Molecular mechanism of silver nanoparticles in human intestinal cells

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Abstract

Silver nanoparticles are used in consumer products like food contact materials, drinking water technologies and supplements, due to their antimicrobial properties. This leads to an oral uptake and exposure of intestinal cells. In contrast to other studies we found no apoptosis induction by surfactant-coated silver nanoparticles in the intestinal cell model Caco-2 in a previous study, although the particles induced oxidative stress, morphological changes and cell death. Therefore, this study aimed to analyze the molecular mechanism of silver nanoparticles in Caco-2 cells. We used global gene expression profiling in differentiated Caco-2 cells, supported by verification of the microarray data by quantitative real-time RT-PCR and microscopic analysis, impedance measurements and assays for apoptosis and oxidative stress. Our results revealed that surfactant-coated silver nanoparticles probably affect the cells by outside-in signaling. They induce oxidative stress and have an influence on canonical pathways related to FAK, ILK, ERK, MAPK, integrins and adherence and tight junctions, thereby inducing transcription factors like AP1, NFκB and NRF2, which mediate cellular reactions in response to oxidative stress and metal ions and induce changes in the cytoskeleton and cell-cell and cell-matrix contacts. The present data confirm the absence of apoptotic cell death. Non-apoptotic, necrotic cell death, especially in the intestine, can cause inflammation and influence the mucosal immune response.

Introduction

Silver nanoparticles are the most commonly used metal nanoparticles across diverse applications, due to their antimicrobial properties (BAM, 2013; nanotechproject.org, 2014). Silver is an ubiquitously occurring element with no known physiological function in humans (Behrens et al., 2002). The estimated dietary intake of silver is 70 to 90 μg per day (Wijnhoven et al., 2009). Silver nanoparticles in food contact materials, drinking water technologies and nutritional supplements can increase the oral uptake of silver. Silver nanoparticles overcome the gastrointestinal digestion process (Böhmer et al., 2013; Walczak et al., 2013). Hence, intestinal cells are the first physiological barrier for ingested silver nanoparticles. Enterocytes constitute the major part of intestinal epithelial cells. The most commonly used in vitro model for intestinal cells is the human Caco-2 cell line (Gitrowski et al., 2014; He et al., 2013; Hubatsch et al., 2007; Lampen et al., 2004; Yu et al., 2013).

Oxidative stress can be defined as a shift of the ratio between oxidized and reduced cell components toward the oxidized species and influences cellular signaling pathways (Harris & Hansen, 2012). The generation of reactive oxygen species plays an important role in silver nanoparticle-induced cell death. In a previous study with Caco-2 cells, we measured oxidative stress, but no induction of apoptosis (Böhmer et al., 2012). By contrast, other studies showed an induction of apoptosis by silver nanoparticles in different human cell lines from other tissues (Arora et al., 2009; Eom & Choi, 2010; Miura & Shinohara, 2009; Piao et al., 2011; Ste˛pkowski et al., 2014); for review see Kim & Ryu (2013). Necrosis is characterized by a breakdown of the plasma membrane and the induction of inflammatory processes around the dying cells. The release of proinflammatory molecules may injure the intestinal epithelium and contribute to the development of inflammatory bowel diseases like Morbus Crohn or Ulcerative Colitis. The question whether ingested silver nanoparticles induce apoptotic or necrotic signaling pathways in intestinal cells may be important for the physiological consequences in people with a genetic susceptibility for these diseases. In the present study we therefore aimed to investigate which molecular and cellular mechanisms are involved in the toxic effects of ingested silver nanoparticles on intestinal cells. We used human Caco-2 cells and employed a full genome transcriptomic approach on cells treated with surfactant-coated silver nanoparticles. For the verification of the results apoptosis, oxidative stress, glutathione content, mitochondrial depolarization and impedance of the cells were assessed.

Materials and methods

Nanoparticles

AgPURE nanoparticles were purchased from Rent a Scientist GmbH (Regensburg, Germany) and contain 10% (w/w) silver stabilized with 4% (w/w) polyoxyethylene glycerol trioleate (trade name Tagat TO) and 4% (w/w) polyoxyethylene (20) sorbitan monolaureate (Tween 20). Comparable material was characterized for the use as a reference material by the Federal Institute for Materials Research and Testing (BAM) in Germany, called
Silver nanoparticle exposure in human intestinal Caco-2 cells

BAM001, which are comparable to the reference material NM-300 available from the Joint Research Centre (JRC) of the European Commission (BAM, 2013; Klein et al., 2011). These particles are about 15 nm in diameter, as determined by small-angle X-ray scattering (SAXS) and about 28 nm in hydrodynamic diameter (intensity weighted), as determined by dynamic light scattering (DLS) (Böhmer et al., 2013). They were diluted in cell culture medium as used for Caco-2 cultivation. The particles did not form a stable suspension in cell culture medium without fetal calf serum (FCS), but were stabilized in cell culture medium containing 10% FCS. In medium, the particles had a diameter of about 15.6 nm (SAXS) and a hydrodynamic diameter of about 50.8 nm (DLS, intensity weighted). Silver ion release was about 5% for 5 μg/mL nanoparticles, determined after 24 h incubation in cell culture medium by ultracentrifugation and atom absorption spectroscopy. For comparison, silver nitrate was used as a source of silver ions.

Cell culture

The human colon adenocarcinoma cell line Caco-2 (European Collection of Cell Cultures (ECACC), Porton Down, UK) was maintained in Dulbecco’s modified Eagle’s medium (DMEM, PAN Biotech, Aidenbach, Germany) supplemented with 1% (v/v) penicillin/streptomycin and 10% (v/v) heat-inactivated FCS (PAA, Cölbe, Germany) for growth or 1% (v/v) ITS (insulin, transferrin, selenium) (PAA) at 37°C in a humidified atmosphere of 5% CO₂. For experiments with proliferating Caco-2 cells, cells were seeded in the desired multi-well plate and allowed to attach for 24 h. For differentiated Caco-2 cells, cells were cultivated for 21 d in the desired multi-well plate and cell culture medium was changed every 2 d.

Viability assays and phase contrast microscopy

Cell viability was assessed using the Promega Cell Titer Blue (CTB) Assay (Alamar blue) (Promega, Mannheim, Germany) and DAPI staining (Sigma Aldrich, Taufkirchen, Germany). Caco-2 cells were plated into 96 well plates at a density of 10 × 10³ cells per well. For treatment, the culture medium was replaced by 100 μL nanoparticle suspension for proliferating or 300 μL nanoparticle suspension for differentiated Caco-2. Cells were exposed to silver nanoparticles for 4, 24, and 48 h (with medium control and corresponding dilutions of surfactant control without silver nanoparticles). Finally, CTB was added, incubated 2 h for proliferating Caco-2 cells or 30 min for differentiated Caco-2 cells, and measured on a micro-plate reader. After the CTB assay, cells were fixed and lysed with methanol. The DNA was stained with 100 μL of 10 μM DAPI per well for at least 30 min. The resulting fluorescence was measured using a micro-plate reader. The medium control was set to 100%. Means and standard deviations were calculated on the basis of at least three independent experiments. Statistical analysis was done with Student’s t-test.

Microscopic images were taken in the 96 well plates with a Zeiss Axio Observer microscope and Zeiss AxioVision.

Microarray and PCR analysis

Differentiated Caco-2 cells were cultivated in 75 cm² cell culture flasks and incubated with 2.5 or 25 μg/mL silver nanoparticles, or with 0.5 μg/mL silver ions (AgNO₃) in 20 mL serum-free cell culture medium for 24 h. Afterwards, the cells were washed twice with PBS and harvested mechanically. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was measured by micro-gel electrophoresis with Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNAs with calculated RIN values above 7 were used.

mRNA samples were analyzed using GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). The analysis was done in triplicates. The data were analyzed using Ingenuity Pathway Analysis software (IPA, Ingenuity® Systems, www.ingenuity.com). The cutoff for the fold change was set to −1.4 ≥ fc ≥ 1.4 and the p value to <0.05. The microarray data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE62253.

PCR array and quantitative real-time RT-PCR

Eighty-nine genes were chosen and a customized PCR array was ordered from SABiosciences (Qiagen). To verify the microarray results, the same RNA samples (assayed in triplicates) as well as RNA samples from an additional biological replicate (assayed in duplicate) were used. A third biological replicate was used for comparison of silver nanoparticles in serum-free and serum-containing cell culture medium. PCR cycler 7900HT Fast real-time PCR system (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany) was used. GAPDH was chosen as housekeeping gene. Results were calculated with the ΔΔCt method. Student’s t-test was used to test for significance. Genes with a fold change −1.4 ≥ x ≥ 1.4 and p value ≤0.05 were considered as regulated.

Transmission electron microscopy

Caco-2 cells were seeded in 12 well Transwell plates (polycarbonate membrane, 3 μm pore size) (Corning, Amsterdam, Netherlands) at a density of 50 000 cells per well. Differentiated cells were incubated for 24 h with 20 μg/mL silver nanoparticles on the apical side. Afterwards, cells were fixed with Karnovsky solution (2% formaldehyde, 2.5% glutaraldehyde in PBS) (Sigma Aldrich), dehydrated, embedded in polymer resin and sliced. The samples were treated with osmium tetroxide (Sigma Aldrich). A Zeiss 10 CR electron microscope and FEI Tecnai G² 20S-TWIN with EDX analysis were used to analyze the samples.

Impedance measurement

For the xCELLigence measurements, Caco-2 cells were transferred to E-plates at a density of 6125 cells per well (this is the same cell density as on the 96 well plates). Subsequently, the culture medium was replaced by 200 μL medium control, nanoparticle dilutions and corresponding surfactant control. Cells were exposed for 48 h and impedance was measured at least every minute. The incubation start and medium control were set to CI = 0.

Apoptosis measurement

Apoptosis was measured using Annexin-V/7AAD staining for proliferating Caco-2 cells and a caspase activity assay for differentiated Caco-2 cells.

Annexin-V/7AAD staining

Apoptosis was quantified by Annexin-V/7AAD (Immuno Tools GmbH, BD Biosciences, Heidelberg, Germany) staining and subsequent FACS analysis. Cells were plated into 12 well plates at a density of 1 × 10⁵ cells per well and allowed to attach for 24 h. Culture medium was replaced by 1 mL particle dilutions, medium control and positive control 10 μM staurosporine (Sigma-Aldrich), and cells were exposed for up to 48 h. Treated cells were harvested, washed with annexin buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂) (Sigma-Aldrich) and stained for
20 min with Annexin-V/7AAD before FACS analysis was performed using BD FACS Canto II.

Caspase assay

Differentiated Caco-2 were incubated with medium control, positive control 2.5 μM staurosporine and silver nanoparticle dilutions in 300 μL for 24 and 48 h. For caspase activity detection, Caspase-Glo 3/7 Assays (Promega) and a microplate reader were used.

Mitochondrial depolarization

Differentiated Caco-2 cells were incubated in 96 well plates with medium control, 2.5 μM staurosporine as a positive control, and different dilutions of silver nitrate or silver nanoparticles (300 μL per well) for 24 h. For the detection of mitochondrial depolarization, Cell Meter JC-10 assays (AAT Bioquest, Sunnyvale, CA) and a microplate reader were used.

Oxidative stress

Oxidative stress was measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate, a glutathione assay, and preincubation with the antioxidant N-acetylcysteine.

Dichlorofluorescein assay

Differentiated Caco-2 cells were incubated in 96 well plates with 100 μL 100 μM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma Aldrich,) in cell culture medium with 1% ITS and incubated for 1 h. The cells were washed with 200 μL PBS and exposed to a medium control, positive control iron (II) sulfate (100 μg/mL Fe²⁺) (Sigma Aldrich) or a particle suspension (300 μL per well) for 24 h. The plates were measured using a microplate reader.

Glutathione assay

Differentiated Caco-2 cells were incubated in 96 well plates with 300 μL per well medium control, the positive control 100 μM buthionine sulfoximine (Sigma Aldrich), and different silver nitrate and nanoparticle dilutions. Measurements were done with the GSH/GSSG-Glo™ assay (Promega) and a microplate reader.

Antioxidant preincubation

Proliferating Caco-2 cells were seeded, cultured and measured as described in the viability and toxicity assay section. Prior to the incubation, the cells were incubated with 10 mM N-acetylcysteine or 100 μM vitamin C (Sigma Aldrich) for 1 h and washed with 100 μL PBS.

Results

Cytotoxicity testing

Surfactant-coated silver nanoparticles as well as silver ions showed concentration-dependent effects on cell viability (Figure 1, and Figures S1 and S2 for differentiated and proliferating Caco-2 cells, respectively). Given on a mass dose base, silver ions were more toxic. The corresponding surfactant controls had no effect. The comparison of serum-containing and serum-free cell culture medium revealed slight differences for proliferating Caco-2 cells and no differences for differentiated Caco-2 cells. The comparison of proliferating and differentiated Caco-2 cells showed higher cytotoxic effects for proliferating cells on a mass concentration base, possibly due to differences in the cell number or stress response. The nanoparticle dilutions for all other assays were chosen based on these results.

Uptake of silver nanoparticles

Transmission electron microscopy was used to visualize the cellular localization of the silver nanoparticles. A number of nanoparticles were found inside the cells (Figure S3).

Gene expression profiling

Gene expression profiling of differentiated Caco-2 cells in response to 24 h exposure to surfactant-coated silver nanoparticles and silver ions was performed by microarrays. The cells were treated with 2.5 and 25 μg/mL silver nanoparticles, or with 0.5 μg/mL silver ions. On the basis of the cytotoxicity tests, these concentrations are non-cytotoxic concentrations for silver nanoparticles as well as silver ions and one additional slightly cytotoxic concentration (approximately EC20) for silver nanoparticles. Genes that were significantly and at least 1.4-fold regulated compared to the untreated medium control are summarized in Table 1 and Figure S4. The number of affected genes was considerably higher in cells treated with a high silver nanoparticle concentration, as compared to the other treatment groups. Gene expression patterns of cells treated with the low silver nanoparticle concentration or with silver ions were similar to untreated cells, whereas the samples treated with the high nanoparticle concentration show clear differences. Additionally, Figure S4 shows the overlap between the different expression profiles: about half of the genes regulated by the low nanoparticle concentration and about 40% of the genes regulated by silver ion treatment were also regulated by the high nanoparticle concentration, whereas only 8 genes were regulated by all three treatments.

The Ingenuity pathway analysis (IPA) software was used for biological interpretation of the microarray results. This analysis revealed an oxidative or electrophilic stress response, as reflected by affected pathways like “NRF2-mediated Oxidative Stress Response”, the highest upregulated genes (different metallothioneins, heat shock proteins and heme oxygenase 1) and the predicted activation of transcription factors like Nrf2 (nuclear factor (erythroid-derived 2)-like 2) and NF-κB (nuclear factor “κappa-light-chain-enhancer” of activated B-cells). IPA further indicated an influence of silver nanoparticles on the cytoskeleton as well as on cell-cell and cell-matrix contacts (predicted influence on “Integrin Signaling”, “Epithelial Adherens Junction Signaling”, “Tight Junction Signaling” and “Actin Cytoskeleton Signaling”). Furthermore, kinase-based signaling and upstream regulators of cell morphology were predicted to be activated, like “FAK Signaling” (focal adhesion kinase, PTK2), “ERK/MAPK Signaling” (extracellular signal-regulated kinases/mitogen-activated protein kinases), “ILK Signaling” (integrin-linked kinase), p38 MAPK (p38 mitogen-activated protein kinases) and Akt (protein kinase B, PKB). Another aspect was the effect on cell death and proliferation. Surprisingly, IPA indicated a decrease in apoptosis.

Based on these predictions, 89 genes were chosen to verify the microarray results by using a PCR array. The selected genes belong to five categories: “stress and inflammation”, “cellular morphology and cellular movement”, “cell death and apoptosis”, “cell cycle and proliferation”, and “cell-cell communication and cellular signaling”. These categories are depicted as networks in the supporting information (Table S2 and Figures S11–S15). Beside the analysis of the same RNA samples that were used for the microarray, a biological replicate was performed using two more dilutions (5 and 10 μg/mL silver nanoparticles) to ensure the observed effects are not driven by cytotoxicity. Table S2 summarizes the results of all transcriptional experiments. The results reveal 80% identity in the regulation of the selected genes between the microarray and the PCR arrays.
Figure 1. Results of the CTB assays to assess cell viabilities and of DAPI staining to assess the cell number of differentiated Caco-2 cells after incubation with surfactant control, surfactant-coated silver nanoparticles, and silver ions for 24h. Ten thousand Caco-2 cells per well were seeded into 96 well plates and differentiated for 21 d. They were incubated in 300μL. Medium control was set to 100%. The experiment was reproduced three times. Asterisks (*) mark significant differences, as compared to the medium control (Student’s t test p ≤ 0.05).

Table 1. Number of genes that were at least 1.4-fold regulated compared to the medium control.

|                         | Silver nanoparticles/control | Silver ions/control |
|-------------------------|-----------------------------|---------------------|
|                         | 2.5 μg/mL       | 25 μg/mL       | 0.5 μg/mL       |
| Upregulated             | 34 ↑           | 2134 ↑         | 15 ↑           |
| Downregulated           | 19 ↓           | 2918 ↓         | 100 ↓          |
| All                     | 53             | 5052           | 115            |

Analysis of cell death mechanisms

The predicted downregulation of apoptosis is at variance with previous reports on nanoparticle-induced cell death (“Introduction” section). Therefore, the mechanisms of silver nanoparticle-induced cellular damage and death were subjected to further investigation.

Exposure of Caco-2 cells to silver nanoparticles led to decreased adherence of the cells, as observed in optical microscope images (Figure 2). Cells detached and adopted a spherical form. Cells at the outer edge of cell clusters were more susceptible, as compared to those inside the cell cluster. This effect was concentration-dependent and well visible for proliferating Caco-2 cells, whereas it was less evident for differentiated Caco-2 cells, due to the structure of the cellular monolayer.

To quantify the time course of morphological changes, a real-time impedance measurement was performed (Figure S5 and S6). The surfactant control had no influence on the cells, whereas silver ions and silver nanoparticles exhibited a concentration- and time-dependent effect on proliferating and differentiated Caco-2 cells. The fast decrease in the measured cell index (CI) showed a cellular reaction to silver nanoparticles and silver ions for proliferating as well as for differentiated Caco-2 cells.

Apoptosis measurements were done using annexin-V/7AAD staining for FACS analysis for proliferating Caco-2 cells and caspase activity detection for differentiated Caco-2 cells. Proliferating Caco-2 cells showed no apoptosis induction by silver nanoparticles, even though the positive control revealed a properly working assay (Figure S7). The same holds true for the differentiated Caco-2 cells in the caspase activity assay (Figure 3). Especially in the FACS analysis, a high proportion of necrotic cells were visible.

The results of the measurements of the mitochondrial depolarization (Figure S8) demonstrate that the positive control showed a clear induction of mitochondrial depolarization, while no effects were detectable for silver nanoparticles and silver ions.
Three different methods were used to measure the oxidative stress: the detection of reactive oxygen species using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (dichlorofluorescein assay), analysis of cellular glutathione content, and a rescue approach by preincubation with antioxidants. The results of the dichlorofluorescein assay are pictured in Figure S9. The silver ions and silver nanoparticles in serum-free cell culture medium produced an increase in reactive

Figure 2. Microscopic images (200-fold magnification) of proliferating (A) and differentiated (B) Caco-2 cells after 24 h of exposure to surfactant-coated silver nanoparticles, silver ions and corresponding controls.
oxygen species levels, whereas the silver nanoparticles in serum-containing cell culture medium showed no influence up to nanoparticle concentrations where the cytotoxic effect takes over. Glutathione determination (Figure 4) revealed a clear shift from reduced to oxidized glutathione as result of the silver nanoparticle exposure. Additionally, the influence of preincubation with vitamin C and N-acetylcysteine on cell viability is shown in Figure S10. Both antioxidant substances reduced the cytotoxic effect of the silver nanoparticles.

Discussion

We investigated the molecular and cellular mechanisms involved in the toxic effects of silver nanoparticles on intestinal cells. Therefore, we incubated differentiated Caco-2 cells with surfactant-coated silver nanoparticles and performed a full genome transcriptomic approach. Regulated pathways, identified by microarray technology combined with Ingenuity Pathway Analysis software (IPA), were verified by PCR analysis and different cellular assays aimed to elucidate cell death and oxidative stress. Our results are visualized in Figure 5.

The upregulation of hemeoxygenase 1 (HMOX1), heat shock proteins, and metallothioneins documents an increase of oxidative stress. This finding was substantiated by the DCF and glutathione assays and is associated with the non-apoptotic death of Caco-2 cells. Indeed, oxidative stress is the most frequently identified effect of silver nanoparticles on cells. Oxidative stress usually causes apoptosis in cells from most human tissues (Ahamed et al., 2008; Gopinath et al., 2010; Lee et al., 2011). Nevertheless, some reports with conflicting results describing toxic effects of silver nanoparticles on intestinal cells can be identified: different groups measured apoptosis in HT29, LoVo, and HCT116 cells, whereas apoptosis could not be detected in HCT116 cells after incubation with silver nanoparticles (Gopinath et al., 2010; Hsin et al., 2008; Miethling-Graff et al.; Satapathy et al., 2013). We confirmed the downregulation of apoptosis with two apoptosis assays. As demonstrated previously by our group, peptide-coated silver nanoparticles increase oxidative stress in Caco-2 cells without inducing apoptosis (Böhmert et al., 2012). Similarly, Bouwmeester et al. measured an influence of silver nanoparticles on the apoptotic pathway in the Caco-2-based M cell model, but without a clear prediction whether it is up- or downregulated (Bouwmeester et al., 2011).

There is evidence that Caco-2 cells may undergo necrosis. Rounded and detached cells were observed as first sign of toxicity after incubation with silver nanoparticles. Furthermore, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), a key enzyme in regulated necrosis, is slightly upregulated by silver nanoparticles as is a number of ubiquitin-conjugating enzymes, which are able to activate RIPK1 (O’Donnell et al., 2007).

To obtain information about the upstream site of silver nanoparticle-induced toxicity, we analyzed the cellular uptake of silver nanoparticles. TEM data show that nanoparticles outside and inside the cells. In connection with the results by Bouwmeester et al., who found a very low transport of just about 0.5% through the Caco-2-based M-cell model after 4 h incubation with silver nanoparticles (Bouwmeester et al., 2011) it can be speculated that just a small amount of nanoparticles is taken up by the cells. An influence of silver nanoparticles from outside the cells may also be important. Many cell surface and extracellular proteins contain oxidizable cysteine and methionine.
residues and respond to variations in the extracellular redox environment (Moriarty-Craige & Jones, 2004). Due to the high affinity of silver to sulphur, a direct interaction of silver nanoparticles and thereof-released silver ions with extracellular and membrane-associated proteins is likely. Other silver species formed in cell culture medium like AgCl and soluble chlorine complexes may also interact. This, in turn, will influence cellular signaling: outside-in signaling pathways influence cellular adhesion, cytoskeletal organization and cellular spreading (Honore et al., 2000). Especially integrins and G protein-coupled receptors can give signals that cause changes in cell morphology (Shen et al., 2012). This fits well with our observations. The IPA software predicted upstream regulators like growth factors, cytokines, G-protein-coupled receptors, and integrins as involved in pathways regulated by silver nanoparticle exposure. Furthermore, the microarray analysis showed an influence of silver nanoparticles on the extracellular matrix as well as on proteins that degrade the extracellular matrix, like metallopeptidases. An induction of metallopeptidase was also detected by Park et al. (2010) after silver nanoparticle treatment of mouse macrophages. The transduction of signals from outside the cell to the nucleus is often mediated by fast enzymatic protein modifications like phosphorylation (Clark & Brugge, 1995; Whitmarsh & Davis, 1996). These steps were not directly detectable by microarray, but predicted by IPA software on the basis of the transcriptomic data. The ERK/MAPK Signaling (p44/p42 MAPK) induced by EGFR phosphorylation is regulated by the extracellular redox state in Caco-2 cells (Nkabyo et al., 2002). MAPK was also found in the microarray analysis of the lung epithelial cell line A459 after incubation with silver nanoparticles (Foldbjerg et al., 2012). Kang et al. (2012) showed that the induction of hemeoxygenase (HMOX1) in the ovarian carcinoma cell line SK-OV3 after silver nanoparticle incubation requires the p38 MAPK signaling pathway.

The morphological changes in silver nanoparticle-treated Caco-2 cells are reflected by the downregulation of genes encoding for tight and adherence junctions. Similarly, an influence of silver nanoparticles on the gap junctions of A549 cells was observed by Deng et al. (2010) and morphological changes due to silver nanoparticle and silver ion incubation were also observed by others (Kawata et al., 2009; Liu et al., 2010; Nowrouzi et al., 2010). Beside the cell–cell contacts, the cell–matrix contacts and the cytoskeleton were also affected, as evidenced by a regulation of the expression of β-actin and tubulin. Changes in the expression of β-actin are also found in rat lung after i.v. administration of silver nanoparticles (Kim et al., 2010).

Oxidative stress activates transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), activator protein 1 (AP-1), hypoxia-inducible factor 1-alpha (HIF-1α), and nuclear factor erythroid-derived 2-like 2 (NRF2) (Reuter et al., 2010). NRF2 is regulated by changes in the extracellular redox state (Imhoff & Hansen, 2009). Especially, NFkB and NRF2 are predicted to be upregulated by silver nanoparticles in Caco-2 cells. The transcription factor NRF2
regulates basal and inducible expression of phase 2 proteins, for example, heme oxygenase 1 (HMOX1), NAD(P)H quinone oxidoreductase 1 (NQO1), or glutathione S-transferase (GSTA2), that protect cells against electrophiles and oxidants (Kang et al., 2004; Simmons et al., 2011). Beside oxidative stress, Nrf2 is induced by disruption of the actin cytoskeleton (Kang et al., 2004). A Nrf2-specific reporter gene assay showed an induction by silver ions in HepG2 hepatoma cells but not in the lung cell line A549, probably due to a high antioxidative capacity of A549 and a mutation in a Nrf2-regulating protein (Simmons et al., 2011). This could be the reason why Foldbjerg et al. did not find this pathway in their analysis (Foldbjerg et al., 2012). The heterodimeric transcription factor AP1 can be activated upon oxidative stress, metal exposure, or as a response to cytokines and growth factors (Valko et al., 2006). Members of the AP1 group are regulated by nanoparticles in the Caco-2 based M-cell model employed by Bouwmeester et al., as well as in your transcriptomic analysis (Bouwmeester et al., 2011).

The pronounced upregulation of metallothioneins is equally important: metallothioneins are small, cysteine-rich, metal-binding proteins which protect cells under numerous stressed states (Lindeke et al., 2010). Recently, Devischer et al. (2014) identified metallothioneins as a danger signal in inflammatory bowel disease, where they attract macrophages and other cells of the immune system and trigger inflammation. Interestingly, based on our transcriptomic data IPA predicted a number of antiviral processes as upregulated. It remains to be elucidated, whether intestinal cells may see the nanoparticle as a kind of virus particle and react with an inflammatory response.

Conclusion

We obtained the following overall picture for oral ingestion of silver nanoparticles: With the exception of a small proportion, the majority of silver nanoparticles are not taken up into differentiated Caco-2 cells. Our data support the hypothesis that silver nanoparticles and silver ions released from the particles interact with membrane-bound and/or extracellular proteins. Silver nanoparticles induce morphological changes and oxidative stress. In contrast to other cell models, we found no induction of apoptosis. Instead, the cells undergo necrosis. Necrotic cell death, especially in the intestine, can trigger inflammation and enhance pathological processes in people predisposed for inflammatory bowel disease.

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Declaration of interest

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Supplementary material available online
Supplementary Figures S1–S15 and Tables S1–S2