Maternal Exposure to Diesel Exhaust Leads to Pathological Similarity to Autism in Newborns

Masao Sugamata,*,[a] Tomomi Ihara,[a] Miho Sugamata,[b] and Ken Takeda[c]

[a]Department of Pathology, Tochigi Institute of Clinical Pathology, 2308–3, Minamiakatsuka, Nogi, Shimotsuga-gun, Tochigi 329–0112, Japan, [b]Department of Pathology, Omori Hospital, Toho University School of Medicine, 6–11–1, Omori-nishi, Ota-ku, Tokyo 143–8541, Japan, and [c]Department of Hygiene Chemistry, Faculty of Pharmaceutical Science, Tokyo University of Science, 2641, Yamazaki, Noda, Chiba 278–8510, Japan

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We have already demonstrated many diesel exhaust particles (DEPs) and some damages in brain tissues (cerebral cortex and hippocampus) of newborn mice whose mothers inhaled DE during pregnancy, and these damages have the possibilities to lead to some disorders of nervous system. In this study, we examined pathological effects on newborn brain by DE-exposure to pregnant mice, especially focused on autism. ICR pregnant mice were exposed to DE and some were exposed to clean air as a comparative control. Brain tissues (cerebellum) were obtained from the mice (housed in a clean air) born from DE-exposure and control pregnant mice, and examined with light and electron microscope. To detect apoptosis, the immunohistochemical staining for caspase-3 was performed, especially; the numbers of positive Purkinje cell in cerebellum were compared between DE-exposure and control. In DE-exposure group, numerous caspase-3 positive cells were diffusely observed and the number of positive Purkinje cells was significant large than in control. Electron microscopically, specific features of apoptosis were found in Purkinje cells in the DE-exposure group. These findings indicate that DE-exposure to pregnant mice has a severe impact on fetal brain development and, especially, numerous apoptotic Purkinje cells cause the innate deficiency of them and would involve the pathogenic backing of autism. Our results would give a grave warning that the maternal inhalation of DE is hazardous to fetuses' health and it is possible that these fetal damages carries a great risk of various disorders of nervous system afterward, such as autism.

*To whom correspondence should be addressed: Department of Pathology, Tochigi Institute of Clinical Pathology, 2308–3, Minamiakatsuka, Nogi, Shimotsuga-gun, Tochigi 329–0112, Japan. Tel.: +81-280-56-1278; Fax: +81-280-56-2792; E-mail: mspathol@beige.ocn.ne.jp

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INTRODUCTION

Diesel exhaust (DE) is a major air pollutant in urban areas and there is a fear of the health impacts of DE inhalation, especially regarding respiratory and circulatory diseases. However, vehicles with diesel engines are widely used not only for their low operating costs and significant power but also because they produce little carbon dioxide that leads to global warming. The smallest DE particles (DEPs) are nanostructures (< 0.1 μm); we have already detected many such nano-sized particles in brain tissues (cerebral cortex and hippocampus) of newborn mice whose mothers inhaled DE during pregnancy.[1)] These prenatal DE-exposed murine brains showed apoptosis of endothelial cells and scavenger cells surrounding blood vessels; these cells have roles in blood-brain barrier (BBB) and degenerative changes in the neighboring parenchyma.[1)] These findings indicated that maternal DE-exposure affects the development of central nervous system in embryo or fetus and induces an innate morphological abnormality of brain tissue in newborn; therefore, there is a possibility that these effects of DE lead to various disorders of the nervous system in offspring. In this study, we found evidence that the morphological alterations in cerebella of newborn mice following maternal exposure to DE have a striking resemblance to those in autism.

MATERIALS AND METHODS

Animals —— Pregnant ICR mice were exposed to DE (0.3 mg DEPs/m^3) during a 12-hr light/12-hr dark cycle (19:00–07:00) from 2 days post-coitus to 16 days post-coitus. Some of the pregnant ICR mice were exposed to clean air as a comparative control group. After DE-exposure, the pregnant mice were housed in a clean cage in a clean room until delivery; brain tissue (from the cerebellum) was collected from the offspring at 11 weeks of age. These cerebella from offspring of DE-exposed pregnant mice (DE group: 10 males and 10 females) and those of control pregnant mice (10 males and 10 females) were examined with light and electron microscopy. All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Immunohistochemical Staining of Caspase-3 and Counting Positive and Negative Purkinje Cells under a Light Microscope —— Tissue samples of cerebella from the DE group and the control group were fixed with 10% buffered formalin and, after routine dehydration, embedded in paraffin. To detect apoptosis in these cerebella under a light microscope, the immunohistochemical staining for caspase-3 (a common enzymatic biomarker of apoptosis) was performed. Paraffin sections 5-μm thick of cerebellum tissue samples were stained immunohistochemically by the streptoavidin-biotin method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The primary antibody used was anti-human/mouse caspase-3 (active) rabbit IgG (R&D Systems, Inc., Minneapolis, MN, U.S.A.).

On the sections of immunohistochemical staining for caspase-3, positive or negative cells per light microscopic field ($\times$ 20 objective, $\times$ 10 ocular) were counted using image analysis software (Image-Pro PLUS; Media Cybernetics, Inc., Silver Spring, MD, U.S.A.) in all fields per section (mean $\pm$ S.D. of examination fields/section; DE group: 34.00 $\pm$ 4.64, control: 32.50 $\pm$ 4.14). The mean numbers and S.D. of positive cell count and total cell number of Purkinje cells were calculated per light microscopic field. Statistical analysis of these data was performed using the Mann-Whitney *U* test and statistical significance was defined as p less than 0.05.

Electron Microscopic Observations —— Cerebellum tissue samples from the DE group and the control group were fixed immediately with 1% glutaraldehyde and 4% formalin over 6 hr at 4°C and rinsed in 0.1 M cacodylate buffer overnight. These tissues were postfixed with 1% osmium tetraoxide and embedded in Epon 812 resin. Ultrathin sections were prepared with a Nova Ultratome (LKB, Bromma, Sweden), double-stained with uranyl acetate and lead citrate, and examined under an electron microscope (Model 1200EX; JEOL, Tokyo, Japan). Apoptotic appearance was evaluated based on findings of the ultrastructural process into the apoptotic body described in our previous report.[2] In that report, the ultrastructural apoptotic process was divided into four stages and characteristic findings could be observed at each stage. One of these findings is called crescent-shaped spaces. From quite an early stage of the morphological process of apoptotic formation, crescent-shaped spaces appear around the nuclear membrane of the cell, which is seemingly normal but has been triggered by apoptotic induction.

RESULTS AND DISCUSSION

In DE group, caspase-3-positive cells were diffusely observed in each cerebellum (Fig. 1A and 1B) and numerous positive Purkinje cells were detected compared with control (Fig. 1C and 1D). The ratio of positive counts in DE group was significant large compared to that in control (control: 2.36 $\pm$ 3.16%, DE group: 57.50 $\pm$ 27.98%, $p < 0.0001$). Moreover, in DE group, some areas on the cerebella showed no detection of Purkinje cells (Fig. 1D, arrows) and the total number of Purkinje cells was fewer than that in control group (control: 31.22 $\pm$ 7.43 cells per field, DE group: 18.59 $\pm$ 5.23 cells per field, $p < 0.0001$). Electron microscopically, specific features of apoptosis[2] were also found in many Purkinje cells in DE group. These findings were observed in all newborn mice of DE group and in none of control. There was no significant difference in the total number of Purkinje cells between males and females of DE group (males: 18.53 $\pm$ 5.26 cells per field, females: 18.65 $\pm$ 5.36 cells per field, $p = 0.89$), however, the ratio of positive counts in males was generally larger than that in females (males: 64.82 $\pm$ 29.05%, females: 50.19 $\pm$ 25.62%, $p = 0.09$).

The cerebella in cases of autism show a common pathology in the decrease of Purkinje cells and the alteration is considered to be a fetal developmental abnormality.[3,4] Our findings indicate that DE-exposure to pregnant mice has a severe impact on development of the fetal cerebellum and an innate cerebellar deficiency from numerous apoptotic

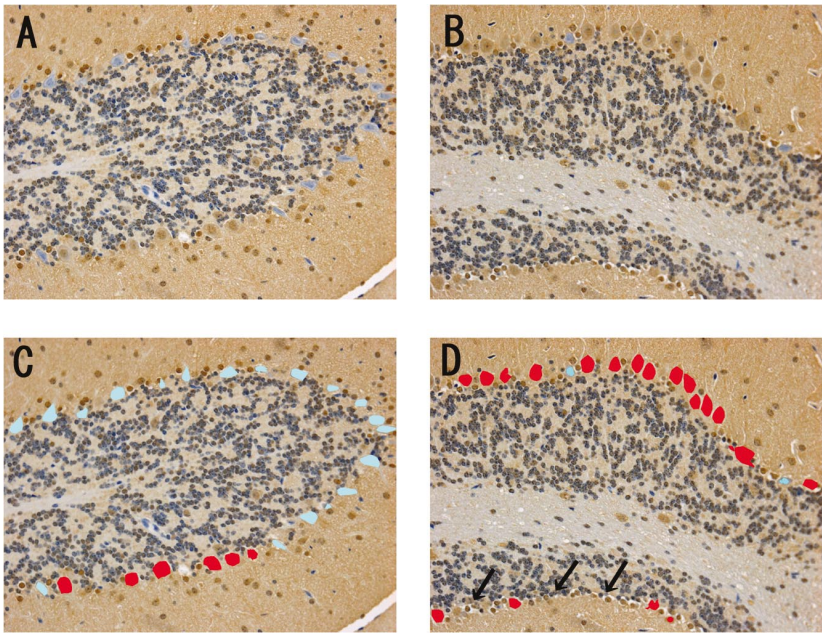

Fig. 1. Immunohistochemical Staining of Caspase-3 in Cerebellum (A: Control, B: DE Group) and Detecting Positive and Negative Purkinje Cells by Image Analysis (C: Control [Same Field to A], D: DE Group [Same Field to B]) Par Field (× 20 Objective, × 10 Ocular)

In C and D, positive (brown color in A and B) Purkinje cells detected by image analysis are red color, and negative (violet color in A and B) are blue. In DE group, numerous positive Purkinje cells are detected and a positive count is significant large compared with that in control. Some area on cerebellum of DE group shows no detection of Purkinje cells (in D; arrows).

Purkinje cells. These alterations found in the newborns of DE-exposed pregnant mice had a striking resemblance to those found in cases of autism. The great possibility exists that this same or more severe damage may occur in human infants secondarily to maternal inhalation of DE because the human placenta consists of two layers while the mouse placenta consists of four layers. The exposure to DE during pregnancy seems to involve the pathogenic mechanism of autism in the offspring. Our results suggest a grave warning that maternal inhalation of DE is hazardous to fetal health and it is possible that the resulting fetal damage carries a great risk of some disorders of the nervous system, such as autism.

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