Expression of steroid-related genes

During perinatal exposure to DE, expression level of ER alpha and ER beta mRNA in the brain of newborns tended to increase. This pattern of expression varied with the concentration of DE or the developmental stage. In particular, the dose-dependent changes in ER alpha mRNA expressions were shown in males on PND-5 during fetal exposure and in both males and females on PND-2 during neonatal exposure. Dose-dependent changes of ER beta mRNA expression were shown in males on PND-5 and females on PND-16 during fetal exposure. These findings suggest that the timing of the changes in expression level of ER alpha and ER beta mRNA after DE exposure was similar. The expression level of aromatase mRNA was affected by DE exposure in the fetal period but not in the neonatal period. However, perinatal DE exposure appeared to have little effect on the expression level of AR mRNA in the newborn brain. From these results, it appears that DE exposure during the perinatal period may affect the steroid hormone system of the brain dependent on the timing of the exposure.

During the perinatal period, estrogen plays an important role in functional development of the cerebrum, cerebral sexual differentiation, and behavioral development (Takahama and Shirasaki 2001). It is thought that sexual differentiation of the rodent brain is induced by expression of androgens during the perinatal period (critical period), and that these androgens are converted to estrogen by aromatase in the brain (Reddy et al. 1974), and interact with the ER, resulting in masculinization of the nervous system through the induced expression of target genes (MacLusky and Naftolin 1981). If there is no signal through the ER, the nervous system feminizes. The estrogen, which is present at high levels in female blood, binds to fetoprotein expressed specifically during the critical period, and cannot cross the blood–brain barrier (Payne and Katzenellenbogen 1979).

The critical period for cerebral sexual differentiation varies for different animals; however, for mice, it occurs approximately from just before birth to 1 week after birth (MacLusky and Naftolin 1981; Barraclough and Leathem 1954; Kimura 1975). It has been reported that hormone imbalances caused by exogenous steroid hormones, loss of reproductive organs, and various inhibitors disrupt cerebral sexual differentiation (Barraclough 1961; Hayashi and Gorski 1974; Vreeburg et al. 1977). In the present study, expression of ER and aromatase was found to be increased in the mouse brain during the critical period in response to DE exposure. This suggests that perinatal exposure to DE could significantly affect cerebral sexual differentiation. It is thought that the level of ER is regulated by estrogen (Brown et al. 1996; Osterlund et al. 1998), and that expression of ER in males with high levels of estrogen is low. Previously, expression level of ER alpha mRNA was found to be highest in the hypothalamus of female rats on PND-6 (Suzuki and Nishihara 2002). In the present study, however, expression level of ER alpha was highest in the female cerebrum on PND-2 and -5, especially during neonatal exposure. Perhaps, the increase in expression level of ER alpha after DE exposure was a part of the cerebral demasculinization phenomenon. Attardi and Ohno (1976) reported that androgen and ERs in brain cytosol from immature mice can be distinguished by their different specificities and developmental patterns across the whole brain. So, it is hypothesized that the high level of ER mRNA expression in the exposure group may be due to the decrease in testosterone concentration in the brain, which could be due to the decrease in estrogen concentration. This could be due to the decreased aromatase activity, or the effect on the brain of an anti-estrogenic substance present in DE. It has been reported that exposure to steroids or dioxins during the perinatal period causes abnormalities in brain aromatase activity (Roselli and Klosterman 1998; Ikeda et al. 2002; Hany et al. 1999). Moreover, expression of ER and a feedback mechanism are effected by estrogen (Rune et al. 2002; Tsai et al. 2001; Nomura et al. 2003; Shupnik et al. 1989). Estrogen treatment during the neonatal period results in high levels of ER beta in rat hypothalamus (Suzuki and Nishihara 2002), and treatment with bisphenol A (BPA) or octyl phenol, which are endocrine disruptors, during the neonatal period results in high levels of ER alpha (Khurana et al. 2000). The implications of this are unclear, but the present data suggest that DE exposure during the perinatal period may affect the estrogen system in the brain.

Expression of thyroid hormone-related genes

Neonatal DE exposure tended to increase BW of newborns, whereas fetal exposure did not tend to increase it. TR alpha mRNA level overall displayed a tendency to increase on both fetal and neonatal DE exposure. From this study, it appears that BW was associated with TR alpha mRNA level only after neonatal DE exposure; however, BW might also be associated with TR alpha mRNA level after fetal DE exposure. These results suggest that BW increase could be caused by the delayed effect after birth and/or by milk components from exposed dams. There is a report that serum thyroid hormone measurements have been performed after prenatal exposure to DEP (Hougaard et al. 2008). Regrettably, no data on serum thyroid hormone level were collected.

In fetal exposure, BW, and BDNF and neurogranin mRNA levels decreased in females on PND-5; however, these levels did not change in males. The development of the brain in females may be inhibited by DE exposure on PND-5. However, this change was not observed during neonatal exposure. The reasons for this are uncertain, but it

is suggested that the fetal exposure, rather than neonatal exposure, affected development of the brain in females and that BW might be strongly related to BDNF and neurogranin mRNA expressions in fetal exposure.

Thyroid hormones are also thought to increase expression of ER. It appears that crosstalk occurs between the thyroid hormone and estrogen systems. If an adult female is treated with thyroid hormone, expression level of ER mRNA in the hypothalamus will increase, and TR can interact with ER on the transfer level of a gene in the brain (Dellovade et al. 1996). Thyroid hormone, along with steroid hormones in the brain, plays an important role in the development of the CNS during the perinatal period (Koibuchi and Chin 2000). In recent years, chemicals such as PCBs have been shown to disrupt the thyroid hormone system, resulting in abnormalities in brain development (Zoeller et al. 2002).

In the present study, DE exposure during the perinatal period caused an increase in TR alpha mRNA expression level in the brain of newborns only. Moreover, DE had very little effect on expression level of BDNF mRNA, and the changes observed might be due to a generalized increase in expression due to growth in all of the groups, including the control groups. Although expression of neurogranin mRNA tended to decrease in females on PND-5 after fetal DE exposure, it is difficult to evaluate the influence of DE exposure, considering that neurogranin mRNA level in both the fetal and neonatal exposure groups at PND-16 showed increased expression dose dependently in most cases. These findings suggest that perinatal DE exposure may affect the thyroid hormone system in the brain. Measurements of serum thyroid hormone concentrations during perinatal exposure to DE are needed to further elucidate this.

There are two mechanisms proposed to underlie the actions of endocrine disrupting chemicals such as PCBs. One is that endocrine interrupters decrease thyroid hormone concentrations, resulting in abnormal brain development. Winneke et al. (2002) found that exposure to PCB and similar substances during the perinatal period caused decreased thyroid hormone concentrations. Moreover, thyroid hormone replacement can correct at least part of the brain disorder (Goldey and Crofton 1998). The other possibility is that transfer regulation of TR across the blood–brain barrier is lowered (Iwasaki et al. 2002). Thyroid hormone can cross the blood–brain barrier and the choroid plexus-cerebrospinal fluid (Hennemann et al. 2001). In the nervous system, thyroid hormone interacts with TR to alter expression of various genes important for brain development including BDNF, neurogranin, and other neurotrophic factors and transfer factors (Iñiguez et al. 1993; Koibuchi et al. 2001; Calzà et al. 1997). Because DE exposure changed expression level of mRNA for these three genes, DE may affect development of the nervous system.

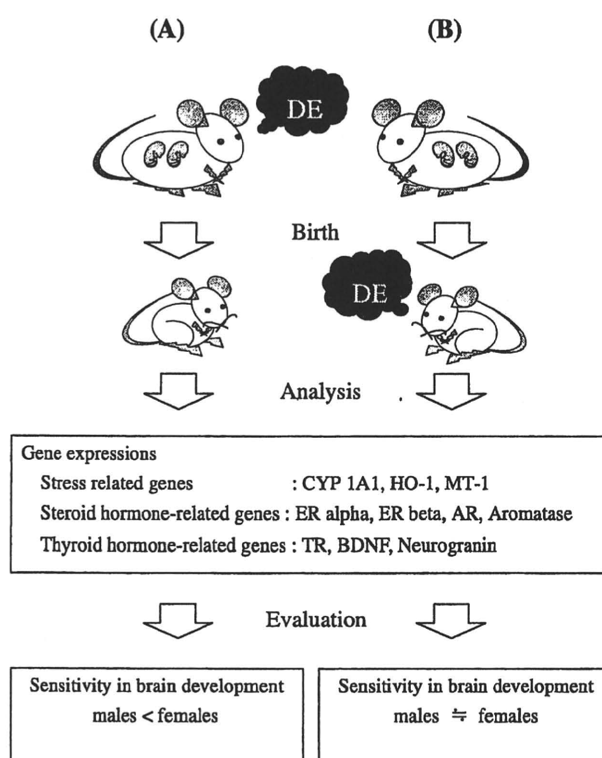

Fig. 1 A diagrammatic representation of the effect of fetal and neonatal DE exposure (**a, b**)

Concerning the difference between males and females, it was discussed that the development of the cerebrum in females might be temporarily affected by DE exposure in the fetal period rather than the neonatal period, but not in males (Fig. 1). Moreover, ER alpha and aromatase mRNA levels on PND-2 in females after fetal exposure were different from those of males. The level of other mRNA species from both exposure groups tended to be similar between males and females as a whole. It is, therefore, suggested that brain development in females showed higher sensitivity to DE after fetal exposure in comparison to males, but for neonatal exposure there was no difference.

The expression of some genes showed linear dose response, whereas that of the others demonstrated inverted U-type response. These results might be explained, at least partly, by the effect of a variety of chemical substances in DE on various complex developmental changes in offspring. Alternatively, endocrine disrupting chemicals with estrogenic, anti-estrogenic, or anti-androgenic activities contained in DE (Furuta et al. 2004; Mori et al. 2002; Taneda et al. 2002; Kizu et al. 2003; Li et al. 2006a, b) might affect each other and induce complicated patterns of dose response. In some cases, these chemicals, as endocrine disruptors in DE, could activate the specific receptors resulting in the inverted U-type response. This could lead to some gene expressions showing different changes.

Evaluation of time course changes in detail or with greater exposure concentrations could find the different results from the results of the present study.

In conclusion, some compounds in DE may reach the brain of newborns during perinatal DE exposure, which results in the disruption of the brain endocrine system, specifically the estrogen system, either directly or indirectly. Further studies, including histological examination of brain tissue, immunohistochemistry, in situ hybridization, and analysis for other mRNA expression are required.

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Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse

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Abstract

Background: Nanotechnology is developing rapidly throughout the world and the production of novel man-made nanoparticles is increasing, it is therefore of concern that nanomaterials have the potential to affect human health. The purpose of this study was to investigate the effects of maternal exposure to nano-sized anatase titanium dioxide (TiO_2) on gene expression in the brain during the developmental period using cDNA microarray analysis combined with Gene Ontology (GO) and Medical Subject Headings (MeSH) terms information.

Results: Analysis of gene expression using GO terms indicated that expression levels of genes associated with apoptosis were altered in the brain of newborn pups, and those associated with brain development were altered in early age. The genes associated with response to oxidative stress were changed in the brains of 2 and 3 weeks old mice. Changes of the expression of genes associated with neurotransmitters and psychiatric diseases were found using MeSH terms.

Conclusion: Maternal exposure of mice to TiO_2 nanoparticles may affect the expression of genes related to the development and function of the central nervous system.

Background

Nanotechnology and the production of novel man-made nanoparticles are increasing worldwide. Titanium dioxide (TiO_2) has a high level of photocatalytic activity, and can be used for air and water purification and self-cleaning surfaces [1]. The activity level of nanoparticles is higher than that of bulk-sized particles [2,3]. TiO_2 has the potential to produce reactive oxygen species (ROS) in its photocatalysis [1] and its possibly detrimental health effects are of concern. It has been reported that a mixture of anatase and rutile TiO_2 nanoparticles induced cytotoxicity against human lung epithelial cells (BEAS-2B), even in the absence of photoactivation [4]. Sayes *et al.* [5] showed that anatase TiO_2 nanoparticles, which can generate more ROS than rutile TiO_2 particles, exhibited a higher level of cytotoxicity against human dermal fibroblasts and human lung epithelial cells (A549) than rutile TiO_2 nanoparticles.

The small size of nanoparticles can bestow unique translocational properties [6,7]. It has been reported that nano-sized elemental carbon particles (36 nm) inhaled by adult rats were translocated into extrapulmonary organs, such as liver [8]. A subsequent study showed that intranasally instilled carbon black nanoparticles can be translocated to the central nervous system, including cerebrum, cerebellum, and olfactory bulb via the olfactory nerve [9]. In a recent study, Takeda *et al.* [10] found that TiO_2 nanoparticles administrated subcutaneously to pregnant mice were transferred from the mother to the fetal brain, and induced apoptosis in the mitral cells of the olfactory bulb of mice exposed maternally to the nanoparticles. Fetal brains are easily affected by blood-borne substances, including nano-sized materials, to a much greater extent than adult brains because the development of the blood-brain barrier in the fetal brains is incomplete [11]. Taking these observations into consideration, functional alterations of the central nervous system induced by maternal exposure to nanoparticles need to be investigated. To analyze the effect of maternal exposure to TiO_2 nanoparticles on the early stages of development of the brain, we used microarray technology and gene expression profiles by functional annotation of genes using Gene Ontology (GO) terms and Medical Subject Headings (MeSH) terms.

Methods

Titanium dioxide nanoparticles

TiO_2 nanopowder (particle size 2570 nm; surface area 2025 m^2/g; crystal form anatase) was purchased from Sigma-Aldrich Japan Inc. (Tokyo, Japan) and used as TiO_2 nanoparticles. The nanopowder was suspended in saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) with 0.05% (v/v) Tween 80 and sonicated for more than 30 minutes immediately before administration.

Animals and treatments

Pregnant ICR mice, purchased from Japan SLC Inc. (Shizuoka, Japan), were housed in a room under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and light (12 h light/12 h dark cycle with light on at 8:00 a.m.) with ad libitum access to food and water. Pregnant mice were transported carefully to minimize stress factors by Sankyo Labo Service Co., Inc (Tokyo, Japan). All animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

A 100 μL volume of TiO_2 suspended at 1 μg/μL was injected subcutaneously into pregnant mice (n = 15) on gestational days 6, 9, 12, and 15 for the exposure group, while 100 μL of vehicle alone was injected into pregnant mice (n = 14) as a control group. Brain tissue was obtained from male fetuses on embryonic day (ED) 16 (n = 8/group) and from male pups on postnatal days 2 (n = 10/group), 7 (n = 10/group), 14 (n = 9/group), and 21 (n = 9/group).

Total RNA extraction

Whole brains were immediately frozen in liquid nitrogen and kept at -80°C. Frozen tissue was homogenized and extracted with Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) while well stirred by a Vortex-Genie 2 (Scientific Industries, Tokyo, Japan). Total RNA was isolated according to the manufacture's protocol and suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Complementary DNA microarray analysis

RNAs for microarray analysis were pooled for each group, purified using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse-transcribed to yield complementary DNA (cDNA) labeled with the fluorescent dye Cy3 or Cy5 using the SuperScript Indirect cDNA Labeling Core Kit (Invitrogen, CA, USA) and the SuperScript Indirect cDNA Labeling System Purification Kit (Invitrogen). Cy3- and Cy5-labeled samples were purified using the CyScribe GFX Purification Kit (GE Healthcare Bio-Sciences, Little Chalfont, UK). The generated targets were mixed and subjected to hybridization to an NIA mouse 15 K Microarray v2.0 (AGC Techno Glass Co. Ltd., Chiba, Japan) consisting of 16,192 gene probes. Microarrays were scanned with two different photomultiplier sensitivities by a ScanArray (Packard BioChip Technologies, MA, USA). The scanner output images were normalized and signal quantification was performed using ScanArray Express (Perkin Elmer, MA, USA) and TIBCO Spotfire (TIBCO Software Inc., CA, USA). Normalization was used so that the overall intensity ratio of Cy3 and Cy5 was equal to 1. Statistical analysis was done with analysis of variance (ANOVA) and the level of statistical significance was set at $P < 0.05$.

Functional analysis of microarray data with gene annotation

A total of 37 GO terms and 66 MeSH terms associated with anatomy, brain development and associated peptides, neurotransmitters, hormones, behavior and psychological phenomena, brain related disorders, oxidative stress, inflammation, and cell death were selected (Table 1, 2); and 2838 and 3625 genes were annotated by GO and MeSH terms, respectively, using the gene reference database PubGene (https://server.pubgene.com/online/PubGene/, Pub Gene AS, Oslo, NOR). These annotations were updated in April, 2008. The genes for which upregulation and downregulation were detected were categorized with GO and MeSH terms. The enrichment factor for each category was defined as $(nf/n)/(Nf/N)$, where nf is the number of differentially expressed genes within the category, n is the total number of genes within that same category, Nf is the number of differentially expressed genes on the entire microarray, and N is the total number of genes on the microarray. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution and the level of statistical significance was set at $P < 0.05$.

Table 1: List of GO terms selected for gene annotation

Category		GO term
biological process	developmental process	brain development
		forebrain development
		midbrain development
		hindbrain development
		generation of neurons
		glial cell differentiation
	biological regulation	cell death
		apoptosis
		neuron apoptosis
		activated T cell apoptosis
		B cell apoptosis
		negative regulation of neuron apoptosis
		apoptotic mitochondrial changes
		induction of programmed cell death
		induction of apoptosis
		anti-apoptosis
		glucocorticoid biosynthesis
		glucocorticoid metabolism
		neurotransmitter metabolism
		neurotransmitter transport
	multicellular organismal process	cognition
		learning and, or memory
	regulation of biological process	regulation of glial cell differentiation
		regulation of nerve growth factor receptor activity
		regulation of glucocorticoid biosynthesis process
	cellular process	mitochondrial fission
		mitochondrial fusion
	response to stimulus	response to oxidative stress
		response to reactive oxygen species
		response to superoxide
		superoxide metabolism
		glutathione biosynthesis
		glutathione metabolism
molecular function		motor activity
		superoxide dismutase activity
		glucocorticoids receptor activity
		brain derived neurotrophic factor binding

Results

Analysis of cDNA microarrays

In the maternal TiO_2 exposure group, the expression levels of 462 genes were changed significantly in the brain of the fetus at ED 16 (upregulation 229 genes; downregulation 233 genes), and those of 864 (upregulation 234; downregulation 630), 417 (upregulation 351; downregulation 66), 738 (upregulation 450; downregulation 288), and 1887 (upregulation 613; downregulation 1274) were changed significantly in the brain of offspring 2, 7, 14, and 21 days old, respectively (Table 3). The number of genes differentially expressed between groups was increased remarkably in the brain of 21 days old pups.

Functional categorization of microarray data

Of the genes expressed differentially in the maternal TiO_2 exposure group, 3, 2, 8, and 4 GO categories were enriched significantly in the brain at 2, 7, 14, and 21 days after birth, respectively (Table 4), while 6, 2, 36, and 28 MeSH categories were enriched significantly at 2, 7, 14, and 21 days after birth (Additional file 1). Eight MeSH categories were also enriched significantly in the fetal brain

Table 2: List of MeSH terms selected for gene annotation

Category	MeSH term	
Anatomy	Blood Brain Barrier Microglia Mitochondria Neuroglia	Neurons Olfactory Receptor Neurons Synapses
Diseases	Alzheimer Disease Anxiety Disorders Attention Deficit Disorder with Hyperactivity Autistic Disorder Cognition Disorders Epilepsy	Inflammation Learning Disorders Memory Disorders Mitochondrial Disease Neurogenic Inflammation Parkinson Disease Schizophrenia
Psychiatry and Psychology	Affective Symptoms Anxiety Cognition Depression Emotions	Memory Memory, Short-Term Motivation Stress, Psychological
Chemicals and Drugs	Apoptosis Inducing Factor Apoptosis Regulatory Proteins Caspases Brain Derived Neurotrophic Factor Glial Cell Line-Derived Neurotrophic Factor Nerve Growth Factor Hormones Glucocorticoids Growth Hormone Thyroid Hormones	Anti-Anxiety Agents Glutathione Glutathione Peroxidase Glutathione Synthase Inflammation Mediators Neuronal Apoptosis-Inhibitory Protein Nitric Oxide Reactive Oxygen Species Superoxides Superoxide Dismutase
Neurotransmitters	Acetylcholine Dopamine Epinephrine gamma-Aminobutyric Acid Glutamic Acid	Norepinephrine Serotonin Receptors, Neurotransmitter Neuropeptides Neurotransmitter Uptake Inhibitors
Biological Science	Apoptosis Cell Death Gene, Mitochondrial Lipid Peroxides	Motor Activity Neural Plasticity Oxidative Stress

at ED 16 (Additional file 1). The largest group of GO categories enriched was those related to cell death 2 21 days after birth; 121 and 64 genes linked to apoptosis at 2 and 7 days after birth, respectively, and 92 and 173 genes linked to "cell death" were identified at 14 and 21 days after birth. "Brain development" was also a large category at 2 (34 genes) and 14 (43 genes) days after birth. GO categories related to oxidative stress, such as "superoxide dismutase activity", were also enriched significantly at 14 and 21 days after birth. The largest MeSH categories enriched were "Mitochondria" at ED 16 (31 genes) and 2 days (56 genes) after birth and "Apoptosis" at 14 (118 genes) and 21 (230 genes) days after birth. The "Mitochondria" category was persistently enriched at 14 (60 genes) and 21 (109 genes) days after birth. MeSH categories related to oxidative stress, such as "Glutathione", "Lipid Peroxidation", and "Reactive Oxygen Species", were also enriched significantly at ED 16 and 14 and 21 days after birth. MeSH categories related to inflammation and neurotransmitters including "Epinephrine", "Norepinephrine", "Serotonin", and "Glutamic Acid" were also highly enriched at 14 and 21 days after birth.

Table 3: The number of genes differentially expressed in maternal TiO_2 exposure group

Age	Upregulated	Downregulated	Total
Embryonic day 16	229	233	462
2 days old	234	630	864
7 days old	351	66	417
14 days old	450	288	738
21 days old	613	1274	1887

Discussion

Nanoparticles have a high level of reactivity with biological tissue, since they have a large specific surface area [6,7]. It has been reported that fullerenes, which are manufactured carbon nanoparticles, induce oxidative stress in the brain of juvenile largemouth bass [12]. Tin-Tin-Win-Shwe *et al.* [13] showed that intranasal instillation of ultrafine carbon black (14 nm) to mice induced a higher level of expression of cytokines and chemokines in the olfactory bulb compared to those induced by the same mass of carbon black (95 nm). The particles used in the exposed pregnant mice group can enter the circulatory system and can transfer to and damage the fetus. Sugamata *et al.* [14] reported that the cytoplasmic granules of granular perithelial cells contain particles of diesel exhaust (DE) and degenerate in both the cerebral cortex and the hippocampus of mice exposed prenatally to DE. A later study [15] showed that maternal DE exposure alters the levels of monoamines and their metabolites in brains and spontaneous motor activity in male mice. Since TiO_2 was detected in the brain of mice maternally exposed to TiO_2 nanoparticles [10], which is the material used in this study, microarray was applied to the analysis of the effects of maternal exposure to TiO_2 nanoparticles on the brain of neonatal mice.

Table 4: Significantly enriched GO categories in maternal exposure group vs. control group

GO term	Enrichment factor	P value
Embryonic day 16		
(None)		
2 days old		
apoptosis	1.04	.05
brain development	1.21	.04
motor activity	1.80	.02
7 days old		
apoptosis	1.11	.01
glial cell differentiation	5.14	.02
14 days old		
activated T cell apoptosis	3.75	.02
brain development	1.48	.00
cell death	1.08	.04
induction of apoptosis	1.28	.01
motor activity	1.58	.05
response to oxidative stress	1.70	.01
response to reactive oxygen species	1.53	.05
superoxide dismutase activity	2.22	.01
21 days old		
anti-apoptosis	1.58	.02
cell death	1.03	.04
glutathione biosynthesis	1.62	.04
superoxide dismutase activity	1.75	.01

In the present study, we used only male fetuses and pups for analysis because the prevalence of some psychiatric disorders in childhood, such as autism and attention deficit hyperactivity disorder, is higher in men than in women. The results of the microarray analysis showed changes in expression of hundreds of genes in the brain at ED 16, and at 2, 7, 14, and 21 days after birth. To interpret the large amount of data generated, functional categorization using GO terms and MeSH terms were performed, which identified potentially important categories on the basis of both a high enrichment factor (>1.00) and statistical significance ($P < 0.05$). MeSH is a controlled vocabulary thesaurus produced by the National Library of Medicine and used for indexing, cataloging, and searching for biomedical and health-related information and documents. Although most researchers use GO for providing annotation to genes, MeSH terms are proposed to be a useful complementary tool for interpretation of microarray data [16]. A subsequent report [17] showed that the use of MeSH has the advantage of producing anatomical and disease information with respect to the genes of interest. In the present study, genes were annotated with the terms related to anatomy, brain development, brain-related disorders, those associated with nanotoxicology (oxidative stress [6,7,12] and inflammation [6,7,13]), and those associated with the effects of maternal exposure to DE or TiO_2 nanoparticles (hormones [18], behavior and neurotransmitters [15,18], and cell death [10,14,19]) for analysis.

As a result, GO terms associated with development of brain were extracted at 2 and 14 days after birth, those associated with cell death, including apoptosis, were extracted 2 to 21 days after birth, and those associated with response to oxidative stress were extracted at 14 and 21 days. Brain development is regulated by neurotrophins such as nerve growth factor, brain-derived neurotrophic factor [20], and glial cell line-derived neurotrophic factor [21], and hormones including growth hormone [22] and thyroid hormone [23,24]. Analysis using MeSH terms showed that alteration of these factors that can lead to abnormal development of the central nervous system was induced by maternal exposure to TiO_2 nanoparticle. It has

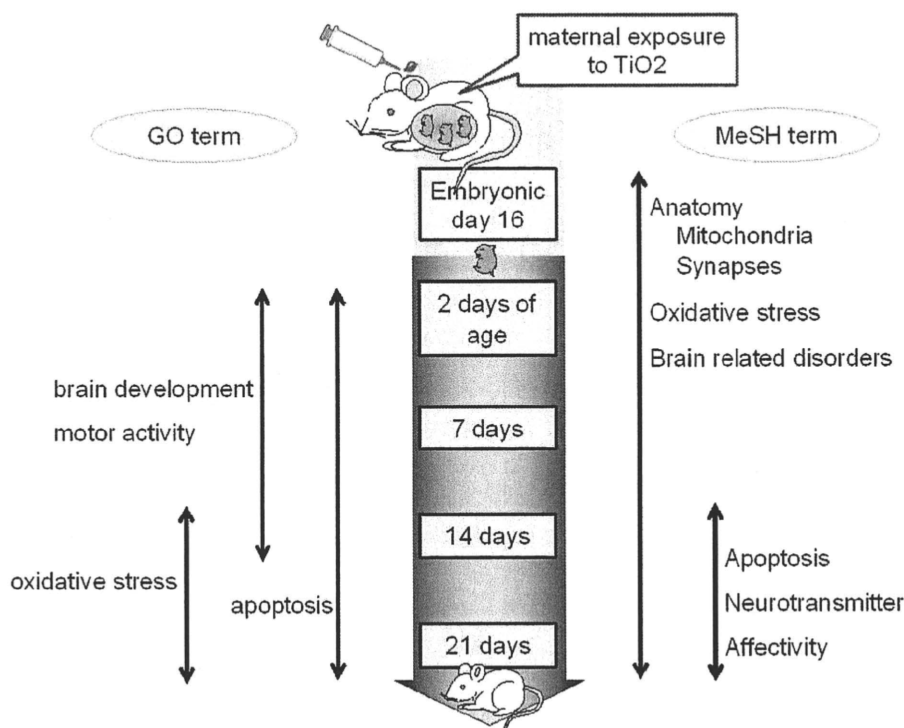

Figure 1
Summary of the extracted terms with genes differentially expressed in the maternal TiO_2 exposure group.

been reported that neuronal cell death, including apoptosis, is essential for elimination of neurons and axons to make correct synaptogenesis in the early stage of brain development [25,26]. The result of functional analysis suggested that disruption of these processes can be caused by maternal exposure to TiO_2 nanoparticles.

It has been reported that the changes of environment surrounding pregnant mice cause abnormal level of neurotransmitters in the brain of the offspring. Meyer *et al.* [27] reported that maternal immune challenge by the viral mimic polyriboinosinic-polyribocytidilic acid causes abnormal fetal dopaminergic development, which is similar to a schizophrenic symptom. Maternal stress also induces altered expression of genes related to the dopaminergic system in the midbrain and causes hyperactivity in adult offspring [28]. The results that MeSH terms associated with neurotransmitters and motor activity were extracted suggest that maternal exposure to TiO_2 nanoparticles causes abnormal levels of neurotransmitters that can lead to altered motor activity.

As for MeSH terms, those associated with diseases were extracted in the functional analysis. Some diseases such as autistic disorder, epilepsy, and learning disorders, occur in childhood, and although Alzheimer's disease, schizophrenia, and Parkinson's disease arise mainly in adulthood or old age, related MeSH terms were extracted in the results from infant mice of mothers exposed to TiO_2. In the early 1990s, Dr David Barker J.P. stated that fetal undernutrition increases the incidence of cardiovascular disease in adult life [29]. Subsequent studies showed the environment that the fetus senses indirectly through the mother can be linked to other diseases in adulthood, and proposed a hypothesis of "early developmental origins of adult disease" [30]. The results of the present study suggest that maternal exposure to nanoparticles can alter gene expression in the neonatal period and might cause the onset of psychiatric disorders even in adulthood. However, the present study did not show how the maternal response to the nanoparticles altered the mother's behavior toward the pups and how this in turn altered gene expression. Further investigations are needed to clarify the critical factor for the gene expression change. Moreover, the changes caused by maternal exposure to TiO_2 nanoparticles should not be limited to the brain. Our published [10] and unpublished data suggest that other organ systems are also affected.

Conclusion

This study showed that maternal exposure to anatase TiO_2 nanoparticle caused the changes in the expression of genes associated with brain development, cell death, response to oxidative stress, and mitochondria in the

brain during the perinatal period, and those associated with inflammation and neurotransmitters in the later stage (Figure 1). Further investigation is needed to clarify the alterations of neurotransmitter levels and motor function. This study showed also that analysis using microarray data with GO and MeSH terms can provide meaningful information, and will contribute to further interpretation of microarray results in toxicological research.

Abbreviations

cDNA: complementally DNA; DE: diesel exhaust; ED: embryonic day; GO: Gene Ontology; MeSH: Medical Subject Headings; ROS: reactive oxygen species; TiO_2: titanium dioxide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT conceived the overall research idea. MS, TO, and KM carried out all procedure for animal experiments. HT, an expert on microarray analysis, had idea to apply GO and MeSH term methods for study of gene expression. MS and HT conducted the microarray analysis. MU participated substantially in the functional analysis of microarray data and drafted the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

***Significantly enriched MeSH categories in maternal exposure group vs. control group.** Additional table.*
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Original Article

Diesel exhaust (DE) aggravates pathology of delayed-type hypersensitivity (DTH) Induced by methyl-bovine serum albumin (mBSA) in mice

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ABSTRACT — Diesel exhaust particles (DEP), a well-known air pollutant, exacerbate type I hypersensitivity conditions, such as asthma and pollen allergy. In this study, we examined the effect of diesel exhaust (DE) exposure on delayed-type hypersensitivity (DTH), a type IV hypersensitivity, induced with methyl-bovine serum albumin (mBSA) in C57BL/6 mice. Mice were exposed to DE containing DEP at a dose of 1.78 mg/m^3 in an inhalation chamber for 14 days. On Day 7, DTH mice and DE-exposed DTH mice were injected s.c. with 200 μl of 1.25 mg/ml mBSA emulsified with CFA in the dorsal region as initial sensitization. On Day 14, mice were injected s.c. into one footpad with 20 μl of 10 mg/ml mBSA dissolved in PBS as challenge. On Day15, footpad thickness and spleen weight were measured. Significant footpad swelling (%) was observed in DTH mice compared with normal control mice, and this swelling was significantly augmented by DE exposure. The levels of pro-inflammatory cytokines, including IFN-γ, TNF-α, and IL-6, in DTH mice were significantly higher than in normal mice, and were also further enhanced by DE exposure. DE exposure increased production of IL-17, which enhances local tissue inflammation through up-regulation of pro-inflammatory cytokines, while production of IL-10, which inhibits local tissue inflammation through suppression of immune cell proliferation, was unchanged. No change was observed in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T regulatory (Treg) cells in splenic lymphocytes following DE exposure. IL-6 production was increased by DE, and this would facilitate the differentiation of naïve T cells to IL-17-producing Th17 cells, while concomitantly suppressing the competing differentiation pathway to IL-10-producing Treg cells. Our results indicate that DE inhalation may, in part, exacerbate the pathological symptoms of DTH and induction of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6 and IL-17.

Key words: DE, DTH, Exacerbation, Pro-inflammatory cytokines

INTRODUCTION

It is well documented that air pollutants exacerbate respiratory diseases, such as asthma (Brunekreef and Holgate, 2002). Diesel exhaust particles (DEP), which are major contributors to the level of airborne particles less than 2.5 micrometers in aerodynamic diameter (PM2.5) in urban areas, consist of a carbon core upon which are absorbed various compounds, including polyaromatic hydrocarbons (PAH), nitroaromatic hydrocarbons, heterocyclics, quinones, aldehydes, aliphatic hydrocarbons and heavy metals (Vouk and Piver, 1983; Draper, 1986). As diesel engines have been modified to obtain higher combustion efficiency, the size of the emitted particles has become smaller and the number of particles per unit mass has become larger. Smaller particles have a

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greater surface area per unit mass and therefore can carry more absorbed chemicals. The smallest particles (with a diameter of < 100 nm) can infiltrate the body through the lungs or nasal membrane, and reach the blood circulation, subsequently appearing systemically (Peters *et al.*, 2006). When inhaled, these particles can translocate into liver (Oberdörster *et al.*, 2002), brain (Oberdörster *et al.*, 2004) and fetus (Sugamata *et al.*, 2006). It has recently been reported that DEP or organic DEP extracts induce oxidative stress and adjuvant effects, leading to modulation of cytokine production, in the respiratory tract and these effects can be blocked by thiol-containing antioxidants such as *N*-acetylcysteine (Sagai *et al.*, 1993; Whitekus *et al.*, 2002). DEP has been shown to exacerbate asthma, accompanied with increases of immunoglobulin E (IgE) and several Th 2 cytokines (Takano *et al.*, 1997). Increase of Th 2 cytokines, such as IL-4, IL-5, IL-6 and IL-10, suppresses Th1 cells, while increase of Th 1 cytokines, such as IFN-γ and TNF-β, suppresses Th2 cells, and disturbance of the Th1/Th2 balance is associated with various immune-related diseases (Shirakawa *et al.*, 1997; Nishimura and Ohta, 1999). It has also been shown that DEP facilitates production of IgE antibodies (Diaz-Sanchez *et al.*, 1994; Tsien *et al.*, 1997) and secretion of Th2 cytokines (Diaz-Sanchez, 1997; Polosa *et al.*, 2002). However, its effect on production of IFN-γ is controversial: in cellular studies DEP suppressed production of IFN-γ *in vitro* (Ohtani *et al.*, 2005) and *in vivo* (Finkelman *et al.*, 2004), but it was found to augment production of IFN-γ in collagen-induced arthritis (CIA) mice (Yoshino and Sagai, 1999).

Delayed-type hypersensitivity (DTH) is induced not via antibody-dependent immune response, but via cellular memory immune response involving neutrophils, macrophages and T cells (Black, 1999; Kobayashi *et al.*, 2001). First, T cells sensitized with antigen generate memory T cells. Second, the recall response of T cells is evoked by challenge. IFN-γ and IL-17, produced by antigen-specific $CD4^+$ T cells, play an important role in the development of DTH response, which reaches a peak 24 to 48 hr after the challenge (Nakae *et al.*, 2002; Schulz *et al.*, 2008). Normalization of the Th1/Th2 balance might attenuate DTH. Th17 cells, which produce IL-17, have recently been identified as pro-inflammatory T helper cells distinct from both Th1 and Th2 cells. These effector cells constitute 1-2% of peripheral $CD4^+$ T cells and specifically express orphan nuclear receptor RORγt. This nuclear receptor, which is a key transcriptional factor that regulates the differentiation of Th17, induces transcription of the gene encoding IL-17 (Ivanov *et al.*, 2006). IL-17 enhanced local tissue inflammation through the up-regulation of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines, thereby enabling activated T cells to penetrate the extracellular matrix (Qian *et al.*, 2007). On the other hand, regulatory T (Treg) cells, which constitute 5-10% of peripheral $CD4^+$ T cells (1-2% of peripheral lymphocytes), play an important role in the maintenance of peripheral immune tolerance and prevention of immune-mediated diseases by suppressing immune responses (Taylor *et al.*, 2001). Failure of Treg cell function is associated with the development of various autoimmune diseases, including experimental allergic encephalomyelitis (EAE) and CIA (Kelchtermans *et al.*, 2005; Mimran and Cohen, 2005). These functional suppressor cells express CD4, IL-2 receptor α-chain (CD25), and the transcriptional factor Foxp3. Foxp3 is specifically expressed in Treg cells, and this distinguishes Treg cells from $CD4^+CD25^+$ T cells, which lack regulatory function (Fontenot *et al.*, 2003). It was suggested that DEP blocks the induction of oral tolerance in the DTH response induced with hen egg lysozyme (Yoshino *et al.*, 1998). Oral tolerance is classically defined as the suppression of immune response to an antigen that has been administered previously via the oral route. Low-dose antigens favor the induction of Th2 and Treg cell subsets that mediate active suppression of antigen-specific T cells (Miller *et al.*, 1992), whereas high-dose antigens favor induction of clonal anergy or deletion (Whitacre *et al.*, 1991; Melamed and Friedman, 1993). Oral tolerance is suppressed in several animal models of autoimmune disease, including arthritis (Thompson and Staines, 1986) and EAE (Higgins and Weiner, 1988), as well as non-autoimmune diseases, such as asthma (Nakao *et al.*, 1998) and DTH (Miller and Hanson, 1979; Mowat *et al.*, 1982). The effects of DEP on these processes remain to be fully established. In the present study, we examined the effect of DE exposure on the pathology symptoms and pro-inflammatory cytokine profiles of DTH induced with methyl bovine serum albumin (mBSA) in C57BL/6 mice. The results indicate that DE inhalation exacerbates the pathological symptoms of DTH systematically through induction of pro-inflammatory cytokines.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6 weeks of age) were purchased from Sankyo Labo Service (Tokyo, Japan), kept for 1 week at animal facility and used at 7 weeks of age. They were housed in wire-mesh cages at a temperature of 22 + 2°C and relative humidity of 55 + 10% under a 12 hr light-dark cycle, and were allowed free access to

tap water and experimental normal diet, Labo MR Stock (Nosan Co., Kanagawa, Japan). Mice divided into a normal group, a DTH-disease control group, a DE exposured normal group and a DE exposured DTH-disease group. Each group consisted of 5 mice. They were treated and handled according to the Guide Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee.

Exposure of mice to DE

Mice were exposed to DE in an inhalation chamber at the Nano-particles Health Science Research Center, Tokyo University of Science (Chiba, Japan) according to the method described elsewhere Umezawa *et al.*, (2008). A 2179-cc diesel engine (Isuzu Automobile, Inc., Tokyo, Japan) was operated at 1,580 rpm with commercial diesel oil. The exhaust was introduced into a stainless steel dilution tunnel and mixed with clean air that was passed through a high-efficiency particulate air filter and a charcoal filter. The diameter distributions of DEP in the exhaust was analyzed using a scanning mobility particle sizer (SMPS, model 3936, TSI Inc., St. Paul, MN, USA). The SMPS apparatus comprised a condensation particle counter (CPC, model 3785) and a differential mobility analyzer (DMA, model 3081). Concentrations of DEP in DE gas were adjusted to approximately 0.1 and 1.78 mg/m^3.

Experimental design for DE exposure and immunization with mBSA (Yoshimoto *et al.*, 2000)

As shown in Fig. 1, mice were divided into 4 groups with or without DE exposure and with or without immunization with mBSA to induce DTH. Normal mice and DTH mice were exposed to DE (0.1 or 1.78 mg DEP/m^3; 8 hr/day) from Day1 to Day 14, or housed with clean air as controls. On Day 7, DTH mice and DE-exposed DTH mice were injected s.c. with 200 µl of 1.25 mg/ml mBSA (Sigma) emulsified with CFA (Chondrex, Redmond, WA, USA) in the dorsal region as initial sensitization. On Day 14, mice were injected s.c. into one footpad with 20 µl of 10 mg/ml mBSA dissolved in PBS. An equal volume of PBS was injected into another footpad as a control. At 24 hr after the challenge, footpad thickness was measured with a digital caliper. DE exposures were conducted at doses of 0.1 and 1.78 mg DEP/m^3 under the same experimental protocol as shown in Fig. 1. In this report, only data obtained at a dose of 1.78 mg DEP/m^3 are presented in this study, since DEP at a dose of 0.1 mg/m^3 appeared to have little effect.

Determination of footpad swelling and splenomegaly

The magnitude of the DTH response was determined as follows: [footpad swelling (mm)] = [footpad thickness of mBSA-injected footpad (mm)] - [footpad thickness of PBS-injected footpad (mm)], [footpad swelling (%)] = ([footpad swelling (mm)]/[footpad thickness of PBS-injected footpad (mm)]) x 100. Splenomegaly was deter-

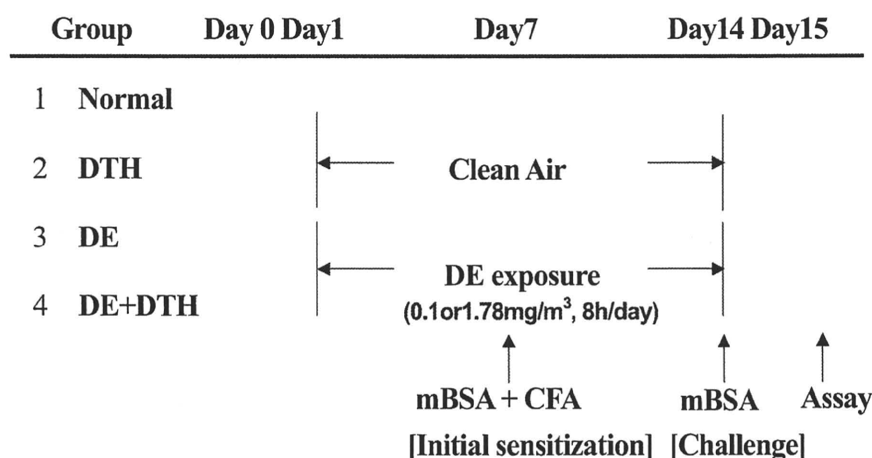

Fig. 1. Experimental design for exposure to DE and immunization with mBSA. Mice of groups 3 and 4 were exposed to DE (1.78 mg DEP/m^3; 8 hr/day) for 14 days. Mice of groups 1 and 2 were housed in clean air as controls from Day1 to Day 14. On Day 7, mice of groups 2 and 4 were injected s.c. with 200 µl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization and on Day14, they were further injected s.c. with 20 µl of 2.5 mg/ml mBSA into one foot-pad as the challenge. At 24 hr after the challenge, footpad thickness and spleen weight were measured.

mined as follows: [splenomegaly (%)] = [spleen weight (g)] / [body weight (g)] x 100.

Reagents

Anti-mouse IFN-γ mAb (XMG1.2), biotin-conjugated anti-mouse IFN-γ mAb (R4-6A2), purified anti-mouse IL-4 mAb (11B11), biotin-conjugated anti-mouse IL-10 mAb (JES5-2A5), purified anti-mouse TNF-α mAB (1F3F3D4), biotin-conjugated anti-mouse TNF-α mAb (MP6-XT22), purified anti-mouse IL-6 mAb (MP5-20F3) and biotin-conjugated anti-mouse IL-6 mAb (MP5-32C11) were purchased from eBioscience (San Diego, CA, USA). Purified anti-mouse IL-10 mAb (JES-16E3), purified anti-mouse IL-17 mAb (TC11-18H10.1), biotin-conjugated anti-mouse IL-17 mAb (TC11-8H4) and recombinant mouse IL-17 were purchased from BioLegend (San Diego, CA, USA). Recombinant mouse IFN-γ, TNF-α IL-6 and IL-10 were purchased from eBioscience. Phycoerythrin (PE)-conjugated anti-mouse CD3 mAb (17A2), which recognizes T cell receptor-associated complex present on all mature T cells; PE-Cy5-conjugated anti-mouse CD4 mAb (H129.19), which recognizes protein on helper T cell; FITC-conjugated anti-mouse CD8a mAb (53-6.7), which recognizes protein on cytotoxic T cells; and FITC-conjugated anti-mouse CD19 mAb (1D3), which recognizes protein on B cells, were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). PE-Cy5-conjugated anti-mouse CD38 mAb (90), which recognizes protein on plasma cells; FITC-conjugated anti-mouse CD25 mAb (PC61.5), which recognizes protein on activated T cells; and PE-conjugated anti-mouse Foxp3 mAb (FJK-16a), which recognize a transcriptional factor specifically expressed in Treg cells, were purchased from eBioscience.

Phenotype of lymphocytes

Immunophenotyping of splenic lymphocytes was carried out using a FACS-LSR flow cytometer (Becton Dickinson, San Jose, CA, USA). Spleens were harvested from normal mice and DTH mice with or without DE exposure on Day 15. Each spleen was minced with scissors, and a single-cell suspension was prepared. Splenocytes were purified by means of hemolysis, counted under a microscope, and suspended in RPMI1640-based buffer (containing 102 mM NaCl, 5 mM KCl, 0.4 mM $CaCl_2$, 0.4 mM $MgSO_4$, 23.8 mM $NaHCO_3$, 5.6 mM Na_2HPO_4, 11.1 mM glucose and 10 mM HEPES-NaOH; pH 7.4) at a concentration of 2 x 10^6 cells/ml for use as samples. Cells were stained with fluorochrome-conjugated antibodies for 30 min, and washed. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson). The percentages of T cells ($CD3^+$), helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), B cells ($CD19^+$) and plasma cells ($CD3^-CD19^-CD38^+$) in splenocytes were calculated.

Analysis of Treg cells

Splenocytes were prepared from normal and DTH mice with or without DE exposure on Day 15. Cells (5 x 10^6) were stained with PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD25 antibodies for 30 min at room temperature, washed with RPMI1640-based buffer, fixed with 4% para-formaldehyde for 10 min on ice, and then treated with 0.1% Triton X-100 for 5 min at 4°C. After having been washed with RPMI1640-based buffer and blocked with 1% BSA/PBS(-) for 30 min at room temperature, the splenocytes were stained with PE-conjugated anti-Foxp3 antibody for 1 hr at room temperature, and washed with RPMI1640-based buffer for assay. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson).

Assays of INF-γ, TNF-α, IL-6 , IL-17 and IL-10

Splenocytes were suspended in RPMI1640 medium containing 10% FBS at a concentration of 7 x 10^6 cells/ml and cultured with mBSA (20 mg/ml) in a 6-well plate. After incubation at 37°C under an atmosphere of 5% CO_2 for 96 hr, the culture supernatant was harvested for assays of IFN-γ, IL-10, TNF-α, IL-6 and IL-17. The concentrations of these cytokines were measured by ELISA as described below. A 96-well plate was coated with purified anti-mouse IFN-γ (XMG1.2) (1:500), IL-10 (JES-16E3) (1:250), TNF-α (1F3F3D4) (1:500), IL-6 (MP5-20F3) (1:500), or IL-17 (TC11-18H10.1) (1:250) mAb. Nonspecific binding was blocked with 1% bovine serum albumin. Culture supernatant was added for 2 hr at room temperature. The plate was washed and anti-mouse biotin-conjugated IFN-γ (XMG1.2) (1:500), IL-10 (JES5-2A5) (1:500), TNF-α (1F3F3D4) (1:1000), IL-6 (MP5-20F3) (1:500), or IL-17 (TC11-8H4) (1:500) mAb was added. The plate was then washed, and avidin-horseradish peroxidase (Sigma, Saint Louis, MO, USA) was added. The plate was washed again, and 3,3',5,5'-tetramethylbenzidine was added and left for a few min. The reaction was stopped by adding 2.5 M H_2SO_4, and the absorbance at 450 nm was measured with an Immuno Reader NJ-2000 (Nihon Inter Med, Tokyo, Japan). These antibodies were purchased from eBioscience. The assay sensitivities were 16-1,000 pg/ml (IFN-γ system), 2-125 pg/ml (TNF-α system), 8-2,000 pg/ml (IL-6 system), 63-4,000 pg/ml (IL-17 system) and 63-4,000 pg/ml (IL-10 system). Values are the average ± S.E.M. of three independent assays.

Statistics

Each value is given as the mean ± S.D. Comparison between two values was performed by using the unpaired Student's t-test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's post hoc analysis. Calculations were done with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA, USA). The criterion of significance was $P < 0.05$.

RESULTS

The particle diameter distribution of the DEP in the DE gas was shown in Fig. 2. The mean diameter of DEP was 104 nm. Though chemicals and gaseous components included in DE were not examined in this study, they would be almost similar to those which were already reported by our research group (Ono *et al.*, 2008). DE exposures were conducted at doses of 0.1 and 1.78 mg DEP/m^3 according to the protocol as shown in Fig. 1. Since DE exposure at the dose of 0.1 mg DEP/m^3 had little effect, only data obtained at the dose of 1.78 mg/m^3 are presented in this section.

Footpad swelling and splenomegaly in normal and DTH mice with or without DE exposure were investigated in order to determine the effect of DE on mBSA-induced DTH. Significant footpad swelling was observed in DTH mice, compared with normal control mice. The footpad swelling in DTH mice was further augmented by DE exposure (Fig. 3A). No significant splenomegaly was observed in any of the groups (Fig. 3B).

Pro-inflammatory cytokines involved in the pathology of DTH were assayed. The levels of IFN-γ and TNF-α in DTH mice were both significantly increased in comparison with those in normal control mice. DE exposure significantly increased the level of TNF-α, but not that of IFN-γ in normal control mice (Figs. 4A and B). However, these values in DE-exposed DTH mice were significantly higher than those in DTH mice. The level of IL-6, which induces activation of B cells and antibody production, was also significantly increased in DTH mice compared with that in normal control mice , and further enhanced by DE exposure (Fig. 4C). Moreover, we examined the effect of DE exposure on the production of IL-17 by Th17 cells. The level of IL-17 in DTH mice was much higher than that in normal control mice, and the level was further significantly augmented by DE exposure (Fig. 5A). It is likely that augmented production of these pro-inflammatory cytokines is involved in the exacerbation of DTH pathology induced by DE exposure. Next, anti-inflammatory cytokine IL-10 was assayed. As shown in Fig. 5B, the level of IL-10 was higher in DTH mice than in normal control mice, but there was no difference in IL-10 production between DTH mice and DE-exposed DTH mice.

To elucidate the involvement of Treg in the DE-induced exacerbation of DTH, changes in the population of Treg cells in CD4$^+$ T cells were examined. CD4$^+$CD25$^+$Foxp3$^+$ T cells were considered as Treg cells in this study. The percentage of CD25$^+$Foxp3$^+$ T cells in CD4$^+$ T cells of DTH mice was slightly, but not significantly, higher than that of normal control mice, and DE exposure had no effect on the value in DTH mice (Fig. 6A). The percentages of helper T cells (CD3$^+$CD4$^+$), cytotoxic T cells (CD3$^+$CD8$^+$), B cells (CD19$^+$) and plasma cells (CD3$^-$CD19$^-$CD38$^+$) in splenocytes in all groups were analyzed. No significant differences were found (Fig. 6B).

DISCUSSION

In this study, we examined the effect of DE exposure in a mouse model of DTH. The significant footpad swelling observed in DTH mice compared with normal control

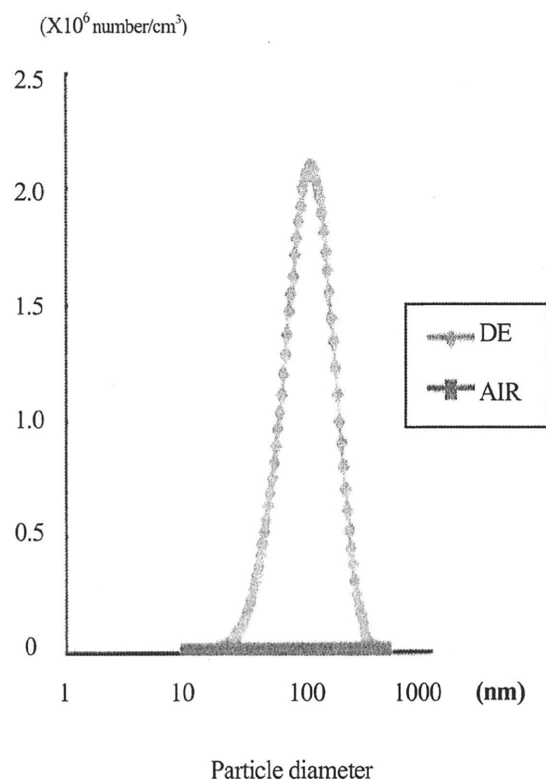

Fig. 2. Particle diameter distribution of diesel exhaust particles in diesel exhaust gas.

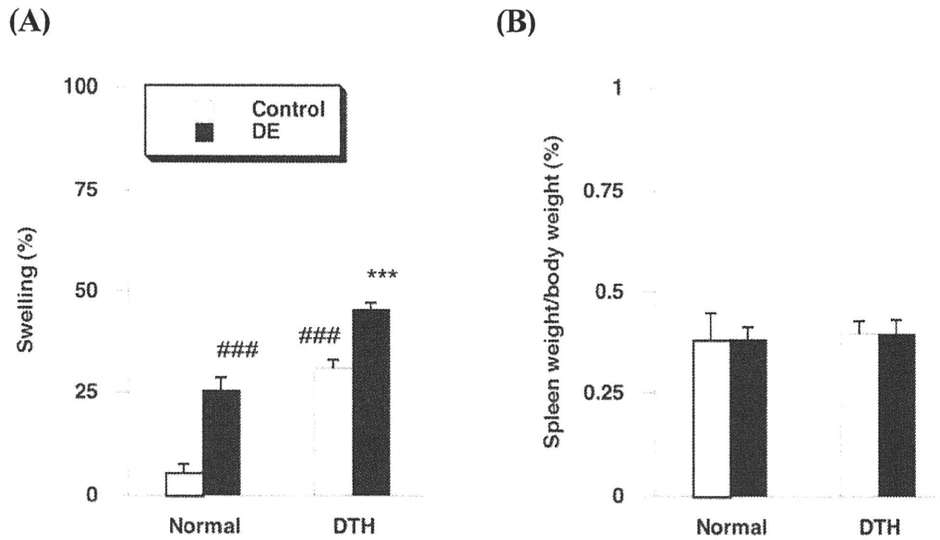

Fig. 3. Effects of DE exposure on footpad swelling and splenomegaly in DTH mice. Mice were exposed to DE (1.78 mg DEP/m^3; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μl of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On Day15, footpad thickness and spleen weight were measured to determine footpad swelling and splenomegaly as described in "Materials and Methods". (A) Footpad swelling and (B) Splenomegaly. Each value represents the mean ± S.D. for five mice. ###Statistically significant difference ($p < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

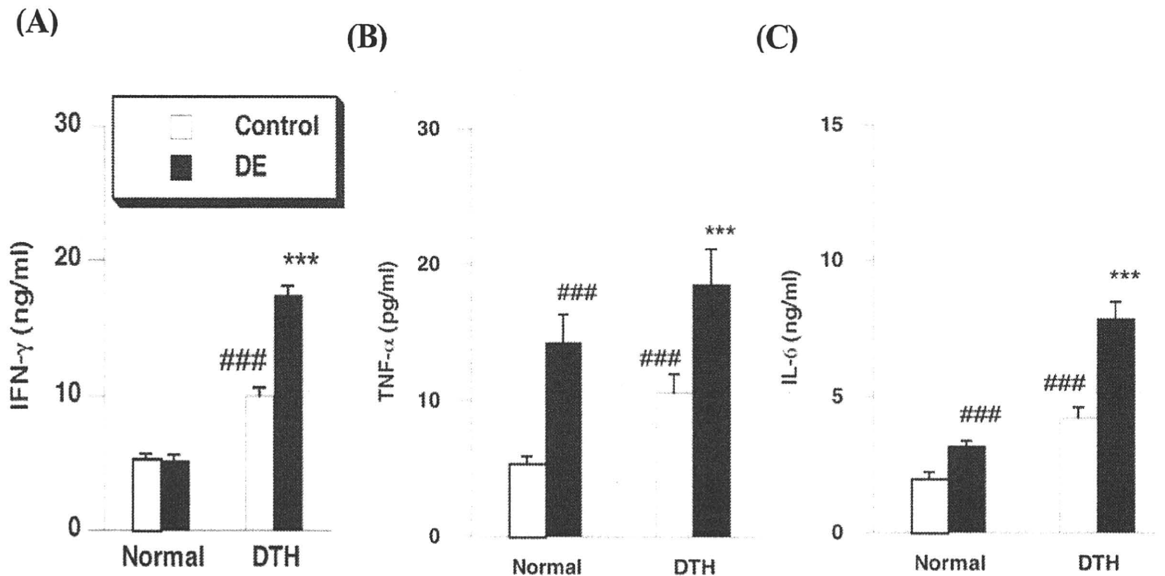

Fig. 4. Effect of DE exposure on production of IFN-γ, TNF-α and IL-6 by splenocytes. Mice were exposed to DE (1.78 mg DEP/m^3; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μl of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day15, the spleen was harvested. Splenocytes (7 x 10^6 cells/ml) were cultured with mBSA (10 mg/ml) for 96 hr in a CO_2 incubator. The supernatants were subjected to assays of IFN-γ, TNF-α and IL-6 by ELISA as described in "Materials and Methods". (A) IFN-γ, (B) TNF-α, and (C) IL-6. Each value represents the mean ± S.D. for four or five mice. ###Statistically significant difference ($P < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

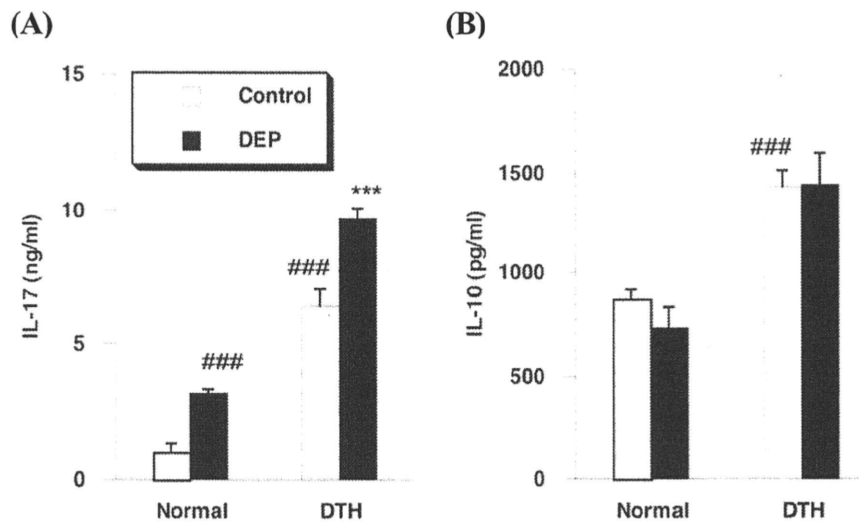

Fig. 5. Effect of DE exposure on production of IL-17 and IL-10 by splenocytes. Mice were exposed to DE (1.78 mg DEP/m^3; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μl of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day15, the spleen was harvested. Splenocytes (7 x 10^6 cells/ml) were cultured with mBSA (10 mg/ml) for 96 hr in a CO_2 incubator. The supernatants were subjected to assays of IL-17 and IL-6 by ELISA as described in "Materials and Methods". (A) IL-17 and (C) IL-10. Each value represents the mean ± S.D. for four or five mice. ###Statistically significant difference ($P < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

mice was further increased by DE (Fig. 3A). Interestingly, footpad swelling was also observed in DE-exposed normal control mice. These results indicate that DE activates the immune system. The immune system should be activated in DTH-mice, and further augmented by DE exposure, resulting in splenomegaly, although splenomegaly, which would reflect activation of immune cells, unexpectedly was not observed in any of the groups (Fig. 2B). The activation and the augmentation should have been evaluated by the assay of proliferation activity, such as a ^{3}H-thymide incorporation study, of splenic lymphocytes. Further studies will be required for full elucidation of these details.

The effect of DE exposure on the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-6, was also examined. These cytokines are involved in the development of DTH. Increased productions of IFN-γ, TNF-α and IL-6 were observed in DTH mice, and were further increased by DE exposure. It was reported that DEP induces mRNAs of IL-6 and chemokines such as monocyte chemoattractantprotein-1 (MCP-1) and keratinocyte-derived chemokine (KC) in TNF-α deficient mice (Saber *et al.*, 2006). IL-6 is considered to play a more important role in DEP-induced inflammation than TNF-α, because it controls the initial steps of the T-cell activation with enhancement of IL-2 responsiveness (Lotz *et al.*, 1988) and Th17 cell differentiation (Bettelli *et al.*, 2006). Naive T helper cells differentiate into Th17 cells and Treg cells under the influence of IL-6 and TGF-β and IL-2 and TGF-β, respectively. Further, IL-6 suppresses the development of Treg cells (Fong and Mosmann, 1989). That is to say, IL-6 regulates the development of Th17 cells and Treg cells. Though IFN-γ plays an important role in the development of DTH, as mentioned above, the pathology of DTH is actually exacerbated in IFN-γ knock-out mice (Irmler *et al.*, 2007). A similar phenomenon has been found in other Th1-dominant autoimmune diseases, such as CIA, EAE and experimental autoimmune uveitis (Caspi *et al.*, 1994; Ferber *et al.*, 1996; Jones *et al.*, 1997). In other words, IFN-γ is indispensable in the development of DTH and other immune-related diseases, but also has a protective effect against them. This seems paradoxical from the viewpoint of the Thl paradigm of DTH. However, the apparent paradox has been resolved by the discovery of Th17 cells, a third subset of T helper cells that secrete IL-17. It is reported that IL-17 and IFN-γ are both involved in the development of these cytokine-producing cells during immune responses, and IL-17 plays a more crucial role in DTH than IFN-γ; IL-17 can induce the expression of a variety of genes encoding many cytokines

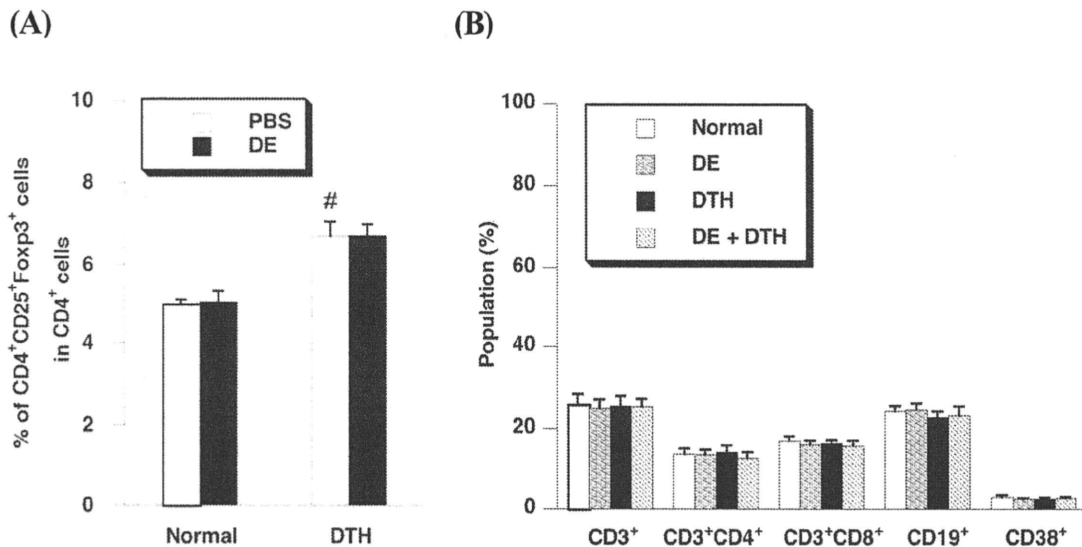

Fig. 6. The percentages of T cells (CD3+), helper T cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), B cells (CD19+) and plasma cells (CD38+) in splenocytes. Mice were exposed to DE (1.78 mg DEP/m^3; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μl of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day 15, the spleen was harvested. Immunophenotyping of splenic lymphocytes was carried out by flow cytometry as described in "Materials and Methods". Each value represents the mean ± S.D. for four or five mice. #Statistically significant difference ($p < 0.05$) compared to the normal control group.

involved in the pathology of DTH (Schulz *et al.*, 2008). In fact, Th17 cells and IL-17 production are known to be increased in DTH mice, and blocking IL-17 can ameliorate DTH (Nakae *et al.*, 2002; Irmler *et al.*, 2007). We found that DEP exposure significantly augmented the production of IL-17 in DTH mice. This result may suggest that increased production of IL-17 is a key mediator of the adverse effect of DE.

Inhibition of oral tolerance has been reported in DEP-administered DTH mice (Yoshino *et al.*, 1998). It can be hypothesized that DEP exposure inhibits Treg cells or activates antigen-specific T cells to exacerbate inflammation in the DTH model. Thus, we measured the percentage of Treg cells in each group; however, no significant difference was observed between DTH mice and DE-exposed DTH mice (Fig. 6A). IL-10, produced by Th2 and Treg cells, is an anti-inflammatory cytokine known to inhibit production of IL-1, IL-4, IL-5, IL-6, IL-8, TNF-α and IFN-γ (Del Prete *et al.*, 1993; Wang *et al.*, 1994). We found no difference in IL-10 production between DTH mice and DE-exposed DTH mice (Fig. 5B). These results may imply that Treg cells are not involved in the DE exposure-induced exacerbation of DTH. Further, we found no significant difference in the percentages of other lymphocytes (CD3+, CD3+CD4+, CD3+CD8+, CD19+, CD38+) between DTH mice and DE-exposed DTH mice (Fig. 6B).

In conclusion, it has been demonstrated that DEP inhalation may, in part, exacerbate the pathological symptoms of DTH and induction of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6, and IL-17.

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