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Thinking Small: New Approaches for Evaluating the Toxicologic Pathology of Nanotechnology Products

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Abstract

Nanotechnology involves matter that is in the nanoscale size range. The products of nanotechnology are increasingly impacting fields of electronics, aerospace engineering, cosmetics and medicine. Toxicologic pathologists are familiar with ultrastructural pathology and, therefore, with biological processes of nanoscale dimensions. Not surprisingly, when particulates are similar in size to biological structures, the *in vivo* toxicity can be very different than for larger particulates with the same chemical composition. In evaluating the toxicologic pathology of these smallest of engineered products, the potential may exist for movement across biological barriers, interaction with subcellular structures, transport within lymphatics and neurons, and changes in the chemical and physical properties of the test agent. This is a brief introduction to considerations for toxicologic pathology studies of nanotechnology products.

Background

The National Nanotechnology Initiative (www.nano.gov) defines nanotechnology as “the understanding and control of matter at the nanoscale, at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications.” The economic impact of products utilizing nanotechnology is predicted to exceed 2 trillion dollars by 2015.[1-3] This economic impact principally stems from the ability to engineer products from the atom up and is having a profound effect on the automotive, aerospace, electronics, construction, food, cosmetics and pharmaceutical industries.[1, 3, 4] Even definitions in the new field of nanotechnology have important economic and regulatory significance and are evolving. The term nanoscale is generally accepted to refer to the size range between 1 and 100 nanometers. However, the term nanoparticle has been variably defined as “a

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nanoscale particle",[5] "a particle with a diameter in the nanoscale size range",[6] or as a "nano-object with all three external dimensions in the nanoscale."[7] The term nano-object is a generic term for nanoscale objects but in occupational health, objects that are small enough to be workplace inhalation, dermal and occular hazards are designated as particulates.[7-9] Similarly, environmental regulations often deal with particulate matter; nanopharmaceuticals are rarely called objects. The impact of nanotechnology for occupational and environmental health and for the pharmaceutical industry focuses on the increasing presence of purposefully engineered particulate matter of nanoscale dimensions. For the purposes of this brief summary of approaches to the toxicologic pathology of nanotechnology products, we will, therefore, use the term nanoparticulate (NP) instead of nano-object for a particulate with one or more dimensions in the nanoscale.

Existing terminology and regulations do not necessarily accurately reflect the new technology. At this time, many guidelines, recommendations and regulations that are specific to reporting or controlling nanotechnology products are still being developed or are in draft form.[10-16] Even the methods for evaluating the economic impact of nanotechnology are still in a developmental stage.[17] Because NPs are produced as a component of many long-established processes, such as combustion, as well as through intentional engineering, it is not always clear which products are byproducts of nanotechnology or are nano-enabled. Thus, the economic impact of nanotechnology is large, but specific details regarding the number and the full spectrum of nanotechnology products is not known.[17] What we do know is that NPs can have very different physiciochemical properties and biological effects than micron-sized particulates with the same chemical composition.[18] We also know that the development of nanotechnology has outpaced the understanding of the health effects of nanontechnology products. Thus, safe harnessing of nanotechnology requires multi-disciplinary teams.[19] Toxicologic pathologists can make important contributions to those multi-disciplinary teams. Here, we summarize fundamental differences between NPs and micron-sized particulates and new approaches which can facilitate the interpretation of NP toxicologic pathology studies. Finally, we summarize the techniques that can help pathologists see NPs and evaluate their movement within tissues and cells.

Nanosizing can Alter Fundamental Physical and Chemical Properties and Biological Interactions

NPs differ from micron-size particulates in several ways that have potential health implications.[20] 1) On a mass basis, the surface area increases. Therefore, for particulates that cause toxicity because of a reactive surface or a soluble component, the effective dose may increase for a given mass of the compound. 2) Quantum phenomena occur in the nanoscale and alter basic properties, particularly for the smallest NPs, including whether the physical state is solid or liquid at a given temperature. 3) Some NPs, such as the fullerenes, have novel chemistry. 4) NPs can traverse intracellular and intracellular barriers that exclude micron-sized particulates. 5) NPs can interact with, and potentially accumulate within subcellular structures.

In particular, the last two of these fundamental differences have implications for toxicologic pathologists. Important biological structures have nanoscale dimensions and the size similarity between these biological structures and NPs alters the way that NPs are distributed within the body and within cells. For example, intercellular junctions, endocytic pathways, nuclear pores, and subcellular organelles are nanoscale biological structures. Nanotechnology products can be engineered to intentionally pass through, or to interact with, biological structures.[21-31] Harnessing critical biological interactions that are facilitated in the nanoscale is a part of the enormous promise of nanotechnology. The ability to engineer pharamaceuticals and medical devices in the nanoscale has created the rapidly expanding field of nanomedicine. In many cases, biological structures can be considered forms of NPs. Thus, nanotechnology may be harnessed in ways that allow drugs to reach tumors and degenerative processes that are currently difficult to target – for example those that are in the brain.[25, 28, 32, 33] However, biological interactions in nanoscale dimensions are often incompletely understood and novel toxicologic effects have been described for some materials that are much less toxic on a mass basis when they are larger.[34-39] Nanosizing can affect the tissue as well as the intracellular distribution of NPs.[37, 40-43] For this reason, the adverse health effects of some nanotechnology products has also created another very important new field, nanotoxicology.[44] Unfortunately, publications in nanotoxicology have lagged far behind publications in nanotechnology and nanomedicine.[43] For toxicologic pathologists, new approaches may be needed to fully evaluate the biological consequences of altered intracellular and tissue distribution in the nanoscale. New approaches which have proven particularly useful include immunofluorescence, confocal and intravital microscopy in combination with high resolution digital imaging technology.[45]

Identifying Fundamental properties of the NP

Toxicology data is most meaningful when the exposure is fully characterized.[46] As mentioned above, nanosizing can alter chemical and physical properties of matter. Even within nanoscale dimensions, there are many different physical and chemical properties that can affect toxicity. For example, multi-walled carbon nanotubes (MWCNTs) can vary in width, the number of walls, the length, the ratio of length to width (the aspect ratio), presence of trace metals, presence or absence of functional groups added to the carbon rings of the MWCNTs, structural defects, and their agglomeration status.[47-54] An additional example is the ability of some NPs to directly pass from the alveolar space into the pulmonary lymphatics, a characteristic that affects biological distribution and is limited by NP size and surface charge.[55] These are all factors which can potentially alter toxicity and target tissues. Therefore, characterizing the physical and chemical properties of the test agent is one of the most important steps in a nanotoxicology study.[56, 57]

Thinking Small: Finding the NP and NP-induced Cytopathology

When entering the body, micron-sized particulates are frequently recognized by the phagocytic receptors which respond to particulates that are usually greater than 500 nm in diameter.[58] Thus, bronchoalveolar lavage cytology is a useful screening test for inhaled toxic agents that cause lung injury through recruitment of phagocytes into the alveolar space.[59] Bronchoalveolar lavage and histopathologic assessment of pulmonary inflammation has also been very useful in identifying lung damage caused by some NPs.[60-62] However, in a recent study comparing pulmonary interstitial fibrosis caused by single-walled and multi-walled carbon nanotubes, interstitial fibrosis was found to correlate with the dose to the alveolar interstitium, as opposed to percent of macrophages phagocytizing the NP.[63] This is not unexpected, since phagocytic receptors are present only on the small percentage of cells in the body that have phagocytic functions, while the endocytic pathways responsible for internalizing particulates by pinocytosis are present in all cells and most of these pathways exclude particulates more than 100 nm in diameter.[31, 64] The pulmonary interstitium would receive NPs that pass through the epithelium, a process that could be accomplished by migrating phagocytes but can also occur when NPs utilize endocytic pathways or traverse intercellular junctions that exclude larger particulates. In addition, non-cationic NPs less than 34 nm in diameter deposited in the alveolar region can enter the pulmonary lymphatics and systemic circulation rapidly and directly without phagocytosis.[55] This suggests that inflammatory infiltrates may not always be present when NPs cause histopathologic alterations.

A concern for pathologists is that these inflammatory infiltrates are not just recovered in screening tests such as bronchoalveolar lavage, these inflammatory infiltrates are often used by pathologists to decide to use higher magnification for evaluating changes during histopathologic assessment of tissue damage. Other triggers, or systematic selection of specific portions of the slide for high magnification evaluation, may be need in NP toxicologic pathology studies. Thus, we first identified migration of a MWCNT through the pulmonary pleura while examining lymphangiectasia in a subpleural lymphatic.[51, 65] Subsequent high magnification morphometry identified that subpleural and pleural migration of MWCNTs was not uncommon and was not always accompanied by a phagocytic response or lymphangiectasia.[66] Changes in lymphatics and lymph nodes are particularly important in toxicologic pathology studies of NPs, since some NPs can alter critical immune responses,[67, 68] some NPs circulate in lymphatics,[69] and high aspect ratio NPs can accumulate near lymphatic capillaries and can cause lymphangiectasia.[51, 70] Similarly, communication with other members of the multi-disciplinary team may suggest the need for selective high magnification or morphometric evaluation of tissue changes. For example, a high magnification evaluation of target cells, particularly non-replicative cells in the heart and brain, would be indicated if a NP is expected to translocate to these cells and there are no known enzymes in those cells that can digest the NP. As pathologists, we are aware of the devastating consequences of storage diseases from undegraded macromolecules when they accumulate in tissues and such macromolecules are similar in size to small NPs.[71] The ability of some NPs to use endocytic networks cells that are not phagocytic may be a key to advances in nanopharmaceuticals but it is also important that NPs are successfully degraded when using pathways which are novel for exogenous particulates. Important toxicologic pathology findings may involve detailed examination of cells and subcellular compartments where NPs may accumulate or interact.[63, 66, 72, 73]

Changes in Microscopes and Photomicroscopy that Facilitate Toxicologic Pathology Studies

Submicron particulates, such as NPs, scatter light. This property can be harness in darkfield evaluations to enhance the detection of NPs in tissue sections using transmission or confocal microscopes.[43, 45, 70, 74] Many nanomaterials, have dimensions less than the wavelength of light, have closely packed atoms in a near crystalline arrangement, and typically have a refractive index significantly different from that of biologic tissues and/or mounting medium. These characteristics produce significantly greater scattering of light by NPs than by the surrounding tissues. The enhanced-darkfield optical system images light scattered in the section and, thus, nanomaterials in the section stand-out from the surrounding tissues with high contrast. Newer, or enhanced, darkfield instruments use illumination focused at an acute angle to the specimen which significantly reduces the residual transmitted illumination. The enhanced illumination results in a nearly dark level of image in the surrounding tissue. NP scattering of light varies in characteristic pattern with wavelength but is sufficiently broad that the light scattered from NPs is generally white. The net results of dark tissue and bright white NPs is a high contrast imaging between the desired subjects of imaging, the NPs, and surrounding tissue. Using this method of imaging, sections can be easily scanned at relatively low magnification to identify NPs that would not be detected by other means. The method has been applied to a number of NPs detection tasks. Applications have included analysis of lung deposition and clearance patterns of titanium nanoparticles,[70, 75] cerium oxide nanoparticles,[76] and MWCNTs,[63, 70] as well as ultra-sensitive detection of systemic transport of single MWCNTs fibers from the lungs to systemic organs after an acute inhalation exposure.[77] This is one emerging tool which facilitates detection of many different types of NPs within tissue sections.

Disclaimer

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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