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Assessment of cytotoxicity and mutagenicity of exfoliated graphene

Katsuhide Fujita[a,b,*], Seiji Take[b], Ryotaro Tani[b], Junko Maru[a,b], Sawae Obara[a,b], Shigehisa Endoh[a,b]

[a] Research Institute of Science for Safety and Sustainability (RISS), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8569, Japan
[b] Technology Research Association for Single Wall Carbon Nanotubes (TASC), Tsukuba, Ibaraki, Japan

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ABSTRACT

Graphene and related materials (GRMs) have unique optical and thermal characteristics and are expected to be adopted for industrial applications. However, there are concerns with respect to their safety to human health. To conduct cytotoxicity and mutagenicity assessments, exfoliated graphene (EGr) dispersed in Tween-20® was diluted in cell culture medium. Rat alveolar macrophage viability significantly decreased after 24 h exposure to 1 and 10 μg/mL EGr. No significant levels of intracellular reactive oxygen species were detected in the 2′,7′-dichlorodihydrofluorescin diacetate assay after 24 h of exposure to EGr. The levels of the pro-inflammatory cytokines macrophage inflammatory protein-1α, interleukin (IL)-1β, IL-18, macrophage chemoattractant protein-1, and tumor necrosis factor α were significantly higher in cells treated with 10 μg/mL EGr for 24 h than in untreated controls. Transmission electron microscopy confirmed that EGr was present in the cytoplasm of the cells. Many genes were upregulated by EGr treatment, and significantly overrepresented gene ontology categories included the biological processes “response to external stimulus”, “response to stress”, “cell-cell signaling”, “biological adhesion”, and “cell proliferation”. EGr did not induce genetic mutations in *E. coli* or cause micronucleus induction in mouse bone marrow cells. The results suggest that EGr cytotoxicity should be carefully considered.

1. Introduction

Graphene is a six-membered ring sheet formed by bonding of carbon atoms through sp2 hybrid orbitals. Graphene oxide (GO) is a product obtained by surface oxidization during the separation of graphene from graphite (Hummers and Offeman, 1958; Chen et al., 2009). Reduced graphene oxide (rGO) has similar physicochemical properties to graphene (Tkachev et al., 2012). Materials with functional groups covalently or noncovalently bonded to these surfaces have also been developed. The physicochemical properties of graphene and related materials (GRMs) vary greatly. For example, graphene and rGO are less dispersible in aqueous solution and tend to aggregate (Lotya et al., 2009; Zu and Han, 2009), whereas dispersibility can be improved with GO (Konkena and Vasudevan, 2012).

Several studies have reported on the toxicity of environmental GRMs to microorganisms and vertebrates (Ahmed and Rodrigues, 2013; Pretti et al., 2014). In particular, inhalation toxicity and mutagenicity of graphite, GO, and rGO have been described. A 5-day inhalation toxicity test of graphene produced by thermal shocking of GO increased the levels of lavage markers indicative of inflammatory responses, beginning at graphene exposure concentration of 10 mg/m^3. Consistent with the changes in lavage fluid, microgranulomas were observed after the exposure to 10 mg/m^3 graphene (Ma-Hock et al., 2013). Graphene is composed solely of carbon atoms, without functional groups or bonds that could be targeted by metabolic/degrading enzymes *in vivo*. For this reason, graphene that reaches the alveoli likely remains there for a relatively long time without being metabolized or degraded. Park et al. confirmed that graphene nanoplatelets remained in mouse lungs for 28 days after a single administration (Park et al., 2015).

Comet assays demonstrated that pristine graphene, rGO, and graphite caused DNA damage and genotoxicity in human U87 glioblastoma cells, whereas GO did not (Hinzmann et al., 2014). Liu et al. demonstrated that GO (mean sheet diameter 156.4 nm) intravenously administered to mice at 4 mg/kg for 5 consecutive days clearly induced the formation of micronucleated polychromatic erythrocytes (MNPCEs) in mice, suggesting that GO may promote mutagenesis (Liu et al., 2013).

Exfoliated graphene (EGr) peels off graphite by a liquid phase process that results in the formation of graphene. EGr has similar features to GO and rGO, namely, small breakdown of sp2 structure, high

* Corresponding author at: Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan.
E-mail address: ka-fujita@aist.go.jp (K. Fujita).

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mobility and conductivity, and high thermal conductivity. Because of these characteristics, EGr could be used in conductive resins, electrodes for next generation ion batteries, conductive inks, and in other applications. However, to the best of our knowledge, the cytotoxicity and mutagenicity of EGr have not been elucidated. In this study, we assessed EGr cytotoxicity and mutagenicity by examining cell viability, intracellular reactive oxygen species (ROS) levels, pro-inflammatory cytokine expression, cellular EGr uptake, and gene expression profiles in NR8383 rat alveolar macrophages. We also assessed the induction of genetic mutations in *Escherichia coli* and micronucleus induction in mouse bone marrow cells.

2. Materials and methods

2.1. Test materials and their preparation

EGr obtained from the Technology Research Association for Single-Wall Carbon Nanotubes (Tsukuba, Japan), was dispersed into polyoxyethylene sorbitan monolaurate (Tween-20®, Sigma-Aldrich) using a magnetic stirrer. The samples were diluted in deionized water, and the resulting supernatant containing 0.4% Tween-20® was filtered through a test sieve with a 20 μm mesh (AS ONE Corporation, Japan). The filtrates were used as 100 μg/mL stock suspensions and diluted in cell culture medium for use in cytotoxicity tests. Stock suspensions diluted to required concentration were used for mutagenicity tests.

2.2. Characterization of EGr

A transmission electron microscopy (TEM) system at 75 kV (H-7600; Hitachi, Japan) was used to observe EGr in stock suspensions. The particle diameter of EGr dispersed in stock suspensions was measured 24 h after preparation by a laser diffraction particle size analyzer (SALD-2300; Shimadzu Co., Kyoto, Japan).

2.3. Cell culture and exposure to EGr

Stock suspensions of EGr were diluted 10-fold in Ham's F-12 K (Kaighn's) culture medium (Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hana-Nesco Bio, Tokyo, Japan), 100 units/mL penicillin, and 100 units/mL streptomycin (F-12 K + FBS medium). These working solutions were used for *in vitro* cell-based assays. NR8383 rat alveolar macrophage cells obtained from the American Type Culture Collection (CRL-2192; ATCC, USA) were cultured at 37 °C in 75-cm^2 cell culture flasks in F-12 K + FBS medium in a humidified atmosphere of 95% air and 5% CO_2. NR8383 cells were seeded into individual wells of 96-well plates (Becton, Dickinson & Company). After centrifugation, F-12 K + FBS medium was removed and replaced with the working solutions. Cell density at the time of working solution replacement was approximately 3×10^5 cells/mL.

2.4. Assessment of cell viability

The 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1)-based assay (Takara Bio Inc., Otsu, Japan) was used to detect mitochondrial dehydrogenase activity as indicator of cell viability. After treating cells with the working solutions in quadruplicate, the supernatant was removed. WST-1, diluted 1:10 (v/v) with F-12 K + FBS medium, was added to each well, and the samples were incubated for 1 h at 37 °C. Sample absorbance at 450 nm was measured in a Model 680 microplate reader (Bio-Rad Laboratories, Tokyo, Japan), using the absorbance at 750 nm as reference. Data were normalized to the absorbance values of untreated control cells. Pyocyanin (100 μM) was used as positive control.

2.5. Measurement of intracellular ROS

Intracellular ROS were detected using 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA; Sigma-Aldrich) dissolved in dimethyl sulfoxide to 5 mM. After treatment, the working solutions containing 10 μM DCFH-DA were added to NR8383 cells and incubated for 30 min at 37 °C in quadruplicate. The cells were washed once with phosphate-buffered saline (PBS) and resuspended in PBS. Samples were excited with a 488-nm argon laser in a Guava Flow Cytometer with CytoSoft™ software containing Guava ExpressPro module (Merck Millipore, Billerica, MA, US). The emission of 2′,7′-dichlorofluorescein was measured at 525 nm. Data were collected from 1000 gated events. Pyocyanin (100 μM) was used as positive control.

2.6. Cytokine assays

The levels of interleukin (IL)-1α, macrophage inflammatory protein-1 α (MIP-1α), IL-1β, IL-18, macrophage chemoattractant protein-1 (MCP-1), tumor necrosis factor α (TNF-α), and secreted phosphoprotein 1 (SPP1, also known as osteopontin) in cell suspensions were measured using a MILLIPLEX® MAP Rat Cytokine/Chemokine Magnetic Bead Panel or Rat Bone Magnetic Bead Panel 2, and a Luminex 200 System (Merck Millipore).

2.7. Quantification of cellular EGr uptake

Quantitative analysis of intracellular EGr uptake was performed based on our previously described protocol (Zhang et al., 2018). Approximately 3×10^5 NR8383 cells/well were seeded in 6-well dishes and incubated for 24 h, after which the medium was replaced with EGr-containing medium (10 μg/mL) in four wells and fresh F-12 K + FBS medium in another four wells. After further incubation for 24 h, the medium was removed, and the cells were rinsed with PBS and detached from the culture dishes with 0.25% trypsin-ethylenediaminetetraacetic acid. Light microscopy confirmed that EGr attached to the cell surface can be removed. The cell suspensions were centrifuged, and the pellets were dispersed in 1 mL of a 1:1 mixture of CelLytic M Cell Lysis Reagent (Sigma) and 5% sodium dodecylbenzenesulfonate and sonicated for 10 min with a horn-type sonicator (~300 W; VC-750, Sonics & Materials Inc., CT). After measuring the absorbance at 750 nm on a UV–Vis-NIR spectrometer (Lambda 19, PerkinElmer Japan Co., Ltd., Tokyo), the concentration of EGr in cell lysates was estimated based on the calibration curves established by measuring the absorbance of various known concentrations of EGr (0, 0.025, 0.1, 0.25, 1.0, 2.0, 10, and 20 μg/mL) at 750 nm. The cellular uptake ratio was calculated as follows:

$$\text{Cellular uptake ratio (\%)} = \text{EGr in cell lysate (mg)} / \text{concentration of EGr in working solution (mg/mL)} \times \text{volume (mL)} \times 100$$

After the incubation with EGr-containing medium and washing with PBS, cells were observed with a microscope (Axio Vert.A1, Carl Zeiss Inc.). We believe that EGr particles strongly adhered to the cell surface and could not be removed by washing. We considered that this was evidence of uptake, as the latter defines not only internalization but also absorption of substances by living tissue.

2.8. TEM observations

After treatment, cells were fixed sequentially in 1.2% (*v*/v) glutaraldehyde for 1 h at 20 °C, in 1% osmium oxide solution for 1 h at 4 °C, dehydrated in ethanol, and embedded in a commercially available epoxy resin (TAAB Laboratories Equipment Ltd., Reading, England). Samples were transferred to fresh resin in capsules and polymerized at 60 °C for over 48 h. A TEM system at 75 kV (H-7600; Hitachi, Japan)