An OMICs Approach for Assessing the Safety of Single-Walled Carbon Nanotubes in Human Skin and Lung Cells

Mary Jane Cunningham[*], Mrinal Shah[*], Carolina Lema[*], Scott R. Magnuson[**], Michael T. Falduto[**], Leandro Balzano[***] and Daniel E. Resasco[****]

[*]Houston Advanced Research Center, 4800 Research Forest Drive, The Woodlands, TX 77381, mjcunningham@harc.edu
[**]GenUs BioSystems, Inc., 1808 Janke Dr., Northbrook, IL 60062
[***]SouthWest Nanotechnologies, 2360 Industrial Blvd., Norman, OK 73069
[****]University of Oklahoma, School of Chemical, Biological and Materials Engineering, 100 East Boyd St., Norman, OK 73019

ABSTRACT

High-throughput OMICs biotechnologies combined with a systems biology approach were used to screen for the toxicity of nanomaterials. Profiling of the macromolecular interactions of nanomaterials with human cells was done with mRNA and microRNA (miRNA) expression microarrays (genomics) and two-dimensional gel electrophoresis annotated by mass spectrometry (proteomics). Primary human epidermal keratinocytes (HEK) and primary human bronchial epithelial cells (NHBE) were exposed *in vitro* to single-walled carbon nanotubes (SWNT) and other nanoparticulate substances. A four-tiered data analysis approach was used which included statistical analysis, similarity profile matching, biomarker identification and pathways analysis. By comparing the miRNA, mRNA and protein profiles, a more complete description of the interactions on the macromolecular and cellular level after exposure to nanomaterials can be obtained.

Keywords: nanotubes, microarray, proteomics, gene expression, microRNA

1 INTRODUCTION

Safety assessment of nanomaterials is just beginning to be evaluated. The approach outlined here is a combination of using global screening OMICs technologies combined with a systems biology approach. OMICs technologies encompass gene expression (genomics), protein expression (proteomics), single-nucleotide polymorphisms or SNPs (genotyping, pharmacogenomics) and metabolic profiling (metabonomics). Each of these disciplines uses leading-edge technologies to gather a comprehensive view of cellular processes. For example, genomics takes advantage of gene expression microarrays to evaluate the expression levels of tens of thousands of genes (whole genomes) in a single experiment. The results are data in the form of gene expression profiles (GEP). Proteomics by using two-dimensional gels annotated by mass spectrometry or by protein microarrays offers a simultaneous comprehensive view of what proteins are present and how they are expressed within a set of cells or tissues. Genotyping analyzes the single-base variations within genes which may dictate why adverse reactions occur in some individuals and not in others. Metabonomics takes advantage of the recent advances in mass spectrometry in combination with either nuclear magnetic resonance spectroscopy or liquid chromatography to analyze real time metabolic profiles of compounds *in situ*. All of the above OMICs technologies can in themselves address a "systems biology" approach, but by combining information from each discipline (genomic, proteomic and metabonomic), an overall systems biology perspective can take shape and be evaluated by creating a "virtual cell". More information can be obtained in previously published reviews [1-4].

1.1 Nanomaterials

Recent advances in nanotechnology have made possible the manufacturing of nanomaterials on a commercial scale. Nanomaterials range considerably in their composition, size, commercial application and their environmental exposure [5]. The current accepted definition of nanomaterials encompasses both anthropogenic (naturally occurring) and engineered materials whose size is below 100 nm [6]. Anthropogenic nanomaterials have gained attention as suspected contributing factors to environmental pollution and accompanying human disorders. For example, ultrafine particles generated by manufacturing plants and diesel exhaust engines have shown to contribute to respiratory and cardiovascular disorders [7-8].

Engineered nanomaterials are made using the basics of fullerene chemistry. These nanomaterials, which include nanocrystals, nanoparticles, nanotubes, nanohorns, and nanowires, are now being developed for use in medical, diagnostic, energy, component and

communications industries [9-10]. The properties of these engineered materials supercede their micro- and macro-counterparts due to the increased surface area compared to their size making the possibilities of their applications almost limitless.

Nanomaterials are being developed to provide certain advantages not seen with classic larger-sized materials. C_{60} molecules (fullerenes or buckyballs) are being derivatized with monoclonal antibodies for passage within the body to expand medical uses. Nanowires and nanotubes are being developed and manufactured for use in fuel cells and communications. In addition, the tensile strength of some nanotubes preparations has been shown to be in excess of what is now available with fiberoptics.

1.2 Toxicity of Engineered Nanomaterials?

Few safety assessment studies of nanomaterials have been published to date and have varied as to their conclusions of the toxicity of nanomaterials. Some examples are described below. Two early studies with carbon nanotubes done at the Warsaw University showed no inflammation or toxicity when SWNT was exposed to the lungs of guinea pigs or came in contact with human and rabbit skin [11-12]. No toxicity was observed at 1-10 μM levels when human 3T6 cells were exposed to SWNT in culture [13]. However, when an unpurified SWNT preparation was exposed to an immortalized human epithelial keratinocyte cell line, cytotoxicity and oxidative stress was seen [14]. The authors do comment that the high content of iron (30%) carried over from the manufacturing process could be a contributing factor.

Recent *in vivo* studies gave opposing conclusions as well. Carbon nanotubes may be more toxic than quartz dust (a known causative factor in silicosis) in a study where mice were exposed to SWNT [15]. A similar study with rats found granulomas (a similar histopathological finding) but interpreted this observation as "inconclusive" and may be "artifactual" [16]. Even though 15% mortality was observed in the rats, it was concluded that the SWNT agglomerates led to the physical occlusion of the animals' airways causing suffocation and not due to the toxicity of the SWNT themselves.

Two *in vitro* studies have shown that carbon nanotubes do have adverse effects on cultured human cells. One study exposed HEK293 (transformed human embryo kidney cells) to SWNT and found apoptosis was induced as well as decreased cellular adhesion. Results from a 100-gene microarray showed cell cycle, signal transduction, and cellular adhesion genes down-regulated while genes involved in cell cycle arrest and apoptosis were up-regulated [17]. Another study cited that multi-walled carbon nanotubes (MWNT) after treating primary HEK were found localized within the cytoplasmic vacuoles in these cells and that interleukin-8 (IL-8), a molecule highly active in a heightened immune response, was released [18].

Based on these preliminary studies, nanomaterials may cause toxicity. An additional study showed another class of engineered nanomaterials, fullerenes, to cause oxidative stress in fish [19]. However, in each set of experiments described above, different compositions of nanomaterials and different experimental designs (i.e. assays, exposure routes, positive controls, negative controls) were used. In addition, most experiments were performed with animal models.

No comprehensive study has been performed to date. Most of references above cite experiments using either cell lines or transformed cells or non-pure preparations of SWNT. In the experiments detailed here, non-immortalized, nontransformed primary human cells were exposed to a purified preparation of SWNT. Labeled probes from harvested cell pellets were run on both gene and miRNA microarrays to obtain mRNA and miRNA expression profiles. Cell lysates were run on two-dimensional gel electrophoresis and annotated by mass spectrometry to obtain protein expression profiles. These technology platforms allow investigators to survey the macromolecular information on a global scale. By comparing these profiles of SWNT-exposed cells to those profiles of known nanomaterial-exposed cells, an assessment as to whether the macromolecules activated are associated with toxicity or not can be made. A preliminary report has been made [20].

MATERIALS AND METHODS

Primary HEK and primary NHBE were obtained free of any infectious contamination. Both cell strains were cultured using aseptic technique in serum-free culture media. The cells were subpassaged using the trypsin/EDTA and trypsin neutralizer solutions and maintained in a humidified incubator at 5% CO_2 and 37°C.

Titanium dioxide (TiO_2, nanopowder), carbonyl iron (CI, ferronyl iron), silica (SiO_2, α-quartz, crystalline silica, Min-U-Sil®5) and carbon black (CB, Printex® 90) were chosen as appropriate controls. Crystalline silica is a known lung irritant and carcinogen and is used as a positive control [21]. Carbonyl iron was obtained of a grade and purity used in medical applications and is used as the negative control. Single-walled carbon nanotubes (SWNT) were manufactured by SouthWest NanoTechnologies, Inc. using a modified chemical vapor deposition method, CoMoCAT™, invented by the laboratory of Dr. Daniel Resasco at the University of Oklahoma [22].

Either the MTT or XTT assays (Promega Corporation, Roche, respectively) were used to test for cell viability. These assays are based on a previously developed dye method [23]. Both HEK and NHBE were seeded 16 hr. prior to treatment at preconfluent cell numbers per well.

HEK and NHBE were preconfluent at the time of the nanomaterial exposures. Cell pellets were snap frozen

and total RNA isolated at a later time. The total RNA was used to manufacture labeled cRNA or miRNA probes to hybridize onto mRNA or miRNA microarrays. The arrays were scanned and image and data analysis performed. Cells were also lysed in a protein lysate buffer and run in two-dimensional gel electrophoresis. The resulting spots were annotated for mass and tryptic peptide sequence by mass spectrometry. Analysis of all data files was done using a tiered approach which included statistical analysis, similarity profiling, biomarker identification, pathway analysis and network profiling.

RESULTS AND CONCLUSIONS

Each nanoscale or low micron-scale particulate (TiO_2, CI, SiO_2, CB, and SWNT) formed a heterogeneous suspension of particles in the culture medium. A preliminary experiment exposed HEK to TiO_2, SiO_2 and CB for 24 hours. No interference of particles was observed in the microarray process and gene expression profiles (GEP) were obtained. A more comprehensive time course with a total of 8 time points within a 24-hour time frame was used to investigate both noncytotoxic and cytotoxic doses of CI-, SiO_2-, CB- and SWNT-exposed HEK cells. At the noncytotoxic doses, the GEP for SWNT was found to be most similar to the GEP for CI (negative control). At cytotoxic doses, the GEP for SWNT was most similar to GEP for SiO_2 (positive control). In the case of NHBE exposures, the GEP for SWNT at the noncytotoxic dose matched more closely with the GEP for CI than SiO_2. Preliminary data also shows differences between miRNA and protein expression profiles of silica-treated NHBE and untreated NHBE.

In the HEK experiments, hundreds of genes were significantly-expressed. Several genes known to be involved in immune response (e.g. cytokines) and in membrane integrity and remodeling (e.g. vimentin) were expressed with exposure to silica and these findings correlated with the literature. In the NHBE experiments, several genes were significantly-expressed but this magnitude was about 10 times lower than the values seen with HEK. Genes active in immune response and membrane integrity were also significantly-expressed in silica-exposed NHBE as well.

While genes were significantly expressed with SWNT-exposed HEK and NHBE, the overall number of genes expressed was low and comparable to the negative control, CI. The results and findings here are in agreement with some of the earlier studies in that SWNT does not appear to have adverse toxic effects on human cells at doses are not overtly toxic. It may be that the highly purified preparation of SWNT and the tightly-controlled experimental design coupled with the ability to globally survey expression of three different levels of macromolecules (mRNA, miRNA and protein) may be very predictive.

ACKNOWLEDGEMENTS

This work was supported by the funding from the National Sciences Foundation SGER BES-0436366 and BES-0536679. A special thanks to HARC and its primary contributor, the Mitchell family, for their additional financial support.

REFERENCES

[1] Cunningham MJ. 2000. Genomics and proteomics: the new millennium of drug discovery and development. J. Pharmacol. Toxicol. Meth. 44: 291-300.

[2] Cunningham MJ and Dao D. 2005. Fabricating microarrays. In: J. Fuchs and M Podda, eds. "Encyclopedia of Diagnostic Genomics and Proteomics", Marcel Dekker, New York, NY.

[3] Cunningham MJ and Shah M. 2007. Toxicogenomics. In: Gad, SC, ed. "Handbook of Pharmaceutical Biotechnology", John Wiley & Sons, Inc., NJ, in press.

[4] Cunningham MJ. 2007. Gene-Cellular Interactions of Nanomaterials: Genotoxicity to Genomics. In: Monteiro-Riviere NA and Tran L, eds. "Nanotoxicology: Characteristics, Dosing and Health Effects on Target Organs", Taylor & Francis/Informa Healthcare, New York, NY, in press.

[5] Roco MS and WS Bainbridge, eds. 2001. Societal Implications of Nanoscience and Nanotechnology. In: National Science Foundation, NSET Workshop Report, Kluwer Academic Publishers, Norwell, MA.

[6] Oberdorster G, Oberdorster E and Oberdorster J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113: 823-839.

[7] Mauderly JL. 2001. Diesel emissions: is more health research still needed? Toxicol. Sci. 62: 6-9.

[8] Salvi S and Holgate S. 1999. Mechanisms of particulate toxicity. Clin. Exp. Allergy 29: 1187-1194.

[9] Salata OV. 2004. Applications of nanoparticles in biology and medicine. J. Nanobiotechnol. 2: 3.

[10] Jortner J and Rao CNR. 2002. Nanostructured advanced materials. Perspectives and directions. Pure Appl. Chem. 74: 1491-1506.

[11] Huczko A, Lange H, Calko E, Grubek-Jaworska H and Droszcz P. 2001. Physiological testing of carbon nanotubes: are they asbestos-like? Fullerene Sci. Technol. 9: 251-254.

[12] Huczko A and Lange H. 2001. Carbon nanotubes: experimental evidence for a null risk of skin irritation and allergy. Fullerene Sci. Technol. 9: 247-250.

[13] Pantarotto D, Briand JP, Prato M and Bianco A. 2004. Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem. Commun. (Cambridge) 1: 16-17.

[14] Shvedova AA, Castranova V, Kisin ER, Schwegler-Berry D, Murray AR, Gandelsman VZ, Maynard A and Baron P. 2003. Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. J. Toxicol. Environ. Health A 66: 1909-1926.
[15] Lam C, James JT, McCluskey R and Hunter RL. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci. 77: 126-134.
[16] Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GAM and Webb TR. 2004. Comparative pulmonary toxicity assessment of single wall carbon nanotubes in rats. Toxicol. Sci. 77: 117-125.
[17] Cui D, Tian F, Ozkan CS, Wang M and Gao H. 2005. Effect of single wall carbon nanotubes on human HEK293 cells. Toxicol. Lett. 155: 73-85.
[18] Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY and Riviere JE. 2005. Multi-walled carbon nanotubes interactions with human epidermal keratinocytes. Toxicol. Lett. 155: 377-384.
[19] Oberdorster E. 2004. Manufactured nanomaterials (fullerenes, C_{60}) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health. Perspect. 112: 1058-1062.
[20] Cunningham MJ, Magnuson SR, Falduto MT, Balzano L and Resasco D. 2005. Gene expression profiling of nanoscale materials using a systems biology approach. Toxicol. Sci. 84(1-S): 9.
[21] World Health Organization and the International Agency for Research on Cancer. 1997. Silica. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 68: 41.
[22] Ivarez WE, Kitiyanan B, Borgna A and Resasco DE. 2001. Synergism of Co and Mo in the catalytic production of single-wall carbon nanotubes by decomposition of CO. Carbon 39: 547-558.
[23] Mossman T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 55-63.