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Effects of nano-scaled particles on endothelial cell function *in vitro*: Studies on viability, proliferation and inflammation

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Recent studies give support for a connection between the presence of inorganic particles (of μm and nm size) in different organs and tissues and the development of inflammatory foci, called granulomas. As the potential source of particles (e.g. porcelain dental bridges) and the location of particle detection were topographically far apart, a distribution via the blood stream appears highly probable. Thus, endothelial cells, which line the inner surface of blood vessels, would come into direct contact with these particles, making particle–endothelial interactions potentially pathogenically relevant.

The objective of this study was to evaluate the effects that five different nano-scaled particles (PVC, TiO_2, SiO_2, Co, Ni) have on endothelial cell function and viability. Therefore, human endothelial cells were exposed to different amounts of the above-mentioned particles. Although most particle types are shown to be internalised (except Ni-particles), only Co-particles possessed cytotoxic effects. Furthermore, an impairment of the proliferative activity and a pro-inflammatory stimulation of endothelial cells were induced by exposure to Co- and, to a lesser extent, by SiO_2-particles. If a pro-inflammatory stimulation of endothelial cells occurs *in vivo*, a chronic inflammation could be a possible consequence.

1. Introduction

The release of particulate matter by wear and corrosion of implants is an important aspect in the pathogenesis of periprosthetic osteolysis and aseptic implant loosening. Studies regarding these effects have focused principally on the local effects in the peri-implant tissues (reviewed in Revell *et al*. [1]). However, recent studies indicate the presence of particles disseminated within different human tissues/organs in patients affected by diseases of unknown origin (e.g. showing cryptogenic granulomas) [2]. Since all the randomly selected samples of these patients showed the presence of inorganic particles of varying chemical composition, a direct association between the particles (of μm and nm size) and the development of inflammatory foci, called granulomas, was postulated. As the potential source of particles (e.g. porcelain dental bridges) and the location of particle detection were far apart, a distribution via the blood stream must have occurred. Thus, endothelial cells, which line the inner surface of blood vessels, will have had direct contact with these particles.

Endothelial cells are important in inflammation and wound healing. Upon pro-inflammatory stimulation of the endothelium adhesion molecules are expressed on the cell surface, thus mediating leukocyte attachment. Furthermore, endothelial cells are able to release cytokines, such as interleukin-8 (IL-8, a key factor in neutrophil chemotaxis). Thus, these features contribute to the pro-inflammatory endothelial phenotype that permits the transmigration of leukocytes from the blood into the perivascular space (reviewed in Michiels [3]). Enhanced endothelial permeability supports leukocyte transmigration; this permeability is actively regulated by the actin-cytoskeleton among other mechanisms [4].

The objective of the study was to evaluate the effects of five different nano-scaled particles on endothelial viability and function. Particles of PVC, TiO_2, SiO_2, Co, and Ni were examined with respect to the cellular internalisation and their influence on cell viability, proliferative activity, and the pro-inflammatory endothelial phenotype.

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2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma (Germany) if not otherwise indicated.

2.2. Cell culture

Human dermal microvascular endothelial cells (HDMEC) were isolated from juvenile foreskin and cultured in endothelial cell basal medium MV (PromoCell, Germany) supplemented with 15% fetal calf serum (Invitrogen, Germany), basic fibroblast growth factor (bFGF, 2.5 ng/ml), sodium heparin (10 µg/ml), penicillin/streptomycin (10 000 units penicillin/ml, 10 000 µg streptomycin sulphate/ml; Invitrogen, Germany), cultivated in a humidified atmosphere at 37 °C (5% CO_2) and used in passage 4.

2.3. Particles

SiO_2- and TiO_2-particles were produced by flame spray pyrolysis (TAL Materials Inc., USA). The size spectrum of SiO_2-particles was between 4 and 40 nm with 14 nm mean particle size. The size of TiO_2-particles was between 20 and 160 nm with 70 nm mean particle size. The size of Co-particles (Sigma Chemicals) was between 50 and 200 nm (mean particle size 120 nm). Ni-particles were made by closed circuit pulverising (University of Bologna, Italy). Their mean crystallite size was 50 nm (mostly agglomerated to clusters of 40 to 420 µm). The poly-vinyl-chloride particles possessed 130 nm mean particle size with a size distribution between 60 and 170 nm (European Vinyls Corporation International, UK). The particles were added to the cell culture medium and tested at three different concentrations (0.5, 5, and 50 µg/ml culture medium).

2.4. Transmission electron microscopy (TEM)

Cells were seeded onto fibronectin-coated Thermanox coverslips (Nunc, Germany). Exposure to particles was performed two days after seeding (50 µg/ml particle concentration). After 48 h incubation cells were fixed in cacodylate-buffered glutaraldehyde (2.5%) and embedded in Agar100 (Plano, Germany). Ultrathin sections were made with the Ultracut E microtome (Leica, Germany). TEM was performed with Phillips 410 EM (Phillips, Germany).

2.5. Cytotoxicity assay

For the evaluation of cytotoxicity the CellTiter 96® AQ_{ueous} non-radioactive assay (Promega, Germany) was performed according to the manufacturer's instructions. This assay gives a measure of the enzymatic conversion of a tetrazolium salt (MTS reagent) by mitochondrial dehydrogenase and thus indirect evidence for cell viability.

2.6. Detection of Ki67 expression

Cells were seeded onto fibronectin-coated 96-well microtitre plates (6500 cells/well) and grown to subconfluence. Afterwards cells were exposed to particles (particle amounts see Section 2.3) and cultivated for an additional 24 h. The cells were fixed with methanol : ethanol (2 : 1, 15 min, room temperature) and permeabilised with buffered 0.1% Triton X-100 (5 min, room temperature).

Ki67, a protein expressed in the nucleus of proliferating cells, was detected with mouse anti-human Ki67-antibody (Dako, Germany). The secondary antibody was a peroxidase-conjugated rabbit anti-mouse-antibody (Dako, Germany). Staining reaction was performed by addition of the peroxidase-substrate (o-phenylenediamin-dihydrochloride) for 15 min at 37 °C. The staining reaction was stopped with 3 M HCl. Light extinction was determined with a microtitre plate photometer (ThermoLab Systems, Germany) at 492 nm.

2.7. Quantification of IL-8 release in cell culture supernatant

Cells were seeded onto fibronectin-coated microtitre plates (13 000 cells/well), grown for 24 h and exposed to particles and TNFα (300 U/ml; inflammatory control), respectively. Cell culture supernatants were collected 24 h after substance or particle exposure. The IL-8 content in supernatants was assayed using human IL-8 immunoassay/ELISA (Hiss Diagnostics, Germany) according to the manufacturer's instructions.

2.8. Fluorescence staining

Human dermal microvascular endothelial cells were seeded onto fibronectin-coated glass chamber-slides (Nunc, Germany). After 48 h, cells were exposed to particles (50 µg/ml), incubated for an additional 24 h and fixed with buffered 3.7% paraformaldehyde (15 min, room temperature). Staining for F-actin was performed with fluorochrome-conjugated Phalloidin (F-actin binding molecule, Phalloidin-AlexaFluor594, Molecular Probes/MoBiTec, Germany). Nuclear staining was performed with Hoechst 33342. Fluorescence labelled cells were covered with GelMount (Biomeda/Natutec, Germany).

3. Results

Ultrastructural studies (TEM) from perpendicular sections of non-treated HDMEC monolayers (control) demonstrated a flattened cell phenotype. The cytoplasm contained numerous organelles and vacuoles (Fig. 1(a), arrowheads: vacuoles with autophagic function). When HDMEC were exposed to different particles an internalisation of most particles was observed (except Ni-particles). The particles were mainly detected within the autophagic vacuoles, which also contained amorphous cellular material and membranes. Whereas the exposure of particles of PVC, SiO_2, and TiO_2 (Fig. 1(b), arrow TiO_2-particles) did not lead to further ultrastructural alterations in HDMEC, the exposure of Co-particles induced changes in the vacuolar system (Fig. 1(c)). Here, a number of vacuoles appeared enlarged. The large number of whorl-shaped membrane complexes within the large vacuoles indicated vacuole fusion as the cause

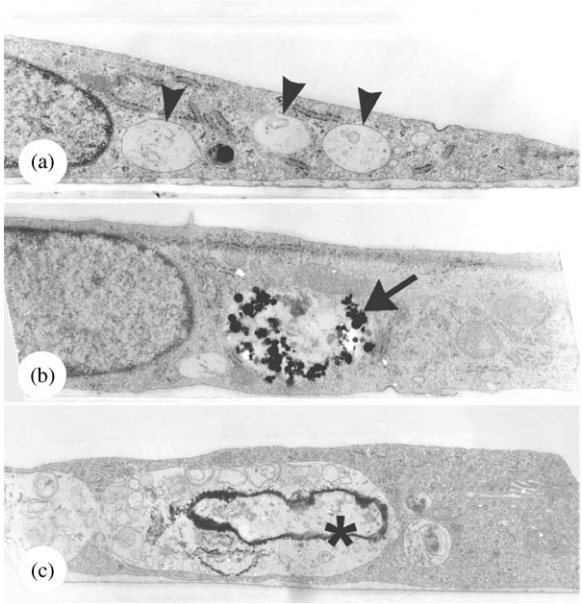

Figure 1 Perpendicular sections of HDMEC. (a) Non-treated control, (b) TiO_2-particle exposed, (c) Co-particle exposed. Arrowheads: autophagic vacuoles; arrow: vacuole internalised TiO_2-particles; asterisk: Co-particle agglomeration. Magnification 17 500 × , TEM).

for enlargement. Striking was the presence of annular-shaped electron-dense material within these vacuoles which are most likely Co-particle agglomerations. Individual Co-particles were not detectable.

The evaluation of particle-induced cytotoxic effects was performed by the photometric detection of enzymatic tetrazolium salt (MTS) conversion by mitochondrial dehydrogenase. This assay revealed a decreased enzymatic conversion in HDMEC when exposed to high amounts of Co-particles (50 and 5 µg/ml) for 72 h, indicating a decrease in cellular viability in a concentration-dependent manner (Fig. 2). Lower Co-particle amounts and all other particles did not induce significant effects.

Detection of the cell cycle-associated antigen Ki67 by immunocytochemistry gives a precise insight into the particle-induced effects on endothelial proliferation (Fig. 3, Ki67 expression 24 h after particle exposure). Particles of SiO_2 (highest amount 50 µg/ml, about 10% reduction) and Co (50 and 5 µg/ml, about 50% and 13% reduction) induced lower Ki67 expression, thus indicating a reduction of cells participating in the active part of the cell cycle. All other particles induced minor, non-significant effects.

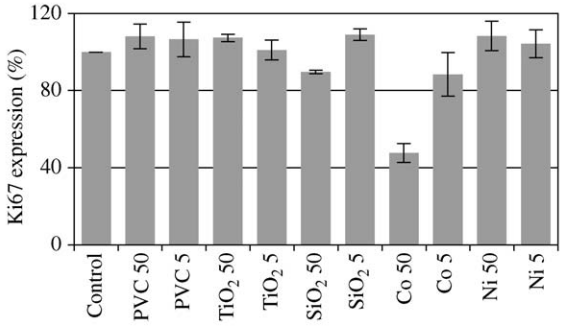

Figure 3 Ki67-expression in non-treated (control) and particle-exposed HDMEC after 24 h ($n = 3$, mean values with standard deviations, untreated control set as 100%, shown are the results for 5 and 50 µg particles/ml culture medium).

In order to evaluate the pro-inflammatory potential of the different particles we examined the release of IL-8 into cell culture supernatants 24 h following particle exposure by ELISA (Fig. 4). The release of IL-8 was enhanced by exposure to high amounts of Co-, SiO_2- and TiO_2-particles (50 µg/ml). The increase of release was more marked for Co- than for SiO_2-particles. TiO_2-particles induced only a minor increase in IL-8 release. Lower particle amounts (5 and 0.5 µg/ml) did not induce changes.

As described above, the F-actin cytoskeleton of endothelial cells plays an active role in the regulation of endothelial permeability during inflammation. Therefore, we stained non-treated (control) and particle-treated HDMEC for F-actin to determine its localisation (Fig. 5). Whereas the non-treated control cells showed the F-actin-staining mainly within the so-called dense peripheral actin-ring (Fig. 5(a), arrows: exemplary actin-rings), cells treated with a pro-inflammatory factor (tumour necrosis factor/TNFα, 300 U/ml, 24 h, inflammatory control) led to the development of cytoplasm-spanning F-actin, indicating stress fibre formation (Fig. 5(b), arrowheads). Furthermore, the peripheral actin-rings were extensively dissolved and a few intercellular gaps were observed (asterisks, Fig. 5(b)). The exposure of HDMEC to large amounts of Co-particles also induced actin reorganisation comparable to the TNFα-treatment, with intercellular gaps appearing (Fig. 5(c)). However, the phenotype did not completely

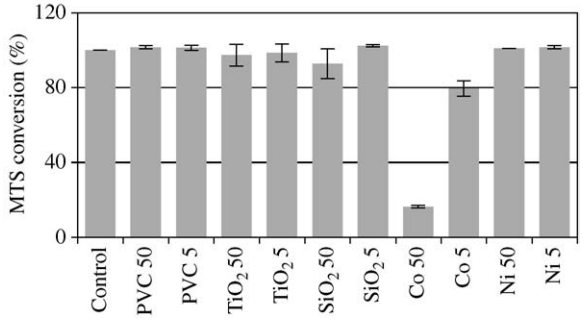

Figure 2 MTS-conversion (cytotoxicity assay) of non-treated (control) and particle-exposed HDMEC after 72 h ($n = 3$, mean values with standard deviations, untreated control set as 100%, shown are the results for 5 and 50 µg particles/ml culture medium).

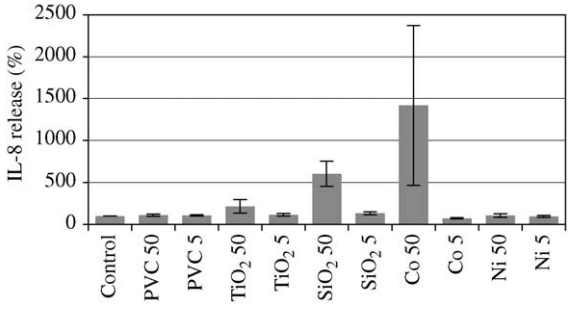

Figure 4 IL-8-release of non-treated (control) and particle exposed HDMEC after 48 h ($n = 3$, mean values with standard deviations, untreated control set as 100%, shown are the results for 5 and 50 µg particles/ml culture medium).

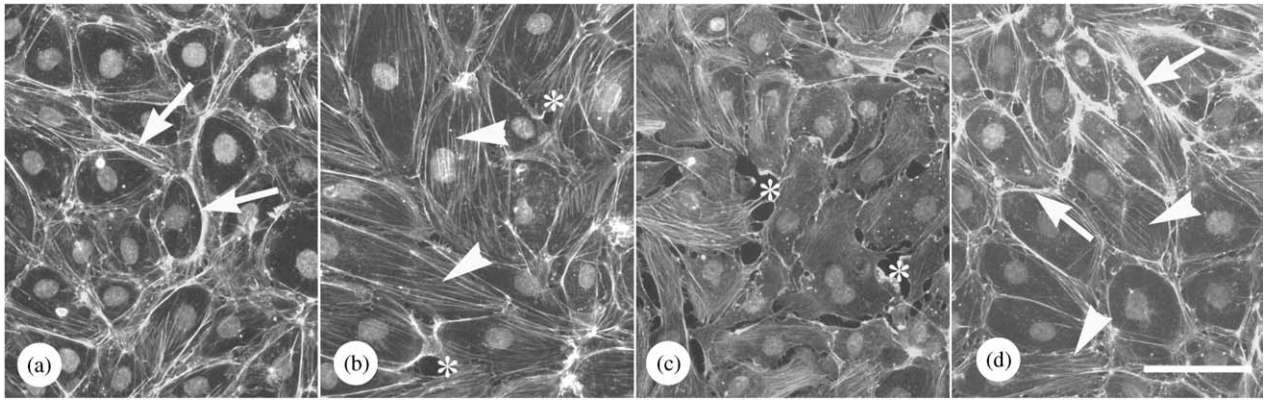

Figure 5 F-actin cytoskeleton and nuclei of HDMEC (a) non-treated control, (b) TNFα-treated pro-inflammatory control, (c) Co-particle exposed, (d) SiO_2-particle exposed. Arrows: dense peripheral actin-rings; arrowheads: actin stress fibres; asterisks: intercellular gaps; fluorescence microscopy, digital overlay, scale bar = 50 μm).

match that of TNFα-treatment: partially dense peripheral actin-rings remained and the actin-stress fibre formation was not as pronounced. All other particles induced no or only minor deviations in F-actin distribution compared to the control. An example of this is shown in Fig. 5(d) for the exposure of HDMEC to SiO_2. Dense peripheral rings were detectable (arrows) and interendothelial gaps did not emerge. However, the number of stress fibres appeared slightly increased (arrowheads, Fig. 5(d)). Due to biological variance this aspect is difficult to quantify.

4. Discussion

The role of particulate matter has received increasing attention in the past years. In the literature the investigations were targeted to the incorporation of particles by inhalation of polluted air and abrasion from implanted materials. These particles have been shown to integrate within different organs and tissues [2, 5, 6]. In recent studies particles smaller than 100 nm were examined since it has been shown that particles below this size were able, for example, to cross the air–blood-barrier (consisting of epithelial and endothelial cells) of the lung and pass into the circulation [7]. It was suggested that this particle transmigration via the lung into the circulation contributed to cardiovascular pathology [8].

Our study has shown that human endothelial cells possess a large capacity for the internalisation of particulate matter in the nanometre scale. The tested particles (PVC, TiO_2, SiO_2, Co) were incorporated for the most part into vacuoles. In addition, it appeared that the particle-containing vacuoles have an autophagic function, since these vacuoles also contained varying amounts of amorphous cellular material and membranes [9]. Although in most cases large amounts of particles were detected only minor changes in organelle ultrastructure appeared. Only the exposure of HDMEC to Co-particles led to the significant enlargement of vacuoles. Since these large vacuoles contained a number of circular membrane fragments the enlargement appeared as a result of vacuole fusion.

Pro-inflammatory effects in HDMEC were shown after exposure to Co-, SiO_2-, and TiO_2-particles (enhanced release of IL-8). The IL-8 release following Co- and SiO_2-particle exposure was pronounced and TiO_2-particles induced only minor effects. The other particles did not induce a pro-inflammatory stimulation. The observed deviations upon Co-particle exposure were attributed to a release of Co-ions by the particles, since the exposure of endothelial cells with divalent Co-ions leads to the described effects (impaired endothelial viability, pro-inflammatory stimulation) [10]. Moreover, the effects of high amounts of SiO_2-particles on IL-8 release and proliferation of cells without the appearance of cytotoxicity indicated a pro-inflammatory state. Since it has been shown that SiO_2-particles can cause chronic inflammatory lung disease by inhalation [11] a comparable mechanism might be present during pro-inflammatory activation of endothelial cells. The SiO_2-induced inflammation was shown to be induced by the activation of the transcription factors NF-κB (nuclear factor-κB) and AP-1 (activator protein-1), which are both involved in the regulation of inflammation. Additionally, the SiO_2-induced AP-1 activation plays an important role in neoplastic transformation and tumour promotion (reviewed in Ding *et al*. [12]).

Interestingly, even TiO_2 nano-scaled particles, deriving from a very biocompatible material, revealed a slight but reproducible pro-inflammatory effect (IL-8 release) in endothelial cells. This supports the hypothesis that a material possesses divergent features when tested as fine or nano-scaled particles compared to large test specimens. For example, Donaldson *et al*. [13] showed that nano-scaled particles of TiO_2 exhibited a higher amount of free radical activity on the surface than larger TiO_2-particles.

The absence of cytotoxic, pro-inflammatory, and cell cycle influencing effects for the other tested particles (PVC and Ni) does not exclude further effects, since the time frame of this study did not exceed 72 h. However, additional long-term experiments should give a more precise insight into particle-induced effects regarding their cytotoxic, pro-inflammatory, and genotoxic capacity. This is the subject of a separate study.

If a pro-inflammatory stimulation of endothelial cells by nano-scaled particles occurs *in vivo*, a chronic inflammation (such as granulomatosis) could be a possible consequence. Since endothelial proliferation is a prerequisite for blood vessel formation (e.g. during wound healing), an impairment of endothelial prolifera-

tion could indicate a reduced capacity for blood vessel formation (angiogenesis) *in vivo*.

Acknowledgments

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