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The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells

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ABSTRACT

Nanomaterials and nanoparticles have received considerable attention recently because of their unique properties and diverse biotechnology and life sciences applications. Nanosilver products, which have well-known antimicrobial properties, have been used extensively in a range of medical settings. Despite the widespread use of nanosilver products, relatively few studies have been undertaken to determine the biological effects of nanosilver exposure. The purpose of this study was to evaluate the toxicity of nanosilver and to elucidate possible molecular mechanisms underlying the biological effects of nanosilver. Here, we show that nanosilver is cytotoxic, inducing apoptosis in NIH3T3 fibroblast cells. Treatment with nanosilver induced the release of cytochrome *c* into the cytosol and translocation of Bax to mitochondria, indicating that nanosilver-mediated apoptosis is mitochondria-dependent. Nanosilver-induced apoptosis was associated with the generation of reactive oxygen species (ROS) and JNK activation, and inhibition of either ROS or JNK attenuated nanosilver-induced apoptosis. In nanosilver-resistant HCT116 cells, up-regulation of the anti-apoptotic proteins, Bcl-2 appeared to be associated with a diminished apoptotic response. Taken together, our results provide the first evidence for a molecular mechanism of nanosilver cytotoxicity, showing that nanosilver acts through ROS and JNK to induce apoptosis via the mitochondrial pathway.

1. Introduction

Nanomaterials, defined as particles ranging from 1 to 100 nm in at least one dimension, have become widely utilized because of their unique physicochemical properties (Hood, 2004). Many of these nanomaterials, including quantum dots (Azzazy et al., 2007), composite nanodevices (Balogh et al., 2007), gold nanorods (Eghtedari et al., 2007), and nanosilver (Fu et al., 2006), have been used in biomedical settings. It has long been known that silver possesses antibacterial properties. Importantly, these properties are retained when silver is synthesized in nanoparticulate formulations, such as silver-containing multilayer film for surface modification (Fu et al., 2006), silver-loaded bone cement (Alt et al., 2004), and silver complexes in nanofiber mats (Melaiye et al., 2005). With increased application of nanosilver products, however, comes the inevitable possibility of effects on the health of humans exposed to the products. Previous studies have suggested that elementary silver exhibits little cytotoxicity at low concentrations (Hardes et al., 2007; Kinoshita et al., 2002). Similarly, minimal toxicity was induced in HL-60 cell grown in direct contact with a metal silver plate (Yamazaki et al., 2006). However, accumulating evidence suggests that nanosilver formulations may be cytotoxic, as indicated by recent studies using rat liver cells (Hussain et al., 2005), and mammalian germline stem cells (Braydich-Stolle et al., 2005). Given the increasing use of nanosilver products, it has become crucial to develop a fundamental understanding of the cellular responses to nanosilver exposure.

Cell survival and cell death are two major toxicity endpoints that can be rapidly and effectively measured using *in vitro* experimental models employing cultured mammalian cells. Of particular interest is apoptotic cell death, which represents a biologically essential, regulated sequence of events. Apoptosis is a complex process that occurs in response to a variety of stress stimuli including metal exposure (Thiébault et al., 2007; Wang et al., 2008; Yang et al., 2007) and oxidative stress (Lin and Beal, 2006; Ott et al., 2007; Sharma

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et al., 2007; Ueda et al., 2002). A mitochondria-dependent mechanism has been identified as an intrinsic pathway, characterized by translocation of Bax to mitochondria, Bax/Bak oligomerlization in the mitochondrial membranes, regulation by Bcl-2 family (Nomura et al., 1999; Nechushtan et al., 2001; Shimizu et al., 1999; Yi et al., 2003) and release of cytochrome *c* from mitochondria into the cytoplasm (Liu et al., 1996; Iverson and Orrenius, 2004; Jiang and Wang, 2004; Kluck et al., 1997; Yang et al., 1997). These mitochondrial alterations subsequently activate a caspase cascade that induces an ordered series of events culminating in degradation of the cell (Kakkar and Singh, 2007; Nuñez et al., 1998; Thress et al., 1999; Vander Heiden and Thompson, 1999).

The purpose of this study was to assess nanosilver's biological effects and elucidate the molecular mechanisms associated with its toxicity. Our results suggest that nanosilver increases the production of intracellular ROS and activates JNK, leading to mitochondria-dependent apoptosis. The presumed safety of nanosilver products exemplified by their widespread use, however, stands in sharp contrast to the toxic effects we report in this *in vitro* study. As nanosilver cytotoxicity may be associated with adverse health effects, these results may provide helpful guidance on the future use of nanosilver particles.

2. Materials and methods

2.1. Materials

Nanosilver powders were obtained from Ching-Tai Resin (Taichung, Taiwan, ROC) and Sun-Lan International Biotechnology (Taipei, Taiwan, ROC). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). The anti-PARP, anti-JNK, anti-phosphorylated JNK, anti-phosphorylated p53, anti-Bcl-2, and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-cytochrome *c* and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-β-actin antibody was purchased from Chemicon International, Inc. (Tamekula, CA, USA). The anti-Bax antibody was from Upstate (Lake Placid, NY, USA). The secondary anti-mouse-conjugated FITC antibody and Mitotracker were from Molecular Probes Inc. (Eugene, OR, USA). The silver powder (<250 μm), anti-p53, anti-mouse IgG antibodies, and other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified.

2.2. Cell culture

NIH3T3 (mouse fibroblast) and A10 (rat vascular smooth muscle) cells were cultured in DMEM containing 10% fetal bovine serum. HCT116 (human colon cancer) cells were grown in McCoy's 5A medium with 10% fetal bovine serum. All media contained 100 units/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and medium was replaced every 2–3 days.

2.3. Transmission electron microscopy

The structure of nanosilver particles was examined using a Transmission Electronic Microscope (JEM-2010, JEOL Ltd., Japan). After diluting in purified water, samples were placed over a copper grid coated with carbon film followed by staining with 2% phosphotungstic acid. The samples were air dried prior to placement in the TEM instrument for analysis.

2.4. Cell viability assays

Cells were seeded onto 96-well culture plates at 8×10^3 cells per well and permitted to adhere overnight at 37 °C. After incubation, cells were treated with a 0.5 mg/ml solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 100 μl/well) for 3 h at 37 °C. The number of viable cells was determined by uptake of MTT, assayed at 570 nm. All experiments were performed at least in triplicate on three separate occasions. Data are presented as mean ± S.D.

2.5. Apoptosis assays

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit (BD PharMingen, San Jose, CA, USA). Cells cultured in 6 cm dishes were trypsinized and collected by centrifugation. The cell pellet was washed, resuspended in 1× binding buffer and stained with annexin V-FITC as recommended by the manufacture. Cells were also stained with propidium iodide to detect necrosis. Apoptosis was analyzed by flow cytometry using a Beckman Coulter FC500.

2.6. Measurement of reactive oxygen species

Cells (1×10^5) were incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) in DMSO for 30 min. Cells were collected by trypsinization and centrifugation, washed with PBS and centrifuged at 200 × *g* for 5 min and analyzed immediately using a Beckman Coulter FC500 flow cytometer.

2.7. Western blot analysis

HeLa cell extracts were prepared in lysis buffer (20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 2 mM PMSF, 10 ng/ml leupeptin and 10 μg/ml aprotinin), and volumes of extract containing equal amounts of proteins (40 μg) were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked, washed, and probed with primary antibody. After washing to remove primary antibody, membranes were incubated with HRP-conjugated secondary antibody for 2 h. The blots were washed again, and developed using enhanced chemiluminescemce (ECL) according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

2.8. Silver uptake assay

NIH3T3 and HCT116 cells were treated with 50 and 100 μg/ml of nanosilver, respectively, and harvested after 1 min or 8 h. Cells were sonicated at 12 W for 10 s (Sonifier s-450D, Branson, Danbury, CT, USA), digested with 70% nitric acid in a microwave oven for 15 min at 180 °C. Silver concentration in the cell lysates was determined using a graphite furnace atomic absorption spectrophotometer (Z2000, Hitachi, Tokyo).

2.9. Flow cytometric light scatter assay

Cells treated with Ching-Tai for different time periods were trypsinized and suspended in medium. The amount of Ching-Tai up taken by the cells was analyzed using a Beckman Coulter FC500.

2.10. Confocal microscopy

Cells were grown overnight on coverslips, treated with the indicated concentrations of nanosilver for 24 h, and incubated with Mitotracker (Molecular Probes Inc.) for 10 min. Cells were then washed, fixed in 2% paraformaldehyde, washed with PBS, and permeabilized with 0.1% Triton X-100. Coverslips were stained with anti-cytochrome *c* or anti-Bax antibodies. Immunofluorescent images were acquired on a confocal microscope (TCS SP5, Leica Microsystems USA, Bannockburn, IL, USA).

2.11. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from HCT116 cells was isolated using TRIzol reagent (GIBCO, Carlsbad, CA, USA). The first strand cDNA was synthesized from 1 μg of total RNA using superscript II (Life Technologies, Rockville, MD, USA). The sequences of primer sets used were: Bcl-2 sense, 5′-GCGAATTCATGGCGCAAGCCGGGAG and antisense, 5′-GCAAGCTTTCACTTGTGGCCCAGGTATGC; β-actin sense, 5′-GATGATGATATCGCCGCGCT and antisense, 5′-TGGGTCATCTTCTCGCGGTT. Reaction conditions consisted of 30 cycles at 94 °C for 15 s and 61 °C for 6 min, followed by a final extension of 10 min at 72 °C. Electrophoresis was performed in 0.8% agarose gel.

2.12. Statistics

All data are expressed as the mean ± S.D. of three independent experiments. The differences between the control and the treatment groups were calculated by one-way ANOVA and posttests were analyzed using Dunnett's test to evaluate the significant levels.

3. Results

3.1. Nanosilver induces cytotoxicity

Silver powder (<250 μm) purchased from Sigma–Aldrich and two commercially available nanosilver powders were assessed in this study. The shapes and sizes of nanosilver particles were determined using transmission electron microscopy, which showed that particles diameters in both preparations were in the nanosize range (1–100 nm) (Fig. 1) whereas much larger particles diameters were observed for the non-nanosilver preparation (data not