Toxicology in Vitro 31 (2016) 12–22

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Gold nanoparticles induce apoptosis, endoplasmic reticulum stress events and cleavage of cytoskeletal proteins in human neutrophils

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ARTICLE INFO

Article history:
Received 21 May 2015
Received in revised form 9 September 2015
Accepted 6 November 2015
Available online 7 November 2015

Keywords:
Gold nanoparticles
Neutrophils
Apoptosis
Caspases
Endoplasmic reticulum
Cytoskeleton

ABSTRACT

Gold nanoparticles (AuNPs) are promising candidates for developing nanomedicines, for the treatment of different disorders, including inflammatory diseases. However, how AuNPs could alter the biology of human neutrophils, key player cells in inflammation, is a poorly documented area of research. Here we found that, although AuNP of 20 nm ($AuNP_{20}$) could be internalized in cytosolic vacuoles but that $AuNP_{70}$ were localized at the cell membrane, both induced apoptosis similarly by a caspase-dependent mechanism. AuNPs induced degradation of the cytoskeletal proteins vimentin, lamin B_1 and gelsolin, but, unexpectedly, did not increase their cell surface expression. Consequent with caspase-4 processing, AuNPs were found to activate endoplasmic reticulum (ER)-stress, as evidenced by activation of the three ER sensors, IRE1 (inositol-requiring protein-1), ATF-6 (activating transcription factor-6) and PERK (protein kinase RNA (PKR)-like ER kinase). AuNPs are novel human neutrophil proapoptotic agents indicating that they are toxic to these cells. However, the fact that they do not induce cell surface expression of cytoskeletal proteins could decrease potential adverse effects and toxicity of AuNPs by limiting, for example, the production of autoantibody against cytoskeleton components.

1. Introduction

Utilization of nanoparticles (NPs) becomes constantly more important in nanomedicine. Because of their size similar to cellular components, NPs can directly interact with the cell and their organelles. Utilization of NPs is of great interest for health care because of their numerous benefits like the ability to specifically target cancerous cells or improve the efficiency of certain drugs (Bharali and Mousa, 2010; Pan et al., 2007; Ventola, 2012). Good candidates in nanomedicine are gold nanoparticles (AuNPs). They are currently being used widely because of their optical and photothermal properties (Daniel and Astruc, 2004; Eustis and El-Sayed, 2006). Indeed, the optical properties of AuNPs allow specific targeting of cancer cells and that could be specifically destroyed because of their photothermal properties (Huang and El-Sayed, 2010; Zhang et al., 2011). Recently, it has been proposed that the use of AuNP carriers can improve the delivery and safety of immunotherapy agents in cancer (Almeida et al., 2014).

Among the adverse effects of NPs described in the literature, inflammation is certainly the most reported one, based especially on exacerbation of in vivo models of airway of inflammation and on the increased proinflammatory cytokine production by different cell types (Aalapati et al., 2014; Brandenberger et al., 2013; Hussain et al., 2011; Skuland et al., 2014). However, in general, the effects of naked AuNPs are anti-inflammatory (Sumbayev et al., 2013; Tsai et al., 2007) and when inflammation is reported the effects appear to be only transitory. Moreover, AuNPs were found to have great potential as carriers for local drug delivery and as a primary therapeutic for treatment of inflammation (Labens et al., 2013), including arthritis (Tsai et al., 2007). Of note, it has been showed in vivo that polyethylene glycol-coated AuNPs of 10 nm and 60 nm induced a recruitment of polymorphonuclear neutrophil cells (PMNs) while the same particles at a diameter of 5 nm and 30 nm decreased such recruitment (Zhang et al., 2011).

PMNs, the predominant leukocyte type in human blood, are key player cells in inflammation. They are known to be the first cell type to migrate at an inflammatory site. A huge number of PMNs are released from the bone marrow; this has been estimated at ~5×10^{10} cells on a daily basis in a normal adult (Ward et al., 1999). They have a limited life span and have the shortest half-life (~12 h in circulation) among all cells of the immune system. Cell turnover must therefore be rigorously controlled to avoid adverse effects. Fortunately, PMNs undergo constitutive or spontaneous apoptosis explaining why the number of PMNs remains relatively stable in healthy individuals. Importantly, elimination of apoptotic PMNs by professional phagocytes is a major step for the resolution of inflammation (Ward et al., 1999).

Cytoskeleton rearrangement is important for many PMN functions (Torres and Coates, 1999). We have previously shown that some cytoskeletal proteins are cleaved and expressed at the surface of PMNs during spontaneous apoptosis as well as in response to some agents,

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http://dx.doi.org/10.1016/j.tiv.2015.11.003
0887-2333/

including the potent proapoptotic plant lectin *Viscum album* Agglutinin-I (VAA-I) (Lavastre et al., 2002; Moisan and Girard, 2006; Savoie et al., 2000). Also, it had been shown that cytoskeletal proteins played a role during inflammation. For example, gelsolin, a microfilament-associated protein, is known to regulate PMN apoptosis and induce an inflammatory response (Li et al., 2012). Moreover, the cell surface expression or the secretion of some cytoskeletal proteins is associated with the presence of autoantibodies against these proteins and with several autoimmune inflammatory disorders (Dieudé et al., 2002; Thebault et al., 2002).

Despite the above observations, the effects of AuNPs on PMN apoptosis and cytoskeleton rearrangements have never been reported. In this study, we demonstrated that AuNPs of 20 nm ($AuNP_{20}$) and of 70 nm ($AuNP_{70}$) increased the basal PMN apoptotic rate by a caspase-dependent mechanism. Both AuNPs activate endoplasmic reticulum (ER) stress mediated cell death as evidenced by activation of the three ER sensors IRE1, PERK and ATF-6. AuNPs also induced the degradation of some cytostokeletal proteins, but did not induce cell surface expression of cytoskeletal proteins in apoptotic PMNs.

2. Materials and methods

2.1. Chemicals

The $AuNP_{20}$ and $AuNP_{70}$ (PELCO® Tannic BioPure™ Gold Colloids) were purchased from Ted Pella (Redding, CA). The caspase inhibitor N-benzyloxy-carbonyl-V-A-D-O-methylfluoromethyl ketone (z-VAD-FMK) was purchased from Calbiochem (Pasadena, CA). The plant lectin *V. album* agglutinin-I (VAA-I), trypan blue, tunicamycin, bovine serum albumin (BSA), phosphate buffered saline solution (PBS), monoclonal anti-gelsolin, anti-β-actin and dextran were purchased from Sigma Aldrich Ltd. (St. Louis, MO). Phospho-IRE-1 and total IRE-1 antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) and from Pierce (Rockford, IL), respectively. Ficoll-Hypaque was purchased from GE Healthcare (Uppsala, Sweden). RPMI-1640, HEPES, penicillin, streptomycin, Annexin-V-FITC, propidium iodine (PI) and Hank's Balanced Salt Solution (HBSS) were from Life Technologies (Grand Island, NY, USA). The goat polyclonal anti-lamin B_1 (C-20), mouse monoclonal anti-vimentin (clone V9), phospho-PERK, PERK, ATF-6α, caspase-4 and anti-GAPDH (FL-335) specific antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). FITC-mouse anti-human CD16 mAb was purchased from BD Pharmingen (Mississauga, Ontario, Canada). HRP-labeled goat anti-mouse IgG, HRP-labeled goat anti-rabbit IgG and HRP-labeled rabbit anti-goat IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). The 30% acrylamide/BIS solution, polyvinylidenedifluoride (PVDF) membrane and Clarity TM Western ECL substrate were purchased from Bio-Rad (Hercules, CA). Rabbit anti-caspase-3, caspase-7 and caspase-9 antibodies were from Cell Signaling Technology Inc. (Danvers, MA). The Hema 3 stain kit was from Fisher Scientific Company L.L.C. (Kalamazoo, MI), dry milk from ©Nestle and propidium iodide (PI) from Life Technologies (Eugene, OR).

2.2. Size distribution and zeta potential measurements

The size distribution (intensity) and polydispersity index of $AuNP_{20}$ and $AuNP_{70}$ were determined by DLS, and surface charge by zeta potential, using a Malvern Zetasizer Nano-ZS (ZEN3600) (Malvern Instruments Inc., Westborough, MA).

2.3. PMN isolation

PMNs were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque as previously described (Babin et al., 2013; Poirier et al., 2014). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures.

2.4. Cell viability

Freshly isolated human PMNs (10×10^6 cells/mL in RPMI-1640 HEPES-P/S, supplemented with 10% heat-inactivated autologous serum) were treated for 24 h with or without increasing concentrations of AuNPs ranging from 0 to 100 μg/mL. Cell viability was monitored by trypan blue exclusion and remained ≥95% (*data not shown*).

2.5. Assessment of apoptosis by cytology and by flow cytometry

PMNs (10×10^6 cells/mL suspension in RPMI-1640 supplemented with 10% autologous serum) were incubated at 37 °C in 5% CO_2 in 96-well plates for 24 h with the indicated agonists. At this time point, normally 30–50% of cells are in apoptosis (Moisan and Girard, 2006; Poirier et al., 2014). For some experiments, cells were pre-treated 30 min with or without z-VAD-FMK (50 μM). For cytology, cells were cytocentrifuged on microscope slides, stained with the Hema 3 staining kit and examined by light microscopy at ×400 final magnification. Apoptotic neutrophils were defined as cells containing one or more characteristic darkly stained pyknotic nuclei (Poirier et al., 2014). Results were expressed as the percentage of PMNs in apoptosis. For the flow cytometric procedure, PMNs were stained with FITC annexin-v or FITC anti-human CD16. Ten thousand cells were analyzed by FACSscan (Becton-Dickinson, San Jose, CA) using CellQuest program (BD Biosciences, San Jose, CA, USA).

2.6. Cell morphology and cellular uptake of AuNPs

Freshly isolated human PMNs (10×10^6 cells/mL in RPMI-1640 HEPES-P/S, supplemented with 10% heat-inactivated autologous serum) were treated for 24 h with or without increasing concentrations of AuNPs ranging from 0 to 100 μg/mL. The morphological cell shape changes were observed under light microscopy (×400 magnification), and photomicrographs were taken using a Nikon Eclipse TS100 camera (Melville, NY) as previously published (Girard et al., 1997). Evaluation of cell size and inner complexity was determined by flow cytometry (Suzuki et al., 2007). Briefly, freshly isolated human PMNs (10×10^6 cells/mL in RPMI-1640 HEPES-P/S) were treated with or without 100 μg/mL AuNPs for 30 min and data for cell size (forward scattered light height (H)) and inner complexity (side scattered light height (H)) were acquired by flow cytometry using a FACScan (BD Biosciences, San Jose, CA) as we documented previously (Poirier et al., 2014).

2.7. Transmission electron microscopy

To visualize potential cell internalization of the NPs, freshly isolated PMNs were incubated (10×10^6 cells/mL) with 100 μg/mL of $AuNP_{20}$ or $AuNP_{70}$ or with the equivalent volume of HBSS for 1 h or 24 h. Cells were then fixed overnight with 2.5% glutaraldehyde in PBS. After several washes in PBS with 3% sucrose, cells were fixed with 1.3% OsO_4 for 2 h and embedded in Spurr resin. Thin slices were prepared with an ultramicrotome and stained with 5% uranyl acetate and filtered lead citrate. Examinations of ultrathin sections (total of 50–70 cells/sample) were examined using a Hitachi H-7100 transmission electron microscope as described (Poirier et al., 2014; Simard et al., 2015a).

2.8. Western blot analysis

Cells (10×10^6 cells/mL) were incubated with the indicated agonists for the specified periods of time and then harvested for the preparation of cell lysates in Laemmli's sample buffer. Aliquots corresponding to 500,000 cells were loaded and subjected to 7.5–10% SDS-polyacrylamide gel electrophoresis and transferred from the gel to PVDF or nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in 5% milk in TBS-Tween (25 mM Tris–HCl, pH 7.8, 190 mM NaCl, 0.15% Tween-20). After washing, a dilution of