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Toxicity of tannic acid-modified silver nanoparticles in keratinocytes: potential for immunomodulatory applications

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ABSTRACT

Hydrolyzable tannins are known to exhibit anti-inflammatory activity, which can be used in combination with silver nanoparticles (AgNPs) for dermal uses. In this study, we investigated the effects of tannic acid-modified 13, 33, 46 nm and unmodified 10–65 nm AgNPs using the human-derived keratinocyte HaCaT and VK2-E6/E7 cell lines in the form of stationary and spheroids cultures. After exposition to tannic acid-modified AgNPs, VK2-E6/E7 cells showed higher toxicity, increased production of reactive oxygen species (ROS) and activity of JNK stress kinase, while HaCaT cell line demonstrated less ROS production and activation of ERK kinase. AgNPs internalization was detected both in the superficial and internal layers of spheroids prepared from both cell lines. Tannic acid modified AgNPs sized above 30 nm did not induce DNA breaks in comet assay performed in both cell lines. Tannic acid-modified but not unmodified AgNPs down-regulated TNF-α and LPS-triggered production of IL-8 in VK2-E6/E7 but not in HaCaT cells. In summary, tannic acid-modified AgNPs sized above 30 nm show good toxicological profile both in vitro and possess immunomodulatory properties useful for potential dermal applications in humans.

1. Background

The skin covers the body surface and consists of three main layers: epidermis, dermis, and the subcutaneous tissue. It forms a barrier preventing water loss, but also provides an important immunological barrier constituting the first line of defense against potentially pathogenic microorganisms. The epidermis is the uppermost skin layer consisting of keratinocytes, which represent approximately 90% of skin cells, Langerhans cells, monocytes/macrophages, γδ T cells, melanocytes and Merkel cells. Exposure to pathogens, wounds or other pathologic conditions promotes activation of keratinocytes, which release inflammatory mediators, such as interleukin (IL)-1, IL-3, IL-6, IL-8, TNF, G-CSF, GM-CSF and M-CSF (Freedberg et al., 2001; Rai et al., 2009; Webb et al., 2004). The cytokines produced by keratinocytes attract and activate several types of immune cells. Toll like receptors (TLRs) are a class of pathogen recognition receptors (PRRs) responsible for recognition of various components of microorganisms. Human keratinocytes express TLR 1–6 and 9 (De Wild et al., 2003). Stimulation of keratinocytes through TLRs results in increased production of TNF-α, IL-8, CCL2 and up-regulation of ICAM-1, HLA-DR, HLA-ABC (Miller, 2008). Therefore, an intense crosstalk between keratinocytes and immune cells can play a crucial role in the maintenance of physiological skin homeostasis.

Although elemental silver is considered non-toxic, non-allergic, and not cumulative, the interaction of AgNPs preparations with the skin is currently not fully understood. In comparison to other routes of exposure (inhalation and ingestion), dermal exposure to AgNPs has been shown to be safer. Penetration of silver nanoparticles into the skin is low but detectable, and it increases within the damaged skin (Larese et al., 2009). Upon application onto undamaged skin, approx. 70–80% of AgNPs remain in the upper layers of the stratum corneum (Vogt et al., 2014), but AgNPs are able to penetrate intact human skin in vivo beyond the stratum corneum and can be found as deep as the reticular dermis (George et al., 2014). However, when used in wound dressings upon the damaged skin, AgNPs can exhibit systemic effects (Trop et al., 2006). Dermal toxicity of AgNPs has been linked with changed cell morphology, increased oxidative stress, decreased proliferation,

Abbreviations: AgNPs, silver nanoparticles; DLS, dynamic light scatter; IL, interleukin; NPs, nanoparticles; TNF, tumour necrosis factor; ROS, reactive oxygen species; TEM, transmission electron microscopy.

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increased DNA damage or genotoxicity (AshaRani et al., 2009; Carlson et al., 2008; Orlowski et al., 2013; Samberg et al., 2010). Therefore, we can assume that dermal exposition to AgNPs can elicit not only the local toxicity but also local immune response.

There are numerous reports indicating that AgNPs promote wound healing and show antimicrobial properties against a broad range of bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (Kim et al., 2007; Morones et al., 2005; Panacek et al., 2006).

Tannins are water-soluble phenol derivatives naturally synthesized and accumulated by higher plants as secondary metabolic products. From the chemical point of view, tannins are polyphenols with molecular weights between 500 and 3000 Da (Erdelyi et al., 2005). Hydrolyzable tannins have been demonstrated to exhibit diverse biological effects ranging from anti-inflammatory, antioxidant to anti-microbial effects (Akiyama et al., 2001; Bakondi et al., 2004; Scalbert et al., 2005). In recent years, many studies have been focused on the use of tannins in skin applications such as wound and burn healing (Agyare et al., 2011; Buzzini et al., 2008; Natarajan et al., 2013). Tannic acid (penta-m-digalloyl glucose) is the simplest and principal hydrolysable tannin shown to exert anti-oxidative, anti-mutagenic and antimicrobial properties (Buzzini et al., 2008; Coppo and Marchese, 2014).

In our previous paper we demonstrated that toxicity of tannic acid-modified AgNPs was cell-type dependent, with monocytes but not keratinocytes, producing reactive oxygen species (ROS) upon exposition to AgNPs (Orlowski et al., 2013). Tannic acid-modified AgNPs significantly increased production of TNF-α, but not IL-1β in keratinocytes (Orlowski et al., 2013). Taking into account the ability of AgNPs to penetrate skin and interact with the skin immune system, but also the immunomodulatory effects of tannic acid, we can assume that combining AgNPs with tannic acid allows to prepare a new type of nanomaterials with the immunomodulatory activity of both tannic acid and metallic silver. Ideally, such combination should allow to restrict excessive inflammation caused by the skin inflammatory disorders, but also by microorganisms invading both acute and chronic wounds.

Since two modes of nanoparticle application are expected - skin and mucosa, we decided here to use two relevant cells lines – HaCaT and VK2-E6/E7 to investigate the toxicity and immunomodulatory effects of tannic-acid-modified AgNPs synthesized by our own method of chemical reduction. HaCaT keratinocytes cell line constitutes a recognized model for assessing in vitro the toxicological potential of chemicals that can cause skin damage (Gibbs, 2009), while VK2-E6/E7 cell line can be used as a model of mucosal exposure to chemicals (Zalenskaya et al., 2011). We demonstrated that higher sizes of tannic acid-modified AgNPs show good toxicological profile in vitro and can exhibit anti-inflammatory potential when applied in the inflammatory conditions.

2. Materials and methods

2.1. Silver nanoparticles

Tannic acid modified and unmodified silver nanoparticles of 13, 33, 46 and 10–65 nm were synthesized as described previously (Orlowski et al., 2013). Nanoparticles were characterized for size distribution after each synthesis by Dynamic Light Scattering (DLS) using Nano ZS zetasizer system (Malvern Instruments, Worcestershire, United Kingdom) and by transmission electron microscopy (TEM), as described previously (Orlowski et al., 2013).

2.2. Cell culture and treatment

Human VK2-E6/E7 vaginal epithelial cells were obtained from American Type Culture Collection (ATCC CRL-2616, Rockville, MD, USA) and propagated in Keratinocyte-Serum Free Medium (SFM) with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, 0.4 mM calcium chloride with 10 U/ml penicillin and 100 μg/ml streptomycin (GIBCO by Thermo Fisher Scientific, Carlsbad, CA, USA).

Human HaCaT keratinocytes (Boukamp et al., 1988) were a gift from Department of Clinical Virology, University of Gothenburg, Göteborg, Sweden and propagated in Dulbecco's modified EMEM (DMEM) supplemented with 10% fetal calf serum, 10 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). The cells of both lines were seeded into 24 well plates at the density of 5×10^4/ml cells and cultured for 48 h before exposure to nanoparticles at the concentrations range of 0.5–10 μg/ml for 24 h. All plasticware used in experiments were from Falcon (Falcon®, Corning, NY, USA), if not stated otherwise.

2.3. Toxicity tests

Apoptosis in single cell suspensions was detected using Annexin V-Apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The annexin V-positive, propidium iodide negative cells, were scored as apoptotic cells, while all propidium iodide positive cells were considered to be necrotic. The stained cells were analyzed in FACS Calibur using CellQuest software (Beckton Dickinson, Franklin Lakes, NJ, USA). Changes in the mitochondrial potential were measured by staining with a cationic dye, 5.5′,6,6′-tetrachloro1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) (Sigma-Aldrich) (Salvioli et al., 1997), as described previously (Orlowski et al., 2013).

2.4. ROS production

The ROS generation was determined by a fluorometric assay with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich), as described previously (Orlowski et al., 2013). The dye-loaded cells were harvested and the fluorescence was measured at 520 nm emission wavelengths in FACS Calibur flow cytometer.

2.5. Cell morphology assessment by TEM

After 24 h of incubation with AgNPs, the cells were fixed with prewarmed 2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1 M NaCl, pH 7.5 for 20 min, washed with cacodylate buffer and post-fixed with 1% aqueous osmium tetroxide in the same buffer for 30 min. Next, the samples were treated and the images were acquired as described previously (Orlowski et al., 2013).

2.6. Generation of spheroids and AgNPs treatment

12-well culture plates were coated with 1 ml of 1% sterile agarose to obtain a non-adhesive substrate. The cells were detached and the single cell suspension (10^5/well) was prepared in an appropriate complete culture medium. Every second day, a half of the culture medium was replaced. After 10 days, the grown spheroids were treated for 48 h with 5 μg/ml AgNPs. For transmission electron microscopy, after treatment spheroids were collected and fixed with 2.5% glutaraldehyde with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH = 7.2) for 15 min, then washed with cacodylate buffer and mounted in 4% agarose. Small pieces of agarose-mounted spheroids were post fixed with 1% aqueous osmium tetroxide for 45 min and processed as described above. For histology, spheroids were frozen for cryo-sectioning and cut to 5 μm slides. Sections were stained with hematoxylin, dehydrated and mounted in DPX. Image capture, analysis and processing were performed using the Zeiss Axio Scope.A1 microscope and ZEN software (Zeiss).

2.7. Antibodies and immunostaining

After 24 h of incubation with AgNPs, intra-cellular Ki 67 antigen was detected using Cytofix/Cytoperm fixation/permabilisation kit (BD