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Nanoparticles up-regulate tumor necrosis factor-α and CXCL8 *via* reactive oxygen species and mitogen-activated protein kinase activation

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ABSTRACT

Evaluating the toxicity of nanoparticles is an integral aspect of basic and applied sciences, because imaging applications using traditional organic fluorophores are limited by properties such as photobleaching, spectral overlaps, and operational difficulties. This study investigated the toxicity of nanoparticles and their biological mechanisms. We found that nanoparticles, quantum dots (QDs), considerably activated the production of tumor necrosis factor (TNF)-α and CXC-chemokine ligand (CXCL) 8 through reactive oxygen species (ROS)- and mitogen-activated protein kinases (MAPKs)-dependent mechanisms in human primary monocytes. Nanoparticles elicited a robust activation of intracellular ROS, phosphorylation of p47phox, and nicotinamide adenine dinucleotide phosphate oxidase activities. Blockade of ROS generation with antioxidants significantly abrogated the QD-mediated TNF-α and CXCL8 expression in monocytes. The induced ROS generation subsequently led to the activation of MAPKs, which were crucial for mRNA and protein expression of TNF-α and CXCL8. Furthermore, confocal and electron microscopy analyses showed that internalized QDs were trapped in cytoplasmic vesicles and compartmentalized inside lysosomes. Finally, several repeated intravenous injections of QDs caused an increase in neutrophil infiltration in the lung tissues *in vivo*. These results provide novel insights into the QD-mediated chemokine induction and inflammatory toxic responses *in vitro* and *in vivo*.

Introduction

Colloidal semiconductor nanoparticles, or quantum dots (QDs), are single crystals with diameters of a few nanometers. Nanoparticle size can be precisely controlled by varying the reaction time, temperature, and ligand molecules. Recently, QDs have emerged as promising fluorescent markers for *in vitro* and *in vivo* imaging, because in contrast to organic fluorescence dyes, they have unique optical properties such as bright and photostable fluorophores with a broad excitation but narrow emission wavelength range (Bruchez, 2005; Medintz et al., 2005). The use of QDs for tracking noninvasive intracellular events facilitates basic research on underlying mechanisms as well as improves the clinical translation of basic research findings (Kiessling, 2008). However, an important issue concerning the toxicity of nanoparticles has recently arisen, because there is little information about the toxicological effects or detailed biological mechanisms such as the induction of inflammatory toxic responses. Therefore, a study about the toxicity of nanoparticles is essential to validate their biological application *in vivo*.

In general, QDs are composed of periodic groups II–VI (e.g., CdSe) or III–V (e.g., InP) materials (Alivisatos et al., 2005). Although the sizes of QDs can be significantly controlled by synthetic conditions or surface modifications, their diameters can range from 1 to 25 nm (Clift et al., 2008). Recent technical progress in the field of QD development has shown that water-soluble and highly luminescent nanoparticles can be easily obtained (Medintz et al., 2005; Yu et al., 2006; Biju et al., 2008). Nevertheless, much work remains to be done to examine potential inflammatory and cytotoxic properties of QDs in human cell systems and the effects of the size and raw materials of QDs on their potential toxicity.

The relatively recent introduction and rapid expansion of the nanotechnology field may contribute to the incidence of adverse

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health effects, although controlled epidemiological studies are basically non-existent (Li et al., 2008). As foreign particles, nanoparticles are able to affect host immune responses such as the release of inflammatory cytokines and matrix metalloproteinases (MMPs) and the generation of reactive oxygen species (ROS) (Wan et al., 2008). A recent study has demonstrated that inflammatory cytokine tumor necrosis factor (TNF)-α mRNA expression was increased significantly in nano-sized carbon black-treated mice (Tin-Tin-Win-Shwe et al., 2008). In addition, titanium dioxide nanoparticles have been shown to induce pulmonary toxicity and to up-regulate chemokines that may be responsible for pulmonary emphysema development and alveolar epithelial cell apoptosis (Chen et al., 2006).

The cytotoxicity of QDs can be attributed to the leaching of harmful metals from their nanocrystal core (QD core degradation), their ability to induce generation of ROS, or interactions of QDs with intracellular components leading to loss of function (Hardman, 2006). Redox-responsive signaling pathways induced by nanoparticle-mediated oxidative stress have been implicated in the development of adverse effects through the activation of ubiquitously expressed mitogen-activated protein kinases (MAPKs) (Beck-Speier et al., 2005; Donaldson et al., 2003). MAPKs are involved in signal transduction *via* the activation of numerous cellular proteins and transcription factors (Seger and Krebs, 1995). Therefore, the oxidative potential of nanoparticles is an important parameter for evaluating toxicity and triggers of inflammatory or immunological responses in a variety of cells and tissues.

Many intracellular targeting studies have revealed that Tat peptide-conjugated QDs are tethered to inner vesicular surfaces and are actively transported along microtubule tracks to microtubule organizing centers (Ruan et al., 2007). A recent study has demonstrated that QDs are endocytosed by dendritic cells and compartmentalized inside the cytoplasm (Sen et al., 2008). In addition, QDs were found to be localized within intracellular vacuoles in human epidermal keratinocytes (Ryman-Rasmussen et al., 2007). Although accumulating data provide new insights into the intracellular uptake and active transport of nanoparticles, it is not clear whether water-soluble QDs would undergo the same processes of cellular uptake and transport. Furthermore, there is very limited knowledge about the potential toxicity of water-soluble QDs (Biju et al., 2008).

This study investigated the potential toxicity of water-soluble nanoparticles (mercaptoacetic acid-conjugated CdSe), because QDs are most frequently used in aqueous solution (Aldana et al., 2001). We determined the effects of QDs on the generation of ROS and the production of TNF-α and CXC-chemokine ligand (CXCL) 8 in human primary monocytes. We also investigated the molecular mechanisms of the QD-mediated TNF-α and CXCL8 production by human primary monocytes. QDs clearly induced the phosphorylation of MAPKs, a crucial step for inflammatory responses and cellular activities, in a ROS-dependent manner. The study shows that the internalized QDs are trapped in endocytic vesicles in the cytoplasm before they are trafficked to lysosomal compartments. Finally, *in vivo* administration of QDs by intravenous injection resulted in an increased recruitment of neutrophils to lung tissues of mice, further suggesting a role for QDs in the induction of inflammatory responses.

Materials and methods

Synthesis of quantum dots and its surface modification. The QDs (CdSe/ZnS, core-shell nanoparticles) prepared in this work were synthesized in a three-component coordinating solvent mixture of hexadecylamine, trioctylphosphine (TOP), and trioctylphosphine oxide (TOPO) (Bruchez et al., 1998). The ZnS precursor, diethylzinc $(CH_2CH_3)_2$, was added to hexamethyl (disilanthaine)($(TMS)_2S$) at a 1:1 ratio in TOP, before adding to a TOPO-functionalized CdSe solution at 270 °C. This solution was sequentially washed with methanol and chloroform. The synthetic nanoparticles were stored in chloroform at 4 °C. The critical step to control particle size was manipulated with reaction times ranging from 30 s to 2 h. In this study, the particles (QD645) were obtained with a reaction time of 90 min.

The resulting organic-soluble CdSe/ZnS could be converted into water-soluble nanoparticles through ligand exchange with mercaptoacetic acid (MAA). In brief, the TOPO-capped CdSe/ZnS QDs (5 mg/ml) were mixed with a 1 M MAA. The transparent mixture was sonicated for 200 min at 60 °C. To eliminate the excess MAA, the aqueous solution of QDs was centrifuged after the addition of phosphate-buffered saline (PBS) (20 mM, pH 7.4). Finally, the recovered water-soluble QDs, with an average size of 10.5 nm, were stored in PBS at 4 °C. Preparations of QDs used in the experiments were tested for lipopolysaccharide (LPS) contamination by a *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD) and contained less than 20 pg/ml at the concentrations of QDs used in the following experiments.

Isolation and culture of human monocytes and cell lines. This study was reviewed and approved by the Institutional Research Board of Chungnam National University Hospital, and written informed consent was obtained from each participant. Venous blood was drawn from the healthy subjects into sterile blood collection tubes, and peripheral blood mononuclear cells were isolated by density sedimentation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated for 1 h at 37 °C, and nonadherent cells were removed by pipetting off the supernatant. Adherent monocytes were collected as previously described (Jung et al., 2006). Human monocyte and THP-1 (ATCC TIB-202) cells were maintained in RPMI 1640 complete medium (Gibco-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, nonessential amino acids, penicillin G (100 IU/ml), and streptomycin (100 μg/ml). THP-1 cells were treated with 4 nM phorbol-12-myristate-13-acetate (Sigma) for 24 h to induce differentiation into macrophage-like cells and then washed three times with PBS.

Cell viability assays. Cell viability of human monocytes was determined using a cell count assay kit (CCK8; Dojindo Molecular Technologies, Gaithersburg, MD), which measures the reduction of WST-8, a water-soluble tetrazolium salt, by dehydrogenases in viable cells. Human monocytes (100 μl of 200,000 cells ml^{-1}) were seeded in each well of a 96-well culture plate and allowed to grow overnight at 37 °C with 5% CO_2. AC toxin was added, and the cells were incubated at 37 °C for the indicated times. CCK8 solution (10 μl) was then added and incubated for 1 h at 37 °C. Absorbance was measured at 450 nm using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT). Viable cells were determined as a percentage of control cells.

Antibodies and reagents. Specific antibodies against phospho-(Thr202/Tyr204)-extracellular signal-regulated kinases (ERK) 1/2 and phospho-(Thr180/Tyr182)-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-(Ser345)-p47phox antibody, as described previously (Yang et al., 2007), was kindly provided by Dr. J. El-Benna (Inserm, Paris, France). LPS (*Escherichia coli* 026:B6) was purchased from Sigma. Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (v/v) as a solvent control. The specific nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenylene iodium (DPI), the ROS scavenger *N*-acetyl-L-cysteine (NAC), the xanthine oxidase inhibitor allopurinol, the p38 inhibitor SB203580, the MEK1/2 inhibitor U0126, and the JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA, USA).

Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. A sandwich ELISA was used to detect human and mouse TNF-α and CXCL8 (BD PharMingen Inc, San Diego, CA, USA) in culture supernatants. Assays were performed as recommended by the