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RESEARCH ARTICLE

Engineered nanoparticle respiratory exposure and potential risks for cardiovascular toxicity: Predictive tests and biomarkers

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Abstract

The most attractive properties of engineered nanomaterials for technological applications, including their small size, large surface area, and high reactivity, are also the main factors for their potential toxicity. Based on ambient ultrafine particle research, it is predicted that nanosized particles may have deeper pulmonary deposition, higher biological activity, and a tendency for extrapulmonary translocation compared to larger particles. In this regard, nanoparticle exposure, by direct or indirect mechanisms, may lead to unexpected distant responses, involving the immune system, cardiovascular system, liver, kidney, and brain. The systemic effects may induce or modify the progression of existing diseases such as cardiovascular disease. Current experimental toxicity evaluation of engineered nanomaterials, specifically carbon nanotubes, demonstrated that deposition of these materials in the lung leads to inflammation and fibrosis. The local toxicity is associated with cardiovascular effects related to atherosclerosis. Although translocation of carbon nanotubes into the systemic circulation is hypothetically possible, there is no current evidence to support this hypothesis. However, studies pointed out that carbon nanotube-induced lung inflammation results in a release of inflammatory mediators and activation of blood cells which can contribute to cardiovascular adverse effects. Furthermore, complex protein and gene expression blood analysis can help in development of biomarkers for application in human screening of nanoparticle exposure. Future studies to evaluate the systemic effects of carbon nanotube exposure under workplace or environmental exposure paradigms should be conducted.

Keywords: *Atherosclerosis; biomarkers; blood gene expression; inflammatory cytokines; nanomaterials; nanotoxicology; predictive tests*

Air pollution and cardiovascular diseases—A lesson for nanotoxicology

The lung is a complex organ consisting of a series of branching tubules and alveoli that are highly vascularized to provide a large gas exchange surface. The gas exchange region comes in direct contact with inhaled particles and pathogens, a challenge that is overcome by multiple layers of intrinsic defense systems including epithelial cell barrier, mucociliary clearance, numerous host defense proteins, such as lysozyme, defensins, and surfactant proteins, professional phagocytic cells, cytokines, and chemokines. Pathogens or hazards that escape these defense mechanisms may induce tissue injury involving disruption of the alveolar epithelial and endothelial integrity, leading to inflammatory response and loss of compartmentalization (Deng and Standiford 2005). Thus, damage of the endothelial or epithelial side of the alveolar–capillary interface will facilitate the leakage of inflammatory mediators and hazard components into the circulation. The tissue injury, as well as inflammatory response, is associated with alterations of procoagulant signaling mechanisms in the lung (Chambers 2008). The disturbed homeostasis in the lung may lead, through multiple mechanisms, to extrapulmonary effects involving the cardiovascular system.

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Many recent epidemiological studies have demonstrated that air pollution caused significant adverse cardiovascular outcomes (Kunzli and Schindler 2005; Miller et al. 2007; Mills et al. 2008; Pope et al. 2004). Although the putative biological mechanisms and factors linking air pollution to heart diseases are not well understood, it is accepted that airborne particulate matter (PM) plays a significant role. Short-term exposure to elevated PM has been associated with increased acute cardiovascular mortality, particularly with at-risk population subsets, while prolonged exposure has been considered a causative factor for atherosclerosis and reduced life expectancies (reviewed Brook et al. 2004). The epidemiological data were supported by human and animal experimental studies. In this regard, hyperlipidemic rabbits exposed to PM_{10} (diameter $\leq 10\,\mu m$) or ApoE-/- mice exposed to $PM_{2.5}$ (diameter $\leq 2.5\,\mu m$) develop advanced coronary atherosclerosis (Calvano et al. 2005; Sun et al. 2005). Additionally, transient exposure of healthy volunteers to diesel exhaust, a common air pollutant with complex chemical composition including PM, resulted in impaired endogenous fybrinolytic function and endothelial vasomotor dysfunction (Mills et al. 2005; Tornqvist et al. 2007). Furthermore, the same group found that diesel exposure promotes myocardial ischemia and inhibits endogeneous fibrinolytic capacity in men with stable coronary heart disease (Mills et al. 2007).

A number of pathophysiological mechanisms have been proposed to explain the adverse cardiovascular effects of PM, linking their ability to stimulate oxidative stress, inflammation, thrombosis, and ischemic events (Brook et al. 2004; Mills et al. 2007). Direct effects through translocation of particles or constituents, such as organic chemicals and transition metals, as well as indirect effects secondary to pulmonary inflammation and/or autonomic nervous system activity, might contribute to cardiovascular toxicity following particle exposure. Increasing evidence demonstrates that the smallest particles in the urban environment are the most dangerous since they have higher pulmonary deposition and biological activity compared to larger particles (reviewed in Oberdorster et al. 2005). For example, previous studies showed $PM_{2.5}$ has greater cardiovascular effects when compared to PM_{10} (Brook et al. 2004; Miller et al. 2007; Pope et al. 2004). Furthermore, a recent study utilizing atherosclerotic prone ApoE-/- mice showed exposures to PM < 0.18 μm had an increased proatherogenic potential compared to larger $PM_{2.5}$, consistent with a greater propensity of PM < 0.18 μm to generate systemic oxidative stress and to interfere with the anti-inflammatory capacity of plasma HDL (Araujo et al. 2008). The smallest inhaled particles can be deposited in the deepest regions of the lung and currently the potential systemic translocation to other organs, such as liver or the heart, is under debate. Recent human studies with ultrafine carbon particles labeled with technetium (Tc)-99*m* demonstrated that these particles can accumulate in the lung but systemic translocation at 48 h postexposure is not significant (Moller et al. 2008). It has been reported that some nanoparticles treated with albumin and/or surfactant proteins cross the alveolo–capillary barrier to gain access to the systemic circulation (Kato et al. 2003; Oberdorster et al. 2005). The proximity between epithelial type I and endothelial cell caveolar membrane structures might play a role in the particle translocation mechanisms (Heckel et al. 2004). The major unresolved question is whether particles translocate in sufficient numbers to exert a significant influence on vascular endothelial or myocardial function (Mills et al. 2008).

Inhaled particles can provoke a lung inflammatory response with a consequent release of inflammatory and prothrombotic mediators into the circulation, which can contribute to cardiovascular pathology. Increased circulating cytokine levels (e.g., interleukin [IL]-6, IL-8, IL-1β, granulocyte–macrophage colony-stimulating factor [GM-CSF]) have been found as a result of pulmonary exposure to asbestos, zinc oxide, PM, and endotoxin (Agusti et al. 2003; Copeland et al. 2005; van Eeden et al. 2001). Furthermore, pulmonary inflammation in humans due to chronic obstructive pulmonary disease (COPD) and other disorders associated with reduced lung function was a strong risk factor for adverse cardiovascular events (Mannino et al. 2008). COPD patients with elevated C-reactive protein (CRP) have a higher risk of cardiac events than those with normal CRP (Fimognari et al. 2008). The roles of systemic inflammation and oxidative stress have been closely related to the pathogenesis of atherosclerosis and related ischemic cardiovascular events (Libby 2006; Libby et al. 1999; Ross 1999). In the light of these studies, many traditional cardiovascular risk factors, including high cholesterol levels, diabetes mellitus, and hypertension, as well as nontraditional risk factors, including concomitant infections, systemic autoimmune diseases, and chemical exposure, have been investigated for induction of vascular inflammation and thus promotion of atherogenesis and its complications (Fischer et al. 2004; Hollan et al. 2007; Simeonova and Luster 2004). Identification and prevention of any triggers of vascular inflammation will then contribute to reduction of the incidence and mortality from cardiovascular diseases (Libby 2002).

Engineered materials and potential for cardiovascular toxicity

Engineered nanosized particles (NP; a generalized abbreviation) are new materials of emerging technological importance in different industries (Colvin 2003; Hood 2004). The U.S. National Science Foundation estimated that millions of workers would be needed to support nanotechnology industries worldwide within 15 years, citing the necessity for toxicological assessment. The concerns that NP can induce local and systemic toxicity are related to the toxicology of air pollution. Ambient ultrafine particles are mostly derived from combustion sources and are heterogeneous in size, aggregation state, and chemical composition, including a solid core made of either inorganic material or soot surrounded by a layer of adsorbed or condensed semivolatile organic chemicals, all of which can contribute to

ROS generation and toxicity (Quinton et al. 2004). Thus, the significant body of toxicological knowledge on air pollution gives insight but cannot be directly translated to understanding the potential heath effects of the new engineered nanomaterials with more defined chemical composition and purity. The main biological impact of NP is dependent on size, chemical composition, surface structure, solubility, shape, and aggregation. The unique biological properties of NP may differ from the base materials or chemical compounds from which they are manufactured. Also, due to high energetic adhesive forces close to the surface, the particles are highly reactive, thus easily agglomerating or attaching to other available surfaces (reviewed in Borm et al. 2006). The initial experimental toxicological studies on engineered NP demonstrated that with pulmonary exposure larger agglomerates rather than individual NP were deposited into the lung. However, it is known that aggregates do not behave like a geometric particle of equal size because the greater surface area of the individual particles is mostly retained in the aggregate and may contribute to the toxicity (reviewed in Borm et al. 2006). If NP pulmonary exposure occurs and results in biopersistent lung accumulation, inadequate mucociliary and macrophage clearance, induction of oxidative, ischemic, inflammatory, and coagulation perturbations, and pulmonary as well as systemic toxicity can be expected. This paradigm should be investigated under experimental conditions in order to prevent potential NP toxicity resulting from human exposures.

Carbon nanomaterials—Toxicity studies

One direction of the nanomaterial industries is developing new carbon nanomaterials. Carbon atoms can be arranged into diverse geometries, forming a number of stable nanostructures. For example, carbon atoms, usually using a metal catalyst, can be aligned to form a long single-walled carbon nanotube (SWCNT) with a diameter of ~1 nm. Bare carbon atoms can also be organized into spherical structures called fullerenes ("buckyballs") with the most stable and readily available fullerene, C_{60}, having an average diameter of 0.72 nm. In addition to these single-layer structures, larger nanotubes and fullerenes can also be synthesized, forming multiwalled carbon nanotubes (MWCNT) or onion-like clusters, respectively (Park et al. 2003). Fullerenes, because of their strong electronegativity, can also be combined with metals and other molecules to form metallofullerenes. Global revenues from carbon nanomaterials in 2006 are estimated at $230 million, which provides potential for workplace and general exposure (Donaldson et al. 2006). Specifically, concerns have been raised over occupational carbon nanotube (CNT) exposure because adverse effects related to lung deposition have been found in the first animal studies (Lam et al. 2004; Li et al. 2007; Mangum et al. 2006; Mercer et al. 2008; Muller et al. 2005; Shvedova et al. 2005; Warheit et al. 2004).

Initial animal studies demonstrated that CNT respiratory exposure resulted in acute pulmonary inflammation and chronic responses, including granuloma formation around larger agglomerates and fibrotic responses associated with both the granulomas and the more dispersed materials. These findings, based on the oxidative and inflammatory hypothesis of atherosclerosis and the air pollution cardiovascular research, suggested that CNT exposure should be evaluated as a potential cardiovascular risk factor. Thus, we studied the cardiovascular toxicity of SWCNT (CNI, Houston, TX), purified by acid treatment to remove metal contaminants, using the exposure settings described previously to induce both inflammatory and fibrogenic pulmonary responses in a mouse model (Shvedova et al. 2005). First, the activation of HO-1 gene expression in cardiovascular tissues, a biomarker of oxidative stress (Prawan et al. 2005), was evaluated using *Ho1-luc* reporter mice; second, mitochondrial homeostasis, a sensitive marker of oxidative insults (Ballinger et al. 2002), was evaluated by measuring aortic mitochondrial DNA damage, protein oxidation, and glutathione levels in C57BL/6 mice. We demonstrated that exposure to purified SWCNT by single intrapharyngeal instillation induced dose-dependent cardiovascular oxidative modifications including mitochondrial perturbations in mice (Li et al. 2007). Mitochondria have been reported to be highly susceptible to oxidative stress, mediated by metabolic defects and environmental insults, and mitochondrial dysfunction is emerging as an important pathophysiological factor in a number of cardiovascular diseases including atherosclerosis (Ballinger 2005). The combination of multiple cardiovascular risk factors working through similar processes, such as mitochondrial dysfunction, may lead to synergistic acceleration of atherosclerosis progression and precipitation of its complications. Consistently, atherosclerosis was accelerated in SWCNT-exposed ApoE-/- mice primed with a high-fat diet (Li et al. 2007). Although SWCNT-exposed ApoE-/- mice did not have altered lipid profiles, they had exacerbated plaque development in the aorta and brachiocephalic arteries. Thus, chronic pulmonary toxicity of SWCNT lung deposition was associated with cardiovascular effects through mitochondrial oxidative perturbations, which can result in altered vessel homeostasis. In addition to mild inflammation, recently it has been suggested that inefficient metabolism in blood vessels as a result of ischemic events can cause vascular diseases through mitochondrial dysfunction (Bernal-Mizrachi et al. 2005). Mediators, released from the lung into the systemic circulation, or ischemic events, associated with altered pulmonary function after SWCNT exposure, may lead to vascular oxidative modifications. Additionally, SWCNT exposure might mediate cardiovascular effects through platelet activation in the lung circulation. The pulmonary circulation is considered a site for platelet maturation (Kurahashi et al. 1999), and recently, it has been demonstrated that SWCNT can directly stimulate platelet aggregation in vitro (Radomski et al. 2005). Furthermore, an acute systemic prothrombotic response, a potential contributor to an adverse cardiovascular outcome, was shown following CNT-induced lung inflammation (Nemmar et al. 2007). These findings were of sufficient significance to warrant further studies to evaluate test approaches for prediction

of potential systemic toxicity of respiratory exposure to various forms of nanomaterials.

Predictive toxicity tests and biomarkers

The toxicological testing of NP should include physicochemical characterization of NP, in vitro assays, and in vivo studies (Oberdorster et al. 2005). The preferred approach of the National Toxicology Program to test chemical toxicity is a predictive scientific model that focuses on target-specific, mechanism-based biological observations (Quinton et al. 2004). Thus, evaluation of potential cardiovascular effects, presuming that NP will go beyond the entry port, will include in vitro assessment of target cells such as endothelial and smooth muscle cells. It has recently been demonstrated that inflammation of human aortic endothelial cells (HAEC) following acute exposure to metal oxide NP depends on the particle composition and concentration, rather than only the particle size (e.g., Y_2O_3 and ZnO NP induced endothelial cell inflammation, in contrast to Fe_2O_3 NP) (Gojova et al. 2007). Other cell culture experiments using rat aortic smooth muscle cells demonstrated signs of cell toxicity when exposed to purified high-concentration SWCNT and the effect was not related to the aggregated tubes (Raja et al. 2007). Further, we have demonstrated that in cultures of HAEC, raw (~30% iron) but not purified, SWCNT dose-dependently induced low-density lipoprotein (LDL) oxidation, a risk factor for atherosclerosis (Simeonova et al. 2007). Consistent with the "LDL oxidative modification hypothesis," free transition metal ions, such as copper and iron, have been shown to stimulate lipoprotein oxidation by vascular cells in vitro (Li et al. 2007). In general, the initial in vitro studies confirmed that if sufficient concentrations of NP escape into the systemic circulation, direct effects may occur dependent not only on the nanosize but also on complex physical characteristics including chemical composition, purities, and aggregation.

Although the in vitro models are intriguing to predict possible target toxicity of NP exposure, the complete understanding of the potential risks requires appropriate animal screening models. Recently, we tested the hypothesis that NP-induced pulmonary and systemic effects might be predicted using blood biomarkers measured during an acute exposure. Prior to analyzing different models of occupational particle exposure, the systemic effects of LPS pharyngeal aspiration as well as intraperitoneal (ip) injection (a positive control for systemic effects) were characterized (Erdely et al. 2007). Expectedly, ip LPS resulted in a marked inflammatory response in the blood, heart, and lung and pharyngeal aspiration of LPS resulted in significant lung inflammation. Interestingly, whole blood gene expression for some inflammatory mediators showed similar induction following LPS by pharyngeal aspiration or ip injection. The pulmonary response to aspirated LPS resulted in increases of many inflammatory mediators in the blood at 4 h postexposure. Although it has been previously reported that extrapulmonary tissues were mostly unaffected by LPS lung instillation, surprisingly we found increased vascular expression of inflammatory mediators and adhesion molecules, which play a role in endothelial dysfunction.

Based on the established time points and findings of the LPS study, we analyzed the acute effects of SWCNT and MWCNT. We applied a select panel of genes (TaqMan array), known to play important roles in the molecular

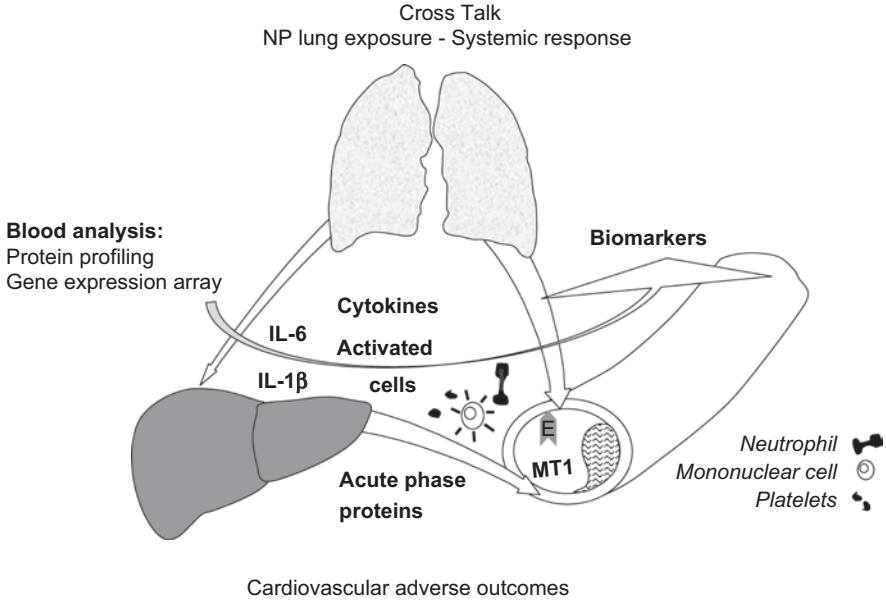

Figure 1. Diagram of a cross-talk between lung and systemic circulation related to carbon nanotube respiratory exposure. The acute lung insult results in a release of inflammatory cytokines and activated blood cells, which trigger systemic tissue activation. Aortic endothelium responds with increased expression of E-Selectin (E) and other stress-responsive genes (e.g., metallothionin, MT-1). The vascular response together with the blood pro-inflammatory and pro-thrombotic alterations might be a prerequisite for cardiovascular adverse outcomes. The combined blood protein and gene expression analysis characterizes the local and systemic toxicity and can find application for development of biomarkers of exposure.

mechanisms of tissue injury, such as inflammation, oxidative stress, endothelial function, and coagulation, to evaluate the simultaneous expression of these genes in the lung, circulating whole blood cells, and cardiovascular tissue upon pulmonary deposition of particles. Observed alterations in gene expression were then compared to changes in inflammatory and prothrombotic blood proteins (Erdely et al. 2009). These studies demonstrated that upon CNT deposition in the lung, acute local and systemic inflammatory as well as pro-thrombotic responses were activated, and the effect was represented by the complex gene expression and protein blood analyses. The acute respiratory CNT exposure resulted in the lung expression of many genes coding mediators of inflammation, oxidative stress, remodeling, and thrombosis; these effects were enhanced in the MWCNT compared to SWCNT treated mice. The lung response resulted in corresponding alterations in the systemic circulation, including soluble biomarkers of inflammation and coagulation, as well as activated blood inflammatory cells, measured by total blood gene expression. Interestingly, this response was paralleled with a representative systemic tissue response, including the expression of genes related to hypoxia, oxidative stress, and inflammation. Furthermore, CNT-induced systemic distress at the level of aorta included the activation of E-selectin, an endothelial-specific cell adhesion molecule that facilitates recruitment of leukocytes into the vessel wall. Selectin expression in the systemic vasculature, together with an impaired blood inflammatory and coagulation balance, might be a prerequisite for endothelial dysfunction. The findings are summarized schematically in Figure 1. Studies in progress, including varying particle types, will determine the specificity of this response regarding exposure agents and pathophysiological outcomes. Thus, the combination of blood gene expression analysis and circulating soluble proteins provides insight into the mechanisms of particle toxicity and novel biomarkers that can find application in human clinical screening and epidemiological studies. This approach will foster the development of predictive tests for estimation of the toxicity of new nanomaterials based on their physiochemical characteristics and potential to induce oxidative stress, inflammation, and specific pulmonary and systemic toxicity.

Conclusions

Based on ambient ultrafine particle research, it is predicted that engineered nanosized particles may have deeper pulmonary deposition, higher biological activity, and a tendency for extrapulmonary translocation compared to larger particles. In this regard, nanoparticle exposure, by direct or indirect mechanisms, may lead to unexpected distant responses, involving the immune system, cardiovascular system, liver, kidney, and brain. The systemic effects may induce or modify the progression of existing diseases such as cardiovascular disease. Blood screening methods for evaluation of the systemic responses to particle-respiratory exposure will help in developing biomarkers of exposure and prediction of potential toxicity of emerging new nanomaterials.

***Declaration of interest*:** The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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