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Inflammasome formation and IL-1β release by human blood monocytes in response to silver nanoparticles

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ABSTRACT

In this study, the immunological effect of silver nanoparticles on innate immunity was investigated using primary human monocytes. After exposure to silver nanoparticles, production of IL-1β, a critical cytokine involved in induction of innate immunity, significantly increased as particle size decreased. These results suggest that silver nanoparticles may evoke an immunologically active state. The size effect of silver nanoparticles on IL-1β production was also further investigated. 5 nm and 28 nm silver nanoparticles induced inflammasome formation and subsequent caspase-1 activation. Using inhibitors, we found exposure to silver nanoparticles caused leakage of cathepsins from lysosomes and efflux of intracellular K^+. These two events induced superoxide within mitochondrial membranes, leading to inflammasome formation. 5 nm silver nanoparticles produced more hydrogen peroxide and were more cytotoxic than 28 nm silver nanoparticles, suggesting the balance between superoxide and hydrogen peroxide governs cell fate, death or activation. Moreover, these findings also suggest that the immunological significance of silver nanoparticles should be considered with respect to their capacity to synergistically activate immune responses.

1. Introduction

Silver nanoparticles are widely used in biological fields including many medical applications [1–3]. With regard to immune responses, 5 nm silver nanoparticles induced the expression of IL-8, a neutrophil-recruiting chemokine, in macrophages; a process mediated by reactive oxygen species (ROS) [4,5]. When individuals are exposed to nanoparticles, many particles translocate to the blood or enter the systemic circulation from the entry site [6,7], where they are detected and internalized by macrophages or monocytes [8].

Innate immunity, which initiates adaptive immune responses, can be activated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs include microbial antigens such as lipopolysaccharide (LPS) of Gram (−) bacteria and viral genomes. DAMPs include molecules originating from the host such as heat shock proteins or monosodium urate [9]. In response to sensing PAMPs or DAMPs, immune cells secrete a variety of cytokines with different functions. IL-8 is a chemokine mainly involved in local inflammation, which functions by recruiting neutrophils during the acute stage, whereas IL-1β is a prototypic pro-inflammatory cytokine [10]. IL-1β also participates in innate immunity, but exerts more influence on immune cells, such as in activating macrophages and endothelial cells to produce cytokines or adhesion molecules. In addition, IL-1β has systemic effects, such as fever induction or triggering hepatocytes to secrete acute phase proteins. IL-1β production is strictly regulated at the transcriptional and post-translational levels through the control of both its maturation and secretion. The maturation of IL-1β is regulated by inflammasomes [11], and more specifically by NLRP3 [12,13]. Because nanoparticles, such as carbon black [14], polystyrene [15], silica [16], double-walled carbon nanotubes [17], and TiO_2 [16] induce inflammasome formation, we speculate that silver nanoparticles may also trigger inflammasome formation.

This study investigated the immunological impact of silver nanoparticles in the induction of innate immunity, with a focus on human blood monocytes. Both inflammasome formation and the release of mature IL-1β were assessed after treatment with different sized silver nanoparticles. In addition, the mechanisms leading to inflammasome formation were characterized based on the type of ROS involved and the roles of cathepsins and K^+ efflux. Collectively, the results of this study will help to understand the activation of innate immunity after exposure to silver nanoparticles.

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2. Materials and methods

2.1. Silver nanoparticles and their characterization

Silver nanoparticles in water suspension were provided by I&C (5 nm & 28 nm, Seoul, Korea) or purchased from ABC Nanotech (100 nm, Daejeon, Korea). All silver nanoparticles were round and PVP-coated. Particles were tested for contamination using the Pyrogene Recombinant Factor C Assay (Cambrex Bioscience, Walkersville, MD, USA) and were negative for endotoxin (less than 0.01 EU/mL). For cell culture, dispersions of different concentrations of silver nanoparticles were prepared in RPMI 1640 medium with 2 mM L-glutamine supplemented with 10%-FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Primary particle diameter was determined by transmission electron microscopy (TEM, model JEM-1011, JEOL, Tokyo, Japan). Agglomeration states of nanoparticles in 10%-FBS RPMI 1640 medium at 10 μg/mL concentrations were analyzed using dynamic light scattering DLS (Novato, CA, USA).

2.2. Cell culture and purification of peripheral blood mononuclear cells (PBMCs) and monocytes

100 mL of blood were each obtained from ten healthy donors after acquiring internal review board approval and informed consent (No: 4-2012-0088). PBMCs were separated by Ficoll-Hypaque Density Gradient Centrifugation (density = 1.070–1.074) at 1600 rpm for 25 min. PBMCs were cultured in RPMI 1640 medium containing 10% FBS and streptomycin/penicillin (each 100 IU/mL) at 37 °C in a moisturized 5% CO_2 incubator. Although endotoxin was not detected in silver nanoparticles used in this study, polymyxin B (InvivoGen, San Diego, CA, USA) at a concentration of 10 ng/mL was added as an endotoxin neutralizer. To purify monocytes, PBMCs were incubated for 2 h in RPMI 1640 containing 1% FBS at 37 °C in a moisturized 5% CO_2 incubator and adherent cells were obtained after discarding non-adherent cells. Staining for CD14 (monocyte marker) was performed to identify monocyte population and usually 88–90% of cells were positively stained.

2.3. Reagents

Inhibitors were pre-treated for 1 h at 20 μM zVAD-FA-fmk (ALX-260-154; Enzo Life Sciences Farmingdale, NY, USA) to inhibit cathepsins, 50 mM KCl (Sigma–Aldrich, St Louis, MO, USA) to complement K^+ efflux, and 10 mM N-acetyl cysteine (NAC, Sigma–Aldrich) to scavenger ROS. LPS (*E.coli* 026:B6, Sigma–Aldrich) was treated at 50 pg/mL for 2 h for PBMCs and 1 h for monocytes before nanoparticle exposure.

2.4. Cytotoxicity assay

Cell viability was assessed using the colorimetric cell counting kit-8 (CCK-8) (Dojindo laboratories, Kyoto, Japan). CCK-8 is based on a colorimetric assay utilizing a highly water soluble tetrazolium salt, WST-8[2-(2-methyxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-^{2}H-tetrazolium, monosodium salt]. The wells were then treated with 200 μL of silver nanoparticles solutions diluted in cell culture medium. After 6 h, 15 μL of CCK-8 reagent was added to each well and then incubated at 37 °C for 2 h. After centrifugation, 100 μL of supernatant was transferred to 96-well microtiter plates and optical densities (O.D.) were measured at 450 nm with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to avoid optical interference caused by silver nanoparticles.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to assess IL-1β in culture supernatant. PBMCs or monocytes were plated in 24-well plates at 2×10^5 cells per well in 200 μL of 10%-FBS RPMI 1640 medium. Silver nanoparticles in cell culture media were added to each well, making a final volume of 400 μL per well. After 6 h, the cell culture supernatants were collected and stored at −80 °C. ELISA was performed with a human cytokine IL-1β assay kit (BD Biosciences, San Jose, CA, USA), which uses biotinylated anti-IL-1β detection antibodies and streptavidin-horse radish peroxidase. The O.D. was read at 450 nm.

2.6. Western blot

For western blot analysis, 1×10^6 monocytes were seeded into 60 mm dishes (SPL, Pocheon, Korea). Cells were then treated with silver nanoparticles for 90 min. Cells were harvested and lysed at 4 °C for 2 h in 200 μL of lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM tris pH 8, 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail). The cell lysates were centrifuged at 13,000 rpm for 15 min at 4 °C and supernatants were stored at −20 °C. Protein concentrations of the lysates were measured by the Bradford assay. From each sample, 50 μg of protein was boiled for 5 min and loaded on a 10% and 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, proteins in the gels were transferred onto nitrocellulose membranes (Amersham, Glattbrugg, Switzerland). After being blocked with 5% skim milk (BD Biosciences, Franklin, NJ, USA), membranes were reacted with anti-caspase-1 primary antibody (Cell signaling technology, Danvers, MA, USA) at 1:1000 dilution in 5% bovine serum albumin (BSA) (Sigma–Aldrich) at 4 °C overnight. After washing, the membranes were further reacted with secondary antibodies (peroxidase-conjugated affinipure goat anti-rabbit IgG, Jackson ImmunoResearch, West Grove, PA, USA) at 1:2000 dilution in 5% skim milk at room temperature for 2 h. Protein bands were detected using a West-save up western blot detection kit (Ab frontier, Seoul, Korea). As an internal control, anti-β-actin antibody (Cell signaling technology) was assessed. To analyze cleaved caspase-1 in culture supernatants, each 150 μL of culture supernatant was centrifuged at 5000 g in Nanosep devices (3k omega) (Port Washington, NY, USA) to a final volume of 30 μL. Then, 6 μL loading buffer was added to each sample, followed by boiling for 10 min before SDS-PAGE and western blot was done with caspase-1 p20 (Asp297) antibody (D57A2; Cell Signaling Technology).

2.7. Caspase-1 substrate assay

After treatment with 5 nm, 28 nm, or 100 nm silver nanoparticles for 3 h, caspase-1 activity in culture supernatant was determined with a caspase-1 colorimetric kit (BioVision, MountainView, CA, USA) by adding 5 μL of the caspase-1 substrate YVAD-*p*NA. Caspase-1 activity was quantified by spectrophotometric detection of free *p*NA (at 405 nm) after cleavage from YVAD-*p*NA, using a multi-well plate reader. The "fold" was calculated as the O.D. value of monocyte culture supernatant after exposure to nanoparticles divided by the O.D. value of monocyte culture supernatant without treatment with nanoparticles (control cells).

2.8. Staining with MitoSOX, CM-H_2DCFA and JC-1

Monocytes were treated with silver nanoparticles (0.9 μg/mL for 5 nm and 28 nm particles, 1.25 μg/mL for 100 nm particles) for 40 min. After treatment with silver nanoparticles, blood monocytes were stained with fluorescent dyes (MitoSOX for 15 min and CM-H_2DCFA for 30 min) diluted in Hank's balanced salt solution at 37 °C in the dark. 2.5 μM MitoSOX (Invitrogen, San Diego, CA, USA) was treated to detect mitochondrial superoxide and 2.5 μM CM-H_2DCFA (Invitrogen) to detect hydrogen peroxide. After exposure to same concentrations of silver nanoparticles, 2 μM of JC-1 staining (Invitrogen) was performed to determine mitochondrial depolarization. Flow cytometric analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). After staining cells with Mitosox, CM-H_2DCFA, and JC-1, flow cytometric analysis was performed at 580 nm, 510 nm, and 590 nm. Results were analyzed using WinMDI software.

2.9. TEM analysis of cells

Monocytes were treated with 1.15 μg/mL of silver nanoparticles for 15 min were fixed with Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$) for 6 h. After washing with PBS for 2 h cells were treated with 1% OsO_4 in 0.1 M PBS buffer for 2 h. Dehydration of samples was performed gradually with 50%, 60%, 70%, 80%, 90%, 95%, and 100% alcohol for 10 min each, followed by a final addition of propylene oxide for 10 min. Samples were then treated with a mixture of EPON (EPON 812, MNA, DDSA, DMP30) and propylene oxide (1:1) for 18 h and heated in an embedding oven at 35 °C for 6 h, 45 °C for 12 h, and 60 °C for 24 h. The cell block was trimmed and cut as 0.25 μm using an ultramicrotome (Leica Ultracut UCT, Leica Microsystems GmbH, Wetzlar, Germany). These ultra-thin sections were stained with 1% toluidine blue and put on a copper grid. The samples were then stained with uranyl acetate (6%) and lead citrate and analyzed using a TEM (JEM-1011, JEOL).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) and Student's independent *t*-tests were conducted. $P < 0.05$ was considered to be significant.

3. Results

3.1. Characterization of silver nanoparticles

The silver nanoparticles used in this study had diameters of 5 nm, 28 nm and 100 nm, were coated with polyvinylpyrrolidone, and were synthesized by the reduction of $Ag(NO_3)_3$. The physicochemical properties were previously reported in part [4,5]. The individual sizes of silver nanoparticles were determined using TEM. All particles were round in shape. 5 nm silver nanoparticles were relatively uniform, but 28 nm and 100 nm silver nanoparticles showed a range of different sizes. The average size was 7.9 ± 5.3 nm for 5 nm silver nanoparticles, 18.3 ± 10.3 nm for 28 nm silver nanoparticles, and 81 ± 42.1 nm for 100 nm silver nanoparticles (Fig. S1). No aggregation was observed under TEM. DLS analysis showed that the mean diameters of the 5 nm, 28 nm, and 100 nm silver nanoparticles were