trachea of mice in a spray or mist shape, witch well mimics the most plausible human

Table 5. Experiment 2; body and organ weights, and leucocyte typing and counting of dams

Items	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
Number of dam	10	10	15	5
Body weight on day 9 of the gestation	33.0 ± 2.0	33.6 ± 2.8	33.8 ± 2.8	32.5 ± 2.2
on day 18 of the gestation	55.4 ± 3.1	58.4 ± 5.5	59.1 ± 6.9	45.1 ± 4.5*
Organ weight				
Liver (g)	2.80 ± 0.27	2.73 ± 0.36	3.05 ± 0.31	2.44 ± 0.11
Kidney (mg)	454 ± 52	431 ± 60	457 ± 54	422 ± 28
Heart (mg)	155 ± 10	161 ± 14	162 ± 15	150 ± 9
Lung (mg)	157 ± 14	168 ± 10	197 ± 51	228 ± 47**
Spleen (mg)	136 ± 22	122 ± 29	149 ± 40	158 ± 35
Thymus (mg)	19.9 ± 7.5	16.4 ± 5.3	18.9 ± 5.5	13.9 ± 8.9
Tracheobronchial lymph node (mg)	4.2 ± 3.6	6.8 ± 5.2	6.2 ± 4.3	8.7 ± 2.3
Leucocyte count ($10^2/\mu l$)				
Total	37.5 ± 6.4	49.5 ± 11.3	51.6 ± 11.5*	51.3 ± 10.6*
Lymphocyte	21.0 ± 4.4	30.0 ± 8.2	26.5 ± 7.4	22.5 ± 6.4
Neutrophil	14.7 ± 4.5	17.4 ± 9.7	20.3 ± 11.2	25.4 ± 11.5
Eosinophil	0.7 ± 0.9	1.1 ± 0.7	2.7 ± 2.5	1.6 ± 1.1
Monocyte	1.2 ± 0.7	1.0 ± 0.5	2.2 ± 1.4	1.7 ± 0.4

Values are the means ± S.D. Asterisks represent that values are significantly different from the control value (* or ** indicating $p < 0.05$ or 0.01, respectively).

Table 6. Experiment 2; incidences of malformations

Items	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
External malformation				
Number of litters with malformed fetuses/examined (percentages in the parentheses)				
	0/10(0)	0/10(0)	5/14(35.7)*	2/5(40.0)*
Percent incidence of malformations#	0	0	15.6 ± 27.9	5.6 ± 8.2
Number of malformed fetuses/examined	0/113	0/133	15/158***	3/44**
Number of fetuses with				
short or absent tail	0	0	12**	3**
reduction deformity of limb	0	0	7*	0
Skeletal malformation				
Number of litters with malformed fetuses/examined (percentages in the parentheses)				
	0/10(0)	1/10(10.0)	6/14(42.8)*	4/5(80.0)*
Percent incidence of malformations#	0	0	39.9 ± 48.4*	61.9 ± 38.2*
Number of malformed fetuses/examined	0/113	1/133	56/158***	31/44***
Number of fetuses with				
fusion of ribs	0	0	8*	10***
fusion of vertebral bodies and arches	0	0	54***	25***
hypophalangia	0	0	10*	1
hyperphalangia	0	1	0	1

Calculated by averaging the percentage in each litter (*i.e.*, number of malformations/fetuses) and shown as the means ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

exposure situation of the inhalation. The highest dose of 5 mg/kg body weight must have been too high, because it caused the apparent maternal toxicity, and it agglomerated in the lung (data not shown). It is clearly indicated, however, that MWCNT is teratogenic, because the malformations in the fetuses were significantly induced by the middle dose of 4 mg/kg body weight that did not cause the apparent maternal toxicity.

It is known that methyl cellulose of a certain length has nephrotoxicity but, in this study, no adverse effect on kidney of dam given 2% CMCNa solution (control) nor suspension of MWCNT in 2% CMCNa (dosed groups) was observed.

Mechanisms underlying the teratogenicity of MWCNT are still obscure. Sargent *et al.* (2009) has demonstrated that single-wall carbon nanotube induces aneuploidy in cultures primary and immortalized human airway epitherial cells by the disruption of the mitotic spindle. In that study, the association of nanotubes with cellular and mitotic tubulins as well as chromatins within the nuclei is demonstrated, and the similarity of nanotube bundles to microtubules in size of microtubules is considered to play roles, because it may make nanotubes incorporated into the mitotic spindle apparatus. Recently, Takahashi *et al.* (2010) has reported that MWCNT also induces polyploidy, suggesting that MWCNT may exert similar effects on microtubules to the situation of single-wall carbon nanotube. If it is a case, the disruption of the mitotic spindle and the fragmentation of the centrosomes may inhibit subsequent cell division, which results in the embryonic death in early phase and the malformation of the reduction type. Further studies are apparently warranted, and especially a passage of MWCNT through the placenta and the reach to the fetus should be evidenced.

Another possible factor involved in the teratogenicity may be the chronically persisting inflammation caused by the exposure to MWCNT, which is frequently considered to participate in the biological effects of nanomaterials (Takagi *et al.*, 2008; Sakamoto *et al.*, 2009; Erdely *et al.*, 2009; Hubbs *et al.*, 2011). The present results of the increments of the numbers of leucocyte and related hemocytes, and of the weight of the spleen might support this possibility.

The present intratracheal study gives no-observed-adverse-effect level (NOAEL) of 3 mg/kg body weight for external and skeletal malformations. Although the human exposure level of MWCNT has not as yet clearly determined, the interim report for the risk assessment of MWCNT by the National Institute of Advanced Industrial Science and Technology (AIST, 2009) roughly estimated the quantity of MWCNT exposure of workers as 0.53- 6.20 μg/kg/day. Comparing with these values, the above NOAEL for external and skeletal malformations are approximately 480-5,660 times high. It is thus tentatively evaluated that the present results may not necessarily or immediately indicate a human risk. Needless to say, however, more detailed and careful investigations including those for the teratogenicity must be conducted to complete the risk assessment of MWCNT.

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Original Article

No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats

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ABSTRACT — Three female Crl:CD(SD) rats/group were dosed with single wall carbon nanotube (SWCNT) or multi wall carbon nanotube (MWCNT) four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals). Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects, and thus the oral LD_{50} values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw, in rats respectively. Five or ten Crl:CD(SD) rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). Six or twelve Crl:CD(SD) rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 50 mg/kg bw/day groups). Based on no toxicological effects, the no observed adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day (the highest dose tested), respectively. It was suggested that SWCNT and MWCNT dosed by gavage reached the gastro-intestinal tract as agglomerates and were mostly excreted via feces.

Key words: Single wall carbon nanotube, Multi wall carbon nanotube, Acute oral toxicity, Repeated oral dose toxicity, Rat

INTRODUCTION

Nanomaterials possess different physico-chemical properties from bulk materials. Therefore, it is necessary to develop specialized approaches to testing their effects on human health and on the environment. Our study group has worked on the establishment of a human health risk assessment methodology of nanomaterials since 2005. As part of efforts, our co-researchers reported carcinogenic potential of intraperitoneal administration of multi wall carbon nanotube (MWCNT) in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009) and also suggested that nano-sized particles can be transferred to other organs. Subsequently, Sakamoto *et al.* (2010) showed that expression of renal carcinoma/mesothelin can be used as a biomarker of mesothelial proliferative lesions induced by intrascrotal administration of MWCNT.

In parallel of the establishment of our study group, the OECD Working Party on Manufactured Nanomaterials (WPMN) was established to assess the safety of the use of nanomaterials for human health and the environment in 2006, and the Sponsorship Programme on the Testing on Manufactured Nanomaterials was launched in 2007 (OECD, 2011). The aim of the Sponsorship Programme is to fill data gaps between existing data and a desired data set for 13 nanomaterials including single wall carbon nanotubes (SWCNTs) and MWCNTs. Japan has volunteered to act as the Joint Lead Sponsors with the US for the evaluation of mammalian toxicology for fullerenes (C60), SWCNTs and MWCNTs in the Sponsorship Programme. We recently reported no toxicological effects of gavage doses of fullerene C60 up to 1,000 mg/kg bw/day in rats (Takahashi *et al.*, 2012). After the 29-day

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administration period, blackish feces and black contents of the stomach and large intestine were observed. Fullerene C60 was not detected in the liver, spleen or kidney at the end of administration period. This study indicated that fullerene C60 dosed by gavage was excreted via feces and not distributed in major organs.

We also conducted acute and repeated dose toxicity studies of SWCNT and MWCNT by gavage, target endpoints of the Sponsorship Programme. In some past studies, the physical-chemical properties of tested materials are not clear due to the diversity of carbon nanotubes (CNTs) with respect to purity, production methods, purification methods or surface treatments/ coatings (Kobayashi *et al.*, 2009; OECD, 2011). The clear characterization and good control of the size and shape of nano-sized particulate materials are essential for ensuring the reproducibility and reliability of tests. Therefore, well defined Nikkiso SWCNT and MWCNT were set as principal samples in this Sponsorship Programme to examine mammalian toxicity.

Major current uses of MWCNTs are electronics applications such as super-capacitors and batteries and structural composite applications such as sporting equipments and conductive sheets (OECD, 2010a) and the future applications of MWCNTs include medical care and fabrics (Kobayashi *et al.*, 2009; MHLW, 2010). Major expecting uses of SWCNTs in future are super-capacitors, high speed transistor, fuel cells, super high strength wires (OECD, 2010b). A total volume of production and import of MWCNTs was 500 tons in 2008 in Japan, and it is expected to increase in future (MHLW 2010). Oral exposure to CNTs may occur through the migration from food contact products or agricultural foods that uptake CNTs from environment (Magnuson *et al.*, 2011).

Many toxicity studies have been available for SWCNTs or MWCNTs dosed by pharyngeal aspiration (Erdely *et al.*, 2009; Shvedova *et al.*, 2008), intravenous injection (Yang *et al.*, 2008), intratracheal instillation (Inoue *et al.*, 2009; Elgrabli *et al.*, 2008; Inoue *et al.*, 2008) and inhalation (Shvedova *et al.*, 2008) in rats and mice, but this will be the first report to show the results of acute and repeated dose toxicity of SWCNT and MWCNT by gavage in rats according to the OECD guidelines (TG 423 and TG 407). Although a gavage dose is not likely to be representative of the anticipated exposure scenario, the findings of our studies will be useful to characterize the feature of CNTs toxicity and for risk assessment in humans.

MATERIALS AND METHODS

Acute and repeated dose toxicity studies for SWCNT or MWCNT were performed in the Gotemba Laboratory, Bozo Research Center Inc. or the Safety Research Institute for Chemical Compounds Co., Ltd., respectively. These studies were conducted in compliance with the OECD Guideline 423; Acute Oral Toxicity, the OECD Guideline 407; Repeated Dose 28-Day Oral Toxicity Study in Rodents and the Guideline for 28-Day Repeated Dose Toxicity Test in Mammalian Species (Chemical Substances Control Law of Japan) under GLP. The SWCNTs studies were conducted in compliance with the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006) and the Guidelines for Proper Conduct of Animal Experiments (June 1, 2006). The MWCNT studies were conducted in compliance with the Guidelines for Animal Experimentation (May 22, 1987), along with the above described Acts and Standards.

Animals

Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Female rats (SWCNT: 8 weeks old; MWCNT: 9 weeks old) were used for acute toxicity studies, and male and female rats (SWCNT: 6 weeks old; MWCNT: 5 weeks old) were used for repeated dose toxicity studies. Rats were individually housed in metallic cages with wire mesh bottoms and reared on a basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. Rats were maintained at room temperature, a humidity of 50 ± 20%, 10-15 air changes per hour and a 12 hr dark/12 hr light cycle.

Chemicals and dosing

Principal single-wall carbon nanotubes (SWCNT: purity > 95%; Lot No.: SW1859/SW1860/SW1865) and principal multi-wall carbon nanotubes (MWCNT: purity: > 98%; Lot No.: 04-12/10#1-(4)) supplied by Nikkiso Co., Ltd. (Shizuoka, Japan) were used. These chemicals are principal samples in the OECD Sponsorship Programme on the Testing on Manufactured Nanomaterials. The structure of SWCNTs is a honeycomb carbon lattice rolled into a cylinder, and the basic morphology is in sheet form consisting of entangle SWCNTs (with a diameter of around 2 nm) bundles with diameters of several decade nanometers. The structure of MWCNTs is honeycomb carbon lattices rolled into a multi-layer tubular shape,

and the basic morphology is particles consisting of entangled MWCNTs with a diameter of around 30 nm. Both test materials were not coated or modified. The test materials were stored in a polycarbonate bottle with an airtight stopper to prevent dissemination at room temperature, and were accurately weighed and added to gum acacia (vehicle). This vehicle was chosen based on the results of the preliminary investigation with commonly used vehicles, in which CNTs showed the best dispersion state in 5% gum acacia of aqueous solution. The mixture of test materials was homogenized using an ultrasonic homogenizer (SWCNT: UR-200P, TOMY Seiko Co., Ltd., Tokyo, Japan; MWCNT: VC-130, Sonics & Materials Inc., Newtown, CT, USA) and a compact ultrasonic cleaning bath (SWCNT: US-1, As One Co., Ltd., Tokyo, Japan; MWCNT: USC-6, Iwaki Glass Co., Ltd., Chiba, Japan). The homogeneity of test suspensions was confirmed microscopically (Figs. 1 and 2).

Acute toxicity studies for SWCNT and MWCNT were conducted in a stepwise procedure. As a first step, three female rats/group were dosed with SWCNT or MWCNT four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals), respectively. Because oral toxicity of the test materials was expected to be low, dosage levels were determined based on the maximum doses which could be prepared and administered. The concentrations of 0.625 mg/ml SWCNT and 2.5 mg/ml MWCNT were confirmed to be the limit to prepare. In addition, ethically, a dosing volume of 20 ml/kg was limit for gavage dosing and four times seemed to be the limit for the number of dosing times. In the first step, no deaths or adverse effects were found for either SWCNT or MWCNT dosing. Therefore, a second step was carried out with the same regimen to confirm the acute toxicity of the test materials.

Five or ten rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days. Five animals/sex at 0 and 12.5 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. Six or twelve rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days. Six animals/sex at 0 and 50 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. A high dose was set with the maximum doses which could be prepared, as described above, and the middle and low

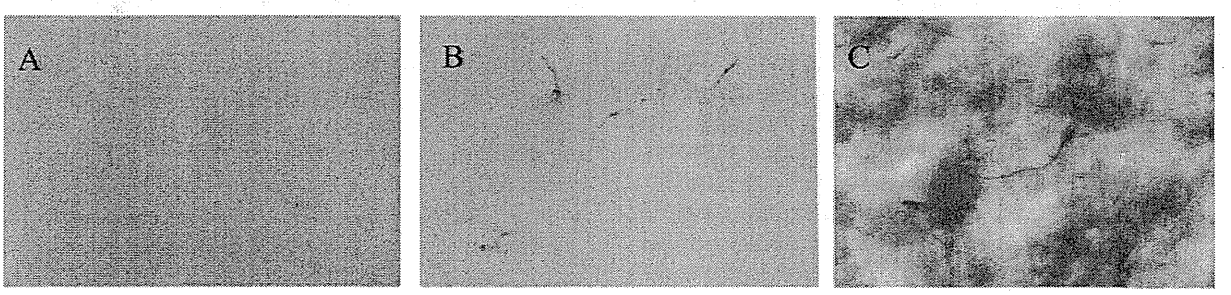

Fig. 1. Microscopic views of SWCNT suspensions (× 400). (A) 0.125 mg/kg bw/day (0.00625 mg/ml); (B) 1.25 mg/kg bw/day (0.0625 mg/ml); (C) 12.5 mg/kg bw/day (0.625 mg/ml).

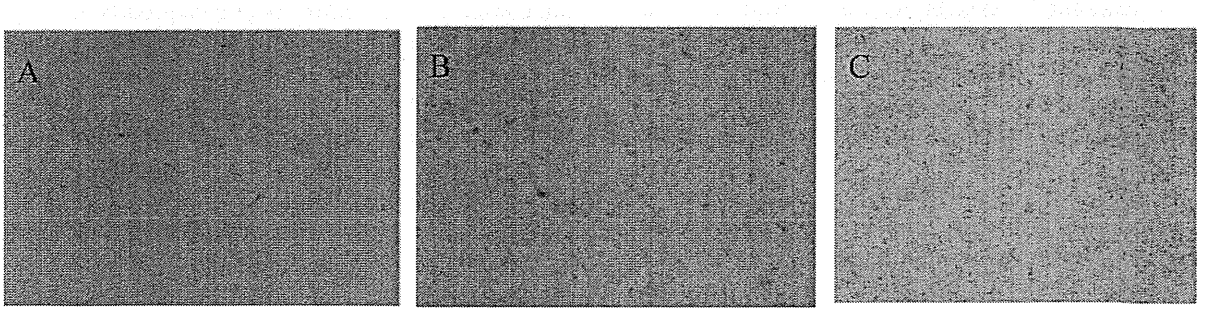

Fig. 2. Microscopic views of MWCNT suspensions (× 400). (A) 0.5 mg/kg bw/day (0.025 mg/ml); (B) 5.0 mg/kg bw/day (0.25 mg/ml); (C) 50 mg/kg bw/day (2.5 mg/ml).

doses were set with a common ratio of 10.

Observations

As for the acute toxicity studies, rats were observed for 14 days. Clinical observation was performed consecutively for several hours after administration and once or twice a day from the next day of administration. Animals were weighed just prior to administration, and 1, 3, 5, 7, 10 and 14 days (MWCNT) or 1, 3, 7 and 14 days (SWCNT) after administration. Necropsy was performed 14 days after administration.

As for the repeated dose studies, all males and females in the MWCNT study were observed twice per day, every day during administration and recovery periods, and in the SWCNT were observed three times per day, every day during the administration and once a day during the recovery period. A detailed clinical observation was carried out one day before administration, on days 7, 14, 21 and 28 days of the administration period and days 7 and 14 of the recovery period in the MWCNT study, and once a week in the SWCNT study. A functional examination was carried out in the fourth week of the administration period and in the second week of the recovery period. Body weight was measured on days 1, 4, 7, 14, 21 and 28 of the administration period and on days 7 and 14 of the recovery period in the MWCNT study, and on days 1, 4, 7, 10, 14, 17, 21, 24 and 28 of the administration period and on days 1, 3, 7, 10 and 14 of the recovery period. Food consumption was measured on days 1, 7, 14, 21 and 28 of the administration period, and on days 7 and 14 of the recovery period. Hematological examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Clinical chemistry examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Necropsy was performed under anesthesia on the day following the end of the administration or recovery period. The external surfaces of rats were examined and a gross internal examination was performed. Organ weights were measured and histopathological evaluations were performed on the organs. Urinary samples were collected for 3 or 4, and 20 hr in the fourth week of the administration period, and in the second week of the recovery period. Urine volume was calculated and a urinary examination was conducted.

Data analysis

For the SWCNT study, continuous data from the administration period were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, data were analyzed by the Dunnett test, whereas heterogeneous data were analyzed by the Dunnett-type mean rank test between the control group and individual treatment groups. For the recovery group data, homogeneity of variance was tested by the F-test. When homogeneity was recognized, the difference in mean values between the control group and treatment groups was analyzed by a Student's t-test, whereas heterogeneous data were analyzed by an Aspin-Welch's t-test.

For the MWCNT study, continuous data were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, the Dunnett test was conducted for comparison between the control group and individual treatment groups after a one-way layout analysis of variance. If not homogenous, the data were analyzed using the Kruskal-Wallis test followed by a Mann-Whitney's U-test. Qualitative data were analyzed by the Kruskal-Wallis test followed by Mann-Whitney's U-test.

RESULTS AND DISCUSSION

Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects on the clinical observation and body weight. Thus, the oral LD_{50} values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw in rats, respectively (data not shown).

A 28-day dose of SWCNT caused no death in both sexes. There were no differences in the clinical observation, detailed clinical observation, body weight and food consumption (Table 1) or histopathological examination (Table 2). In the functional examination, significantly low values of landing foot splay were observed in all the treatment groups in males at the end of the administration period (90 ± 11, 61 ± 15, 64 ± 18 and 75 ± 10 mm at 0, 0.125, 1.25 and 12.5 mg/kg bw/day, respectively). However, it was due to the high value in the control group and was not considered to be toxicological effects. In urinalysis, a significantly low urine volume was observed during the administration period in females at 1.25 mg/kg bw/day and above (Table 3). However, these values were within the historical background data of the test facility (Mean ± S.D.: 8.3 ± 4.0 ml/24 hr), and these were not dose dependent. In the hematological examination, significantly high erythrocyte counts in females and lymphocyte counts and basophil counts in males were observed at 12.5 mg/kg bw/day at the end of the administration period (Table 4). However, these changes were considered to be incidental because there were no changes in related parameters. In the serum biochemistry examination, significantly high alanine aminotransferase and triglyceride levels were observed in females in the 0.125 mg/kg bw/day group but not in the high dose groups at