Toxicology 328 (2015) 84–92

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages

Yuta Arai [a], Takamitsu Miyayama [b,c], Seishiro Hirano [a,b,*]

[a] Graduate School of Pharmaceutical Sciences, Chiba University, Japan
[b] Environmental Nanotoxicology Project, RCER, National Institute for Environmental Studies, Japan
[c] Department of Hygiene and Public Health I, School of Medicine, Tokyo Women's Medical University, Japan

ARTICLE INFO

Article history:
Received 25 November 2014
Accepted 14 December 2014
Available online 16 December 2014

Keywords:
Silver nanoparticle
Silver ion
Lysosome
Lung
Macrophages
Metallothionein

ABSTRACT

The health effects of silver nanoparticles (AgNPs) have not been well investigated, despite AgNPs now being widely used in consumer products. We investigated the metabolic behavior and toxicity of AgNPs in comparison to silver nitrate ($AgNO_3$) both *in vivo* and *in vitro*. AgNPs (20 nm diameter) suspended in 1% albumin solution or $AgNO_3$ solution was injected into the mouse lung. Less than 1% of the initial dose of AgNPs and more than 7% of the initial dose of $AgNO_3$ was recovered in the liver 4 h after administration, suggesting that the ionic form of silver was absorbed by the lung tissue and entered the systemic circulation more efficiently than AgNPs. The pro-inflammatory cytokine, IL-1β, and neutrophils in bronchoalveolar lavage fluid (BALF) increased following intratracheal instillation of AgNPs or $AgNO_3$. $AgNO_3$ recruited more neutrophils in the alveolar space than did AgNPs. In the *in vitro* study, $AgNO_3$ was more cytotoxic than 20, 60, or 100 nm diameter AgNPs in a mouse macrophage cell line (J774.1). To investigate the intracellular distribution of Ag in detail, J774.1 cells were exposed to $AgNO_3$ or 20 nm AgNPs and the distribution of Ag to cytosolic proteins was investigated using HPLC-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). Ag was mainly distributed to metallothioneins (MT) and to high molecular weight proteins in $AgNO_3$- and AgNPs-exposed cells, respectively. Confocal laser microscopic examination of LysoTracker®-labeled cells indicated that AgNPs were colocalized with lysosomes in J774.1 cells. These results suggest that AgNPs were transported to lysosomes and only gradually dissolved in the macrophages, causing milder inflammatory stimulation in the mouse lung compared to $AgNO_3$.

1. Introduction

Silver (Ag) has antibacterial properties, and silver nanoparticles (AgNPs) are currently used in consumer products such as electronics, cosmetics, medical supplies, and pharmaceuticals (Ahamed et al., 2008). There is thus a high probability of exposure to AgNPs through ingestion, skin contact, and inhalation, which raises a potential health risk of silver in humans. It has been shown that Ag is deposited in the skin, eyes, and other organs in workers processing silver-containing materials (Lansdown, 2006; Atiyeh et al., 2007). Argyria and argyrosis are chronic disorders of skin microvessels and eyes in humans, and these disorders reportedly develop following extended oral and inhalational exposure to $AgNO_3$ (Stafeeva et al., 2012), silver oxide (Moss et al., 1979; Rosenman et al., 1979), and particulate and colloidal Ag (Atiyeh et al., 2007). Excessive nasal exposure to a Ag-protein complex resulted in a blue-grey pigmentation in the skin due to intradermal silver deposition (Lansdown, 2010).

A single intratracheal instillation of AgNPs (average diameter, 243.8 ± 176.7 nm) caused helper type 2-dominant inflammatory responses, pro-inflammatory cytokine production, and lung tissue damage in mice (Park et al., 2011). Subchronic inhalation exposure of Sprague-Dawley rats to different concentrations of AgNPs (average diameter 18–19 nm) showed that AgNPs were mainly distributed to the lungs and liver, and caused lung inflammation and bile-duct hyperplasia (Sung et al., 2009). The no-observable-adverse-effect level (NOAEL) was calculated to be 100 μg/m^3 in this study.

Exposure to $AgNO_3$ *in vitro* decreased the cell viability dose-dependently in various types of cells such as rat hepatocytes (Baldi et al., 1988), human dermal fibroblasts (Hidalgo and Domínguez, 1998), Jurkat cells (Eom and Choi, 2010), human leukocytes

* Corresponding author at: Environmental Nanotoxicology Program/Health Risk Research Section, RCER, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan. Tel.: +81 29 850 2512; fax: +81 29 850 2512.
E-mail address: seishiro@nies.go.jp (S. Hirano).

http://dx.doi.org/10.1016/j.tox.2014.12.014
0300-483X/

(Jansson and Harms-Ringdahl, 1993), and neuronal PC12 rat pheochromatocytoma cells (Powers et al., 2010). Silver ion interacts with a variety of biomolecules, such as nucleic acids, cell wall components, and sulfhydryl groups of metabolic enzymes, metallothioneins (MTs), and glutathione (GSH), which leads to cellular dysfunction (Arora et al., 2009; Greulich et al., 2011). The toxicity of $AgNO_3$ and AgNPs arises in part from their inhibitory effect on mitochondrial function and cellular energy metabolism (Carlson et al., 2008; Miyayama et al., 2013). The cytotoxic effects of $AgNO_3$ and AgNPs seem to be associated with oxidative stress (Hussain et al., 2005; Miyayama et al., 2013) and apoptosis signaling (Almofti et al., 2003; Wilkinson et al., 2011).

MTs (MT-I, -II, -III, and -IV) are low-molecular weight proteins (MW ~7 kDa). MT-I and MT-II can bind to a variety of essential and toxic metals, such as copper, zinc, cadmium, mercury, and Ag (Kagi and Schaffer, 1988; Maret, 2000; Nordberg and Nordberg, 2000; Haq et al., 2003). The induction of MTs protects cells against heavy metal toxicity by chelating those metals with cysteine residues and by reducing reactive oxygen species (ROS) generation (Fu et al., 2010). It has been reported that $AgNO_3$ and AgNPs were incorporated into cells, where the Ag ion induced *de novo* synthesis of MT-I and MT-II (Lansdown, 2002). Contradictory results have been reported regarding induction of MTs; AgNPs induced MTs at 24 h in rat astroglia-rich primary culture cells (Luther et al., 2012) and AgNPs did not induce MTs within 24 h in human hepatoma HepG2 cells (Kim et al., 2009).

One of the major toxicological interests regarding Ag used in commercial products is the difference in cellular uptake, tissue distribution, and toxicity between ionic (Ag^+) and nanoparticulate forms (AgNPs). In the present study, we report the tissue distribution of Ag and the inflammatory responses of the lung following intratracheal instillation of $AgNO_3$ and AgNPs in mice. We also report the cytotoxicity, induction of MTs, and intracellular distribution of Ag in $AgNO_3$- or AgNPs-exposed J774.1 murine macrophage cells.

2. Materials and methods

2.1. Characterization of AgNPs

AgNPs (<100 nm) coated with polyvinylpyrrolidone (Sigm–Aldrich, St. Louis, MO) were used to study the stability of AgNPs in solutions containing different concentrations of bovine serum albumin (Sigma–Aldrich). The AgNPs were suspended in deionized water containing 10% albumin by ultrasonication for 30 min at 4 °C (a 15-s sonication with a 15-s interval) (Bioruptor® UCD-250, CosmoBio Co., Tokyo). The suspension was diluted in serum-free RPMI 1640 medium and the hydrodynamic size of the AgNPs was measured by dynamic light scattering (DLS, ELS-Z, Otsuka Electronics, Osaka, Japan). For the following *in vitro* and *in vivo* toxicity tests suspensions of citrate-capped 20, 60, and 100 nm AgNPs purchased from nanoComposix (San Diego, CA) were used throughout. The AgNPs suspensions were evaporated to dryness using a vacuum concentrator (Concentrator Plus, Eppendorf, Hamburg, Germany), sterilized by autoclaving (121 °C for 20 min), and re-suspended in deionized water (18.2 MΩ cm) containing 10% albumin. An aliquot of the AgNPs suspension was dropped onto a collodion-membrane attached grid (JEM-2010, NISSIN EM, Tokyo) and the geometric sizes of the particles were observed using a transmission electron microscope (TEM, JEM-2000FX, JEOL, Tokyo). The hydrodynamic diameter and the zeta-potential of those citrate-capped 20, 60, and 100 nm AgNPs were measured by DLS in RPMI 1640 medium containing 1% albumin.

2.2. Cell culture and cytotoxicity assay

J774.1 murine macrophage cells were passaged in RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS. The cells were cultured to early confluence in a 96-well culture dish and were exposed to various concentrations of $AgNO_3$ or AgNPs for 24 h in 1% albumin-containing RPMI 1640 culture medium. In the cytotoxicity experiment the cells were also pre-treated with 0.5 mM L-buthionine sulfoximine (BSO, Sigma–Aldrich), an inhibitor of glutathione synthesis, for 4 h and were exposed to $AgNO_3$ in the presence of BSO for the following 24 h. The cell monolayers were gently washed twice with PBS and cell viability was evaluated using a WST-8Cell Counting kit (Dojindo, Osaka, Japan). After incubation with the WST-8 reagent for 40 min, an aliquot of chromophore-developed solution was carefully transferred to a fresh 96-well culture dish to obtain a particle-free reaction solution, then the reaction was terminated by adding one-tenth volume of 0.1 mol/L HCl solution. The optical density (O.D.) at 450 nm was measured using a microplate reader (POLARstar OPTIMA, BMG Labtech, Offenburg, Germany).

2.3. Animals

Specific pathogen-free male ICR mice (5 weeks old) were purchased from Clea Japan (Tokyo, Japan). Mice were housed in a clean and air-conditioned room (temperature: 22 ± 0.5 °C, relative humidity: 50 ± 5%) and given free access to conventional laboratory chow and tap water. The animal study was approved by the Ethics Committee for Animal Care and Experimentation of the National Institute for Environmental Studies (Japan).

2.4. Animal experiment 1 with bronchoalveolar lavage (BAL)

At 6 weeks of age the animals were anesthetized with 2.5% halothane in a carrier gas (nitrous oxide:oxygen = 1:2). Groups of 3 mice were intratracheally instilled with a 50-μL aliquot of sterile deionized water containing 1% albumin solution (vehicle control), 20 nm AgNPs suspension, or $AgNO_3$ solution at a dose of 10 μg Ag/mouse. Four and 24 h after administration, the animals were killed by exsanguination through the abdominal aorta under pentobarbital anesthesia (50 mg/kg body weight) and the thoracic cavity was opened to collapse the lung. The trachea was cannulated and the lungs were lavaged 4 times by infusion and withdrawal of 150 mM Tris–HNO_3 (pH 7.4) buffer (0.035 mL/g body weight). The bronchoalveolar lavage fluids (BALF) were centrifuged (4 °C, 400 × *g*) for 5 min to separate the supernatant from the cellular pellet. The first BALF supernatant was used for measurement of interleukin 1β (IL-1β) concentration using an IL-1β ELISA kit (Thermo Fisher Scientific K.K., Yokohama, Japan) and Ag concentration. The total cellular pellets were re-suspended in 1.0 mL Tris–HNO_3 buffer (150 mM, pH 7.4) and the number of cells was counted using a hemacytometer. An aliquot of the cell suspension was cytospun and stained using Diff-Quik® (International Reagents, Kobe, Japan). The rest of the BALF cells, an aliquot of the BALF supernatant, and the whole lung tissue were wet-digested with analytical grade nitric acid (0.75 mL) and H_2O_2 (0.25 mL) at 135 °C for 24 h. The digested samples were diluted with deionized water and concentrations of Ag were measured by inductively coupled plasma-mass spectrometry (ICP-MS) (7500c, Agilent Technologies, Tokyo) at *m*/*z* 107.

2.5. Animal experiment 2 with urine collection

In a separate experiment, groups of 8 animals were intratracheally instilled with a 50-μL aliquot of 1% albumin solution