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Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes

Alexandra Jaeger[a,c], Dieter G. Weiss[a], Ludwig Jonas[b], Ralf Kriehuber[a,c,*]

[a] University of Rostock, Institute of Biological Sciences, Cell Biology and Biosystems Technology, Albert-Einstein-Straße 3, D-18059 Rostock, Germany
[b] Electron Microscopic Centre, Department of Pathology, University of Rostock, Strempelstraße 14, D-18055 Rostock, Germany
[c] Department of Safety and Radiation Protection, Radiation Biology Unit, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany

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ABSTRACT

Since nano-sized particles (NPs) are increasingly used in various fields of innovative biomedicine and industrial technologies, it is of importance to identify their potential human health risk. We investigated whether ROS-induced mitochondrial DNA damage is the mode of action of titanium dioxide-NPs (TiO_2-NPs; ≤20 nm) to induce cytotoxic and genotoxic effects in human HaCaT keratinocytes *in vitro*. We showed that TiO_2-NPs accumulate at the cell surface and are taken up by endocytosis. Micronucleus (MN) formation was found to be significantly maximal increased 24 h after treatment with 10 μg/ml and 48 h after treatment with 5 μg/ml TiO_2-NPs about 1.8-fold respectively 2.2-fold of control. Mitochondrial DNA damage measured as "common deletion" was observed to be significantly 14-fold increased 72 h after treatment with 10 μg/ml TiO_2-NPs when compared to control. Four hours after treatment with 5 and 50 μg/ml TiO_2-NPs the level of ROS in HaCaT cells was found to be significantly increased about 7.5-fold respectively 16.7-fold of control.

In conclusion, for the first time we demonstrate the induction of the mitochondrial "common deletion" in HaCaT cells following exposure to TiO_2-NPs, which strongly suggests a ROS-mediated cytotoxic and genotoxic potential of NPs. However, the effects of the modification of TiO_2-NPs, such as agglomeration, size distribution pattern and exposure time have to be further critically examined.

1. Introduction

Nano-sized titanium dioxide (TiO_2-NPs, <100 nm) is used in a wide application range, especially in sun protectors and cosmetics and was mostly classified as inert and biocompatible, although a number of studies showed potentially harmful risks to human health, especially when NPs are inhaled, ingested or applied to body surfaces (Seaton et al., 2010). When evaluating the biopersistence and related dose–effect relationships of inhaled particles of largely different sizes, the particle surface area rather than the mass of the retained particles appears to be the most relevant dose parameter (Oberdörster et al., 1994). Compared to macro-sized particles (MPs, >100 nm) of the same composition NPs have a much larger surface area per unit mass and hence a high surface to volume ratio, at which a greater proportion of atoms/molecules are found at the surface compared to those inside (Beck-Speier, 2005; Yang and Watts, 2005). Increased surface-to-volume ratio results in the increase of the particle surface energy, which may render NPs more biologically reactive (Oberdörster, 1996; Cassee et al., 2002; Huang et al., 2004; Warheit, 2004; Yang and Watts, 2005). Surface potential and size of particles can influence their bioavailability. TiO_2-NPs were found to be highly mobile in the pH region 2.5–5.9, where electrostatic forces between TiO_2-NPs aggregates are supposed to be strong (Lecoanet et al., 2004). This enables NPs to bind to organic or toxic compounds, carry them to cell surfaces and subsequently to subcellular structures, potentially leading to the generation of ROS (Oberdörster et al., 2005).

Titanium dioxide is found in three modifications: rutile, anatase and brookit and its macro-sized particular form is widely used as white pigment, which efficiently absorb Ultraviolet irradiation (UV) (Stiller, 2003). Studies showed, that NPs are taken up in rat and mouse cells *via* phagocytosis or via the bulbus olfactorius and are transported by blood and lymph fluid to bone marrow, liver, heart and lymph nodes as well as to the central nervous system (CNS) (Oberdörster et al., 2005). NPs cause an increase of ROS as a result of an inflammatory response which correlates with increased cytotoxicity and genotoxicity (Nel et al., 2001; Donaldson and

* Corresponding author at: Department of Safety and Radiation Protection, Radiation Biology Unit, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany. Tel.: +49 02461 61 4054; fax: +49 02461 61 2166.
E-mail addresses: a.jaeger@fz-juelich.de (A. Jaeger), dieter.weiss@uni-rostock.de (D.G. Weiss), ludwig.jonas@gmx.de (L. Jonas), r.kriehuber@fz-juelich.de (R. Kriehuber).

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Stone, 2003). Furthermore, ROS are produced by photo-activated NPs, chemicals on the surface of NPs, or as a consequence of the interaction between NPs and cellular components (Dreher, 2004; Hirakawa et al., 2004; Xia et al., 2006). Moreover, the disruption of cell membrane and mitochondrial membrane by NPs lead to the production of additional ROS and spontaneously ROS production after dispersion was also reported (Nel et al., 2001). ROS pose a significant threat to cellular integrity in terms of damage to DNA, lipids, proteins and other macromolecules (Cooke et al., 2003; Slupphaug et al., 2003). As a consequence, this oxidative damage results in ROS-induced DNA base modifications, single- and double-strand breaks, and the formation of apurinic/apyrimidinic lesions, many of which are toxic and/or mutagenic (Girard and Boiteux, 1997). Additionally, TiO_2 particles are assumed to bind directly to DNA or repair enzymes leading to the generation of strand breaks (Hartwig, 1998; Jha et al., 1992).

DNA damage was observed to persist 5- to 10-fold longer in mitochondrial DNA than in nuclear DNA, because of limited repair mechanisms, less accuracy of replication and presence of respiratory enzymes (Yakes and Van Houten, 1996). A conclusive indicator for DNA damage is the "common deletion" (CD) of mitochondrial DNA (mtDNA) which is the most frequent and best characterized mutation in the mtDNA. This large deletion of 4977 bp on human mtDNA is considered to be a sensitive and early marker for mitochondrial mutations in dividing cells and is primarily induced by enhanced levels of ROS (Shoffner et al., 1989). Petersen et al. (2000) showed in UVA-exposed HaCaT cells the induction of common deletion in conjunction with intracellular ROS generation. Combined exposure with TiO_2 and UVA in human skin fibroblasts induced increased phototoxic effects like DNA damage depending on particle size and particle modification (Wamer et al., 1997). Furthermore, it was shown, that TiO_2-NPs induce micronuclei and apoptosis in Syrian Hamster Embryo Fibroblasts (Rahman et al., 2002). Zhao et al. (2009) identified TiO_2 particles to activate the intrinsic apoptosis pathway in JB6 cells, followed by significant changes in mitochondrial membrane permeability. Activation of intrinsic apoptosis leads to DNA fragmentation, nitration of proteins, lipid peroxidation, lysosomal membrane destabilization and cathepsin B release, directly causing proteolysis or activating other proteases (Hussain et al., 2010; Juedes and Wogan, 1996).

Previous studies, by several groups, have shown on the basis of different biological endpoints that TiO_2-NPs can induce cyto- and genotoxic effects depending on the particle concentration and exposure time *in vitro* and *in vivo*. Nevertheless, a conclusive mechanism of MN formation and induction of apoptosis by NPs has yet not been demonstrated. However, a ROS-associated mechanism is widely assumed but not proven.

In order to clarify whether a ROS-mediated mechanism is involved we examined the cytotoxic and genotoxic potential of TiO_2-NPs in comparison to TiO_2-MPs and UVA. The latter was used as positive control, since UVA is known to cause oxidative stress *via* ROS (Henri et al., 2012) and induces loss of cell-to-cell contacts, nuclear condensation and surface blebbing in human keratinocytes, which is assumed to be the consequence of an alteration in the redox equilibrium of the cells *in vitro* as well as *in vivo* (Giacomoni et al., 1998). We extensively studied micronucleus formation and apoptosis induction in human HaCaT keratinocytes. Furthermore, we investigated the cellular uptake of TiO_2-NPs employing transmission electron microscopy (TEM) and applied the quantification of the mitochondrial "common deletion", to examine ROS-driven DNA-damage. To our knowledge, the common deletion has not been investigated in this context before and we demonstrated successfully for the first time, that TiO_2-NPs induce the common deletion and that this is correlated to an increased cellular ROS level.

2. Materials and methods

2.1. Cell culture

The spontaneously immortalized human HaCaT keratinocyte cell line (Human adult low Calcium high Temperature Keratinocytes), established by Professor N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany (Boukamp et al., 1988) out of the periphery of a primary malignant melanoma, were maintained in a monolayer culture in 95% air and 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom, Germany), 1% Modified Eagle's Medium with nonessential amino acids (PAA, Germany) and 1% penicillin/streptomycin (penicillin 10,000 Units/ml, streptomycin 10 mg/ml; PAA, Germany). The non-tumorigenic, permanent HaCaT cell line is characterized by a highly preserved differentiation capacity and a high genetic stability (Boukamp et al., 1988). These adherent growing, spindle-shaped and fibroblast-like, flat cells are almost completely filled by the nucleus and form a nearly regular epidermal architecture.

2.2. Chemicals

An anatase–rutile mixture of 20 nm sized TiO_2-NPs (P-25, 70% anatase/30% rutile; Degussa, Germany) and TiO_2-MPs anatase of 200 nm size (Sigma–Aldrich, Germany) were provided by Prof. D. Schiffmann (Institute of Biosciences, University of Rostock). The N_2-BET specific surface area of TiO_2-NPs in DMEM was determined to be at $52.7 \pm 3.6\ m^2/g$ (Long et al., 2006) respectively. The studied TiO_2-NPs possess an average primary particle size of 21 nm (Degussa) and a primary crystallite size of ~30 nm (Kirchnerova et al., 2005). The Zeta potential of TiO_2-NPs, measured in DMEM (pH 7.5), is -11.6 ± 1.2 mV (Long et al., 2006).

TiO_2 particles were sterilized by heating to 120 °C for 2 h, suspended in sterilized phosphate-buffered saline (1× PBS; PAA, Germany) to a 12.5 mM stock solution (1 mg/ml) and kept at 4 °C until used. Before application to the cells, the particle suspension was treated with an ultrasonicator for 3 min at cycle count 20% and 70% of maximum power.

2.3. UVA exposure

UVA exposure was performed using a Waldmann UV-181 device (Waldmann, Germany) equipped with five UV-A lamps (PUVA, 36 W, 320–400 nm; Waldmann, Germany). HaCaT cells were grown for 24 h in Petri dishes (10 cm; TBB, Switzerland). Prior irradiation the cell culture medium was discarded and cells were washed with pre-warmed PBS. PBS was removed and Petri dishes were placed inverted on the Waldmann device for exposure. UVA exposure was performed at room temperature (RT). After irradiation fresh medium was added, and the cells were incubated at 37 °C for subsequent analysis.

2.4. Scanning electron microscopy and energy-dispersive X-ray element analysis

Cells were seeded onto coverslips (21 mm × 26 mm; Menzel-Gläser, Germany) placed in 6-well-plates (TPP, Switzerland) at a density of about 10^5 cells/ml medium in each well. 24 h after seeding cells were exposed to TiO_2 particles at a concentration of 5 μg/ml (equates to 1.66 $\mu g/cm^2$). After 1 h exposure cells were fixed in 4% glutaraldehyde at 4 °C for 1 h. Fixed samples were dehydrated, sputtered with gold particles and scanning electron microscopy (SEM) was performed (DSM 960 A, Zeiss, Germany). The element content of titanium (Ti) and oxygen (O) in single particles was determined using an energy dispersive X-ray device (KEVEX, USA) attached to the SEM.

2.5. Transmission electron microscopy

HaCaT cells were seeded into Petri dishes (6 and 10 cm; TPP, Switzerland) and exposed to 10 μg/ml (equates to 2.26 $\mu g/cm^2$) TiO_2 for 1 h. Afterwards cells were trypsinized, resuspended in FBS-containing culture medium and centrifuged. Medium was then decanted, cells were washed thrice with serum free medium and fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 1 h at 4 °C. After incubation in 1% OsO_4 in 0.1 PBS for 1 h at 4 °C, the samples were washed in PBS, dehydrated in a graded series of acetone, and embedded in the epoxy resign Araldite (Fluca Buchs, Germany). Ultrathin sections were cut with the Ultramicrotome Ultracut S (Leica, Germany), deposited on copper grids, stained with uranyl acetate and lead citrate and studied in a transmission electron microscope (EM 902 A; Zeiss, Germany).

2.6. Phase contrast microscopy

Morphological changes of HaCaT cells after treatment with TiO_2 and UVA was investigated during culturing of the cells by phase contrast microscopy (Leica DME light microscope, Germany).

2.7. Flow cytometry measurement of apoptosis induction

HaCaT cells exposed to 10 μg/ml (equates to 2.16 $\mu g/cm^2$) TiO_2 respectively 4 J/cm^2 UVA were washed twice with 37 °C pre-warmed PBS, incubated