The Effects of *in Utero* Exposure to a Migrant, 4,4′-Butylidenebis(6-*t*-butyl-*m*-cresol), from Nitrile-Butadiene Rubber Gloves on Monoamine Neurotransmitter in Rats

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4,4′-Butylidenebis(6-*t*-butyl-*m*-cresol) (BBBC) can be eluted from disposable gloves made of nitrile-butadiene rubber and possibly also detected in food. We have reported that BBBC is an androgen and estrogen antagonist. In this report, BBBC (0.1, 1.0 mg/kg body weight (bw)/day) was subcutaneously administered to pregnant rats from gestation days 11 through 18 and the effects on male offspring (postnatal day (PND) 102) were examined. Body weight at lactation and brain weight at PND 102 were decreased in the 1.0 mg/kg bw BBBC-treated group. Altered levels and turnover of the monoamines dopamine (DA), serotonin (5-HT), and noradrenalin (NA) as well as their metabolites were detected. In the prefrontal cortex DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA) levels were significantly increased, but homovanillic acid (HVA)/DA was decreased. In the striatum NA level, DOPAC/DA and HVA/DA were significantly increased, but 3-methoxy-4-hydrophenylglycol hemipiperazinium (MHPG) level and MHPG/NA were decreased. In hippocampus MHPG level was significantly decreased. In hypothalamus 5-HIAA level and 5-HIAA/5-HT were significantly increased. These results suggested that prenatal exposure to BBBC affects the central nervous system of male rat offspring, and BBBC may be an endocrine disrupting-chemical during the fetal periods.

Key words 4,4′-butylidenebis(6-*t*-butyl-*m*-cresol); central nerve; endocrine disruptor; dopamine; serotonin; monoamine metabolite

Disposable nitrile-butadiene rubber (NBR) gloves are now widely used for food-contact uses such as cooking and serving in Japan, having replaced polyvinyl chloride (PVC) gloves. Recently in Japan, disposable gloves made of PVC were prohibited for food-contact because di(2-ethylhexyl)-phthalate (DEHP), a widely used plasticizer, was detected in packed lunches for sale at convenience stores and in-patients' meals in hospitals.[1,2] Mutsuga *et al.*[3,4] isolated and identified chemicals eluted from NBR gloves including 4,4′-butylidenebis(6-*t*-butyl-*m*-cresol) (BBBC, CAS No. 85-60-9, Fig. 1) *etc.*, and reported that the amount of BBBC eluted from NBR gloves was 4.22 μg/cm^2. Disposable gloves used during cooking or packaging are sprayed with 68% (w/w) ethanol for disinfection. Alcohol sprayed onto disposable gloves increased the level of chemicals eluted in foods 8—41 times (possibility 33.8—173.0 μg/cm^2).[2]

Endocrine-disrupting effects are suspected for phthalate diesters (including DEHP) and phthalic acid monoesters, a metabolite of phthalic acid,[5] and we have studied the *in vitro* androgenic and estrogenic effects of phthalate diesters and phthalic acid mono esters. However, hormonal effects of DEHP and mono(2-ethylhexyl)phthalate, a mono ester of DEHP, were not observed[6] or were very weak endocrine-disrupting chemicals.[7] Furthermore, we examined the endocrine-disrupting effects of chemicals eluted from NBR gloves using *in vitro* assays, and reported that BBBC has the strongest androgen (AR) antagonist and estrogen (ER) antagonist activities (in the 10^{-6} M range of IC_{50} values).[8] The strength of these activities was about 5 times higher than that of a known AR antagonist, 1,1′-(2,2dichloroethylidene)bis[4-chlorobenzene] (*p,p′*-DDE), and similar to that of bisphenol A (BPA), which has strong AR antagonist activity and ER agonist activity.[9,10] We have examined the hormonal effects using *in vivo* assays[11,12] some of the chemical that became strong positive using *in vitro* assays.[6,8—10,13—16] For example, styrene trimers had antiandrogenic-like and estrogenic-like activity *in vitro*,[14] and effects of the chemicals on the offspring of pregnant rats included changes in the relative weights of the brain, and levels of several serum hormones, *etc.*[12] Prenatal and neonatal exposure to BPA in mice changed the expression of central dopamine (DA) receptors.[17,18] Furthermore, exposure to BPA during embryonic/fetal life and infancy decreased the brain weight[19] and Kabuto and Shishibori[20] reported that that exposure to BPA, which is suspected to have an endocrine disrupting-effect, during the fetal to infantile periods resulted in low brain weights in mice and promotion of monoamine metabolism in the brain.

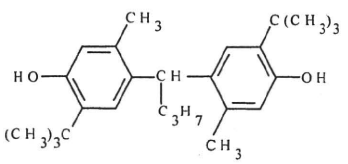

Fig. 1. The Structure of 4,4′-Butylidenebis(6-*t*-butyl-*m*-cresol)

Endocrine disruptors (EDs) show some adverse effects on development and/or function of the reproductive system and the nervous system, particularly when exposure occurs in fetus or neonates. Evidence indicates that certain behaviors are exquisitely responsive to ED exposure during gestation, and that one index of susceptibility may be reflected in the

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differing responses of male and female offspring, a phenomenon termed sexual dimorphism.[21] Weiss introduces the possibility that current tissue stores of EDs in the bodies of women may present serious risks to the optimal development of their children's brains. When the effects of BBBC on the offspring of pregnant rats were examined, the weights of rat brains exposed to 1.0 mg/kg body weight (bw)/day BBBC were significantly decreased, but the change in the brain weight of 0.1 mg/kg bw BBBC-treated rats was not admitted.[8,22] In this report, we studied the effects of prenatal exposure to BBBC (1.0 mg/kg bw/day) in male rats similarly to our previous experiment, and measured levels of the monoamines DA, serotonin (5-HT), and noradrenalin (NA) as well as their metabolites in the brains of exposed rats to clarify the influence of BBBC on the central nervous system.

MATERIALS AND METHODS

Reagents BBBC was purchased from Tokyo Kasei Industry, Co., Ltd. (Tokyo, Japan, purity: >97% by HPLC).[8] Isoproterenol (ISO), DA, 5-HT, homovanillic acid (HVA), 3-methoxytyramine (3-MT), normetanephrin (NM), 3-methoxy-4-hydrophenylglycol hemipiperazinium (MHPG), and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). NA and adrenaline (Ad) were purchased from Nakalai Tesque Inc. (Kyoto, Japan) and 3,4-dihydroxyphenylacetic acid (DOPAC) was from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Methanol was used for HPLC (Kanto Kagaku, Tokyo, Japan). Other reagents used in the study were of the highest grade commercially available.

Animals and Experimental Protocol Pregnant specific pathogen-free Sprague-Dawley rats (9 weeks old, $n=24$) purchased from Charles River Japan (Kanagawa, Japan) were received on day 7 of gestation, and individually housed with woodchip bedding (Clea-chip, CLEA Japan Ins., Tokyo, Japan), and maintained under controlled conditions at 22—24 °C, 45—65% humidity, 12-h lighting, and 10 ventilations per hour. The animals were given free access to general food (CE-2, Clea Japan Inc., Tokyo, Japan) and water. Observation of the general condition and measurements of body weight were performed daily. All animal studies were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Experimental protocol was determined as described.[12,22] After a housing acclimatization period of 4 d, rats were divided into three groups. BBBC was dissolved in ethanol, and this solution was diluted with saline (1% ethanol solution). BBBC ($n=8$, 0.1, 1.0 mg/kg bw/day saline solution at a volume of 1.0 ml/100 g bw; respectively) or 1% ethanol–saline solution at a volume of 1.0 ml/100 g bw ($n=8$, control) was subcutaneously administered to pregnant rats once a day for 8 d from gestation days 11 through 18 (during which monoamine nervous systems develop and mature). Four male rat offsprings born from one mother were selected at random and kept with the litters until weaning (0.1, 1.0 mg/kg bw BBBC-treated rats $n=32$, respectively; control rats $n=32$). Male rat offspring were individually housed in stainless cages from postnatal day (PND) 25 (day of birth=PND 0). The body weight of male rat offspring was recorded at frequent intervals until the terminal day. Male rat offspring were sacrificed on PND 102 (after maturation) as described[12] under ether anesthesia, and brains were removed, weighed, and stored at −80 °C until measurement. There were no differences in liver and kidney weight, and the histological change between BBBC-treated rats and control rats. The difference of serum levels at aspartate aminotransferase, alanine aminotransferase, cholesterol, triglyceride, and glucose *etc.* was not admitted.

Preparation of Homogenates The homogenate of brains of the 1.0 mg/kg bw BBBC-treated group with significantly light weight was prepared. Brain regions including the prefrontal cortex, striatum, hippocampus, hypothalamus, and amygdale, were dissected out from frozen brains with a brain blocker. Dissected tissues were frozen immediately in liquid nitrogen and stored at −80 °C until used. These tissues were homogenized in 0.2 M perchloric acid (Nacalai Tesque, Kyoto, Japan) containing 100 mM EDTA·2Na and 1 μl ISO (100 ng/μl) as an internal standard. Homogenates were centrifuged for 15 min at 0 °C and 20000×g. The pH of each supernatant was adjusted to 3.0 with 1 M sodium acetate and supernatants were then used to determine monoamine levels. The protein concentration of each precipitate was determined with an Advanced Protein Assay (Cytoskeleton, Denver, CO, U.S.A.).

Quantification of Monoamine Levels by HPLC Each sample (10 μl) was injected into an HPLC system (HTEC-500) with electrochemical detection (ECD-300) (EICOM, Kyoto, Japan) with an ODS column (EICOMPAC SC-5 ϕ3.0 mm×150 mm, EICOM Inc., Kyoto, Japan). The mobile phase was sodium acetate–citric acetate buffer (pH 3.5)/methanol/sodium octane-1-sulfonate (85%/15%/0.21 g), the flow rate was maintained at 0.5 ml/min, and the temperature of the column was 25 °C. Tissue levels of DA, 5-HT, and NA as well as their metabolites (DOPAC, 3-MT and HVA as DA metabolites; 5-HIAA as a 5-HT metabolite; and MHPG, NM and Ad as NA metabolites) were measured. Each of the standard solutions for the 9 catecholamines (DA, NA, Ad, DOPAC, HVA, 3-MT, NM, MHPG and ISO) and 2 indoleamines (5-HT and 5-HIAA) was prepared at a concentration of 0.1 ng/μl. Each standard (10 μl) was analyzed by HPLC, and the standard chromatographic peaks per 1 ng for each sample were obtained.

The amount of each monoamine was determined with peak-area ratios using HPLC chromatogram analysis software, eDAQ Power Chrom (eDAQ, New South Wales, Australia). Values were normalized for the amount of protein in each sample. Moreover, metabolic turnover was calculated as the ratio of each metabolite to its specific monoamine to estimate the activity of each metabolic pathway.

Statistical Analysis Statistical analysis was performed using Excel 2003 (Microsoft, U.S.A.). Differences between groups were assessed for statistical significance by Student's t test (body and brain weights) and by Mann–Whitney's U test (monoamines and their metabolites levels), respectively. Differences with $p<0.05$ were considered significant. Data are expressed as means±S.D. (body and brain weights) and means±S.E.M. (monoamines and their metabolite levels).

RESULTS

Effects of BBBC-Treated on Pregnancy Rats There

was no difference on birth number per one mother. No effect on male/female ratio was found for BBBC. The treatments induced no maternal toxicity.

General Condition No abnormal behavior and appearance or diarrhea was noted at PND 102 in any of the control or BBBC treatment groups (0.1, 1.0 mg/kg bw), and all animals survived until completion of administration.

Body Weight The body weights were measured during the PND 2—102 period. Body weight at lactation decreased in the 1.0 mg/kg bw BBBC-treated group (data not shown). Significant decreases were observed at PND 2 (7.79±0.68 g, $p=0.025$, $n=32$) and PND 17 (42.44±3.10 g, $p=0.025$, $n=32$) compared with the corresponding control values at PND 2 (8.06±0.72 g, $n=32$) and PND 17 (44.48±3.39 g, $n=32$). Although the body weights in the 1.0 mg/kg bw BBBC-treated group at other PND were lower than control, the differences were not statistically significant. Significant differences in body weight after delactation were not observed. Body weights in the 0.1 mg/kg bw BBBC-treated group during the PND 2—102 were not changed.

Brain Weight Significant decreases ($p=0.01$) in brain weights from 2.25±0.07 g (control, $n=32$) to 2.19±0.05 g (exposed to 1.0 mg/kg bw BBBC, $n=32$) were observed in rat offspring prenatally exposed to BBBC (Table 1). However, the relative weights per 100 g bw were not changed (1.0 mg/kg bw BBBC-treated: 419±34 mg, control: 423±19 mg). Brain weights in the 0.1 mg/kg bw BBBC-treated group were not changed.

Monoamines and Their Metabolites Levels DA, DOPAC, 3-MT, and HVA levels as well as monoamine turnover were measured in the prefrontal cortex, striatum, hippocampus, hypothalamus, and amygdale regions of the 1.0 mg/kg bw BBBC-treated group (Table 2). All tables show means±S.E.M. and all figures indicate relative values in which each control value was assumed to be 100%. In the prefrontal cortex, DA (162%) and DOPAC (153%) levels were significantly increased in the BBBC-exposed group, but DA turnover (HVA/DA 52%) was significantly decreased. HVA level and DOPAC/DA in the prefrontal cortex showed no significant differences between the two groups. In the striatum, DOPAC/DA (118%) and HVA/DA (120%) were significantly increased, but DA, DOPAC, 3-MT, HVA, and 3-MT/DA showed no significant differences between the two groups. In the hippocampus, hypothalamus, and amygdala there were no significant differences between the two groups.

The 5-HT and 5-HIAA levels as well as 5-HT turnover were measured in the prefrontal cortex, striatum, hippocam-

Table 1. The Weight of Brain in Male Rat Offspring Prenatally Exposed to BBBC

Dose of BBBC (mg/kg bw/d)	$n^{a)}$	Body weight (g)	Brain weight (mg) (relative weight)$^{b)}$
0 (Control)	32	531.9±26.6	2248±73 (423±19)
0.1	32	529.9±41.6	2255±82 (429±31)
1.0	32	525.9±43.2	2190±53* (419±34)

BBBC (0.1, 1.0 mg/kg bw) was administered subcutaneously to pregnant SD rats as described in Materials and Methods. The control group received 1% ethanol in saline. *a*) Number of animals; *b*) absolute brain weight/100 g body weight. Each value is the mean±S.D. *$p<0.01$.

Table 3. Serotonin (5-HT) and 5-HT Metabolite Levels, and Values for 5-HT Turnover in Various Brain Regions

Brain region	Group	Content (pg/mg protein)		Turnover
		5-HT	5-HIAA	5-HIAA/5-HT
Prefrontal cortex	Control	68.1±3.9	72.2±4.2	1.08±0.05
	BBBC	84.0±5.7*	90.8±4.9**	1.11±0.05
Striatum	Control	125±11	158±12	1.28±0.04
	BBBC	130±11	169±11	1.36±0.06
Hippocampus	Control	30.9±2.6	63.0±4.6	2.11±0.11
	BBBC	32.6±3.8	68.6±6.9	2.15±0.09
Hypothalamus	Control	148±6	145±7	0.984±0.023
	BBBC	137±4	162±9*	1.20±0.07**
Amygdala	Control	83.7±6.6	68.4±5.8	0.817±0.028
	BBBC	69.8±5.1	60.4±4.3	0.878±0.035

BBBC (1.0 mg/kg bw) was administered subcutaneously to pregnant SD rats as described in Materials and Methods. The control group received 1% ethanol in saline. Brain regions for analysis were dissected out of 102-d-old male offspring. 5-HT and its metabolites were measured by HPLC-ECD. Moreover, metabolic turnover was calculated as the ratio of metabolite to monoamine. Data are represented as mean±S.E.M. *$p<0.05$, **$p<0.01$, Mann–Whitney's *U* test. In control group, $n=16$ for prefrontal cortex, hippocampus, hypothalamus and amygdala, $n=14$ for striatum. In BBBC group, $n=16$ for each region. 5-HIAA, 5-hydroxyindole-3-acetic acid.

Table 2. Dopamine (DA) and DA Metabolite Levels, and Value for DA Turnover in Various Brain Regions

Brain region	Group	Content (pg/mg protein)				Turnover		
		DA	DOPAC	3-MT	HVA	DOPAC/DA	3-MT/DA	HVA/DA
Prefrontal cortex	Control	106±14	25.9±3.8	N.D.	56.5±7.0	0.253±0.029		0.612±0.090
	BBBC	172±37*	39.5±4.4*	N.D.	45.5±6.4	0.275±0.030		0.317±0.048***
Striatum	Control	6430±350	707±50	220±12	248±20	0.113±0.004	0.036±0.001	0.040±0.002
	BBBC	5650±320	740±36	207±11	263±12	0.133±0.005**	0.037±0.001	0.048±0.002*
Hippocampus	Control	38.4±3.6	21.5±1.6	N.D.	24.1±2.5	0.581±0.027		0.670±0.071
	BBBC	41.0±3.7	24.0±2.9	N.D.	22.2±3.0	0.578±0.032		0.540±0.057
Hypothalamus	Control	246±14	39.0±2.6	N.D.	67.5±7.1	0.160±0.007		0.277±0.025
	BBBC	246±16	42.2±4.7	N.D.	75.0±7.9	0.167±0.015		0.304±0.023
Amygdala	Control	222±26	31.3±3.5	N.D.	43.2±5.8	0.146±0.007		0.223±0.035
	BBBC	191±24	27.3±2.8	N.D.	30.4±4.1	0.155±0.012		0.197±0.033

BBBC (1.0 mg/kg bw) was administered subcutaneously to pregnant SD rats as described in Materials and Methods. The control group received 1% ethanol in saline. Brain regions for analysis were dissected out of 102-d-old male offspring. DA and its metabolites were measured by HPLC-ECD. Moreover, metabolic turnover was calculated as the ratio of metabolite to monoamine to estimate the activity of each metabolic pathway. Data are reported as mean±S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Mann–Whitney's *U* test. N.D., not detectable. In control group, $n=16$ for prefrontal cortex, hippocampus, hypothalamus and amygdala, $n=14$ for striatum. In BBBC group, $n=16$ for each region. DOPAC, 3,4-dihydroxyphenylacetic acid. 3-MT, 3-methoxytyramine. HVA, homovanillic acid.

Table 4. Noradrenalin (NA) and NA Metabolite Levels, and Values for NA Turnover in Various Brain Regions

Brain region	Group	Content (pg/mg protein)		Turnover
		NA	MHPG	MHPG/NA
Prefrontal cortex	Control	169±9	257±29	1.52±0.14
	BBBC	177±9	242±23	1.40±0.14
Striatum	Control	66.9±16.0	198±19	3.77±0.53
	BBBC	82.6±9.7*	142±17*	1.91±0.93***
Hippocampus	Control	87.0±8.2	79.9±5.2	1.01±0.11
	BBBC	99.4±13.6	63.3±5.3*	0.801±0.138
Hypothalamus	Control	738±70	246±22	0.347±0.027
	BBBC	616±52	240±23	0.461±0.086
Amygdala	Control	147±13	136±16	0.966±0.100
	BBBC	121±10	105±8	0.927±0.084

BBBC (1.0 mg/kg bw) was administered subcutaneously to pregnant SD rats as described in Materials and Methods. The control group received 1% ethanol in saline. Brain regions for analysis were dissected out of 102-d-old male offspring. NA and its metabolites were measured by HPLC-ECD. Moreover, metabolic turnover was calculated as the ratio of metabolite to monoamine. Data are represented as mean±S.E.M. *$p<0.05$, ***$p<0.001$, Mann–Whitney's U test. In control group, $n=16$ for prefrontal cortex, hippocampus, hypothalamus and amygdala, $n=14$ for striatum. In BBBC group, $n=16$ for each region. MHPG, 3-methoxy-4-hydrophenylglycol hemipiperazinium.

pus, hypothalamus, and amygdale region (Table 3). 5-HT (123%) and 5-HIAA (126%) levels in the prefrontal cortex as well as 5-HIAA level (112%) and 5-HT turnover (5-HIAA/5-HT 122%) in the hypothalamus were significantly increased in the BBBC-exposed group. However, in the striatum, hippocampus, and amygdala, there were no significant differences between the two groups.

The NA, MHPG, HM, and Ad levels as well as NA turnover were measured in the prefrontal cortex, striatum, hippocampus, hypothalamus, and amygdale regions (Table 4). In the striatum, the NA level (123%) was significantly increased but levels of its metabolites, the MHPG level (71%), and NA turnover (MHPG/NA 51%) were significantly decreased. In the hippocampus, the MHPG level (79%) was significantly decreased. In the prefrontal cortex, hypothalamus, and amygdala, there were no significant differences between the two groups. NA turnover, NM, and Ad levels were below the detection limit.

DISCUSSION

We previously performed several *in vitro* studies and found that BBBC exhibited an anti-androgenic effect stronger than that of *p,p'*-DDE and an anti-estrogenic effect comparable to that of BPA.[8] We also found that exposure of rats to BBBC (1.0 mg/kg bw) during the fetal period (11—18 d of gestation during which monoamine nervous systems develop and mature) resulted in a significantly lower brain weight in males after growth.[22] Exposure to BPA during mice's embryonic/fetal life and infancy decreased the brain weight,[19] and Kabuto and Shishibori[20] reported that DOPAC+HVA/DA and 5-HIAA/5-HT values were elevated in mice exposed to BPA during the fetal period over infancy, suggesting that excess activity of the monoamine nervous system induces hyperactivity. BPA is suspected to have an endocrine disrupting-effect.[20] To investigate that BBBC could affect the monoamine nerve system, monoamines (DA, 5-HT, and NA) in the brains of 1.0 mg/kg bw BBBC-treated male rats during the fatal period were measured after maturation.

Generally, the synthetic and metabolic pathways of the monoamines DA, 5-HT, and NA are described as follows. DA biosynthesized from tyrosine is metabolized to DOPAC mainly by monoamine oxidase (MAO) present on the mitochondrial outer membrane in neurons, and to 3-MT by catechol-*O*-methyltransferase (COMT) present on the postsynaptic neuronal membrane. DOPAC is further metabolized by COMT, and 3-MT by MAO to HVA. Thus, DOPAC mostly exists as free DA metabolites in nerve endings. 5-HT biosynthesized from tryptophan in the brain is metabolized by MAO to 5-HIAA *via* 2 steps. 5-HIAA accounts for almost 100% of 5-HT metabolites in the brain. NA is biosynthesized from DA through metabolism by dopamine-β-hydroxylase (DBH). NA is metabolized, to MHPG, Ad, and NM, by MAO and COMT, and is degraded by pathways present in nerve endings and outside neurons.

In the prefrontal cortex, DA and DOPAC levels were significantly increased but the HVA level remained unchanged, significantly reducing HVA/DA. In the 5-HT system, 5-HT and 5-HIAA levels were significantly increased. In the NA system, no change was detected. These findings suggest that BBBC treatment induced DA- and 5-HT synthesizing enzymes and DOPAC- and 5-HIAA-producing metabolic enzymes, and inhibited DA metabolic turnover. Moreover, BBBC may have caused excess accumulation of DA because DOPAC is produced in nerve endings. Because the levels of DA and DOPAC risen, it is suggested that the hyperactivity occurs in BBBC-treated rat's brain. Since the HVA level was not changed, BBBC may have affected the DA release process. Antkiewicz-Michaluk *et al.*[23] hypothesized that increased DOPAC levels promote radical production and cytotoxicity. We have previously reported that BBBC actually showed strong cytotoxicity[8] *in vitro*, this cytotoxicity was thought to be the one by the rise of the DOPAC level.

In the striatum region, no changes were noted in DA or its metabolite levels, but DOPAC/DA and HVA/DA rose, showing enhanced metabolism. These findings suggest that BBBC promoted release of DA into the synaptic clefts, thereby activating the nigrostriatal DA nerve pathway. BBBC treatment increased metabolic enzyme activity in nerve endings and resulted in DOPAC/DA elevation that may have occurred concurrently with an increase in HVA/DA. In this case, the amount of DA released into the synaptic clefts may have been decreased with a concomitant reduction of DA system activity. Changes in the nigrostriatal DA pathway may have affected motor function.[24] No influence of BBBC was noted in the 5-HT system. In the NA system, an increase in the NA level and decreases in the MHPG level and MHPG/NE were detected. The metabolic turnover of the NE system was reduced despite the elevation of the NA level, suggesting that BBBC induced the NA-synthesizing enzyme DBH and activated the re-uptake process, and inhibited the metabolizing enzyme MAO. Based on the 2 metabolic pathways of NE described above, the increased NA level may have increased release into synaptic clefts, but feedback by autoreceptors may have limited this release because of reduced metabolism. Since drugs that increase the extracellular NA level in the brain cause hyperthymia and those that decrease its level induce depression,[25] it is likely that BBBC exposure during the fetal period has some influence on the integrating function of

the brain and affects behavior.

In the hippocampus, BBBC only influenced the NA system. The NA level did not change, but the level of its metabolite, MHPG, was decreased. This finding suggests that BBBC inactivated a metabolic enzyme. BBBC exposure could affect hippocampus monoamine nervous systems and change stress sensitivity.[26] BBBC exposure during the fetal period may influence learning and memory because the hippocampus is associated with learning and memory formation.

In the hypothalamus, BBBC affected only the 5-HT system. The 5-HIAA level and 5-HIAA/5-HT were increased, suggesting that BBBC activated this region. It may also be necessary to investigate whether BBBC inhibits appetite and elevates body temperature.

Body weight was reduced in male suckling rats exposed to BBBC during the fetal period and various irreversible influences were noted in the nervous system, suggesting that BBBC somehow affected development. The action mechanism of EDs in the central nervous system is complex. Endocrine disruption caused by ingestion of trace amounts of chemicals induces abnormal thyroid metabolism and inhibits functional development of the brain in children, and this may be a cause of mental retardation and behavioral abnormalities.[21,27,28] Our results suggested that BBBC is an endocrine disrupting-chemical during the fetal periods. Then, we are planning to investigate the influence of prenatal BBBC exposure on behavior by measuring spontaneous movement in offspring. It is also necessary to confirm by *in vivo* microdialysis whether similar changes actually occur in the prefrontal area *in vivo*. Monoamines and metabolites in the brain were measured after sexual maturation in male rats exposed to 1.0 mg/kg BBBC during the fetal period, but the body weight of suckling rats was lower than control group. This difference disappeared after weaning, and a similar tendency of the body and brain weight was also noted in female rats exposed during the fetal period (data not shown). Thus, investigation of the brain in the suckling period is also necessary. No observable adverse effect level and low observable adverse effect level of BBBC have not been determined and the level of eluted BBBC has not been detected in food. However, it is thought to be necessary to clarify the effect of BBBC-treated rats from the viewpoint of preventing before a problem happens. We are investigating prenatal exposure to same dose and high dose BBBC in rats to further clarify whether BBBC is an ED chemical and/or a neurochemical, and measure the levels of several serum hormones. Based on these data, we are planning to measure some enzyme activity levels of the BBBC-treated brain.

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REPRODUCTIVE TOXICOLOGY

Detrimental effects of prenatal exposure to filtered diesel exhaust on mouse spermatogenesis

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Abstract We recently showed that prenatal exposure to diesel exhaust (DE) disrupts spermatogenesis in mouse offspring. This study was undertaken to determine whether filtered DE in which 99.97% of diesel exhaust particles >0.3 μm in diameter were removed affects spermatogenesis in growing mice. After prenatal exposure to filtered DE for 2–16 days postcoitum, we examined daily sperm production (DSP), testicular histology, serum testosterone levels and mRNA expression of hormone synthesis process-related factors. In the filtered DE exposed group, DSP was markedly reduced at 12 weeks compared with the control group; clean air exposed group. Histological examination showed multinucleated giant cells and partial vacuolation in the seminiferous tubules of the exposed group. Testosterone was elevated significantly at 5 weeks. Moreover, luteinizing hormone receptor mRNA at 5 and 12 weeks, 17α-hydroxylase/C17-20-lyase and 17β-hydroxysteroid dehydrogenase mRNAs at 12 weeks were significantly elevated. These results suggest that filtered DE retains its toxic effects on the male reproductive system following prenatal exposure.

Keywords Diesel exhaust · Diesel exhaust particles · Testosterone · Spermatogenesis · Multinucleated giant cell · Prenatal exposure

Introduction

Air pollutants are widely associated with respiratory and cardiovascular diseases (Bremner et al. 1999; Donaldson and Stone 2003), and diesel exhaust (DE) is a major contributor to ambient air particles, particularly in Japan. The effects of suspended particulate matter (SPM) have recently

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been studied and reported. Particulate matter with diameters of <2.5 μm (PM2.5), of which diesel exhaust particles (DEP) are primarily composed, are associated with increases in daily mortality (Ostro et al. 2006). Moreover, ultrafine particles (UFP: diameters <100 nm) are apparently associated with increased health risks (Delfino et al. 2005; Gwinn and Vallyathan 2006; Lambert et al. 2003). It is suggested that this is because UFP may penetrate deeply into the lung and rapidly translocate across the respiratory epithelium (Frampton 2001; Stearns et al. 2001). Another suggestion is that the reactive surface properties of the particles, with their complex arrays of metal moieties and organic compounds, may produce changes in the protective lining fluids or airway surface epithelium, leading to oxidative stress (Ghio et al. 1998; Stohs and Bagchi 1995).

Although it is extensively studied about the effect of DE on respiratory and cardiovascular diseases, there are few reports concerned about male reproductive system. In these days, reduced sperm count and reduced numbers of morphologically normal sperm among youth are major concerns in many countries (Selevan et al. 2000; Jørgensen et al. 2002). Moreover, a number of studies reported reduced semen quality among men occupationally exposed to various chemicals, heat and prolonged automobile driving (De Celis et al. 2000; Kolstad et al. 1999; Alexander et al. 1996; Eskenazi et al. 1991; Thonneau et al. 1998; Raymond 1993; Laven et al. 1988; Figà-Talamanca et al. 1996). We have already reported that 6 months of DE exposure impairs male reproductive function in adult mice (Yoshida et al. 1999).

There is mounting evidence that the fetus and infant are significantly more sensitive to a variety of environmental toxicants than adults. For example, it was reported that lower fertility in mice exposed in utero to benzo (a) pyrene, a mutagenic polycyclic aromatic hydrocarbon (MacKenzie and Angevine 1981) and lower sperm counts following prenatal tobacco exposure (Jensen et al. 2005). Our previous studies revealed that exposure to DE in utero reduces the expression of mRNAs required for normal male reproductive tract development in fetal mice (Yoshida et al. 2002), and that prenatal exposure to DE impairs spermatogenesis (Ono et al. 2007). In the present study, we examined the effects of prenatal exposure to filtered DE, that represented approximate total removal of DEP, on male reproductive function in mice.

Materials and methods

Animals

Twenty-eight pregnant (plug positive) ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were divided into 2 groups of 14, and were housed in separate inhalation chambers. On postnatal day (PND) 5, the number of pups per litter was adjusted randomly to twelve. Although all male pups were raised, female pups without acceptable numbers were eliminated from the study. The young mice were weaned on PND 21, and female pups were culled. At the same time, maternal body weights were measured. The weights in the control group and the filtered DE-exposed group were 46.97 ± 1.46 and 45.36 ± 1.32 g, respectively. After the weighing, dams were sacrificed. All mice were allowed ad libitum access to a commercial stock diet CE2 (Japan Clea Co., Tokyo, Japan) and water. Study protocols were approved by the ethics and safety committees for animal experimentation at the National Institute of Environmental Studies.

Diesel exhaust exposure

A four-cylinder light-duty diesel engine, 4JG-2-type (3,059 cc) (Isuzu Automobile Co., Tokyo, Japan), was connected to an Eddy current dynamometer (ECDY; Meidensha, Tokyo, Japan). The engine was operated using standard diesel fuel at a speed of 1,500 rpm under a torque load of 10 kg × m. This corresponds to an approximate speed of 40 km/h. Standard diesel fuel was purchased from a local fuel station (Nippon Oil Corporation, Tokyo, Japan). The sulfur content of the fuel used in the present study was less than 20 ppm. The monitored gas components in DE were nitric oxide (NO_X), sulfur dioxide (SO_2) and carbon dioxide (CO_2), which were measured using a chemiluminescence NO–NO_2–NO_X analyzer model 42 (Thermo Environmental Instruments, Inc., Franklin, MA, USA), ML 9850 SO2 (Teledyne Monitor Labs, Inc., Englewood, CO, USA) and CGT-10-3A (Shimazu Co, Kyoto, Japan), respectively. DEP was measured using particulate counters (AP-632TM&MR-632; SIBATA SCIENTIFIC TECHNOLOGY LTD, Tokyo, Japan): the data of DEP (mg/m^3) were calculated based on the data of count per minute (cpm). DE was diluted with clean air to a constant particle density of 1.0 mg/m^3. Immediately after dilution, diluted DE was passed through HEPA filters which consists of DUSTRON Filter (DST-18-85; NIPPON MUKI CO., LTD. Tokyo, Japan) and ATM perfect filter (ATM-22-P-A; NIPPON MUKI CO., LTD. Tokyo, Japan) as described by Fujimaki and Kurokawa (Fujimaki and Kurokawa 2004). Mice were exposed to filtered DE or to clean air (control). DE exposure began on day 2 postcoitum (p.c.; the day the plug was observed was considered to be day 0 p.c.) and continued until day 16 p.c. Pregnant mice were housed in stainless-steel wire-mesh cages during the exposure period; postpartum offspring would immediately drop away from the cages. Day 16 p.c. is considered to be the end of tera-

togenic period in mice; hence, the designated exposure period was from day 2 p.c. to day 16 p.c. Animals were housed in a chamber maintained at 24 ± 1°C with 60 ± 1% humidity and a 12-h light/dark cycle. The exposure period was 12-h a day (10:00 p.m.–10:00 a.m.). After day 16 p.c., but before euthanasia, all animals were moved to a regular animal housing room maintained at 22 ± 1°C with 50 ± 5% humidity and a 12-h light/dark cycle.

Body and organ weights, testicular morphology

Body weight was recorded on days 5, 8, 16, 21, 35 and 84. Testis, epididymis and accessory organ (including prostate, seminal vesicle and coagulating gland) weights were bilaterally measured for each animal, and relative weights (weight of organ/body weight) were calculated on the same days as body weight calculations. After weighing, a sample from the right testis was fixed with neutrally buffered formalin and embedded in paraffin for histological observation. Sections were stained with hematoxylin and eosin (HE). The remainder of the right testis was frozen at −80°C, until thawing for sperm counts. The left testis was frozen at −80°C until use.

Daily sperm production

Testicular tissue was thawed and weighed after trimming of any extra capsular materials (In the control group, the weight of testicular tissue at 5 and 12 weeks were 22.49 ± 2.16 and 60.96 ± 12.87 mg, respectively. In the filtered DE-exposed group those were 20.46 ± 1.84 and 49.17 ± 2.69 mg, respectively.). They were homogenized for 2 min in 0.5–1 mL of saline containing 0.05% Triton X-100 (Nacalai Tesque, Inc., Kyoto, Japan) and 0.2% Eosin Y (Merck & Co, Inc., Whitehouse station, NJ, USA). The number of sperm nuclei in each suspension was determined using a hemocytometer, as described by Amann (1981).

Serum testosterone

Serum testosterone was measured by EIA (TESTOSTERONE ELISA KIT, Cat. No. 1880, Alpha Diagnostics Intl, Inc., San Antonio, TX, USA).

Real-time RT-PCR

Testicular tissue was homogenized using ISOGEN (Nippon Gene, Tokyo, Japan). Reverse transcription of total RNA into cDNA was carried out as described elsewhere (Yoshida et al. 1998). Amplification and detection were performed using the ABI PRISM® 7700 Sequence Detector System (Applied Biosystems, Foster, CA, USA). PCR conditions were as described previously (Ono et al. 2007). The primer pairs and TaqMan probes were designed using Primer Express Software (Applied Biosystems, Foster, CA, USA) to amplify specific small fragments as shown in Table 1. Expression of the following mRNAs encoding gonadotropin and sex steroid hormone receptors, as well as factors in the sex steroid hormone biosynthesis pathway were analyzed; androgen receptor (AR), estrogen receptor (ER)(α, luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), steroidogenesis acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17-20-lyase (P450c17), 17β-hydroxysteroid dehydrogenase (17β-HSD) and cytochrome P450 aromatase (P450arom). The murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined for normalization of expression levels of target genes as an internal control.

Statistical analysis

Data were expressed as mean ± SE. Serum testosterone levels were analyzed by Mann–Whitney *U* test, and the sex ratio of offspring was tested using Fisher's exact test.

Table 1 Sequences (5′–3′) of primers and probes used for quantification of gene expression by PRISM

	Forward primer	Reverse primer	Probe
AR	CCAGATGGCGGTCATTCAGT	GAAGGACCGCCAACCCA	TTCCTGGATGGGACTGATGGTATTTGCC
ERα	CCAGCAGTAACGAGAAAGGAAAC	TCATTGCACACGGCACAGTA	TGATCATGGAGTCTGCCAAGGAGACTCG
LHR	GGTGCTGGCAATGCTGG	CGCAGTCGCAGGGCTC	TCTCCAGAGTTGTCAGGGTCGCGC
FSHR	GTCTCCTTGCTGGCATTCTTG	TCGGTCACCTTGCTATCTTGG	ATCTGGATGTCATCACTGGCTGTGTCATTG
StAR	TCACTTGGCTGCTCAGTATTGAC	TGGTTGGCGAACTCTATCTGG	TGGCTGCCGAAGACAATCATCAACC
P450scc	ACTAGCAGTCCTAGGTCCTTCAATGA	TGGATTTTCTGTGTGCCACTCC	CTGGCGACAATGGTTGGCTAAACCTGT
3β-HSD	ATTCCCAGGCAGACCATCCTA	TGAGCTGCAGAAGATGAAGGC	TCTGAAAGGTACCCAGAACCTATTGGAGGC
P450c17	CTCATCCCACACAAGGCTAACA	TTATCGTGATGCAGTGCCCA	TTGCCATCCCGAAGGACACACATGT
17β-HSD	AGCCTATTCATTTGAGTTGGCC	TGGTCCTCTCAATCTCTTCTGCA	AGCCGGACACTGGAAAAGCTACAGACCA
P450arom	CTGTACTTCATGTTACTTCTCGTCGC	TCGATCTTTATGTCTCTGTCACCC	ATCCAGAGGTCGAAGCAGCAATCCTG
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	CAGAAGACTGTGGATGGCCCCTC

Table 2 Mean concentrations of particulate matter and major gaseous components in DE during experiment

	Control	Filtered-DE
Particulate matter		
DEP (mg/m^3)	0.004 ± 0.00002	0.004 ± 0.00005
DEP (cpm)	2.09 ± 0.01	2.69 ± 0.04
Gaseous components		
NO (ppm)	ND	10.22 ± 0.07
NO_2 (ppm)	ND	4.09 ± 0.05
SO_2 (ppm)	ND	0.184 ± 0.002
CO_2 (ppm)	408.8 ± 6.9	4647.7 ± 45.9

Values are means ± SE

ND not detected

Student's t test was used for other experiment. A P value of <0.05 was considered to be significant.

Results

Components of filtered DE

The concentrations of major components were shown in Table 2. Particulate matter concentration of DE before filtration set up as 1.0 mg/m^3, and that after filtration was not significant different compared with the clean air exposed group as the control (Table 2). On the other hand, the concentrations of gaseous compounds remained unchanged compared with non-filtered DE-exposed group (data not shown) (Ono et al. 2007).

Number and sex ratio of offspring

In both groups, pregnant (plug positive) ICR mice did not die during the exposure period. However, 2 of 14 were not delivered in the filtered DE-exposed group. Litter sizes ranged from 7 to 17 pups (control group: 12.9 ± 0.7, filtered DE-exposed group: 13.3 ± 0.8) and the total offspring were 180 in the control group and 160 in the filtered DE-exposed group, respectively. It was attributed the difference in the total offspring to the number of dams. There were no significant changes in number of offspring and the sex ratio. The number and the sex ratio of offspring were shown in Table 3.

Body and reproductive organ weights

In order to determine the general toxicity of filtered DE, body and reproductive organ weights were measured. The filtered DE-exposed group had significantly lower testis weight at 16 days (−86.7% relative to controls) (Table 4). However, there were no significant changes in body weight or the weight of other reproductive organs (Table 4).

Testicular morphology

In the seminiferous tubules, filtered DE exposure induced an increase in multinucleated giant cells (Fig. 1a, c) and a reduction in germ cells (Fig. 1b). In the control group, multinucleated giant cells were observed in two out of ten each animal (zero to one giant cells per animal) at 5 weeks and in only one of nine each animal (zero to one giant cells per animal) at 12 weeks. In contrast, in the filtered DE-exposed group, multinucleated giant cell were observed four out of eight each animal (1–17 giant cells per animal) at 5 weeks (Fig. 1c) and in four of seven each animal (two to three giant cells per animal) at 12 weeks (Fig. 1a). In addition, filtered DE-exposed Sertoli cells showed marked vacuolization (Fig. 1b).

Daily sperm production

In both groups, DSP increased from 5 until 12 weeks (Fig. 2). In the filtered DE-exposed group, DSP had decreased by 25.3% at 12 weeks (Fig. 2).

Serum testosterone

In both groups, serum testosterone levels increased with age. However, serum testosterone levels in the filtered DE-exposed group were more than 2.9-fold higher at 5 weeks (Fig. 3).

Real-time RT-PCR

In the filtered DE-exposed group, expression of several mRNAs was significantly higher. LHR mRNA expression was 126.0% that of controls (Table 5) at 5 weeks, while LHR, P450c17 and 17β-HSD mRNA expression was 125.9%, 134.2 and 126.6%, respectively, at 12 weeks (Table 5).

Table 3 Number and sex ratio of offspring

	Number of dam	Number of offspring			Total offspring	Sex ratio (%) (male/(males + females) × 100)
		Male	Female	Total		
Control	14	7.29 ± 0.60	5.57 ± 0.51	12.86 ± 0.73	180	56.67
Filtered-DE	12	6.17 ± 0.52	7.17 ± 0.63	13.33 ± 0.76	160	46.25

Values are means ± SE

Table 4 Effect of filtered-DE exposure on body weight and weight of reproductive organ

		5 days			8 days	16 days	3 weeks	5 weeks	12 weeks
		Male	Female	Total					
Numbers of animal examined	Control	102	78	180	10	9	9	9	9
	Filtered-DE	74	86	160	10	9	7[a]	7	7
BW (g)	Control	3.56 ± 0.14	3.40 ± 0.15	3.49 ± 0.14	5.03 ± 0.16	6.92 ± 0.45	10.17 ± 0.85	29.26 ± 0.86	42.31 ± 0.93
	Filtered-DE	3.42 ± 0.08	3.38 ± 0.12	3.41 ± 0.10	4.87 ± 0.25	6.50 ± 0.48	10.34 ± 1.51	29.96 ± 1.46	43.29 ± 0.91
Testis (mg)	Control	-	-	-	7.24 ± 0.38 (1.49 ± 0.04)	19.20 ± 1.23 (2.79 ± 0.11)	35.51 ± 4.13 (3.42 ± 0.12)	133.39 ± 7.23 (4.55 ± 0.18)	250.43 ± 10.51 (5.91 ± 0.16)
	Filtered-DE	-	-	-	6.78 ± 0.37 (1.34 ± 0.07)	15.63 ± 1.11* (2.42 ± 0.07*)	39.60 ± 7.85 (3.69 ± 0.19)	123.27 ± 10.97 (4.08 ± 0.21)	243.87 ± 12.99 (5.62 ± 0.2)
Epididymis (mg)	Control	-	-	-	3.03 ± 0.20 (0.61 ± 0.04)	5.44 ± 0.21 (0.80 ± 0.03)	9.21 ± 1.17 (0.89 ± 0.04)	38.99 ± 1.25 (1.33 ± 0.03)	94.10 ± 3.18 (2.22 ± 0.04)
	Filtered-DE	-	-	-	2.79 ± 0.08 (0.56 ± 0.02)	5.19 ± 0.41 (0.80 ± 0.02)	9.69 ± 1.77 (0.90 ± 0.06)	39.24 ± 1.89 (1.31 ± 0.03)	94.21 ± 3.78 (2.18 ± 0.09)
Accessory gland (mg)	Control	-	-	-	1.02 ± 0.15 (0.20 ± 0.03)	2.07 ± 0.10 (0.30 ± 0.02)	3.38 ± 0.61 (0.32 ± 0.03)	84.02 ± 10.70 (2.82 ± 0.29)	288.39 ± 17.43 (6.82 ± 0.41)
	Filtered-DE	-	-	-	0.73 ± 0.09 (0.14 ± 0.02)	1.88 ± 0.16 (0.29 ± 0.02)	4.50 ± 1.02 (0.26 ± 0.09)	68.17 ± 6.54 (2.26 ± 0.15)	299.80 ± 6.92 (6.94 ± 0.21)

Values are means ± SE

Data within parenthesis indicate relative weight [mg/body weight (g)]

BW body weight

[a] The numbers of Accessory gland examined was 5

* $P < 0.05$ versus control

Discussion

Numerous epidemiological studies have suggested that air pollution is a major causative factor in several diseases (Michelozzi et al. 1998; Souza et al. 1998). Of all such pollutants, SPM is of the major concern. Recent studies have revealed that inhalation of SPM increases the risk of respiratory disease, cardiovascular disease, lung cancer and myocardial infarction (Bremner et al. 1999; Donaldson and Stone 2003; Souza et al. 1998; Garshick et al. 2004; Mills et al. 2005; Nemmar et al. 2002; Peters et al. 2004). Moreover, some investigators have reported that traffic-related air pollution has detrimental health effects in children. For example, the relevance of SPM in asthma, bronchitis symptoms and chronic otitis media is supported (Brauer et al. 2006; Delfino et al. 2003; Kim et al. 2004; Zhang et al. 2002).

We previously reported that DE exposure affects the male reproductive system in mice (Yoshida et al. 1999). In that paper, we observed a decline in DSP and degeneration in Leydig cells after exposure to DE in male adult mice. In growing rats, DE exposure caused changes in the levels of reproductive hormones and led to a reduction in spermatogenesis (Watanabe and Oonuki 1999). Moreover, DE exposure in utero induces endocrine disruption and accelerates male puberty (Yoshida et al. 2006). In addition, we recently revealed that prenatal DE exposure damages mouse spermatogenesis (Ono et al. 2007).

In the present study, pregnant (plug positive) ICR mice did not die during the exposure period. And there were no significant difference in maternal body weights compared with the control group. In addition, the sign of maternal toxicity associated with filtered DE was not observed in autopsy findings. Thus, the effects of filtered DE exposure might be mild for dams.

Exposure to filtered DE did not affect body weight or reproductive organ weight of offspring except for testicular weight at 16 days. In a previous study, prenatal DE exposure at 1 mg/m^3 significantly decreased body weight at 8 days and 5 weeks, although there were no significant differences in the weight of reproductive organs (Ono et al. 2007). Recent reports have demonstrated the endocrine-disrupting activity of DE and/or DEP (Furuta et al. 2004; Li et al. 2006a, b; Mori et al. 2002; Takeda et al. 2004; Taneda et al. 2004); specifically, estrogenic and anti-androgenic actions were observed. In addition, typical endocrine-disrupting chemicals induced lower testicular weight and DSP (Li et al. 2001; Sharpe et al. 1995). Thus, the reduction in both testicular weight and DSP might have been due to the endocrine-disrupting activity of DEP.

In the present study, DSP was decreased at 12 weeks after filtered DE exposure. We previously reported marked reductions in DSP at 5 and 12 weeks in mice prenatally

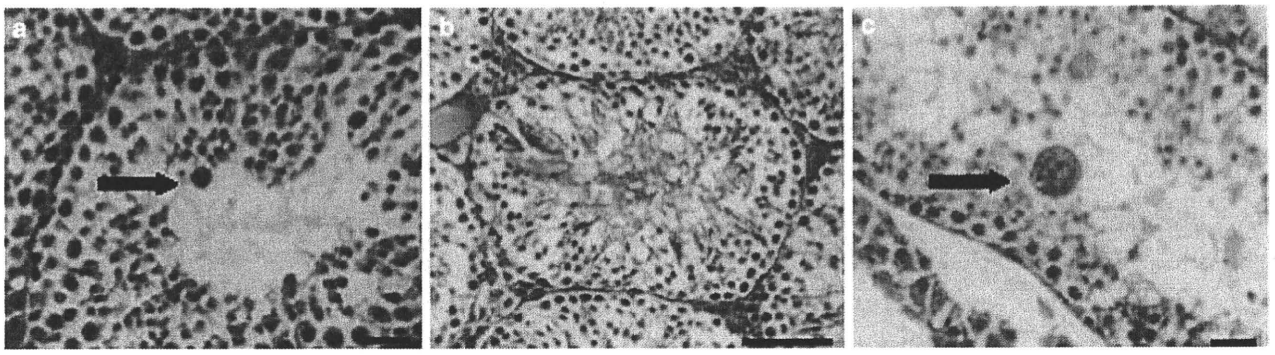

Fig. 1 Effects of filtered DE on testis morphology: **a** multinucleated giant cell (*arrow* 12 weeks), **b** abnormality in seminiferous tubules (12 weeks), **c** multinucleated giant cell (*arrow* 5 weeks). *Bar*100 μm

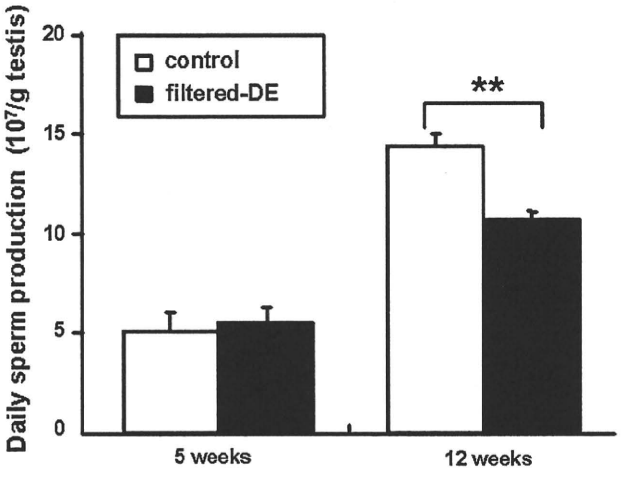

Fig. 2 Effects of filtered DE exposure on daily sperm production. Control, $n = 9$; filtered DE, $n = 7$ (5 weeks). Control, $n = 9$; filtered DE, $n = 7$ (12 weeks). Values are means ± SE. ** $P < 0.01$ versus control

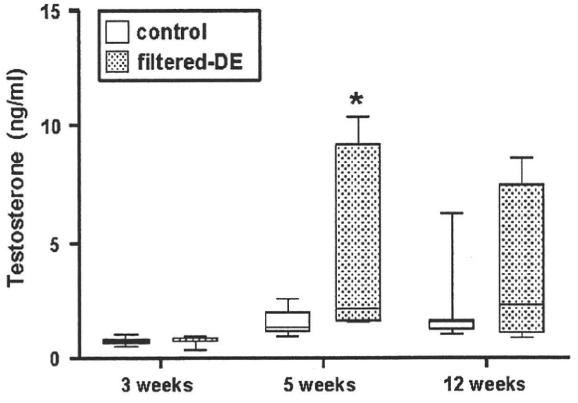

Fig. 3 Effects of filtered DE exposure on serum testosterone concentrations. Control, $n = 9$; filtered DE, $n = 7$ (3 weeks). Control, $n = 9$; filtered DE, $n = 7$ (5 weeks). Control, $n = 9$; filtered DE, $n = 7$ (12 weeks). Values are means ± SE

exposed to DE (Ono et al. 2007). Watanabe showed that the reduction of DSP in mature rats exposed to diesel exhaust in utero was caused by the loss of Sertoli cells (Watanabe 2005). In mice, Sertoli cell proliferation begins on embryonic day 14 and ends early in postnatal life (postnatal days 12–17, depending on strain) with no additional proliferation in adulthood. Consequently, this finding suggests that the reduction in DSP following prenatal exposure to DE is probably due to decreased Sertoli cell number. This observation is supported by observed abnormalities in testicular tissue in the filtered DE-exposed group. However, no significant differences were seen in DSP at 5 weeks, possibly due to the fact that the physiological machinery for spermatogenesis on postnatal day 35 is not yet fully functional (Janca et al. 1986).

Multinucleated giant cells containing more than two nuclei were seen in the seminiferous tubules of the testis. Although the number of multinucleated giant cells is typically small in the seminiferous tubules, it is possible that prolonged exposure to filtered DE induces morphological damage in the spermatogonia and/or primary spermatocytes during spermatogenesis. Such abnormalities in testicular tissue were observed in prenatal DE-exposed mice (Ono et al. 2007) as well as in the present study. It is likely that the appearance of vacuoles is partly due to the absence of germ cells. Because the accumulation of vacuoles in conjunction with chemical disruption of spermatogenesis is a common (Allenby et al. 1990; Cho and Park 1994), nonspecific response to a variety of insults on the testis, this may or may not be a direct consequence of exposure to filtered DE.

Serum testosterone levels were elevated at 5 weeks, thus suggesting feedback regulation at the hypothalamus/pituitary level. We also observed increased serum testosterone levels after prenatal DE exposure (Ono et al. 2007; Yoshida et al. 2006). As described above, DE and/or DEP has endocrine-disrupting activity. The increased serum testosterone after filtered DE exposure may therefore be due to this activity.

In the present study, LHR mRNA expression was significantly increased at 5 and 12 weeks. Moreover, mRNA

Table 5 Effect of filtered-DE exposure on mRNA expression in testis

		5 weeks	12 weeks
AR	Control	1 ± 0.03	1 ± 0.05
	Filtered-DE	1.05 ± 0.02	1.03 ± 0.04
ERα	Control	1 ± 0.05	1 ± 0.07
	Filtered-DE	1.02 ± 0.03	1.15 ± 0.05
LHR	Control	1 ± 0.08	1 ± 0.09
	Filtered-DE	1.26 ± 0.07 *	1.39 ± 0.10 *
FSHR	Control	1 ± 0.07	1 ± 0.06
	filtered-DE	1.21 ± 0.08	1.01 ± 0.05
StAR	Control	1 ± 0.14	1 ± 0.10
	Filtered-DE	1.02 ± 0.06	1.27 ± 0.12
P450scc	Control	1 ± 0.11	1 ± 0.09
	Filtered-DE	1.07 ± 0.06	1.15 ± 0.05
3β -HSD	Control	1 ± 0.09	1 ± 0.14
	Filtered-DE	0.78 ± 0.07	1.14 ± 0.11
P450c17	Control	1 ± 0.09	1 ± 0.06
	Filtered-DE	1.01 ± 0.07	1.34 ± 0.06 **
17β -HSD	Control	1 ± 0.14	1 ± 0.08
	Filtered-DE	0.85 ± 0.14	1.27 ± 0.07 *
P450arom	Control	1 ± 0.10	1 ± 0.07
	Filtered-DE	0.91 ± 0.10	1.12 ± 0.05

Control, $n = 9$; filtered DE, $n = 7$ (5 weeks)

Control, $n = 9$; filtered DE, $n = 7$ (12 weeks)

Values are means ± SE

*$P < 0.05$, ** $P < 0.01$ versus control

expressions of P450c17 and 17β-HSD were also significantly increased at 12 weeks. Zhang et al. (2001, 2004) reported that the levels of P450c17 and 17β-HSD were reduced in LHR knockout (LuRKO) male mice. Thus, the increase in P450c17 and 17β-HSD mRNA expression may have occurred via alteration of LHR mRNA expression.

Exposure to materials known to have anti-androgenic effects induces increased expression of LHR and P450c17 mRNA (Kubota et al. 2003; Svechnikov et al. 2005). Some reports have suggested that several compounds contained in DE have anti-androgenic effects (Li et al. 2006a; Taneda et al. 2004). Thus, the increased mRNA expression in the present study may have been caused by DE-mediated endocrine disruption. In prenatal DE-exposed mice, StAR protein mRNA expression was significantly increased at 12 weeks (Ono et al. 2007); however, although the elimination of particles of >0.3 μm in diameter had similar effects on DSP, testicular tissue and serum testosterone, changes in mRNA expression differed between the exposure groups. Consequently, the site of action may vary with particle size.

The particle reduction system used in the present study was filtration. After filtration, DEP concentration was 0.004 mg/m^3, in short, almost particle of <0.3 μm in diameter was removed by HEPA filters. Because UFP (particle diameter <100 nm) and/or gaseous components pass through the filter, the detrimental effects observed in the present study may have been caused by UFP and/or gaseous components. Several studies have observed an association between UFP and respiratory (Chalupa et al. 2004) and cardiovascular diseases (Nemmar et al. 2002). Such small particles may penetrate deeply into the lung and readily translocate across the respiratory epithelium. It was not peculiar to respiratory system, other organs (including fetus) might have also been affected.

The present study suggests that UFP and/or gaseous components exposure in utero have detrimental effects on mouse spermatogenesis. Further studies are needed to clarify the mechanisms of these effects. The gaseous concentrations used in this experiment were higher than those found in the average urban area. Experiments with dose exposure of same level as urban area will be also necessary to provide reasonable estimates of the human risks of exposure to UFP and/or gaseous components.

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Short Communication

Cytokine and chemokine expression in a rat endometriosis is similar to that in human endometriosis

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ABSTRACT

The pathogenesis of endometriosis, a gynecologic disorder associated with infertility, appears to involve immune responses. However, the details involved have not been clarified. In this study, we analyzed expression levels of interleukin (IL)-6, IL-10, monocyte chemoattractant protein-1, eosinophil chemotactic protein, macrophage inflammatory protein-1α, and regulated on activation normal T cell expressed and secreted (RANTES) and CC chemokine receptor 1 in endometriotic lesions in a rat model in which endometrium is autotransplanted onto peritoneal tissue and found that they were remarkably increased, while those of IL-2, IL-4, and interferon-γ were not. These results were obtained in a rat model induced by autologous, not allogeneic, transplantation of endometrial epithelium to the peritoneum. Expression of these factors is consistent with that of endometriosis in humans. Therefore, this model may be useful in the investigation of the pathogenesis and treatment of endometriosis.

1. Introduction

Endometriosis, which is characterized by the growth of endometrial tissue outside the uterine cavity, is a common gynecologic disorder that affects 6–10% of women of reproductive age [1]. This disorder frequently causes pelvic pain, decreases quality of life and is likely related to infertility. Although the etiology of endometriosis is not clear, Sampson's hypothesis [2], which proposes the involvement of retrograde seeding of endometrial cells during menstruation, is widely accepted. Ectopic endometrial tissue occurs mainly in the pelvic peritoneum as well as the ovaries and the rectovaginal septum, and rarely in the pericardium, pleura, and brain [1]. However, the presence of ectopic endometrial tissue in sites well removed from the pelvic peritoneum cannot be explained by this hypothesis.

To clarify the pathogenic factors of this disorder, animal models have been created. Vernon and Wilson [3] developed the surgical technique of autotransplantation of uterine tissue for the induction of endometriosis in a rat model. Uchiide et al. [4] reported hyperplasia of interstitial stromal cells and degranulation of mast cells in the peritoneum adjacent to autotransplanted endometrial tissue, consistent with human endometriosis [5]. In the present study, we determined the expression of the cytokines interleukin (IL)-2, IL-4, IL-6, IL-10, and interferon (IFN)-γ, the chemokines monocyte chemoattractant protein (MCP)-1, eosinophil chemotactic protein (eotaxin), macrophage inflammatory protein (MIP)-1α, and regulated on activation normal T cell expressed and secreted (RANTES) and CC chemokine receptor 1 (CCR1) in the rat endometriosis model and compared the expression with that in endometriotic lesions in humans.

2. Materials and methods

2.1. Animals and treatments

Female Sprague–Dawley rats were purchased from Japan SLC Inc. (Shizuoka, Japan) and used for the induction of endometriosis at 8 weeks of age. A 5 mm × 5 mm piece of uterine tissue was attached to each side of the peritoneum by surgical autotransplantation. Tissue from the endometriotic lesions ($n = 2$ per rat) was obtained on days 4 ($n = 17$ rats) and 7 ($n = 14$ rats) after transplantation. As a control, normal peritoneal tissues were obtained from sham-operated rats on days 4 ($n = 9$) and 7 ($n = 11$) after sham surgery. Blood samples were also obtained from rats with endometriosis and sham-operated rats. All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

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2.2. Total RNA extraction and reverse transcription

Tissue samples were immediately frozen in liquid nitrogen and homogenized with a Digital Homogenizer (As One Inc., Osaka, Japan) in 1 mL Isogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan), and total RNA was isolated with chloroform. RNA was precipitated in isopropanol, washed with ethanol and resuspended in RNase-free water. Total RNA (2000 ng) resuspended in 16 μl of water was treated with DNase (Promega Corp., Madison, WI) in the presence of RNase inhibitor (Promega) in buffer (Promega). DNase-treated total RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen Corp., Carlsbad, CA), GeneAmp dNTP Mix (Applied Biosystems Japan Ltd., Tokyo, Japan), and Random Primer (Takara, Shiga, Japan) in buffer (Invitrogen) for 60 min at 37 °C.

2.3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) analysis was performed the following tissue samples: endometriotic lesions on days 4 ($n = 8$) and 7 ($n = 18$) and normal peritoneal tissues on days 4 ($n = 8$) and 7 ($n = 7$). IL-2, IL-4, IL-6, IL-10, IFN-γ, MCP-1, eotaxin, MIP-1α, RANTES, and CCR1 mRNA expression levels were examined by quantitative real-time PCR with a PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) and an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Real-time PCR was performed on individual cDNA samples from each endometriotic lesion and from control tissue. cDNA samples from endometriotic lesions were diluted serially for use as standards. PCRs were prepared as a final volume of 25 μl containing either 5 μl of the 2.5-fold diluted reverse-transcribed sample (cDNA) or diluted standards, 12.5 μl of Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), 0.2 μM TaqMan probe (Applied Biosystems Japan) and 0.6 μM of each forward and reverse primer (Nippon EGT, Toyama, Japan). PCR was performed at 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Detected relative expression levels of IL-2, IL-4, IL-6, IL-10, IFN-γ, MCP-1, eotaxin, MIP-1α, RANTES, and CCR1 mRNAs were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.4. Enzyme-linked immunosorbent assay

Blood was obtained via cardiac puncture on days 4 and 7 after autotransplantation. Serum MCP-1 levels were measured with the use of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bio Source International, Inc., Camarillo, CA) according to the manufacturer's instructions.

2.5. Statistical analysis

Normalized expression levels of cytokines, chemokines and CCR1 and serum MCP-1 levels are shown as mean ± SEM. Mann–Whitney U test was used to analyze differences in values between groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Expression of cytokine and chemokine mRNAs in endometriotic lesions

Expression levels of IL-6, IL-10, MCP-1, eotaxin, RANTES, MIP-1α, and CCR1 mRNAs are shown in Fig. 1. mRNA expression of these molecules was significantly increased in endometriotic lesions compared to the level in controls on days 4 and 7. RANTES

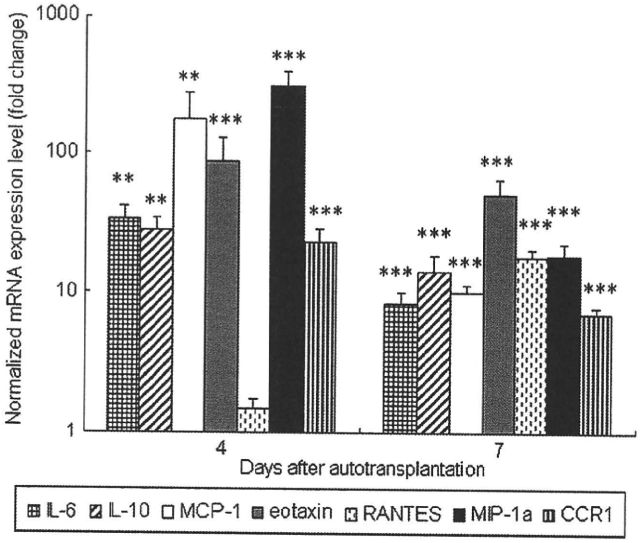

Fig. 1. Cytokine, chemokine, and CCR1 mRNA expression in endometriotic lesi in rats. mRNA expression of IL-6, IL-10, MCP-1, eotaxin, RANTES, MIP-1α, and C in endometriotic lesions were analyzed by quantitative RT-PCR. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. Abbreviations: CCR1, CC chemokine receptor 1; eotaxin, eosinophil chemotactic protein; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; RANTES, regulated on activation normal T cell expressed and secreted.

mRNA expression was significantly increased in endometriotic lesions on day 7 but not on day 4. mRNAs for these cytokines and chemokines were highly detected in almost all (89%) endometriotic lesions. However, mRNAs for IL-2, IL-4, and IFN-γ were undetectable in 37–100% of the lesions (Table 1) and at very low levels when detectable.

3.2. Serum MCP-1 levels in rats with endometriotic lesions

Serum MCP-1 levels in rats with and without endometriotic lesions are shown in Fig. 2. The serum MCP-1 level was significantly higher in rats with endometriosis than in those without on day 4 after autotransplantation ($P < 0.01$).

4. Discussion

Autotransplantation of endometrium onto peritoneal tissue resulted in accretion with surrounding tissues and the development of proliferative lesions. Our rat endometriosis model was similar to that of Uchiide et al. [4] by histopathologic observation (data not shown). In the present study, we analyzed the cytokines and chemokines, which were reported to increase in human endometriosis and predicted their role in pathogenesis of endometriosis from the observations of the previous report [4], and showed that expression levels of IL-6, IL-10, MCP-1, eotaxin, RANTES, MIP-1α, and CCR1 in the endometriosis model were increased compared to

Table 1
Percentage of lesions showing detectable cytokine levels

	Day 7	
	Normal peritoneum	Endometriosis model
IL-4	0% (0/4)	13% (1/8)
IFN-γ	14% (1/7)	63% (10/16)
IL-2	0% (0/4)	25% (2/8)

Cases in which no mRNA was detected by 40 quantitative real-time PCR cycles were regarded as undetectable.
Abbreviations: IFN-γ, interferon-gamma; IL, interleukin.

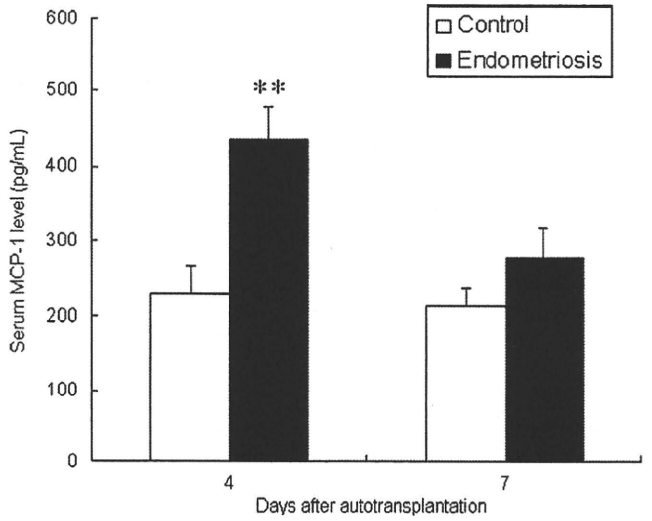

Fig. 2. Serum MCP-1 level in rat with endometriosis model. Serum MCP-1 levels were analyzed by ELISA. Data are shown as mean ± SEM. $^{**}P < 0.01$ vs. control. Abbreviations: CCR1, CC chemokine receptor 1; eotaxin, eosinophil chemotactic protein; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; RANTES, regulated on activation normal T cell expressed and secreted.

normal peritoneal tissue. These findings are similar to those in endometriosis in humans. The results presented herein were obtained by using normal peritoneal tissues from sham-operated rats as control, and similar results were obtained when we used normal tissues from non-operated rats as control to confirm with excluding the invasive surgical effects (unpublished data).

IL-6 was initially purified as B-cell stimulatory factor-2, which induces the final differentiation of B cells into high-rate Ig-secreting cells [6]. Several studies have reported increased expression of IL-6 in endometriosis [7–9]. Uchiide et al. [4] reported the presence of plasma cells and degranulation of mast cells induced by the interaction of mast cell Fc epsilon receptors (FcεRs) with IgE produced by plasma cells in this model. IL-6 may stimulate mast cells by activating Ig-secreting plasma cells. Rier et al. [10] reported that the expression of IL-6 receptor decreased in ectopic endometrial tissue (endometriotic lesions) compared to eutopic endometrial tissue and that this decreased expression caused resistance of endometriotic lesions to growth inhibition induced by IL-6. These findings support the hypothesis that IL-6 plays a role in the pathophysiology of endometriosis.

Increased expression of IL-10 has been shown in human ovarian endometrioma [7], and increased peritoneal fluid IL-10 has been shown in patients with endometriosis compared to women without [11]. IL-10 also induces the growth and differentiation of B cells [12] and thus may contribute to the pathogenesis of endometriosis.

Activation of plasma cells and mast cells associated with type I allergies is often followed by activation of T helper 2 (Th2) cells. A recent study reported that the T helper 1 (Th1)/Th2 balance in the peritoneal cavity shifts to Th2 dominance and IFN-γ and IL-2 levels in peritoneal fluid are decreased in patients with endometriosis compared to women without [13]. To investigate whether Th2 responses contribute to the pathogenesis of the present endometriosis model, mRNA expression levels of the Th2 cytokine IL-4 and the Th1 cytokines IFN-γ and IL-2 were examined and were detected at low levels. Low expression of IFN-γ and IL-2 is consistent with human endometriosis [13]. mRNA expression of IL-4 was also low in this model. Th2-dominant cytokine expression following increased expression of IL-4 in human endometriosis has been reported in several studies [13–15]. Odukoya et al. [7] reported that mRNA expression of IL-6 and IL-10 is increased, but that of IL-4 is not in ovarian endometrioma. Further investigation is required to determine whether Th2 cells contribute to the pathogenesis of endometriosis.

MCP-1 is secreted by a number of cell types, including macrophages, endothelial cells, fibroblasts, peritoneal fluid mesothelial cells and endometrial cells [16–18] and attracts monocytes, eosinophils, T lymphocytes and natural killer cells [19–23]. MCP-1 also contributes to Th2 polarization [24]. In the present study, we observed increased mRNA expression for MCP-1 was increased in the endometriosis model compared to normal peritoneal tissue and an increased serum MCP-1 level in rats with endometriosis compared to those without, consistent with several reports of human endometriosis [25–28]. MCP-1 expression is induced in human endometrial stromal cells by adhesion of these cells to extracellular matrix proteins via integrin signaling [29]. MCP-1 significantly increases the production of Fas ligand in cultured endometrial stromal cells, and interestingly, this increased production does not increase apoptosis of endometrial cells but increases apoptosis of T lymphocytes [30]. This may result in the development of immunotolerance by increasing apoptosis of leukocytes and thereby supporting the survival of ectopic endometrial cells [30].

We also analyzed serum levels of IL-4, IL-6, and IL-10. There was no significant difference in serum levels of IL-4 and IL-10 between rats with and without endometriosis model, and serum IL-6 level was below detection limit (<8 pg/mL) in both groups of rats with and without endometriosis. The fact that only serum MCP-1 level was significantly increased in rats with endometriosis model than in those without is notable and indicates a potential of serum MCP-1 level for non-invasive marker of presence of endometriosis.

Eotaxin is a selective chemoattractant for eosinophils [31]. Eosinophils are located in the submucosa, especially in the digestive tract and uterus. Eotaxin is constitutively expressed in endometrial epithelial cells and contributes to the homing of eosinophils to uterine tissue [32]. Ectopic migration of eosinophils contributes to type I allergies, such as asthma [33,34]. Degranulation of eosinophils in human endometriosis [35] and increased peritoneal fluid eotaxin level in patients with endometriosis compared to women without have been reported [36]. In the present study, increased mRNA expression of eotaxin in the endometriosis model compared to normal peritoneal tissue was observed. Infiltration of eosinophils reported previously in this model [4]. These findings support the similarities between this endometriosis model and human endometriosis.

RANTES is a chemokine that attracts natural killer cells, T lymphocytes and eosinophils [37–39]. It is secreted by fibroblasts, epithelial cells and macrophages, and its secretion is promoted by macrophage-derived growth factors and cytokines such as TNF-α, IFN-γ, and IL-1 [40]. The peritoneal fluid RANTES level is increased in women with endometriosis and is correlated with its severity of endometriosis [41]. In the present study, increased mRNA expression of RANTES in the endometriosis model compared to normal peritoneal tissue was observed on day 7 after autotransplantation but not on day 4. This data suggests that RANTES expression is possibly promoted after accumulation and activation of macrophages by increased expression of MCP-1 in endometriosis model. Previous studies have shown increased numbers and activity of peritoneal macrophages in women with endometriosis [42]. Activated macrophages produce growth factors that stimulate the proliferation of endometriotic lesions [40,43,44]. Uchiide et al. [4] reported the infiltration of macrophages in their rat endometriosis model. The ectopic, local expression of RANTES induced by macrophage-derived cytokines produced by ectopic endometrial cells may activate inflammatory reactions in endometriotic lesions.

MIP-1α is produced by macrophages, dendritic cells, and lymphocytes and plays an important role in allergic reactions [45]. The expression of CCR1, a MIP-1α receptor, by peritoneal macrophages is increased in patients with endometriosis compared to women without [46], suggesting that MIP-1α and CCR1 contribute to the pathogenesis of endometriosis. In the present study, increased mRNA expression of MIP-1α and CCR1 in the endometriosis model compared to normal peritoneal tissue was observed. Interestingly, interaction of MIP-1α and CCR1, which is also expressed on mast cells, activates histamine secretion from mast cells and basophils [47]. This interaction may promote mast cell degranulation in endometriosis.

Advantages of the rat model include the fact that endometrial epithelium is transplanted autologously, not allogeneically, allowing investigation of allergic reactions. Several studies induced lesions by allogeneic or heterologous transplantation of endometrial epithelium. However, immune responses in these cases would differ from those in lesions induced by autotransplantation. It is surprising that an allergic reaction involving mast cell degranulation is induced even by autologous transplantation of endometrial tissue. Our present findings in the rat endometriosis model are in agreement with findings of endometriosis in humans. Ectopic and local expression of these cytokines and chemokines may contribute to the formation and penetration of endometriotic lesions. Control of cytokine expression may be a novel strategy for the treatment and/or prevention of endometriosis.

In conclusion, we observed increased expression of cytokines and chemokines in the rat endometriosis model, consistent with findings of endometriosis in humans. These findings suggest that these factors and the immunoinflammatory processes in which they participate contribute to the formation of endometriotic lesions. This information will be helpful in further investigations of the pathogenesis and treatment of endometriosis in humans.

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Diesel Exhaust Exposure Enhances the Persistence of Endometriosis Model in Rats

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Diesel exhaust (DE) is known to be one of the main causes of air pollution. Several studies have suggested that DE causes lung cancer, cardiovascular disease, abnormal reproductive function, and central nervous system damage as well as type I allergy in the airway. Type I allergy also plays a role in pathogenesis of endometriosis. In the present study, we examined the effect of exposure to DE on a rat model of endometriosis. Endometriosis was induced by autotransplantation of endometrium to the peritoneum in female Sprague-Dawley rats exposed to DE during prenatal and postnatal periods. Endometriotic lesions, normal peritoneum, and blood samples were obtained on days 4, 7, and 14 after autotransplantation. The extent of stromal proliferative lesions in the endometriosis model was greater in the rats of the DE exposure group than in those of the control group on day 14. Serum monocyte chemoattractant protein (MCP)-1 level was significantly higher in rats with endometriosis in the DE exposure group than in those in the control group on day 14. Results of this study suggest that DE exposure enhances the histologic and molecular pathology of endometriosis in rats.

Key words——endometriosis, diesel exhaust, monocyte chemoattractant protein-1, stromal proliferation

INTRODUCTION

Air pollution is a serious problem in urban areas all over the world. One of the major air pollutants is diesel exhaust (DE), which contains carbon monoxide, sulfur oxides, nitrogen oxides, and DE particles (DEPs). Numerous studies have indicated that DE and DEPs have various detrimental effe on health; it has been reported that DE in humans causes lung cancer,[1] chronic obstructive pulmonary disease,[2] cardiovascular disease,[3–6] and in mice, reduced male reproductive function,[7–10] abnormal fetal development of female reproductive function,[11] abnormal expression of immune-related genes in the placenta,[12] and central nervous system damage.[13] It has also been reported that DE exposure promotes allergic reactions in the airway and exaggerates the pathology of respiratory allergic diseases. Diaz-Sanchez *et al.*[14,15] reported that *in vivo* nasal challenge with DEPs enhances local expression and production of cytokines and CC chemokines in humans. Matsumoto *et al.*[16] showed that repeated exposure to low-dose DE (100 μg DEP/m^3) increased airway hyper-responsiveness and exaggerated allergic responses in ovalbumin-induced asthmatic mic

Endometriosis is a common gynecological disorder, the prevalence of which is 6–10% in women of reproductive age[17] and which is now on the rise. It is well known that retrograde seeding of endometrial cells during menstruation partially contributes to the pathogenesis of endometriosis;[18] however, the details involved in such pathogenesis have not been clarified. To identify the pathogenic factors of this disorder, we created a rat endometriosis model by autotransplantation of endometrium to peritoneal tissue. Uchiide *et al.*[19] found interstitial stromal hyperplasia and degranulation of mast cells in a endometriosis model induced by autotransplantation of endometrial tissue to peritoneum. We previously reported increase of local expression of cytokines and chemokines in the rat endometriosis model, consistent with the characteristics of endometriosis in humans.[20] These observations suggest that

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