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Titanium dioxide nanoparticles induce an adaptive inflammatory response and invasion and proliferation of lung epithelial cells in chorioallantoic membrane

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) studies have been performed using relatively high NPs concentration under acute exposure and limited studies have compared shape effects. We hypothesized that midterm exposure to low TiO_2 NPs concentration in lung epithelial cells induces carcinogenic characteristics modulated partially by NPs shape. To test our hypothesis we synthesized NPs shaped as belts (TiO_2-B) using TiO_2 spheres (TiO_2-SP) purchased from Sigma Aldrich Co. Then, lung epithelial A549 cells were low-exposed (10 $\mu g/cm^2$) to both shapes during 7 days and internalization, cytokine release and invasive potential were determined. Results showed greater TiO_2-B effect on agglomerates size, cell size and granularity than TiO_2-SP. Agglomerates size in cell culture medium was 310 nm and 454 nm for TiO_2-SP and TiO_2-B, respectively; TiO_2-SP and TiO_2-B induced 23% and 70% cell size decrease, respectively, whilst TiO_2-SP and TiO_2-B induced 7 and 14-fold of granularity increase. NO_x production was down-regulated (31%) by TiO_2-SP and up-regulated (70%) by TiO_2-B. Both NPs induced a transient cytokine release (IL-2, IL-6, IL-8, IL-4, IFN-γ, and TNF-α) after 4 days, but cytokines returned to basal levels in TiO_2-SP exposed cells while TiO_2-B induced a down-regulation after 7 days. Midterm exposure to both shapes of NPs induced capability to degrade cellular extracellular matrix components from chorioallantoic membrane and Ki-67 marker showed that TiO_2-B had higher proliferative potential than TiO_2-SP. We conclude that midterm exposure to low NPs concentration of NPs has an impact in the acquisition of new characteristics of exposed cells and NPs shape influences cellular outcome.

1. Introduction

The development of nanotechnology and the application of engineered nanomaterials (NM) have dramatically increased in the last decade (Forloni, 2012; Roco, 2011). NM are defined as a natural, incidental or manufactured material containing at least 50% of the particles with one or more external dimensions in the size range 1–100 nm, in an unbound state or as an aggregate or agglomerate (European Union). Among NM, titanium dioxide nanoparticles (TiO_2 NPs) is the one of the most manufactured NM in worldwide with 10,000 t of annual production (Piccinno et al., 2012). TiO_2 NPs are widely used in industry for plastics, papers, inks, medicines, food products, cosmetics, toothpastes and skin care products, among others. In spite of oral and dermal are important routes of NM exposure in occupational settings, the

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effects of inhalation have gained special concern (Ponce, 2013). In fact, International Agency for Research of Cancer (IARC, 2010) has classified the TiO_2 in the group 2B as a *possible carcinogenic to human*, and National Institute for Occupational Safety and Health (NIOSH, 2011) has determined that TiO_2 is *potential occupational carcinogen* by inhalation.

The effects induced by TiO_2 NPs exposure are currently under investigation in several experimental models, which includes acellular assays, bioaccumulation, and toxicity in different tissues after inhalation, dermal and oral exposure. The studies also take into account cellular alterations in different types of cells including lung cells, fibroblast, kidney, hepatic and blood cells, but also side effects after TiO_2 NPs interaction with microorganism such as *Daphnia magna* (Tan and Wang, 2014b). Direct effects after acute exposure are critical to understand the mechanism of TiO_2 NPs toxicity; nevertheless, effects after midterm exposure and long-term exposure are less explored, particularly in *in vitro* systems. In addition, acquirement of new properties beyond TiO_2 NPs toxicity has even been less investigated.

On the other hand, TiO_2 NPs production is extending to new shapes, including fibers, wires and belts (TiO_2-B). Remarkably, NPs shaped as belts has distinctive impact because their promising properties (Wang et al., 2014), for example, TiO_2-B can be used to coat transition-metal sulfide nanosheets that are used for electrode materials (Mao et al., 2014). TiO_2-B can also be used to form heterostructures with graphene oxide showing better photocatalytic activity (Sang et al., 2014) and also with cerium dioxide to form heterostructures with enhanced activity to degrade organic pollutants (Tian et al., 2013). However, the exact impact of TiO_2-B in human health has not investigated and indeed, the exact TiO_2 mechanism to act as a possible carcinogen has not completely described. What is known is that after inhalation, TiO_2 NPs causes inflammation (Ponce, 2013; Bonner et al., 2013; Xia et al., 2013; Baisch et al., 2014) with increase in interleukin (IL)-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ release (Müller et al., 2010; Sun et al., 2012a; Liu et al., 2013) and retention in alveolar regions (Ferin et al., 1990, 1992; Oberdörster et al., 1990). In lung tissue, cells can internalize TiO_2 NPs in vesicles in the cytoplasm (Simon-Deckers et al., 2008) with a concomitant generation of reactive oxygen species (ROS) (Montiel-Dávalos et al., 2012) and triggering apoptosis (Tang et al., 2013). Cellular effects of TiO_2-B have less been described, but there is some information about their toxicity including inflammation (Silva et al., 2013), apoptosis and cell cycle arrest (Tilton et al., 2014). In an overall view, TiO_2-B seem to display greater effects than TiO_2-SP (Silva et al., 2013) because shape of NPs has an impact on biological effects (Kettler et al., 2013). However, in terms of *in vitro* experiments, most of the studies have performed using relatively high concentration of NPs (1–200 μg/mL) and effects have been evaluated after short term of exposure (24–72 h). Even if *in vitro* experiments cannot be compared to real exposure, it is accepted that in occupational settings TiO_2 NPs exposure occurs in low levels and longer periods and a better approach could be reached using low concentration of NPs and midterm exposure. In this regard, cells exposed under those conditions may not exhibit toxicity; however some alterations may impact cellular function of exposed cells. Based on the above information, we aimed to investigate whether two relevant shapes of TiO_2 NPs for industry induce changes in cell size and release of inflammatory cytokines and if exposure promotes new characteristics such as invasive and proliferative capabilities in lung epithelial cells after a low and midterm exposure. We want to underline that midterm exposure in this study refers to a continuous TiO_2 NPs exposure for 7 days, which is different from previous studies usually performed by less than 72 h of NPs treatments. Midterm exposure could be compared in some extent to sub-chronic term, which refers usually *in vivo* experiments for several months but less than the lifetime of the exposed organism. In this study, invasive and proliferative capabilities of exposed cells were evaluated in chick chorioallantoic membrane (CAM) assay, which is a more suitable model to study invasive and proliferative potential of cancer cells (Cimpean et al., 2008; Lokman et al., 2012). We want to highlight that this model consist of the chorionic epithelium separated from the underlying allantoic membrane by connective tissue and extracellular matrix proteins including collagen type I, integrin alpha (v)beta3, fibronectin and laminin (Giannopoulou et al., 2001), which mimics physiological basement membranes that cancer cells need to break through during invasion process.

2. Materials and methods

2.1. TiO_2 NPs characterization

TiO_2-SP and TiO_2-B size and morphology were observed by scanning electron microscopy (JEOL 5800-LV, Japan) at 5000×, 15 kV. Agglomerate size and morphology were analyzed by the transmission electron microscopy (JEOL JEM 1010, Japan) and images were taken at 75,000× 60 kV. Agglomerate size was measured by dynamic light scattering (DLS) (NS-Zeta sizer Malvern, UK) and Zeta Potential of TiO_2-SP and TiO_2-B suspensions in F12K medium supplemented with 10% fetal bovine serum (FBS) were measured by Zeta Plus meter Brookehaven (USA). Raman spectra of NPs were measured in 300–2000 cm^{-1} spectral region with an Almega XR dispersive Raman spectrometer. An Olympus microscope (100× and 0.90 NA: numerical aperture) was used both for focusing the laser on solid samples and for collecting the scattered light in a 180° backscattering configuration. In addition, the Raman spectra were accumulated over 25 s with a resolution of ~4 cm^{-1}, the excitation source was 532 nm radiation from a Nd:YVO_4 laser (frequency-doubled) and the laser power on the sample was 2.5 mW.

2.2. TiO_2-B synthesis

To synthesize belts, which are not commercially available yet, 0.5 g of anatase titanium dioxide spheres (Aldrich, cat# 637254) were added to 35 mL of 10 M NaOH solution. The solution was stirred in ultrasonic for 10 min, then was poured into a steel-container and sealed. To the hydrothermal treatment, the container was heated in a muffle at 200 °C for 24 h. Wet TiO_2 was obtained and was repeatedly washed with 0.1 M HCl and distilled water until pH 7, then was centrifuged at 3.5g for 7 min. TiO_2 pellet was calcinated at 700 °C for 30 min at a ramp rate of 1 °C/min (Hamilton et al., 2009).

2.3. Cell culture and TiO_2-SP and TiO_2-B treatments

The lung adenocarcinoma epithelial cell line (A549 cell line) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cells were cultured with F12K medium (In vitro S.A., ME-038) supplemented with 10% FBS. Cell culture was maintained in an incubator at 37 °C with humidity 95% and 5% CO_2. After 80% of confluence, 2.5×10^5 cells were seeded in plates (24 cm^2). TiO_2-SP and TiO_2-B stocks of 1 mg/mL were prepared in F12K medium supplemented with 10% FBS and sonicated at 60 Hz for 30 min to TiO_2-SP and 60 min to TiO_2-B. Immediately, cell culture was exposed to final concentration of 1, 5 and 10 μg/cm^2 of TiO_2-SP and TiO_2-B for 7 days.