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Letter

Effect of fetal exposure to titanium dioxide nanoparticle on brain development − brain region information

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ABSTRACT — The production of man-made nanoparticles is increasing in nanotechnology, and health effect of nanomaterials is of concern. We previously reported that fetal exposure to titanium dioxide (TiO_2) affects the brain of offspring during the perinatal period. The aim of this study was to extract candidate brain regions of interest using a specific group of Medical Subject Headings (MeSH) from a microarray dataset of the whole brain of mice prenatally exposed to TiO_2 nanoparticle. After subcutaneous injection of TiO_2 (total 0.4 mg) into pregnant mice on gestational days 6-15, brain tissues were collected from male fetuses on embryonic day 16 and from male pups on postnatal days 2, 7, 14 and 21. Gene expression changes were determined by microarray and analyzed with MeSH indicating brain regions. As a result, a total of twenty-one MeSH were significantly enriched from gene expression data. The results provide data to support the hypothesis that prenatal TiO_2 exposure results in alteration to the cerebral cortex, olfactory bulb and some regions intimately related to dopamine systems of offspring mice. The genes associated with the striatum were differentially expressed during the perinatal period, and those associated with the regions related to dopamine neuron system and the prefrontal region were dysregulated in the later infantile period. The anatomical information gave us clues as to the mechanisms that underlie alteration of cerebral gene expression and phenotypes induced by fetal TiO_2 exposure.

Key words: Titanium dioxide, Nanoparticle, Brain region, Medical Subject Headings, Microarray, Dopamine system

INTRODUCTION

Nanocrystalline titanium dioxide (nano-TiO_2) is an important material used in commerce and can be found in paints, cosmetics, food additives and implanted biomaterials. The activity level of nanoparticles is higher than that of fine or bulk-sized particles (Beydoun *et al*., 1999; Jang *et al*., 2001; Sager *et al*., 2008), and its possibly detrimental health effects are of concern (Borm *et al*., 2006). With the increase in large scale production of manufactured nanoparticles, the potential occupational and public exposure to manufactured nanoparticles has aroused concern because of their large surface areas and the ability to deposit in the body (Oberdorster *et al*., 2005). Evaluating the mechanisms underlying the hazards associated with TiO_2 nanoparticles is vital for risk assessments (Johnston *et al*., 2009).

TiO_2 has three structural isoforms, anatase, rutile and brookite. Since the anatase form of TiO_2 was reported to be toxic than the rutile form (Sayes *et al*., 2006), we previously examined the effects of fetal exposure to anatase-formed nano-TiO_2 on the central nervous system in a mouse model. Transfer of nano-TiO_2 from pregnant mother mice to fetal brains was determined using field emission-scanning electron microscope/energy dispersive X-ray spectroscopy in 2009 (Takeda *et al*., 2009). A subsequent study showed that exposure of pregnant mice to nano-TiO_2 altered central dopaminergic system in offspring (Takahashi *et al*., 2010). Transfer of nano-TiO_2 administered to pregnant mice to fetal brain as well

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as fetal liver was also reported (Yamashita *et al*., 2011). A previous study conducted microarray analysis of gene expression change the whole brain of neonatal mice (ED 16-PND 21) by fetal nano-TiO_2 exposure, and showed dysregulation of genes associated with apoptosis, brain development, oxidative stress and neurotransmitters (Shimizu *et al*., 2009). However, since the data was provided from samples of whole brains, it was not possible to obtain information on the brain regions that were of importance.

Here, we propose that a method of analysis using a selected group of Medical Subject Headings (MeSH) vocabulary, a controlled vocabulary produced by the National Library of Medicine (Bethesda, MD, USA), which can provide anatomical information from dysregulated gene group. In the present study, the genes dysregulated in the brain by fetal TiO_2 exposure were categorized by MeSH that indicates brain regions. The aim of the present study was to show candidate brain regions of interest for the effects of maternal TiO_2 exposure on the development of central nervous system based on a microarray data.

MATERIALS AND METHODS

Titanium dioxide nanoparticle

Anatase TiO_2 nanopowder (particle size 25-70 nm; surface area 20-25 m^2/g; Sigma-Aldrich Japan Inc., Tokyo, Japan) was suspended at 1 mg/ml in saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) containing 0.05% (v/v) Tween 80. The suspension was sonicated for 30 minutes immediately before administration.

Animals and treatments

Twenty-nine pregnant ICR mice (Japan SLC Inc., Shizuoka, Japan) were housed in a room under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and light (12 hr light/12 hr dark cycle with light on at 8:00 a.m.) with *ad libitum* access to food and water. All animals were handled in accordance with national guidelines for the care and use of laboratory animals and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee. They were randomly divided into fetal TiO_2 exposure group (n = 15) and control group (n = 14). TiO_2 (100 μg/time) suspension was injected subcutaneously into pregnant mice of the exposure group four times on gestational days 6, 9, 12 and 15 for exposure group, while vehicle alone (100 μl/time) was injected into those of the control group.

Preparation of microarray data

Brain tissues were collected from male fetuses on embryonic day (ED) 16 and from male pups on postnatal days (PNDs) 2, 7, 14 and 21. From the whole brain samples, total RNA was isolated and pooled for each group, purified and reverse-transcribed to yield complementary DNA and then labeled with the fluorescent dyes Cy3 and Cy5. The generated targets were hybridized to an NIA mouse 15 K Microarray v2.0 (AGC Techno Glass Co., Ltd., Chiba, Japan). The microarray scan output images were normalized and signal quantification was performed according to the MIAME guidelines (Brazma *et al*., 2001). Statistical analysis was performed using analysis of variance (ANOVA); $P < 0.05$ was considered statistically significant.

Analysis of microarray data with Medical Subject Headings

A total of 87 MeSH associated with brain regions were selected (Supplementary Table 1) and these 83 MeSH were mapped to the 2,037 genes on the microarray using the gene reference database PubGene (Pub Gene AS, Oslo, Norway), which can determine the literature co-occurrences between genes and the medically functional terms (MeSH) (Jenssen *et al*., 2001). The annotation was updated in October, 2009. The genes for which dysregulation was detected were categorized with MeSH. 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 categorized by any categories on the entire microarray; and N is the total number of genes categorized by any categories on the microarray. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution and the level of statistical significance was set at $P < 0.05$. The method was based on a principle of gene set enrichment analysis to interpret complex microarray data (Subramanian *et al*., 2005).

RESULTS

Microarray analysis

2,037 genes on the microarray were annotated by 83 MeSH related to brain regions by the PubGene. Significant expression changes were detected in 78 genes in fetal brains (23 upregulated genes; 55 downregulated genes) at ED 16, and 158 genes (31 upregulated; 127 downregulated), 70 genes (64 upregulated; 6 downregulated), 138 genes (70 upregulated; 68 downregulated) and 262 genes (181 upregulated; 81 downregulated) in the brains of off-

Table 1. Significantly enriched MeSH categories of dysregulated genes in the maternal TiO_2-exposed group vs. control group

Age at specimen collection	MeSH category	Enrichment factor	*P* value
Embryonic day 16			
	Corpus Striatum	1.48	0.04
Postnatal day 2			
	Cerebral Aqueduct	6.45	0.03
	Olfactory Bulb	1.39	0.03
	Entorhinal Cortex	1.59	0.03
	Hippocampus	1.10	0.04
Postnatal day 7			
	Basal Ganglia	3.64	0.004
	Lateral Ventricles	2.97	0.02
	Frontal Lobe	2.39	0.03
	Neostriatum	2.39	0.03
	Hypothalamic Area, Lateral	2.60	0.03
	Substantia Nigra	1.77	0.04
Postnatal day 14			
	Olfactory Bulb	1.75	0.003
	Trigeminal Caudal Nucleus	5.54	0.01
	Caudate Nucleus	2.25	0.02
	Hippocampus	1.16	0.03
	Cerebrum	4.03	0.03
	Neostriatum	1.82	0.03
	Corpus Striatum	1.31	0.04
	Amygdala	1.64	0.04
Postnatal day 21			
	Prefrontal Cortex	1.86	0.02
	Entorhinal Cortex	1.52	0.02
	Trigeminal Nucleus, Spinal	1.13	0.03
	Cerebellar Cortex	1.44	0.03
	Hippocampus	1.06	0.04
	Olivary Nucleus	1.88	0.04
	Corpus Striatum	1.16	0.04
	Pyramidal Cells	1.27	0.04
	Hypothalamus, Anterior	1.16	0.05

The enrichment factor for each category was defined as $(nf/n)/(Nf/N)$, as described in the Materials and Methods. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution.

spring at PNDs 2, 7, 14 and 21, respectively.

Categorization of microarray data using brain-region specific MeSH

Of the genes expressed differentially in the fetal TiO_2 exposure group, one MeSH category was significantly enriched in the brain at ED 16, whereas 4, 6, 8 and 9 MeSH categories were significantly enriched at PNDs 2, 7, 14 and 21, respectively (Table 1). "Corpus Striatum" was enriched on ED 16 and on PND 14 and 21. The largest group was "Hippocampus" on PND 2 (62 genes), 14 (57 genes) and 21 (99 genes). The second largest group was "Olfactory Bulb" on PND 2 (20 genes) and 14 (22 genes). "Basal Ganglia", "Frontal Lobe", "Neostriatum" (PND 7) and "Cerebrum" (PND 14) were strongly enriched (enrichment factor > 2) (Table 1, Fig. 1). The categories "Corpus Striatum" (ED 16), "Basal Ganglia", "Frontal Lobe", "Substantia Nigra" (PND 7) and "Neostriatum" (PND 14) were significantly enriched of upregulated genes in the fetal TiO_2-exposed group (Supplementary Table 2). "Olfactory Bulb" (PND 2 and 14), "Hippocampus" (PND 2, 14 and 21) and "Cerebrum" (PND 14) were significantly enriched of downregulated genes in the exposure group (Supplementary Table 3).

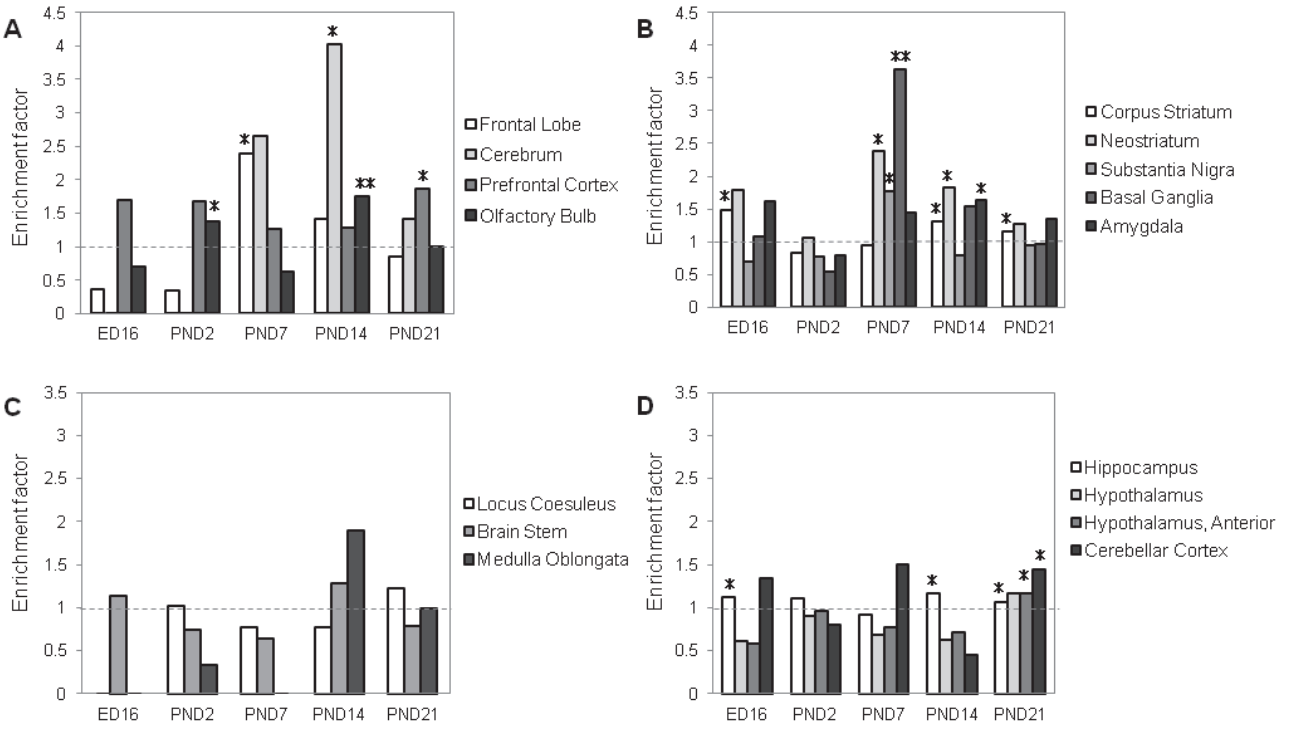

Fig. 1. Time-dependency of enrichment of each MeSH from genes dysregulated by maternal TiO_2 exposure. MeSH data indicating brain regions closely associated with (A) telencephalon, (B) dopamine neuron system, (C) noradrenaline and serotonin neuron system and (D) others of strong interest. The enrichment factor for each category was defined as $(nf/n)/(Nf/N)$, as described in the Materials and Methods.

DISCUSSION

The developmental toxicity is one of the major emerging issues on the hazard of nanomaterials (Fujitani *et al*., 2012). Previous animal experiments suggested that the administration of a suspension of nano-TiO_2 to pregnant mothers affects the various organs including central nervous system of offspring (Takeda *et al*., 2009; Takahashi *et al*., 2010; Yamashita *et al*., 2011; Shimizu *et al*., 2009; Hougaard *et al*., 2010). The present study employed a method for determining which MeSH categories indicating brain regions were enriched of genes differentially expressed by fetal nano-TiO_2 exposure. As a result, the categories associated with the striatum (ED 16, PND 7 and 14), olfactory bulb (PND 2 and 14) and cerebral cortex (PND 7, 14 and 21) were enriched as expected (Figs. 1A, B). Pathological evaluation showed apoptosis of mitral cells in the olfactory bulb and an accumulation of TiO_2 particles in the olfactory bulb and the cerebral cortex in a previous study (Takeda *et al*., 2009). A later study showed an increase in the levels of a neurotransmitter, dopamine, and its metabolites in the striatum and the cerebral cortex (Takahashi *et al*., 2010). The observation was consistent with the data of the present study which showed an association with the MeSH terms of the regions closely related to the dopamine system; the substantia nigra (PND 7), basal ganglia (PND 7) and amygdala (PND 14) (Fudge and Emiliano, 2003) (Fig. 1B). The results suggest that gene expression changes in the brains of developing offspring mice may precede the changes in pathology (Takeda *et al*., 2009) and monoamine levels (Takahashi *et al*., 2010) observed in 6-week-old offspring mice. Other terms related to the noradrenaline and serotonin neuron system such as the locus coeruleus, brainstem and medulla oblongata were not enriched at any point through ED16-PND21 (Fig. 1C). Additionally, an enrichment analysis was conducted using sets of upregulated and downregulated genes separately. The results showed that downregulated genes were enriched in the olfactory bulb (PND 2 and 14), whereas upregulated genes were enriched in the dopamine-related regions including corpus striatum, neostriatum, basal ganglia and

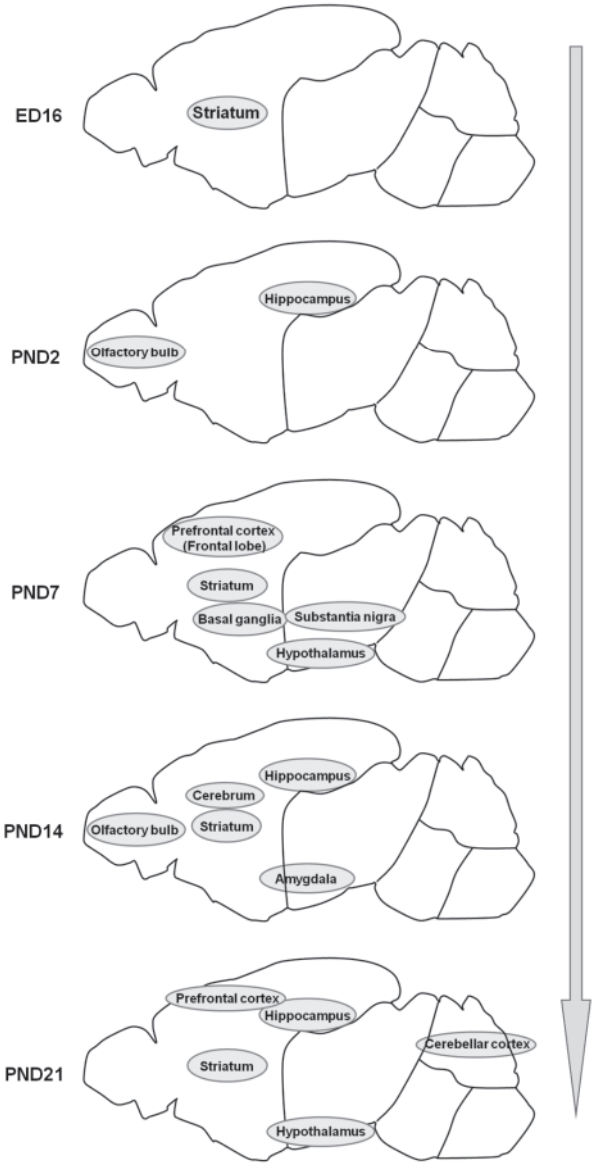

Fig. 2. Summary of the extracted MeSH terms indicative of brain regions among dysregulated genes in the maternal TiO_2 exposure group.

substantia nigra (ED 16, PND 7, 14 and 21). The analysis also revealed that the genes associated with the striatum were altered during the perinatal period, and those associated with regions related to the dopamine neuron system and the prefrontal region were dysregulated in the later infantile period (Fig. 2). These data gave us some clues as to the mechanisms that underlie cerebral gene expression changes by fetal TiO_2 exposure.

The method presented in this paper showed interesting novel categories that deserve further exploration including the hippocampus, hypothalamus and cerebellar cortex (Fig. 1D). There are reports that inhaled or injected particulate matter enters the systemic circulation (Takenaka *et al*., 2001) and reaches various tissues including the brain (Kreyling *et al*., 2002; Oberdorster *et al*., 2002). The hippocampus has been reported as a target region of the effects of fetal exposure to environmental particulate matter (diesel exhaust particles) in a pathological study (Sugamata *et al*., 2006a) and by the evaluation of monoamine levels (Suzuki *et al*., 2010). The cerebellum is another target of particulate matter including inhaled diesel exhaust particles (Sugamata *et al*., 2006b) and carbon nanoparticles (Oberdorster *et al*., 2004). The hypothalamus lacks an effective blood-brain barrier and its fenestration allows for the easy passage of substances in the blood (Harre *et al*., 2002). However, the enrichment factors of MeSH terms related to these brain regions were relatively low (< 2) and therefore the regions were less likely to be targets of fetal nano-TiO_2 exposure. The difference in the regions affected between both nanoparticles of diesel exhaust particles and TiO_2 may be caused by their difference in chemical composition.

In conclusion, the present study showed anatomical information extracted from a dataset of gene expression in the whole brain of mice prenatally exposed to nano-TiO_2 using a specific group of MeSH related to brain regions. The result showed that the principle of analysis using a selected group of MeSH can provide anatomical information in the interpretation of microarray results. The analysis provides data to support the hypothesis that maternal TiO_2 exposure results in alteration to the cerebral cortex, olfactory bulb and the regions intimately related to dopamine systems of offspring mice.

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