Introduction

Silver ions possess anti-microbial activity preventing microbial growth, silver based medicaments were historically used for wound and burns healing, being safe in limited doses for humans [1-3]. Nowadays, when viral, bacterial and fungal resistance to drugs is a potential and increasing problem, scientists take an ancient knowledge into consideration, looking for a new form of solid silver mattery – silver nanoparticles (AgNPs). Nanoparticles by definition are particles smaller than 100 nm. Because of nano-size their physicochemical properties differ from the bulk substances [4-6]. There are different synthesis techniques of noble metals nanoparticles. The main methods are chemical reduction of salts and electric non – explosive method where electrodes made of high purity metals are used. Physical method provides cheap and large quantities of AgNPs being a mixture of different sizes (5-500 nm) and shapes. There are more and more commercially available – products containing AgNPs.

Monocytes constitute 5-8% of all circulating leucocytes. During inflammation monocytes migrate to tissues where they become macrophages or dendritic cells. Macrophages are known as the most phagocytic cells disposing of cell debris or pathogenic bacteria and viruses. Moreover, macrophages play an important role during wound healing being major producers of proangiogenic growth factors. Healed skin in macrophagy deficient mice showed minimal scar formation in consequence of the reduced inflammatory response, however, depletion of myeloid cells during early stages of repair process significantly reduced formation.

Toxicity of silver nanoparticles in monocytes and keratinocytes: potential to induce inflammatory reactions

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Abstract

Silver nanoparticles are of interest to be used as antimicrobial agents in wound dressings and coatings in medical devices, but potential adverse effects have been reported in the literature. The possible local inflammatory response to silver nanoparticles and the role of cell death in determining these effects are largely unknown. Effects of the mixture of silver nanoparticles of different sizes were compared in in vitro assays for cytotoxicity, caspase-1 and caspase-9 activity and bax expression. In all tested concentrations, silver nanoparticles were more toxic for RAW 264.7 monocytes than for 291.03C keratinocytes and induced significant caspase-1 activity and necrotic cell death. In keratinocytes, more significantly than in macrophages, silver nanoparticles led to increase of caspase-9 activity and apoptosis. These results indicate that effects of silver nanoparticles depend on the type of exposed cells. In addition, the potency of silver nanoparticles to induce necrosis and caspase-1 activity in monocytes indicates their possible immunotoxic inflammatory potential.

Key words: silver nanoparticles, toxicity, monocytes, keratinocytes, apoptosis.

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of vascularized granulation tissue [7, 8]. Depletion of macrophages during tissue formation cause severe hemorrhage in the wound tissue and prevent tissue maturation [8].

Skin is the critical surface barrier of a body, it is constantly exposed to physical, chemical and biological factors. In addition, it is essential in maintaining body temperature and physical contact to the environment. Skin has evolved into a complicated and tightly regulated tissue, consisting of many layers. The epidermis is the outermost layer of the skin, it consists of keratinocytes, which contribute to 95% of skin cells; dendritic cells (DCs); Langerhans cells (LCs); macrophages and mast cells, belonging to the immune system; melanocytes providing the pigment essential for protection against UV radiation; and Merkel cells being capable of sensor light touching [9-11]. Previously, keratinocytes were believed to only create a physical barrier to the environment. In the last decade, after discovery that keratinocytes express Toll like receptors (TLRs) 1, 2, 3, 5, 9, 10, it has been shown that keratinocytes are potent source of chemokines (CXCL8), cytokines (GM-CSF, IL-6), antimicrobial peptides, nitric oxide and are able to express the class II MHC. It has been demonstrated that keratinocytes, together with immune cells present in epidermis, are one of the crucial element initiating innate immune response in the skin [12-15]. Skin is the most subjected to contact with nanoparticles. Macrophages present in skin may internalize nanoparticles penetrating in to deeper layers of skin. Any disturbance of skin cells may lead to serious disorders, i.e. local inflammation, overproduction of extracellular matrix or increased neovascularization [16].

Recently, there an increasing number of reports indicating potential toxic characteristics of AgNPs. Braydich-Stolle (2005) and co-workers demonstrated that commercially produced in pulsed – plasma reactor AgNPs changed cell morphology, physically produced AgNPs changed cell morphology, and even apoptosis resulting in varying levels of cytotoxicity. Furthermore, changes in shape and dimension and even a method of production may affect AgNPs influence on cell structure, viability and function.

In this study, we compared toxicity of commercially available, physically produced AgNPs hydrocolloids in RAW 264.7 monocyte cell line and 291.03C keratinocyte cell line. Additionally, we tested possible immunotoxic reaction induced in murine monocytes by silver nanoparticles.

Material and methods

Preparation and characterization of nanoparticles

Hydrosols of silver nanoparticles (50 ppm) were produced by electric non-explosive patented method (Polish patent 380649) from high purity silver (99.9999%) and high purity demineralized water (Nano-Tech, Poland). Silver nanoparticles present in the hydrosol were tested using transmission electron microscope (TEM) with JEM – 2000EX (JEOL, Japan). Observations revealed mixture of sizes and shapes of nanoparticles. It can be seen that the nanoparticles were almost monodisperse. Potential Zeta, measured with light scattering methods, was ~9.2 mV and the size of nanoparticles was ranging 44-106 nm.

Cell culture and treatment

Mouse RAW 264.7 monocytes were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) in standard conditions (37°C, 5% CO2). The mouse keratinocyte cell line 291.03C was kindly provided by M. Kulesz-Martin (Department of Dermatology, Oregon Health and Science University, Portland, OR, USA). The 291.03C cells were cultured in Minimum Essential Medium Eagle α modification (Sigma), supplemented with FBS and antibiotics, as above (Sigma), in standard conditions. The cells were seeded into 24 well plates at density of 5 × 10^4/cells and cultured for 24 h before exposure to nanoparticles at different concentrations.

Cell toxicity assays

Neutral red uptake

Briefly, 24 h (RAW 264.7) or 48 h (291.03) after seeding the cells, medium was discarded and different concentrations of AgNPs suspended in fresh medium were added. After another 24 h medium was collected and the cells were washed with phosphate buffered saline (PBS, Sigma) and the fresh medium containing 10% neutral red solution was added. Cells were returned into incubator for another 2 hours. Next, the cells were washed in PBS and incorporated dye was liberated from the cells according to manufacturer’s protocol. Absorbance was measured at 540 nm in Tecan Infinite M200 microplates reader (Tecan Systems, San Jose, CA, USA). The viability of cells was expressed as the percentage of the control, untreated cells (100%).
Cell viability

Cell viability was assessed by carboxyfluorescein diacetate (6-CFDA) and propidium iodide (PI) staining. After exposure to nanoparticles, the cells were collected, washed with PBS, and then suspended in staining solution containing 6-CFDA (500 μM) and PI (1 μg/ml), incubated for 10 min in 4°C and then analysed in FACS Calibur using CellQuest programme (Beckton Dickinson, Franklin Lakes, NJ, USA). Apoptotic cells were defined as percentage of CFDA (−) cells, while necrotic cells as percentage of PI (+) cells.

Mitochondrial potential

Following experimental treatments, harvested cells were stained with a cationic dye, 5,5’;6,6’-tetrachloro-1,1’;3,3’-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Sigma). Briefly, cells were incubated with culture medium (RPMI-1640 or DMEM) containing 10% FBS and 5 μg/ml JC-1 at 37°C for 15 min. Following incubation, cells were washed two times in PBS and analysed in FACS Calibur using CellQuest programme (Beckton Dickinson) for the percentage of cells with a decrease in the red to green fluorescence intensity ratio.

Cell morphology assessment

Cells were seeded into glass coverslips in six well plates then following experimental treatments, the cells were rinsed with PBS pre-warmed to 37°C and then fixed with pre-warmed 3% glutaraldehyde in PBS for 20 min. Next, cells were washed with PBS and sealed with DPX mounting medium (Sigma). Phase contrast images were acquired using camera-equipped Zeiss AxioX10 microscope (Zeiss, Jena, Germany).

Antibodies and immunostaining

Intracellular antigens were detected using Cytofix/Cytoperm fixation/permeabilization kit (Beckton Dickinson) according to the manufacturer’s protocol and by using the following antibodies: mouse anti-bax monoclonal antibody, goat anti-cleaved caspase-1 (p10) polyclonal antibody, goat anti-cleaved caspase-9 (p10) polyclonal antibody, rabbit anti-MAPLC3 polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA), and mouse anti-ki67 monoclonal antibody (Becton Dickinson). Following incubation with primary antibodies, appropriate anti-mouse or anti-goat PE – conjugated were used (Becton Dickinson). The stained cell suspensions were analyzed in FACS Calibur for the percentage of positively stained cells and/or mean intensity of fluorescence.

Statistical methods

Quantitative data were presented as means ± SEM. In the case of normal distribution of values, confirmed by Shapiro’s test, statistical comparisons were performed using the Student’s t-test. With non-Gaussian distributions, non-parametric Kruskal-Wallis and Wilcoxon tests were applied. In every analysis values of \( p = 0.05 \) were considered significant.

Results

Cell morphology

To assess the influence of AgNPs on the cell morphology we exposed cells for 24 h to 2.5 μg/ml AgNPs. Comparing to untreated control (Fig. 1A), RAW 264.7, showed typical necrotic morphology with cytoplasm fragmentation, membrane permeabilization and pyknotic nucleus. Some cells showed typical apoptotic morphology with blebbing and cell shrinkage (Fig. 1B). 291.03C keratinocytes exposed to AgNPs showed more dense cytoplasm with more vacuolization and nuclear fragmentation (Fig. 1C and D).

Neutral red uptake assay

We used neutral red assay to investigate the ability of viable cells to incorporate the dye into lysosomes. Figure 2A shows higher sensitivity of monocytes to 2.5-10 μg/ml AgNPs in comparison to keratinocytes. All tested AgNPs concentrations significantly reduced RAW 264.7 viability defined as percent of neutral red positive cells in comparison to untreated control (\( p \leq 0.05 \)). Silver nanoparticles at the concentration of 5 μg/ml reduced monocytes viability by more than 50% (37.5 ±6.5%) whereas 291.03C keratinocytes showed higher viability (80%) for the same concentration (81.2 ±3.5) (Fig. 2A). Both cell lines visibly decreased dye uptake when treated with 10 μg/ml of nanoparticles (5.14 ±0.5% for RAW 264.7 and 10.8 ±2% for 291.03C).

Mitochondrial potential

Decreased neutral red uptake and a leakage of dye deposition in acid compartments of cells may be caused by blockage of ATP synthesis. We used the JC-1 assay to evaluate the mitochondrial function (Fig. 2B). In RAW 264.7, treatment with 2.5 and 5 μg/ml AgNPs did not significantly affect mitochondrial transmembrane potential (\( p \leq 0.05 \)), however while, 10 μg/ml of AgNPs significantly increased up to 70 ±0.02% the percentage of cells with the loss of mitochondrial potential (Fig. 2B). 291.03C keratinocytes treated with 5 and 10 μg/ml showed 17.2 ±0.4% and 40.25 ±0.02%, respectively, of the cells with the mitochondrial dysfunction (\( p \leq 0.05 \)).

Apoptosis/necrosis staining

Because of possible metabolism collapse by AgNPs treatment we assessed the possibility of apoptosis/necrosis (Fig. 2C and D). Increasing concentrations of AgNPs caused concentration dependent cytotoxicity in both lines. Although necrosis was the dominating cell death for monocytes, in
For RAW 264.7 we observed a significant ($p \leq 0.05$) decrease in the percentage of apoptotic cells reaching 2.5 ±1.2% at 2.5 μg/ml in comparison to control for all tested concentrations. On the other hand, the percentage of necrotic cells was significantly increasing with the higher concentrations of nanoparticles reaching more than 90% for 10 μg/ml ($p \leq 0.05$) (Fig. 2D). In keratinocytes the percentages of apoptotic cells were significantly increasing in concentration dependent manner, reaching 19.6 ±0.01% at 10 μg/ml ($p \leq 0.05$). The percentages of necrotic keratinocytes were significantly increased for 5 and 10 μg/ml, reaching maximally 7.77 ±0.01% for 10 μg/ml ($p \leq 0.05$).

**Intrinsic pathway of apoptosis**

Because the high concentrations of AgNPs affected mitochondrial potential in both cell lines and apoptosis was the dominating type of cell death in keratinocytes we assessed the possibility of apoptosis induction by mitochondrial-dependent pathway. Caspase-9 is one of the components of apoptosome and it is activated in the consequence of cytochrome c release from damaged mitochondria [18]. All concentrations of AgNPs significantly ($p \leq 0.05$) increased percentages of cells with active form of caspase-9 in both cell lines. Although the percentage of cells with active form of caspase-9 in keratinocytes increased in concentration depending manner reaching a two – fold increase in comparison to control untreated cells at 10 μg/ml (71.7 ±7% and 35.5 ±2.2%, respectively) (Fig. 3A). This corresponded to our previous observations of apoptosis levels. In monocytes we observed a higher percentage of caspase-9 – positive cells for 1 μg/ml (32.5 ±2.8%) than for 5 and 10 μg/ml (18 ±2.3% and 23.4 ±0.9%, respectively). We also investigated the intensity of bax expression to estimate the involvement of this protein in the observed apoptosis. Bax is one of the proapoptic cytosol proteins, whose action leads to destabilization of mitochondrial membranes and release of cytochrome c, one of apoptosome components. Neither monocytes nor keratinocytes show any significant increase in bax expression, except for keratinocytes at 10 μg/ml AgNPs ($p \leq 0.05$) (Table 1).
Inflammatory reaction

Active caspase-1 as a major inducer of activation and secretion of IL-1β and IL-18 during pyroptosis [19-22]. As we observed massive lysosome dysfunction and cell death induction – pyroptosis features, we tested for activation of caspase-1. Figure 3B shows that in both cell lines subjected to all tested concentrations of AgNPs the percentages of cells with active caspase-1 form were significantly elevated in comparison to untreated control (\(p \leq 0.05\)). However, monocytes were more sensitive than keratinocytes (Fig. 3B). In RAW 264.7, the percentages of positive cells were increased in concentration dependent manner reaching a four-fold increase at 10 μg/ml in comparison to untreated cells (74 ±6% and 16.9% ±2.8% respectively). Similarly, in exposed 291.03C, the highest percentage of cells with the active caspase-1 form was observed for 10 μg/ml (33.6 ±3.7%) (Fig. 3B).

Discussion

Silver nanoparticles are used in applications such as textiles, coatings in catheters, wound dressings and even prostheses [23, 24]. Skin is one of the most exposed tissues to contact with nanoparticles. Immune cells present in skin ensure protection against infections and tolerance to normal skin microflora. Tightly regulated interplay between immune cells and keratinocytes may be disturbed by toxicants and lead to non-healing wounds or atopic dermatitis.

Therefore, the aim of this study was to investigate the immunotoxicity level of commercially available, physically produced, AgNPs on mouse monocyte cell line RAW 264.7 and 291.03C cell lines at 24 h of exposition to AgNPs. A) Neutral red uptake assay. Percentage of cells uptaking neutral red in comparison to control, untreated cells treated as 100% of living cells. B) Mitochondrial potential expressed as percentage of cells with green JC-1 fluorescence; C, D) Staining for apoptotic and necrotic cells. Apoptotic cells were expressed as % 6-CFDA/PI negative cells (C), while necrotic cells are PI – positive cells (D); \(^{*} p \leq 0.05\)
264.7 and cytotoxicity level on mouse keratinocyte cell line 291.03C in vitro. Moreover, we tried to clarify potential toxicity mechanism of AgNPs in two distinct cell lines, phagocytic and non-phagocytic. To estimate the toxicity level we used assays for metabolic activity, mitochondrial transmembrane potential, apoptosis and necrosis as well as for inflammatory potential. Our studies revealed that in both cell lines AgNPs were toxic in concentration-dependent manner. We observed high immunotoxicity potential of AgNPs to relatively low concentrations. Silver nanoparticles were toxic for both cell lines however 291.03C cells were more resistant to nanoparticles treatment (Fig. 2). Necrosis were the dominating cell death mechanism in monocytes while in keratinocytes we observed rather apoptosis than necrosis. Exposure to the highest concentration of AgNPs of 10 μg/ml resulted in mitochondrial transmembrane potential collapse in both cell lines. Park et al. (2011) sowed size-dependent immunotoxicity of AgNPs coated with polyvinylpyrrolidone (PVP) to macrophages. In their work 4 nm AgNPs caused 36% cells viability to 3.12 μg/ml of nanoparticles whereas 70 nm nanoparticles were nontoxic up to 50 μg/ml and apoptosis were indicated as a death mechanism [25]. Zanette and co-workers (2011) reported that AgNPs coated with PVP caused reduction of mitochondrial function but did not lead to necrosis in HaCaT human keratinocyte line [26]. What is more, the work of Hussain et al. (2005) indicated mitochondria as primary targets of nanoparticles [27]. In our studies we used non-modified AgNPs stabilized with citrate, being a mixture of different shapes and sizes. High immunotoxicity observed in RAW 264.7 might be a pleiotropic effect of different sizes of nanoparticles. Our observations of mitochondria potential showed no significant changes in monocyte cell line to lower concentrations whereas in keratinocytes turnover of acidified compartiments in cytoplasm. Low pH within compartments may led to release of silver ions from surface of nanoparticles, which can disturb cellular membrane and in consequence cause necrosis [28].

Inflammatory response is one of the most important process during eradication of local infection. Macrophages are able to produce plethora of inflammatory cytokines that facilitate killing of invading pathogens. Herein we demonstrated activation of caspase-1 in monocyte cell line in

**Table 1.** Mean fluorescence of bax staining in RAW 264.7 and 291.03C cell lines at 24 h of exposition to different concentrations of AgNPs; \(^*p \leq 0.05\)

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>1 μg/ml</th>
<th>5 μg/ml</th>
<th>10 μg/ml</th>
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<tbody>
<tr>
<td><strong>RAW 264.7</strong></td>
<td>15.2 ±2.11</td>
<td>17.74 ±2.38</td>
<td>15.72 ±0.3</td>
<td>14 ±0.12</td>
</tr>
<tr>
<td><strong>291.03C</strong></td>
<td>17.9 ±1.35</td>
<td>19.22 ±2.9</td>
<td>21.17 ±4.66</td>
<td>26.63* ±0.92</td>
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**Fig. 3.** Activation of caspase-9 and caspase-1 in RAW 264.7 and 291.03C cell lines at 24 h of exposition to AgNPs. Percent cells with active form of caspase-9. B) Percent cells with active form of caspase-1; \(^*p \leq 0.05\)
response to tested AgNPs (Fig. 2B). Active caspase-1 is one of the proteins involved in forming of inflammusome complex. Activation of caspase-1 leading to pyroptosis – a conserve program of cell death [29, 30]. The features of pyroptosis include rapid formation of plasma membrane pores and DNA damage, resulting in cell lysis and release of inflammatory intracellular contents. It has been shown exogenous materials such as silica or aluminum adjuvant can activate inflammusome [31, 32]. As we observed cell morphology could be a result of pyroptosis which correlates to activation of caspase-1 in monocytes.

We showed a caspase-9 activations in keratinocyte cell line, which may suggest a mitochondrial dependent pathway of apoptosis (Fig. 2A). Nonetheless the level of bax, major protein involved in cytochrome c release, was significantly increased only for 10 μg/ml suggesting an alternative way of cytochrome release. Salnikov et al. (2007) reported that 3 nm gold nanoparticles can penetrate outer mitochondrial membrane through voltage dependent anion channels [33]. There are multiple possible ways of penetration of mitochondrial membranes and transmembrane potential withdrawal by nanoparticles: permeability transition pores [34, 35], apoptosis related ceramide pores [36] or protein import channels [37, 38]. Mean size value of AgNPs was 3.5 nm, according to work of Salnikov et al., tested AgNPs might penetrate outer mitochondrial membrane through voltage dependent anion channels and endure transmembrane potential, leading to release cytochrome c and caspase-9 activation.

In our study, the type of apoptotic pathway induced by silver nanoparticles depended on the type of the exposed cells. Murine keratinocytes exposed to silver nanoparticles underwent apoptosis characterized by a high activity of caspase-9 – an indicator of mitochondrial apoptotic pathway. In contrast, murine monocyte RAW 264.7 cell line showed more necrosis than apoptosis and high activity of caspase-1 – an enzyme involved in inflammatory reactions. Taken together the inflammatory potential of necrotic cell death and activation of caspase-1 induced by AgNPs in monocytes we can conclude that exposition of monocytes to silver nanoparticles may lead to immunotoxic inflammatory reactions.

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References