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Original Article

Genotoxicity in Cell Lines Induced by Chronic Exposure to Water-Soluble Fullerenes Using Micronucleus Test

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Abstract

Objectives: Nanomaterials have numerous potential benefits for society, but the effects of nanomaterials on human health are poorly understood. In this study, we aim to determine the genotoxic effects of chronic exposure to nanomaterials in various cell lines.

Methods: Chinese hamster ovary (CHO) cells, human epidermoid-like carcinoma (Hela) cells and human embryonic kidney 293 (HEK293) cells were treated with the water-soluble fullerene $C_{60}(OH)_{24}$ for 33–80 days. Cell proliferation, cytotoxic analysis and micronucleus tests were performed.

Results: When treated with $C_{60}(OH)_{24}$ (0, 10, 100, or 1000 pg/ml) for 33 days, both the HEK293 and Hela cells showed increased cell proliferation, but cellular lactate dehydrogenase (LDH) activity was not affected. After long-term exposure (80 days) to $C_{60}(OH)_{24}$ (0, 10, 100, or 1000 pg/ml), the CHO, Hela and HEK293 cells showed increased genotoxicity on the micronucleus test.

Conclusion: This study suggests that nanomaterials, such as $C_{60}(OH)_{24}$, have genotoxic effects.

Key words: water-soluble fullerene, genotoxicity, micronucleus test

Introduction

Environmental health studies have focused on the relationship between health outcome and ambient levels of PM10 and PM2.5, which are particles having aerodynamic diameters <10 and 2.5 μm, respectively. Recently, however, epidemiological studies have begun focusing on ultrafine particles (UFPs) having a diameter of <100 nm, which are abundant but account for a small proportion of total particle mass. UFPs are important with regard to adverse health effects due to their high alveolar deposition fraction (1–3).

Biomedical applications under development include targeted drug delivery systems for brain and tumor tissues, as well as intravascular nanosensor and nanorobotic devices for imaging and diagnosis. However, the potential adverse effects or humeral response following the introduction of nanomaterials into an organism remain unknown (4–6). After inhaled nanomaterials are deposited in the respiratory tract, their small size allows cellular uptake and transcytosis into the vascular and lymphatic systems.

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Our experiments have demonstrated that both carbon black (CB) and water-soluble fullerene [$C_{60}(OH)_{24}$] exhibit cytotoxicities, such as decreased cell density and cell growth, and that CB facilitates autophagic cell death in human umbilical vein endothelial cells. Furthermore, CB and $C_{60}(OH)_{24}$ up-regulate the expression of inflammation- and ubiquitin-proteasome-related genes, indicating that exposure to CB or $C_{60}(OH)_{24}$ represents a risk of atherosclerosis and ischemic heart disease (7, 8).

The results of epidemiologic and animal studies have suggested that exposure to nanoparticles plays roles in cardiovascular diseases such as atherosclerosis and myocardial infarction (2, 3, 9–12), and in genetic damage to cells or tissues (13–16). However, how exposure to airborne particulate matter induces genetic changes in germ lines or tissues is poorly understood. The small size of nanoparticles may allow them to be inhaled into the respiratory system, where they can pass into the bloodstream, ultimately reaching the germ lines. Nanomaterials then have the potential to adversely affect these cells.

Nanomaterial cytotoxicity in cells varies with chemical characteristics and surface properties, including hydrohobicity, hydrophilicity, and surface area per molecule (1, 5). Experimental studies have shown that $C_{60}(OH)_{24}$ may stimulate reactive oxygen species production in cells or tissues, and may inhibit cell proliferation or induce cell death (14). In contrast, other experimental studies have shown that $C_{60}(OH)_{24}$ may scavenge produced reactive oxygen species, and inhibit cell proliferation (17). The aim of the this study is to investigate the cytotoxic

and genotoxic effects of long-term exposure to $C_{60}(OH)_{24}$ in cultured cells. In particular, we focused on genotoxicity in germ cell [Chinese hamster ovary (CHO) cells: short G1 phase], somatic cell [human embryonic kidney 293 (HEK293) cells], and adult cell [human epidermoid-like carcinoma cells (Hela) cells: long period of G1 phase] models.

Materials and Methods

Materials

Hydroxyl fullerene {$C_{60}(OH)_{24}$; Tokyo Progress System, Tokyo, Japan} was used as described (7, 8), and its diameter was 7.1±2.4 nm.

Cell culture

All cell lines (CHO, Hela, HEK293) were obtained from Dainippon Seiyaku (Osaka, Japan) and were cultured in DMEM with 10% fetal bovine serum (Hyclone, Utah, USA). In experiments, cells were cultured in DMEM with 2% FBS and sonicated $C_{60}(OH)_{24}$ (0, 10, 100, or 1000 pg/ml or 20, 100 ng/ml). Cells were passaged every 3–4 days. Representative photomicrographs of CHO, Hela and HEK293 cells treated with $C_{60}(OH)_{24}$ had taken. CHO, Hela and HEK293 cells at ~30% confluence were treated with $C_{60}(OH)_{24}$ (0, 20, or 100 ng/ml) for 3 or 6 days.

LDH assay

Lactate dehydrogenase (LDH) activity was analyzed using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) in accordance with the manufacturer's protocols. Cells at 50–60% confluence were treated with $C_{60}(OH)_{24}$ for 33 days. LDH activity in the culture medium was evaluated on the basis of absorbance at 490 nm using a microplate reader (ARVO; PerkinElmer, Japan). Cytotoxicity was expressed relative to basal LDH release rate in untreated cells.

Proliferation assay

Cell proliferation assay was performed using a Cell Counting-8 kit (Dojindo laboratories, Kumamoto, Japan) in accordance with the manufacturer's protocols. Cells were cultured in 12-well plates and treated with $C_{60}(OH)_{24}$ (0, 10, 100, or 1000 pg/ml) for 33 days. An assay solution was added in each well, and after incubating for 3 h, the media were transferred to 96-well plates. Cell growth was measured on the basis of absorbance at 450 nm using an ARVO microplate reader.

Micronucleus test

A micronucleus test was performed in accordance with the method of Matsushima et al. (18), with slight modifications. Cells were cultured in six-well plates for 24 h, and then exposed to $C_{60}(OH)_{24}$ (0, 10, 100, or 1000 pg/ml) for 80 days. After exposure to $C_{60}(OH)_{24}$, the cells were washed 3 times with PBS, and suspended in a hypotonic solution (75 mM KCl) for 10 min at room temperature. The cells were then resuspended in cold methanol containing 25% acetic acid. After fixation, the cells were then suspended in methanol containing 1% acetic acid and spotted onto a glass slide. The cells were then air-dried and mounted with 4',6-diamido-2-phenyindole dilactate (DAPI)-containing medium. The nucleus was observed under a fluorometry microscope at 200× magnification. The number of micronucleated cells per 1000 cells was determined.

Statistical analysis

Data are presented as mean±SEM. Statistical evaluation was performed using an unpaired Student's t test. p values of <0.05 were considered to be statistically significant.

Results

We first analyzed the acute effects of exposure to relatively high doses of $C_{60}(OH)_{24}$ (0, 20, and 100 ng/ml) on cells by treating CHO, Hela and HEK293 cells with $C_{60}(OH)_{24}$ for 3 or 6 days (Fig. 1). The numbers of CHO and HEK293 cells decreased significantly in a dose-dependent manner, but the number of Hela cells did not significantly change for the 3-day exposure. The cytotoxic effects of $C_{60}(OH)_{24}$ in CHO and HEK293 cells were suppressed after 6 days; Hela cells exhibited a significantly decreased cell growth rate in a dose-dependent manner. We believe that $C_{60}(OH)_{24}$-sensitive cells died within 3 days, but $C_{60}(OH)_{24}$-resistant CHO and HEK293 cells were able to survive for 6 days. In contrast, CHO and HEK293 cells were more sensitive to ng/ml doses of $C_{60}(OH)_{24}$ than Hela cells.

To analyze the effects of very low doses of $C_{60}(OH)_{24}$ (0, 10, 100, and 1000 pg/ml), we investigated cell proliferation rate, cytotoxicity, and mitogenic effects in the three cell lines. After $C_{60}(OH)_{24}$ exposure for 33 days, the cell proliferation rates of HEK293 (peak, 1.3 fold) and Hela (peak, 2.0 fold) cells significantly increased in a dose-dependent manner (Fig. 2a). Moreover, cytotoxic effects were not observed, rather than suppressed in Hela cells under this condition (Fig. 2b). In addition, cell morphology did not change after $C_{60}(OH)_{24}$ exposure for 33 days (data not shown). These results suggest that $C_{60}(OH)_{24}$ has mitogenic rather than cytotoxic effects with long-term exposure at very low concentrations. Hela cells were more sensitive to $C_{60}(OH)_{24}$ than HEK293 cells with regard to cell growth efficiency. These results may differ owing to the cell cycle period; the G1 phase period of CHO and HEK293 cells is shorter than that of Hela cells. We hypothesized that if nanomaterials have mitogenic activity when cells are chronically exposed to $C_{60}(OH)_{24}$, abnormal nuclei such as micronuclei would be observed. After treating the cells with very low doses of $C_{60}(OH)_{24}$ for 80 days, the number of micronuclei that were stained with DAPI were determined (Fig. 3, arrowheads). The number of micronuclei was significantly higher after exposure to $C_{60}(OH)_{24}$ for 80 days than control (Fig. 4). The ratio (‰) of cells with micronuclei was also significantly high in CHO, Hela and HEK293 cells. CHO and HEK293 cells were more sensitive to $C_{60}(OH)_{24}$ than Hela cells in terms of the ratio of cells with micronuclei.

These results demonstrate that the effects of nanomaterials, such as $C_{60}(OH)_{24}$, which may have toxic, mitogenic, or mutagenetic activity, may contribute to cardiovascular diseases as well as other diseases such as cancer.

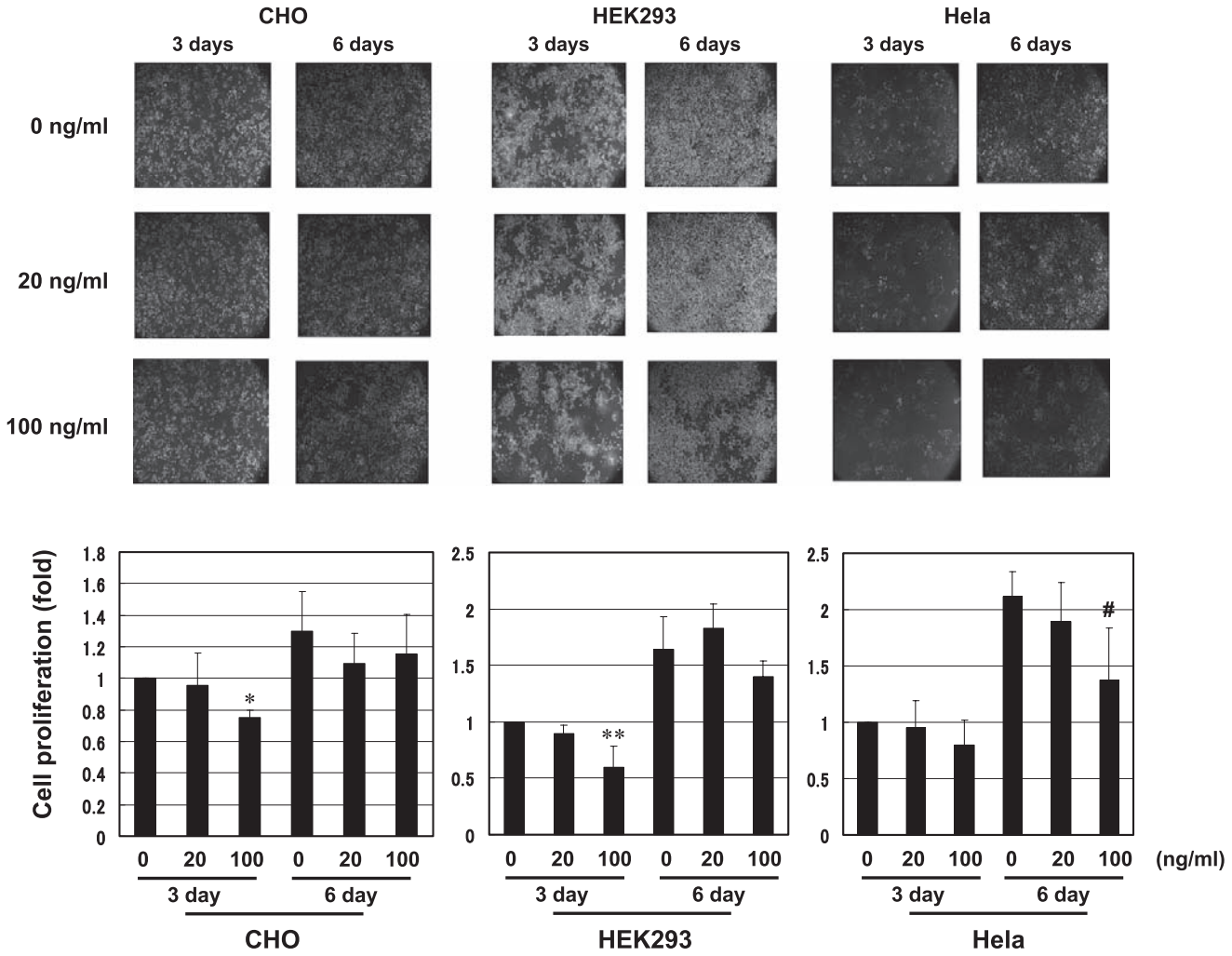

Fig. 1 Representative photomicrographs of CHO, Hela and HEK293 cells treated with $C_{60}(OH)_{24}$. CHO, Hela and HEK293 cells at ~30% confluence were treated with $C_{60}(OH)_{24}$ (0, 20, 100 ng/ml) for 3 or 6 days (upper panels). Cell number was calculated using a hemocytometer (n=4). Results are shown relative to those of controls (lower panel). * $p<0.02$, ** $p<0.03$, # $p<0.05$.

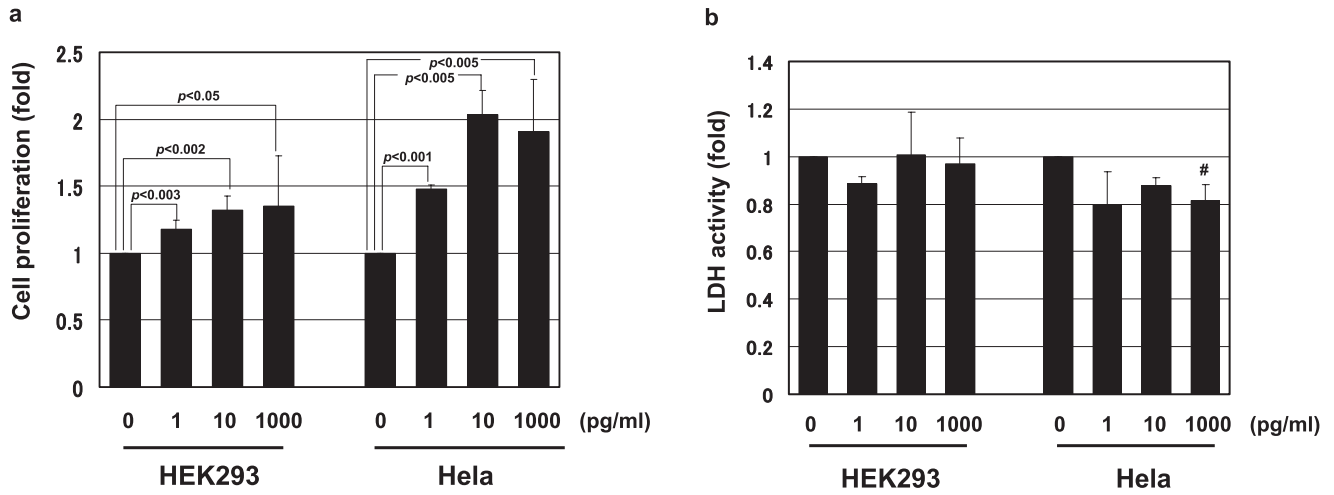

Fig. 2 $C_{60}(OH)_{24}$-induced cell growth in dose-dependent manner. HEK293 and Hela cells at ~30% confluence were treated with $C_{60}(OH)_{24}$ (0–1000 pg/ml) for 33 days. (a) The number of cells was determined using water-soluble tetrazolium salt (WST-8). (b) $C_{60}(OH)_{24}$ did not induce cytotoxic injury in HEK293 or Hela cells. Cells at ~30% confluence were treated with $C_{60}(OH)_{24}$ (0–1000 pg/ml) for 33 days. The amount of lactate dehydrogenase released into culture medium was measured. Cytotoxicity was calculated as the fold-change relative to that of the controls (n=4). Results are shown relative to those of the controls. *p* values indicate significance.

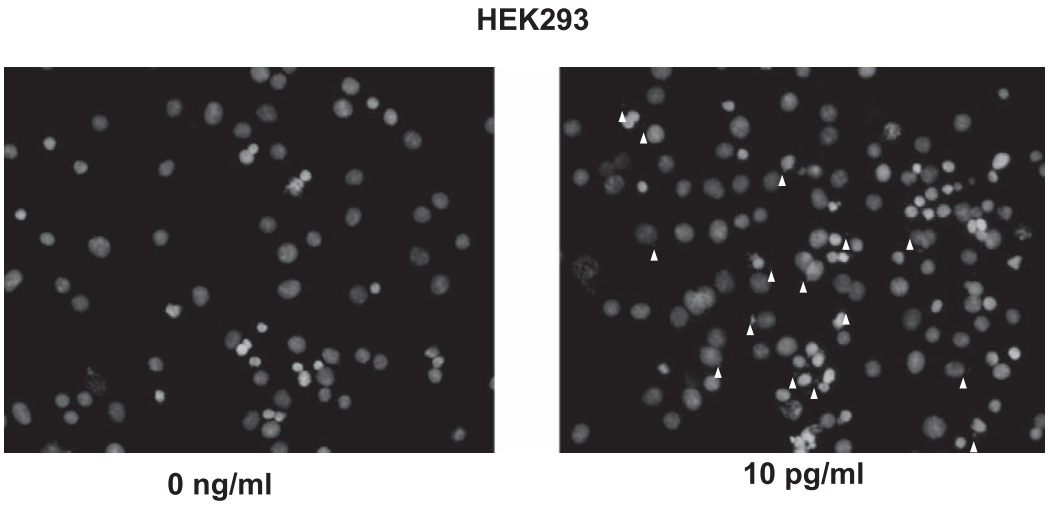

Fig. 3 The number of cells with micronuclei among HEK293 cells exposed to $C_{60}(OH)_{24}$ (0 or 10 pg/ml) for 80 days was elevated.

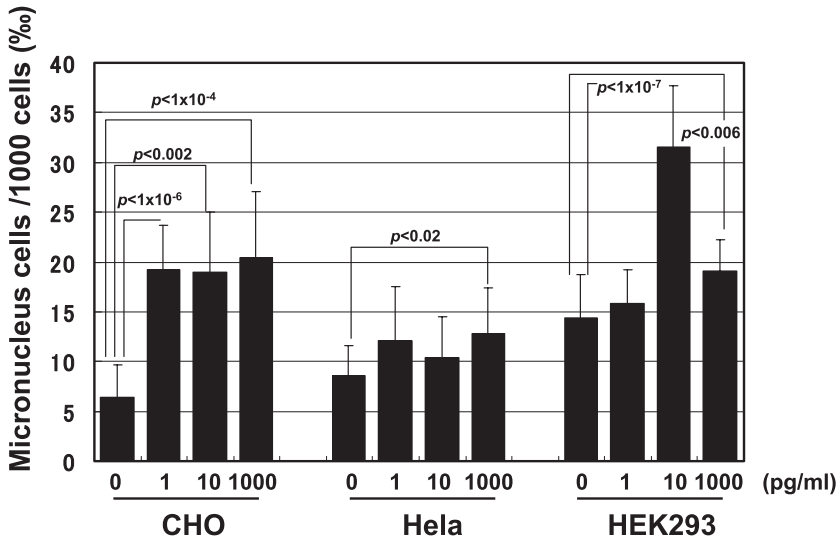

Fig. 4 Formation of micronuclei (‰) in CHO, Hela and HEK293 cells after treatment with different concentrations of $C_{60}(OH)_{24}$ for 80 days. *p* values indicate significance.

Discussion

The aim of this study is to clarify effects of chronic exposure to very low doses of nanomaterials, such as $C_{60}(OH)_{24}$, particularly with regard to genotoxicity *in vitro*. There is no evidence that pg/ml doses of $C_{60}(OH)_{24}$ have oncogenic or antioncogenic activity. Short-term exposure to relatively high doses of $C_{60}(OH)_{24}$ in the ng/ml range may induce antioncogenic functions, such as cell growth suppression (Fig. 1); however, long-term exposure to pg/ml doses of $C_{60}(OH)_{24}$ may induce cell growth (Fig. 2a). Recent studies have shown that ng/ml doses of $C_{60}(OH)_{24}$ have antioncogenic activity in various cancer cell lines (4, 19). In contrast, very low concentrations of $C_{60}(OH)_{24}$ are able to stimulate micronucleus production after long-term exposure. In this study, the cells (CHO, Hela, and HEK293) probably experienced DNA damage via an unknown mechanism (Fig. 4).

We believe that micronucleus generation caused by $C_{60}(OH)_{24}$ is not the result of chromosomal DNA damage by genotoxic molecules, such as reactive oxygen species, but is rather due to unsuccessful chromosomal DNA division during cell division during the M phase (20–22). Indeed, micronucleus production is thought to be caused when one daughter cell becomes trisomic and the other monosomic owing to aberrant segregation. The lagging chromosome may then form a micronucleus. Micronuclei can contain an entire chromosome that lags at mitosis or chromosome fragments that are not incorporated into daughter nuclei during cell division owing to kinetochore dysfunction. In addition, LDH activity was suppressed depending on the concentration of $C_{60}(OH)_{24}$ in culture medium (Fig. 2b). This shows that $C_{60}(OH)_{24}$ may scavenge reactive oxygen species, and protect against cell death. Fullerene derivatives were previously used as scavengers for reactive oxygen species, such as O_2^- and nitric oxide (17). On the basis of these results, reactive oxygen species were determined not to be involved in micronucleus generation in these experiments.

We used commercial $C_{60}(OH)_{24}$ containing about 2% (w/w) organic solvents, such as toluene. We analyzed the cytotoxic

effects of a 3-week exposure to toluene in cultured cells. At 2%, toluene did not exert any cytotoxic effects on cells (data not shown). We thus believe that $C_{60}(OH)_{24}$ stimulated a signaling pathway that influenced cell division, particularly during the period between the G2 phase and the M phase.

The results of other experiments suggest that although $C_{60}(OH)_{24}$ was used at more than 100-fold higher concentrations, both CB and $C_{60}(OH)_{24}$ induce nonapoptotic cell death mediated by the accumulation of polyubiquitinated proteins in autophagosomes, and the expression of inflammatory genes (*MCP-1*, *ICAM-1*, and *E-cadhelin*) or ubiquitin-proteasome system genes [*HECT* (a COOH-terminal catalytic homologous to E6-AP-COOH), *C2- and WW-domain-containing E3 ubiquitin protein ligase 2*, *ubiquitin-specific protease 31*, *ubiquitin-specific protease 32* and *ubiquitin-conjugating enzyme E2*] in human umbilical vein cells (7, 8). Furthermore, we demonstrated that CB and $C_{60}(OH)_{24}$ facilitate the uptake of oxidized LDL in macrophages to form foam like cells and induce the expression of the oxidized LDL receptor LOX-1 (in submission).

The kinetics of nanomaterials *in vivo* have not yet been determined. Inhaled nanoparticles are thought to be removed by alveolar macrophages via phagocytosis. By using radiolabeled water-soluble fullerene administered intravenously to rats, in which most fullerenes moved rapidly to the liver (within 1 h), it was found that fullerenes are distributed in various other tissues, including the spleen, lung, kidney, heart, and brain (23). Removal is apparently slow, with more than 90% being retained after one week, thus raising concerns about chronic toxicity. The effects of chronic exposure to nanomaterials *in vivo* thus require further examination.

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