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Matrix metalloproteinase-2 and -9 are induced differently by metal nanoparticles in human monocytes: The role of oxidative stress and protein tyrosine kinase activation

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Abstract

Recently, many studies have shown that nanoparticles can translocate from the lungs to the circulatory system. As a particulate foreign body, nanoparticles could induce host responses such as reactive oxygen species (ROS) generation, inflammatory cytokine and matrix metalloproteinase (MMP) release which play a major role in tissue destruction and remodeling. However, the direct effects of nanoparticles on leukocytes, especially monocytes, are still unclear. The objective of the present study was to compare the ability of Nano-Co and Nano-TiO₂ to cause alteration of transcription and activity of MMPs and to explore possible mechanisms. We hypothesized that non-toxic doses of some transition metal nanoparticles stimulate an imbalance of MMP/TIMP that cause MMP production that may contribute to their health effects. To test this hypothesis, U937 cells were treated with Nano-Co and Nano-TiO₂ and cytotoxic effects and ROS generation were measured. The alteration of MMP-2 and MMP-9 expression and activity of MMP-2 and MMP-9 after exposure to these metal nanoparticles were subsequently determined. To investigate the potential signaling pathways involved in the Nano-Co-induced MMP activation, the ROS scavengers or inhibitors, AP-1 inhibitor, and protein tyrosine kinase (PTK) inhibitors were also used to pre-treat U937 cells. Our results demonstrated that exposure of U937 cells to Nano-Co, but not to Nano-TiO₂, at a dose that does not cause cytotoxicity, resulted in ROS generation and up-regulation of MMP-2 and MMP-9 mRNA expression. Our results also showed dose- and time-related increases in pro-MMP-2 and pro-MMP-9 gelatinolytic activities in conditioned media after exposure of U937 cells to Nano-Co, but not to Nano-TiO₂. Nano-Co-induced pro-MMP-2 and pro-MMP-9 activity increases were inhibited by pre-treatment with ROS scavengers or inhibitors. We also demonstrated dose- and time-related decreases in tissue inhibitors of metalloproteinases 2 (TIMP-2) in U937 cells after exposure to Nano-Co, but not to Nano-TiO₂. However, neither Nano-Co nor Nano-TiO₂ exposure led to any transcriptional change of TIMP-1. The decrease of TIMP-2 after exposure to Nano-Co was also inhibited by pre-treatment with ROS scavengers or inhibitors. Our results also showed that pre-treatment of U937 cells with AP-1 inhibitor, curcumin, or the PTK specific inhibitor, herbimycin A or genistein, prior to exposure to Nano-Co, significantly abolished Nano-Co-induced pro-MMP-2

and-9 activity. Our results suggest that Nano-Co causes an imbalance between the expression and activity of MMPs and their inhibitors which is mediated by the AP-1 and tyrosine kinase pathways due to oxidative stress.

Keywords

Metal nanoparticles; Matrix metalloproteinases; Human monocyte cell line U937; Tissue inhibitors of metalloproteinases

Introduction

Nanotechnology includes the design, characterization, production, and application of structures, devices and systems by controlling shape and size at the nanometer scale (Chow et al., 2005; Owen and Depledge, 2005). These technologies directly improve our lives in areas as diverse as engineering, information technology, and diagnostics. Nanomaterials are the building blocks of this new technology and comprise a range of different morphologies including nanotubes, nanowires, nanofibers, nanodots and a range of spherical or aggregated dendritic forms. With the development of nanotechnology, a large number of transition metal nanoparticles have been or will be developed and produced as new formulations with surface properties to meet novel demands.

It is well known that exposure to metal particles such as metal fume can cause pulmonary fibrosis, pneumonitis, and bronchial asthma in exposed workers (Hartung et al., 1982; Kusaka et al., 1986). Transition metals also play a key role in the health problems induced by particulate matter (PM) air pollution (Donaldson et al., 2003; Ghio et al., 2002 and Kodavanti et al., 2000). We and others doing toxicology studies in rats have demonstrated that metal nanoparticles instilled into the lungs caused a greater inflammatory response when compared to larger particles of identical chemical composition at equivalent mass concentrations (Brown et al., 2000; Dick et al., 2003; Ferin et al., 1992; Li et al., 1999; Hamoir et al., 2003; Zhang et al., 2000a, 2000b, 2003). Thus, surface properties appear to play an important role in nanoparticle toxicity (Zhang et al., 1998a; Gilmour et al., 1996; Nemmar et al., 2003; Oberdoster et al., 1994, 2001; Wilson et al., 2002). Nanoparticles are also not as readily phagocytized by alveolar macrophages as larger particles and can penetrate much more rapidly through the epithelium to the endothelium. They may even enter the blood circulation and translocate to extra-pulmonary tissues (Ferin et al., 1990, 1992; Nemmar et al., 2001, 2002a, 2002b; Oberdoster et al., 2002; Oberdoster and Utell, 2002; Takenaka et al., 2001). Previous studies in dogs exposed to PM showed that PM particles were found not only in alveolar type I and II cells, interstitial macrophages, and intravascular macrophage-like cells, but also in endothelial cells (Calderon-Garciduenas et al., 2001). Myocardial and vascular dysfunction induced by inhaled PM could be due to passage of some particles from the lungs into the systemic circulation (Calderon-Garciduenas et al., 2001) due to a modification of epithelial and endothelial permeability induced by inflammatory mediators (Donaldson et al., 1996, 1998, 2001, 2003; Hamoir et al., 2003). The translocation of particles also depends on the exposure route, dose, particle diameter, and surface chemical characteristics. Our previous *in vitro* and *ex-vivo* studies showed that metal nanoparticles (nickel and cobalt) could induce cytokine and NO release by rat blood leukocytes (Mo et al., 2008). This suggests that the adverse effects of metal nanoparticles may involve activation of blood leukocytes, raising the intriguing possibility that nanoparticles could enter the circulation and produce direct or indirect effects on leukocytes. Recent studies in humans showed that exposure to nano-size carbon altered peripheral blood leukocyte distribution and expression of adhesion molecules (Frampton et al., 2006). However, there are few reports to study the direct effects of nanoparticles on leukocytes, especially monocytes, when they are translocated to the

circulation. Monocytes/macrophages are among the first cells to home to inflammatory sites, and they play a key role in the immune response. Several studies have suggested that human monocytes, such as U937 cells, exposed to metal ions such as Co^{2+} , Cr^{3+} and Ni^{2+} , experienced altered activity of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) via oxidative stress activation of protein tyrosine kinases (PTK) (Nakashima et al., 2002; Luo et al., 2005). MMPs belong to a family of zinc- and calcium-dependent enzymes that are employed in numerous physiological and pathological processes. Most MMPs are secreted as inactive pro-enzymes; their proteolytic activity is regulated by zymogen activation by extracellular proteases or other MMPs or by inhibition of specific TIMPs or other non-specific protease inhibitors. This implies that the balance between MMP and TIMP levels is a critical determinant of the net proteolytic activity at any given time.

The goal of the present study was to compare the ability of Nano-Co and Nano-TiO₂ to alter transcription and activity of MMPs. We hypothesized that non-toxic doses of some transition metal nanoparticles stimulate MMP production that may contribute to their health effects. We investigated the following: (1) whether exposure of U937 cells to metal nanoparticles caused an alteration in their transcription and activities of two gelatinases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B) and in the transcription of their specific inhibitors TIMP-1 and TIMP-2; (2) whether MMP-2 and MMP-9 release is related to oxidative stress caused by nanoparticles; (3) the potential role of ROS scavengers or inhibitors in the prevention of nanoparticle-induced MMP release; and (4) if the increase in MMP activity induced by nanoparticles is regulated by activator protein 1 (AP-1) and the protein tyrosine kinase signaling pathways related to redox signaling.

Materials and methods

Metal nanoparticles and their characterization

Nano-Co and Nano-TiO₂ with a mean diameter of 20 nm were made by the process of vacuum vapor deposition and were provided by INABTA and Co., Ltd., Vacuum Metallurgical Co., Ltd., Japan. The microstructure and composition of Nano-Co and Nano-TiO₂ were characterized by transmission electron microscopy (TEM) (Hitach H-8000) (Zhang et al., 1998a) and ancillary techniques involving selected-area electron diffraction (SAED) and energy-dispersive (X-ray) spectrometry (EDS). The characterization of these nanoparticles has been reported in our previous studies (Zhang et al., 1998a; Mo et al., 2008). Briefly, the specific surface area is 47.9 m²/g for Nano-Co and 45.0 m²/g for Nano-TiO₂. Nano-Co is composed of 85 to 90% metal Co and 10 to 15% Co₃O₄, and Nano-TiO₂ is composed of 90% Anatase and 10% Rutile.

Nano-Co and Nano-TiO₂ were freshly prepared and dispersed in PBS and were ultrasonicated for about 30 min prior to each experiment. The solubility of Nano-Co has been described in other studies (Kyono et al., 1992).

Chemicals and reagents

2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was obtained from Molecular Probe (Eugene, OR). A stock solution of H₂DCF-DA (20 mM) was prepared in 100% ethanol and stored under N₂ at -20 °C in the dark. *N*-Acetyl-L-Cysteine (NAC) was obtained from Fisher Chemical (Pittsburgh, PA). Catalase and curcumin were from MP Biomedicals (Solon, OH). Gelatin was from Acros Organics (Morris Plains, NY). Genistein and herbimycin A were purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Fisher Chemical (Pittsburgh, PA) unless otherwise detailed. All chemicals used were of analytic grade.

Cell culture and treatment

Human U937 monocytes were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and cultured in RPMI 1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Inc., Manassas, VA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Mediatech, Inc., Manassas, VA). U937 cells were exposed to Nano-Co or Nano-TiO₂ at the doses and times indicated in each experiment at 37 °C in the presence of 5% CO₂. For MMP-2 and -9 analysis, cells were cultured in 1% FBS for 24 h to minimize the selective activation of MMPs. For some experiments, U937 cells were pre-treated with catalase (1000 U/ml), NAC (20 mM), curcumin (20 µM), genistein (50 µM), or herbimycin A (1 µM) prior to exposure to Nano-Co or Nano-TiO₂. Unexposed U937 cells were served as controls. The supernatant from serum-free cultured HT1080 cells (ATCC) was used as a molecular weight marker of pro-MMP-2 and pro-MMP-9 as described elsewhere (Das et al., 2008; Dagnell et al., 2007; Togawa et al., 1999).

Cell viability

CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) (MTS assay) was used to assay the cytotoxic effects of Nano-Co and Nano-TiO₂ on U937 cells according to the manufacturer's instruction. Briefly, 5×10⁵ cells in 100 µl culture medium were seeded into each well of 96-well plates and incubated overnight. After cells were treated with various concentrations of Nano-Co or Nano-TiO₂ for 24 h, 20 µl of the combined MTS/PMS solution was added to each well of the 96-well plate. After incubation at 37 °C for 3 h, the absorbance at 490 nm was recorded using multi-detection microplate reader (Synergy HT, BioTek, Vermont).

Measurement of intracellular ROS generation

ROS production was determined using 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCF-DA) as described previously (Zhang et al., 2005, 2008). Nonfluorescent H₂DCF-DA can be converted by intracellular esterases to H₂DCF, which is oxidized by ROS to DCF, a highly fluorescent compound. 1×10⁵ U937 cells were seeded in each well of 96 well plates and cultured overnight. After H₂DCF-DA was added to make a final concentration of 5 µM and cells were pre-incubated at 37 °C for 2 h, cells were treated with 0, 0.625, 1.25, 2.5 and 5.0 µg/ml of Nano-Co or Nano-TiO₂ for another 12 h. Fluorescence (Excitation: 485 nm, Emission: 528 nm) was measured by multi-detection microplate reader (Synergy HT, BioTek, Vermont).

To examine the role of antioxidants on ROS generation in U937 cells, a density of 1×10⁵ cells were cultured in each well of 96 well plates. ROS scavengers or inhibitors, NAC (20 mM) or catalase (1000 U/ml), were added 2 h prior to adding H₂DCF-DA. Fluorescence intensity was recorded as described above.

Analysis of gene expression by semi-quantitative RT-PCR

Total RNA was extracted from U937 cells using TRIZOL Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. 2 µg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI) in a total volume of 25 µl. A total of 1 µl of cDNA, 0.5 µl of 10 mM dNTP, 1 µl of 5 µM each primer, and 1.25 U of HotMaster Taq DNA polymerase (Promega, Madison, WI) were used in each PCR reaction at a final volume of 25 µl. The PCR reaction was performed on a Mastercycler (Eppendorf, Westbury, NY) using 32 cycles at 94 °C for 45 s, at 62 °C for 45 s, and at 72 °C for 45 s for MMP-2 and MMP-9; 30 cycles at 94 °C for 45 s, at 58.5 °C for 45 s, and at 72 °C for 45 s for TIMP-1 and TIMP-2; and 23 cycles at 94 °C for 45 s, at 56 °C for 45 s, and at 72 °C for 45 s for GAPDH. The primers for human MMP-9 were: forward 5'-CGG TGA TTG ACG ACG CCT TT-3' and reverse 5'-CGC TGT CAA AGT TCGAGGTGGTA-3'; for human MMP-2

were: forward 5'-ATT TGG CGG ACT GTG ACG-3' and reverse 5'-GCT TCA GGT AAT AGG CAC-3'; for human TIMP-1 were: forward 5'-AAT TCC GAC CTC GTC ATC AG-3' and reverse 5'-GTT TGC AGG GGA TGG ATA AA-3'; for human TIMP-2 were: forward 5'-CTG GAC GTT GGA GGA AAG AA-3' and reverse 5'-GTC GAG AAA CTC CTG CTT GG-3'; for human GAPDH were: forward 5'-AGC CAC ATC GCT CAG ACA C-3' and reverse 5'-TGG ACT CCA CGA CGT ACT C-3'. After separation on a 1.2% agarose gel, PCR products were visualized and photographed (Polaroid, 667) on an ultraviolet screen. Pictures were scanned and analyzed by NIH Image J software. Intensities of target gene products will then be normalized by that of human GAPDH to obtain the relative densities.

Gelatin zymography assay

MMP-2 and MMP-9 activities were measured by gelatin zymography as previously described (Swallow et al., 1996). Briefly, U937 cells were seeded at a density of 1×10^6 cells/well in a 6-well plate in RPMI1640 medium containing 1% FBS for 24 h before exposure to Nano-Co or Nano-TiO₂. For some experiments, U937 cells were pre-treated with NAC (20 mM), catalase (1000 U/ml), curcumin (20 μ M), genistein (50 μ M) or herbimycin A (1 μ M) prior to treating with Nano-Co or Nano-TiO₂. The conditioned media of U937 cells were collected and subjected to electrophoresis on 10% SDS-PAGE copolymerized with 0.5 mg/ml gelatin (Acros, Morris Plains, NJ) which was used as the substrate under non-reducing conditions. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 (Sigma Chemical., St. Louis, MO) solution for 1 h, and then incubated at 37 °C overnight with Tris-HCl buffer (pH 7.4) containing 10 mM CaCl₂ and 0.05% Brij solution (Bio-Rad, Hercules, CA). The gels were then stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) and destained in methanol: acetic acid: water (volume, 30:10:60) until clear bands were observed against the background of Coomassie blue-stained gelatin. Enzyme activity was quantified by using NIH image J software. HT1080 cells were cultured in serum-free DMEM for 2 days, then the supernatants were collected and used as a molecular weight marker of pro-MMP-2 and pro-MMP-9 as described elsewhere (Das et al., 2008; Dagnell et al., 2007; Togawa et al. 1999).

Statistical analysis

Values were expressed as the means and standard errors. For dose-response studies, differences among groups were evaluated with two-way analysis of variance; if the F-value was significant, groups were then compared at each dose by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. If a *p* value was less than 0.05, a difference was considered significant. Statistical analysis was carried out using Sigma Stat (Jandel Scientific, San Raphael, CA).

Results

Cytotoxic effects of Nano-Co and Nano-TiO₂ on U937 cells

To determine the cytotoxicity of Nano-Co and Nano-TiO₂, U937 cells were exposed to various concentrations of Nano-Co or Nano-TiO₂ for 24 h. Cell viability was measured by MTS assay as described above. The results showed a dose-response increase in the cytotoxicity of Nano-Co on U937 cells; exposure U937 cells to 7.5 μ g/ml of Nano-Co caused significant cell death, while exposure to 5 μ g/ml or less of Nano-Co did not cause significant cytotoxicity (Fig. 1). Exposure of U937 cells to any dose from 0 to 20 μ g/ml of Nano-TiO₂ did not cause any cytotoxic effects (Fig.1). Therefore, in order to compare the effects other than cytotoxicity of Nano-Co and Nano-TiO₂ on U937 cells, a non-toxic dose, 5 μ g/ml, of Nano-Co was used in the subsequent experiments. The same dose was chosen for Nano-TiO₂ since they have similar diameters.

U937 cells exposed to Nano-Co, but not to Nano-TiO₂, generate ROS

The fluorophore H₂DCF was used to detect ROS generation in U937 cells after exposure to Nano-Co or Nano-TiO₂. A dose-response increase in the DCF fluorescence was observed after 12 h exposure to 0 to 5 µg/ml of Nano-Co (Fig. 2A). However, exposure U937 cells to Nano-TiO₂ did not cause ROS generation. The increase in DCF fluorescence with exposure to Nano-Co was abolished by pretreatment of U937 cells with NAC (20 mM) and was markedly attenuated by pre-treatment with catalase (1000 U/ml) (Fig. 2B).

Effects of metal nanoparticles on the expression of MMP-2 and MMP-9 mRNAs and the activities of pro-MMP-2 and pro-MMP-9

The effects of Nano-Co and Nano-TiO₂ on MMP-2 and MMP-9 gene expression in U937 cells were investigated at the transcriptional level using semi-quantitative RT-PCR analysis. The results showed that there was a dose-response increase on MMP-2 and MMP-9 mRNA levels after exposure to Nano-Co from 0 to 5 µg/ml for 12 h, whereas Nano-TiO₂ did not induce any significant expression changes (Fig. 3A and B). Our results also showed a time-response increase on MMP-2 and MMP-9 mRNA levels after exposure to Nano-Co from 1 h to 12 h (data not shown). Again, Nano-TiO₂ did not cause a time-response increase in the MMP-2 and MMP-9 mRNA levels (data not shown). The expression of MMP-2 and MMP-9 mRNA results were further confirmed by gelatin zymography assay. We evaluated MMP-2 and MMP-9 activities in U937 cells after treatment with Nano-Co or Nano-TiO₂ at different doses and time points. The results showed that MMP-2 and MMP-9 activities were significantly higher in conditioned media from cells exposure to Nano-Co for 12 h (Fig. 4A and B). Pro-MMP-9 (92 KD gelatinase) activity was 1.7 and 2.2 times and MMP-2 (72 KD gelatinase) activity was 1.3 and 1.9 times greater than that in controls when U937 cells were treated with 2.5 and 5 µg/ml of Nano-Co, respectively (Fig. 4A and B). In the time course studies, the results showed that when U937 cells were exposed to 5 µg/ml of Nano-Co for 6 h, 12 h, 24 h and 48 h, there were significant increase of pro-MMP-2 and pro-MMP-9 activities as compared with those in controls, and the activities reached peak after 24 h exposure (data not shown). However, similar to the RT-PCR results, exposure to Nano-TiO₂ did not cause any dose- or time-response increases in the pro-MMP-2 and pro-MMP-9 activities.

Effects of metal nanoparticles on the transcription of TIMP-1 and TIMP-2 mRNAs

To examine whether the imbalance between MMPs and their inhibitors was involved in the different response of Nano-Co and Nano-TiO₂ on pro-MMP-2 and pro-MMP-9 mRNA expression and activities, we also determined the expression of TIMP-1 and TIMP-2 by RT-PCR. Our results showed that exposure of U937 cells to Nano-Co caused a dose-response decrease in TIMP-2 mRNA expression, reaching statistical significant at an exposure of 5 µg/ml Nano-Co (data not shown). However, Nano-TiO₂ did not have any effects on TIMP-2 mRNA expression. Exposure of U937 cells to either Nano-Co or Nano-TiO₂ did not cause any change on TIMP-1 mRNA expression. Moreover, treatment with Nano-Co caused a slightly time-response decrease in TIMP-2 mRNA expression; TIMP-2 mRNA expression decreased at 6 h, 12 h and 24 of Nano-Co treatment (data not shown). Treatment with Nano-TiO₂ did not cause a time-response decrease in TIMP-2 mRNA expression (data not shown).

Effects of ROS scavengers or inhibitors on MMP activities and TIMP expression in U937 cells exposed to Nano-Co

To investigate the role of ROS in Nano-Co-induced MMP and TIMP transcription, U937 cells were pre-treated with NAC (20mM) or Catalase (1000 U/ml) for 2 h, then cells were grown in the presence or absence of Nano-Co (5 µg/ml) for another 12 h. Semi-quantitative RT-PCR results showed that pre-treatment with NAC (20 mM) or catalase (1000 U/ml) resulted in inhibition of Nano-Co-induced MMP-2 and MMP-9 expression (Fig. 5A and B). These results

were further confirmed by using gelatin zymography analysis; the results showed that pre-treatment with NAC or catalase attenuated the activities of pro-MMP-2 and pro-MMP-9 (Figs. 6A and B).

Our results also demonstrated that pre-treated with NAC or catalase prevented the TIMP-2 down-regulation induced by exposure to Nano-Co (data not shown).

Involvement of AP-1 and PTK in Nano-Co-induced MMP-2 and MMP-9 expression

To determine whether Nano-Co-induced MMP activation through oxidative stress via AP-1 and/or PTK pathways, we used the AP-1 specific inhibitor, curcumin (20 μ M), and PTK specific inhibitors, genistein (50 μ M) and herbimycin A (1 μ M) to pre-treat U937 cells for 4 h for curcumin, and 24 h for genistein and herbimycin A prior to exposure to Nano-Co for 12 h. Our results showed that Nano-Co-induced MMP-2 and MMP-9 expression was completely inhibited by curcumin (Fig. 7A and B), genistein (Fig. 8A and B) and herbimycin A (Fig. 8A and B).

Discussion and conclusions

Inhalation exposure in human and animal studies have shown that nanoparticles can penetrate rapidly through the epithelium, reach the endothelium and enter the blood circulation (Nemmar et al., 2001, 2002a, 2002b; Oberdorster et al., 2002; Oberdorster and Utell, 2002). The translocation of nanoparticles may result in adverse effects on extra-pulmonary organs and tissues. Monocytes/macrophages are among the first immune cells recruited to a site of invasion, and come into contact with the foreign agents. Activation of monocytes/macrophages can release a variety of cytokines, toxic oxygen metabolites and proteinases that can cause cellular damage. As a particulate foreign body, nanoparticles can induce a host inflammatory response which could play a major role in tissue destruction and remodeling. In fact, our previous studies showed that exposure to Nano-Ni and Nano-Co caused cytokine and NO release from rat peripheral blood neutrophils (Mo et al., 2008). In this study, we analyzed the expression and the secretion of two gelatinases, MMP-2 and MMP-9, and their specific tissue inhibitors, TIMP-1 and TIMP-2, in human monocyte U937 cells after exposure to Nano-Co and Nano-TiO₂. We explored the possible mechanisms involved in the regulation of MMP expression and activity after exposure to these metal nanoparticles.

Our previous results showed that exposure to Nano-Co caused greater lung injury and inflammation than Nano-TiO₂, although they have similar diameters (Zhang et al., 1998a). Our present study demonstrated a dose-response toxic effect on U937 cells after exposure to Nano-Co at concentrations ranging from 0 to 20 μ g/ml. Nano-Co had higher toxic effects than Nano-TiO₂ when the dose was greater than 5 μ g/ml. It is difficult to estimate the degree of human health effects from these dose-response studies. However, toxicology has always been informed by dose-response studies, and under studying of nanoparticle toxicity is hampered by insufficient understanding of their mechanisms and effects. Exposure to a dose that is lower than a cytotoxic dose can help us to identify potential health effects of metal nanoparticle other than those due to cytotoxicity. Our results also showed that Nano-Co caused significant ROS generation, which may partially explain the higher toxic effects of Nano-Co. While Nano-TiO₂ and Nano-Co have similar particle size, and Ti and Co belong to the same element group, they are dramatically different in their ability to induce cytotoxicity. The difference in their ability to generate ROS may underlie their different cytotoxicity.

MMPs are zinc-dependent endopeptidases involved in the remodelling of extracellular matrix and play important roles in morphogenesis, angiogenesis, arthritis, skin ulcer, tumor invasion, metastasis, and wound healing. Regulation of MMPs may occur at multiple levels, either by gene transcription and synthesis of inactive pro-enzymes, post-translational activation of pro-

enzymes, or via the interaction of secreted MMPs with their inhibitors called TIMPs (Murphy et al., 1994; Oum'hamed et al., 2004; Parson et al., 1997). According to their structure and substrate specificity, MMPs can be divided into five subgroups: collagenases, stromelysins, gelatinases, membrane type (MT)-MMPs, and others (Vihinen and Kahari, 2000). Among the MMPs, the 72 kDa gelatinase A (or MMP-2) and the 92 kDa gelatinase B (or MMP-9) are believed to be the critical enzymes for degrading type IV collagen, a major component of basement membrane. It is believed that the secretion of gelatinases having specificity for type IV collagen would endow endothelial cells with an advantage for degradation of the extracellular matrix and subsequent migration across the basement membrane. Understanding how metal nanoparticles induce changes in the MMP/TIMP system that result in matrix breakdown may lead to interventions that delay or prevent potential health effects of metal nanoparticles. Our results demonstrated that exposure to Nano-Co at non-toxic doses caused up-regulation of MMP-2 and MMP-9, but down-regulation of TIMP-2. The effects of Nano-Co on MMP and TIMP expression effectively amplified the degradation arm of the MMP/TIMP system that could lead to net matrix breakdown. It has been reported that exogenous hydrogen peroxide and endogenous ROS can induce MMP expression in endothelial cells, cardiac fibroblasts, macrophages and breast cancer cells (Belkhiry et al., 1997; Siwik et al., 2001; Rajagopalan et al., 1996; Zhang et al., 2002; Hemmerlein et al., 2004). To investigate the role of ROS in Nano-Co-induced MMP/TIMP expression imbalance, ROS scavengers or inhibitors such as NAC and catalase were used to pretreat U937 cells. Our results clearly showed that pre-treatment with NAC or catalase significantly inhibited Nano-Co-induced up-regulation of MMP-2 and MMP-9 mRNA expression and activities.

Activation of MMPs is a complex process, which is tightly regulated by their natural inhibitors, TIMPs. Therefore we also investigated the transcriptional levels of TIMP-1 and TIMP-2. Our results demonstrated that exposure to Nano-Co induced down-regulation of TIMP-2 and this effect was completely inhibited with catalase and NAC, suggesting that ROS is also involved in the regulation of TIMP-2, in agreement with previous studies (Haorah et al., 2007). Unlike TIMP-2, TIMP-1 was not induced by exposure to Nano-Co.

ROS have been shown to activate latent MMPs in conditioned media (Tyagi et al., 1993, 1995). Analysis of the MMP/TIMP promoters has identified essential response elements including activator protein-1 (AP-1) elements and multiple Ets elements (Benbow et al., 1997). In addition, MMP-9 has the nuclear factor-kappaB (NF- κ B) element (Sato et al., 1993), and TIMP-1 has multiple transforming growth factor- β (TGF- β) inhibitory elements (Benbow et al., 1997). Regulation of MMP and TIMP gene expression involves an interplay between all of the transcription factors that bind to these response elements (Benbow et al., 1997). Therefore, ROS generation by exposure to Nano-Co may activate MMP-2 and MMP-9 through intracellular signal pathways via NF- κ B and/or AP-1. Our results demonstrated that pre-treatment with curcumin, an AP-1 inhibitor, significantly inhibited the up-regulation of MMP-2 and -9 in U937 cells exposed to Nano-Co.

Oxidative stress has been shown to up-regulate transcription and activity of MMPs in several types of cells, including hepatic stellate cells (Galli et al., 2005), human monocytes (Lu and Wahl, 2005), and human coronary smooth muscle cells (Valentin et al., 2005). To investigate whether Nano-Co-induced increase of MMP transcription and activities are mediated by the protein tyrosine kinase pathway, the PTK inhibitors, genistein and herbimycin A were used to pre-treat U937 cells prior to exposure to Nano-Co. Pre-treatment with genistein significantly inhibited Nano-Co-induced activation of MMP-2 and -9. Previous studies have shown that PTK signaling pathway may link oxidative stress and MMPs. Redox-associated signal transduction mediates PTK activation (Nakashima et al., 2002), and oxidative stress was shown to regulate PTK activity (Whisler et al., 1994; Liu et al., 1996). Previous studies showed that both receptor and non-receptor PTK signaling are involved as second messengers in regulation of MMP

activation *in vivo* (Lucchesi et al., 2004) and *in vitro* (Matsumoto et al., 2004). This study does not *clarify* the mechanism by which Nano-Co-induced oxidative stress mediates PTK activation or regulate PTK activity. However, previous studies showed that modification of specific cysteine residues of MMPs represents a direct model of signaling for ROS (Finkel et al., 2003; Force et al., 2007; Salvi et al., 2005). The oxidant burst is often associated with an increase in tyrosine phosphorylation, one attractive candidate for ROS-mediated effects. Protein tyrosine phosphatases are particularly attractive candidates because they contain reactive cysteine residues within their active sites. Such reactive cysteine residues are easily oxidized by even mild oxidants such as hydrogen peroxide (Xu et al., 2002; Finkel et al., 2003). The oxidation of a single cysteine residue in MMP-9, followed by formation of a stable sulphonate intermediate, *leads* to activation of the protease.

In conclusion, we have demonstrated that under certain culture conditions, at a noncytotoxic concentration, Nano-Co acts as a toxic agent by increasing the transcription and activities of MMP-2 and MMP-9 in U937 cells. The AP-1 and PTK signaling pathways were involved in this process via oxidative stress. These findings have important implications for understanding the potential health effects of metal nanoparticle exposure.

Abbreviations

H₂DCF-DA, 2',2'-dichlorodihydrofluorescein diacetate; Nano-Co, Nano-size cobalt; Nano-TiO₂, Nano-size titanium dioxide; ROS, Reactive oxygen species; MMP-2, Matrix metalloproteinases-2; MMP-9, Matrix metalloproteinases-9; NAC, N-Acetyl-L-Cysteine; TIMP-1, Tissue inhibitors of metalloproteinases 1; TIMP-2, tissue inhibitors of metalloproteinases 1.

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Fig. 1.

Cytotoxic effects of Nano-Co and Nano-TiO₂ on U937 cells. U937 cells were treated with different doses of Nano-Co and Nano-TiO₂ for 24 h and cytotoxicity were determined with MTS assay. U937 cells without treatment were used as control. Values are mean±SE of six experiments. *Significant difference from control group, $p < 0.05$; #Significant difference from the same dose of Nano-TiO₂-treated group, $p < 0.05$.

Fig. 2.

(A, B) Effects of ROS generation in U937 cells treated with different doses of Nano-Co and Nano-TiO₂ and effects of ROS scavenger or inhibitor on Nano-Co-induced ROS generation. U937 cells were pre-treated with H₂DCF-DA for 2 h prior to exposure to different doses of metal nanoparticles (A). For antioxidant experiments, NAC or catalase was added 2 h prior to adding H₂DCF-DA and Nano-Co (B). *Significant difference compared with control, $p < 0.01$; #significant difference from the same dose of Nano-TiO₂-treated group, $p < 0.01$; **significant difference as compared with Nano-Co alone, $p < 0.01$.

Fig. 3.

Dose-response induction of gene expression after exposure of U937 cells to different doses of Nano-Co or Nano-TiO₂. Total RNA was extracted from U937 cells after treatment with Nano-Co or Nano-TiO₂. MMP-2 and MMP-9 gene expression was measured by RT-PCR as described in Materials and Methods. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of PCR results. *Significant difference as compared with control, $p < 0.05$; #significant difference from the same dose of Nano-TiO₂-treated group, $p < 0.05$.

Fig. 4.

Dose-response analysis of gelatinolytic activities of pro-MMP-2 and pro-MMP-9 after exposure of U937 cells to Nano-TiO₂ or Nano-Co. Conditioned medium samples were collected from control U937 cells and U937 cells treated with 2.5 or 5.0 µg/ml of Nano-TiO₂ or Nano-Co. Pro-MMP-2 and pro-MMP-9 activities were measured by gelatin zymography. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments ± SE of gelatin zymography results. *Significant difference compared with control, $p < 0.05$; #significant difference from the same dose of Nano-TiO₂-treated group, $p < 0.05$.

Fig. 5.

Effects of NAC or catalase on Nano-Co-induced MMP mRNA expression. Total RNA was extracted from U937 cells with or without pre-treatment with NAC or catalase for 2 h prior to treating with 5.0 $\mu\text{g}/\text{ml}$ of Nano-Co. MMP-2 and MMP-9 mRNA expression was measured by RT-PCR. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of PCR results.

*Significant difference compared with control, $p < 0.05$; **significant difference as compared with Nano-Co treatment alone, $p < 0.05$.

Fig. 6. Effects of NAC or catalase on Nano-Co-induced pro-MMP-2 and pro-MMP-9 activities in U937 cells. Conditioned medium samples were collected from control U937 and U937 cells with or without pre-treatment with NAC or catalase for 2 h prior to adding Nano-Co. Pro-MMP-2 and pro-MMP-9 activities were measured by gelatin zymography. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of gelatin zymography results. *Significant difference compared with control, $p < 0.05$; **significant difference compared with Nano-Co treatment alone, $p < 0.05$.

Fig. 7.

Effects of curcumin on Nano-Co-induced pro-MMP activities. Conditioned medium samples were collected from control U937 cells and U937 cells with or without pretreatment with 20 μ M curcumin for 4 h prior to treatment with 5.0 μ g/ml of Nano-Co. Pro-MMP-2 and pro-MMP-9 activities were measured by gelatin zymography. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of gelatin zymography results. *Significant difference as compared with control, $p < 0.05$; **Significant difference compared with Nano-Co alone, $p < 0.05$.

Fig. 8.

Effects of PTK inhibitors on Nano-Co-induced MMP activities. Conditioned medium samples were collected from control U937 cells and U937 cells with or without pretreatment with genistein (50 μ M) or herbimycin A (1 μ M) for 24 h, prior to treatment with 5.0 μ g/ml Nano-Co. Pro-MMP-2 and pro-MMP-9 activities were measured by gelatin zymography. (A) Shows the results of a single experiment. (B) Represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of gelatin zymography results. *Significant difference compared with control, $p < 0.05$; **significant difference compared with Nano-Co alone, $p < 0.05$.