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Characterizing the inhibitory action of zinc oxide nanoparticles on allergic-type mast cell activation

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ABSTRACT

The development of nanoparticles (NPs) for commercial products is undergoing a dramatic expansion. Many sunscreens and cosmetics now use zinc oxide (ZnO) or titania (TiO_2) NPs, which are effective ultraviolet (UV) filters. Zinc oxide topical creams are also used in mild anti-inflammatory treatments. In this study we evaluated the effect of size and dispersion state of ZnO and TiO_2 NPs, compared to "bulk" ZnO, on mast cell degranulation and viability.

ZnO and TiO_2 NPs were characterized using dynamic light scattering and disc centrifugation. Rat basophilic leukaemia (RBL-2H3) cells and primary mouse bone marrow-derived mast cells (BMMCs) were exposed to ZnO and TiO_2 NPs of different sizes (25–200 nm) and surface coatings at concentrations from 1 to 200 μg/mL. The effect of NPs on immunoglobulin E (IgE)-dependent mast cell degranulation was assessed by measuring release of both β-hexosaminidase and histamine *via* colorimetric and ELISA assays. The intracellular level of Zn^{2+} and Ca^{2+} ions were measured using zinquin ethyl ester and Fluo-4 AM fluorescence probes, respectively. Cellular viability was determined using the soluble tetrazolium-based MTS colorimetric assay.

Exposure of RBL-2H3 and primary mouse BMMC to ZnO NPs markedly inhibited both histamine and β-hexosaminidase release. This effect was both particle size and dispersion dependent. In contrast, TiO_2 NPs did not inhibit the allergic response. These effects were independent of cytotoxicity, which was observed only at high concentrations of ZnO NPs, and was not observed for TiO_2 NPs.

The inhibitory effects of ZnO NPs on mast cells were inversely proportional to particle size and dispersion status, and thus these NPs may have greater potential than "bulk" zinc in the inhibition of allergic responses.

1. Introduction

Metal oxide ZnO and TiO_2 NPs are currently used in various commercial products, such as sunscreens and cosmetics (Cross et al., 2007; Popov et al., 2005; Turney et al., 2012). The global production of these engineered NPs in sunscreen products was estimated to be up to 1000 tonnes during 2003 alone, although there has been some decline in recent years partially due to formulation costs and largely unsubstantiated public safety concerns (Borm et al., 2006). The formulation of NPs into these products is often commercially sensitive, but it is not uncommon for sunscreen preparations to contain surfactant and other dispersants that prevent ZnO and TiO_2 NPs from aggregating (Monteiro-Riviere et al., 2011; Smijs and Pavel, 2011), to ensure that the NPs are dispersed and well-spread on the skin to give a more transparent and desirable appearance. There has been much discussion of nanoparticle health risk assessments for these metal oxide containing nanosunscreen preparations Nohynek et al., 2007), though the more recent evidence suggests that penetration through the skin is minimal, even for very small NPs (Leite-Silva et al., 2013). Whilst safety is clearly paramount, these ZnO NPs are like many other nanostructures (Nazir et al., 2014), in that they exhibit unique bioactivities that can be exploited to directly manipulate cellular systems (Saptarshi et al., 2013).

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Many studies have focused on investigation of the cytotoxicity of ZnO and TiO_2 NPs in cancer cell lines (Landsiedel et al., 2010; Nohynek and Dufour, 2012; Shen et al., 2013; Schilling et al., 2010). However, there have been few studies conducted in primary (non-cancerous) cells, or involving a direct comparison with respective cancer cell lines. Akhtar et al. (2012) studied the cytotoxicity of ZnO NPs in three different human cancer cells lines (hepatocellular carcinoma (HepG2), lung adenocarcinoma (A549), and bronchial epithelial (BEAS-2B) cells) and two primary rat cell types: astrocytes and hepatocytes. Although they did not directly compare between primary and cancer cells from the same species, they did report that ZnO NPs induced death of cancerous cells, whilst not causing cytotoxicity in primary cells at the same concentrations. Similarly, Taccola et al. (2011) demonstrated that ZnO NPs induced cell death in proliferating pluripotent mesenchymal stem cells, but did not exhibit cytotoxic effects to non-proliferating osteogenically differentiated mesenchymal stem cells. In addition, it has been observed that ZnO NPs and Zn^{2+} increased the cellular uptake of anticancer drugs by a human leukaemia (K562) cell line (Guo et al., 2008). The focus of most of the literature has been the induction of cytotoxicity using relatively high concentrations of ZnO, and whilst immune modulation is often observed (Sahu et al., 2014), it is often strongly correlated to cell death. It is interesting that whilst ZnO has been historically used as a topical anti-inflammatory agent for skin rashes and inflammation (Prasad, 2008), there are few published studies on the effects of ZnO or TiO_2 NPs related to their potential immunomodulatory effects on mast cells. Very recently, Kim et al. (2014) demonstrated the potential of ZnO NPs to inhibit cytokine production in human mast cells, findings that are very complementary to the study presented here.

Widely used *in vitro* models of IgE-mediated responses in mast cells include the RBL-2H3 and HMC-1 cancer cell lines, and primary BMMCs (Elbaz et al., 2012; Kalesnikoff and Galli, 2011). Degranulation assays are commonly used to quantify the biological activity of mast cells initially sensitized by a recombinant IgE monoclonal antibody and subsequently challenged with a multivalent antigen. The β-hexosaminidase, a lysosomal enzyme, is stored in mast cell granules and released concomitantly with histamine, following mast cell activation by cross-linking surface receptor-bound IgE with antigens (Tkaczyk et al., 2006). Therefore, β-hexosaminidase release into cell culture media is a very useful *in vitro* marker for assessing mast cell activation and degranulation (Xia et al., 2011). Interestingly, the role of β-hexosaminidase in allergic diseases is not well understood (Tomasiak et al., 2008). The second biomarker of mast cell degranulation, histamine, has been more thoroughly studied and is a directly responsible for inducing the major symptoms of type I allergic responses, such as bronchoconstriction, mucous secretion, vasodilation, vascular permeability, itching, atopic dermatitis and asthma (Huang et al., 2009). Measuring both of these degranulation markers together may provide a better insight into their functional differences, as well as providing internal validation of a given set of mast cell experimental data.

Preliminary examinations of the effects of ZnO NPs on mast cell lines have been reported. A study by Yamaki and Yoshino (2009) found that aggregated ZnO NPs inhibited the release of β-hexosaminidase from RBL-2H3 cells activated with anti-ovalbumin-IgE plus antigen. In a related study, Chen et al. (2012) observed that TiO_2 NPs directly stimulated histamine release from RBL-2H3 cells *via* a Ca^{2+} dependent pathway, but they did not investigate the effects of TiO_2 NPs on IgE-activated mast cells. Neither of these studies took into consideration the potential differences in responses that may arise in primary mast cells, nor the potential effects of NP dispersion on mast cell degranulation.

The mechanism of ZnO NP's inhibitory effects on mast cells is also unclear, although there is some indication that this is related to intracellular Ca^{2+} levels. For example, Hide and Beaven (1991) found that Zn^{2+} inhibited IgE-activated mast cell degranulation through inhibition of Ca^{2+} influx. In contrast, Yamaki and Yoshino (2009) postulated that ZnO NP inhibitory effects were not due to Ca^{2+} ion inhibition, but were a result of the inhibition of protein phosphorylation.

In this study, we sought to determine whether Zn-modulated suppression of mast cells could be enhanced through the use of nanoparticulate ZnO. We report the effects of size and dispersion state of ZnO NPs, as well as $ZnCl_2$ (as a control for Zn^{2+} ion exposure) and two different crystalline forms of TiO_2 NPs (as non-Zn containing NP controls), on degranulation activity and cytotoxicity in both the RBL-2H3 cancer cell line and primary mouse BMMCs.

2. Materials and methods

2.1. Chemicals and reagents

This study used the following chemicals and reagents: 0.05% (w/v) trypsin–EDTA (Invitrogen, USA); conditioned medium from the mouse monoclonal IgE (mIgE) secreting cell line TIB-142 (ATCC TIB-142™); 2,4,6-trinitrophenyl hapten conjugated to bovine serum albumin (TNP-BSA) (BioSearch Technology, USA); Triton-X 100, p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG), glycine and zinquin (Sigma Aldrich, USA); Fluo-4, AM fluorescence probe (Invitrogen); histamine-ELISA kit (Life Science Format, Oxford Biomedical Research, USA); and CellTiter 96® AQueous Solution (MTS) kit (Promega, USA).

2.2. Nanoparticle characterization

The nanoparticles and particulates (bulk form), with and without surfactant dispersant (polyacrylate copolymer, Orotan 731 DP), used in this study included: pristine zinc oxide at $d_{90} = 36 \pm 4$ nm and 250 ± 50 nm (ZnO 30 and ZnO 200) and their surfactant-treated analogues (sZnO 30 and sZnO 200) (Micronisers Pty. Ltd., Melbourne, Victoria, Australia); 34 nm rutile titania (TiO_2 R) and 25 nm anatase (P25) titania (TiO_2 P25) (Evonik Industries, Dandenong, VIC, Australia). Nanoparticle size within the cell growth media was estimated using a DC1800 disc centrifuge (CPS instruments, Stuart, FL, USA), with an RPMI-1640 media (Invitrogen, USA) and sucrose gradient. Size distribution and agglomeration of nanoparticle suspensions were measured in both RPMI-1640 media and RPMI-1640 media plus 10% FBS (Sigma–Aldrich, USA).

2.3. Culture of RBL-2H3 cells

The RBL-2H3 cells (American Type Culture Collection, USA) were cultured in RPMI-1640 media containing 10% (v/v) FBS with gentamycin, glucose, pyruvate, 2-mercaptoethanol and L-glutamine supplementation (Invitrogen, USA), maintained in a humidified incubator at 37 °C and 5% CO_2. When cultures reached 90% confluence, cells were detached by 0.05% trypsin-EDTA and subcultured into fresh media, with subsequent passaging every 2 days.

2.4. Preparation of BMMCs

The femurs of six control mice (C57Bl6) were donated from unrelated experiments, and the bone marrow cells flushed from the excised femurs using RPMI-1640 and a 1 mL syringe and 20 G needle (Terumo). Cells from each mouse were cultured separately in RPMI-1640 medium, supplemented with 10% (v/v) FBS, gentamycin, glucose, pyruvate, 2-mercaptoethanol, L-glutamine and interleukin-3 (5 ng/mL) in a humidified incubator at 37 °C and 5% CO_2. The cells in suspension were removed from the adherent cells in the flask, centrifuged and replenished with fresh media and