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Altered protein expression profile associated with phenotypic changes in lung fibroblasts co-cultured with gold nanoparticle-treated small airway epithelial cells

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ABSTRACT

Despite the availability of toxicity studies on cellular exposure to gold nanoparticles (AuNPs), there is scarcity of information with regard to the bystander effects induced by AuNPs on neighboring cells not exposed to the NPs. In this study, we showed that exposure of small airway epithelial cells (SAECs) to AuNPs induced changes in protein expression associated with functional effects in neighboring MRC5 lung fibroblasts in a co-culture system. Uptake of 20 nm size AuNPs by SAECs was first verified by focused ion beam scanning electron microscopy. Subsequently, pretreated SAECs were co-cultured with unexposed MRC5 lung fibroblasts, which then underwent proteome profiling using a quantitative proteomic approach. Stable-isotope labeling by amino acids in cell culture (SILAC)–based mass spectrometry identified 109 proteins (which included 47 up-regulated and 62 down-regulated proteins) that were differentially expressed in the lung fibroblasts co-cultured with AuNP pretreated SAECs. There was altered expression of proteins such as Paxillin, breast cancer anti-estrogen resistance 1 and Caveolin-1, which are known to be involved in the cell adhesion process. Morphological studies revealed that there was a concomitant increase in cell adhesion and altered F-actin stress fiber arrangement involving vinculin in the lung fibroblasts. It is likely that phenotypic changes observed in the underlying lung fibroblasts were mediated by AuNP-induced downstream signals in the pretreated SAECs and cell–cell cross talk.

1. Introduction

Gold nanoparticles (AuNPs) are inert and display unique plasmonic properties that make them potentially well suited for a myriad of biomedical and industrial applications [1]. For instance, using AuNPs for target delivery of recombinant human tumor necrosis factor alpha (rhTNF) in cancer treatment has entered a Phase I clinical trial [2]. However, with the advancement of nanotechnology, potential safety and health issues raised have become a concern to the community. Nanoparticle (NP)-related toxicity has been extensively evaluated as part of the risk assessment procedure and for determining the safe exposure limit for occupational workers, consumers and environment [3–5]. Thus far, there are *in vitro* and *in vivo* studies that have shown contradictory and discordant results, possibly due to differences in the experimental settings and parameters used [6,7].

We have previously reported that AuNPs exert cytotoxic and genotoxic effects in lung cells *in vitro*, in parallel with an increase in oxidative stress and the induction of autophagy [8–10]. We have also established that rats exposed to AuNPs by inhalation showed deposition of the NPs in lungs [11]. *In vitro* analyses using the monolayer cell culture system, is a relatively easy method for the assessment of NP-related toxicity and useful for molecular biology-related studies. However, extrapolation of *in vitro* studies in animal models are fraught with difficulties [12], with many *in vivo* studies revealing minimal or mild effects resulting from NP exposure [13–15]. On the other hand, it is difficult to conduct mechanistic

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studies in *in vivo* models (using rodents, zebra fish and the drosophila fly) due to complexity of the biological systems [16–18].

Despite the abundance of toxicity studies on the cellular exposure to NPs, little is known with regard to the bystander effects induced by AuNPs on neighboring cells that are not exposed to the NPs. Hence, we adopted a SAEC-MRC5 lung fibroblast co-culture system (which mimics the respiratory tract), as SAECs are lung epithelial cells which serve as the first line of contact with inhaled NPs, and lung fibroblasts constitute the stromal component surrounding the epithelial cells in the lung [19]. We also used a state of the art Stable Isotope Labeling by Amino acids in Cell culture (SILAC) proteomics approach, a robust tool which allows quantification and thus, proteome comparisons [20], to analyze changes in the proteome of the lung fibroblasts. In addition, functional effects associated with the altered proteins, in particular cell adhesion and changes in the cytoskeleton, were also examined.

2. Materials and methods

2.1. Synthesis of AuNPs

AuNPs (20 nm in size) were synthesized through the reduction of Au-containing tetrachloroauric acid (HAuCl4) by trisodium citrate dehydrate as described previously following Turkevich's method [8–10]. Colloidal AuNP suspension was then washed twice with ultra pure water by centrifugation at 9000 rpm for 20 min before coating with fetal bovine serum (FBS).

2.2. Cell culture

SAECs (CC–2547S), purchased from Lonza (Basel, Switzerland), were cultured using Small Airway Growth Medium (SAGM, Gibco) supplemented with BulletKit (cc-3119). MRC5 human fetal lung fibroblasts (ATCC® CCL-171™) were purchased from the American Type Culture Collection. MRC5 cells were cultured in Rosewell Park Memorial Institute (RPMI 1640) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 100 units/ml penicillin/100 μg/ml streptomycin.

2.3. Pretreatment of SAECs with AuNPs

SAECs were treated with 1 nM of AuNPs in 6-well culture plates for 72 h. The dose of AuNPs used, which is known to induce cytotoxicity and genotoxicity, was pre-optimized from our earlier reported studies in lung cells [8–10].

2.4. Focus ion beam scanning electron microscopy (FIB-SEM)

AuNP-treated SAECs were fixed with 2.5% glutaraldehyde, washed and stained with 0.2% osmium tetroxide. After dehydration with a graded series of alcohol, specimens were embedded in epoxy resin, which was allowed to polymerize at 60 °C overnight. Polymerized resin blocks were trimmed and polished with a glass knife and mounted on aluminum SEM specimen stub. A Zeiss Auriga 60, CrossBeam instrument (Carl Zeiss Microscopy, Oberkochen, Germany) was employed for milling and imaging. Backscattered electron (BSE) imaging was performed using electron beam at an acceleration voltage of 2 kV with a 1.2 nA probe current. The BSE signal was collected by an in-column Energy-selective Backscattered (EsB) detector at a filtering grid bias of 1350 V.

2.5. Co-culture of SAEC-MRC5 lung fibroblasts

SAECs were co-cultured in the upper chamber of a Transwell polycarbonate membrane with SILAC-labeled MRC5 lung fibroblasts in the lower chamber (Fig. 1).

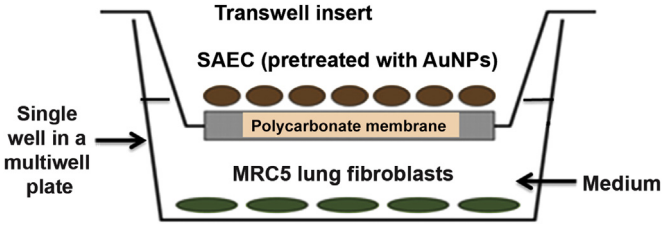

Fig. 1. Schematic drawing of the SAEC-MRC 5 lung fibroblast co-culture system. SAECs (treated with and without AuNPs) were seeded in the upper chamber of a Transwell polycarbonate membrane with the SILAC-labeled MRC5 lung fibroblasts plated in the lower chamber.

2.6. SILAC labeling of lung fibroblasts

MRC5 lung fibroblasts were initially grown for at least 4 passages, to allow maximum metabolic incorporation of substituted stable isotopic nuclei (deuterium, 13C, 15N) in selected amino acids into proteins, prior to co-culture. The lung fibroblasts were grown in DMEM without Lysine and Arginine amino acids, supplemented with either "light" or with "heavy" forms of the two amino acids isotopes (with13C615N2-L-lysine and 13C615N4-L-arginine), 10% dialyzed FBS and 1% penicillin/streptomycin. An incorporation check for labeling efficiency was performed using MS analysis after the fourth passage and a median incorporation rate of 97% was observed. The forward experimental set is defined as lung fibroblasts (control without treatment) that were incubated with light isotopes of arginine and lysine (also labeled as "light" or "L" cells) and conversely, the reverse set was incubated with heavy isotopes of arginine and lysine (regarded as "heavy" or "H" cells).

2.7. Mass spectrometry analysis

Whole proteomes of the MRC5 lung fibroblasts were extracted after 72 h, and the "light" and "heavy" protein lysates were mixed in a 1:1 ratio according to the weight of the proteins. After 4–12% gradient gel separation, protein bands were digested with the trypsin enzyme overnight at 37 °C. Extraction of tryptic peptides were carried out before LC-MS/MS analysis using the LTQ-Orbitrap mass spectrometer. Protein identification and quantitation were performed using the Mascot (version 2.2; Matrix Science, London, UK) software against the International Protein Index (IPI) human protein database (V3.68). Fold change was obtained from the normalized ratio of peptides which were quantified in the experimental MRC5 cells labeled with heavy (K8R10) isotopes compared to control MRC5 cells that had been labeled with light (K0R0) isotopes and statistical significance was calculated by MaxQuant version 1.0.13.13.

2.8. Gene ontology and IPA analysis

Gene Ontology (GO) annotations enrichment analysis was performed by importing the gene ID into the program and IPA pathway analysis (www.ingenuity.com) by importing the IPI number into the program, with automatic mapping of the pathways.

2.9. Cell adhesion assay

MRC5 lung fibroblasts were resuspended in medium at a concentration of 5×10^5 cells/ml. 100 μl of cell suspension (5×10^4 cells) was seeded into microwell plates (pre-coated with Collagen I or Fibronectin). The plated MRC5 cells were allowed to incubate for 30 min. Non-adherent cells were removed and the well was washed with PBS. Unwashed wells served as loading controls. The number of adherent cells was quantified by a colorimetric method using the MTS tetrazolium compound.

2.10. Confocal microscopy

Cells grown in glass chambers were fixed in 4% paraformaldehyde. Permeabilization of the cell membrane was performed using 0.2% PBS-triton ×100 thrice for 5 min at room temperature. For actin filament and vinculin staining, cells were incubated with phalloidin (1:50, Merck, Milipore) and anti-vinculin antibody (1:200, Milipore) in PBS for 1 h in dark, rinsed with PBS before mounting.

2.11. Statistical analysis

Differences in the values between control and treated groups were analyzed by the Student *T*-test using GraphPad Prism version 4.00. Error bars indicate standard error of the mean and *p*-values less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Intracellular localization of AuNPs in SAECs

For verification of uptake of AuNPs by SAECs, micrographs were taken from the milled sections of araldite embedded with AuNP-exposed SAECs using FIB-SEM. A micrograph of SAEC revealing ultrastructural details such as a double membrane nucleus with a nucleolus, and cytoplasmic organelles that include endoplasmic reticulum, lysosomes and endosomes is shown in Fig. 2A. The milled and raw image of the lung fibroblast as shown in Fig. 2B was then subjected to contrast reversal, whereby, AuNP clusters were observed as dark deposits (Fig. 2C).