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Genotoxic and cell-transforming effects of titanium dioxide nanoparticles

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ABSTRACT

The *in vitro* genotoxic and the soft-agar anchorage independent cell transformation ability of titanium dioxide nanoparticles (nano-TiO_2) and its microparticulated form has been evaluated in human embryonic kidney (HEK293) and in mouse embryonic fibroblast (NIH/3T3) cells. Nano-TiO_2 of two different sizes (21 and 50 nm) were used in this study. The comet assay, with and without the use of FPG enzyme, the micronucleus assay and the soft-agar colony assay were used. For both the comet assay and the frequency of micronuclei a statistically significant induction of DNA damage, was observed at the highest dose tested (1000 μg/mL). No oxidative DNA damage induction was observed when the comet assay was complemented with the use of FPG enzyme. Furthermore, long-term exposure to nano-TiO_2 has also proved to induce cell-transformation promoting cell-anchorage independent growth in soft-agar. Results were similar for the two nano-TiO_2 sizes. Negative results were obtained when the microparticulated form of TiO_2 was tested, indicating the existence of important differences between the microparticulated and nanoparticulated forms. As a conclusion it should be indicated that the observed genotoxic/tranforming effects were only detected at the higher dose tested (1000 μg/mL) what play down the real risk of environmental exposures to this nanomaterial.

1. Introduction

Nanosized titanium dioxide particles (nano-TiO_2), as well as other nanomaterials, are increasingly used in many industrial, food and healthy applications and for this reason its presence into the environment is spectacularly rising. As human exposure certainly occurs in this scenario, deep information on their mechanisms of action is required to better understand their potential exposure associated risks (Skocaj et al., 2011). Although the bulk form of TiO_2 is biologically inert, the nanosized forms present interesting physic, chemical and biological properties associated to size. Thus, when nanoparticles have diameters ≤ 30 nm, dramatically changes occurs enhancing their interfacial reactivity (Auffan et al., 2009) and modulating the toxicological properties (Xiong et al., 2013).

With respect to the possible biological risks of nano-TiO_2 exposure many authors have focused their attention on the interactions with genetic material. In fact, several studies have been recently published indicating genotoxic properties of nano-TiO_2, independently of the assay used or the biological system employed. Thus, positive results in the comet and in the micronucleus (MN) assays in HEp-2 cells have been reported (Osman et al., 2010), as well as in the MN and sister-chromatid exchanges (SCE) assays in Chinese hamster ovary (CHO-K1) cells (Di Virgilio et al., 2010). In the human lung cancer (A549) cell line strand breaks and DNA oxidative lesions, together with a impair cell ability to repair DNA by inactivation of both NER and BER pathways were reported (Jugan et al., 2012), in addition to oxidative stress, apoptosis and micronuclei induction (Srivastava et al., 2013). Furthermore, positive results in the comet assay in human amnion epithelial (WISH) cells have also been obtained (Saquib et al., 2012). A recent

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review has been published on the genotoxicity of nano-TiO_2 (Chen et al., 2014) showing that many studies have been conducted until know trying to identify the potential genotoxic risk associated to nano-TiO_2 exposure with contradictory results.

In spite of the large amount of studies looking for genotoxicity of nanomaterials, few studies have been carried out to prove its ability to induce carcinogenesis processes by using *in vitro* approaches. In fact, only two previous studies have focused on the detection of cell transformation induction, by measuring the promotion of anchorage-independent growth in soft-agar, as a pertinent tool. One study detected the transforming potential of alumina nanoparticles (Dey et al., 2008) and the other was performed with nano-TiO_2 (Huang et al., 2009). In this last study changes in cell morphology and in the number of foci were observed in NIH-3T3 cells, just after one week of exposure. A recent study has also used this approach to detect the transforming potentials of nano-cobalt in mouse embryonic fibroblasts (Annangi et al., 2014). Although anchorage-independent growth is one of the hallmarks of cell transformation, other approaches have also been used to measure the transforming potential of nanometals, as occurs with the mouse fibroblast cell line (Balb/3T3) model (Corvi et al., 2012). In this assay, after exposure to potential carcinogenic agents, cells become tumorigenic forming morphological transformed colonies called foci. Interesting, a recent study using this approach and multiwall carbon nanotubes (MWCNT) has shown that, in spite that MWCNT did not induced genotoxicity as measured by the MN assay, the transforming potential of MWCNT was evident (Ponti et al., 2013).

In this context, in the present study we extend the genotoxicity studies on nano-TiO_2 using the comet and the micronucleus assays to detect the induction of both primary and fixed DNA damage. The comet assay was complemented with the use of FPG enzyme detecting oxidized DNA bases to detect if nano-TiO_2 produces genotoxic damage via oxidative stress. The effects have been evaluated in two different cell lines: the human embryonic kidney (HEK293) and the mouse embryonic fibroblast (NIH/3T3) cell-lines to determine possible cell-line associated effects. To further explore the potential cell-transforming ability of nano-TiO_2, we have evaluated the promotion of anchorage-independent growth on soft-agar, as a pertinent tool for detecting malignant cell-transformation (Dey et al., 2008; Huang et al., 2009). For comparisons, the micro-sized form of TiO_2 was used to disciminate wheter the obtained results were due to TiO_2 itself or to the nano-sized form.

2. Materials and methods

2.1. HEK293 and NIH/3T3 cells

Human embryonic kidney (HEK293) and mouse embryonic fibroblast (NIH/3T3) cell lines were obtained from the American Type Culture Collection (ATCC, CRL 1573) and Child Health Centre in Warsaw (Poland), respectively. HEK293 and NIH/3T3 cells were grown at 37 °C, in a 5% CO_2 atmosphere, and were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; ingredients: 1 g/L D-glucose, 2 mM L-glutamin, 1 mM sodium pyruvate) supplemented with 10% heat inactivated fetal bovine serum (Sigma F 9665), 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.2. Chemicals

Nano-TiO_2 (21 nm) (≥ 99.5% purity; CAS no. 13463-67-7) and the microparticulated form (TiO_2, crystalline form, 99% to 100.5% purity; CAS no. 13463-67-7) were from Sigma Chemical Co. (St. Louis, MO, USA). Nano-TiO_2 (50 nm) (≥ 98% purity; CAS no. 13463-67-7) was from MK Impex Corp. (Mississauga, Ontario, Canada). Both nano-TiO_2 were in anatase crystalline form. All the other compounds used in the different tests were provided from Sigma Chemical Co.

2.3. Nano-TiO_2 characterization

Transmission electron microscopy (TEM), dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) methodologies were used to characterize nano-TiO_2 (Jacobsen et al., 2010). TEM methodologies were carried out on a JEOL JEM-2011 instrument to determine size and morphology. DLS and LDV were performed on a Malvern Zetasizer Nano-ZS zen3600 instrument for the characterization of hydrodynamic size and zeta potential, respectively. For these measures nano-TiO_2 were dispersed at the concentration of 2.56 mg/mL prepared in a 0.05% bovine serum albumin (BSA) in water. The use of BSA for dispersion has been the agreement proposal obtained in the frame of the Nanogenotox UE project (Nanogenotox, 2011). From the different approaches used this one was the best in front a wide set of nanomaterials. For dispersion, nano-TiO_2 were subjected to ultrasonication (S-250D, Branson, Digital Sonifier®) at 20 kHz for 16 min in an ice-cooled bath prior its addition to the culture media.

2.4. Comet assay

In order to avoid false positive responses, in the comet assay it is recommended that more than 75% of cells must be viable in the applied treatments (Henderson et al., 1998). Cell viability measurements were performed after nano-TiO_2 exposure using a mix of fluorescein diacetate (FDA) and ethidium bromide (EtBr). Cells were stained with a 1:1 solution of 80 μg/ml FDA plus 50 μg/ml EtBr and observed under a fluorescence microscope (Nikon Eclipse E200) with an excitation filter of 488 nm (blue light). Living cells were stained in green while dead cells exhibit their nucleus stained in orange. The survival % was obtained dividing the number of living cells by the total number of cells (Strauss, 1991). Two hundred cells were scored for viability in each treatment. To carry out the comet assay, HEK293 and NIH/3T3 cells were detached using trypsin/EDTA solution (0.05%/0.2%, w/v) centrifuged at 500*g* for 2 min, and the pellet was resuspended in DMEM (5×10^4 cell in 1 mL). Each nano-TiO_2 was dispersed in distilled water, and three concentrations of each were tested. Different concentrations of each nano-TiO_2 and their microparticulated forms (1, 10 and 100 μg/mL) were chosen based on preliminary studies (Theogaraj et al., 2007; Reeves et al., 2008; Ghosh et al., 2013; Dobrzyńska et al., 2014; Tavares et al., 2014). Aliquots of 10 μL of each solution were added to the cultures for 1 h at 37 °C. Ethyl methanesulfonate (EMS, 5 mM) was included as a positive control.

The assay was performed using the standard protocol (Sing et al., 1988) with slight modifications. Cell samples were carefully resuspended in 75 μL of 0.75% low-melting agarose (LMA) prepared in PBS. Cells and agarose were gently mixed by repeated pipetting, and layered onto microscope slides pre-coated with 0.75% normal melting agarose (NMA) (dried for 25 min). Slides were immediately covered with cover-slips and kept on ice for 5 min to solidify the agarose. After solidification, cover slips were carefully removed and 75 μL of molten 0.75% LMA prepared in PBS was spread onto the slides. Slides were again covered with cover-slips and kept on ice for 5 min. Then, cover-slips were removed and slides were immersed in cold, freshly made lysing solution, for 2 h at 4 °C in a dark chamber. Slides were placed for 40 min in a horizontal gel-electrophoresis tank filled with cold electrophoresis buffer to allow DNA unwinding. Electrophoresis was carried out in the same buffer for 30 min at 25 V (1 V/cm) and 300 mA. The unwinding and electrophoresis were done at 4 °C. After