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Zinc oxide nanoparticles induce migration and adhesion of monocytes to endothelial cells and accelerate foam cell formation

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ABSTRACT

Metal oxide nanoparticles are widely used in industry, cosmetics, and biomedicine. However, the effects of exposure to these nanoparticles on the cardiovascular system remain unknown. The present study investigated the effects of nanosized TiO_2 and ZnO particles on the migration and adhesion of monocytes, which are essential processes in atherosclerogenesis, using an *in vitro* set-up of human umbilical vein endothelial cells (HUVECs) and human monocytic leukemia cells (THP-1). We also examined the effects of exposure to nanosized metal oxide particles on macrophage cholesterol uptake and foam cell formation. The 16-hour exposure to ZnO particles increased the level of monocyte chemotactic protein-1 (MCP-1) and induced the migration of THP-1 monocyte mediated by increased MCP-1. Exposure to ZnO particles also induced adhesion of THP-1 cells to HUVECs. Moreover, exposure to ZnO particles, but not TiO_2 particles, upregulated the expression of membrane scavenger receptors of modified LDL and increased cholesterol uptake in THP-1 monocytes/macrophages. In the present study, we found that exposure to ZnO particles increased macrophage cholesterol uptake, which was mediated by an upregulation of membrane scavenger receptors of modified LDL. These results suggest that nanosized ZnO particles could potentially enhance atherosclerogenesis and accelerate foam cell formation.

Introduction

Evidence based on epidemiological and toxicological studies indicates that high concentrations of particle masses <2.5 μm (PM2.5) are associated with high risk of pulmonary complications, cardiovascular events, and death from cardiovascular disease (Araujo et al., 2008; Mar et al., 2000; Miller et al., 2007; Mills et al., 2007; Peters et al., 2001). The recent explosion in the field of nanotechnology provides promising potential applications of manufactured nanomaterials in a variety of areas (Donaldson et al., 2004). It has been demonstrated that the relative surface area of ultrafine carbon black or titanium dioxide (TiO_2) particles correlates with the degree of their toxicity (Hohr et al., 2002; Sager et al., 2008; Yamamoto et al., 2006). Some kinds of nanomaterials are also considered to generate reactive oxidant species, resulting in the induction of oxidative stress and inflammation (Nel et al., 2009; Xia et al., 2006). Oxidative stress is known to be involved in the pathogenesis of cardiovascular diseases, such as hypertension and atherosclerosis (Noma et al., 2007; Taniyama and Griendling, 2003). Therefore, there is a concern that nanomaterials could have a major impact on the cardiovascular system. However, the effects of exposure to newly developed metal oxide nanoparticles on the cardiovascular system remain elusive.

Atherosclerosis is a disease of the vasculature characterized by a chronic inflammation of the arterial wall and the formation of fibrotic plaques in the major arteries (Lusis, 2000). The process of atherosclerogenesis is initiated by the activation of endothelial cells, with subsequent migration of mononuclear cells and expression of adhesion molecules for inflammatory cells (Berk, 2008; Libby et al., 2009). In addition, a critical factor in the progression of atherosclerogenesis is the development of an oxidizing environment caused by the activation of macrophages that become loaded with oxidized low-density lipoprotein (LDL) and other lipids (Tsimikas and Miller, 2001). The formation of foam cells is crucial in the initiation and progression of atherosclerosis, and one of the critical steps in foam cell formation is the uptake of modified LDL by macrophages *via* scavenger receptors (Moore and Freeman, 2006).

Abbreviations: PMA, 12-myristate 13-acetate; DLS, dynamic light scattering; THP-1, human monocytic leukemia cells; HUVECs, human umbilical vein endothelial cells; ICP-MS, inductively coupled plasma mass spectrometry; ICAM-1, intracellular adhesion molecule-1; LDL, low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; PdI, polydispersity index; TiO_2, titanium dioxide; ZnO, zinc oxide.

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TiO_2 and zinc oxide (ZnO) are widely used in paints, pharmaceutical, and cosmetic industries. The rapidly developing field of nanotechnology becomes a source for human potential exposures to engineered nanoparticles by different routes: inhalation (respiratory tract), ingestion (gastrointestinal tract), dermal (skin), and injection (blood circulation) (Oberdörster et al., 2005). The present study investigated the effects of nanosized metal oxide particles on the migration and adhesion of monocytes, which are essential processes in atherosclerogenesis, using an *in vitro* set-up of human umbilical vein endothelial cells (HUVECs) and human monocytic leukemia cells (THP-1). We also examined the effects of exposure to nanosized metal oxide particles on macrophage cholesterol uptake and foam cell formation.

Methods

Nanoparticle preparation and characterization. TiO_2 nanoparticles (AEROXIDE TiO_2 P25; Degussa AG, Dusseldorf, Germany) with a primary diameter of 21 nm, and ZnO nanoparticles (MKN-ZnO-020; mkNANO, Mississauga, ONT, Canada) with a primary diameter of 20 nm were used in the present study. Nanoparticles were suspended in culture media and dispersed using sonicator (BRA NSON Sonifier model 450, Danbury, CT, 80% pulsed mode, 100 W, 15 min), as described previously (Wu et al., 2013). The hydrodynamic sizes of the particles in media were measured four times after 1 h on standing using the dynamic light scattering (DLS) technology with a Zetasizer Nano-S (Malvern Instruments, Worcestershire, UK). Dispersion status was described by the intensity-weighted hydrodynamic average diameter (z-average) and polydispersity index (PdI), which reflect the broadness of the size distribution (scale range from 0 to 1, with 0 being monodispersion and 1 being polydispersion) (Murdock et al., 2008).

Cell culture. HUVECs from Lonza Group (Basel, Switzerland) were cultured in endothelial basal medium-2 containing endothelial growth media supplement bullet kit (Lonza Group) at 37 °C in 5% CO_2. Cells were passaged with trypsin-EDTA, trypsin neutralizing solution and HEPES buffering solution (Lonza Group) every 2–3 days and experiments were performed between passages 3 and 4. THP-1 cells from the American Type Culture Collection (ATCC) (Rockville, MD) were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) containing 10% FBS with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2. For the differentiation of THP-1 monocytes into macrophage-like cells, THP-1 cells were treated with 162 nM 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO) for 16 h in RPMI 1640 medium containing 10% FBS.

Cell viability assay. HUVECs were seeded at 1.5×10^4 cells per well on 96-well plates overnight prior to the experiment. Nanoparticles were dispersed in complete medium, the final concentrations of nanoparticles ranged from 1 to 100 μg/ml. Cell viability was determined after incubation of suspended nanoparticles for 24 h by MTS assay as indicated by the CellTiter 96 AQueous One Solution (Promega, Madison, WI), which measures mitochondrial function and correlates with cell viability. After exposure, the cells were incubated with fresh medium (phenol red-free) containing MTS reagent for 1 h before absorbance measurements at 490 nm. The effect of nanoparticles on cell proliferation was calculated as the percentage of inhibition of cell growth with respect to the controls. THP-1 monocytes were seeded at 1.5×10^4 cells per well on 96-well plates, exposed to nanoparticles at a concentration ranging from 1 to 100 μg/ml for 3 h, and then treated with 162 nM PMA for 16 h in RPMI 1640 medium containing 10% FBS. Cell viability was determined by MTS as described above.

Inductively coupled plasma mass spectrometry. To evaluate the uptake of nanoparticles by the cells using inductively coupled plasma mass spectrometry (ICP-MS), confluent HUVECs in 150 mm dishes were exposed to 1, 5 and 10 μg/ml of TiO_2 or ZnO particles. After 16 h, cells were washed with Hanks' Balanced Salt Solution (HBSS; Invitrogen), detached using trypsin, and harvested with complete cell culture media. The cell suspensions were centrifuged at 200 ×*g* for 3 min at 4 °C. The cell pellet was suspended in 1 ml of HBSS, and the number of cells was calculated. The solutions were mixed with concentrated nitric acid (HNO_3; Wako, Osaka, Japan) to reach the final HNO_3 concentration of 3%, and then heated to 80 °C until cell content was dissolved, as described previously (Gojova et al., 2007). A blank control solution was prepared for ICP-MS reaction (without cells) by mixing 1 of the HBSS with the same amount of concentrated HNO_3; this solution was processed the same way as the sample solutions. Finally, the dissolved solutions were adjusted to a volume of 10 ml with 3% HNO_3 in water and used for ICP-MS analysis. The concentrations of Ti and Zn were determined using Agilent ICP-MS 7700× (Agilent Technologies, Santa Clara, CA).

Detection of intracellular free zinc ion. Intracellular free zinc ion was visualized using a fluorescent, membrane permeable probe Zinquin ethyl ester (Dojindo, Kumamoto, Japan) as described previously (Zalewski et al., 1994). Briefly, HUVECs (1×10^5 cells/ml) were seeded on 24-well plates. After 16 h, cells were exposed to 10 μg/ml of TiO_2 or ZnO for 16 h. After washing with HBSS three times, the cells were treated with 25 μM of Zinquin ethyl ester for another 30 min at 37 °C. Cells were washed with HBSS and observed using a fluorescent microscope; FSX100 (Olympus, Tokyo, Japan).

Monocyte chemotaxis assay. HUVECs (1×10^5 cells/ml) were seeded on 24-well plates and allowed to adhere to the plate for overnight. HUVECs were exposed to TiO_2 or ZnO (both at 1, 5, 10 μg/ml) particles for 16 h, centrifuged (10,000 ×*g*, 10 min) to remove any suspended nanoparticles, and then the supernatant was obtained. The supernatants were harvested and used as the chemoattractant in the lower chamber of Cell Culture inserts (8.0 mm pore size, 24-well plates; BD Falcon, Franklin, NJ), as described previously (Shaw et al., 2011) with minor modification. THP-1 cells were loaded into the upper chamber ($n = 3$, 2.5×10^5 cells/well) and incubated for 2 h at 37 °C (Fig. 1A). Cells that had actively migrated through the membrane were fixed (100% ethanol; 5 min) and stained with crystal violet (Sigma Aldrich). Transmigration was quantified by counting the number of cells present in four randomly selected fields using a light microscope at a magnification of ×200. After exposure of HUVECs to different doses (1, 5, or 10 μg/ml) of TiO_2 or ZnO particles for 16 h at 37 °C, monocyte chemotactic protein-1 (MCP-1) levels were measured using ELISA (eBioscience, San Diego, CA) according to the protocol supplied by the manufacturer.

Cell adhesion assay. The adhesion of THP-1 cells to HUVECs was assessed as described previously (Choi et al., 2003) with minor modifications. HUVECs (1.5×10^4 cells) were grown overnight in 96-well plates at 37 °C. The cells were exposed to different doses (1, 5, or 10 μg/ml) of TiO_2 or ZnO particles for 16 h at 37 °C and prior to the adhesion assay, washed three times with HBSS containing 0.1% BSA. THP-1 cells were suspended at a density of 1.0×10^6 cells/ml of 0.1% BSA/HBSS and labeled with 1 μM of calcein-AM (BD Bioscience, Franklin Lakes, NJ) by 30 min incubation at 37 °C, followed by three washings with 0.1% BSA/HBSS. Labeled THP-1 cells were then incubated with HUVECs exposed to nanoparticles for 30 min at 37 °C. Nonadherent cells were removed by careful three-time washings with 0.1% BSA/HBSS. The adherence of calcein-labeled THP-1 cells was quantified by counting the number of endothelial monolayers using a fluorescent microscope; FSX100.

For western blot analysis, HUVECs were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Santa Cruz, Dallas, TX). The concentration of extracted protein was measured in triplicate using the Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA). Protein