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Attenuation of delayed-type hypersensitivity by fullerene treatment

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ABSTRACT

Expansion and commercialization of nanotechnology mean that it is important to understand the potential health hazards of manufactured nanoparticles. Here, we focused on the effect of fullerene, a type of nanoparticle already in commercial use, on delayed-type hypersensitivity (DTH) induced by methyl-bovine serum albumin (mBSA). Delayed-type hypersensitivity was induced with methyl-bovine serum albumin in female C57BL/6 mice. A colloidal suspension of crystalline C_{60} (nano-C_{60}; average particle size 165 nm; 200 μL; 5.5 μg/mL) was injected intravenously twice, just before immunization and challenge with mBSA.

Nano-C_{60} treatment significantly attenuated footpad swelling, compared with that in DTH-disease control mice. Cytokine analysis indicated that nano-C_{60} treatment switched the cytokine balance towards Th1-dominance. Pro-inflammatory cytokines IL-6 and IL-17 were significantly increased in DTH mice, and these increases were significantly suppressed by nano-C_{60} treatment. Suppression of IL-17 by nano-C_{60} was confirmed in an *in vitro* splenocyte culture. However, production of TNF-α was increased in DTH mice, and the increase was significantly enhanced by nano-C_{60} treatment. The ratio of regulatory T (Treg) cells to total T ($CD4^+$) cells was also significantly increased by nano-C_{60} treatment, compared with that in DTH-disease control mice.

Nano-C_{60} treatment showed significant immunomodulatory effects in a mouse DTH model: IL-6 and IL-17 production was down-regulated, and the Treg cell ratio was up-regulated, concomitantly with attenuation of the pathology of DTH.

1. Introduction

Nanoparticles often exhibit new or enhanced size-dependent properties compared with larger particles of the same material, and are expected to have practical applications in electronics, environmental science, medical science and many other fields (Emerich and Thanos, 2003; Moghimi et al., 2005; Satoh and Takayanagi, 2006). However, there is a risk of internal pollution with manufactured nanoparticles (Brown et al., 2001; Oberdörster et al., 2005). Since the diameter of nanoparticles is very small, their surface area is extremely large, and they may be much more toxic than larger particles. Furthermore, nanoparticles that enter the respiratory system may be distributed to the whole body via blood. It has been reported that diesel exhaust particles (DEP), which are nanoparticles often used as a model air pollutant, exert adjuvant effects on the response of humans and mice to inhaled or instilled allergens through the generation of oxidative stress (Ichinose et al., 1995; Diaz-Sanchez, 1997; Diaz-Sanchez et al., 1997; Fujimaki et al., 1997; Takano et al., 1997; Haley and Drazen, 1998). However, in general, little is known about the immunomodulatory effects of nanoparticles, or the mechanisms involved. Given the widespread potential applications of nanoparticles and their impending commercialization, human and environmental exposure to nanoparticles is likely to increase markedly in the near future. Therefore, early evaluation of the health effects of nanoparticles is important.

Fullerenes are a family of carbon allotropes composed entirely of carbon, in the form of hollow spheres, ellipsoids, tubes, or planar structures. The existence of carbon molecular structures was predicted by Osawa (1970), and fullerene (buckminsterfullerene; IUPAC name, C_{60}-I_h) was subsequently discovered in 1985 (Kroto et al., 1985). The structure of C_{60} resembles a soccer ball composed of twenty hexagons and twelve pentagons, and atoms composing the pentagons are more reactive than those composing the hexagons because of strain energy. The van der Waals diameter of a C_{60} molecule is about 1 nanometer (nm), and the nucleus-to-nucleus distance is about 0.7 nm. Fullerene has unique properties, and is expected to have practical applications in various fields (Da Ros and Prato, 1999; Tagmatarchis and Shinohara, 2001; Bosi et al.,

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2003). Because of its radical scavenging activity, fullerene has been introduced as a component of cosmetics, which are now commercially available. Golf clubs, rackets, etc., that contain fullerene are also available. Thus, large-scale industrial production of fullerene has begun for a diverse and growing range of commercial applications within the last few years. Another biologically important property of C_{60} is the ability to form a long-lived triplet excited state upon photosensitization, so that it has the potential to generate singlet oxygen ($^1O_2$), a highly reactive form of molecular oxygen (Guldi and Prato, 2000). Singlet oxygen reacts with a wide variety of biological targets including lipids, proteins, nucleic acids and carbohydrates, and is known to be involved in cellular signaling and in cell damage (Briviba et al., 1997). In 2004, Oberdörster reported that C_{60} aggregates, prepared using a solvent extraction methodology, elevated lipid peroxidation levels and glutathione production in the brain and liver of largemouth fish (Oberdörster, 2004). Later, Sayes et al. (2005) reported increased lipid peroxidation and total glutathione in human cultured cells exposed to C_{60}. We speculated that fullerene might exert adjuvant effects on the response of mice sensitized with allergens through the generation of oxidative stress, like DEP. An understanding of the toxicity of fullerene is important for defining and constraining its possible biomedical applications.

Delayed-type hypersensitivity (DTH) is classified as type IV hypersensitivity, and is not antibody-dependent, but rather is a cell-mediated immunity involving antigen-specific effector Th1 and Th17 cells (Black, 1999; Särnstrand et al., 1999; Kobayashi et al., 2001). DTH reactions can be classically subdivided into tuberculin-type, Jones–Mote type and contact hypersensitivity. The tuberculin-type reaction was assayed in this study as a typical DTH reaction. DTH develops in two phases: in the sensitization phase, T cells are sensitized and antigen-specific effector Th1 and Th17 cells are formed. Next, in the elicitation phase, recall responses of these T cells are induced upon secondary challenge with the antigen. These effector T cells release cytokines, including IFN-γ and IL-17, that trigger recruitment of inflammatory cells such as neutrophils and macrophages (Nakae et al., 2002; Kariyone et al., 2003). The inflammation generally reaches a maximum at 24–48 h after secondary challenge.

In this study, we show that fullerene has an immunomodulatory action in a delayed-type hypersensitivity model in C57BL/6 mice. The mechanisms involved were examined, focusing on changes in inflammatory cytokines and Th17 cells, as well as regulatory T cells (Treg), which have a critical function in the regulation of autoimmune diseases, including DTH (Fontenot et al., 2003; Sakaguchi, 2004; Sato et al., 2006).

2. Materials and methods

2.1. Preparation of colloidal fullerene (nano-C_{60})

C_{60} was suspended in water according to the method of Deguchi et al. (2001), as follows. C_{60} (99.5%, Aldrich) was dissolved in tetrahydrofuran (THF) (Fisher Scientific) at a concentration of 100 mg/L. This solution was sparged with nitrogen and stirred overnight in the dark, then filtered through a 0.22 μm nylon filter (Osmonics, Fisher Scientific). An equal volume of MilliQ water was added to the solution of C_{60} in THF at a rate of 1 L/min. The resulting solution was dialyzed to eliminate the THF using Seamless Cellulose Tubing (Sanko Junyaku Tokyo, Japan). The final solution was stored overnight and then filtered through a 0.22 μm nylon filter to yield a colloidal suspension of crystalline C_{60} in water, which we refer to as nano-C_{60}.

2.2. Characterization of nano-C_{60}

The particle size distribution of nano-C_{60} was evaluated by means of dynamic light-scattering measurements (DLS, NICOMP 380 ZLS). The UV–vis spectra of nano-C_{60} suspensions were scanned within the wavelength range of 200–550 nm using a UV–vis spectrophotometer U-1100 (Hitachi). All UV–vis measurements were carried out at 20 °C, being automatically corrected for the suspending water.

2.3. Animals

Female C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 5–6 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 10% under a 12 h light–dark cycle. They were allowed free access to tap water and experimental normal diet, CE-2 (CLEA Co., Tokyo, Japan). 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.

2.4. Induction and evaluation of DTH

DTH was induced by immunization with mBSA (Sigma, St. Louis, MO, USA). Briefly, mice were injected SC with 200 μL of 1.25 mg/mL mBSA emulsified with Complete Freund's Adjuvant (CFA) (Chondrex, Redmond, WA) (immunization). Seven days after immunization, they were injected SC into one footpad with 20 μL of 10 mg/mL mBSA in PBS (challenge). Animals were injected with an equal volume of PBS into another footpad as a control. Evaluation of the responses was performed as described elsewhere (Särnstrand et al., 1999; Yoshimoto et al., 2000). At the indicated times after challenge, footpad thickness was measured with a digital caliper (Mitutoyo, Tokyo, Japan). 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)]) × 100. Footpad swelling (%) was calculated as a percentage of the mean value of the control group.

2.5. Dosing schedule of nano-C_{60}

Mice of the nano-C_{60} treated group were administered IV with 200 μL of 5.5 μg/mL nano-C_{60} twice, just before immunization and challenge with mBSA. The dose of nano-C_{60} was determined with reference to previous reports (Oberdörster, 2004; Sayes et al., 2005). Control groups received only 200 μL of PBS on the same schedule as the nano-C_{60} treated group.

2.6. Reagents

The following antibodies were used for cytokine assay: purified anti-mouse TNF-α mAb (1F3F3D4), biotin-conjugated anti-mouse TNF-α mAb (MP6-XT22), purified anti-mouse IFN-γ mAb (XMG1.2), biotin-conjugated anti-mouse IFN-γ mAb (R4-6A2), purified anti-mouse IL-6 mAb (MP5-20F3), biotin-conjugated anti-mouse IL-6 mAb (MP5-32C11), purified anti-mouse IL-4 mAb (11B11), biotin-conjugated anti-mouse IL-4 mAb (BVD6-24G2) (eBioscience, San Diego, CA). Recombinant mouse TNF-α, IFN-γ, IL-4 and IL-6 were purchased from eBioscience (San Diego, CA). 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). For immuno-fluorescence studies, the following mAbs were used: 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 cells; FITC-conjugated anti-mouse CD8a mAb (53-6.7), which recognizes protein on cytotoxic T cells; FITC-conjugated anti-mouse CD19 mAb (1D3), which recognizes protein on B cells; FITC-conjugated anti-mouse CD25 mAb (PC61.5), which recognizes activated T and B lymphocytes; PE-Cy5-conjugated anti-mouse CD38 mAb (90), which recognizes protein on mature B cells and plasma cells; PE-conjugated anti-mouse Foxp3 mAb (FJK-16s), which recognizes Foxp3 expressed in Treg cells (these antibodies were purchased from eBioscience, San Diego, CA).

2.7. Phenotyping of lymphocytes

Immunophenotyping of splenic lymphocytes was carried out using a FACS-LSR flow cytometer (Becton Dickinson, San Jose, CA). Spleens were harvested from normal mice and DTH mice with or without nano-C_{60} treatment. 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 2×10^6 cells/mL. Cells were stained with fluorochrome-conjugated antibodies for 30 min, then washed. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson, San Jose, CA, USA).

2.8. Determination of IFN-γ, IL-4, TNF-α, IL-6, and IL-17

Splenocytes were prepared from normal mice and DTH mice with or without nano-C_{60} treatment. The cells were suspended in RPMI1640 medium containing 10% FBS at a concentration of 7×10^6 cells/mL and cultured with mBSA (20 mg/mL) in 96-well plates. After incubation at 37 °C under an atmosphere of 5% CO_2 for 96 h, the culture supernatant was harvested for determination of IFN-γ, IL-4, TNF-α, IL-6,

and IL-17. The concentrations of IFN-γ, IL-4, TNF-α, IL-6, and IL-17 were measured by ELISA as follows. A 96-well plate was coated with purified anti-mouse IFN-γ (1:500), IL-4 (1:250), TNF-α (1:500), IL-6 (1:500), or IL-17 (1:250) mAb, and incubated overnight at 4 °C. The wells were washed with PBS containing 0.05% Tween-20, and nonspecific binding was blocked with PBS containing 1% bovine serum albumin for 1 h at room temperature. The plate was washed, and the culture supernatant was kept for 2 h at room temperature. The plate was washed again, and anti-mouse biotin-conjugated TNF-α (1:1000), IFN-γ (1:500), IL-4 (1:1000) IL-6 (1:500), or IL-17 (1:500) mAb was added for 1 h at room temperature. The plate was further washed, and avidin-horseradish peroxidase (Sigma, St. Louis, USA) was added. The plate was incubated for 30 min at room temperature, then washed, and 3,3′,5,5′-tetramethylbenzidine was added for 10–30 min. The reaction was stopped by adding 2.5 M H_2SO_4, and the absorbance at 450 nm was measured with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). Standard curves were established with recombinant mouse IFN-γ, IL-4, TNF-α, IL-6, and IL-17, and the concentrations were estimated from the standard curves.

2.9. Effect of nano-C_{60} on cytokine productivity of splenocytes prepared from normal mice

Splenocytes were harvested from normal and DTH mice in the same way as previously described. T cells in splenocytes were activated by plate-bound anti-CD3 mAb (1 mg/mL) for 24 h and cultured with or without 30 μL of mBSA (10 mg/mL) and 100 μL of nano-C_{60} (5 mg/mL) at a concentration of 2×10^6 cells/mL in a 24-well plate at 37 °C for 96 h. After incubation, cells were harvested and cytokines in the culture supernatant were measured with ELISAs.

2.10. Analysis of regulatory T (Treg) cells

Splenocytes were prepared from normal and DTH mice with or without nano-C_{60} treatment. Cells (5×10^6 cells) were stained with PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD25 antibodies for Treg cell analysis 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 samples were stained with PE-conjugated anti-Foxp3 for Treg cells for 1 h at room temperature, and washed with RPMI1640-based buffer. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson, San Jose, CA, USA).

2.11. Statistical analysis

Values are given as mean ± S.E. Comparison between two values was performed by use of the unpaired Student's *t*-test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's *post hoc* analysis. The criterion of a significant difference was set at $P < 0.05$. Calculations were done with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA).

3. Results

The C_{60} particles were crystalline (simple hexagonal) with an estimated mean diameter of 165 nm (Fig. 1A), and their UV spectrum was similar to that of commercial C_{60} (peaks at 270 nm, 330 nm; Fig. 1B), although there was a slight displacement of peaks.

DTH was induced in C57BL/6 mice by immunization with mBSA. The footpad swelling reached maximum at 24 h after challenge (data not shown). Normal mice and mice treated only with nano-C_{60} showed no swelling. The footpad swelling was significantly reduced in DTH mice treated with nano-C_{60}, compared with DTH-disease control mice (Fig. 2), suggesting that nano-C_{60} treatment attenuates the pathology of DTH.

Production of cytokines, including IFN-γ, IL-4, TNF-α, IL-6, and IL-17, was then examined (Fig. 3). IFN-γ production was significantly increased in DTH mice compared with the normal-control group, and nano-C_{60} treatment had no effect on this. IL-4 production was significantly increased in DTH mice, and this increase was significantly suppressed by nano-C_{60} treatment. The levels of IL-6 and IL-17 were also significantly increased in DTH mice, and nano-C_{60} treatment significantly suppressed both increases. Production of TNF-α was increased in DTH mice, and the increase was significantly further elevated by nano-C_{60} treatment (Fig. 4). The suppression of IL-17 level by nano-C_{60} was confirmed in an *in vitro* culture assay (Fig. 5). There was no significant difference in production of other cytokines between the *in vitro* assay systems with and without nano-C_{60} (data not shown).

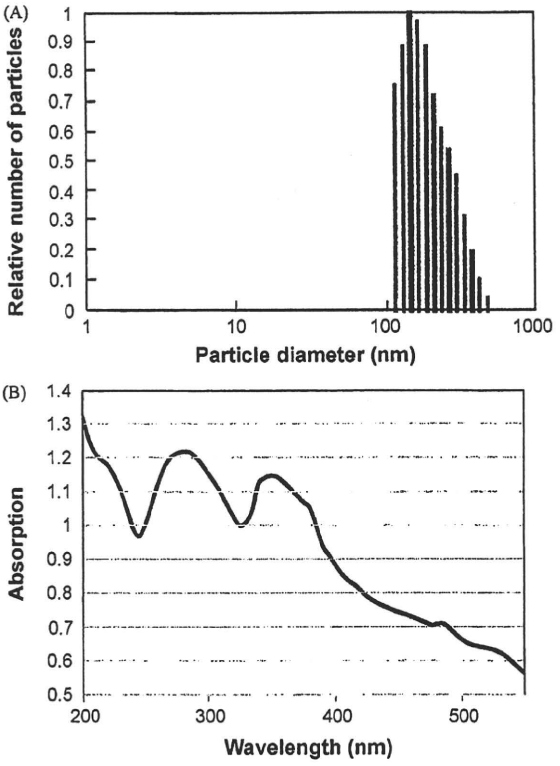

Fig. 1. Particle diameter distribution (A) and UV–vis absorbance spectrum (B) of nano-C_{60} aggregates in water.

Nano-C_{60} treatment had no significant effect on various populations of splenic lymphocytes in normal or DTH mice, as shown in Fig. 6. However, when changes in the population of Treg cells were examined, the elevated population ratio observed in the DTH-disease control group was significantly further increased by nano-C_{60} treatment (Fig. 7). These results indicate that nano-C_{60} induces up-regulation of Treg cells via the suppression of IL-17 production.

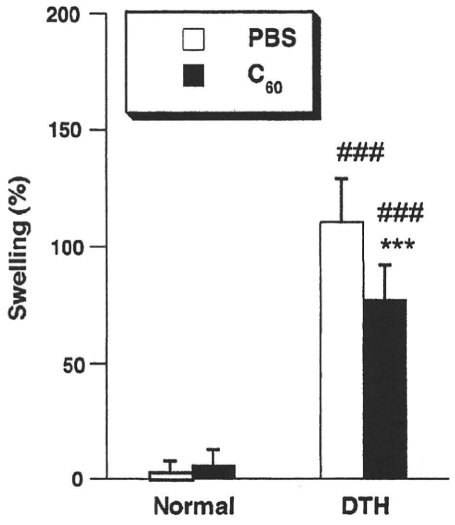

Fig. 2. Footpad swelling in DTH-disease control and nano-C_{60}-treated DTH-mice. Each value represents the mean ± S.E. ($n = 5$). DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; C_{60}, nano-C_{60}. ###Statistically significant difference ($P < 0.005$) compared to the normal control group. ***Statistically significant difference ($P < 0.005$) compared to the DTH-disease control group.

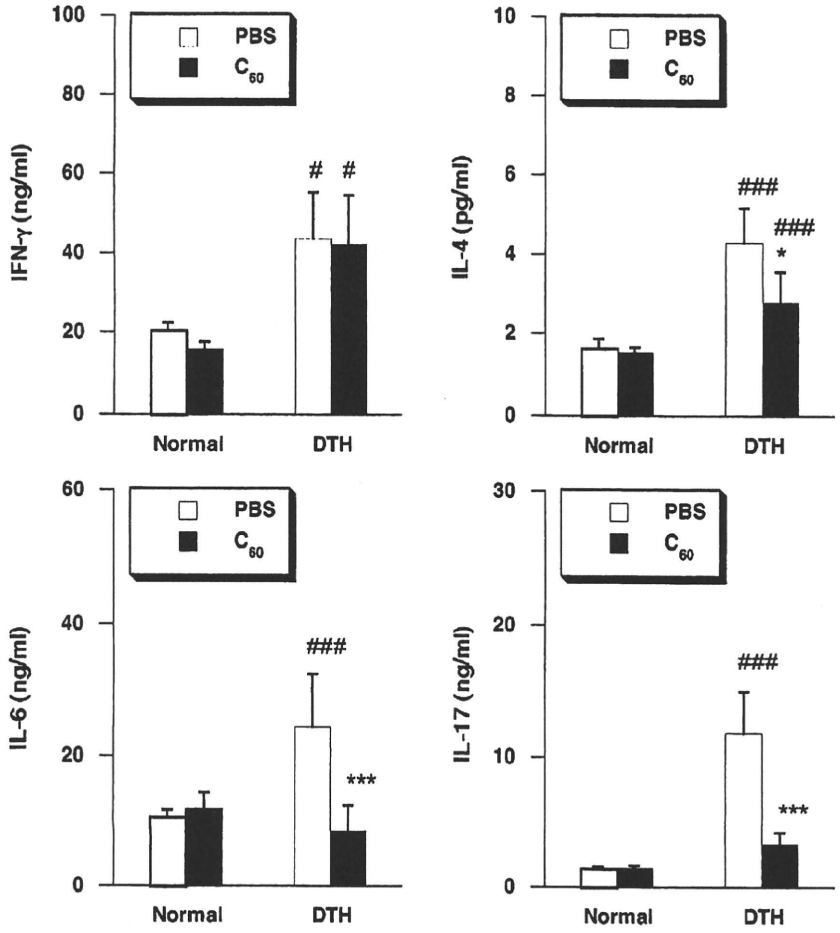

Fig. 3. Changes in cytokine production of splenocytes. Each value represents the mean ± S.E. ($n = 5$). DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; C_{60}, nano-C_{60}. Statistically significant differences compared to the normal control group: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$. Statistically significant differences compared to the DTH-disease control group: $^{*}P < 0.05$, $^{***}P < 0.005$.

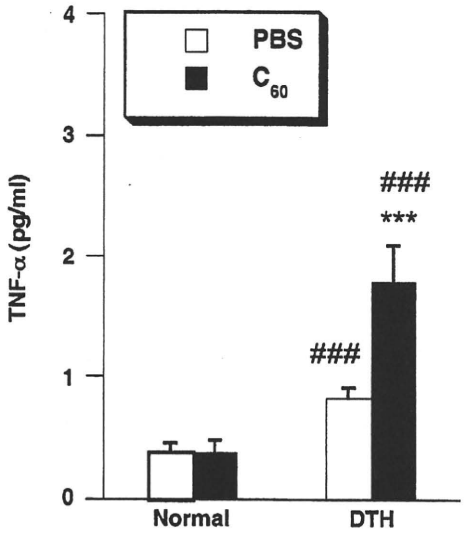

Fig. 4. Effect of nano-C_{60} treatment on the elevated TNF-α level observed in DTH-disease control mice. Each value represents the mean ± S.E. ($n = 5$). DTH, delayed type hypersensitivity; PBS, phosphate-buffered saline; C_{60}, nano-C_{60}. $^{\#\#\#}$Statistically significant difference ($P < 0.005$) compared to the normal control group. *Statistically significant difference ($P < 0.05$) compared to the DTH-disease control group.

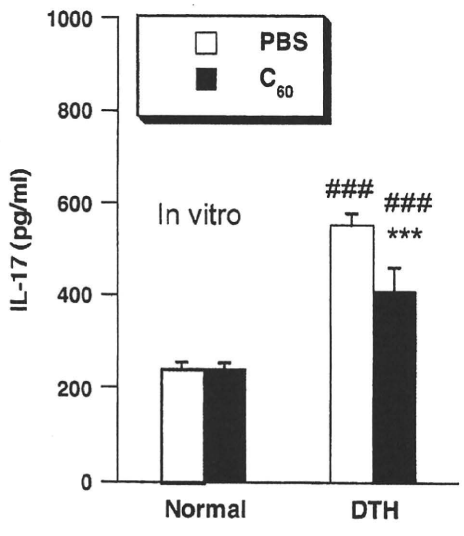

Fig. 5. Effect of nano-C_{60} on *in vitro* IL-17 production by splenocytes. Each value represents the mean ± S.E. ($n = 5$). PBS, phosphate-buffered saline; C_{60}, nano-C_{60}. $^{\#\#\#}$Statistically significant difference ($P < 0.005$) compared to the normal control group. ***Statistically significant difference ($P < 0.005$) compared to the DTH-disease control group.

4. Discussion

Fullerene acts as a photosensitizer, generating highly reactive singlet oxygen, which reacts with a wide range of biological targets, including lipids, proteins, nucleic acids and carbohydrates. This activity may prove to be an important constraint on its potential biomedical applications. We hypothesized that fullerene might exert adjuvant effects on the delayed hypersensitivity response of

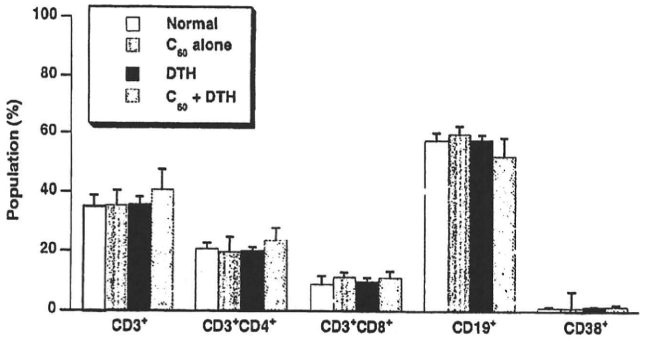

Fig. 6. Populations of splenic lymphocytes in normal, nano-C_{60} alone, DTH, and nano-C_{60}-DTH-groups. Phenotypes were analyzed by flow cytometry. DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; C_{60}, nano-C_{60}.

mice through the generation of oxidative stress, and in this study, we investigated the effect of nano-C_{60} in a mouse model. Contrary to our expectation, footpad swelling in DTH mice was significantly suppressed by nano-C_{60} treatment.

After antigen presentation by antigen-presenting cells (APC), undifferentiated helper T cells (naive T cells) differentiate into effector T cells, which can be classified into two different types, Th1 and Th2, based on the kinds of cytokine they produce. The Th1 cytokines promote cellular immunity by activating macrophages, cytotoxic $CD8^+$ T lymphocytes, etc., while the Th2 cytokines enhance humoral immunity, including activation and class switching of antibody-producing B cells. The direction of naive T cell differentiation is determined by the kind of antigen and immunocyte with which the T cell interacts. An imbalance of cytokines produced by helper T cells is believed to play an important role in the development of allergy and autoimmune diseases (Charlton and Lafferty, 1995). It is generally accepted that Th1 cells play a pathogenic role in the development of DTH. IFN-γ, which is a representative Th1 cytokine produced by antigen-specific $CD4^+$ T cells, promotes DTH responses by enhancing Th1 cell development. Development of DTH in mice immunized with mBSA is therefore associated with elevation of pro-inflammatory cytokines such as IL-6, IL-17 and TNF-α. Interestingly, the elevations of pro-inflammatory cytokines, except for TNF-α, in our DTH mice model were significantly suppressed by nano-C_{60} treatment, in accordance with the observed inhibitory effect of nano-C_{60} on the pathological paw swelling. However, the elevated production of TNF-α in DTH mice was further increased by nano-C_{60} treatment. TNF-α plays an important role in the elicitation phase of inflammation, so this finding seems in conflict with the observed attenuation of footpad swelling. Further study is needed to understand the mechanisms involved. As regards Th1/Th2 balance, the increased production of IFN-γ in DTH mice was not suppressed by nano-C_{60} treatment, but that of IL-4 was suppressed, resulting in a switch of the balance toward Th1-dominant immunity. Though IFN-γ plays an important role in the development of DTH, as mentioned above, the clinical severity and pathology of DTH were reported to be exacerbated in IFN-γ knockout mice (Jones et al., 1997). This phenomenon was found not only in DTH, but also in other Th1-related autoimmune diseases, including collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) (Caspi et al., 1994; Ferber et al., 1996). Namely, though IFN-γ is indispensable for development of DTH, it also appears to have a protective effect against these diseases. This seems paradoxical from the viewpoint of the Th1 paradigm of DTH. However, the apparent paradox could be resolved by considering the role of Th17 cells. These cells are a newly identified helper T cell subset, distinct from Th1 and Th2 cells (Harrington et al., 2006); they express CD4 and orphan nuclear receptor RORγt, and produce IL-17. It was reported that DTH responses induced by mBSA were impaired in IL-17-deficient mice; IL-17 was required for allergen-specific T cell activation in the sensitization phase during DTH (Afzali et al., 2007). IL-17 enhances the production of pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α, and chemokines, and acts together with these cytokines, though it has no direct pathological effect (Furuzawa-Carballeda et al., 2007). Nano-C_{60} treatment markedly suppressed the production of IL-17 in DTH mice (Fig. 3), and this finding was confirmed *in vitro* (Fig. 5), suggesting that nano-C_{60} may act directly on IL-17 synthesis in Th17 cells.

Treg cells also constitute a distinct subset that ameliorates immune pathology through suppression of pathogenic T cells (Tanaka and Sakaguchi, 2005). In contrast with the aggravating function of Th17 cells on autoimmune diseases, Treg cells have a critical function in the regulation of autoimmune diseases, including DTH (Fontenot et al., 2003; Sakaguchi, 2004; Sato et al., 2006). Treg cells express CD4, IL-2 receptor α chain (CD25), and transcriptional factor Foxp3 (Vicki, 2003). The suppressive action of Treg is due to release of inhibitory cytokines and cellular interaction. The percentage of Treg in $CD4^+$ T cells was significantly increased by nano-C_{60} treatment in DTH disease control mice (Fig. 7). Both Th17 cells and Treg cells are differentiated from naive $CD4^+$ T cells, and the pathways are mutually exclusive. Naive $CD4^+$ T cells will differentiate to Th17 cells in the presence of TGF-β and IL-6, and to Treg cells in the presence of TGF-β and absence of IL-6 (Bettelli et al., 2006; Mangan et al., 2006). In addition, IL-6 is an inflammatory cytokine that is involved in the development of DTH. Our results indicate that nano-C_{60} treatment significantly suppressed the elevation of IL-6 in DTH disease-control mice (Fig. 3). These results would suggest that a decrease of IL-6 production by splenic lymphocytes plays a significant role in the inhibitory effect of nano-C_{60} on DTH. Further study to examine the mechanisms in detail is under way.

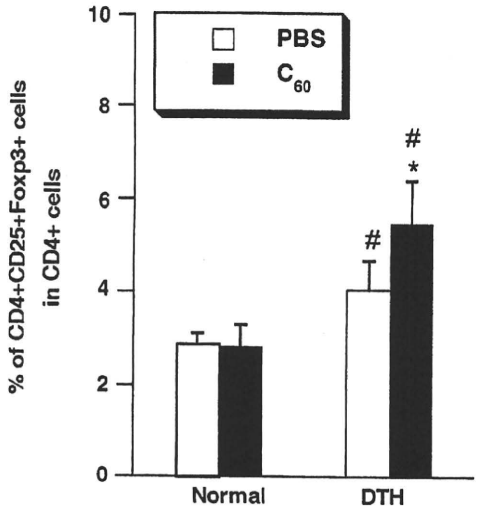

Fig. 7. Up-regulation of regulatory T cells in DTH mice treated with nano-C_{60}. The percentage of $CD4^+CD25^+Foxp3^+$ was analyzed by flow cytometry. Each value represents the mean ± S.E. ($n = 5$). PBS, phosphate-buffered saline; C_{60}, nano-C_{60}. #Statistically significant difference ($P < 0.05$) compared to the normal control group. *Statistically significant difference ($P < 0.05$) compared to the DTH-disease control group.

Conflict of interest

None.

Acknowledgments

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ナノマテリアルの次世代健康影響

——脆弱性集団への影響

Nanoparticles affect the health of next generation——Vulnerable populations to nanoparticles

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◎ナノテクノロジーの基盤材料であるナノマテリアルは，産業発展のため飛躍的な応用が期待される一方で，健康への影響が懸念されている．小さいゆえの物理化学的な特性から，もし体に入ったらどのような作用をもたらすかという点で，未知な部分が多い．著者らは脆弱性集団であるマウスの胎仔期に焦点をあて，意図的に生産される酸化チタンやカーボンブラックナノ粒子，非意図的にナノ粒子が生産されるディーゼル排ガスなどを曝露し，発達の段階で脳神経系や生殖系などにどのような影響が現れるか検討した．ここ10年近くにわたる著者らの研究および国内外で蓄積されつつある研究報告を通し，ナノ粒子が潜在的にさまざまな疾患の原因や増悪化の要因になりうると考えられるようになった．

Key word：ナノマテリアル，ナノ粒子，次世代影響，脳神経系，生殖系

ナノテクノロジーはわが国の将来を担う重要な基盤技術として大きな期待が寄せられている．すでに，さまざまなナノマテリアルの開発・製造・応用が開始され，工業分野，医薬分野などにおいて革新的な素材として注目されている．しかし最近，ナノマテリアルの健康影響を懸念する研究結果があいついで報告されるようになってきた．ナノサイズの物質の生体影響評価というまったく新しい分野であるために，リスク評価手法の標準化ができていないなど，さまざまな問題が存在する．そこで，わが国のナノテクノロジー産業が世界をリードしていくためには，ナノマテリアルの健康への影響に関し十分な基礎研究を行い，安全性を確認したうえで円滑に社会に受け入れられることが求められている．

一方，大気中の浮遊粒子状物質(直径10μm以下の粒子状物質)の健康影響が危惧されてきたが，最近，浮遊粒子状物質のなかには無数のナノサイズの粒子が含まれていることが明らかになった．都市圏の幹線道路沿道では浮遊するナノ粒子の大部分はディーゼル車由来といわれている．

著者らは脆弱性集団，とくに胎仔がナノマテリアルの曝露を受けたとき，出生後の成長の過程でどのような影響を受けるかについて研究を行ってきた．著者らの研究結果を中心に，意図的・非意図的に生産されるナノマテリアルの健康影響について紹介したい．

意図的に生産されるナノマテリアルの健康影響

1．ナノマテリアルの種類と性状

ナノマテリアルとしては炭素素材のカーボンブラック，カーボンナノチューブ，フラーレンなどがナノテクノロジー基盤材料のトップランナーとしてさらなる応用が期待されている．とくにカーボンブラックは，タイヤの高品質化や黒色顔料として使用量がもっとも多い．カーボンナノチューブは導電性や強度が注目され，今後の利用拡大が期待されている．また，金属酸化物のナノマテリ

アルとして酸化チタンは化粧品や光触媒として，酸化亜鉛は化粧品としてすでに汎用されている．さらに，銀，アルミナ，白金などが日用品や食品に利用されるなど，多くの種類のナノマテリアルが実用化されている．

これらのナノマテリアルでは結晶のサイズが小さくなることにより電子の状態が変化し，通常の大きな物質にはないような性質が現れる．たとえば，化学的な反応性が高まる点があげられる．化学反応は基本的に物質の表面で起こるが，物質がナノサイズになることにより単位質量当りの表面積が大きくなり，この比表面積の増大が化学的反応性を高めることになる．その他，小さくなることで多くの物理化学的変化の起こることが知られているが，身体の中での生物学的な作用はかならずしも明らかになっていない．

2. ナノマテリアルの体内への侵入経路

ナノマテリアルが体内へ侵入する場合，その経路として吸入(経気道)，浸透(経皮)，摂取(経口)などが想定されている[1]．しかし，侵入経路に関する定量的な解析はまだほとんどされていない．実験動物を用いた研究により，さまざまな曝露法による各種組織への影響に関する報告がみられるようになってきた[2]．しかし，物質の種類，サイズ，表面性状，曝露量，曝露方法など試験法が統一されていないことから，ナノマテリアルの体内への侵入経路についての全体像は得られていない．

3. ナノマテリアルの体内動態

ナノマテリアルが生体内に入ったときにどのような挙動を示すかについて，DDSや毒性評価を目的とした体内動態に関する研究が報告されている．しかし，統一された見解が得られているわけではない．

Semmler-Behnkeらによるラットへの金ナノ粒子静脈投与試験によると，投与後24時間後において18 nmの粒子は大半が肝(93.9%)に存在し，ごくわずかに脾(2.2%)に検出されたが，1.4 nmの粒子は肝(47.5%)腎(5.5%)血液(3.7%)など全身に検出された．いずれの粒子も脳には検出されなかった[3]．一方，De Jongらによるラットへの金ナノ粒子静脈投与試験では10，50，100，250 nm金ナノ粒子のすべてについて，24時間後には大半が肝，脾に検出されたが，とくに10 nmの粒子はさまざまな組織(血液，肝，脾，腎，精巣，胸腺，心臓，肺，脳)に検出された[4]．また，Fabianらによるラットへの酸化チタンナノ粒子静脈投与試験では，20〜30 nmの粒子が用いられ，血液，脳，リンパ液には検出されなかったが，肝，肺，脾，腎の順に高濃度に酸化チタンが検出された．肺と腎は14日でコントロールレベルに濃度が低下したが，肝は28日後も高レベルで，脾はわずかに減少していた[5]．マウスにおいてはSugibayashiらによる酸化チタンナノ粒子静脈投与試験により，投与後5分で血液や各種組織における15 nmの粒子濃度が上昇したが，脳には検出されないことが示された．酸化チタンは肝にもっとも多く検出され，1カ月で30%しか減少しなかった[6]．また，Yangらによる14 nmのQ-dot静脈投与においては，28日後においてほぼ100%が体内に残存していた[7]．以上の報告は，体内に侵入し血流に乗ったナノ粒子はクリアランスに非常に時間がかかることを示している．

4. 酸化チタンの胎仔期曝露による産仔の脳，精巣への移行と影響

上記の研究は成獣を用いたものであり，脳にはほとんど移行しないが，静脈投与によって，10 nm以下のナノ粒子であれば，わずかながら血液脳関門を通過し，脳へ到達する可能性が示された[4]．

著者らは，血液脳関門，精巣関門などバリアー機能の未完成な胎仔期に妊娠母体を経由してナノ粒子を曝露することにより血液胎盤関門の通過の可能性および胎仔の脳や精巣への移行と影響に関する研究を行った．酸化チタンナノ粒子を皮下投与した妊娠マウスからの出生仔について6週齢時に，精巣組織の透過電子顕微鏡(TEM)観察，および電界放出型走査電子顕微鏡(FE-SEM/EDS)による解析を行ったところ，酸化チタン粒子が検出・同定された．曝露群の精巣組織ではSertoli細胞の減少やミトコンドリアの損傷が認められた．また，曝露群の1日精子産生数に有意な低下が認められた．脳組織についての同様の解析では嗅球，大脳皮質，海馬などの部位に酸化チタン粒子が検出・同定された．さらに組織学的検討を行ったところ，末梢血管に多発性の微小梗塞の所見が認め

られ，曝露群の組織で嗅球僧帽細胞のカスパーゼ3(アポトーシスのマーカー)の発現が亢進していることが明らかとなった[8]．この結果，ナノ粒子の胎仔期曝露により，胎盤を経由して胎仔に到達し，未完成な血液脳関門，精巣関門を通過し，脳や精巣に沈着し，さらに組織の機能に影響を及ぼすことが示された．

非意図的に生産されるナノマテリアルの健康影響

1. 浮遊粒子状物質(SPM)とPM0.1

微粒子の健康影響については，非意図的に産生され大気中に浮遊する粒子状物質(suspended particle matter：SPM)によるものが従来から研究されてきた．この知見はナノマテリアルの健康影響を考えるうえでおおいに参考になる．SPMは大気中を漂う粒径10 μm以下の粒子(PM10)と定義付けられるものであり，呼吸器や循環器系に影響することが明らかになった．近年，より小さな粒径2.5 μm以下の粒子の健康影響が懸念され，PM2.5の健康影響の研究が行われてきた．さらに最近では，測定機器の発達に伴って粒径0.1 μm以下の粒子(ナノ粒子)の存在が明らかになり，個数的には大部分を占めることから，健康に大きく影響している可能性が注目されるようになった[9]．

SPMの発生源は工場から排出される煤塵や粉塵，自動車排気ガスなどの人為的なもののほかに，土壌の飛散などの自然現象もある．量的にもっとも寄与が大きいのは自動車排気ガスであり，SPMの約半分を占めるといわれている[10]．そのなかでもディーゼル車はガソリン車に比べて燃料の不完全燃焼が起こりやすく，粒子状物質(particle matter：PM)が発生しやすい．平成16年(2004)に環境省が報告した調査結果[11]により自動車から排出されるPMのうちほぼ100%近くがディーゼル車由来であることが示されている．最近，ディーゼル排ガス規制が施行された地域ではSPM汚染が大きく改善されつつある[10]．しかし，ナノ粒子が大きな生体影響を及ぼしうると考えられている一方で，大気中のナノサイズの粒子(PM0.1を含む)についてはこれをモニターする方法さえ確立されておらず，注意が必要である．

環境省は微小粒子状物質は総体として人びとの健康に一定の影響を与えると結論づけ(平成20年4月，微小粒子状物質健康影響評価検討会報告)，現在，同省中央環境審議会大気環境部会が微小粒子状物質の測定法の検討を進めている．

2. ディーゼル排ガス微粒子の組成

ディーゼル排ガスはガス成分と微粒子成分とに大別され，後者はディーゼル排気微粒子(diesel exhaust particle：DEP)ともよばれる．DEPにはナノ粒子(粒径100 nm以下)に相当する画分が非常に多い．とくに粒径5～50 nmの粒子は質量では全体のほんのわずかにすぎないが，個数では大部分を占める[12]．化学成分としては元素状炭素を核にもち，その外側にカルシウムや鉄などの金属成分，さらに有機炭素が付着している[13]．その由来は未燃燃料や潤滑油の不完全燃焼または熱分解生成物であり，有機炭素としてはベンゾ(a)ピレン，ニトロアレーン，ダイオキシンやフタル酸エステル類など，数百種類以上の物質が付着している．培養細胞を用いた研究によりDEPは細胞に酸化ストレスを誘導することが示されているが[14]，DEPのうちでも表面に金属成分が多く存在するものがより強い酸化ストレスを与えることが明らかになっている[13]．

3. ディーゼル排ガス(微粒子)胎児期曝露の健康影響と“成人病胎児期起源説”

ディーゼル排ガスの曝露により引き起こされる健康影響は，吸入により排ガスと接触する気道を中心によく研究されてきた．一方で，著者らは曝露による影響を受けやすい時期に焦点をあて，気道・呼吸器以外の器官に生じる影響について研究を行ってきた．とくに注目して研究を行ってきたのは，胎児期に曝露を受けることによる健康影響である．

著者らは，環境基準値程度の微粒子を含むディーゼル排ガスを妊娠中のマウスに吸わせ，生まれた仔に生じる影響を検討してきている．この研究で，排ガス由来と思われるナノサイズ(100 nm以下)の黒い粒子状物質が仔の脳血管周囲顆粒細胞内の消化顆粒に蓄積していることを世界に先がけて明らかにした[15]．また，脳内の神経伝達物質のモノアミン代謝や行動試験にも異変が認めら

れた[16]．母体の子宮内で発達する胎児は血液胎盤関門によって外界からの異物の侵入はある程度制限されている．しかし，器官の未発達な胎児では血液脳関門などの防御機能が不完全であるため，化学物質の曝露がわずかであっても影響を受けやすい一面ももつ．

器官形成段階である胎児期に受けた影響が不可逆的な変化として表れてしまう場合，一度受けた影響が出生後も生涯にわたって残り，疾病の発症につながってしまうことが考えられる．著者らは，ディーゼル排ガスの胎仔期曝露の実験系において，雄性生殖器[17-19]，雌性生殖器[20]，胎盤[21]，子宮内膜症病変[22]などにも影響が及ぶことを明らかにしている．類似した実験により，ディーゼル排ガス微粒子の胎仔期曝露が産仔の呼吸器に影響するという結果も報告されている[23,24]．

イギリスの Barker 博士は疫学調査の結果に基づいて 1986 年に“成人病胎児期起源説”を唱えている[25]．胎児期における栄養不足は臓器の十分な発育を妨げる要因になっているが，それが生活習慣病とよばれている成人病の発症につながるという説である．この説によると，胎児期や乳幼児期にその素因が形成され，出生後の環境要因によって成人病などの疾患にかかりやすくなるという．胎児期にディーゼル排ガスの曝露を受けると，出生後の発達によって脳神経系や生殖系，その他の臓器でさまざまな異常が表れるという著者らの研究結果は彼の説を支持している[26,27]．著者らは現在，生活習慣病といわれている疾患群の増悪化にも微粒子の胎児期曝露が寄与しているという作業仮説に基づいて，さらなる研究を進めている．

おわりに

胎仔期にナノマテリアルの曝露を受けると産仔の脳において病理学的に，また，神経伝達物質のモノアミン代謝や行動，遺伝子発現などにも異変が認められることから，著者らは最近増加している自閉症や多動症など微細脳機能障害が原因と考えられている脳神経疾患との関連性に注目して研究を進めている．雄の生殖系にも組織学的にまた機能的にさまざまな影響が及ぶことを観察している．

著者らのここ 10 年近くにわたる研究，また，国内外で蓄積されつつある研究報告を通してナノ粒子は潜在的にさまざまな疾患の原因や増悪化の要因になりうると考えるようになった．ナノ粒子の毒性からいかに身を守るか，粒子の除去，無毒化など健康予防対策も今後の課題である．

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Prenatal Diesel Exhaust Exposure Causes Neurodegenerative Diseases in Adults

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Summary

We examined the influences on the risk of neurodegenerative diseases in adult of prenatal diesel exhaust particles (DEPs) exposure. All mice with DEPs exposure showed swelling of astrocytes' endfoot, apoptosis of endothelial cells, and diffuse obstruction of capillaries. Some cytoplasmic granules of scavenger cell had DEPs. Specifically, mice at 40 weeks have the abnormal structure, which it supposes with neurofibrillary tangles, and showed positive for components of paired helical filaments. These findings suggest that prenatal DEPs exposure might effect fetal brain development, carry atrophy in the offspring and be one of risk factors on some neurodegenerative diseases in adults. The damages of fatal brain make influences reach not only in infants and youths but also in adults, the risk that suffers from Alzheimer's diseases etc. must be higher.

Introduction

Diesel exhaust (DE) is a major air pollutant in urban areas and the smallest DE particles (DEPs) are nanostructures (<0.1μm); we have already detected nano-sized particles and some damages in brain tissues of newborn mice whose mothers exposed DE during pregnancy[1-3]. These findings indicated that prenatal DEPs exposure affects the development of central nervous system in embryo or fetus and induces an innate morphological abnormality of infants' brain; therefore, there is a possibility that these effects of DEPs lead to various disorders of the nervous system in offspring. In this study, we examined the influences on the risk of neurodegenerative diseases in adult mice following prenatal DEPs exposure.

Material and Methods

Pregnant ICR mice were exposed to 0.1 mg/time of DEPs by subcutaneous injec-

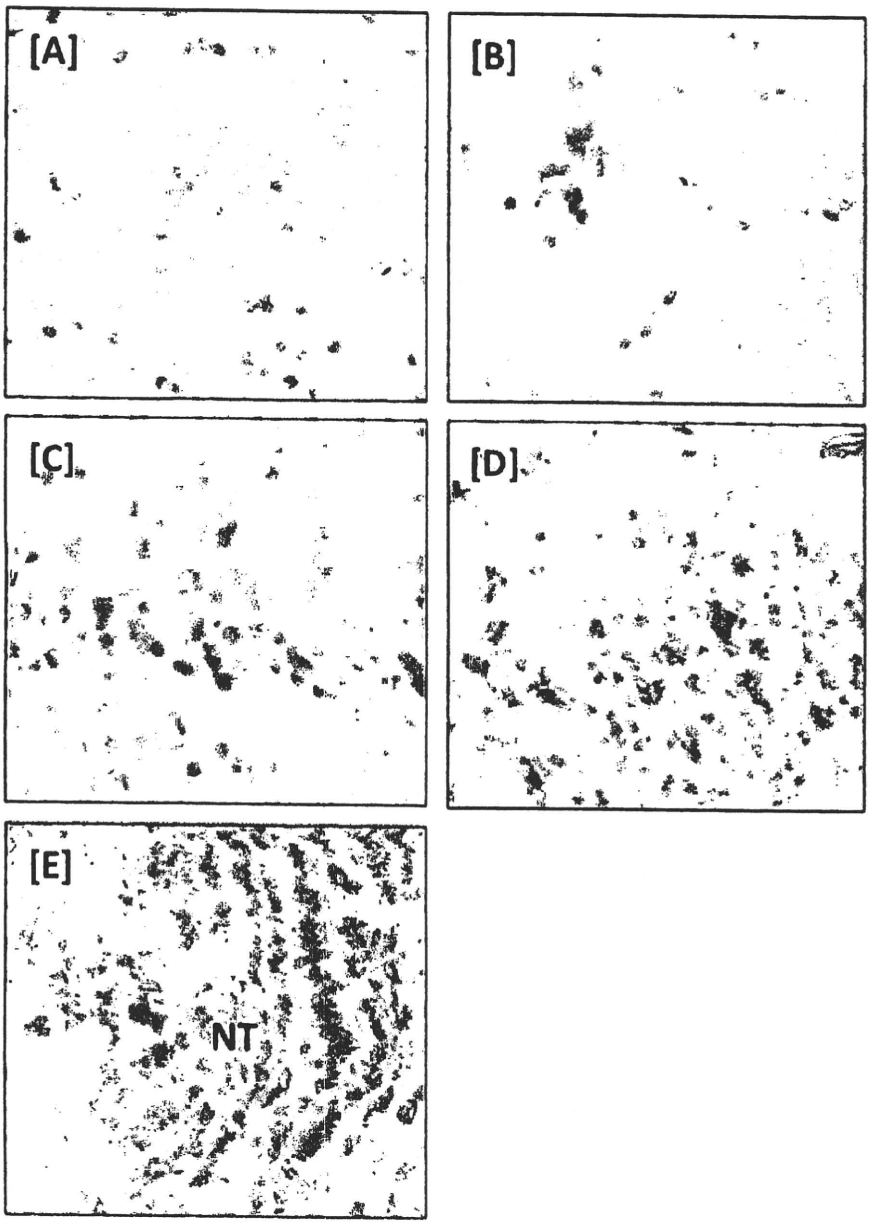

Fig.1 Detection of the abnormal structure in prenatal DEPs-exposed mice of 40 weeks age.
(A, B) Immunohistchemical stain for Aβ using anti-Aβ40 antibody. (x400) Brainstem (pons), Positive reaction: Brown color. A: Control group, B: DEPs exposed group.
(C, D) Immunohistchemical stain for tau using anti-tau phosphorylayed antibody. (x400) Olfactory bulb, Positive reaction: Brown color. C: Control group, D: DEPs exposed group.
(E) Electron micrograph of brain tissue. Abnormal structures, which it supposes with neurofibrillary tangles (NT), were observed in olfactory bulb.

tion five times on gestational day 6, 9, 12, 15 and 18 for maternal exposure group. Control pregnants were treated with vehicle in the similar method. After exposure, all pregnant mice were housed in clean air and were delivered of babies. Brain tissues were obtained from the mice (in clean air) born from DEP-exposed and control pregnant mice at 12 and 40 weeks old.

These brain tissues were examined by light and electron microscopy, we compared morphologically between with and without DEPs exposure. To detected amyloid β and phosphorylayed tau in these tissues, congo red stain and immunohistochemical stain were performed. Specifically, apoptotic appearance was evaluated under electron microscope based on an ultrastructural characteristic in our previous report [4].

Results

The common findings, which observed in prenatal DEPs-exposed mice of 12 and 40 weeks age, were as follows. Brain tissues showed swelling of astrocytes' endfoot, apoptosis of endothelial cells and scavenger cells surrounding blood vessels; these cells have roles in blood-brain barrier, and degenerative changes in the neighboring parenchyma. The cytoplasmic granules of scavenger cell had nano-sized particles, which appeared to be DEPs. These findings weren't observed in normal mice at all.

The characteristic findings in prenatal DEPs-exposed mice of 40 weeks age were detection of amyloid β Aβ (Fig.1-A, B), strong positive reaction for phosphorylated tau compared with control group (Fig.1-C, D), and neurofibrillay tangles in olfactory bulb under electron microscope (Fig.1-E).

Conclusions

These findings suggest that prenatal DEPs exposure might effect fetal brain development and carry atrophy of whole brain tissue in the offspring. The damages of fatal brain make influences reach not only in infants and youths but also in adults, and the accumulation of abnormal structures (Aβ, tau, etc.) due to the aging accelerate. As the result, even if they live in clean environment after birth, the risk that suffers from Alzheimer's diseases etc. must be higher.

Moreover, these phenomena were deriver from our observations on mice who have fetal exposure to DEPs; however, the human placenta consists of two layers while that of mouse consists of four layers. Furthermore, the length of human gestation is considerably longer than that of mice. Therefore, the possibility exists that more severe effects could occur in human than in the mice analyzed in this study.

Our result should be a grave warning that prenatal DEPs exposure is one of risk factors on some neurodegenerative diseases in adults.

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未知なる遭遇—ナノマテリアルの健康影響
次世代影響を中心に

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1　はじめに

ナノマテリアルの健康影響に関する研究は，欧米諸国が先陣を切り，大きくリードしている．米国では，クリントン大統領時代に国策として国家ナノテクノロジー戦略を設定し，ナノテクノロジー産業の育成を図るとともに負の側面のリスクにも着目して一定の予算を割き，安全性に関する研究をスタートさせている．先進欧州諸国でも同様な政策がとられたことから，様々な研究機関から多数の研究結果が報告されるようになってきた．したがって既に多くの論文，総説があり，[1~4] また，それらを紹介する優れた訳書が出版されている．[5~7] そこで，現在まで行われてきた研究の全体的な把握は成書に譲り，主に我々が行ってきた研究を紹介しながら，ナノマテリアルがもたらすであろう健康影響の本質について考えてみたい．

我々は最近，ディーゼル排ガス中の超微小粒子(ナノ粒子)が母マウスから胎仔脳に移行し，出生後脳の特定な細胞の特定なオルガネラに蓄積され周辺の細胞に影響を及ぼすこと，[8] さらに，化粧品や光触媒として汎用される酸化チタンナノ粒子も同様に母マウスから仔の脳や精巣に移行し，様々な異常を生じさせることを明らかにした．[9] 他のナノマテリアルに関しても研究を進めているが，本稿では次世代脳神経系及び生殖系への影響について我々の結果の一部を話題として紹介したい．なお，ナノマテリアルのリスク評価については今回は触れず，有害性とその予防策についてのみ言及したい．

2　非意図的に産生される微小粒子状物質の健康影響—浮遊粒子状物質及びディーゼル排ガスの健康影響

ナノマテリアルの健康影響を論ずるには，既に非意図的に産生され，大気中に浮遊する粒子状物質及びディーゼル排ガスの健康影響についての研究を無視することはできない．それらの結果を念頭において研究を進めることが重要と考える．浮遊粒子状物質やディーゼル排ガスにはナノサイズの超微小粒子が多量に含まれており，動物実験だけでなく多くの疫学調査並びにヒトにおけるボランティア介入実験の結果が報告されている．

初期において粒径10 μm以下の粒子状物質(PM 10)の健康影響が調べられていたが，近年では更に小さな粒子が問題視され，粒径2.5 μm以下の微小粒子状物質(PM 2.5)の健康影響の研究が行われてきた．その結果，主として欧米で行われた疫学研究から，以下のことが明らかになっている．

1) PM 2.5あるいはPM 10への短期曝露と死亡数との関係について，複数の都市の解析結果から，粒子濃度の上昇と1日以内(1日ラグ)に増加する死者数の間に関連性が認められている．

2) 短期曝露と外因死を除くすべての疾患による死亡(全死亡)，循環器系疾患による死亡，呼吸器系疾患による死亡との関連性が報告されている．さらに，心筋梗塞，COPD(慢性閉塞性肺疾患；chronic obstructive pulmonary disease)等，個別の疾患による死亡との関連性を報告しているものもある．

3) 循環器系疾患の死亡数増加に関する結果については，基本的には不整脈，急性心筋梗塞，冠動脈疾患，脳血管疾患等の病態を悪化させ，重篤な場合は死に至るという説明が可能である．

4) PM 2.5 あるいは PM 10 への短期曝露と医療機関への呼吸器系疾患や循環器系疾患で入院・受診する患者数との関連性が世界各国でみられている．

これらの疫学研究の結果は，動物実験及びヒト志願者による介入実験で明らかにされた呼吸器系への刺激や自律神経機能への影響等を介した作用，生理活性物質や過酸化物の増加等を介した作用，血液凝固系の活性化や血栓形成の誘導等を介した作用等で部分的に説明することが可能である．[10]

大気汚染による曝露は，すべての人々に及ぼしうるものであり，地域により，また職業によっては，更に高濃度に曝露される危険性が考えられる．環境衛生の立場からも，ナノ粒子を多量に含む微小粒子の健康影響を軽視することは決してできない．

3 ディーゼル排ガス(超微小粒子)の胎仔期曝露による脳神経系への影響

従来，浮遊粒子状物質の健康影響として粒子状物質 PM 10 及び PM 2.5 の健康影響の研究が行われてきたが，今後，粒径 0.1 μm(100 nm)以下の粒子，すなわちナノ粒子の健康影響である PM 0.1 の本格的な研究が必須である．都市圏では浮遊粒子状物質のうち，微小粒子あるいは超微小粒子の多くはディーゼル車由来といわれている．実際，当研究センターが所有するディーゼルエンジン排ガス曝露装置内に浮遊する粒子の粒径分布を調べてみると，100 nm 前後の粒子が，数では最も多く占めていた．

我々はディーゼル車が排出するガス(diesel exhaust；DE)を妊娠中の母マウスに吸わせ，生まれてきた仔の生殖系，脳神経系などへの影響を検討してきている(CREST 2000～2005 年，代表武田)．この研究で，排ガス由来と思われるナノサイズ(100 nm 以下)の黒い粒子状物質が仔の脳血管周囲顆粒細胞内の消化顆粒に蓄積していること，脳内に様々な異常が認められることを世界に先駆けて明らかにした．[8] 排ガス中のこのナノ粒子は，母体の胎盤を通過して胎仔に移行し，さらに血流に乗り未発達の脳血液関門(blood brain barrier)を通過して脳内に移行し，脳血管周囲顆粒細胞に取り込まれたものと考えられる．

ディーゼル排ガス中の粒子(超微小粒子)

曝露装置内のディーゼル排ガス粒子(diesel exhaust particle；DEP)の粒径分布は，走行状態で異なる．当研究センターの曝露装置ではアイドリング時は小さな(個数ピークは 60 nm 前後)，高速運転時に大きな(個数ピークは 110 nm 前後)粒径分布を呈していた．炭素の球状物質がコアとなり，芳香族炭化水素や硫酸塩，金属など，様々な化学物質が付着して DEP が形成されると考えられている．実際，粒子表面を有機溶媒で洗浄すると粒子表面の鉄イオンが多くなり，毒性が増すことが明らかになった．[11] ディーゼル排ガス曝露装置の希釈トンネルから採取された粒子状物質の電子顕微鏡下での観察では，球状の粒子とともに繊維状(カーボンナノチューブ様)物質も多数認められた．

脳の病理所見

光学顕微鏡所見として，DE 曝露群では，血管周囲に浮腫及び小血管の閉塞が認められた．

この所見は，DE曝露群のすべての脳の神経組織全体にび慢性に(ひろがって)分布していた．さらに電子顕微鏡下で，閉塞小血管の内皮細胞は欠損しているか，あるいはアポトーシス形成過程の内皮細胞が認められた．これら内皮細胞の変化は大脳皮質，海馬すべてに認められ，平滑筋が存在しない微小血管ではつぶれて閉塞していた．これらは，病理学的にび慢性の多発性微小梗塞と判定される．また，光学顕微鏡下での血管周囲の浮腫様形態は，血管周囲に伸びたアストロサイト(神経膠星状細胞)のエンドフットが異常に膨化したことによる．この細胞質内には，しばしばミエリン様物質が認められた.[8]

以上のことから，次のような傷害が示唆される．①末梢血管そのものの傷害，②血管から栄養を得ているアストロサイトの機能不全による神経細胞の傷害，③末梢血管閉塞による閉塞部位から先の細胞の傷害の3点である．これらは脳の萎縮につながる事象である．

さらに，大脳皮質にび慢性のアポトーシス像(カスパーゼ3陽性細胞)や小脳プルキンエ細胞のアポトーシス像も観察された.[12]

モノアミン代謝の変動と行動異変

電子顕微鏡による観察とともに，神経伝達物質のモノアミンの代謝や行動試験にも異変が認められたことから,[13] 最近，増加している微細脳機能障害による脳神経疾患との関連性についても研究を進めている．ディーゼル排ガス由来の微粒子(DEP)画分を妊娠期に投与した研究においても，産仔の脳神経系への影響が認められた．以上の研究は大気に存在する超微小粒子(あるいはガス成分)が母から子に伝わり，子の生育発達に伴って様々な疾患を引き起こす要因になることを示唆している．

4 ディーゼル排ガス胎仔期曝露による生殖系への影響

我々は，ディーゼル排ガス胎仔期曝露の実験系において，雄性生殖,[14~18] 胎盤,[19] 子宮内膜症[20]などに影響が及ぶことを明らかにした．紙面の関係でそれらの紹介は割愛したい．

5 意図的に生産されるナノマテリアルの健康影響―ナノマテリアルの胎仔期曝露による脳神経系への影響

現在，国際的にナノテクノロジーの基盤材料であるナノマテリアルの毒性の有無と，その程度が議論され始めている．我々は非意図的に産生されるディーゼル排ガス微粒子以外にカーボンブラック，カーボンナノチューブ，フラーレン，酸化チタンなど意図的に工業的に生産される様々なタイプのナノマテリアルの健康への影響，特に次世代を担う子供たちへの影響を中心に研究を進めてきている．影響を及ぼす部位やその傷害の程度に差はあるものの，調べたナノマテリアルは，基本的には上記DEPと同様な所見を示すことがみいだされつつある．酸化チタンを妊娠マウス皮下に投与すると，酸化チタンナノ粒子が産仔の脳に移行し，脳末梢血管周囲に異常を引き起こし，脳の特定の部位に限局してアポトーシス像が認められた.[9] さらに神経伝達物質のモノアミン系の代謝異常も認められた．また，網羅的遺伝子発現解析並びに選択的遺伝子発現解析の結果からも様々な異常が明らかになってきた．

ナノマテリアルの脳への移行についてOberdörsterらは，ラットの吸入実験において鼻部上皮に沈着した^{13}Cナノ粒子が，嗅覚神経を介して，脳へアクセスすることを実証した.[21] 一方，我々は酸化チタンナノ粒子が妊娠期の母から仔に移行し，出生後の成長期にも脳内に取り込まれていること，病理学的に，また機能的に様々な影響を及ぼすことを世界に先駆けて明らかにした(図1~3).[9]