Short communication

Fullerene (C₆₀) Is Negative in the *In Vivo* Pig-A Gene Mutation Assay

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Carbon nanoparticles, such as carbon nanotubes and fullerene (C₆₀), are potential candidates as leading substances in nanotechnological fields, but little is known about their safety. Here we examined *in vivo* genotoxicity of C₆₀, by performing the *Pig-A* gene mutation assay in the peripheral blood of male C57BL/6Cr mice. Mice were given single intraperitoneal injection of 3 mg of C₆₀ particles in 0.5 mL suspension containing 0.1%-Tween80-saline. As a positive control for the *Pig-A* gene mutation assay, mice were given a single oral administration of *N*-nitroso-*N*-ethylurea. At 2 and 8 weeks after treatments, we analyzed CD24-negative and -positive red blood cells in peripheral blood and calculated *Pig-A* mutant frequencies. As a result, we detected no significant differences in the mutant frequencies between C₆₀ treated and non-treated mice, indicating that C₆₀ is negative for genotoxicity *in vivo* in the limited target tissues assessed in this study. For the full assessment, we need comprehensive whole body survey on the genotoxicity of C₆₀.

Key words: carbon nanoparticle, *in vivo* genotoxicity, *Pig-A* gene mutation assay, fullerene

Introduction

Manufactured nanomaterials are important substances in nanotechnology, and the potential human and environmental risks need to be investigated for risk assessment and management.

There are several reports on the toxicities induced by carbon nanoparticles, such as single-wall carbon nanotubes (SWCNTs), multi-wall carbon nanotubes (MWCNTs) and fullerene (C₆₀). Intraperitoneal application of MWCNTs induced mesothelioma in p53^{+/-} mouse (1) and intrascrotal administration of MWCNTs induced mesothelioma in wild-type rats (2). Reports on the *in vivo* genotoxicity of C₆₀, however, are conflicting. It was reported that intratracheal instillation of C₆₀ increased both mutation frequency detected by *gpt*-assay

and DNA damage detected by comet assay in lung (3). Nevertheless another group showed that treatment with C₆₀ by gavage has no genotoxic effect in ICR mice, using *in vivo* micronucleus test in bone marrow cells (4). These discrepancies could have been caused by differences in administration route, test method, or target organ.

Here we examined the *in vivo* genotoxicity of C₆₀ using a different test system—the recently established *Pig-A* gene mutation assay (5,6). The *Pig-A* assay, a powerful tool for the evaluation of *in vivo* genotoxicity, is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient erythrocytes and has been shown to be applicable across species from rodent to monkey (5–8). With this method, we need no transgenic animals to test *in vivo* genotoxicity, but need only 1–2 μ L peripheral blood (5,6). Additionally, long-term, accumulated *in vivo* genotoxic effects could be evaluated (9).

Materials and Methods

Test chemicals: Fullerene (C₆₀, Nanom purple SUH; purity >99.9%, Frontier Carbon Corporation, Tokyo, Japan) was obtained and prepared as previously described with some modifications (1). Briefly, C₆₀ was suspended to physiological saline (Ohtsuka Pharmaceutical Co., Tokyo, Japan) and autoclaved. After addition of Tween 80 (Polysorbate 80 (HX), NOF Corporation, Tokyo, Japan) at a final concentration of 0.1%, solutions were subjected to sonication by ultrasonic homogenizer (VP30s, TAITEC Co. Japan). C₆₀ was prepared at a final concentration of 6 mg/mL. *N*-nitroso-*N*-ethylurea (ENU, Sigma) was dissolved in PBS (pH

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6.0) at 10 mg/mL as previously described (5).

Animal treatment: Mice were treated as described previously (1). In brief, 6 male wild-type C57BL/6Cr mice (SLC, Shizuoka, Japan) at the age of 9–11 weeks were given single i.p. injection of 3 mg/head suspension (0.5 mL) of C₆₀. Vehicle solution (0.5 mL) was given to 6 mice as negative controls. As a positive control of this study, 5 mice were given single oral administration of ENU (40 mg/kg). Peripheral bloods were withdrawn from tail vein of mice and analyzed by the *Pig-A* gene mutation assay. All mice were housed individually under specific pathogen-free conditions, with a 12 h light-dark cycle at the animal facility of NIHS. All mice were given tap water and gamma-ray irradiated CRF-1 pellets (Oriental Yeast Co., Ltd.) *ad libitum*. Animal experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, Japan.

Antibodies: Anti-mouse TER119 antibody for erythroid cells staining (clone TER-119, PE-Cy7-conjugated) and anti-mouse CD24 antibody (clone M1/69, FITC-conjugated) were obtained from BioLegend.

***Pig-A* gene mutation assay in mice:** Mice *Pig-A* gene mutation assay was performed as previously described (5,8), with some modifications. In brief, EDTA/2K was dissolved in distilled water to make a 12% solution, and used as an anticoagulant. Eighteen μ L of peripheral blood were mixed with 2 μ L of EDTA solution. Two μ L of blood/EDTA mixture was suspended in 0.2 mL of PBS, and the cells were labeled with 1 μ g of each anti-mouse TER119 and anti-mouse CD24 antibodies. After incubation for 1 h in the dark at room temperature, the cells were washed once by centrifugation (500 $\times$ g, 5 min), resuspended in 2 mL of PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences). After gating for single cells, about 1,000,000 TER119-positive cells were analyzed for the presence of CD24 on their surface. The data were statistically compared with the corresponding solvent control using the Student's t-test.

Results

***Pig-A* gene mutation assay with mice peripheral blood:** Recent works provided that the erythrocyte-based *Pig-A* gene mutation assay is applicable across species (5–8). According to these reports, we modified the original *Pig-A* gene mutation assay and performed it with mice peripheral blood. To classify white blood cells (WBCs) and red blood cells (RBCs) in mice peripheral blood, RBCs were stained with anti-TER119 antibody. Anti-CD24 antibody was used to detect GPI-anchored protein as previously reported (8,10). The gating strategy that was used to score GPI anchor deficient RBCs population was shown in Fig. 1. Single cells in-

cluding RBCs and WBCs were gated by light scatter (Fig. 1A). To exclude WBCs from this population, TER119-positive cells (Fig. 1B) were analyzed further for the presence on the cell surface of either the GPI-anchored CD24 (Fig. 1C and 1D). The gate used for CD24-negative cells was established by blood cell samples prepared without the fluorescent reagents.

***In vivo* genotoxicity tests on fullerene (C₆₀) analyzed by the *Pig-A* gene mutation assay:** At 2 and 8 weeks after the injection of C₆₀ (3 mg/head) and ENU, we analyzed CD24-negative and -positive RBCs in peripheral blood. At both 2 and 8 weeks after the injection, higher amounts of CD24 deficient RBCs were observed in the ENU treated mice (Fig. 1D) as compared with the solvent control (not shown) and C₆₀ treated mice (Fig. 1C), respectively. Frequencies of CD24-negative RBCs were summarized in Fig. 2. Frequency of CD24-negative RBCs was significantly increased in ENU treated mice (2 weeks after treatment; $30.12 \pm 3.54 \times 10^{-6}$, and 8 weeks after treatment; $36.64 \pm 15.71 \times 10^{-6}$). However, we detected no obvious differences in frequency of CD24-negative RBCs between C₆₀ treated and non-treated mice ($0.25 \pm 0.30 \times 10^{-6}$ versus $0.42 \pm 0.19 \times 10^{-6}$ after 2 weeks and $0.82 \pm 0.54 \times 10^{-6}$ versus $1.87 \pm 1.51 \times 10^{-6}$ after 8 weeks).

These results indicated that although the *Pig-A* gene mutation assay with mouse peripheral blood was appropriately performed, C₆₀ was negative for genotoxicity *in vivo* in the RBCs assessed in our study.

Discussion

We demonstrated here that C₆₀ (3 mg/head) given intraperitoneally to male C57BL/6Cr mice was negative in the *Pig-A* gene mutation assay using peripheral blood, suggesting that C₆₀ was not mutagenic to erythroid precursor cells or hematopoietic stem cells.

The *Pig-A* gene mutation assay is based on detections of GPI-anchored protein on the cell surface of RBCs. The *Pig-A* gene is involved in the synthesis of GPI anchors that link various protein markers to the cell surface. It is known that paroxysmal nocturnal hemoglobinuria (PNH) is caused by somatic *PIG-A* mutations in hematopoietic stem cells (HSCs) and Aero-lysin-resistant HSCs from a patient with PNH exhibited clonal *PIG-A* mutations (11,12). Additionally, it is considered that the absence of GPI-anchored protein of RBCs is caused by mutations occurred in the *Pig-A* gene of nucleated erythroid precursors and/or of HSCs (6). These observations suggested that expression of GPI-anchored CD24 of RBCs is depending on the *Pig-A* gene mutations happened in erythroid precursors and/or of HSCs in bone marrows. According to this, we considered that our results, shown here using peripheral blood of mice, reflected genotoxicity of C₆₀ on bone marrows.

Fig. 1. Analysis of mouse peripheral blood by flow cytometry. (A) Single cell populations were gated and further analyzed with anti-TER119 antibody. (B) TER119-negative white blood cells were excluded from the gated single cell population. TER119-positive red blood cells (RBCs) were gated and further analyzed with anti-CD24 antibody. (C) Approximately 1×10^6 TER119-positive RBCs were analyzed for CD24 expression. CD24-negative RBCs (GPI-Anchor -) were scored as *Pig-A* mutants. In here, there were no obvious features in RBCs derived from C_{60} treated mice. (D) TER119-positive cells derived from ENU-treated mice were analyzed for CD24 expression.

Our data are consistent with the finding that C_{60} administered by gavage to ICR mice is negative in the *in vivo* bone marrow micronucleus test (4). These reports and our result suggest that intraperitoneal injection and gavage of C_{60} are negative for genotoxicity on bone marrow cells including erythroid precursors and HSCs. In both studies, however, the bone marrow was not exposed to C_{60} directly. A recent report showed that intratracheal instillation of C_{60} increased both mutation frequency (*gpt* assay) and DNA damage (comet assay) in the lung (3). From the mutation spectra, it was suggested that oxidative DNA damage might be involved in mutagenicity of C_{60} (3). C_{60} -phagocytized macrophages and granulomatous formations were also observed in the lung (3). Additionally, intratracheal instillation of

C_{60} could induce inflammatory responses in the lung (13). It is known that reactive oxygen species (ROS) generation by nanoparticles could be due to particle-cell interactions, especially in the lungs where there is a rich pool of ROS producers like the inflammatory phagocytes, neutrophils and macrophages (14). According to these observations, it is possible that both direct exposure to the target tissue and inflammatory response are important factors in the evaluation of the genotoxicity of C_{60} .

On the other hand, details of inflammatory responses were unclear, but intraperitoneal application of C_{60} induced no obvious change on exposed area except for black patchy deposits on the serosal surface in $p53^{+/-}$ mouse (1). Therefore it is expected that ROS generation

Fig. 2. Frequency of CD24-negative RBCs. At 2 and 8 weeks after mice were treated with C₆₀ (3 mg/animal), ENU (40 mg/kg), or solvent, peripheral blood was withdrawn from the tail vein and RBCs were analyzed by flow cytometry for CD24 expression. Values are the mean $\pm$ SD of data from 6 animals (C₆₀ and solvent) or 5 animals (ENU). *P*-values less than 0.0005 are indicated by asterisks.

by inflammatory responses might not occur and we detected negative genotoxicity in our case.

Recent reports including our results about genotoxicity of C₆₀ are discrepant. However, it is known that C₆₀ have an ability to quench and generate ROS (15,16). These discrepancies about genotoxicity of C₆₀ may be caused by a duality of C₆₀ itself. At this time, we cannot explain the mechanism(s) of C₆₀ genotoxicity in detail, but we suspect that it is complex and includes oxidative DNA damages, inflammation, and other biological factors. To assess the genotoxicity of C₆₀ more fully, we need a comprehensive whole body survey.

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