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Feasibility of Biomarker Studies for Engineered Nanoparticles: What Can Be Learned from Air Pollution Research

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Abstract

Objective—Occupational exposure to engineered nanoparticles (NP) may pose health risks to the workers. This article is to discuss the feasibility of identifying biomarkers that are associated with NP exposure.

Methods—Scientific literature on the adverse health effects of ambient ultrafine particles (UFP) and NP was reviewed to discuss the feasibility of conducting biomarker studies to identify NP-induced early biological changes.

Results—Various approaches for biomarker studies have been identified, including potential injury pathways that need to be considered and the methodologies that may be used for such studies.

Conclusions—Although NP may have novel mechanisms of injury, much can be learned from our experience in studying UFP. Oxidative stress-related pathways could be an important consideration for identifying NP-associated biomarkers and one of the most effective approaches for such studies may be proteome profiling.

Introduction

The introduction of nanotechnology has brought great benefits to a wide span of areas in today's society and will continue to do so in the future, but it also brings many unknowns about its potential adverse health effects. The specific physicochemical characteristics of engineered nanoparticles (NP) may introduce health risks which differ significantly from fine particles of the same chemical composition (1). Therefore, it is important to realize that certain groups of people, such as workers in nanotechnology-related fields, are at higher risk than the general population because of their close and constant contact with these materials and begin to take protective measures before an outbreak of serious clinical outcomes.

One of the strategies for preventing serious nanotoxicity from happening is to identify early biological events associated with exposure to harmful NP, then use that information for prevention. This can be achieved through biomarker studies in NP target organs/tissues or

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preferably in the biological fluid. While biomarker studies for NP toxicity are currently at their early stage, our experience in biomarker research for the incidental or ambient nanoparticles, a.k.a. ultrafine particles (UFP), could be used to facilitate this process due to some similarities between UFP and certain NP. One of the injury mechanisms that are common to UFP and certain NP is the induction of oxidative stress and inflammatory responses by particles. In this communication, the feasibility of conducting NP-associated biomarker studies, based on what has been learned from air pollution research, will be discussed.

Importance of biomarker studies for NP-related occupational safety

NP are less than 100 nm in size and are intentionally produced with specific characteristics required for their applications. Due to their unique size and physicochemical properties, such as surface area, shape, crystallinity, surface charge, reactive surface groups, dissolution rate, state of agglomeration or dispersal, etc., NP are potentially more dangerous than larger particles of the same composition and may cause unanticipated adverse health effects to people who are exposed to these particles (1).

NP exposure can take place in almost all economic sectors, but occupational exposure in research laboratories and industries that manufacture, handle, use and dispose these particles place the workers at potentially higher risk (2–4). Although there has been no report that link NP exposure to a definitive disease outcome, epidemiological studies have found hazardous respiratory effects through occupational exposure to carbon black (CB) and fumed silica (5–7). Another example of potential occupational hazard is the exposure to metal or metal oxide NP (8–10). Metal oxide NP are often used as industrial catalysts and increased levels of these particles have been found in areas surrounding factories (8). There has been reported incidence of bronchitis, metal fume fever, changes in lung function and increased lung infection among welders (9, 10). Metal fume fever is a clinical syndrome that is presented as a flu-like illness characterized by self-limiting inflammation and oxidative stress response in the lung (11). It has been suggested that this condition is caused by inhalation of highly concentrated metal oxide particles, particularly ZnO (11–15). Given the growing use of NP and so many unknowns about their potential health effects, it is imperative to develop effective methods for assessing health risks associated with NP exposure. This is particularly important for the health surveillance and monitoring of workers who may be exposed to NP in the occupational setting.

Due to the short history of nanotechnology, currently, there is no published report that has established a definitive link between a disease outcome and exposure to a specific type of NP in humans. As it is almost certain that the growth of nanotechnology will outpace epidemiological studies, instead of waiting for these reports, an active approach would be to take precaution now so that the people at higher risk can be properly protected. One effective strategy to achieve this goal is to identify biomarkers associated with NP exposure. A “biomarker” is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (16). Therefore, the role of biomarkers in assessing the health effects of NP is to link exposure to the disease outcomes by providing mechanistic indicators that are associated with early adverse effects of NP (Figure). Although it is expected that it may take a long period of time to develop a panel of biomarkers that could be used as indicators of exposure-specific disease outcomes, identification of early biological responses related to injury pathways, based on our knowledge in air pollution research, would be a good starting point at this time.

Oxidative stress-related inflammation as an injury mechanism for the toxicity of UFP and certain NP

In the last few years, in response to the rapid growth of nanotechnology, nanotoxicology has emerged as a unique field to study the toxicity of engineered nanomaterials (ENM) including NP and to understand the injury mechanisms that are specifically related to the scale, dimension and physico-chemical characteristics of these materials (17, 18). The concept of nanotoxicology is basically evolved from air pollution research, especially that on the incidental nanoparticles (i.e. UFP). Although there are significant differences between UFP and NP in many aspects of their characteristics, there are certain similarities in the mechanisms of action and potential to produce adverse health effects between these two types of nanoscaled particles (Table) (1, 12, 19).

For example, two mechanisms that are common to UFP and several types of NP are the induction of oxidative stress and inflammatory response (1, 12, 19, 20). That particle-induced oxidative stress is one of the major mechanisms for the adverse biological effects of UFP has been demonstrated in cellular, animal as well as human studies (19, 21–33). Inhaled UFP are capable of inducing oxidative stress in the lung as well as in systemic circulation. Particle-generated ROS and subsequent oxidative stress have been shown to be involved in many pathological conditions associated with respiratory and cardiovascular disease outcomes including lung inflammation, asthma exacerbation, atherosclerosis and thrombosis (19, 30, 32, 34–38). Similarly, increasing evidence from cellular and animal studies has indicated that a number of NP also exert their pro-inflammatory and toxic effects through the same mechanisms (39–42).

The pro-oxidative and pro-inflammatory properties have been observed in a number of metal oxide NP. TiO_2 NP, which have a number of industrial applications, are capable of generating ROS, inhibiting reduced glutathione (GSH), activating several Nrf2-mediated antioxidant enzymes (i.e., heme oxygenase-1, thioredoxin reductase, glutathione transferase and catalase) and upregulating inflammatory cytokine gene expression in human airway epithelial cells (BEAS-2B) and in rats (39, 43–46). These pro-oxidative and pro-inflammatory effects of TiO_2 are correlated to particle size, surface area as well as composition (8, 39, 43, 44, 46–48). CuO NP that also have widespread applications have been shown to cause oxidative stress-mediated toxicity in a number of cultured cells (8, 49–51). Exposure of airway epithelial cells to CuO NP induced a significant increase in 8-isoprostanes and the ratio of oxidized to total glutathione in these cells, which was accompanied by decreased viability; this pro-oxidative effect of CuO NP could be effectively inhibited by co-exposure to antioxidant resveratrol (8). Moreover, antioxidants including N-acetyl cysteine (NAC) and catalase could significantly attenuate the effect of CuO NP on the expression of plasminogen activator inhibitor-1 (PAI-1), a protein involved in several cardiovascular diseases, in mouse pulmonary microvascular endothelial cells (52). ROS generation by ZnO NP, the NP that are considered responsible for the metal fume fever, in mouse macrophage and human bronchial epithelial cells could lead to oxidant injury, inflammatory response and cell death (53). It has been suggested that the pro-oxidant activity of ZnO NP is the result of particle dissolution (54). Prevention of ZnO NP dissolution through Fe doping could effectively reduce the pro-oxidative and pro-inflammatory effects of these particles (54). In animal studies, long-term inhalation exposure to nickel hydroxide NP induced oxidative stress and inflammation in the lung and cardiovascular system in hyperlipidemic apolipoprotein E-deficient ($\text{ApoE}^{-/-}$) mice (55). Intra-tracheal instillation of iron oxide NP in mice could lead to a significant decrease in GSH and an increase in pro-inflammatory cytokines in the bronchoalveolar lavage fluid (BALF) during acute response while formation of microgranuloma, an indicator of chronic inflammation, was observed 28 days after exposure (56). In addition to metal or metal oxide

NP, other NP such as silica, cationic polystyrene and C60 fullerene have also been reported to exert pro-oxidative and pro-inflammatory *in vitro* and *in vivo*, including increased ROS production, induction of oxidative stress, activation of antioxidant and signaling pathways and apoptosis (57–59).

It is necessary to point out that not all NP cause inflammation via a mechanism involving oxidative stress (60, 61). For example, it has been reported that while purified single-walled carbon nanotubes (SWCNT) failed to generate ROS in cultured mouse macrophages, pharyngeal aspiration of these materials could induce progressive fibrosis and granuloma formation in mouse lung (60). Recently, Crouzier et al have demonstrated that intranasal instillation of purified double walled carbon nanotubes (DWCNT) elicited an inflammatory response in mice, which was accompanied by a decreased ROS production (61). Nonetheless, currently available evidence suggests that there are quite a few types of NP that exert their adverse health effects through similar mechanisms as UFP including generation of oxidative stress and induction of inflammation. Thus, it is not impossible to initiate biomarker studies for these NP by focusing on these two well-defined injury pathways.

Feasibility of biomarker studies to assess NP exposure-associated adverse health effects

From the long history of air pollution research, various biomarkers linking air pollution exposure to its adverse effects in the respiratory and cardiovascular systems have been identified in human studies and most of these biomarkers are associated with two major toxicological response pathways, oxidative stress and inflammation (62, 63). For example, increased 8-isoprostane and melondialdehyde in exhaled breath condensate (EBC) have been reported as biomarkers for local oxidative stress in the lung, whereas systemic oxidative stress markers include alteration in the levels of antioxidant enzymes and GSH in the blood (64–67). While increased levels of pro-inflammatory cytokines, cytokine receptors and C-reactive protein have been considered the biomarkers associated with air pollution-induced systemic inflammation, platelet activation and increased expression of adhesion molecules have been identified as the biomarkers for the adverse cardiovascular effects of air pollution (62, 64, 65, 68). For the NP that share similar injury mechanisms (i.e. oxidative stress and inflammation) with UFP, it is theoretically feasible to conduct biomarker studies starting with similar approaches. For example, to assess the early events associated with exposure to these NP, the choice of potential biomarkers to be studied could include the changes that indicate local and systemic oxidative stress, systemic inflammation and inflammatory response in NP target organs such as those in respiratory, cardiovascular and immune system.

Currently there is no report of any definitive human disease that is caused by NP exposure. Therefore, it would not be practical and efficient to begin NP-associated biomarker identification in human studies. A more effective strategy would be using the step-wise approach to evolve NP-associated biomarker identification from cellular to animal and eventually to human studies; the same approach that has been used for studying air pollution-associated biomarkers. The advantage of cellular studies is that they will allow us to rapidly identify NP-induced early changes at biochemical and molecular levels, which may not be detected as disease endpoints in animal or in human studies, but may provide valuable information about the mechanistic basis for disease outcomes and help to guide further studies. In addition, cellular studies may also provide great potential for developing high throughput screening methods to accelerate biomarker studies. As the second step of studying NP exposure-related biomarkers, animal studies could further validate the findings from *in vitro* studies and have the advantage of being more physiologically relevant to disease outcomes in humans. Finally, biomarkers identified by cellular and animal studies

will be validated in human studies, which have the ability to directly demonstrate the “real-life” disease endpoints and to guide the development of surveillance strategies for the workers who are potentially at higher risk of exposure to the adverse health effects of NP.

Use of proteomics to identify biomarkers associated with NP exposure

While many biomarker studies are still carried out using traditional biochemical and immunological assay methods, the technologies of mass spectrometry, high throughput screening, cell- and tissue-based DNA microarrays and proteomics have provided great potential to accelerate this process. Among these new techniques, use of proteomics has been shown to be an effective approach for studying biomarkers induced by air pollutants including ambient UFP (69–72). Proteomics uses high-throughput methodologies to study the complete profile of proteins in a given cell or tissue (73). Its ability to analyze global cellular response has made it possible to identify potential biomarkers that are associated with exposures to various environmental stimuli and stress (74). Thus, the discovery of new biomarkers by proteomics, combined with the traditional biological response endpoints, could become a powerful tool to assess the health effects and susceptibility factors related to environmental pollutants including certain NP (73).

Proteomics has been used as an analytical approach for identifying markers that are linked to exposures to environmental agents as well as in disease conditions in both animals and humans and proteome changes related to oxidative stress and inflammation have been identified under many of these conditions (71, 75–78). Our own experience of using proteomics to study the biological effects of particulate air pollutants has allowed us to develop a oxidative stress response model that may explain the adverse health effects of particulate matter (PM) in the respiratory, cardiovascular and immune system and to identify potential biomarkers associated with the adjuvant effect of UFP on allergic airway inflammation (71, 77). Using this technology we are able to study the biochemical and immunological changes associated with exposure to incidental nanoparticles (i.e DEP and UFP) focusing on oxidative stress and inflammatory response. We have demonstrated that organic DEP extract is capable of inducing stratified oxidative stress responses in mouse macrophages and human bronchial epithelial cells that include the activation of antioxidant and detoxification defense systems, inflammation and toxicity in cultured cells (71). This series of response is in parallel with a linear increase in newly expressed proteins measured by 2D-gel electrophoresis (71). By liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, more than thirty proteins were identified as responsive to DEP-induced oxidative stress, suggesting that some of these proteins may serve as markers for exposure to pro-oxidative DEP chemicals. (71, 79). Other DEP-induced proteome changes include protein modification by nitrotyrosine, activation of the unfolding protein response (UPR), and increased expression of ATF4, an endoplasmic reticulum (ER) stress-associated transcription factor (69, 71). In animal studies, we are able to identify oxidative stress-induced proteome changes in the BAL fluid and lung tissue in mouse asthma models (72, 77). Our most recent study demonstrates that the expression of polymeric immunoglobulin receptor, complement C3, neutrophil gelatinase-associated lipocalin, chitinase 3-like protein 3 (Ym1), chitinase 3-like protein 4 (Ym2), and acidic mammalian chitinase (AMCase) in the lung is associated with the adjuvant effect of UFP on the primary immune response (allergic sensitization) and particulate oxidant potential (29, 77). Increased Ym1 expression is also associated with the boosting effect of UFP on the secondary immune response in the “real-life” inhalation exposure study conducted near downtown Los Angeles (27). Moreover, our most recent study on NP has demonstrated oxidative stress-associated proteome changes in the BAL fluid from C57BL/6 mice that were exposed to ZnO NP via pharyngeal aspiration, suggesting that proteomics may also be used to identify biomarkers related to the exposure of certain NP (Unpublished data). As it is evident that oxidative stress and inflammatory

responses are also responsible for the toxicity of a number of NP, there is a great potential to use the technology of proteomics to identify the biomarkers associated with exposure to those NP that exert their adverse effects through these two injurious pathways.

In summary, the complicated physico-chemical characteristics of ENM have brought an urgent need to study their potential adverse health effects, especially among workers who are exposed to these materials through daily work. While it will take a long period of time to link human disease outcomes to specific ENM exposures, we can take the advantage of our experience in air pollution research and available new technologies to study NP exposure-associated biological responses at biochemical, molecular and cellular levels, a process known as biomarker studies. The ideal biomarkers for assessing environmental and occupational exposures should be able to provide strong mechanistic, molecular or biochemical basis for the diseases, be exposure-specific, reflect early adverse health effects, have clinical relevance and easy to use. Although we are not able to identify the biomarkers that meet all these criteria at this time, it is feasible to study NP exposure-associated early biological events focusing on well defined injury mechanisms such as oxidative stress and inflammation, which may be used as indicators of exposure to the hazardous NP.

Clinical Significance

Biomarker studies will provide valuable information to identify early biological events associated with the adverse health effects of engineered nanomaterials before the manifestation of clinical outcomes. This is particularly important for the health surveillance of workers who may be at higher risk due to their occupational settings.

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Figure.

The role of biomarkers in assessing the adverse health effects of NP is to link particle exposure to the clinical disease outcomes. Biomarkers are the detectable characteristic changes that reflect exposure-associated early events usually at biochemical and molecular levels. Biomarker studies may provide mechanistic explanation to the adverse health effects of NP as well as the potential to develop high throughput screening methods for medical surveillance of workers who are exposed to NP in occupational settings.

Table**Comparison of UFP and NP**

Particle Properties	UFP	NP
Source	Incidental (i.e. combustion)	Engineered (controlled synthesis)
Size	< 100 nm	< 100 nm
Surface area/volume	High	High
Uniformity	Low	High
Organic chemical content	High	Low
Metal content	High	High to low
Ability to generate ROS	Strong	Varies
Other contributing factors to toxicity	Content of other chemicals Climate variation Local traffic activity	Surface area and shape Crystallinity Surface charge Reactive surface groups State of dissolution, aggregation or dispersal
Exposure routes	Inhalation	Inhalation, skin, ingestion, medical use
Adverse health effects	Yes	Potential
Major risk factors	Distance to source Time to commute	Occupational exposure (manufacture, handling, waste disposal, research laboratories) Commercial use