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Long-term exposures to low doses of silver nanoparticles enhanced in vitro malignant cell transformation in non-tumorigenic BEAS-2B cells

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ABSTRACT

To predict carcinogenic potential of AgNPs on the respiratory system, BEAS-2B cells (human bronchial epithelial cells) were chronically exposed to low- and non-cytotoxic dose (0.13 and 1.33 μg/ml) of AgNPs for 4 months (#40 passages). To assess malignant cell transformation of chronic exposure to AgNPs, several bioassays including anchorage independent agar colony formation, cell migration/invasion assay, and epithelial-mesenchymal transition (EMT) were performed in BEAS-2B cells. Chronic exposure to AgNPs showed a significant increase of anchorage independent agar colony formation and cell migration/invasion. EMT, which is the loss of epithelial markers (*E*-Cadherin and Keratin) and the gain of mesenchymal marker (*N*-cadherin and Vimentin), was induced by chronic exposure to AgNPs. These responses indicated that chronic exposure to AgNPs could acquire characteristics of tumorigenic cells from normal BEAS-2B cells. In addition, caspase-3, *p*-p53, *p*-p38, and *p*-JNK were significantly decreased, while *p*-ERK1/2 was significantly increased. MMP-9 related to cell migration/invasion was upregulated, while a MMP-9 inhibitor, TIMP-1 was down-regulated. These results indicated that BEAS-2B cells exposed to AgNPs could induce *anti*-apoptotic response/anoikis resistance, and cell migration/invasion by complex regulation of MAPK kinase (p38, JNK, and ERK) and p53 signaling pathways. Therefore, we suggested that long-term exposure to low-dose of AgNPs could enhance malignant cell transformation in non-tumorigenic BEAS-2B cells. Our findings provide useful information needed to assess the carcinogenic potential of AgNPs.

1. Introduction

Unique physicochemical properties of nanoparticles led to development for biomedical and industrial application. Among various nanoparticles, silver nanoparticles (AgNPs) with antibacterial properties have been widely used in industrial and consumer products including antimicrobial applications, biosensor, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components (Ahamed et al., 2010). As a result of the widespread applications of AgNPs, there are growing concerns regarding adverse effects on humans via various routes of exposure, including inhalation, ingestion, and dermal exposure (Chen and Schluesener, 2008; Wijnhoven et al., 2009).

Nano size Ag (range of 1–100 nm in diameter) can pass through biological membranes, penetrate even the very small capillaries throughout the body, thereby accumulating in cells (Ji et al., 2007; Luoma, 2008). AgNPs administered by subcutaneous injection in rats were distributed throughout various organs, including kidney, liver, spleen, brain, and lung (Tang et al., 2009). In addition, repeated intravenous administration of AgNPs in rats resulted in accumulation in the liver, lung, and spleen (Lankveld et al., 2010). Based on these reports, the effect of long-term and repeated uses of low-dose AgNPs on human health must be considered. In particular, the lung, as a major port of entry by inhalation, could be a target organ for toxicity after repeated exposure (Wijnhoven et al., 2009; Lankveld et al., 2010).

In the Integrated Risk Information System of the US EPA (2014, IRIS, http://www.epa.gov/iris/subst/0099.htm), carcinogenic potential of silver (Ag) has been reported as Group D (not classified as human carcinogenicity) according to limited animal studies as follows: Chronic subcutaneous administration of colloidal silver in rats led to an increase of malignant tumors (Schmaehl and Steinhoff, 1960), however intramuscular injection of silver (~300 mesh) in rats did not induce cancer

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(Furst and Schlauder, 1977). According to nanotechnology development, nanoscale Ag particles with a diameter ranging from 1–100 nm, has been used as a commercial application. The nanoparticles could have different physicochemical characteristics from larger size particles and could induce high reactivity due to high surface area (Wijnhoven et al., 2009). Therefore, it is possible to induce different results of AgNPs on carcinogenicity from previous research studies (Furst and Schlauder, 1977).

Against this background, in the current study, we evaluated the in vitro malignant transformation on the respiratory system by low-dose and long-term exposure of human bronchial epithelial cells to AgNPs. The lung epithelium plays an important role as a barrier, protecting the respiratory tract from toxic particulates. BEAS-2B cell line, derived from normal human bronchial epithelium of non-cancerous individuals, is immortalized but not tumorigenic as human cells. BEAS-2B cell line has been used a good model for predicting the toxicity and carcinogenic effects for lung-targeting in humans (Reddel et al., 1988; Klein-Szanto et al., 1992; van Agen et al., 1997; Li et al., 2009). Therefore, BEAS-2B cells were chosen as a cell model to predict carcinogenic potentials of AgNPs. Colony forming efficiency (CFE) assay and crystal violet (CV) assay were used to obtain a non-cytotoxic range of AgNPs. BEAS-2B cells were chronically exposed to AgNPs for 4 months (#40 passage) at a non-cytotoxic range obtained by CFE and CV. Anchorage independent colony agar assay, cell migration/invasion assay, and gene expression related to *anti*-apoptosis, Epithelial-Mesenchymal Transition (EMT), and invasion/migration were performed to assess the characteristics of in vitro malignant tumorigenic transformation in BEAS-2B cells that were chronically exposed to AgNPs. This study is first reports to assess malignant cell transformation potentials in BEAS-2B cells by low-dose and long-exposure of AgNPs.

2. Materials and methods

2.1. Materials

Silver nanoparticles (AgNPs), benzo(a)pyrene (B(a)P), diethyl pyrocarbonate (DEPC), isopropanol, ethidium bromide (EtBr), and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich (St Louis, MO, USA). B(a)P (CAT# B1760) was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and stored at −20 °C until needed.

2.2. AgNPs sample preparation and characterization

AgNPs (CAT# 576832) were homogeneously dispersed in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, NY, USA) supplemented with 5% Fetal Bovine Serum (FBS, Hyclone, TU, USA) by sonication for 30 min (Bioruptor UCD-200T, Cosmobio Corp., Tokyo, Japan) and were filtered through a cellulose membrane (0.45 μm pore size; Advantec, Tokyo, Japan). The concentration of filtered AgNPs, measured by ICP (Opima 7300 DV, Perkin Elmer, USA), was 10.60 ± 0.44 μg/ml. The purity of AgNPs was assessed by Energy Dispersion X-ray Spectrum analysis (EDS, EMAX, Horaba, Japan). A transmission electron microscope (TEM; JEM 2100F, Jeol, USA) and scanning electron microscope (SEM; Hitachi, Tokyo, Japan) were used to evaluate the size and shape of AgNPs. The size distribution of AgNPs dispersed in 5% FBS-DMEM at the highest concentration (10.60 μg/ml) was measured by a dynamic light scattering technique (DLS; NANOPHOX particle analyzer, SYMPATEC GmbH, Germany), and the zeta potential was measured using ELS-Z (Otsuka Electronics Co., Inc., Osaka, Japan).

2.3. Cell culture conditions and chemical treatment

BEAS-2B cells (human normal bronchial epithelial cells), obtained from American Type Culture Collection (Manassas, VA, USA), were grown in 5% FBS-DMEM, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37 °C in a 5% CO_2/95% air atmosphere. The cells were exposed continuously to B(a)P (as a positive control, 1 μg/ml) or AgNPs (0.13 and 1.33 μg/ml) for 4 months (passage #40) in 5% FBS-DMEM.

2.4. Cell viability test by crystal violet assay and colony forming efficiency assay

BEAS-2B cells at approximately 80% of confluence were trypsinized and suspended in 5% FBS DMEM medium. Then, the cells were seeded in 6-well plates (3×10^3 cells/well/1.5 ml) or 60 mm dish (200 cells/4 ml) of 5% FBS-DMEM medium. After 24 h incubation, the medium of the cells was changed by 5% FBS-DMEM medium containing various concentrations of AgNPs (from 0.13 to 10.60 μg/ml). The cells exposed to AgNPs for 72 h were washed two times with PBS and were replaced with fresh 10% FBS DMEM medium. After another 3 days (CV assay) or 5 days (CFE assay), the cells were fixed and stained by 0.1% CV or 0.04% Giemsa solution to assess cell viability by CV and CFE assay, respectively. In CV assay, stained cells were extracted with 1.5 ml of extraction solution (50: 49: 1 of ethanol: distilled water: 1 M HCl) and the optical density of each well was measured at 540 nm. In CFE assay, stained colonies (>50 cells or >2 mm in diameter) were scored in accordance to the image and stereological analysis by optical system, which is attached to an upright microscope (Olympus cx31, Japan) and a CCD digital camera (IMTscan cooled model, Germany).

2.5. Cell migration ability in BEAS-2B cells exposed to AgNPs

After BEAS-2B cells were exposed to AgNPs (0.13 and 1.33 μg/ml) for 4 months, the migration assay was performed using a transwell chamber (6.5 mm diameter, 8 μm pore size, BD Biosciences, MA, USA) assay and wound-healing assay. Exposed cells suspended in the serum free medium were seeded at a density of 5×10^5 cells/300 μl/well in the upper chamber, and the lower chamber contained 5% FBS-DMEM. After 24 h incubation, non-migrated cells were scraped from the upper surface of the membrane with a cotton swab, and migrated cells, which remained on the bottom surface, were stained with crystal violet. Stained cells were extracted with 1.5 ml of extraction solution (50:49:1 of ethanol:distilled water:1 M HCl) per well for 10 min under shaking. The optical density of each well was measured at 540 nm. For the wound-healing migration assay, exposed cells were seeded at a density of 5×10^5 cells/ml/well in a 12-well plate. When the cells reached to confluence, a wound was prepared by scraping off the cells using a 100 μl tip. Detached cells were washed off and the remaining cells were treated with fresh media without FBS for 44 h. The wound closure was observed through the migration of cells by a microscopic examination (Olympus cx31, Japan).

2.6. Cell invasion ability in BEAS-2B cells exposed to AgNPs

To assess the invasion of cells exposed to AgNPs (0.13 and 1.33 μg/ml) for 4 months (#40 passages), the upper surface of the transwell chamber (6.5 mm diameter, 8 μm pore size, BD Biosciences, MA, USA) was coated with 70 μl matrigel (Corning, MA, USA). Exposed cells, which were suspended in a serum free medium, were seeded at a density of 5×10^5 cells/ml/well in the upper chamber, and the lower chamber contained 5% FBS-DMEM. After 48 h incubation, non-invaded cells were scraped from the upper surface of the membrane with a cotton swab, and the invaded cells that remained on the bottom surface were stained with crystal violet and extracted extraction solution.

2.7. mRNA expression of apoptosis-related genes using real-time PCR in BEAS-2B cells exposed to AgNPs

The total RNA was extracted from exposed BEAS-2B cell using Trizol Reagent (Gibco BRL, Grand Island, NY, USA) in accordance to the manufacturer's protocol, and stored at −80 °C until needed. The first-