Cell-based Assays for Cytotoxic and Pro-inflammatory Effects of Gold Nanoparticles

Theresa Knight[1], Venkata G.R. Chada[4], Sandra S. Wise[2,3], Michael D. Mason[4], W. Douglas Thompson[1,3], John Pierce Wise Sr.[1,2,3], and Ah-Kau Ng[1,3]

[1]Department of Applied Medical Sciences, University of Southern Maine, Portland, ME 04103, [2]Wise Laboratory of Environmental & Genetic Toxicology, [3]Maine Center for Toxicology & Environmental Health, [4]Dept. of Chemical & Biological Engineering, Inst. for Molecular Biophysics, University of Maine.

ABSTRACT

Nanoparticles are widely used in industrial products and have great potential in medical applications, however, their toxicity and effects on health are unknown. This study has explored the *in vitro* cytotoxic and proinflammatory effects of 15 nm gold nanoparticles. Little to no cytotoxicity was observed in RAW264.7 murine macrophages, Jurkat T lymphocytes, RAJI B lymphocytes, and WHTBF-6 lung fibroblasts after 24 hour treatment with up to 2.5 μg/cm^2 nanoparticles. The ability for RAW264.7 cells to internalize fluorescent latex beads was not significantly inhibited by 24 hr treatment with nanoparticles. An increase in the production of the cytokine TNF-α was observed in RAW264.7 cells after treatment with 2.5 μg/cm^2 nanoparticles. TNF-α levels in freshly isolated human peripheral blood mononuclear cells were elevated after treatment with 1.25, 2.5 and 5.0 ug/cm^2 nanoparticles. An increase in phosphorylation of the MAP kinase proteins SAPK and JNK, was observed in RAW264.7 cells after treatment with 0.25 to 2.5 μg/cm^2 nanoparticles.

***Keywords*:** nanoparticles, cytotoxicity, nanotechnology, inflammation, immunotoxicolgy

1 BACKGROUND

Nanoparticles are defined as having at least one dimension of 100 nm or less. Because nanoparticles exist in the quantum scale, they exhibit mechanical, magnetic, electronic, and color properties unachievable or unseen by these chemicals at a larger size scale. They are currently used in commercial products and as drug delivery systems. Nanotechnology is expected to become a 1 trillion dollar industry within the next ten years, yet the potential health effects of nanomaterials have not been adequately addressed [1-2].

Gold and other noble-metal nanoparticles have many potential applications. They may be used in solar cells and as nanowires in electronics [3]. Gold nanoparticles may also be used in inks, colorants, paints, pharmaceuticals and liquid crystal displays [4]. Large scale production will be required for their use in catalysts for pollution control, chemical manufacturing, and fuel cells [5-6]. In the biomedical industry, they have great potential for use in biomedical assays and in the targeted destruction of disease-causing cells.

The immune system is crucial to good health as it provides protection from pathogens and cancer. However, abnormal or uncontrolled immune activities can be detrimental to certain tissues. The effects of nanoparticles on the immune system are largely unknown and are of particular interest when considering this important network of immune cells and organs. Because of their small size, one likely route of exposure to nanoparticles will be through the lungs. The outcome of pulmonary exposure will depend on the overall responses of lung cells and resident immune cells to the inhaled nanoparticles. It is conceivable that nanoparticle exposure could result in inflammatory response--a first-line defense mechanism in a host--leading to cell signaling (e.g. MAPK activation) [7-8] and up-regulation of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) [9].

In this study, we compared the cytotoxicity of 15 nm gold nanoparticles on cultured murine macrophages (RAW 264.7 cells), human T cells (Jurkat), B cells (RAJI) and lung fibroblasts (WHTBF-6), and investigated their effects on endocytosis and MAPK signaling in RAW 264.7 as well as TNF-α production by RAW 264.7 and human peripheral blood mononuclear cells (PBMC).

2 METHODS

2.1 Chemicals and Reagents

DMEM/F-12 was purchased from Mediatech Inc. (Herdon, VA). Fetal bovine serum was purchased from Omega Scientific, Inc. (Tarzana, CA). Cosmic calf serum was purchased from Hyclone (Logan, UT). Trypsin-EDTA, sodium pyruvate, penicillin-streptomycin, Dulbecco's PBS, and L-glutamine were purchased from Invitrogen Corporation (Grand Island, NY). Tissue culture dishes, flasks, and plasticware were purchased from Corning Inc. (Acton, MA). Cell growth determination kit (MTT based), CelLytic M cell lysis reagent, protease inhibitor cocktail A, Histopaque, 1 μm fluorescent latex beads, bacterial lipopolysaccharide, i**onomycin** and **Phorbol 12-myristate 13-acetate** (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Matrix IsoPrep was purchased from Robbins Scientific Corporation (Sunnyvale, CA). Mouse and human TNF-α DuoSet ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN). SDS-PAGE gel casting supplies, protein standards, electrophoresis apparatuses, PVDF membrane, and western

immunoblot equipment were purchased from Bio-Rad Laboratories, Inc (Richmond, CA). MAP kinase and β-actin primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). Goat anti-rabbit secondary antibody was a gift from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). ECL kit was purchased from GE Healthcare (Piscataway, NJ).

2.2 Synthesis of Gold Nanoparticle (Au NP)

The nanoparticles used here have been produced in M. D. Mason's lab at the University of Maine's Institute for Molecular Biophysics (IMB). Unmodified gold nanospheres are synthesized using single-pot redox solution chemical techniques (sodium citrate reduction) according to published methods. With careful control a broad range of nanoparticle sizes (2.5-62 nm), and geometries have been synthesized. Detailed characterization of all nanoparticles is performed as follows.. Nanoparticle sizing and geometry (volume and surface area) is determined using a combination of TEM, UV-Visible Absorption Spectroscopy, light scattering, and darkfield micro-spectroscopy according to published methods. The nanoparticles used here are 15 nm unmodified gold nanospheres in 0.16 mg/ml sodium citrate that were dialyzed to remove sodium citrate.

2.3 Cell Cultures

WHTBF-6 cells, human lung fibroblasts immortalized with the hTERT transgene, are cultured in a 50/50 mix of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium supplemented with 10% calf serum, 2mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin, and 0.1mM sodium pyruvate. Murine monocyte/macrophage RAW264.7 cells are cultured in 50/50 mix of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin/100 μg/ml streptomycin. Human RAJI B lymphoblasts and human Jurkat T leukemia cells are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin and 0.1mM sodium pyruvate. All cultures are grown in a 37°C incubator with 5% CO_2, except for RAW 264.7 culture which requires 10% CO_2.

Human PBMCs were isolated from approximately 15 ml of anticoagulated blood collected by venipuncture from healthy donors. Blood was centrifuged at 2000 RPM for 10 min. The buffy coat layer was suspended in 40 ml of Dulbecco's PBS and 10 ml of Ficoll solution was carefully layered under the suspension. After centrifugation at 2000 RPM for 15 minutes at room temperature the PBMC layer at the interface was harvested, washed twice and resuspended in assay medium for cell count using a hematocytometer or Coulter counter.

2.4 Cytotoxicity Assays

The MTT assay was used to measure cytotoxicity utilizing a commercial kit according to the manufacturer's instructions. Cells in log phase of growth were treated with 15 nm gold nanoparticles in sodium citrate or sodium citrate alone. Although the Au NP stock suspension was dialyzed to remove much of the sodium citrate, it was unclear as to the final concentration of sodium citrate. Therefore, the concentration of sodium citrate in the suspension before dialysis (0.16 mg/ml) was used to treat negative control cells where indicated. This concentration had little to no effect on the cell lines used here compared to untreated cells (data not shown). The volume of sodium citrate used to treat control cells was equal to the volume of Au NP suspension used to treat test cells. After 24-hour exposure to Au NP or sodium citrate, the cells were removed, washed, counted on a Coulter Multisizer III, and reseeded onto a 96-well plate at 1000 or 2000 cells per well depending on the cell line. The cells were allowed to grow for several days. Next, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to the culture medium and the cells incubated for 2-4 hours. The mitochondrial dehydrogenase in viable cells converts yellow MTT to blue formazan crystals by cleaving its tetrazolium ring. 0.1 N HCl in isopropanol was then added to dissolve the formazan crystals. The plate was read at 570 nm on a spectrophotometer with a reference wavelength of 690 nm.

2.5 Phagocytosis Assay

In this assay, the ability for RAW 264.7 murine macrophages to internalize 1μm fluorescent latex beads after exposure to different doses of 15 nm gold nanoparticles was analyzed. Cells growing in log phase were treated for 24 hours with nanoparticles in sodium citrate or an equal volume of sodium citrate. The cells were washed and counted, then reseeded onto wells of a new plate. Then 1 μm fluorescent latex beads were added to each well in a 1:10 cell to bead ratio. The cells were incubated with the beads for 24 hours and then read on a flow cytometer (BD FACSCalibur) at 488 nm excitation and 530 ± 15 nm emission.

2.6 Enzyme-linked Immunosorbent Assays (ELISAs) for TNF-α

RAW264.7 cells were seeded at 80,000 cells/cm^2 in 100 mm dishes and incubated for two days. Then culture medium was replaced and cells were treated with 0.25, 1.25, or 2.5 μg/cm^2 15 nm Au NP in sodium citrate or sodium citrate alone for 24 hours. For the last 20 minutes, one dish of cells was treated with 100 ng/ml baterial lipopolysaccharide (LPS). Supernatants were then harvested and stored at -70°C for future use. Supernatants were tested for murine TNF-α using a commercial ELISA kit.

Freshly isolated human PBMCs were seeded in 12 well plates at 2 million cells/ml. PBMC were treated with 1, 5, 10, or 20 μg/cm^2 15nm Au-NP in sodium citrate, sodium citrate alone, or a combination of 1 ng/ml PMA and 130 nM ionomycin stimuli for 24 hours, in a total volume of 1 ml. Then

supernatants were collected and frozen at -70°C until future testing for human TNF-α by ELISA as described above.

2.7 MAP Kinase Activation Assays

The ability of gold nanoparticles to stimulate the mitogen activated protein (MAP) kinase pathway was assessed using a western immunoblotting method. RAW264.7 cells growing in log phase were treated with 0.25, 1.25, or 2.5 μg/cm^2 15 nm gold nanoparticles for 24 hours or with bacterial lipopolysaccharide for the last 20 minutes (see method used for TNF-α analysis). A protease inhibitor cocktail was added to CelLytic M lysis reagent before cells were lysed according to suggested manufacturer's instructions. The optical densities at 280 nm of the cytoplasmic extracts were recorded to ensure equal protein loading before separation on an SDS-PAGE gel. After separation, proteins were electro-blotted onto PVDF membrane and membranes were probed for phosphorylated SAPK/JNK according to the manufacturer's instructions. Goat anti-rabbit-HRP was used as a second antibody. Bands were visualized using the ECL system. Then membranes were stripped at 56°C with stripping buffer (62 mM Tris, 100 mM 2-mercaptoethanol, 2% SDS, pH 6.7) and reprobed for total SAPK/JNK protein.

2.8 Statistical Analysis of Data

The dose-response relationship was assessed using multiple linear regression. Included as independent variables in the analysis were two indicators for distinguishing the three experiments and either three-five indicators for comparison of each of the levels of exposure to the control condition or a single quantitative measure of exposure. The statistical significance of the effects of exposure was evaluated on the basis of two-tailed t-tests, and 95% confidence intervals were constructed around the point estimates. All analyses were conducting using SAS, Version 9.

3 RESULTS

3.1 Cytotoxicity of 15 nm Gold Nanoparticles

The standard MTT assay was used to monitor cell proliferation at optimal culture time following 24-hour gold nanoparticle treatment. No significant cytotoxicity was seen in the WHTBF-6 lung fibroblast cells when treated with Au NP at 0.025-2.5 μg/cm^2 compared to untreated controls (not shown) and cells treated with sodium citrate alone (Figure 1). In RAJI B lymphocytes and murine macrophages RAW264.7, the highest dose of Au NP, 2.5 μg/cm^2, significantly inhibited the growth of cells to 75% and 90% relative survival, respectively. Interestingly, the lowest dose, 0.025 μg/cm^2, significantly inhibited the growth of RAJI B cells to 80%. Jurkat T cells appear the most sensitive to Au NP treatment, showing 80% and 60% relative survival when treated at 1.25 and 2.5 μg/cm^2 concentrations, respectively.

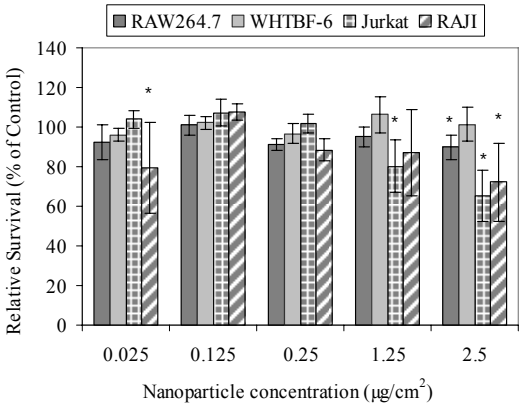

Figure 1. Cytotoxicity of 15 nm Gold Nanoparticles in Established Cell Lines
Cells were treated with various doses of 15 nm gold nanoparticles for 24 hours. Then cells were reseeded into 96 well plates and allowed to recover for several days. Cell viaibility was determined by MTT assay. For each cell line, data represents an average of 3 experiments ± standard error of the mean. Asterisk denotes significance ($P < 0.01$).

3.2 Phagocytosis by Au NP Treated RAW264.7

The ability of RAW264.7 murine macrophages to ingest 1 or more 1 μm latex beads was not significantly inhibited by Au NP at the concentrations used (data not shown). Under observation with a light microscope, RAW264.7 cells do appear to ingest Au NP (data not shown). The phagocytic ability of these macrophages after exposure to Au NP was tested in flow cytometry with 1 μm fluorescent latex beads representing inert cellular debris. A slight decrease in the percent of cells ingesting at least 1 bead was seen at the highest concentration of Au NP used but this was not significant.

3.3 TNF-α Production by Au-NP Treated Cells

15 nm Au NP induction of TNF-α production in RAW264.7 cells and human PBMC was determined by ELISAs. A significant increase in TNF-α production of almost 2-fold was observed in RAW264.7 cells treated with 2.5 μg/cm^2 Au NP compared to a sodium citrate treated control (Figure 2A). No significant increase was seen with the other Au NP concentrations used. The LPS treated RAW264.7 cells were used as a positive control and produced 5 times the amount of TNF-α as the negative control cells. Au NP treated human PBMCs produced elevated amounts of TNF-α in three separate experiments (Figure 2B). PBMCs treated with the potent agonists PMA and ionomycin were used as positive controls; these cells produced levels of TNF-α over 25 fold that of the negative control Cells.

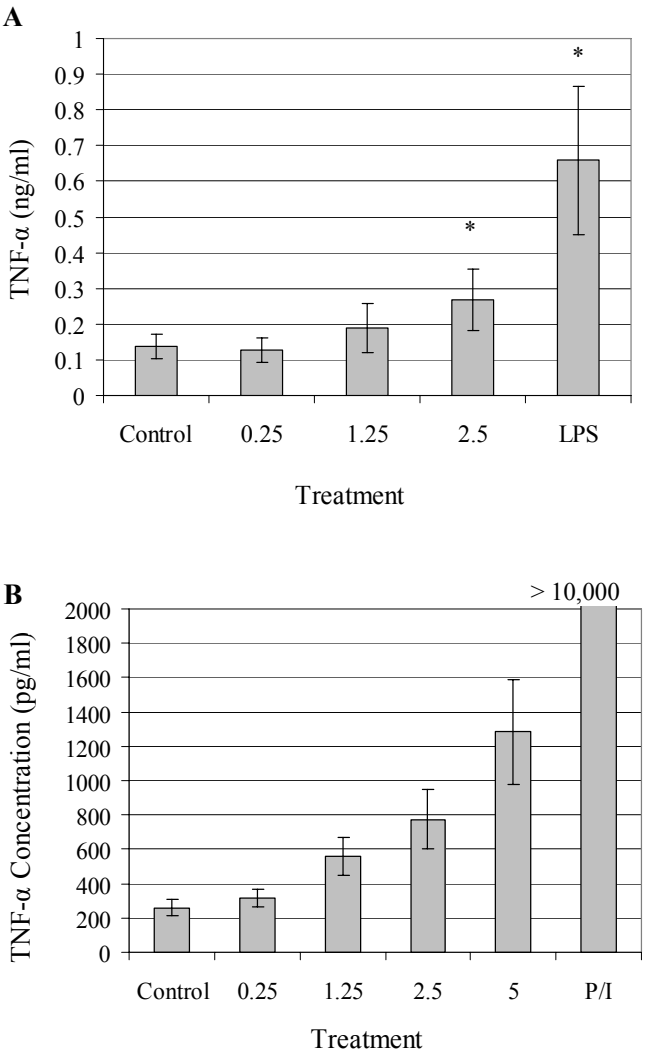

Figure 2. TNF-α production by Au NP Treated Cells RAW264.7 macrophages (A) and human PBMCs (B) were treated for 24 hours with various doses of 15 nm Au NPs (μg/cm^2) in sodium citrate, sodium citrate alone (Control), 100 ng/ml bacterial lipopolysaccharide (LPS), or a combination of the mitogens phorbol myristate acetate and ionomycin (P/I). Asterisk denotes significance ($P < 0.05$). Averages of 3 experiments ± SEM. .

3.4 MAP kinase activation in 15 nm Au NP treated RAW264.7

The ability of 15 nm Au NP to activate the mitogen activated protein (MAP) kinase pathway was determined by western immunoblotting. Relative levels of the phosphorylated forms of the MAP kinase proteins SAP kinase (SAPK) and Jun kinase (JNK) were measured. Cell lysates from treated and control cells were fractionated by SDS-PAGE and electro-transferred to a membrane for immunoblotting. The blots were probed for the active, phosphorylated forms of SAPK and JNK (Figure 3). A dose dependent increase in phosphorylation of SAPK and JNK was observed in two experiments. The LPS treated positive control cells have shown a consistently higher degree of phosphorylation for these MAP kinases compared to the negative control.

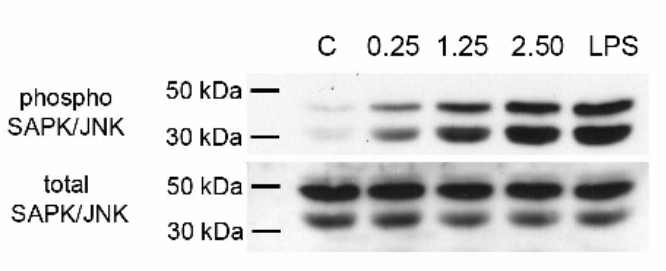

Figure 3. MAPK Activation of RAW264.7 Cells by Au NP Cells were treated with dH_2O, 100 ng/ml bacterial lipopolysachharide (LPS), or various doses of 15 nm Au NP (ug/cm2). Cell proteins were extracted and separated by SDS-PAGE. Proteins were electro-transferred to PVDF membrane and immunoblotted for phosphorylated SAPK (46 kDa) and JNK(54 kDa). Membranes were stripped and reprobed for total SAPK/JNK protein.

4 CONCLUSION

Overall, the results demonstrate that the MTT proliferation/ cytotoxicity assay, flow cytometric phagocytosis assay and ELISA for cytokines represent cell-based assays suitable for preliminary assessment of biological effects of nanomaterials. The immunoblot assay for MAP kinase molecules remains to be optimized; a more efficient (i.e. higher throughput) assay for these signaling molecules should be developed. Our results also clearly indicate that 15 nm gold particles have differential cytotoxic effects on cultured cells of different tissue origins but consistent stimulating effect on the production of the proinflammatory cytokine TNF-α in the cultured murine macrophage line RAW264.7, without significantly altering the latter's phagocytosis activity.

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