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 ($p < 0.05$).

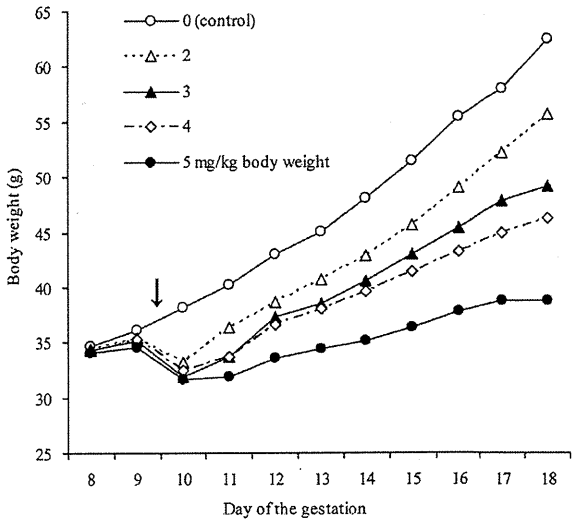

Fig. 1. Experiment 1; changes of the maternal body weights. The arrow represents the timing of the intraperitoneal administration of MWCNT.

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 ($10^2/\mu l$)					
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 $p < 0.05$).

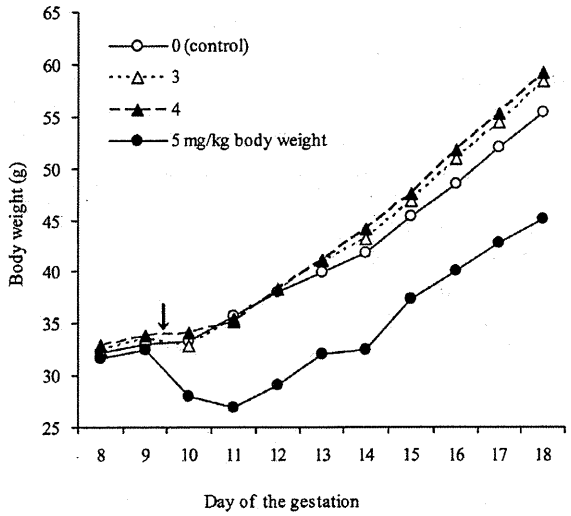

Fig. 2. Experiment 2; changes of the maternal body weights. The arrow represents the timing of the intratracheal administration of MWCNT.

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

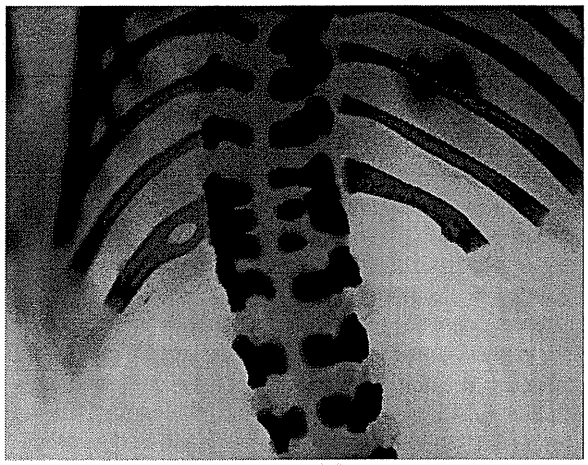

Fig. 4. Experiment 2; an 18-day-old fetus, showing the fusion of vertebrae and ribs, from a dam intratracheally administered MWCNT at a dose of 4 mg/kg body weight on day 9 of the gestation.

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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Time-dependent variation in the biodistribution of C_{60} in rats determined by liquid chromatography–tandem mass spectrometry

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ABSTRACT

We examined the biodistribution of C_{60} in rats after tail vein administration using LC-MS/MS. C_{60} was detected in various tissues, such as brain, kidneys, liver, lungs, and spleen of rats. On the other hand, no C_{60} was found in blood. The highest C_{60} concentration was observed in the lungs, followed by spleen, liver, kidneys, and brain. These results suggested that C_{60} injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. Moreover, C_{60} not being detected in the blood indicates that clearance of C_{60} from the blood by filtration might effectively occur in the lungs. The time-dependent variation in the biodistribution of C_{60} was evaluated. A time-dependent decrease in C_{60} concentrations was observed in all tissues, except spleen. Moreover, a decreasing trend of C_{60} levels differed among tissues, which could be due to differences in accumulation. These results suggest that unmodified C_{60} and/or C_{60} metabolites by metabolic enzymes could be excreted into feces and/or urine. In further studies, the metabolic and excretion pathways of C_{60} should be evaluated to understand the toxicokinetics of C_{60}.

1. Introduction

Recent progress in the field of nanotechnology has resulted in the development of various newly engineered nanomaterials. Engineered nanomaterials are commonly produced in a wide variety of types, including fullerenes (C_{60}), carbon nanotubes (CNT), metal and metal oxide particles, polymer nanoparticles, and quantum dots. These materials have been applied to various fields of science and technology, and have increasingly been used for commercial purposes, such as fillers, opacifiers, catalysts, semiconductors, and personal care products (cosmetics and drugs) (Nel et al., 2006). On the other hand, there is insufficient information about the human health and environmental impact of nanomaterials, and concern about exposure to nanomaterials and the hazard that they pose is rising (Colvin, 2003; Moore, 2006).

Fullerene, a carbon nanomaterial, is a third allotropic form of carbon (after graphite and diamond). Unlike other carbon structures, fullerene is a closed cage carbon molecule with a truncated icosahedron structure that resembles a soccer ball with 12 pentagons and 20 hexagons (Kroto et al., 1985). Because of their unique structures and properties, fullerenes and their derivatives (endohedral fullerenes and chemically modified fullerenes) exhibit widely differing activities and therefore have attracted considerable attention. Since the water solubility of unmodified C_{60} is low, several studies have been performed to increase its water solubility by surface chemical modification and the formation of complexes with water soluble molecules (Bosi et al., 2003; Nakamura and Isobe, 2003), and as a result of these studies, a number of biological applications, such as free radical scavengers (Dugan et al., 1997), photoinduced DNA cleavage agents (Tokuyama et al., 1993), inhibitors of HIV-1 protease (Friedman et al., 1993), and cytotoxic agents to human cells (HDF, HepG2, and NHA) by lipid peroxidation (Sayes et al., 2005), for fullerene derivatives have been discovered. Rapid commercialization of fullerenes and their derivatives has increased the risk of occupational and environmental human exposure to these nanomaterials via oral, dermal, and inhalation uptake. However, little is known about the potential impact induced by exposure to nanomaterials (fullerenes and their derivatives) to human health, and comprehensive studies on the toxicology and biodistribution of fullerenes and their derivatives have been insufficient. To accurately evaluate the toxic effects of fullerenes and their derivatives by *in vivo* and *in vitro* assays, it is necessary to use an analytical chemical approach coupled with a biological approach. Several methods for analyzing fullerenes and their derivatives have been reported. However, only a few studies have

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examined fullerenes and their derivatives in biological samples (Moussa et al., 1997; Xia et al., 2006), and the sensitivity of detection was insufficient and there was interference from the biological matrix in the analysis of samples. Several studies have investigated the biodistribution of fullerenes in rodents (Yamago et al., 1995; Cagle et al., 1999). However, the fullerenes used in these studies were water-soluble fullerene derivatives, and physical properties of these derivatives differ significantly from unmodified fullerene, especially water solubility; thus, it would be expected that the biodistribution characteristics of modified fullerene derivatives would also differ from unmodified fullerene. Furthermore, there are very few reports on the biodistribution of unmodified fullerene (including radiolabeled fullerene, such as ^{14}C-labeled C_{60} where the label is part of the C_{60} cage) (Bullard-Dillard et al., 1996) in rodents. In this study, we describe the biodistribution of unmodified C_{60} in rats after tail vein administration using a sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) analytical method and liposomes as a carrier of unmodified C_{60}. Liposomes are known as one of the most effective drugs carrier. Moreover, the time-dependent biodistribution variation of unmodified C_{60} in rats was investigated, and the behavior of C_{60} after accumulated in tissues is discussed.

2. Methods

2.1. Chemicals and reagents

C_{60} (nanom purple SUH; purity >99.9%) was obtained from Frontier Carbon Corporation (Tokyo, Japan). C_{70} with a purity of 99.5% was purchased from Materials Technologies Research (Cleveland, USA). C_{70} was used for recovery correction of C_{60} extraction. HPLC grade acetonitrile and toluene were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Analytical grade acetic acid, disodium hydrogen phosphate, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium dodecylsulfate, and chloroform were also purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-α-Phosphatidyl-choline and 3-sn-phosphatidyl-L-serine were purchased from Sigma–Aldrich (St. Louis, USA). Stock standard solutions were prepared by dissolving C_{60} (10 mg) and C_{70} (10 mg) in toluene (10 mL) with sonication and agitation and were stored at −20 °C until use. Working standard solutions were diluted with toluene from stock standard solution for the LC–MS/MS analysis.

2.2. Instrumentation

LC–MS/MS analysis was performed using a Waters Alliance 2695 HPLC system (Waters, Milford, USA) interfaced to a Waters Micromass Quattro Micro API triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface (Waters, Milford, USA). System control and data handling were carried out by Waters MassLynx version 4.0.

2.3. Chromatographic and mass spectrometric conditions

Analysis of C_{60} was conducted as described previously (Kubota et al., 2009). The chromatographic separation was performed in isocratic mode at a flow rate of 1 mL/min with a mobile phase of 70% toluene and 30% acetonitrile. Fullerenes were separated using a Develosil RPFULLERENE column (5 μm, 4.6 mm × 250 mm) (Nomura Chemical Co., Ltd., Seto, Japan) at 30 °C (column oven temperature). The autosampler was kept at 10 °C and the injection volume was 20 μL. The mass spectrometer was operated in the APCI negative ion mode with multiple reaction monitoring (MRM). The APCI corona current was 15 μA, and the temperatures of the source and APCI probe were set to 120 °C and 400 °C, respectively. The desolvation and the cone gas flow rates were adjusted to 600 L/h and 50 L/h, respectively. The inter-scan delay was set to 0.1 s, and the inter-channel delay was set to 0.05 s. The dwell time was 0.5 s. Quantitation was performed using MRM of the reaction transitions of m/z = Q1 720 → Q3 720 for C_{60} and m/z = Q1 840 → Q3 840 for C_{70} (used for recovery correction of C_{60} extraction) with a collision energy of 60 eV and a cone voltage of 120 V. Multiple reaction monitoring (MRM) chromatogram of a 200 μg/L standard mixture of C_{60} and C_{70} was shown in Fig. 1.

2.4. Experimental animals

20 male Wistar rats (Slc: Wistar (SPF)) were purchased from Japan SLC, Inc. (Shizuoka, Japan) at six weeks of age. The rats were kept under Specific Pathogen Free (SPF) conditions with a 12 h light–dark cycle at the animal facility of the National Institute of Health Sciences (NIHS), Tokyo, Japan and were given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum*. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of NIHS.

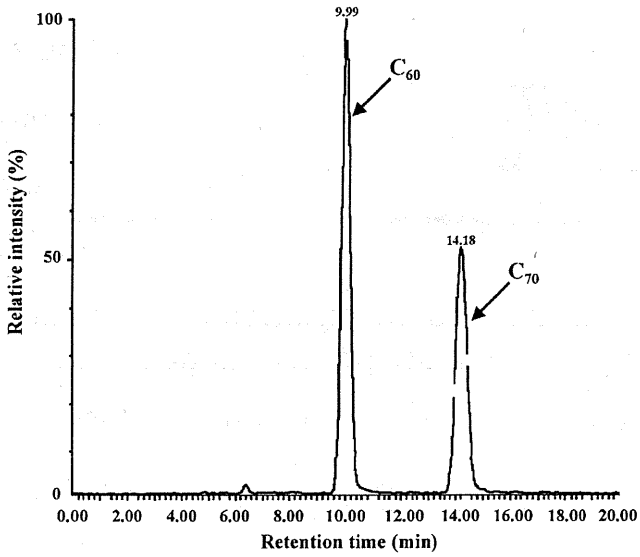

Fig. 1. Multiple reaction monitoring (MRM) chromatogram of a 200 μg/L mixture of C_{60} and C_{70}. Retention times (min) from top: C_{60} (9.99) and C_{70} (14.18).

2.5. Preparation of administration solution (C_{60}-liposome solution)

Because of the solubility of unmodified C_{60} is low, we used liposomes as a carrier of unmodified C_{60}. An administration solution for tail vein injection in rats was prepared as follows. L-α-Phosphatidyl-choline (PC) and 3-sn-phosphatidyl-L-serine (PS) were dissolved in chloroform as 25 mg/mL stock solutions and were stored at −80 °C until use. C_{60} was dissolved in toluene as a 2.5 mg/mL stock solution and was stored at 4 °C until use. A PC and PS mixture was prepared such that each component had a concentration of 0.5 mg/mL (PC:PS = 1:1 (w/w)) in chloroform. 1 mg/mL C_{60} solution (mixture of toluene and chloroform) was prepared by diluting the 2.5 mg/mL C_{60} stock solution with the lipid mixture and the mixture was gently volatilized with a stream of nitrogen gas. After volatilization, 1× PBS buffer (pH 7.4), an amount equivalent to the mixture of toluene and chloroform, was added, and the mixture was vortexed for a few seconds. The liposomes containing C_{60} were sonicated using a bath sonicator for 10–15 min at 60 °C and were centrifuged at 1000 rpm for 10 s, and the supernatant (at room temperature) was used for tail vein administration. The supernatant was given to rats immediately and the solution was sonicated using a bath sonicator before each treatment to rats.

2.6. Treatment of experimental animals and sample collection

20 male Wistar rats at six weeks of age (five per group) were given repeated tail vein injections of 5 mL/kg body weight (one injection). A total of four tail vein injections (once per day) were performed (total injected C_{60}: ca. 929.1 μg). Rats were sacrificed on days 1, 7, 14, and 28 after completion of the injections. Brain, kidneys, liver, lungs, and spleen were collected from each rat and were rinsed with 1 × PBS buffer (pH 7.4). Moreover, blood (taken from the heart) was collected from each rat group. The collected tissues and blood samples were stored at −80 °C until analyzed.

2.7. Sample preparation

Extraction of C_{60} from tissues and blood of rats was performed according to the method of Kubota et al. (2009) with modifications. Freshly harvested whole tissues were weighted and placed in polypropylene copolymer (PPCO) centrifuge tubes. Tissues were frozen at −80 °C, and frozen tissues were freeze-dried over night. Each freeze-dried tissue was weighted and completely homogenized. In the case of small tissues (<0.5 g dry wt., brain, kidneys, spleen, and lungs), 0.2 M SDS solution (1 mL) and acetic acid (1 mL) were added to the centrifuge tubes, and the centrifuge tubes were vortexed and sonicated using a bath sonicator. An internal standard solution (C_{70} toluene solution, 0.5 mL) and 3.5 mL toluene were added to the centrifuge tubes, and they were shaken for 5 h at room temperature in the dark. After shaking, the centrifuge tubes were centrifuged for 30 min at 3500 rpm. 1 mL of supernatant was removed and placed in glass vials to be used for analysis. In the case of blood samples, untreated whole blood (2 mL, taken from the heart) was used for the extraction. Because the dry tissue weight of the liver (ca. 3 g dry wt.) was heavier than other tissues, a six-fold amount of each solution was used for the liver extraction. The limit of quantification (LOQ) in analytical solution was determined by analyzing

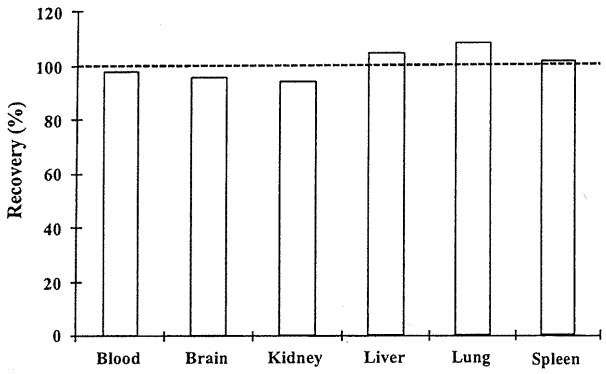

Fig. 2. Recovery of C_{60} from tissues and blood of rat.

the lowest level standard at least 5 times. The LOQ was calculated as 10-fold the standard deviation of these determinations. The LOQ value in analytical solution was 10 μg/L. Moreover, LOQ in each tissue was calculated by LOQ value in analytical solution, volume of toluene used for extraction, and tissue weight of each tissue. The LOQ for each tissue was 0.026 μg/g wet wt. for liver, 0.026 μg/g wet wt. for kidneys, 0.09 μg/g wet wt. for spleen, 0.046 μg/g wet wt. for lungs, and 0.023 μg/g wet wt. for brain, respectively.

2.8. Statistical analyses

Statistical analyses were performed using the program Excel Statistics (Ekuseru-Toukei 2008) (Social Survey Research Information Co., Ltd., Tokyo, Japan). Kolmogorov–Smirnov's test showed that some variables were not normally distributed. Therefore, non-parametric test was used for statistical analysis. Kruskal–Wallis test was used for validation of difference in the C_{60} concentrations in four tissues among treated groups. Where appropriate, Mann–Whitney's *U*-test, Scheffe's test, or Student's *t*-test was conducted to verify the difference in the C_{60} concentration in four tissues among each treated group.

3. Results

3.1. Recovery of C_{60} from tissues and blood of rat

A spike recovery test was conducted to evaluate the validity of the analytical method for determination of C_{60} in the tissues and blood of rat (Fig. 2). The recovery of C_{60} was determined by spiking samples collected from untreated rat. No C_{60} was observed in tissues and blood of untreated rat. 8 μL of C_{60} toluene solution (10 mg/L) was added to previously treated (tissues were freeze-dried, homogenized, added 0.2 M SDS and acetic acid, vortexed and sonicated.) tissues and blood (whole blood) giving a 20 μg/L final concentration, and was mixed. Thereafter, internal standard solution (toluene) and toluene were added and subsequent processes were conducted as described in Section 2. The recovery percentage was calculated by comparing the sample peak areas with those of the C_{60} standard solution at the same concentration (final concentration: 20 μg/L). Recovery percentages of C_{60} were 98.1% for blood, 95.7% for brain, 94.0% for kidneys, 105.0% for liver, 108.3% for lungs, and 101.4% for spleen, respectively. In the case of all tissues and blood as well as the internal standard, good recoveries were obtained. These results suggested that our method was valid for determining C_{60} concentrations in biological samples.

3.2. C_{60} concentrations in five tissues and blood of rats

C_{60} concentrations in tissues and blood of rats after tail vein injection were determined. Although no C_{60} was detected in the blood, C_{60} was observed in almost all of the tissues examined in this study (Table 1). For the Day 1 and Day 7 groups, C_{60} was detected in all samples from the five collected tissues of rats. C_{60} concentrations (mean ± SD) in tissues of the Day 1 rat group were 254 ± 114 μg/g wet wt. for lungs, 53.0 ± 18.8 μg/g wet wt. for spleen, 25.5 ± 5.56 μg/g wet wt. for liver, 1.30 ± 0.68 μg/g wet wt. for kidneys, and 0.08 ± 0.01 μg/g wet wt. for brain. C_{60} concentrations (mean ± SD) in tissues of the Day 7 rat group were 199 ± 88.4 μg/g wet wt. for lungs, 45.3 ± 10.7 μg/g wet wt. for spleen, 22.6 ± 9.63 μg/g wet wt. for liver, 0.42 ± 0.11 μg/g wet wt. for kidneys, and 0.04 ± 0.01 μg/g wet wt. for brain. For the Day 14 and Day 28 groups, although C_{60} was detected in all samples of the lungs, spleen, liver, and kidneys, C_{60} was only detected in one specimen of the Day 14 group in brain tissue. C_{60} concentrations (mean ± SD) in tissues of the Day 14 rat group were 109 ± 51.5 μg/g wet wt. for lungs, 59.0 ± 14.4 μg/g wet wt. for spleen, 21.9 ± 4.94 μg/g wet wt. for liver, 0.20 ± 0.02 μg/g wet wt. for kidneys, and 0.04 μg/g wet wt. (n = 1) for brain. C_{60} concentrations (mean ± SD) in tissues of the Day 28 rat group were 133 ± 47.5 μg/g wet wt. for lungs, 70.6 ± 32.0 μg/g wet wt. for spleen, 14.8 ± 3.00 μg/g wet wt. for liver, and 0.16 ± 0.04 μg/g wet wt. for kidneys.

Table 1
Concentrations of C_{60} in five tissues and blood of Wistar rats.

Group	Sample no.	Concentrations (μg/g wet wt.)					
		Lungs	Spleen	Liver	Kidneys	Brain	Blood
Day 1	1	223	46.5	19.9	1.10	0.080	<0.020
	2	207	78.6	20.8	0.807	0.074	<0.020
	3	188	34.8	32.4	1.14	0.062	<0.020
	4	456	38.7	30.2	0.954	0.078	<0.020
	5	197	66.4	24.5	2.49	0.096	<0.020
Day 7	6	152	52.1	23.0	0.542	0.029	<0.020
	7	281	56.8	18.8	0.508	0.034	<0.020
	8	235	45.0	39.1	0.431	0.048	<0.020
	9	259	28.7	15.4	0.292	0.032	<0.020
	10	66.9	43.8	16.9	0.327	0.037	<0.020
Day 14	11	105	68.3	17.8	0.200	<0.023	<0.020
	12	74.0	51.0	27.9	0.235	<0.023	<0.020
	13	67.5	62.1	17.9	0.200	0.035	<0.020
	14	103	75.1	19.3	0.168	<0.023	<0.020
	15	196	38.8	26.7	0.189	<0.023	<0.020
Day 28	16	113	110	11.4	0.129	<0.023	<0.020
	17	204	94.9	16.6	0.111	<0.023	<0.020
	18	142	68.2	18.3	0.156	<0.023	<0.020
	19	133	44.4	12.0	0.191	<0.023	<0.020
	20	74.1	35.1	15.8	0.191	<0.023	<0.020

4. Discussion

4.1. Biodistribution of C_{60} in rats

C_{60} concentrations in tissues and blood of rats after tail vein injection are investigated (Table 1). C_{60} was detected in the lungs, spleen, liver, and kidneys of rats in all groups, ranging from 0.16 ± 0.04 μg/g wet wt. (kidneys, Day 28) to 254 ± 114 μg/g wet wt. (lungs, Day 1). The highest C_{60} concentration was detected in the lungs, followed by spleen, liver, and kidneys. On the other hand, although C_{60} was detected in all of the brains from the Day 1 and Day 7 groups (0.08 ± 0.01 μg/g wet wt. for Day 1; 0.04 ± 0.01 μg/g wet wt. for Day 7), C_{60} was detected in only one brain specimen from the Day14 group (0.04 μg/g wet wt.) and none of the specimens from the Day 28 group. Moreover, no C_{60} was observed in blood samples from any of the groups. Although studies of the biodistribution of unmodified C_{60} are limited, several studies have reported the biodistribution of water-soluble C_{60} derivatives. Yamago et al. (1995) reported the biodistribution of ^{14}C-labeled water-soluble C_{60} in Fischer rats after intravenous injection. After injection, ^{14}C-labeled water-soluble C_{60} was rapidly removed from blood and about 80% of ^{14}C-labeled water-soluble C_{60} was retained

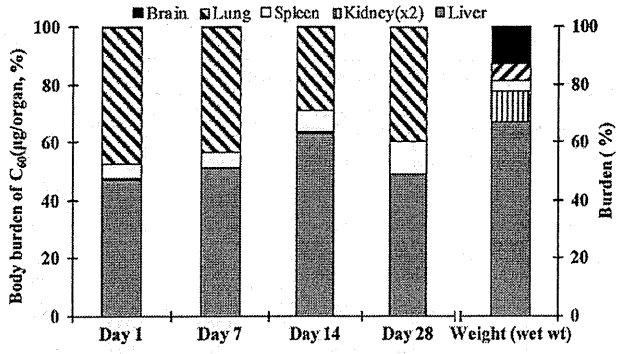

Fig. 3. Comparison of C_{60} distribution in tissues of the four treated rat groups (Days 1, 7, 14, and 28).

in the liver until after 30 h. Furthermore, ^{14}C-labeled water-soluble C_{60} was also detected as a minor component in the spleen, lungs, kidneys, heart, testicles, and brain (Yamago et al., 1995). Bullard-Dillard et al. (1996) reported that a ^{14}C-labeled C_{60} where the label is part of the C_{60} cage after injection in Sprague–Dawley rats was rapidly cleared from the circulation with the majority of the radiolabel accumulated in the liver (90–95%) and ^{14}C-labeled C_{60} was not eliminated from the liver over the 120 h. Moreover, ^{14}C-labeled water-soluble C_{60} derivative was found in liver (>50%) with another 28% distributed between muscle, skin, and lung (Bullard-Dillard et al., 1996). Nikolić et al. (2009) indicated that significantly higher accumulation of radiolabeled C_{60} (^{125}I-nanoC_{60}) was observed in liver as well as spleen, while the accumulation of radiolabeled C_{60} was lower in the lungs, intestines and bone of Wistar rats after intravenous injection. Furthermore, Cagle et al. (1999) showed the selective localization of water-soluble radioactive metallofullerene in the liver of BALB/c mice after intravenous administration. In contrast, although C_{60} was found in the lungs, spleen, liver, kidneys, and brain, in our study the highest concentration of C_{60} was observed in the lungs and the trend differed from the other studies (Yamago et al., 1995; Bullard-Dillard et al., 1996; Cagle et al., 1999; Nikolić et al., 2009). In this study, C_{60}-liposome was suspended in the administration solution, and the C_{60} was an unmodified form. Hence, the difference in the biodistribution of C_{60} between previous studies and this study might be attributable to differences in physical properties, the particle size distribution of C_{60} in the administration solution, and/or period after completion of the injections. Compared with previous studies, the C_{60} particle size in the administration solution for this study seems large (particle size: >100 nm, particle size was obtained from measuring of diluted administration solution by Zetasizer Nano, Malvern Instruments Ltd.) because the C_{60} water solubility is low. We considered that C_{60} injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. C_{60} not being detected in the blood indicates that clearance of C_{60} from the blood by filtration might effectively occur in the lungs. Although C_{60} concentrations were low (0.029–0.096 μg/g wet wt.), C_{60} was detected in the brain. Yamago et al. (1995) and Cagle et al. (1999) have also reported that C_{60} was observed in the brain at low concentrations. These results suggest that a low concentration of C_{60} can pass through the blood brain barrier and accumulate in the brain.

The distribution of C_{60} burden among the five rat tissues was calculated from the product of the C_{60} concentration in each tissue and the weight of each tissue (Fig. 3). Among five tissues, the C_{60} burden was highest in the liver (47.3–63.6%), followed by the lungs (29.0–47.5%), spleen (4.9–11.1%), kidneys (0.1–0.4%), and brain (0–0.03%). Although C_{60} concentrations in the liver were lower than those in the lungs and spleen, the liver showed a higher percentage of C_{60} burden (47.3–63.6%) as a result of the large liver mass (67.0%). On the other hand, although the spleen and lungs accounted for 3.2% and 6.2% of the mass of the five tissues, respectively, the C_{60} burdens were 4.9–11.1% for the spleen and 29.0–47.5% for the lungs, respectively. These three tissues accounted for approximately 100% of the C_{60} burden, indicating that C_{60} was localized to the lungs, spleen, and liver. Because capillary vessels are abundant in these tissues, C_{60} might accumulate by filtration in these tissues. In this study, we focused on the lungs, spleen, liver, kidneys, and brain as major target organs, and other tissues, urine and feces of each specimen were not collected. Although several studies reported the detection of C_{60} derivatives in other tissues (such as, bone, fat, heart, intestines, muscle, and testicles), C_{60} derivatives in these tissues were mostly observed as minor components (Yamago et al., 1995; Bullard-Dillard et al., 1996; Cagle et al., 1999; Nikolić et al., 2009). Further studies should evaluate the metabolic and excretion pathways of C_{60} in experimental animals to understand the toxicokinetics of C_{60}.

4.2. Time-dependent variation in the biodistribution of C_{60} in rats

The time-dependent variation in the biodistribution of C_{60} in rats was examined by comparison of C_{60} concentrations in the five tissues from the four rat groups (Days 1, 7, 14, and 28) (Fig. 4). Although the number of samples for each group was small ($n = 5$), a time-dependent decrease in C_{60} concentrations was observed in all tissues, except the spleen. A significant decrease in C_{60} concentration was found in the kidneys and brain. In the case of the kidneys, significant difference in C_{60} concentration from the four treated groups was found (Kruskal–Wallis test, $p = 0.0007$). C_{60} concentrations from the Day 14 group were significantly lower than those from the Day 1 group (Scheffe's test, $p = 0.0396$). Moreover, C_{60} concentrations from the Day 28 group were also significantly lower than those of the Day 1 group (Scheffe's test, $p = 0.0024$). In the case of the brain, C_{60} concentrations from the Day 7 group were significantly lower than those from the Day 1 group (Student's t-test, $p = 0.0002$). Furthermore, C_{60} concentrations of the Day 14 and Day 28 groups were also lower than those of the Day 7 group. On the other hand, in the lungs and liver, the decreasing trend in C_{60} concentration was slower as compared with the trend in the kidneys and brain. In the case of the lungs, significant difference in C_{60} concentration from the four treated groups was found (Kruskal–Wallis test, $p = 0.0493$) and C_{60} concentrations from the Day 14 group were significantly lower than those from the Day 1 group (Mann–Whitney's U-test, $p = 0.0163$). In the case of the liver, significant difference in C_{60} concentration from the four treated groups was found (Kruskal–Wallis test, $p = 0.0251$) and C_{60} concentrations from the Day 28 group were significantly lower than those from the Day 1 group (Scheffe's test, $p = 0.0298$).

Although with shorter experimental periods than this study, several studies have reported a time-dependent variation in the concentration of C_{60} derivatives in tissues. Yamago et al. (1995) reported the time-dependent change in tissue radioactivity levels using ^{14}C-labeled water-soluble C_{60}. In the liver, about 80% of the total radioactivity was retained after 30 h, and was mostly eliminated (1.6%) after 160 h. This decrease in radioactivity was also observed in the spleen, lungs, and brain. Cagle et al. (1999) also reported the time-dependent decrease of water-soluble radioactive metallofullerene in liver, kidneys, lungs, spleen, and brain after 48 h. Although these studies found a time-dependent decrease of C_{60} derivatives in various tissues, differences in the decreasing trend of the C_{60} derivatives among tissues were not observed. In our study, different decreasing trends of C_{60} among tissues were observed. In the kidneys and brain, a significant decrease in C_{60} concentration was observed. On the other hand, C_{60} concentration

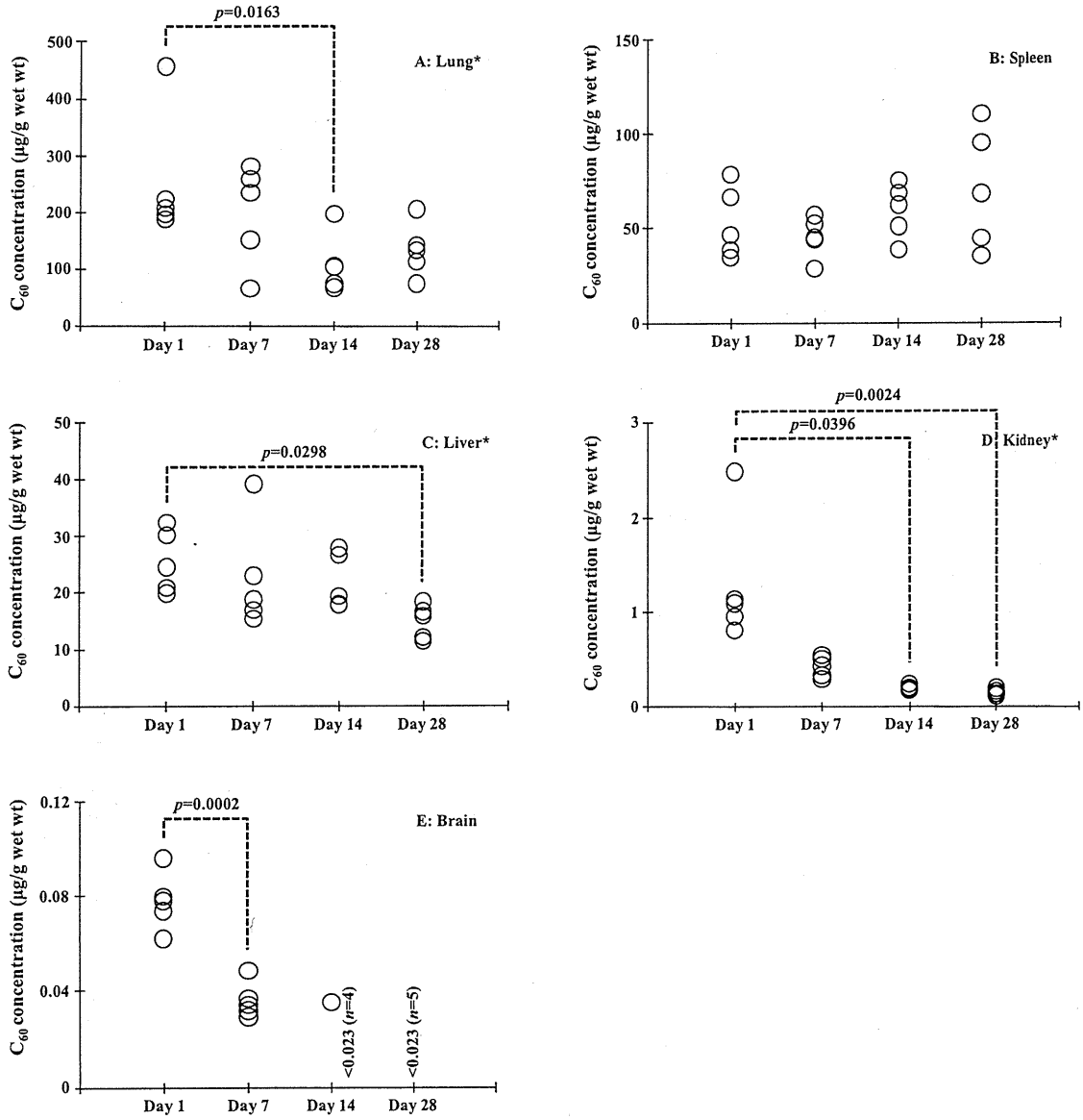

Fig. 4. Comparison of C_{60} concentrations in each tissue of the four treated rat groups (Days 1, 7, 14, and 28). Kruskal–Wallis test was used for validation of difference in the C_{60} concentrations in four tissues among treated groups and significant difference (*) was observed in lungs ($p = 0.0493$), liver ($p = 0.0251$), and kidneys ($p = 0.0007$). (A) C_{60} concentration in lungs (Day 14 vs. Day 1, $p = 0.0163$ (Mann–Whitney's *U*-test)). (B) C_{60} concentration in spleen. (C) C_{60} concentration in liver (Day 28 vs. Day 1, $p = 0.0298$ (Scheffe's test)). (D) C_{60} concentration in kidneys (Day 14 vs. Day 1, $p = 0.0396$ (Scheffe's test); Day 28 vs. Day 1, $p = 0.0024$ (Scheffe's test)). (E) C_{60} concentration in brain (Day 7 vs. Day 1, $p = 0.0002$ (Student's *t*-test)).

in lungs and liver decreased gradually. This difference of decreasing trends among tissues could be due to differences in accumulation levels. The low concentration of C_{60} in the kidneys and brain might be easily decreased compared to the high concentrations of C_{60} in the lungs, liver, and spleen. Although it is not clear whether C_{60} is excreted from the body, we propose some possible mechanisms for the decrease of C_{60} in tissues. The first possible mechanism is redistribution to other tissues via blood. Although only five tissues were examined in this study, C_{60} might be detectable in other organs. Cagle et al. (1999) reported a comparatively high concentration of water-soluble radioactive metallofullerene in bone. Moreover, although the accumulation levels were low, radiolabeled nano C_{60} (^{125}I-nanoC_{60}) was found in intestines and bone of rats (Nikolić et al., 2009). Although the C_{60} concentration in blood was below the limit of detection, the small blood volume used for the analysis could be the reason for this result. If a larger volume of blood were analyzed, C_{60} might be detected. Bullard-Dillard et al. (1996) reported that 0.39% of ^{14}C-labeled C_{60} was detected in blood of rat at 120 h post-injection. The second possible mechanism is metabolism to water-soluble fullerene metabolites and excretion into urine. Although there is no information on the intravital metabolism of C_{60}, it is possible that C_{60} could be metabolized to C_{60} derivatives (e.g. fullerenol ($C_{60}(OH)_n$)) by metabolic enzymes (e.g. cytochrome P450). Hamano et al. (1995) identified the structures of $C_{60}O$, $C_{60}O_2$, and $C_{60}O_3$ formed in P450 chemical model systems and the results support the hypothesis that possibility of a biotransformation of C_{60} into more hydrophilic C_{60} derivative capable of being excreted into the urine. In this study, the renal C_{60} concentration rapidly decreased, which also supports the possibility that C_{60} is excreted from urine. The third possible mechanism is biliary

excretion of unmodified C_{60} and/or C_{60} metabolites. Because C_{60} is lipophilic, if C_{60} is excreted without metabolism, it might be via this route. Consequently, further studies are required to verify the metabolism and excretion of C_{60}. Yamago et al. (1995) reported that virtually all of the excretion of ^{14}C-labeled water-soluble C_{60} occurred via the feces and 5.4% of ^{14}C-labeled water-soluble C_{60} was eliminated into the feces after 160 h in intravenous injection experiment. Moreover, Cagle et al. (1999) also indicated that a water-soluble radioactive metallofullerene was excreted into the feces of rats.

In summary, the current study demonstrates that C_{60} after tail vein administration is widely distributed between various tissues, such as brain, kidneys, liver, lungs, and spleen of rats. Moreover, the large variability in C_{60} concentrations among tissues was found and the highest C_{60} concentration was observed in the lungs, followed by spleen, liver, kidneys, and brain. These results suggested that C_{60} injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. Furthermore, C_{60} not being detected in the blood indicates that clearance of C_{60} from the blood by filtration might effectively occur in the lungs. A time-dependent decrease in C_{60} concentrations was observed in all tissues, except spleen. Moreover, a decreasing trend of C_{60} levels differed among tissues, which could be due to differences in accumulation. These results suggest that unmodified C_{60} and/or C_{60} metabolites by metabolic enzymes could be excreted into feces and/or urine. In further studies, the metabolic and excretion pathways of C_{60} should be evaluated to understand the toxicokinetics of C_{60}.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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Short communication

Fullerene (C_{60}) Is Negative in the *In Vivo Pig-A* Gene Mutation Assay

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Carbon nanoparticles, such as carbon nanotubes and fullerene (C_{60}), are potential candidates as leading substances in nanotechnological fields, but little is known about their safety. Here we examined *in vivo* genotoxicity of C_{60}, by performing the *Pig-A* gene mutation assay in the peripheral blood of male C57BL/6Cr mice. Mice were given single intraperitoneal injection of 3 mg of C_{60} particles in 0.5 mL suspension containing 0.1%-Tween80-saline. As a positive control for the *Pig-A* gene mutation assay, mice were given a single oral administration of *N*-nitroso-*N*-ethylurea. At 2 and 8 weeks after treatments, we analyzed CD24-negative and -positive red blood cells in peripheral blood and calculated *Pig-A* mutant frequencies. As a result, we detected no significant differences in the mutant frequencies between C_{60} treated and non-treated mice, indicating that C_{60} is negative for genotoxicity *in vivo* in the limited target tissues assessed in this study. For the full assessment, we need comprehensive whole body survey on the genotoxicity of C_{60}.

Key words: carbon nanoparticle, *in vivo* genotoxicity, *Pig-A* gene mutation assay, fullerene

Introduction

Manufactured nanomaterials are important substances in nanotechnology, and the potential human and environmental risks need to be investigated for risk assessment and management.

There are several reports on the toxicities induced by carbon nanoparticles, such as single-wall carbon nanotubes (SWCNTs), multi-wall carbon nanotubes (MWCNTs) and fullerene (C_{60}). Intraperitoneal application of MWCNTs induced mesothelioma in $p53^{+/-}$ mouse (1) and intrascrotal administration of MWCNTs induced mesothelioma in wild-type rats (2). Reports on the *in vivo* genotoxicity of C_{60}, however, are conflicting. It was reported that intratracheal instillation of C_{60} increased both mutation frequency detected by *gpt*-assay and DNA damage detected by comet assay in lung (3). Nevertheless another group showed that treatment with C_{60} by gavage has no genotoxical effect in ICR mice, using *in vivo* micronucleus test in bone marrow cells (4). These discrepancies could have been caused by differences in administration route, test method, or target organ.

Here we examined the *in vivo* genotoxicity of C_{60} using a different test system—the recently established *Pig-A* gene mutation assay (5,6). The *Pig-A* assay, a powerful tool for the evaluation of *in vivo* genotoxicity, is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient erythrocytes and has been shown to be applicable across species from rodent to monkey (5-8). With this method, we need no transgenic animals to test *in vivo* genotoxicity, but need only 1-2 μL peripheral blood (5,6). Additionally, long-term, accumulated *in vivo* genotoxic effects could be evaluated (9).

Materials and Methods

Test chemicals: Fullerene (C_{60}, Nanom purple SUH; purity >99.9%, Frontier Carbon Corporation, Tokyo, Japan) was obtained and prepared as previously described with some modifications (1). Briefly, C_{60} was suspended to physiological saline (Ohtsuka Pharmaceutical Co., Tokyo, Japan) and autoclaved. After addition of Tween 80 (Polysorbate 80 (HX), NOF Corporation, Tokyo, Japan) at a final concentration of 0.1%, solutions were subjected to sonication by ultrasonic homogenizer (VP30s, TAITEC Co. Japan). C_{60} was prepared at a final concentration of 6 mg/mL. *N*-nitroso-*N*-ethylurea (ENU, Sigma) was dissolved in PBS (pH

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6.0) at 10 mg/mL as previously described (5).

Animal treatment: Mice were treated as described previously (1). In brief, 6 male wild-type C57BL/6Cr mice (SLC, Shizuoka, Japan) at the age of 9-11 weeks were given single i.p. injection of 3 mg/head suspension (0.5 mL) of C_{60}. Vehicle solution (0.5 mL) was given to 6 mice as negative controls. As a positive control of this study, 5 mice were given single oral administration of ENU (40 mg/kg). Peripheral bloods were withdrawn from tail vein of mice and analyzed by the *Pig-A* gene mutation assay. All mice were housed individually under specific pathogen-free conditions, with a 12 h light-dark cycle at the animal facility of NIHS. All mice were given tap water and gamma-ray irradiated CRF-1 pellets (Oriental Yeast Co., ltd.) *ad libitum*. Animal experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, Japan.

Antibodies: Anti-mouse TER119 antibody for erythroid cells staining (clone TER-119, PE-Cy7-conjugated) and anti-mouse CD24 antibody (clone M1/69, FITC-conjugated) were obtained from BioLegend.

***Pig-A* gene mutation assay in mice:** Mice *Pig-A* gene mutation assay was performed as previously described (5,8), with some modifications. In brief, EDTA/2K was dissolved in distilled water to make a 12% solution, and used as an anticoagulant. Eighteen μL of peripheral blood were mixed with 2 μL of EDTA solution. Two μL of blood/EDTA mixture was suspended in 0.2 mL of PBS, and the cells were labeled with 1 μg of each anti-mouse TER119 and anti-mouse CD24 antibodies. After incubation for 1 h in the dark at room temperature, the cells were washed once by centrifugation ($500 \times g$, 5 min), resuspended in 2 mL of PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences). After gating for single cells, about 1,000,000 TER119-positive cells were analyzed for the presence of CD24 on their surface. The data were statistically compared with the corresponding solvent control using the Student's t-test.

Results

***Pig-A* gene mutation assay with mice peripheral blood:** Recent works provided that the erythrocyte-based *Pig-A* gene mutation assay is applicable across species (5-8). According to these reports, we modified the original *Pig-A* gene mutation assay and performed it with mice peripheral blood. To classify white blood cells (WBCs) and red blood cells (RBCs) in mice peripheral blood, RBCs were stained with anti-TER119 antibody. Anti-CD24 antibody was used to detect GPI-anchored protein as previously reported (8,10). The gating strategy that was used to score GPI anchor deficient RBCs population was shown in Fig. 1. Single cells including RBCs and WBCs were gated by light scatter (Fig. 1A). To exclude WBCs from this population, TER119-positive cells (Fig. 1B) were analyzed further for the presence on the cell surface of either the GPI-anchored CD24 (Fig. 1C and 1D). The gate used for CD24-negative cells was established by blood cell samples prepared without the fluorescent reagents.

***In vivo* genotoxicity tests on fullerene (C_{60}) analyzed by the *Pig-A* gene mutation assay:** At 2 and 8 weeks after the injection of C_{60} (3 mg/head) and ENU, we analyzed CD24-negative and -positive RBCs in peripheral blood. At both 2 and 8 weeks after the injection, higher amounts of CD24 deficient RBCs were observed in the ENU treated mice (Fig. 1D) as compared with the solvent control (not shown) and C_{60} treated mice (Fig. 1C), respectively. Frequencies of CD24-negative RBCs were summarized in Fig. 2. Frequency of CD24-negative RBCs was significantly increased in ENU treated mice (2 weeks after treatment; $30.12 \pm 3.54 \times 10^{-6}$, and 8 weeks after treatment; $36.64 \pm 15.71 \times 10^{-6}$). However, we detected no obvious differences in frequency of CD24-negative RBCs between C_{60} treated and non-treated mice ($0.25 \pm 0.30 \times 10^{-6}$ versus $0.42 \pm 0.19 \times 10^{-6}$ after 2 weeks and $0.82 \pm 0.54 \times 10^{-6}$ versus $1.87 \pm 1.51 \times 10^{-6}$ after 8 weeks).

These results indicated that although the *Pig-A* gene mutation assay with mouse peripheral blood was appropriately performed, C_{60} was negative for genotoxicity *in vivo* in the RBCs assessed in our study.

Discussion

We demonstrated here that C_{60} (3 mg/head) given intraperitoneally to male C57BL/6Cr mice was negative in the *Pig-A* gene mutation assay using peripheral blood, suggesting that C_{60} was not mutagenic to erythroid precursor cells or hematopoietic stem cells.

The *Pig-A* gene mutation assay is based on detections of GPI-anchored protein on the cell surface of RBCs. The *Pig-A* gene is involved in the synthesis of GPI anchors that link various protein markers to the cell surface. It is known that paroxysmal nocturnal hemoglobinuria (PNH) is caused by somatic *PIG-A* mutations in hematopoietic stem cells (HSCs) and Aerolysin-resistant HSCs from a patient with PNH exhibited clonal *PIG-A* mutations (11,12). Additionally, it is considered that the absence of GPI-anchored protein of RBCs is caused by mutations occurred in the *Pig-A* gene of nucleated erythroid precursors and/or of HSCs (6). These observations suggested that expression of GPI-anchored CD24 of RBCs is depending on the *Pig-A* gene mutations happened in erythroid precursors and/or of HSCs in bone marrows. According to this, we considered that our results, shown here using peripheral blood of mice, reflected genotoxicity of C_{60} on bone marrows.