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Original article

Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro

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ABSTRACT

Manufacturing or using nanomaterials may result in exposure of workers to nanoparticles. Potential routes of exposure include skin, lung and gastrointestinal tract. The lack of health-based standards for nanomaterials combined with their increasing use in many different workplaces and products emphasize the need for a reliable temporary risk assessment tool. Therefore, the aim of this work was to explore the effects of different doses of titanium dioxide nanoparticles on human gastric epithelial cells in vitro. We analyzed proliferation by MTT assay, apoptosis by Tunel, migration by injury assay, oxidative stress by determining GSH/GSSG ratio and DNA damage by Comet assay on nanoparticle-treated AGS human gastric epithelial cell line in comparison to controls. We show and discuss the tumor-like phenotypes of nanoparticles-exposed AGS cells in vitro, as increased proliferation and decreased apoptosis. Our results demonstrate for the first time that nanoparticles induce tumor-like phenotypes in human gastric epithelial cells.

1. Introduction

Nanotechnology is one of the fastest growing sectors of the high-tech economy. There are more than 200 separate consumer products alone using nanomaterials with personal, commercial, medical, and military uses [1,2]. Engineered nanomaterials with dimension of 100 nm or less provide us a wide range of novel applications in the electronics, healthcare, cosmetics, technologies and engineering industries. The exploitation of properties inherent to materials at the nanoscale has initiated innovative approaches to technologies which shape our world. Lack of toxicological data on nanomaterials makes it difficult to determine if there is a risk associated with nanomaterials exposure. Thus, there is an urgent need to develop rapid, accurate and efficient testing strategies to assess health effect of these emerging materials [3].

Nano-sized or ultrafine TiO_2 (< 100 nm) is used increasingly in other industrial products, such as toothpastes, sunscreens, cosmetics, pharmaceuticals, and food products [4]. Human exposure may occur during both manufacturing and use. Such widespread use and its potential entry in the body through dermal, ingestion, and inhalation routes suggest that TiO_2 nanoparticles pose a potential exposure risk to humans, livestock, and the ecosystem [4–9].

However, it has been difficult to establish a comprehensive mechanism of nanoparticle cytotoxicity based on previous, and rather inconsistent, observations. For instance, some reports indicated that exposure of cells to TiO_2 leads to lipid peroxidation, DNA damage, caspase activation followed by micronuclei formation, chromatin condensation and eventual cell death via apoptosis. However, other investigators have reported that TiO_2 nanoparticle exposure instead causes plasma membrane damage and decrements in mitochondrial function. There are even reports that TiO_2 exposure does not lead to membrane damage, caspase activation or cell death [10].

These conflicting results are likely caused by variations in experimental procedures. Further differences such as protein adsorption prior to cell exposure and particle dispersion/agglomeration have also been recently shown to play important roles. These input variables are likely related to varied toxicological outputs. It is of paramount importance to identify the mechanistic

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response of exposure-prone cells to nanomaterials as they are not only potential environmental exposure hazards, but are continuously employed in biomedical applications in many different tissues and compartments inside the body [10].

We have therefore carried out a comparative study on the cytotoxic effects of common, widely used TiO_2 nanoparticles on gastric epithelial cells. Two dispersion media were used for this purpose: one protein rich and the other with one type of protein alone. We evaluated proliferation, apoptosis, oxidative stress and genotoxicity of exposed cells. To the best of our knowledge, this is the first report addressing cytotoxicity of nanomaterials on gastric epithelial cells.

2. Materials and methods

2.1. Nanoparticles

TiO_2 nanoparticles (commercial grade, Aeroxide TiO_2 P-25, primary size 21 nm, 80/20 anatase/rutile) were obtained from Degussa Corp. (Parsippany, NJ). TiO_2 nanopowder 637254 (titanium (IV) oxideanatase, < 25 nm) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Particle preparation and characterization

TiO_2 NPs were suspended in two different dispersion media: Milli-Q water and RPMI supplemented with 10% FBS or 2% BSA in phosphate-buffered saline (PBS) and probe sonicated at 30 W for 5 min (1.5 min pulse on and 1 min pulse off for two times and a final pulse of 2 min).

The average hydrodynamic size, size distribution and zeta potential of TiO_2 NPs in water were determined by dynamic light scattering (DLS) and phase analysis light scattering respectively using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN3600, Malvern Instruments Ltd., Malvern, UK).

Before use, TiO_2 NPs stock suspension (150 μg/mL) in medium was serially diluted to desired concentrations in fresh suspension medium. All samples were prepared under sterile conditions.

2.3. Particle treatment

The treatment experimental design consisted of serial concentrations of TiO_2 NPs suspended in two different media: RPMI supplemented with 10% FBS or 2% BSA in phosphate-buffered saline (PBS), applied to cells from a single passage to minimize confounding of comparisons by passage-to-passage variation of the cultured cells. Each multiwell cell culture plate included negative controls.

2.4. Cell line and cell culture

AGS (gastric epithelial cancer) cells were cultured and maintained at 37 °C in a 5% CO_2 humidified atmosphere in RPMI medium (Sigma) with 10% FBS (Sigma) and 1% penicillin/streptomycin (Sigma). Cells were passaged every 5 days. Before treatments with nanoparticles suspended in BSA, cells were allowed to reach 80% confluence before serum-starved for 16 h.

2.5. Trypan blue exclusion assay

The trypan blue exclusion method was used to assess cell viability. AGS cells were plated and incubated until 80% confluency. The cells were treated with TiO_2-nanoparticles. After treatment, the cells were harvested by trypsinization and counted under microscope after trypan blue staining. Three independent experiments were carried out based on the following formula: cell viability percentage = number of cells in drug treatment group/number of cells in control group $\times$ 100%.

2.6. Proliferation assay

The CellTiter 96 AQ nonradioactive cell-proliferation assay (Promega) was used to assess cell viability. The assay is composed of the tetrazolium compound MTS and an electron coupling reagent, PMS. MTS is reduced by viable cells to formazan, which can be measured with a spectrophotometer by the amount of 490 nm absorbance. Formazan production is time-dependent and proportional to the number of viable cells. AGS cells were cultured in 0.1 mL RPMI media in 96-well flat-bottomed plates. Cultures were seeded at 1×10^4 cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μL reagent was added per well, and cells were incubated 1 h before measuring absorbance at 490 nm. Background absorbance from the control wells was subtracted. Studies were performed in triplicate for each experimental condition.

2.7. Apoptosis

Apoptosis in cell cultures was assessed with the in situ cell death detection kit, fluorescein (TUNEL technology) (Roche), analyzed by fluorescence microscopy. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling) was performed according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a magnification 200× field. One thousand nuclei were evaluated. Three independent experiments were performed.

2.8. Oxidative stress assay

Oxidative stress was analysed by evaluation of GSHt, GSH and GSSG levels The intracellular levels of GSH and GSSG in TiO_2-nanoparticle-treated AGS cells were evaluated by the DTNB-GSSG reductase recycling assay, as previously described [11]. After a 3-h treatment with 150 μg/mL TiO_2 nanoparticles, the treated cells were lysed and proteins were precipitated with 5% $HClO_4$. After centrifugation (16,000 g, 10 min, 48 °C), the supernatant obtained was used for the determination of GSHt, GSH and GSSG by spectrophotometry at 412 nm.

2.9. Comet assay

After treatment, cells were washed twice with prechilled PBS (Mg^{2+} and Ca^{2+}-free), centrifuged at 78 × g for 5 min and resuspended in PBS. Cell viability was over 85% for the tested dose in this study as assessed using trypan blue dye-exclusion staining. The alkaline version of the comet assay was performed as described by [12] with minor modifications. Briefly, cells collected by centrifugation (9000 rpm for 3 min) and suspended in 60 μL of 0.6% low-melting-point agarose (LMA) in PBS (pH 7.4) were dropped onto a frosted slide precoated with a layer of 1% normal melting point agarose. Slides were placed on ice for 4 min and allowed to solidify. Coverslips were then removed and slides were immersed in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TrisBase, 0.25MNaOH, pH 10) for 1 h at 4 °C, in the dark. After lysis, slides were placed on a horizontal