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Evaluation of cellular influences induced by stable nanodiamond dispersion; the cellular influences of nanodiamond are small ☆

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ABSTRACT

The increased production of nanoparticles is providing increased investigation of the biological influences they induce. Studies of the biological influences of nanocarbons are also increasing. Some nanocarbons, such as carbon black, fullerene and carbon nanotubes, induce oxidative stress on cultured cells. On the other hand, there are few reports about the cellular influences of nanodiamond. A risk assessment based on accurate evaluation of biological influences is essential for the effective utilization of nanodiamond. In the present study, we prepared nanodiamond culture medium dispersions kept stable for the experimental period and examined their cellular influences. The secondary particle size of nanodiamond in the medium dispersion was 41–103 nm. The dispersion was exposed to HaCaT and A549 cells at concentrations of 1.0, 0.1 and 0.01 mg/mL for 6 and 24 h. After that, mitochondrial activity, apoptosis, intracellular reactive oxygen level, lipid peroxidation, colony formation and cellular uptake were examined. Transmission electron microscopic observations showed cellular uptake of nanodiamond. Slight apoptosis and inhibition of colony formation were shown in the HaCaT cells at a concentration of 1.0 mg/mL. Nanodiamond did not influence cell viability, cell membrane injury, or intracellular oxidative stress. Our results suggest that the cellular influences of nanodiamond are smaller than other nanocarbons.

1. Introduction

Nanoparticle is defined as a particle whose diameter is from 1 to 100 nm (ISO/TS 27687: 2008, Nanotechnologies—Terminology and definitions for nano-objects—nanoparticle, nanofiber, and nanoplate). Recently, many kinds of industrial nanoparticles are being manufactured. Among these industrial nanoparticles, these composed from carbon, such as carbon black, fullerene, carbon nanotube (CNT) and nanodiamond, are known as nanocarbons. Carbon black, fullerene and nanodiamond are classified as nanoparticles, and CNT is classified as a nano fiber. These nanocarbons are already widely applied for industrial use. Nano-scale carbon black is used in various industrial products such as inks, toners, gum additives, food additives and cosmetics. Nanodiamond is used in lubricant agents and is investigated as a novel function material. Although nanocarbon is an important industrial material, it has been reported that some nanocarbons have cytotoxic activity on cultured cells. Oxidative stress is an important factor in the cytotoxicity of nanoparticles, and many nanocarbons induce oxidative stress to cultured cells. For example, fullerene C_{60} induced an increase in the level of intracellular reactive oxygen species (ROS) [1]. Carbon black nanoparticles were taken up into epithelial cells and induced inflammation and oxidative stress [2-5]. Carbon black induced an increase of intracellular ROS level and a decrease of glutathione; however, it did not increase heme oxigenase-1 (HO-1) expression [6,7]. Additionally, it has also been reported that CNT had cytotoxic activity. DNA damage was observed in cells exposed to single wall carbon nanotube (SWCNT) and fullerene C_{60} [1,8]. These investigations suggest that nanocarbons have potential cytotoxic activity. However, among nanocarbons, there are few reports

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of the cellular influences of nanodiamond. There is a study indicating that nanodiamond induces neither oxidative stress nor cell death to culture cells [9]. However, cellular influences induced by nanodiamond are not fully understood.

Understanding about secondary particle size and dispersion stability is of great importance for *in vitro* cytotoxicity evaluation [10,11]. There are many studies about the cytotoxicity of metal oxide nanoparticles, suggesting that metal ion release, protein adsorption and surface activity such as ROS production are important factors in their cytotoxic activities [1,12-16]. For example, NiO and CuO nanoparticles which showed release of nickel and copper ion, respectively, showed strong cellular influences, such as induction of oxidative stress and cell death [1,12,15,17]. Redox reactions induced by CNT were affected by impurities such as iron [18]. Residuals of metallic catalysts which were used in the synthesis of CNT affected the cellular influences of CNT [19]. CNT synthesized with a nickel-yttrium catalyst induced a larger decrease in cell viability than CNT synthesized with an iron catalyst. On the other hand, the cellular influences of insoluble nanoparticles such as TiO_2 and CeO_2 were small [20,21]. Metal ion release is the most important factor in the cellular influences caused by nanoparticles. However, if impurities from catalyst residuals can be excluded, metal ion release need not be considered in the cellular influences of nanocarbons. Additionally, some dispersants such as DPPC affect the cellular responses of *in vitro* examinations [22]. In the present study, we prepared a nanodiamond, ND-A, a culture medium dispersion without any chemical dispersant, for *in vitro* examinations. Instead, bovine serum albumin (BSA) was used as dispersant. The dispersion was very stable during the experimental period, and it included secondary particles whose size was 100 nm or less. We evaluated the cellular influences of nanodiamond using this dispersion.

2. Methods

2.1. Cell culture

Human keratinocyte HaCaT cells were purchased from the German Cancer Research Center (DKFZ, Heidelberg, Germany). Human lung carcinoma A549 cells were purchased from the Riken BioResource Center (Tsukuba, Ibaraki, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Gland Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; CELLect GOLD; MP Biomedicals Incorporated, Solon, OH, USA), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B (Nacalai Tesque Incorporated, Kyoto, Japan). In the present study, this DMEM cocktail was called as "DMEM-FBS". The DMEM cultures were placed in a 75 cm^2 flask (Corning Incorporated, Corning, NY, USA) and incubated at 37 °C in an atmosphere of 5% CO_2. For cellular examinations, cells were seeded in 96-well or 6-well plates (Corning) at 2×10^5 cells/mL and incubated for 24 h. Subsequently, the culture medium was removed and the cells were subjected to nanodiamond dispersion, then incubated for a further 24 h.

2.2. Preparation of nanodiamond-medium dispersion

Three kinds of nanodiamond-water dispersion were provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan). In this study, we called these nanodiamond samples as "ND-A", "ND-B" and "ND-C". The properties of the nanodiamond water dispersions were described as follows, according to the manufacturer's data. ND-A: the averaged secondary particle size was 16 nm, the concentration of nanodiamond was 71 mg/mL (7.1 wt.%), and the zeta potential was −51.66 mV. ND-B: the averaged secondary particle size was 8.4 nm, the concentration of nanodiamond was 78 mg/mL (7.8 wt.%), and the zeta potential was +42.39 mV. ND-C: the averaged secondary particle size was 3.1 nm, the concentration of nanodiamond was 69 mg/mL (6.9 wt.%), and the zeta potential was +45.58 mV. Details of the preparation method of the nanodiamond medium dispersion were described previously [23]. The nanodiamond water dispersion was diluted to 10 mg/mL (1 wt.%) with 10 mg/mL of BSA. Additionally, the nanodiamond BSA dispersion was serially diluted to 10, 100 and 1,000 times with DMEM-FBS. The concentrations of the nanodiamond DMEM-FBS dispersion were 1.0, 0.1 and 0.01 mg/mL. The zeta positive potential of the ND-B and ND-C effectuate excessive adsorption of proteins; thus these nanodiamonds formed large aggregates when the water dispersions were diluted directly into the culture medium. In order to avoid excessive protein adsorption, the nanodiamond water dispersions were diluted to 7.8and 6.9 times, for ND-B and ND-C, respectively, with 1 mM aspartic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dispersions were then further diluted to 2 times with 10 mg/mL of BSA in the water solution. Following that, the nanodiamond BSA dispersions were serially diluted to 10 and 100 times with DMEM-FBS. The final concentration of the nanodiamond DMEM-FBS dispersions were 0.1 and 0.01 mg/mL for ND-B and ND-C, respectively.

2.3. Characterization of nanodiamond-medium dispersions

The nanodiamond DMEM-FBS dispersions prepared by the methods stated above were divided into two parts, and used for simultaneous biological experiments and physical characterization of the dispersion.

The secondary particle sizes of nanodiamond in the DMEM-FBS dispersions and dispersion stabilities were measured by dynamic light scattering (DLS), according to our established protocols [24]. The estimated diameters of the secondary particles were calculated as the mean of three measurements at different wavelengths taken with the following devices: DLS-7000 spectrophotometer (633 nm; Otsuka Electronics Company Limited, Hirakata, Japan), FPAR-1000 fiberoptic particle analyzer (660 nm; Otsuka Electronics), and Nanotrac machine (780 nm; Nikkiso Company Limited, Tokyo, Japan). Undiluted dispersions were used in these measurements. The measurements were carried out at 25.0 ± 0.1 °C with sample concentrations of 50–80 μg/mL. The samples for the particle-size measurements and cytotoxicity assays were obtained 1 cm from the surface of the solutions in static 15-mL tubes. DLS can be used to calculate particle size but has low reliability for calculating particle-size distribution. Therefore, viscosities of the dispersions were measured by an Ubbelohde viscometer number 0 °C (Sibata Scientific Technology Limited, Tokyo, Japan). The zeta potential of metal oxide nanoparticles dispersed in the culture medium was measured by a Zeta-potential & Particle size Analyzer ELS-Z (658 nm; Otsuka Electronics).

2.4. Measurement of the mitochondrial activity, cell membrane damage and colony forming ability of cells

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and water soluble tetrazolium salt (WST-1) assay were conducted to determine mitochondrial activity. In the MTT assay, after the nanodiamond dispersion was removed cells were incubated with fresh DMEM-FBS including 0.5 mg/mL of MTT (Nacalai Tesque Incorporated) at 37 °C for 2 h. Isopropyl alcohol containing 40 mM HCl was added to the culture medium (3:2 v/v) and mixed with a pipette until the formazan was completely dissolved. The optical density of the formazan was measured at 570 nm using a Multiskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). The WST-1 assay was performed by a premix WST-1 cell proliferation assay system (Takara Bio Inc., Otsu, Japan).

Cell membrane damage was detected by a lactate dehydrogenase (LDH) assay. In the LDH assays, LDH release was measured with tetrazolium salt using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The amount of formazan salt formed was measured at 492 nm using a Multiskan Ascent plate reader. The maximum amount of released LDH