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Intrauterine environment-genome interaction and Children's development (2): Brain structure impairment and behavioral disturbance induced in male mice offspring by a single intraperitoneal administration of domoic acid (DA) to their dams

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ABSTRACT — To demonstrate induction of delayed central nervous toxicity by disturbing neuronal activities in the developing brain, we administered a single intraperitoneal dose of domoic acid (DA; 1 mg/kg), a potent glutamate receptor agonist, to pregnant female mice at the gestational day of 11.5, 14.5 or 17.5. The dams had recovered from acute symptoms within 24 hr, followed by normal delivery, feeding and weaning. All male offspring mice after weaning were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. At the age of 11 weeks, our neurobehavior testing battery revealed severe impairment of learning and memory with serious deviances of anxiety-related behaviors. The developed brain of prenatally exposed mice showed myelination failure and the overgrowth of neuronal processes of the limbic cortex neurons. This study indicates that the temporal disturbance of neurotransmission of the developing brain induces irreversible structural and functional damage to offspring which becomes monitorable in their adulthood by a proper battery of neurobehavioral tests.

Key words: Domoic acid, Prenatal exposure, Brain structure, Behavior

INTRODUCTION

Adequate neural activities are necessary for the maturation of neural networks during brain development (Rice and Barone, 2000). Historically, the presence of such plasticity-driven mechanisms has been demonstrated by a series of studies of eyelid suture in kittens or monkeys and corresponding findings reported in young human cataracta patients (Wiesel, 1982; Gu *et al.*, 1989; Fonta *et al.*, 2000). These processes require proper stimuli to the brain that trigger the release of neurotransmitters from the neurons and subsequent receptor-mediated signal transduction (Ooi and Wood, 2008; Greer and Greenberg, 2008; Cohen-Cory, 2002). Therefore, it is highly conceivable that disturbance of neural activities by neuroactive xenobiotics leads to malformation of the fine structure of the brain. Even when the exposure was transient, it would result in anomaly of higher brain functions in adulthood without overt signs of brain damage during maturation.

Glutamate receptors begin to express in the late embryonic stages, and their expression increases with the advance of brain development (Luján *et al.*, 2005; Manent *et al.*, 2005). Prenatal exposure of xenobiotic chemicals that interfere with the glutamate receptor function could induce malformation of the fine structure of the brain which should lead to anomaly of higher brain function that is different from acute neurotoxicity known for such chemicals to induce in adults (Bondy and Campbell, 2005). A marine biotoxin domoic acid (DA) which is structurally related to glutamate, and activates ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kinate subtypes of glutamate receptors (Pulido, 2008) is known to cause acute symptoms of diarrhea, seizures and memory loss in adult human by eating contaminated shellfish (Tryphonas and Iverson, 1990), and DA induced acute neurotoxicity in animal

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model (Chandrasekaran *et al.*, 2004). Additionally, DA is also known to cross the placenta, and enters prenatal brain tissue in rats (Maucher and Ramsdell, 2007). Therefore, prenatal exposure of DA may disrupt the neural activities by excessive stimulation of glutamate receptors, and should induce fine structural and functional disorganization in the developing brain. Here, we report that a transient transplacental DA exposure *in utero* induced alteration of the neurobehavioral parameters and corresponding fine brain structure of the male C57BL/6 mice in their adulthood.

MATERIALS AND METHODS

Animal treatment

All experiments were carried out under approval of Experimental Animal Use Committee of National Institute of Health Sciences, Japan. Pregnant C57LB/6 female mice obtained from Japan SLC, Inc., were individually housed in plastic breeding cages with free access to water and pellet diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) in a 12 hr light-dark cycle conventional condition. Four groups with five pregnant mice each were prepared. All groups received three intraperitoneal injections on gestational day 11.5 (E11.5) as a late embryonic period, 14.5 (E14.5) and 17.5 (E17.5) as early and late fetal period respectively. Group A (Control) received three i.p. shots of saline on E11.5, E14.5 and E17.5. Group B (DA@E11.5) received one shot of DA (Calbiochem, San Diego, CA, USA) at a dosage of 1 mg/kg on E 11.5 and two shots of saline on E14.5 and E17.5. Group C (DA@14.5) received a shot of saline on E11.5, a shot of DA on E14.5 and another saline on E17.5. Group D (DA@E17.5) received two shots of saline on E11.5 and E14.5 , and a shot of DA on E17.5. The pups were weaned at 4 weeks of age, and four male mice per liter were randomly selected and housed in one cage with free access to water and CRF-1 pellet until 11 weeks of age.

Immunohistochemical analysis

Brains (n = 4 male mice per group) were fixed with methacarn fixative (methanol: chloroform:acetic acid, 60:30:10 v/v) and paraffin-embedded sections were prepared. Mouse monoclonal anti-microtubule associated protein 2 (MAP2, sc-32791; Santa Cruz, CA, USA), mouse monoclonal anti-neurofilament-m (NF-M, sc-20013; Santa Cruz, CA, USA), rabbit polyclonal anti-myelin associated glycoprotein (MAG, sc-15324; Santa Cruz, CA, USA), and rabbit polyclonal anti MAP2 (sc-20172; Santa Cruz, CA, USA) were used. Deparaffinized sections were pretreated with HistoVT-One (Nacalai Tesque, Kyoto, Japan.) as previously described (Tanemura *et al.*, 2005) and incubated with primary antibodies. Secondary antibodies were Alexa 568-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Fluorescent images were obtained with an FV-300 confocal laser scanning microscope (Olympus, Tokyo, Japan). For semi-quantitative analysis of images, we calculated the ratio of fluorescence intensity compared to control mice (group A), by using the IMAGE J program (http://rsb.info.nih.gov/ij/index.html. National Institute of Health, Bethesda), after adjusting background noise (n = 4 images per mouse).

Neurobehavioral tests

A battery of neurobehavioral tests were conducted on open field test (OF), light/dark transition test (LD), elevated plus maze test (EP) and contextual/cued fear conditioning test (FZ). Experimental apparatuses and image analyzing softwares were obtained from O'Hara & Co., Ltd., Japan. Image analyzing softwares (Image OF4, Image LD2, Image EP2 and Image FZ2) were developed from the public domain IMAGE J program. All experiments were done with 8 mice per group (32 mice total), and were conducted between 13:30 and 16:30. The level of background noise during behavioural testing was about 50 dBA. After each trial, the apparatuses were wiped and cleaned.

Open field test

The locomotor activity was measured for 10 min using an open field apparatus made of white plastic (50 x 50 x 40 (H) cm).

An LED light system was positioned 50 cm above the centre of the field (50 lux at the centre of field). Total distance travelled (cm), time spent in the central area (30% of the field) (sec), and the frequencies of movement were measured (Tanemura *et al.*, 2002).

Light/dark transition test

The apparatus used for the light/dark transition test consisted of a cage (21 x 42 x 25(H) cm) divided into two chambers by a partition with an opening. One chamber is brightly illuminated (250 lux), whereas the other chamber is dark (2 lux). A mouse is placed into the dark area and allowed to move freely between the two chambers through the opening for 5 min. The latency for the first move to the light area, the total number of transitions and the time spent on each side were measured.

Elevated plus maze test

The plus-shaped apparatus consisted of four arms (25

x 5 cm) connected to a central square area (5 x 5 cm). Opposite two arms are enclosed with 20 cm-high transparent walls and other two are left open. The floor of the maze is made of white plastic plate and is elevated 60 cm above the room floor (200 lux at the centre of the apparatus). A mouse is placed to the central square area of the maze, facing one of the open arms, and the behavior was recorded for 10 min: total distance traveled (cm), total time on open arms and central square area (sec) and the total number of entry to any of the arms (Tanemura *et al.*, 2002).

Contextual/cued fear conditioning test

The apparatus consists of a conditioning chamber (or a test chamber) (17 x 10 x 10 (H) cm) made of clear plastic with ceiling and placed in a sound proof box. The chamber floor has stainless steel rods (2-mm diameter) spaced 5 mm apart for giving electric foot shock (0.1 mA, 3 sec duration) to the mouse. The soundproof box consists of white-coloured wood, and is equipped with an audio speaker and light source (35 lux at the centre of the floor). A CCD camera is positioned 20 cm above the ceiling of the chamber. During the conditioning trial (Day 1), mice are placed individually into the conditioning chamber in the sound proof box and, after 90 sec, they are given three tone-shock pairings (30 sec of tone, 75 dB, 10 KHz followed by 3 sec of electric shock at the end of tone, 0.1 mA) separated by 90 sec. Then they are returned to their home cage. Next day (Day 2), as a "contextual fear test", they are returned to the conditioning chamber without tone and shock for a 6-min. On the third day (Day 3), they are brought to a novel chamber of different make without stainless steel rods place in the sound proof box and, after a period of 3 min, only the conditioning tone is presented for 3 min (no shock was presented, 35 lux at the centre of the floor). The freezing response of mice was defined as a consecutive 2 sec period of immobility. Freezing rate (%) was calculated as [time freezing/session time] x 100 (Tatebayashi *et al.*, 2002).

Statistical analysis

Data were indicated as means ± S.D. Statistical analysis was conducted with student's t-test by using StatView (SAS Institute, Cary, NC, USA). A p-value of < 0.05 compared to the results of control male mice (group A) was considered statistically significant.

RESULTS

Effects on morphology of brain by prenatal exposure to DA

Offspring mice of all groups after weaning up to the age of 11 weeks were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. Routine histological observation of the brain at 11 weeks old by hematoxylin-eosin staining could not reveal difference among the groups (data not shown). By immunohistochemical study on the same brain sections, reduced immuno-reactivity against the MAG, the marker for myelin, was detected in the cortices of group B (DA@11.5) and C (DA@14.5) compared to control (Figs. 1A-D and I). In contrast, increased immuno-reactivity against MAP2, the marker for neuronal dendrite, was indicated in the same area of group B (DA@11.5), C (DA@14.5) and D (DA@17.5) compared to control (Figs. 1E-H and J). Increased immuno-reactivity against MAP2 was also found in lateral area of CA3 hippocampus of group B (DA@11.5), C (DA@14.5) and D (DA@17.5) compared to control, whereas immuno-reactivity for MAP2 showed no significant difference in medial area of CA3 hippocampus among the groups (Figs. 2A-D and I). Immuno-reactivity against NF-M; the marker for neuronal axon, also showed no significant difference in the same area among the groups (Figs. 2E-H and J).

Effects on behavior by prenatal exposure of DA

In the OF test, the distance traveled was not different among the groups (Fig. 3A), the time spent in center area was significantly prolonged in group D (DA@17.5) mice (Fig. 3B). In the LD test, group C (DA@14.5) mice stayed in light area for longer time (Fig. 4A), and latency for the first move to light area was significantly shorter in group C (DA@14.5) and D (DA@17.5) (Fig. 4B). In the EP test, significantly increased distance traveled and time spent in the open area were detected for group B (DA@11.5), C (DA@14.5) and D (DA@17.5) (Figs. 5A and B). In the FZ test, both Day 1 and Day 2 freezing responses of group C (DA@14.5) and D (DA@17.5) were significantly reduced compared to control (Figs. 6A and B).

DISCUSSION

The expression levels of glutamate receptors starts to elevate at the fetal period, i.e. approximately from E14 (Luján *et al.*, 2005; Manent *et al.*, 2005). Exogenous glutamatergic stimuli at this period would affect the for-

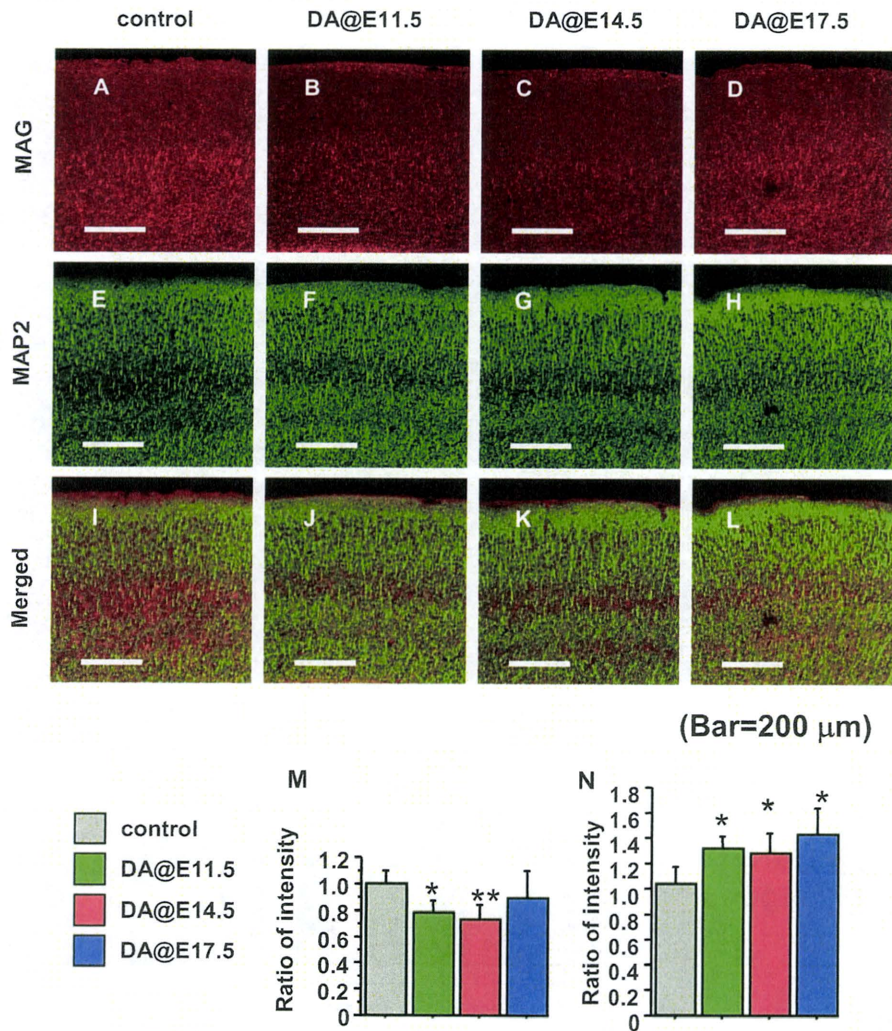

Fig. 1. Delayed effects on cerebral cortex induced by prenatal exposure of DA.
A-D, Immunohistochemical staining against MAG; E-H, immunohistochamical staining against MAP2; I-L, merged images of the cerebral cortex. A, E, I, group A (control), B, F, J, group B (DA@11.5), C, G, K, group C (DA@14.5) and D, H, L, group D (DA@17.5). Scale bar = 200 mm. M, Quantitative analysis in intensity ratio to control of MAG expression, and J, MAP2 expression among the groups (mean ± S.E.M.). Asterisk (**) and (*) indicate significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

mation of the neural circuits. An extreme example to support this hypothesis would be the phenotype of the double knockout mouse of glutamate transportors GLT1 and GLAST (Matsugami *et al.*, 2006). Lack of these transportors is considered to result in abnormally high concentration of glutamate in the brain. In fact, morphological anomaly became apparent in synchronization with the expression of glutamine receptors. In our study, corresponding to the hypothesis, the neurobehavioral symptoms as a whole was severer for those exposed at fetal periods, i.e. E14.5 and E17.5, compared to those at embryonic period, i.e. E11.5 (Fig. 7).

We demonstrated that a prenatal exposure of a relatively low dose of DA induced a spectrum of neurobehavioral anomalies which became monitorable at the adult stage accompanied by alteration in fine brain structures detectable by immunohistochemistry. It is emphasized that this amount of DA did not induce abnormal responses dur-

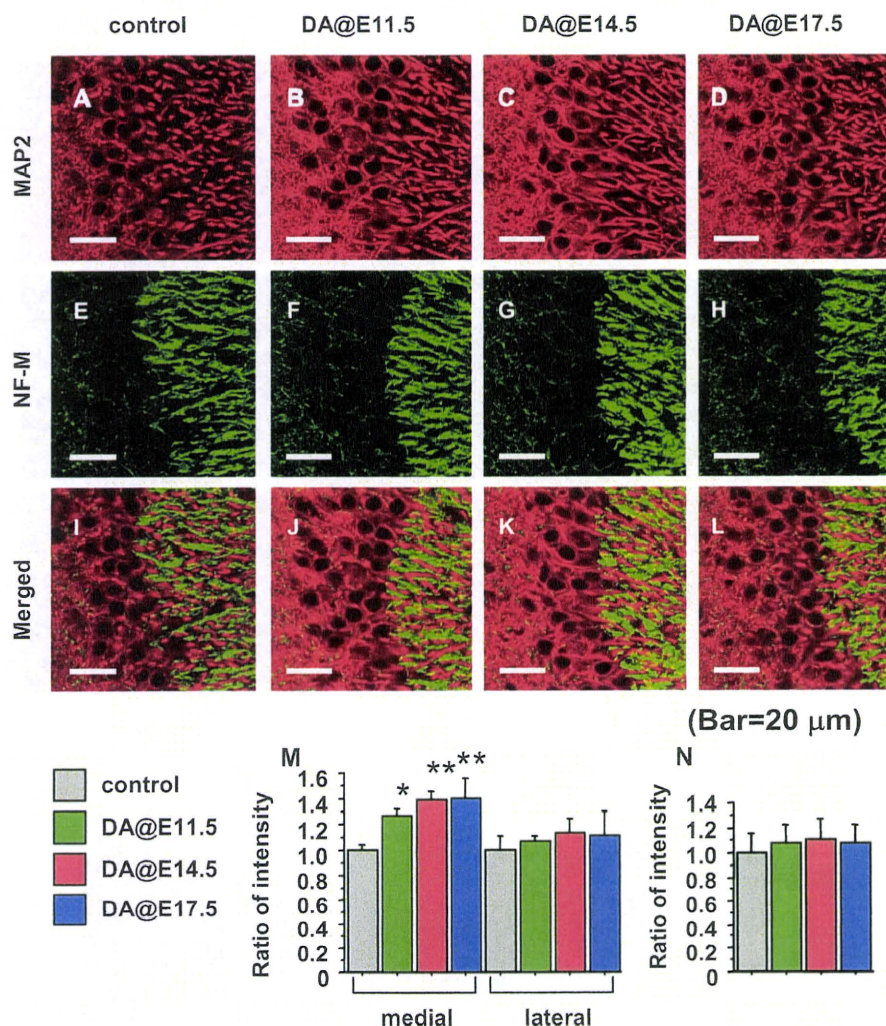

Fig. 2. Delayed effects on hippocampus induced by prenatal exposure of DA.
A-D, Immunohistochemical staining against MAP2; E-H, immunohistochamical staining against NF-M; I-L, merged images, of CA3 hippocampus. A, E, I, group A (control), B, F, J, group B (DA@11.5), C, G, K, group C (DA@14.5) and D, H, L, group D (DA@17.5). Scale bar = 200 mm. M, Quantitative analysis of MAP2 expression, and N, NF-M expression among the groups (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

ing maturation, such as hyperreactivity to handling and to cage mates, and did not present overt malformation of the brain detectable by the routine H&E histology at the age of 2 weeks (data not shown). It is also noted that the spectrum of the neurobehavioral symptoms induced in mice exposed to DA at adulthood was different from those monitored in this study (data not shown).

Although progressive hippocampal neuronal damages were reported to be induced by prenatal administration of DA (0.6 mg/kg intravenous injection to the dam) (Dakshinamurti *et al.*, 1993), we did not find notable neuronal loss or neuronal cell death as the delayed effects in adult mouse brain by prenatal exposure. On the other hand, we found myelination failure (Miller and Mi, 2007) in cortex of group B (DA@11.5) and C (DA@14.5) mice. And we also detected a finding compatible with the overgrowth of neuronal processes in cortex and hippocampus of group B (DA@11.5), C (DA@14.5) and D (DA@14.5)

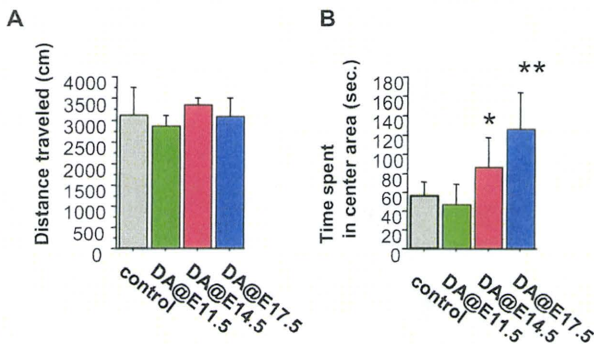

Fig. 3. Delayed effects on locomotor activity (OF test) induced by prenatal exposure of DA.
A, Mean distance travelled (total distances divided by total duration of trial, 10 min) and B, mean time spent in center area (30% of the field) in the open field apparatus (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

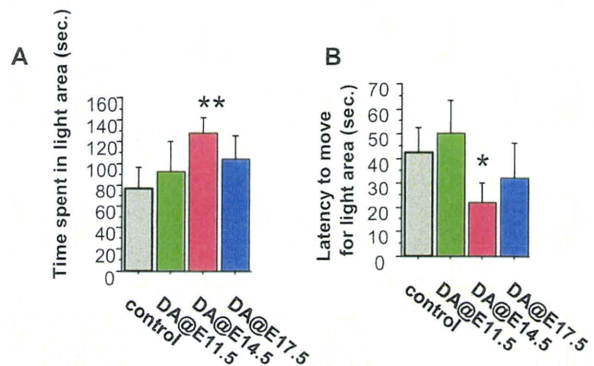

Fig. 4. Delayed effects on anxiety-related behavior (LD test) induced by prenatal exposure of DA.
A, Total time spent in light area, and B, latency time to move to light area in the LD apparatus (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

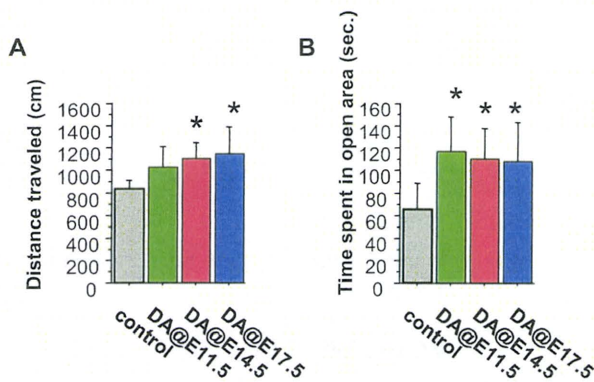

Fig. 5. Delayed effects on anxiety-related behavior (EP test) induced by prenatal exposure of DA.
A, Total distance travelled, and B, total time spent in open area in the elevated plus maze apparatus (mean ± S.E.M.). Asterisk (*) indicated significant difference compared to control ($P < 0.05$).

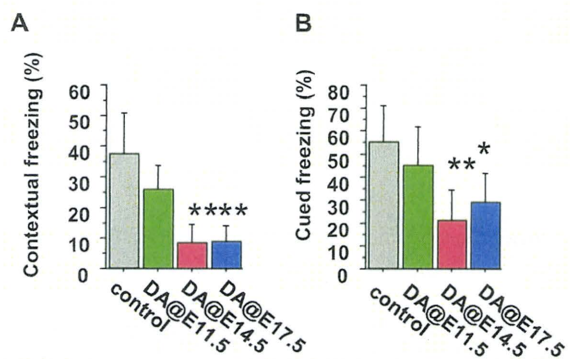

Fig. 6. Delayed effects on learning and memory (FZ test) induced by prenatal exposure of DA.
A, Contextual fear test and B, cued fear test. Memory performance is expressed as a mean percent duration of freezing responses (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

mice by using cytoskeletal marker. These findings indicated that the disorganization of brain was induced by the prenatal exposure of DA, and remained irreversibly up until the maturation period.

Among multiple endpoints of the behavioral test battery we used, serious deviances in anxiety-related behaviors of group C (DA@14.5) and D (DA@17.5) mice were observed. Mice in those groups showed low performances in adaptations for novel circumstances, i.e., strange and broad area in OF test, beamish place in LD test, high and narrow space in EP test. Additionally, we also found severe impairment of learning and memory. Although the low performances of memory task have been reported in rats with prenatal DA exposure (Levin *et al.*, 2005),

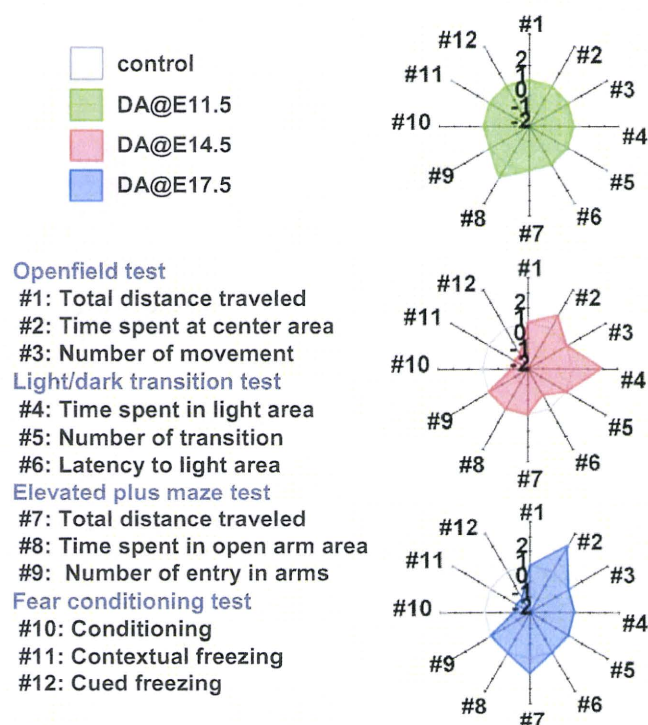

Fig. 7. Summary radar chart of the neurobehavioral battery test results. Radial axis indicates the direction (increase or decrease) of the deviation, and the p value of the endpoints compared to the control (+1 and −1, 0.01 <= p < 0.05, +2 and −2, p < 0.01). Regular dodecagon of radius 0 indicates no deviation from control.

we showed serious deviances about affective (emotional) behaviors additional to severe memory deficit.

In conclusion, we clearly indicated that the disturbance against the adequate neural activity during developmental period when glutamate receptors became active have induced delayed memory defect and unnatural adoptive behaviors that became monitorable at the maturation period in mice. The responsible foci deduced from these behavioral disturbances are the limbic cortex and hippocampus. Our morphological findings are consistent with the interpretation. A combination of neurobehavioral and pathomorphological analysis was shown to be an effective method to assess delayed neurotoxic effects which dose not induce immediate organic brain damage and related symptoms after exposure. Having adopted the hypothesis that exogenous stimuli to neural signaling systems during the development of the brain can be a cause of delayed anomaly of higher brain function, stimuli toward systems other than glutamate receptors should also induce such anomaly of different targets and symptoms in concert with the distribution of the corresponding receptor(s) in the developing brain. Such data on other system would be reported elsewhere.

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Original Article

Enhanced hepatocarcinogenicity by combined inhalation and oral exposures to *N,N*-dimethylformamide in male rats

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ABSTRACT — *N,N*-Dimethylformamide (DMF), a ubiquitous contaminant in living and working environments, enters the human body by inhalation, as well as by oral and dermal routes of exposure. In order to provide bioassay data for carcinogenic risk assessment of humans exposed to DMF by multiple routes of exposure, hepatocarcinogenic effect of combined inhalation and oral exposures of rats to DMF was examined. A group of 50 male F344 rats, 6-week-old, was exposed by inhalation to 0 (clean air), 200, or 400 ppm (v/v) of DMF vapor-containing air for 6 hr/day and 5 days/week during a 104-week period, and each inhalation group was given *ad libitum* DMF-formulated drinking water at 0, 800 or 1,600 ppm (w/w) for 104 weeks. Incidences of hepatocellular adenomas and carcinomas and their combined incidences were significantly increased in the combined-exposure groups compared with the untreated control group or each of the inhalation-alone and oral-alone groups with matching concentrations. Incidences of hepatocellular adenomas and carcinomas induced by the combined exposures were greater than the sum of the two incidences of the hepatocellular adenomas and carcinomas induced by the single-route exposures through inhalation and ingestion. The combined exposures enhanced tumor malignancy. It was concluded that the combined inhalation and oral exposures markedly enhance the incidences and malignancy of hepatocellular tumors, suggesting that the hepatocarcinogenic effect of the combined exposures is greater than the effect that would be expected under the assumption that the two effects of single-route exposures through inhalation and drinking are additive.

Key words: *N,N*-Dimethylformamide, Combined exposure, Inhalation exposure, Rat, Hepatocarcinogenicity

INTRODUCTION

N,N-Dimethylformamide (DMF) has been widely used as a solvent for acrylic fibers and polyurethanes and as a chemical intermediate in chemical manufacturing and pharmaceutical industries (IPCS, 1991). The worldwide production capacity of DMF was estimated to be 125,000 tons /year (IARC, 1999). The annual production of DMF in Japan was reported to amount to 50,000 tons in 2006 (Chemical Daily, 2008). According to U.S. EPA's Toxics Release Inventory Report (2006), total On- and Off-site Disposal or Other Release for DMF amounted to 2,300,000 lbs (1,043 tons). DMF has been observed in ambient air near a fiber plant and in water near waste facilities. Air levels of 0.024 - 0.15 mg DMF/m^3 (0.008 - 0.05 ppm (v/v)) collected in a residential area were reported to originate from neighboring industry (Amster *et al.*, 1983). Concentrations of DMF in public waters were reported to range from 0.037 to 1.5 ppb (w/w), according to a report by the Japan Ministry of the Environment (2006). Ubiquitous presence of DMF in urban air and public waters suggests that the general population may be exposed to DMF at low levels through inhalation and ingestion. On the other hand, workers using DMF in workplaces are at high health risk of exposure to high levels of DMF primarily through inhalation and dermal contact. According to the National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health (NIOSH, 1990), a total of 124,683 workers including 16,011 females were potentially exposed to DMF in workplaces in the USA between 1981 and 1983. Workplace air concentrations of DMF in a textile factory in the

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UK were reported to range from 0.36 to 7.00 ppm (v/v) (1.1 to 21.0 mg/m^3) (Osunsanya *et al.*, 2001).

The International Agency for Research on Cancer (IARC, 1999) made an overall evaluation that DMF is not classifiable as to its carcinogenicity to humans (Group 3), based on both inadequate evidence in humans for carcinogenicity of DMF and evidence suggesting a lack of the carcinogenicity in experimental animals. Malley *et al.* (1994) reported no evidence of carcinogenicity after 2-year inhalation exposure of rats or mice to DMF, and their finding was adopted for the IARC's evaluation. More recently, however, Senoh *et al.* (2004) have demonstrated that 2-year inhalation exposure to DMF produces hepatocellular adenomas and carcinomas in rats and mice and hepatoblastomas in mice. A broad range of *in vitro* and *in vivo* genotoxicity assays showed that genotoxicity of DMF is negative (IARC, 1999). Thus, a non-genotoxic-cytotoxic-proliferative mode of action may be hypothesized for the DMF-induced hepatocarcinogenesis, suggesting that hepatocellular death by toxic insult and subsequent regenerative proliferation of hepatocytes play a crucial role in DMF-induced hepatocarcinogenesis (Butterworth *et al.*, 1992).

It is important to understand to what extent combined exposures of a general population to low levels of environmentally ubiquitous DMF through both inhalation and ingestion enhance hepatocarcinogenic risks. However, it is not yet known whether combined inhalation and oral exposures to DMF in multiple media affect carcinogenicity additively or in a greater than additive manner. Recently, we found that combined inhalation and oral exposures of male rats to DMF for 4 weeks enhanced the proliferative response of hepatocytes in a greater than additive manner (Ohbayashi *et al.*, 2008). The aim of this bioassay study was to provide dose-carcinogenic response data for assessing carcinogenic risks in humans exposed to the environmentally ubiquitous DMF present in multiple media through multiple exposure routes. For this purpose, we examined carcinogenicity by exposing male F344 rats to DMF for 104 weeks by a single route of inhalation in the vapor phase, by oral administration in drinking water or by combined inhalation and oral exposures.

MATERIALS AND METHODS

Chemicals

Reagent-grade DMF (> 99.5%) obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) was used in this study. Gas chromatographic analysis showed that no peaks corresponding to impurities were detected in air containing 200 or 400 ppm (v/v) DMF vapor or in DMF-formulated drinking water containing 800 or 1,600 ppm (w/w).

Animals

Four-week-old, male F344/DuCrlCrj rats (SPF) were purchased from Charles River Japan, Inc. (Atsugi, Japan). Male rats were chosen in this study, since it was found in our 2-year inhalation study (Senoh *et al.*, 2004) that male rats were more sensitive to the DMF-induced hepatocarcinogenicity than female rats. The experiment was started when the rats were 6 weeks old, after 2-week quarantine and acclimation. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996), and this study was approved by the ethics committee of Japan Bioassay Research Center.

Exposure to DMF

The rats were housed individually in stainless steel wire hanging cages in an inhalation exposure chamber maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 15%, with 12 air changes/hr. The inner volume of the exposure chamber was 4.3 m^3. Nine inhalation exposure chambers were installed in two barrier system animal rooms. Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. The rats had free access to a γ-irradiation-sterilized commercial pellet diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan).

The techniques for generating the DMF vapor-air mixture and the system for the combined inhalation and oral exposures to DMF are described in detail in a previous paper (Kano *et al.*, 2002). The study design for the combined exposures and the single-route exposure to DMF through either inhalation or ingestion is presented in Table 1. A group of 50 rats was exposed by inhalation to 200 or 400 ppm (v/v) of DMF vapor-containing air for 6 hr/day and 5 days/week for 104 weeks, and each inhalation group was given DMF-formulated drinking water at 800 or 1,600 ppm (w/w) or vehicle drinking water *ad libitum* for 24 hr/day and 7 days/week, during a 104-week period. A group of 50 rats exposed to clean air together with oral administration of the vehicle drinking water served as an untreated control (Inh-0+Orl-0 ppm group). In addition to the untreated control group, therefore, the following eight different combinations of exposures to DMF were employed in this study: Four combined-exposure groups consisted of inhalation exposure to 200 ppm with oral administration of 800 ppm drinking water (Inh-200+Orl-800 ppm), Inh-200+Orl-1,600 ppm, Inh-400+Orl-800 ppm and Inh-400+Orl-1,600 ppm. Two oral-alone groups consisting of Inh-0+Orl-800 ppm and Inh-0+Orl-1,600 ppm received oral administration of 800

Table 1. An experimental design and time-averaged concentrations of DMF in the inhalation chamber and in the DMF-formulated drinking water

Group Name	Target concentration		Observed concentration (mean ± S.D.)	
	Inhalation (ppm(v/v))	Drinking water (ppm(w/w))	Inhalation (ppm(v/v))	Drinking water (ppm(w/w))
Inh-0+Orl-0 ppm	0	0	0	-
Inh-0+Orl-800 ppm	0	800	0	796 ± 22
Inh-0+Orl-1,600 ppm	0	1,600	0	1,604 ± 46
Inh-200+Orl-0 ppm	200	0	201.3 ± 2.0	-
Inh-200+Orl-800 ppm	200	800	201.8 ± 2.9	800 ± 22
Inh-200+Orl-1,600 ppm	200	1,600	199.7 ± 1.7	1,620 ± 35
Inh-400+Orl-0 ppm	400	0	400.0 ± 3.3	-
Inh-400+Orl-800 ppm	400	800	401.3 ± 2.8	805 ± 24
Inh-400+Orl-1,600 ppm	400	1,600	400.7 ± 2.9	1,616 ± 36

and 1,600 ppm DMF-formulated water without inhalation exposure, respectively. Two inhalation-alone groups consisting of Inh-200+Orl-0 ppm and Inh-400+Orl-0 ppm were exposed by inhalation to 200 and 400 ppm DMF vapor, respectively. Means ± SDs of the observed concentrations of DMF in the inhalation exposure chamber and in the drinking water are also given in Table 1. The selection of dose levels of DMF in inhalation exposure and drinking water was based on our previous findings. First, 2-year inhalation exposure to 400 ppm induced hepatocellular adenomas without hepatocellular carcinomas, while the low concentration of 200 ppm did not increase the incidence of hepatocellular tumors (Senoh *et al.*, 2004). Second, oral administration of 1,600 ppm-formulated drinking water for 4 weeks induced a significant increase in proliferation index of proliferating cell nuclear antigen (PCNA)-positive hepatocytes, while the water concentration of 800 ppm did not increase the number of PCNA-positive hepatocytes. In addition, 4-week combined exposures to 400 ppm in inhalation and 1,600 ppm in water significantly increased the proliferation index of PCNA-positive hepatocytes (Ohbayashi *et al.*, 2008).

Clinical observations, analysis and pathologic examinations

The rats were observed daily for clinical signs and mortality. Body weights and consumption of food and water were measured weekly for the first 14 weeks, every 4 weeks thereafter and at the final 104th week. All rats that died or were killed in a moribund state during the 2-year period, and survived to the end of the 2-year period received complete necropsy. All organs and tissues indicated in the OECD test guideline (OECD, 1981) and the entire respiratory tract including nasal cavity, pharynx and larynx were examined for histopathology in all rats. The organs and tissues for histopathological examination were fixed in 10% neutral buffered formalin and embedded in paraffin. All organs and tissues were cut into 5 μm-thick sections and stained with hematoxylin and eosin (H & E).

Statistical analysis

Statistical comparison was performed between the following groups; a) each DMF-treated group vs. untreated control group; b) each of the two combined-exposure groups vs. each of the oral-alone group with matching concentration; and c) each of the two combined-exposure groups vs. each of the inhalation-alone groups with matching concentrations. We used Dunnett's test for body weights and chi-square test for incidences of neoplastic lesions and survival rates. Two-tailed test was used for all statistics, and in all cases, a P-value of 0.05 was used as the level of significance.

RESULTS

Survival, body weight, clinical and macroscopic observations

There was no statistically significant difference in the survival rate at any time point between the untreated control group and the two inhalation-alone groups, the two oral-alone groups or the four combined-exposure groups (Table 2). The primary cause of deaths was diagnosed on

Table 2. Survival rate, body weight, daily water consumption and estimated amounts of DMF Uptake

Group Name	Initial animal No.	No. of surviving animals week after administration						Terminal body weight (g) (mean ± S.D.)	Time-averaged water consumption (g/day/rat) (mean ± S.D.)	Estimated amount of DMF uptake (mg/kg/day)		
		54	74	84	94	99	104			Inhalation	Drinking water	Total
Inh-0+Orl-0 ppm	50	50	49	47	45	43	41	410 ± 67	19.4 ± 3.1	0	0	0
Inh-0+Orl-800 ppm	50	50	46	43	40	40	34	369 ± 41 ^{a}	19.0 ± 2.5	0	44	44
Inh-0+Orl-1,600 ppm	50	50	48	47	43	43	40	346 ± 44 ^{a}	17.1 ± 1.8	0	82	82
Inh-200+Orl-0 ppm	50	50	47	44	40	39	36	378 ± 26	19.3 ± 1.8	121	0	121
Inh-200+Orl-800 ppm	50	49	45	43	36	36	36	359 ± 18 ^{a,c}	17.4 ± 1.1	121	44	165
Inh-200+Orl-1,600 ppm	50	49	49	46	42	42	41	333 ± 29 ^{a,c}	15.9 ± 0.6	121	84	205
Inh-400+Orl-0 ppm	50	49	48	46	38	38	37	324 ± 41 ^{a}	17.1 ± 1.4	242	0	242
Inh-400+Orl-800 ppm	50	50	50	49	46	45	43	313 ± 20 ^{a,b,c}	16.1 ± 1.5	242	47	289
Inh-400+Orl-1,600 ppm	50	50	47	47	43	41	38	290 ± 29 ^{a,b,c}	15.8 ± 1.4	242	96	338

Symbols *a*, *b* and *c*: significantly different from the untreated control group, and each of the oral-alone and inhalation-alone groups with matching concentrations, respectively, at $p < 0.05$ by Dunnett's test.

the basis of the macroscopic and microscopic findings, and the number of animals that died of liver tumors was 2/50 cases for the Inh-200+Orl-1,600 ppm, 1/50 for the Inh-400+Orl-0 ppm, 4/50 for the Inh-400+Orl-800 ppm and 4/50 for the Inh-400+Orl-1,600 ppm (Table 3). This result revealed that the incidence of liver tumor deaths tended to increase in a dose-related manner, although those increased incidences were not statistically significant. The terminal body weight was significantly decreased in the inhalation-alone group (Inh-400+Orl-0 ppm), both oral-alone groups and all four combined-exposure groups, compared with the untreated control group (Table 2). Water consumption was clearly decreased in the Inh-0+Orl-1,600 ppm group, in the Inh-400+Orl-0 ppm group and in all four combined-exposure groups, compared with the untreated control group or the inhalation-alone groups with matching concentrations (Table 2). No overt clinical sign was observed in any group of DMF-treated rats throughout the 104-week period.

Estimated amounts of DMF uptake into the body are given in Table 2. The uptake of DMF through inhalation was calculated on the basis of the respiratory volume per body weight and the chamber concentration of DMF, assuming 561 ml/min for minute volume per kg body weight (Mauderly *et al.*, 1979) and the lung absorption ratio of DMF as 100% (adopted because of no data available from the literature). For instance, the estimated amount of DMF uptake for 6-hr inhalation exposure to 200 ppm resulted in 121 mg/kg/day by the following calculation (561 ml/min/kg body weight x 60 min x 6 hr/day x 600 mg/m^3 (200 ppm)). Amount of DMF uptake by the oral route was estimated by the following calculation (the daily amount of drinking water consumption x the observed water concentration of DMF/body weight x the gastrointestinal absorption ratio as 100% (adopted because of no data available from the literature)). These three parameters were obtained on a weekly basis, and the weekly multiplied values were averaged over 104 weeks, e.g., the estimated amount of DMF uptake by oral administration of 800 ppm-formulated drinking water resulted in 44 mg/kg/day. Thus, the total amount of DMF uptake in the Inh-200+Orl-800 ppm group was estimated to be 165 mg/kg/day.

Hepatocellular tumors

Table 3 shows that incidences of hepatocellular adenomas and carcinomas and their combined incidences were significantly increased in the four combined-exposure groups, the two inhalation-alone groups and the two oral-alone groups, as compared with the untreated control group and each of the oral-alone and inhalation-alone groups with matching concentrations. As a whole, those incidences of DMF-induced hepatocellular tumors were increased with an increase in total estimated amounts of DMF uptake. It should be noted, however, that the incidences of hepatocellular tumors induced by the combined-exposures tended to exceed the dose-response relation that would be expected under the assumption that the two incidences of hepatocellular tumors induced by the single-route exposures through inhalation and ingestion are additive. As an example, the incidences of hepatocellular adenomas and carcinomas in the two combined-exposure groups of Inh-200+Orl-800 ppm (total estimated uptake of 165 mg/kg/day) and Inh-200+Orl-1,600 ppm (205 mg/kg/day) were greater than those in the single-route exposure group of Inh-400+Orl-0 ppm (242 mg/kg/day), although the total estimated uptakes in the former two groups were less than that in the latter group. Furthermore, the combined exposures were found to produce multiple occurrences of hepatocellular adenomas as compared with the single-route exposures (data not shown).

The incidences of hepatocellular tumors in the combined-exposure groups were compared with the sum of the incidences of hepatocellular tumors in the single-route exposure groups through inhalation and ingestion (Fig. 1) under the same concept as "response additivity" applied to the effects of chemical mixture by the U.S. EPA (2000). The incidences of hepatocellular adenomas in the four combined-exposure groups were greater than the sum of the incidences of hepatocellular adenomas in the single-route exposure groups through inhalation and ingestion (Fig. 1A). Hepatocellular carcinomas induced by single-route exposure through either inhalation or ingestion occurred at low incidences: 1/50 case (2%) for the Inh-200+Orl-0 ppm group, 2/50 cases (4%) for the Inh-400+Orl-0 ppm group and 4/50 cases (8%) for the Inh-0+Orl-1,600 ppm group. As compared with the low incidences of hepatocellular carcinomas induced by the single-route exposures, however, the combined exposures were found to produce significantly increased incidences of hepatocellular carcinomas: 6/50 cases (12%) for the Inh-200+Orl-800 ppm, 14/50 cases (28%) for the Inh-200+Orl-1,600 ppm, 12/50 cases (24%) for the Inh-400+Orl-800 ppm, and 14/50 cases (28%) for the Inh-400+Orl-1,600 ppm. Notably, the incidences of hepatocellular carcinomas in the four combined-exposure groups were greater than the sum of the two incidences of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1B). Comparison of Fig. 1B with Fig. 1A reveals that the increased incidences of hepatocellular carcinomas that are more malignant than adenomas in the combined-exposure groups

Table 3. Number of male rats bearing hepatocellular tumors following combined inhalation and oral exposures or single-route exposures to DMF

Inhalation (ppm)	0			200			400		
Drinking water (ppm)	0	800	1,600	0	800	1,600	0	800	1,600
Total estimated amount of DMF uptake (mg/kg/day)	(0)	(44)	(82)	(121)	(165)	(205)	(242)	(289)	(338)
Number of animals examined	50	50	50	50	50	50	50	50	50
Number of animals dead or found in a moribund state	9	16	10	14	14	9	13	7	12
Hepatocellular adenoma	1	6 [a]	8 [a]	15 [a]	28 [a,b,c]	45 [a,b,c]	26 [a]	43 [a,b,c]	46 [a,b,c]
	(0)	(2)	(2)	(2)	(1)	(4)	(3)	(3)	(9)
Hepatocellular carcinoma	0	0	4 [a]	1	6 [a,b,c]	14 [a,b,c]	2	12 [a,b,c]	14 [a,b,c]
	(0)	(0)	(0)	(0)	(0)	(1)	(0)	(1)	(2)
Hepatocellular adenoma + carcinoma	1	6 [a]	12 [a]	16 [a]	30 [a,b,c]	46 [a,b,c]	26 [a]	45 [a,b,c]	47 [a,b,c]
	(0)	(2)	(2)	(2)	(1)	(5)	(3)	(4)	(9)
Poorly differentiated, hepatocellular carcinoma	0	0	1	0	5 [a,b,c]	5 [a,c]	2	9 [a,b,c]	9 [a,b,c]
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(2)
Number of animals died of liver tumors	0	0	0	0	0	2	1	4	4

Symbols *a*, *b* and *c*: significantly different from the untreated control group, the each oral-alone group and each inhalation-alone group with matching concentrations, respectively, at $p < 0.05$ by chi-square test.
Parenthesized values indicate number of male rats dead and found in a moribund state, bearing hepatocellular tumors on the basis of histopathological examination.
Number of animals died of liver tumors was based on the primary cause of deaths diagnosed on the basis of macroscopic and microscopic findings.

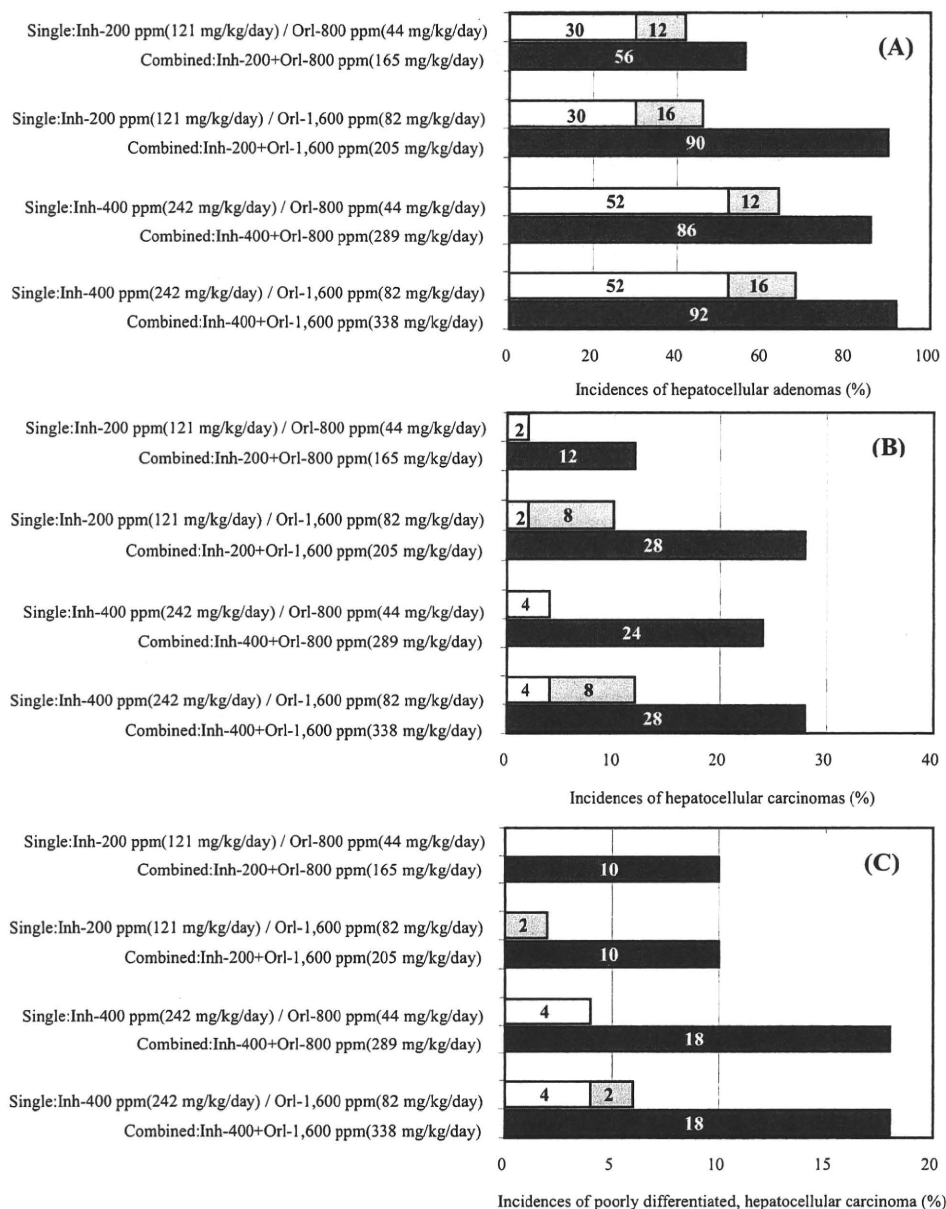

Fig. 1. Comparison of incidences of hepatocellular tumors ((A): hepatocellular adenomas, (B): hepatocellular carcinomas and (C): poorly differentiated hepatocellular carcinoma) in the combined-exposure groups (solid bars) with sum of the compornent incidences of the hepatocellular tumor in the single-route exposure groups through inhalation (open bars) and ingestion (shaded bars). The values in parenthesis indicate the total estimated amount of DMF uptake (mg/kg/day). The values in the bars represent the incidences of (A) hepatocellular adenomas, (B) hepatocellular carcinomas and (C) poorly differentiated, hepatocellular carcinoma.

were more remarkable than those of hepatocellular adenomas that are benign.

DMF-induced hepatocellular carcinomas can be classified into two different types according to the histopathological characteristics described by Senoh *et al.* (2004). The first type of hepatocellular carcinomas is prima-

rily composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei, which are similar to normal hepatocytes in histological appearance (Fig. 2A). The second type of hepatocellular carcinomas is composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei (Fig. 2B). The second type is considered to be more atypical and poorly differentiated, hepatocellular carcinomas, since the appearance of the second type deviates to a greater extent from that of normal hepatocytes than does the first type. Morphological characteristics of the second type became more malignant along the tumor-developmental sequence than those of the first type. The first type of hepatocellular carcinomas was found in all DMF-treated groups including the oral-alone, inhalation-alone and combined-exposure groups. The second type of hepatocellular carcinomas found in single-route exposure groups occurred at low incidences: 2/50 cases (4%) for the Inh-400+Orl-0 ppm group and 1/50 case (2%) for the Inh-0+Orl-1,600 ppm group. However, the combined exposures were found to markedly increase the incidence of the second type of hepatocellular carcinomas: 5/50 cases (10%) for the Inh-200+Orl-800 ppm, 5/50 cases (10%) for the Inh-200+Orl-1,600 ppm, 9/50 cases (18%) for the Inh-400+Orl-800 ppm, and 9/50 cases (18%) for the Inh-400+Orl-1,600 ppm. Notably, the inci-

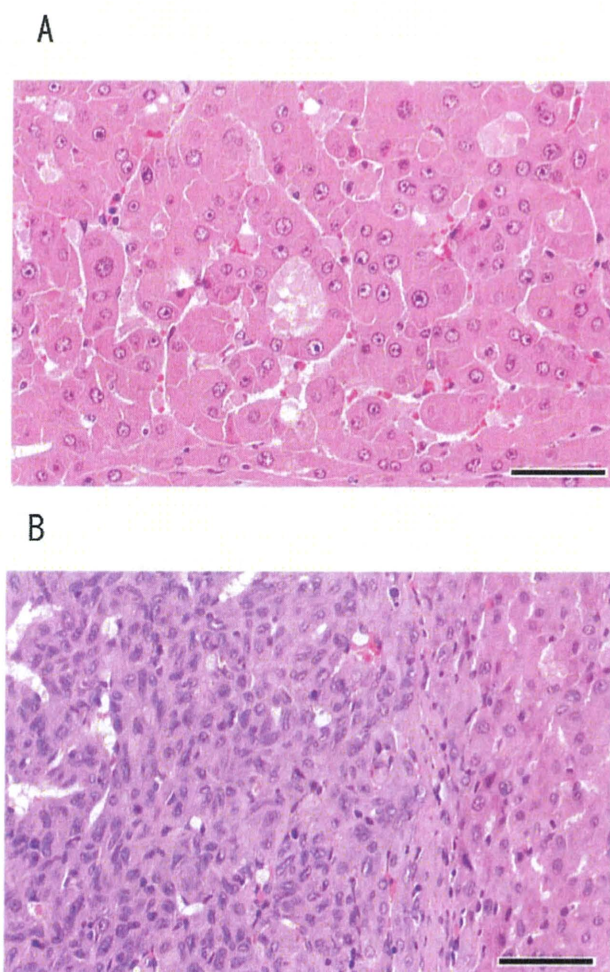

Fig. 2. (A): Hepatocellular carcinomas composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei in a male rat in the Inh-0+Orl-1,600 ppm group. (B): Poorly differentiated, hepatocellular carcinomas composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei in a male rat in the Inh-400+Orl-1,600 ppm group. H & E stain. Bar indicates 100 μm.

dences of the second type of hepatocellular carcinoma in the four combined-exposure groups were markedly greater than the sum of the two incidences of the second type of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the combined inhalation and oral exposures to DMF were found to markedly enhance not only the incidences of hepatocellular adenomas and carcinomas but also their tumor malignancy that might progress from hepatocellular adenomas through carcinomas to poorly differentiated, hepatocellular carcinomas.

It was noteworthy that the DMF-induced hepatocellular carcinoma did not metastasize to any other organs.

DISCUSSION

IARC's overall evaluation that DMF is not classifiable as to its carcinogenicity to humans (Group 3; IARC, 1999) was partly based on experimental evidence indicating lack of carcinogenicity after 2-year inhalation exposure of Crl:CD rats and CD-1 mice to DMF at an inhalation concentration of 25, 100 or 400 ppm (Malley *et al.*, 1994). More recently, however, our carcinogenicity study (Senoh *et al.*, 2004) has demonstrated that 2-year inhalation exposure to DMF vapor at 200, 400 and 800 ppm produces hepatocellular adenomas and carcinomas in F344 rats and BDF_1 mice of both sexes, although the exposure to 200 and 400 ppm primarily elicited benign hepatocellular adenomas. Senoh *et al.* (2004) suggested that the difference in carcinogenicity between Senoh *et al.*'s study and Malley *et al.*'s study was attributed to the strain of rats used. The present study confirms our previous findings, and extends those to the induction of hepatocellular tumors by oral administration of DMF in drinking water. Notably, the present study demonstrated that the combined inhalation and oral exposures of male F344 rats to DMF for 104 weeks markedly increase the incidences of hepatocellular tumors and their malignancy, as compared with the single-route exposures through inhalation and ingestion.

The extent to which the combined exposures markedly enhance the incidences of hepatocellular tumors is characterized by the hepatocarcinogenic effect being greater than the sum of the two hepatocarcinogenic effects of the single-route exposures through inhalation and ingestion. The "greater than additive" effect of the combined exposures on hepatocellular tumors tended to be accompanied by an increase in the malignancy of hepatocellular tumors. That is, the incidences of benign hepatocellular adenomas in the four combined-exposure groups were increased only by 1.3- to 2.0-fold over the sum of the two incidences of hepatocellular adenomas in the single-route exposure groups through inhalation and ingestion (Fig. 1A). The incidences of hepatocellular carcinomas in the four combined-exposure groups were increased by 2.3- to 6.0-fold over the sum of the two incidences of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1B). Furthermore, the combined exposures enhanced by much greater folds the incidences of poorly differentiated, hepatocellular carcinomas, which are more malignant than the commonly observed hepatocellular carcinoma, over the sum of the two incidences of poorly differentiated, hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the present findings suggest that the combined inhalation and oral exposures to DMF enhances the incidence of malignant hepatocellular tumors in a greater than additive manner, when we defined the additivity of carcinogenic responses as the same concept of "response additivity" applied to the effects of chemical mixture by the U.S. EPA (2000).

The characteristic relationship of total doses versus carcinogenic responses expressed as the incidences of hepatocellular tumors can be found in the two combined-exposure groups of Inh-200+Orl-800 ppm and Inh-200+Orl-1,600 ppm, both of which had total estimated DMF uptakes of 165 and 205 mg/kg/day, respectively. It is noteworthy that the incidences of hepatocellular carcinomas were greater in these two combined-exposure groups than in the Inh-400+Orl-0 ppm group having the total estimated DMF uptake of 242 mg/kg/day, although the former two combined-exposure groups had lesser uptakes than the latter single-route exposure group did. This finding also indicates that the hepatocarcinogenic effect induced by the combined inhalation and oral exposures would be enhanced in a greater than additive manner as expected from sum of the two effects induced by the single-route exposure through inhalation and ingestion.

The present carcinogenic effect of the combined exposures is in sharp contrast to that found in an inhalation study by Senoh *et al.* (2004) who reported 13/50 cases of hepatocellular adenomas with null case of hepatocellular carcinomas following 2-year inhalation exposure of male F344 rats to 400 ppm DMF vapor, which corresponded to an estimated DMF uptake of 242 mg/kg/day. Comparison of the estimated DMF dose-carcinogenic response relationships between the present combined-exposure study and Senoh *et al.*'s inhalation study (2004) also indicates that the combined inhalation and oral exposures to DMF produce greater incidences of hepatocellular tumors with higher malignancy than the inhalation-alone exposure

does.

We have not yet obtained any experimental evidence to elucidate the markedly enhanced hepatocarcinogenic effect of the combined inhalation and oral exposures to DMF. However, a clue to understanding the intriguing carcinogenic responses by the combined exposures can be seen in the finding in our previous study (Ohbayashi *et al.*, 2008) that the combined inhalation and oral exposures of male rats to DMF for 4 weeks markedly enhanced the proliferation index expressed as the percentage of PCNA-positive hepatocytes in a greater than additive manner as compared with the sum of the two proliferation indices by the single-route exposures through inhalation and ingestion. Since a broad range of *in vitro* and *in vivo* genotoxicity assays showed negative genotoxicity responses to DMF (IARC, 1999), a nongenotoxic-cytotoxic-proliferative mode of action can be hypothesized to operate for the DMF-induced hepatocarcinogenesis. This hypothesis suggests that hepatocellular death by the toxic insult of DMF and/or its active metabolites and the subsequently increased regenerative proliferation of hepatocytes play a crucial role in DMF-induced hepatocarcinogenesis (Butterworth *et al.*, 1992). Since both *N*-methylformamide (NMF), which was metabolized from DMF through *N*-(hydroxymethyl)-*N*-methylformamide and methyl isocyanate, which was possibly biotransformed from NMF were reported to be the most potent hepatotoxicants (Kestell *et al.*, 1987; Gescher, 1993; Mráz *et al.*, 1989, 1993), these two metabolites might cause severe hepatocellular damage that would result in enhanced cell proliferation in DMF-induced hepatocarcinogenesis. Further quantitative investigations such as physiological based pharmacokinetic modeling in consideration of the hepatic levels of DMF and its toxic metabolites are needed to examine greater than additive effects of the combined exposures to DMF on the hepatocellular tumors.

In conclusion, the present study demonstrated that the combined exposures of male rats to DMF at approximately similar dose levels each through inhalation and ingestion enhance induction of hepatocellular tumors and their malignancy in a greater than additive manner (i.e., possibly synergistic). In addition to the reported nongenotoxicity of DMF suggesting the presence of a threshold level for the tumor induction, sufficient consideration should be paid to delineation of the relationship between the dose levels given through the multiple routes and carcinogenic responses, in order to estimate quantitatively carcinogenic risks of humans exposed to an environmentally ubiquitous carcinogen such as DMF. Indeed, combined exposures to DMF at environmentally relevant levels is anticipated to occur for community residents living near a neighboring factory where DMF is used (Amster *et al.*, 1983) and/or public water contaminated with DMF, although a general population may be exposed to DMF at far lower levels through inhalation and ingestion.

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Original Article

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

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

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

INTRODUCTION

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

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