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Uptake of silver from metallic silver surfaces induces cell death and a pro-inflammatory response in cultured J774 macrophages

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Summary. In clinical medicine metallic silver is used as anti-bacterial coating on various catheters, bandages and prostheses. By means of dissolucytosis, i.e. extracellular macrophage-mediated bio-liberation of metal ions, silver ions are continuously liberated from silver surfaces starting within minutes of exposure. The present study investigates how bio-liberation and subsequent cellular uptake of silver ions affects cell viability and cell signalling within the first 3-24 hours of exposure when J774 macrophages are grown directly on a silver surface. Autometallography (AMG) was applied to demonstrate cytoplasmatic silver uptake and localisation after 1, 3, 12 and 24 hours of exposure to metallic silver. From 12 hours onwards the cells were completely filled with silver enhanced silver-sulphur nanocrystals (AMG-silver grains). At the ultrastructural level, the silver accumulations were located to lysosome-like structures. An immunoassay cell death kit found silver-induced apoptosis after 12 and 24 hours of exposure. Necrosis was seen at the same times. Judged by mRNA analysis silver exposure statistically significantly induces TNF-α and m-CSF gene expression, especially at 3 hours. Furthermore, anti-inflammatory IL-10 transcription is reduced by silver uptake and 24 hours of silver exposure induces massive iNOS-2 gene expression. At the same time silver exposure increases the gene expression of metallothionein (MT-I/MT-II), a cystein-rich protein known for its role in detoxifying heavy metals. Our data suggest that silver ions liberated from metallic silver surfaces accumulate in lysosomes, reduce macrophage viability by apoptosis and necrosis and induce a pro-inflammatory response.

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Introduction

In clinical medicine metallic silver is often used as surface coating on medical devices induced into the body, e.g. catheters used in neurosurgery (Thibon et al., 2000; Bayston et al., 2009; Zegelman et al., 2009) and orthopaedic prostheses (Sudmann et al., 1994). Silver has long been known to have anti-bacterial effects without generating bacterial resistance, making silver attractive as a coating material (Fox et al., 1969; Modak and Fox, 1973; Hartmann et al., 1999; Brett, 2006). Silver particles are thus also used as a content of dressings and foils intended for the treatment of chronic ulcers and burn wounds (Gamelli et al., 1993; Lansdown, 2006; Leaper, 2006). This widespread use of silver coated objects implies that the tissue will come into close contact with metallic silver. Such contact will inevitably result in dissolucytosis, i.e. a macrophage mediated release of silver ions (Larsen et al., 2007, 2008; Locht et al., 2009; Danscher and Locht, 2010). We have previously shown that cultured J774 macrophages take up silver ions from metallic silver after just a few minutes of exposure (Locht et al., 2009). The liberated silver ions bind chemically to sulphide molecules or SH-radicals and accumulate in silver-sulphur nanocrystals that can be traced by autometallography (AMG) (Danscher, 1981; Rungby et al., 1987a,b; Danscher and Stoltenberg, 2006). Such silver-sulphur nanocrystals have been considered harmless (Petering, 1976; Lansdown, 2007; Kristiansen et al., 2008) causing nothing but argyria, i.e. discoloration of the skin following long-term silver exposure (Aaseth et al., 1981;

Wadhera and Fung, 2005). Previous studies have shown that silver compounds like silver lactate have neurotoxic potentials (Rungby and Danscher, 1983a,b) and cause acute coagulation necrosis of cultured mouse peritoneal macrophages (Ellermann-Eriksen et al., 1987; Rungby et al. 1987a).

The aim of the present study is to investigate how silver ions bio-released from metallic silver affect macrophages with regard to 1) cell viability, and 2) the induction of a pro-inflammatory cytokine response.

Materials and methods

Cell cultures

J774 macrophages were grown under standard conditions as previously described (Brunk et al., 1997; Li et al., 2000; Zhao et al., 2000). In brief, the cells were grown in 75 ml Nunc plastic flasks (Nunc A/S, Roskilde, Denmark) in a medium consisting of RPMI 1640 + GlutaMAX™+ 25 mM HEPES (GIBCO®, supplied by Invitrogen, Taastrup, Denmark) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 1% Penicillin-streptomycin (10 U/ml Penicillin G sodium + 10 μg/ml streptomycinsulfate in 0.85% saline) and 1% non-essential amino acids. The cells were incubated at 37.1°C and 5% CO_2 in a Heraeus, Heracell 150 incubator, (Axeb Danmark A/S) and sub-cultivated at confluence every second to third day. When sub-cultivating, the old medium was replaced by 10 ml of fresh medium and the cells were mechanically loosened using a plastic scraper (Nunc A/S, Roskilde, Denmark). Afterwards, 5 ml of this cell suspension was transferred to each of two new cell culture flasks, adding an additional 8 ml of fresh medium to each flask.

Silver exposure

Freshly harvested J774 macrophages were grown directly on metallic silver plates (1.77 cm^2, 99.95% pure silver, Good-Fellow, Cambridge, UK), placed in a 24-well tray (Nunc A/S, Roskilde, Denmark) and covering the entire bottom of the wells. The cells were mechanically loosened from the bottom of the flask by scraping, and 0.5 ml of the cell suspension was added to every well, with a silver plate placed at the bottom, together with 0.5 ml of fresh medium (500,000 cells/ml). The macrophages were grown on the metallic silver plates for 1, 3, 12 and 24 hours, respectively, using five randomly selected wells at each time point. Control cells were grown on thin glass cover slips in similar 24-well trays. All experiments were performed in quadruplicate and repeated three times.

Cell preparation for morphological examination and autometallographic (AMG) silver visualization

After incubation on the metallic silver plate or on glass cover slips (control cultures), 0.5 ml medium was removed and the cells were loosened mechanically and placed on a glass cover slip (1.77 cm^2). 0.5 ml fresh medium was added and the cells were incubated for 3 hours to allow them to adhere to the glass plate before being studied. After morphological evaluation in a phase-contrast microscope the cells were fixated by replacing the medium with 3% glutaraldehyde for 20 minutes. The glutaraldehyde was removed and the fixed cells on the cover slips were rinsed several times in distilled water before AMG development.

The AMG developer

The developer consists of 1) 60 ml gum arabic solution and 2) 10 ml sodium citrate buffer (25.5 g of citric acid $1H_2O$ + 23.5 g sodium citrate 2 H_2O to 100 ml distilled water). Immediately before use 3) 15 ml reductor, 0.85 g of hydroquinone dissolved in 15 ml of distilled water, 40°C, and 4) 15 ml of solution containing silver ions, 0.12 g silver lactate in 15 ml distilled water, 40°C, are added and the AMG developer is thoroughly stirred (Danscher, 1981; Danscher and Stoltenberg, 2006).

AMG development

The glass cover slips were placed in a jar filled with AMG developer and placed in a water bath at 26°C. The set-up was covered with a dark hood and developed for 60 min. The AMG developer was then replaced by a 5% sodium thiosulphate solution for 10 min in order to stop the process, and the cover slips were finally rinsed several times in distilled water while still in the jar. The cell cultures were counterstained with toluidine blue, dehydrated, embedded in DePeX (Bie & Berntsen A/S, Herlev, Denmark) and mounted upside down on a glass slide.

Electron microscopical analysis

Macrophages were grown on silver plates in the same way as the cells destined for analysis in the light microscope. At the end of exposure, 0.5 ml medium from each well was removed and the cells were mechanically loosened. The cell suspensions from three wells were added to 1.5 ml of freshly made medium and placed on a glass slip at the bottom of a plastic bottle (Chamber Slide™ , Nunc A/S, Roskilde, Denmark). After 3 hours of incubation the medium was replaced with 3% glutaraldehyde and the bottles were stored in a refrigerator at 5°C. A drop of Epon was placed on the glass slip and an Epon block was set on top. After 24 hours the Epon block was broken off and 3 μm semi-thin sections were cut, placed on glass slides and stained with uranyl and lead (Danscher, 1981; Danscher and Stoltenberg, 2006).

Cell death ELISA assay

Cell death was determined by the fragmentation of histone-associated-DNA using a photometric enzyme

immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Applied Science). J774 macrophages were placed on a 24-well plate with 200,000 cells per well and exposed 36 hours later to silver (1.77 cm^2, 99.95% pure silver, Good-Fellow, Cambridge, UK) or placed on glass cover slips used as controls. Experiments were performed in quadruplicate. After silver exposure the cells were scraped off with a rubber policeman and centrifuged at 200xg for 10 minutes. Supernatants containing DNA from necrotic cells were removed and stored at 4°C for further analysis. Cell pellets containing DNA fragments were lysed and centrifuged at 200xg for 10 minutes. The supernatant containing the cytoplasmic fraction and the supernatant containing the DNA from necrotic cells were transferred into streptavidin coated microtiter plates in duplicate and incubated with Anti-histone-biotin. The amount of fragmented DNA bound to Anti-DNA-peroxidase was measured by ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) with 405 nm and 490 nm as reference wavelengths.

RNA extraction and cDNA synthesis

J774 macrophages cells were plated on a 24-well plate with 200,000 cells per well and exposed 36 hours later to either silver plates (1.77 cm^2, 99.95% pure silver, Good-Fellow, Cambridge, UK) or placed on glass cover slips used as controls. Experiments were performed in replicates of six. RNA from the cells was extracted using Qiagen RNeasy Mini Kit (VWR, Denmark) and treated with DNase (VWR, Denmark). The RNA quality was controlled on a 1% (v/v) agarose gel stained with ethidium-bromide. A total of 250 ng RNA was reversely transcribed using ImProm-II™ Reverse Transcription System (Promega, Denmark) and oligo dT_{18} primers (TAC, Copenhagen). The cDNA was checked for genomic DNA contamination by PCR analysis using Qiagen HotStarTaq Master Mix Kit (VWR, Denmark) with an intron-spanning primer-set of beta-actin (TAC, Copenhagen). The PCR product was analysed by ethidium bromide staining after electrophoresis in a 1% agarose gel.

Real-time PCR

Quantitative real-time PCR was performed in duplicate with IQ Sybr Green supermix (Bio-Rad, Denmark) in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Denmark). For all reactions a melting curve was included. The results were analysed with iQ™5 Optical System Software, Version 2.0. Starting quantities were calculated from a standard curve. Values were normalised to the geomean of three housekeeping genes (HKGs) (Vandesompele, 2002). The expression of the HKGs were analysed to assure stable expression, and silver seemed not to change the gene expression of the selected HKGs (data not shown).

Primers used for RealTime PCR

Colony stimulating factor transcript variant 1 (CSF –V1) F: 5' CGA GTC AAC AGA GCA ACC AA 3', R: 5' GAG GGG GAA AAC TTT GCT TC 3'. Annealing temp: 57°C. Colony stimulating transcript variant 2 (CSF –V2), F: 5' TCT AGC CGA GGC CAT GTG 3', R: 5' GTC TGT CCT CAT CCT GGG TC 3', Annealing temp: 58°C. Tumor Necrosis Factor-alpha (TNF-α) F: 5' TCT AGC CGA GGC CAT GTG 3', R: 5' GTC TGT CCT CAT CCT GGG TC 3', Annealing temp: 58°C. Interleukin-10 (IL-10)F: 5' TGC TAT GCT GCC TGC TCT TA 3', R: 5' TCC TGC ATT AAG GAG TCG GT 3', Annealing temp: 57°C. Nitric oxide synthase 2 (iNOS2) F: 5' GGC AGA ATG AGA AGC TGA GG 3', R: 5' GAA GGC GTA GCT GAA CAA GG 3', Annealing temp: 59°C. Cyclophilin A (CycA) F: 5' GTG GTC TTT GGG AAG GTG AA 3', R: 5' TTA CAG GAC ATT GCG AGC AG 3', Annealing temp: 58°C. Ubiquitin Conjugase-7 (UBC-7): F: 5' GGA ACT GGG CTG CAA TAA AA 3', R: 5' CCG GAT CAT GTT GTG CTA TG 3'. Annealing temp: 58°C. Hypozanthine-guanine Phosphoribosyltransferase (HPRT): F: 5' AAG CTT GCT GGT GAA AAG GA 3', R: 5' TTG CGC TCA TCT TAG GCT TT 3'. Annealing temp: 57°C. The intron-spanning primers of beta-actin: F: 5'CTA CAA TGA GCT GCG TGT GGC 3', R: 5' GTC CAG ACG CAG GAT GGC ATG 3'. Annealing temp: 65°C. cDNA gives a band of 269 basepair, and genomic DNA gives a band of 732 basepair.

Statistical analysis

Means are shown ± SEM. Comparisons between groups in RT PCR experiments and analyses of DNA fragmentation were made by the Mann-Whitney test. $P<0.05$ was considered statistically significant. The analyses were done with the software GraphPad Prism 5.

Results

Time dependent silver uptake

Light microscopical examination showed that all the macrophages exposed to metallic silver for 3 hours contained small silver-enhanced silver-sulphur nanocrystals (AMG-silver grains) (Fig. 1a). The black AMG-silver grains were not confluent, but surrounded by unstained cytoplasma and the visible nucleus. Morphologically, the silver exposed cells looked like the controls with a well-defined cytoplasm. No signs of cell shrinkage, no blebbing of the membrane and no apoptotic bodies were seen (Fig. 1d). The nucleus was well-defined and showed no chromatin condensation. When increasing the silver exposure to 12 hours the macrophages became heavily loaded with AMG-silver grains (Fig. 1b). The AMG-silver grains were seen to accumulate in the cytoplasm forming silver-containing

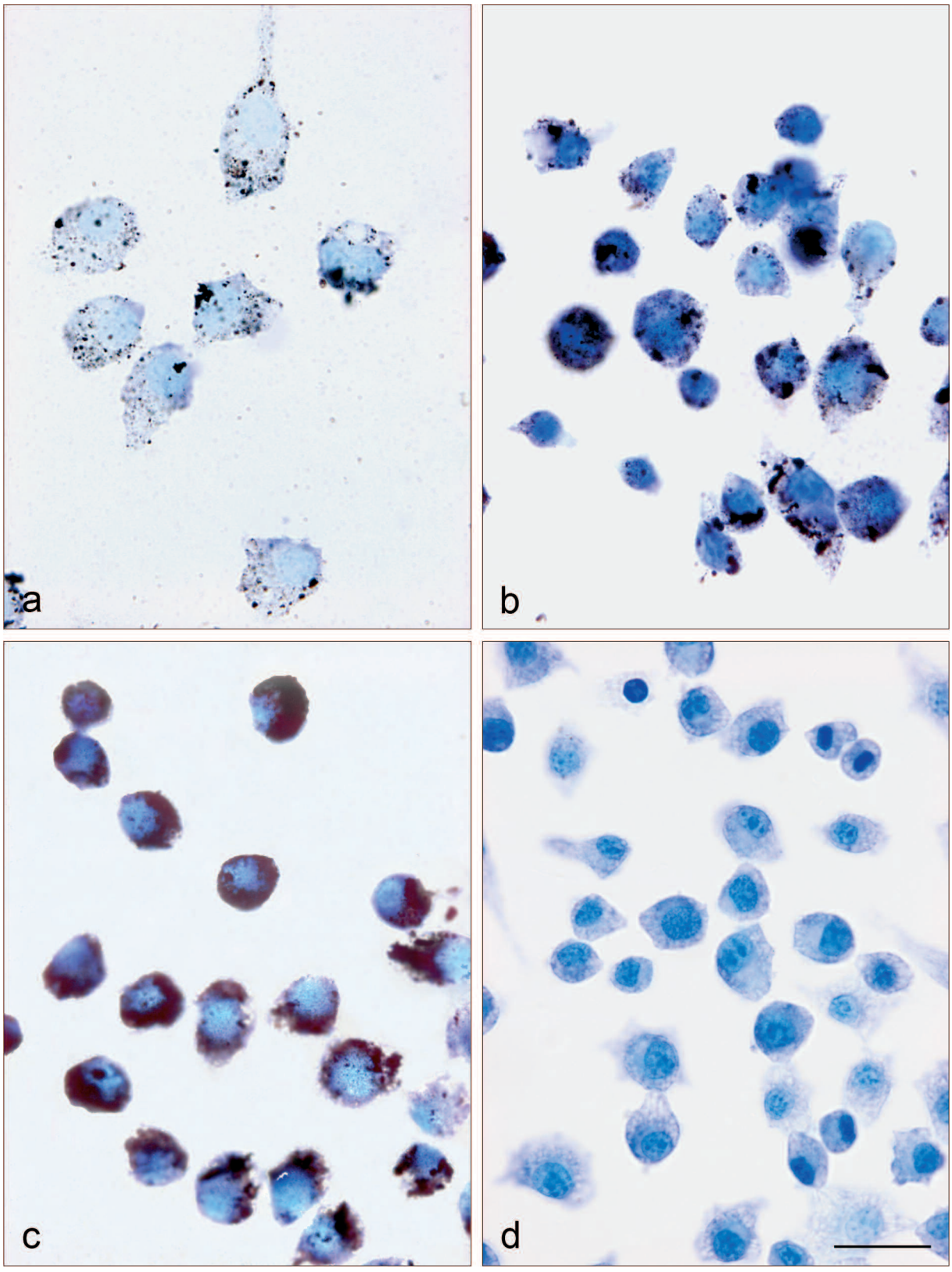

Fig. 1. Silver uptake in cultured J774 macrophages after exposure to metallic silver. Light micrograph of J774 macrophages exposed to metallic silver plates for 3 hours **(a)**, 12 hours **(b)** and 24 hours **(c)**. The cells were fixated with 3% glutaraldehyde before being AMG developed and counterstained with toluidine blue. The AMG enhanced silver-sulphur nanocrystals (AMG-silver grains) are black coloured. Note that the silver uptake increases with exposure time. Control cultures void of staining **(d)**. Scale bar: 20 μm.

conglomerates. Cultured J774 macrophages exposed to metallic silver for 24 hours exhibited a cellular silver uptake and staining pattern resembling what was seen after 12 hours silver exposure (Fig. 1c). Morphologically, the 24 hours silver exposed cells were loosened from the bottom and floated in the medium. The cells adhering to the bottom were irregular, contained dark vacuoles and showed membrane blebbing compared to controls. At the ultrastructural level the AMG-silver grains were found to accumulate in lysosome-like structures in the cytoplasm (Fig. 2d).

Silver induced apoptosis and necrosis after 12 and 24 hours of silver exposure

The potential apoptotic effect of metallic silver exposure on J774 cells was tested and analyses of DNA fragmentation revealed that the cells were apoptotic as well as necrotic after 12 and 24 hours ($p<0.05$), but not after three hours of metallic silver exposure (Fig. 2a,b). The gene expression of Bax/Bcl-2 (an apoptotic pathway) was not induced by metallic silver exposure (Fig. 2c).

Increased gene expression of Macrophage Colony Stimulating Factor (CSF- V1/V2) after short time of silver exposure

The gene expression patterns of CSF-V1 and CSF-V2 in silver exposed cells and controls were similar at all exposure times and the protein products of CSF-V1 and CSF-V2 were similar. CSF-V1 is illustrated as representative for CSF-V1/V2. After the first 3 hours of silver exposure, a statistically significantly increased mRNA expression of CSF-V1/V2 was detected in the exposed macrophages compared to controls (Fig. 3a). In the time span between 3 and 12 hours of exposure the CSF-V1/V2 transcription was markedly reduced in both

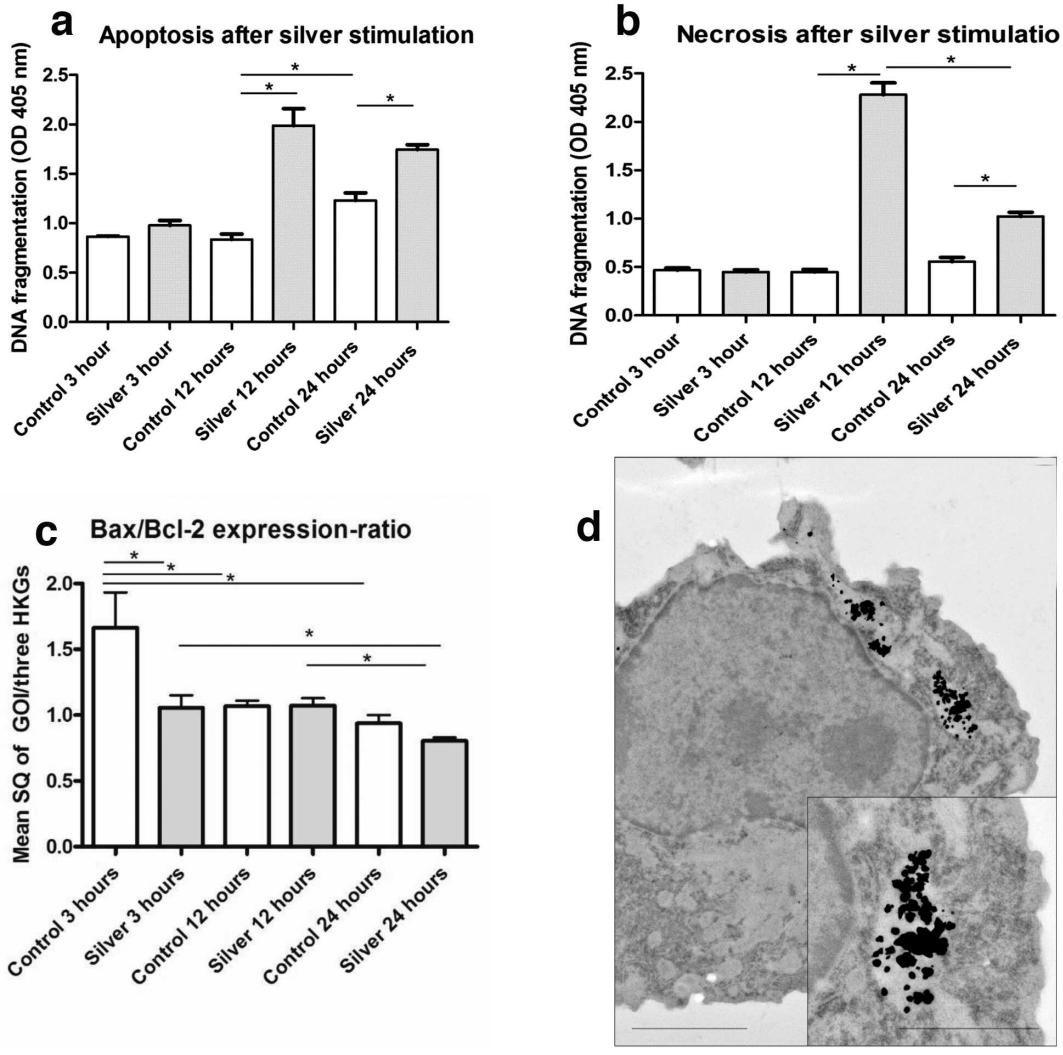

Fig. 2. Detection of apoptosis and necrosis in J774 macrophages related to the accumulation of silver in lysosomes. **a.** Detection of intracellular DNA fragments in J774 macrophages (apoptosis) after 3, 12 and 24 hours of silver exposure. Data are mean and SEM (*$p<0.05$). N=4. **b.** Detection of DNA fragments in medium from J774 macrophages (necrosis) after silver exposure. Data are mean and SEM (*$p<0.05$). N=4. **c.** Bax/Bcl-2 ratio of gene expression. Both genes were normalised to UBC-7, Cyclin A, and HPRT. Mean starting quantity (SQ) of gene of interest (GOI)/Three Housekeeping genes (HKG). Data are mean and SEM (*$p<0.05$). N=6. **d.** Electron micrograph of a J774 macrophage exposed to metallic silver for 1 hour. AMG enhanced silver-sulphur nanoparticles accumulate in lysosomes (insert). n:nucleus. Scale bar: 1 μm.

the silver exposed cells and the control cultures. A statistically significantly increased CSF-V1/V2 exposure was, however, still seen when comparing silver exposed cells to control cells. After 24 hours the gene expression of CSF-V1/V2 increased significantly in both silver exposed cells and controls compared to 12 hour although the gene expression of CSF-V1/V2 remained significantly higher in the silver exposed cells compared to controls.

TNF-α gene expression increases with exposure to metallic silver

At all time points the level of TNF-α gene expression in the silver exposed macrophages was statistically significantly increased compared to the level seen in the control cells (Fig. 3b). Resembling the pattern of CSF-V1/V2 gene expression there was a vast TNF-α response in the early phase after 3 hours of exposure, which was down regulated between 3 and 12 hours of exposure. In the period from 12 to 24 hours of silver exposure the level of TNF-α gene expression was increased significantly.

Metallic silver inhibits gene expression of the anti-inflammatory cytokine, IL-10

The levels of IL-10 gene expression after 3 hours were similar in silver exposed cells and control cultures (Fig. 3c). After 12 and 24 hours of silver exposure the IL-10 transcriptions in the silver exposed macrophages was, however, statistically significantly lower than the IL-10 transcriptions found in the control cultures. The lowest level of IL-10 gene expression was observed in the silver exposed cultures after 24 hours of exposure.

Nitric Oxide Synthase-2 (iNOS-2) increases after 24 hours silver exposure

After 3 and 12 hours a small amount of iNOS-2 gene expression was detected in the silver exposed cells and in the control cultures, although iNOS-2 gene expression was significantly higher in the silver exposed cells compared to controls (Fig. 3d). In contrast, macrophages examined 24 hours after the beginning of the experiment markedly expressed iNOS-2 mRNA. The level of iNOS-2 gene expression was also significantly higher in the

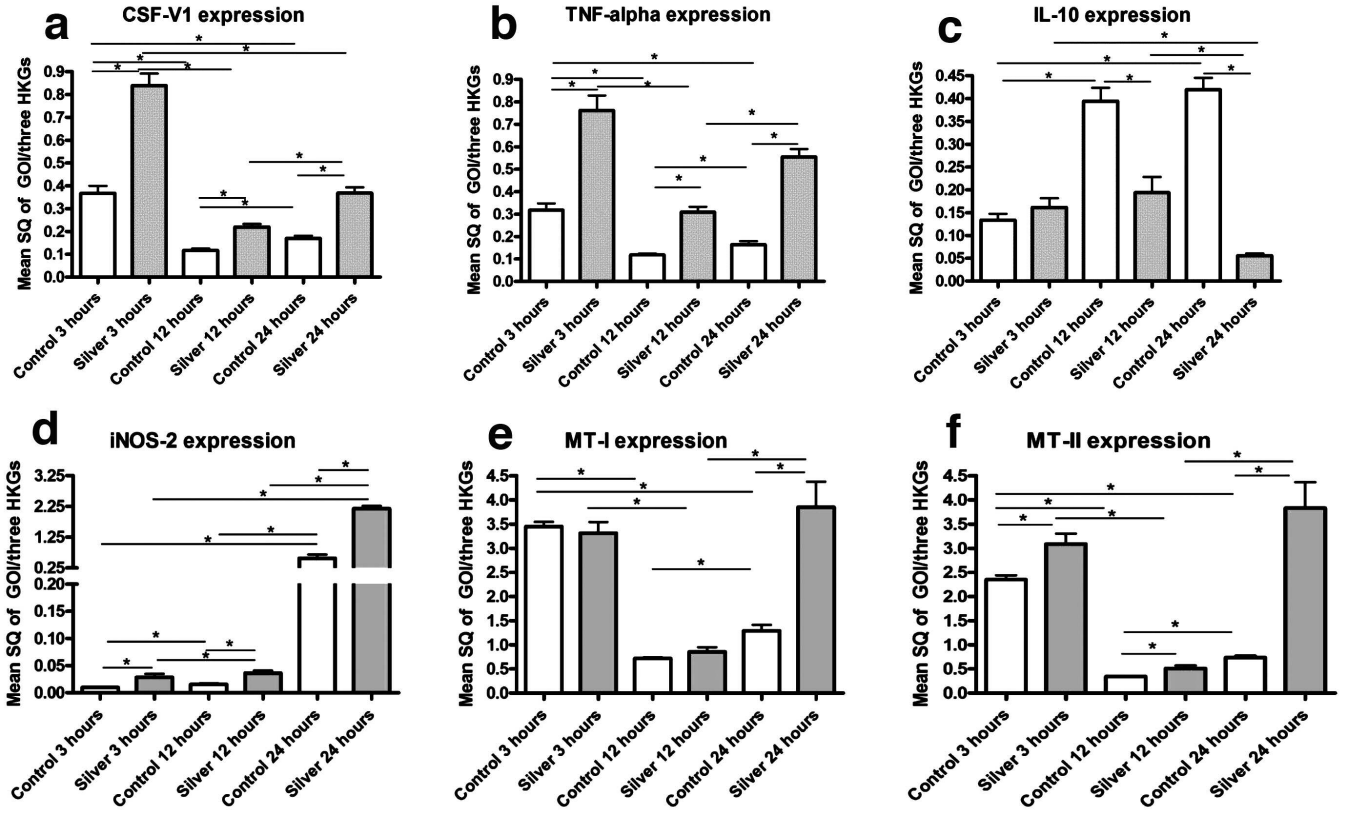

Fig. 3. Relative gene expression of CSF-V1 **(a)**, TNF-α **(b)**, IL-10 **(c)**, iNOS-2 **(d)**, MT-I **(e)** and MT-II **(f)** in J774 macrophages after 3, 12 and 24 hours of metallic silver exposure. Normalised to UBC-7, Cyclin A, and HPRT. Mean starting quantity (SQ) of gene of interest (GOI)/Three Housekeeping genes (HKG). Data are mean and SEM (*p<0.05). N=6.

silver exposed cells at this time point.

Metallic silver exposure induces the gene expression of Metallothionein (MT-I/MT-II)

Following the first hours of the experiment both control and silver exposed cultures exhibited a marked MT-I/MT-II gene expression response. However, a statistically significantly higher level of MT-II gene expression was seen in the silver exposed macrophages compared to controls (Fig. 3e,f). After 12 hours of exposure the levels of MT-I and MT-II were largely down regulated in both the silver exposed cells and control cultures. After 24 hours of silver exposure the MT-I/MT-II gene expression was statistically significantly up-regulated compared to controls.

Discussion

The present study confirms our previous findings (Locht et al., 2009; Danscher and Locht, 2010) that macrophages are able to release and take up silver ions when they are in close contact with a metallic silver surface. This uptake of silver ions is time dependent and after 12 hours of silver exposure large silver conglomerates appear in the cytoplasm. At the ultrastructural level the AMG-silver grains were seen to be located to lysososme-like structures, most likely as silver-sulphur nanocrystals, which is supported by numerous studies (Danscher, 1981; Rungby and Danscher, 1983a,b; Rungby et al., 1987a; Kristiansen, 2008; Locht et al., 2009; Danscher and Locht, 2010). The fact that AMG-silver grains accumulate in lysosome-like structures within the first hour after adhesion to the metallic silver surface (Fig. 2d) shows that lysomal storage is a very early event upon silver uptake. Lysosomes were long considered a stable storage compartment for endocytosed material, but it has become increasingly clear that lysosomes are in fact vulnerable structures (Terman et al., 2006a; Kurz et al., 2008). Larger lysosomes seem more prone to rupture than smaller ones (Ono et al., 2003) with the degree of rupture being the factor that determines whether the ensuing cell death is apoptotic or necrotic (Terman et al., 2006a). It is described that complete lysosomal leakage sets off necrosis, whereas partial leakage can result in apoptosis. In our study, we saw both silver induced apoptosis and necrosis. The lysosomal pathways of cell death are far from understood at present. However, both photo damage, activation of death receptors and accumulation of lysosomotrophic agents have been linked to lysosomally induced apoptosis (Guiccaiardi et al., 2004). Exposure to bismuth, another heavy metal bound to sulphur-crystals within lysosomes has also been shown to cause lysosomal rupture and decreased cell viability (Stoltenberg et al., 2002; Magnusson et al., 2005). Once lysosomal proteases are in the cytosol they can directly or indirectly induce mitochondrial permeability transition (MPT) (Terman et al., 2006b). This induction involves proteolytic activation of the phospholipases or Bid, Bax and Bak. In the present study, the Bax/Bcl-2 index was not found to be affected by silver exposure on an mRNA level, but this does not, however, exclude posttransitional alterations in the Bax/Bcl-2 correlation.

The effect of silver ions on cell viability has previously been demonstrated in silver lactate exposed peritoneal macrophages (Rungby et al., 1987a) and *in vivo* studies have also revealed that this form of silver causes neuronal cell death in the hippocampus of newborn rats (Rungby et al., 1987b). Metallic silver nanoparticles decrease cell viability (Du Toit and Page, 2009; Widgerow, 2010) as well as inducing a pro-inflammatory response (Carlson et al., 2008; AshaRani, 2009; Li et al., 2010). Pro-inflammatory capacities have also been reported in silver nanoparticles used in the treatment of burn wounds (Samberg et al., 2010) indicating that this is a common denominator of various forms of silver. Several others have, on the other hand, reported that silver crystalline dressings have distinct anti-inflammatory effects and that this is of relevance to their use in the treatment of chronic ulcers (Nadworny et al., 2010). There are also conflicting reports on whether the toxic response to silver nanoparticles is size dependent as well (Carlson et al., 2008; Li et al., 2010), as smaller (15 nm sized) particles do seem to generate a larger Reactive Oxygene Species (ROS) response than larger ones (30 nm sized) (Carlson et al., 2008). In this study exposure to bio-liberated silver ions also created a marked pro-inflammatory response in terms of an increased CSF V1/V2 and TNF-α gene expression as early as three hours after silver exposure. This inflammatory response can contribute to toxic effects, such as programmed cell death induced via receptor mediated pathways and TNF-α production of the silver-exposed macrophages. This can contribute to auto- or paracrin induced apoptosis (Silvestris et al., 2003). An additional inflammatory event is the induction of iNOS-2, inducible Nitric Oxide Synthase, which results in NO production as seen e.g. following infectious stimuli (Murakami and Ohigashi, 2007). Nitric Oxide (NO) can by itself contribute to tissue damage during inflammation in many ways, including DNA damage by nitration, nitrosation and oxidation and the formation of the extremely reactive peroxynitrite ($ONOO^-$) when coupling with O_2^- (Szabo and Ohshima, 1997; Murakami and Ohigashi, 2007). In the present study we found that although statistically increased iNOS-2 gene expression is seen when comparing silver-exposed cells to controls at 3 and 12 hours, a massive iNOS-2 gene expression can be seen after 24 hours of silver exposure. It is possible that this event is partly dependent on the production of TNF-α and other pro-inflammatory factors in the hours preceding iNOS-2 induction (Murakami and Ohigashi, 2007). Once NO is liberated this event can contribute to necrotic cell death by inducing mitochondrial permeability, inhibition of mitochondrial respiration and inhibition of glycolysis. NO production

can also result in apoptotic cell death via the p38 pathway, p53 activation or induction of endoplasmatic reticulum stress (Brown, 2010), and both forms of cell death are still very evident after 24 hours of exposure. On the long run in vivo NO induction has been linked to carciogenesis and facilitation of tumor development (Weigert and Brüne, 2008).

In addition to an ongoing inflammatory response silver-exposed macrophages markedly down regulate their gene expression of the anti-inflammatory cytokine IL-10 compared to the control cultures (Fig. 3c). The increases in IL-10 gene expressions seen in controls are probably due to the handling of cells when setting up the experiment. During an inflammatory event, the secretion of IL-10 is known to be delayed within the range of hours (Stenvinkel et al., 2005), and the suppression of IL-10 seen in the silver-exposed cells may be a result of an ongoing strong inflammatory response of the macrophages due to continuous silver exposure. It is very likely that the suppression of IL-10 detected in silver-exposed macrophages causes an additional increase of pro-inflammatory signals e.g. TNF-α, M-CSF and iNOS because one way the IL-10 exhibits its anti-inflammatory effects is through down-regulation of pro-inflammatory signals (Jakobsen et al., 2007). After 24 hours of exposure a burst of MT-I and MT-II transcriptions is seen in the silver- exposed cells which could result from an unmodified pro-inflammatory environment in these cultures, as methallothioneins are known to be induced by stimuli such as oxidative stress and pro-inflammatory cytokines, e.g. IL-6, IL-3, TNF-α, M-CSF and interferon (Penkowa, 2006). This is in contrast to the initial MT-I/MT-II induction after 3 hours of observation which is seen in all types of cultures, making cell harvesting and relocation procedures the most likely cause of activation. Metallothioneins are known to be induced by silver (Lansdown, 2006) and these proteins are involved in the detoxification of heavy metals (Penkowa, 2006; Lansdown, 2007) just as they have been shown to have a protective role in apoptosis (Dutsch-Wicherek et al., 2008). We hypothesize that the increased transcriptions of MT-I and MT-II after 24 hours of exposure corresponds to the selection of a healthy survivor population of MT-I/MT-II expressing cells. Those "healthy survivors" will then try to react towards the metallic silver exposure by increasing their transcription of Colony Stimulating Factor (CSF-V1/CSF-V2) and TNF-α.

Based on our findings we deduce that metallic silver is likely to put a heavy silver load on the organism, in accordance with earlier findings that implanted metallic silver devices release silver ions *in vivo* (Danscher and Locht, 2010). We conclude that silver ions bio-liberated from metallic silver surfaces are taken up by macrophages, followed by a pro-inflammatory response, including iNOS-2 induction and a marked cytotoxic response involving both apoptotic and necrotic cell death.

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