CHAPTER

13

Nanoparticulates

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OUTLINE

1. BACKGROUND

1.1. Definitions

The National Nanotechnology Initiative (NNI) coordinates federal nanotechnology activities in the United States. The Organization for Economic Cooperation and Development (OECD) is an international organization involved in nanotechnology through their Programme on Manufactured Nanomaterials. In addition, the International Organization for Standardization (ISO) is an international organization with a technical committee involved in standardization of nanotechnologies, Technical Committee 229 Nanotechnologies.

The NNI defines nanotechnology as "the understanding and control of matter at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling and manipulating matter at this length scale." This definition of nanotechnology introduces the terminology and the important concept that nanoscale products can accomplish many things not previously possible.

Before addressing the potential benefits of nanotechnology, additional definitions may be helpful. The prefix *nano* commonly confers a meaning of very small or a billionth (Flexner and Hauck, 1993). However, within nanotechnology and related disciplines, the prefix *nano* is often used to refer to dimensions from 1 to 100 nm. Thus, nanomedicine is the medical

Haschek and Rousseaux's Handbook of Toxicologic Pathology, Fourth Edition.
https://doi.org/10.1016/B978-0-443-16153-7.00013-7

application of nanotechnology, and nanotoxicology is the study of the toxicology of the products of nanotechnology.

The terminology for products of nanotechnology is still evolving and the definitions may affect how specific products are regulated. Thus, precise definitions of common terms such as nanoparticle, nanoparticulate (NP), nanomaterial, nanotechnology, and nano-enabled are subject to debate. The ISO defines the nanoscale as "length range from approximately 1 nm to 100 nm" and defines a nanomaterial as a "material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale" (International Organization for Standardization, 2017). The Scientific Committee on Emerging and Newly Identified Health Risks has proposed that a nanoparticle should be defined as a "discrete entity which has three dimensions of the order of 100 nm or less" (Scientific Committee on Emerging and Newly Identified Health Risks (EU) SCENIHR, 2008). Similarly, the ISO defines a nanoparticle as "a nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly" (International Organization for Standardization, 2017). In this chapter, the term NP will be used for a particulate with at least ***one*** dimension in the nanoscale range from 1 to 100 nm. As used here, NPs will encompass both solid and liquid NPs.

1.2. Historical Perspective

NPs have been present in the human environment for centuries. The National Nanotechnology Initiative website (http://www.nano.gov) maintains a Nanotechnology Timeline (http://www.nano.gov/timeline) which begins with the use of colloidal gold and silver in making dichroic glass in the 4th century. It is the ability to engineer in nanoscale dimensions that is a recent phenomenon and led to the new field known as nanotechnology. This is reflected in the rapid increase in PubMed indexed nanotechnology publications during the past decade. Nanotoxicology and nanomedicine have received increasing attention during the past 15 years, but nanotoxicology publications lag far behind nanotechnology publications (Figure 13.1).

Several earlier key discoveries made nanotechnology possible. Among these discoveries were discovery of the electron microscope and the scanning tunneling microscope that led to the 1986 Nobel Prize in physics for Ernst Ruska, Gerd Binnig, and Heinrich Rohrer (Robinson, 1986). The electron microscope revealed ultrastructural details previously unseen by scientists while the scanning tunneling microscope produced atomic scale maps of the surface of biological and inorganic samples and could determine their atomic composition (Binnig and Rohrer, 1987).

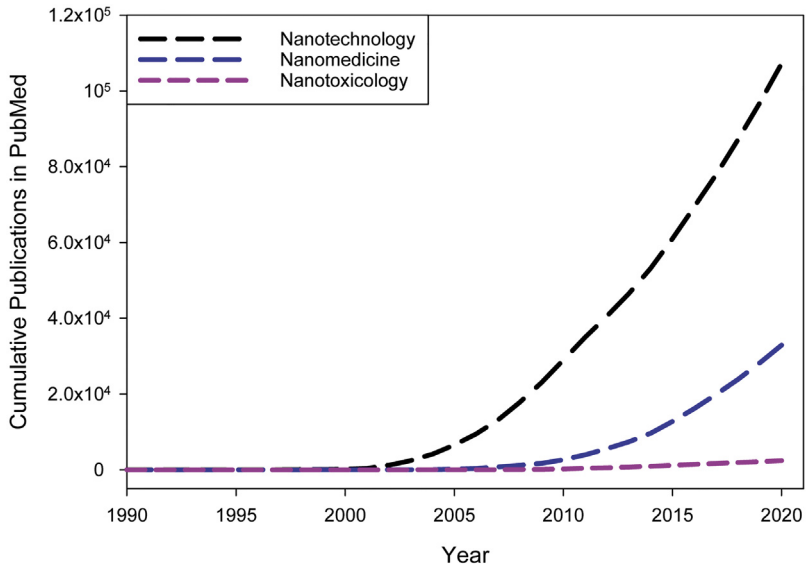

FIGURE 13.1 Publications in nanotechnology (*dashed black line*) began about a decade prior to publications in nanomedicine (*dashed blue line*). Nanotoxicology publications (*dashed purple line*) have lagged in both number and time. *Figure modified from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.1, p. 1374, with permission.*

In this same time frame, other research groups were working on a new form of carbon that would soon revolutionize nanoscale three-dimensional engineering. In 1981, a group of researchers from Rice University discovered buckminsterfullerene, now also known as the buckyball (Kroto et al., 1985). As indicated by its chemical formula, C_{60}, buckminsterfullerene is comprised solely of carbon. Buckminsterfullerene is shaped like a soccer ball with a sheet of carbon atoms analogous to the leather surface of the soccer ball, so that a cagelike internal structure is formed which is suitable for enclosing specific atoms (Curl and Smalley, 1988). The carbon–carbon bonds are the seams of this molecular soccer ball, and like a soccer ball, regular hexagons and pentagons contribute to its three-dimensional symmetry. As with other carbon compounds, each carbon molecule in C_{60} forms four bonds with adjacent carbon molecules. This means that some of the carbon molecules are linked by double bonds. Double bonds are near single bonds and other double bonds, so that sites of double bonds between carbons are destabilized. Thus, the hexagons and pentagons that comprised C_{60} are actually aromatic; a shell of delocalized π electrons surround the internal and external surfaces of C_{60} and add stability to the structure (Figure 13.2A) (Kroto et al., 1985).

C_{60} was the first characterized member of an amazing new class of carbon compounds devoid of hydrogen and known as fullerenes. This class of carbon compounds is as different from previously described carbon compounds as the carbon compound, coal, is from the carbon compound, diamond. The fullerenes form a surface which is one atom thick. That one atom thick carbon surface can form three-dimensional structures of varying shapes. Robert F. Curl, Sir Harold Kroto, and Richard E. Smalley received the 1996 Nobel Prize for this discovery (Curl, 1997; Kroto, 1997; Smalley, 1997).

The group of new carbon compounds soon expanded to include carbon nanotubes, which form rolled sheets of carbon hexagons in the shape of single- or multi-walled tubes with a diameter in the nanoscale and a much greater length (Figure 13.2B) (Iijima, 1991; Iijima and Ichihashi, 1993). Like C_{60}, carbon nanotubes have electrons that are used for double bonds

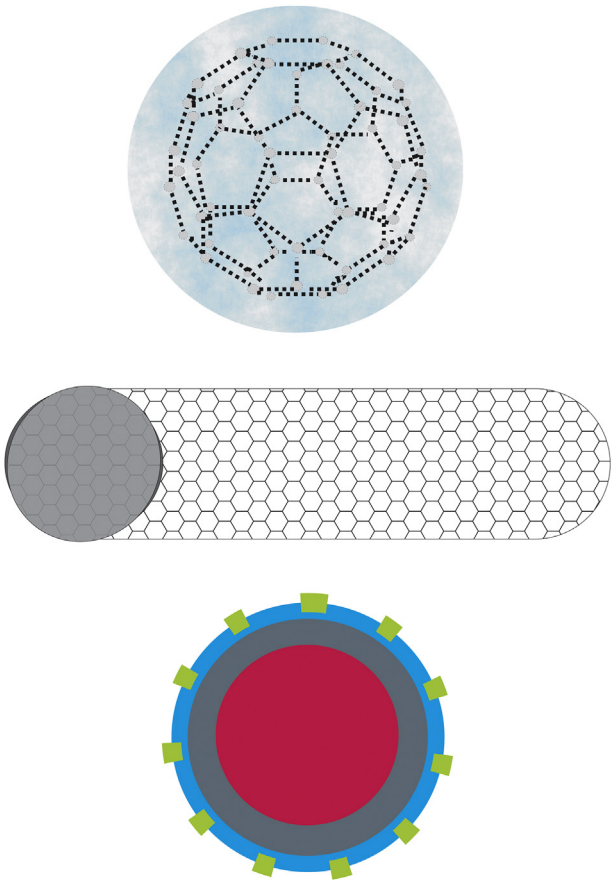

FIGURE 13.2 NPs are a large and diverse group of particulates sharing the common feature of size—at least one dimension in the range of 1–100 nm. The top image is a drawing of one of the early products of nanotechnology, a buckyball, which is comprised solely of carbon arranged into pentagons and hexagons to form a sphere that is surrounded by a cloud of electrons which illustrate the aromatic nature of the buckyball. The middle image is an image of another early product of nanotechnology, a single-walled carbon nanotube, which is principally comprised of carbon molecules arranged into hexagons but also contains variable amounts of metals used as catalysts during synthesis. Single-walled carbon nanotubes tend to behave as polyalkenes, which allow chemical additions to the carbon wall. The bottom image is a hypothetical NP intended to illustrate the concept that modern products of nanotechnology can be complex structures made from many different compounds such as a drug core (shown in *red*) surrounded by a protective coating (shown in *gray*), a tracer for imaging (shown in *blue*), and a ligand for cellular receptors to target the delivery. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.2, p. 1376, with permission.*

and can have aromaticity (Lu and Chen, 2005). The chemistry needed to produce the tubelike shape of the carbon nanotubes also used transition metal catalysts, such as iron, which were a variable contaminant of the carbon sheet which comprised the nanotube (Iijima and Ichihashi, 1993; Charlier et al., 1997). Some considered nanotubes to be fullerenes while others considered nanotubes to be a separate group of carbon-based NP (Smalley, 1997; Government Accountability Office as edited by the journal, 2010). As the understanding of C_{60}, carbon nanotubes, and related carbon NPs evolved, so developed the realization that the curved structure and the presence of five- and four-membered carbon rings made some carbon-based NPs more reactive than most aromatic compounds; chemically many carbon-based NPs behaved more like alkenes than aromatic compounds (Taylor and Walton, 1993).

As a group, the carbon-based NPs demonstrate nanoscale dimensions, chemical and physical properties that include durability, an ability to conduct electricity, and exhibit a diversity of potential shapes which can modify the chemical and physical properties and have the potential for many commercial applications (Law et al., 2004). However, toxicologists and toxicologic pathologists were at first generally unaware of the emergence of these new engineered materials or the unique properties of some products of nanotechnology. Thus, the investigation of their potential toxicity was not initially a major topic of discussion.

1.3. Development of Nanotechnology

The earliest nanotechnology products are known as the first-generation products of nanotechnology and were generally passive structures. The first-generation NPs included C_{60} and the carbon nanotubes but also included other products that demonstrated the principal of engineering in nanoscale dimensions. These included nanotubes formed from cyclodextrins, nanotubes formed from cyclic polypeptides, DNA stick figures, and DNA arranged in a cube. Additional close relatives of the first-generation products of nanotechnology came later but included commercially important products such as modified carbon nanotubes (including carbon nanoribbons and functionalized carbon nanotubes) and nanotubes made of additional elements (such as silicon).

By the early 1990s, a vision of nanotechnology emerged within the scientific community. In November of 1991, the journal Science included a special section called *Engineering a Small World: From Atomic Manipulation to Microfabrication* with related articles (Appenzeller, 1991; Brauman, 1991; Stroscio and Eigler, 1991; Sundaram et al., 1991). Material science, quantum chemistry, and physics had evolved to the point where control of atomic arrangements and variety of molecular devices were envisioned. The similarity in size between synthetic nanostructures and biological structures, such as viruses, proteins, nucleic acids, and cellular organelles, was recognized. It also became apparent that the noncovalent bonds of proteins and nucleic acids played an important role in determining the three-dimensional structure and self-assembly of these natural biological NPs and that these protein and nucleic acid bonds had a potential role in synthesizing engineered NPs (Whitesides et al., 1991). As noted previously, carbon, DNA, and peptides were among the earliest building blocks for nanotechnology products. The biological precedent indeed demonstrated the feasibility of these noncovalent interactions but can now be viewed as an early indicator that the manufactured NPs might interact with biological structures in unintended ways that could produce toxicity.

These early visions of nanotechnology primarily were directed at developing nanodevices. Electronic devices were among the earliest nanodevices. Movement of electrical charge is the basic feature of electrical conductors and movement of electrons is one way to move electrical charge. As mentioned above, the π electrons in C_{60} were destabilized and it is, with some limitations, an aromatic compound. In single-walled carbon nanotubes (SWCNTs), the mobility of the π electrons and the semiconductor properties depend upon the tube diameter and helicity (Hamada et al., 1992; Mintmire et al., 1992; Smalley, 1997; Ajayan et al., 1999). Thus, SWCNTs could behave as semiconductors or as metallic compounds. This was important because electron flow could be improved by connecting electron-rich with electron-deficient semiconductors (Saito et al., 1992). The role

of shape in determining the semiconductor properties also meant that this important property could be modified. In 1997, by combining some of the greatest scientific advances of the 1980s, the scanning tunneling microscope was used to micromanipulate and measure the electrical properties of SWCNTs to describe an early nanodevice, an SWCNT nanodiode (Collins et al., 1997).

1.4. Current and Future Nanotechnology Applications

By 2004, nanotechnology was already in widespread use in the computer and electronics industries (Service, 2004). In 2007, the Environmental Protection Agency (EPA) noted that nanotechnology products predominantly fell into four groups (Environmental Protection Agency, 2007):

(1) Carbon-based materials, such as C_{60} and the carbon nanotubes
(2) Metal-based materials, which are nanomaterials principally comprised of metals
(3) Dendrimers, which are NPs formed by branched polymers
(4) Composites, which contain different NPs or combinations of NPs and larger materials

Within the United States, federal support for research on nanotechnology was $10.5 billion total from fiscal years 2001 through 2009 (Zhu et al., 2003). In 2009, Lux Research, Inc., reported that the automotive industry had the greatest use of "nano-enabled" products with the construction, electronics, healthcare, environment, and energy sectors also using nanotechnology (Lux Research Inc., 2009). The US Patent and Trademark Office reported a 20% annual growth rate for nanotechnology patents between 1985 and 2005. According to StatNano, in 2020, nanotechnology patent applications were 8.5% of the total applications to the US Patent and Trademark Office.

Unfortunately, statistics on the use of specific nanotechnology products are incomplete. There are several reasons for that. Under the Toxic Substances Control Act (TSCA), manufacturers of new chemical substances must provide information to Environmental Protection Agency (EPA) before the chemical substances enter commerce. The majority of the publicly available information is for high production volume chemicals. In an industry, such as nanotechnology, that is rapidly increasing production and where millions of particles can be in a gram of material, existing reporting has undoubtedly missed most of the activity. Reporting requirements have only recently changed to address some of the products of nanotechnology. In 2017, EPA established reporting requirements that specifically address the production of many solid nanoscale materials. The new requirements are for nanoscale materials that at standard atmospheric pressure are solids at 25°C, have 1% of their mass with one dimension between 1 and 100 nm, and have properties not seen in larger particles of the same composition that are a reason for manufacturing or processing at that size. The United States Government Accountability Office has noted the challenges for federal agencies and Congress when trying to ensure safety of any rapidly evolving technology.

Nanomedicine, as mentioned above, is the medical application of nanotechnology. More specifically, as defined by Bawa and coworkers (Bawa et al., 2005), it is "the application of nanoscale technologies to the practice of medicine, namely, for diagnosis, prevention, and treatment of disease and to gain an increased understanding of the complex underlying disease mechanisms." While a search of PubMed revealed only a handful of publications prior to 2005 (Figure 13.1), a more detailed search for all publications and patents revealed that the earliest nanomedicine publications appeared in the 1990s and that a sharp increase in nanomedicine patents began about a decade later.

The reason for the interest is obvious, the potential to improve patient outcomes. Nanoengineering of pharmaceuticals can improve solubility and stability, target delivery, or decrease drug toxicity (also see *Pathology in Nonclinical Drug Safety Assessment*, Vol 2, Chap 4). For example, NPs often have greater solubility than larger particles. Consistent with that greater solubility, the albumin-conjugated nanoparticle form of paclitaxel, Abraxane (Abraxis, Los Angeles, CA), has greater solubility than previous paclitaxel formulations and does not require organic solvents that can cause

hypersensitivity reactions in some patients. In addition to greater solubility, the albumin in Abraxane may be targeting the caveolae. Caveolae are invaginations of the vascular endothelium that are present in very high numbers, react with specific target molecules (such as albumin), and form trafficking vesicles that move the material through the endothelium and into the target tissue (Figure 13.3). This is just one example of how nanotechnology has been used to overcome toxicity and improve delivery. Other examples include NPs designed to scavenge β-amyloid within the vasculature as a potential Alzheimer's disease therapy.

Additional new nanomedical concepts and products are rapidly developing. The development of novel nanomaterials with unique physicochemical characteristics suggests the potential use of some nanomaterials in medical imaging (see *In Vivo Small Animal Imaging*, Vol 1, Chap 13). The small size and large surface area of nanomaterials can facilitate translocation across biological barriers and may enhance cellular and intracellular interactions, features that can be valuable for imaging as well as drug development (Klein et al., 2021). Entire issues of scientific journals have been devoted to therapeutic and diagnostic ("theranostic") nanomedicine. The surface and the core of the NP can each be engineered to contain multiple components (Figure 13.2C). Each of the components can have important properties. Thus, it is possible to construct NPs that target delivery, are contrast agents, and/or contain a therapeutic payload. The development of NPs designed to cross the blood–brain barrier (BBB) can potentially deliver drugs to treat devastating neurologic disorders. However, there is very little published information available on how these new nanomedical products will be degraded within cells, such as neurons, that may not have previously been reached by their therapeutic payload or other by-products of nanopharmaceuticals. In other words, drug delivery to the brain should involve traversing the BBB to deliver the drug *and* safe elimination of the drug. This is also true of any other target tissue or subcellular organelle uniquely being reached by nanopharmaceuticals.

As has been noted in recent reviews, nanomedicine has the potential to overcome some of the greatest medical challenges of our time. Nanotechnology is already providing innovative new products for a variety of nonmedical applications. The challenge is trying to harness this promise as safely as possible. The NPs of today can be made from a virtually infinite number of different compounds and combination of compounds. Part of meeting the challenge for safe development of nanotechnology is understanding how nanosizing alters the toxic effects of particulates. This chapter helps to address some of those issues. However, an important aspect of nanomedicine products is that they should be designed and tested for biocompatibility and potential toxicity before their mass production. The findings from such studies can be proprietary and many more nanomedicine products are in development than have reached the market. Data on nanomedicine safety and toxicologic pathology will undoubtedly increase in the future when more is known about the nanomedicine products that do, and those that do not, reach the market. Thus, much of the available data and risk assessment on human exposures focuses on workplace and consumer NP exposures. However, it is important to understand what is currently known about human exposures to NPs.

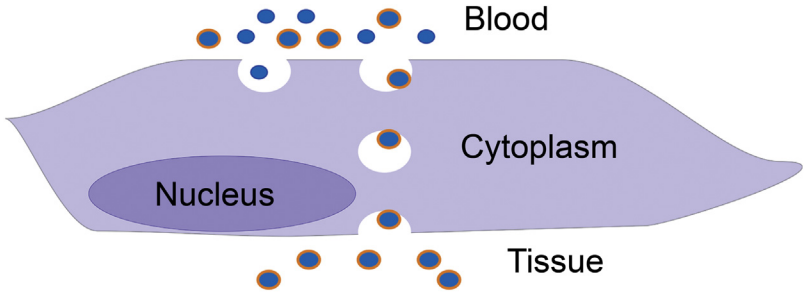

FIGURE 13.3 Caveolae are invaginations of the vascular endothelium that react with target molecules such as albumin and form trafficking vesicles. This permits NPs with surface target molecules (shown here in *orange*) to move across the vascular endothelium. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.3, p. 1378, with permission.*

1.5. Human Exposures

For workers and consumers, inhalation, dermal exposure, and ingestion are major routes of NP exposure. Inhalation exposures can occur when NPs are aerosolized within the workplace, home, or other environments. In addition to inhalation, aerosolized nanoparticles can be deposited on surfaces, which in turn can result in dermal or even oral exposures. Some consumer spray products can release NPs into the breathing zone of consumers. Once inhaled, NPs can reach the nervous system via the olfactory nerves, sensory nerves of the nose and airway, or the vasculature. Additional tissues, such as the spleen, gastrointestinal tract, and liver, can be exposed to NPs that initially enter the respiratory tract and are translocated to other sites. The known pathways for translocation include the lymphatics, the blood vasculature, neuronal transport, and the gastrointestinal tract (Choi et al., 2010; Hubbs et al., 2013; Mercer et al., 2013). However, the magnitude and frequency of parenteral exposure after inhalation remain controversial and may well depend upon the physiochemical characteristics of the NP. The lung itself is a major target of injury from first-generation NPs, including single-walled (SWCNT) and multi-walled carbon nanotubes (MWCNTs). Dermal exposure to engineered NPs can occur during workplace exposure, after environmental contamination, or through topical application of NPs in products such as cosmetics, sun protection lotions, or antibacterial lotions. If NPs are released into the environment, they can be incorporated into food and water, and the gastrointestinal tract then becomes a route of exposure. The gastrointestinal tract can also be exposed through incorporation into foods, since nanomaterials are used for food packaging, in food processing and in nutritional supplements (see *Food and Toxicologic Pathology*, Vol 2, Chap 19). The eyes can also become a route of NP exposure.

NP exposures are difficult to measure. Since the lung is a major target for NP toxicity, NP concentrations in air need to be measured. Standard measurements of particulate exposures in air are based upon mass and may then be divided into size classifications. Thus, for environmental exposures, the concentrations usually measured are for total particulates, particulate matter less than 10 micron (PM10), and/or particulate matter less than 2.5 micron (PM2.5). For occupational exposures to aerosolized particulates not otherwise regulated (PNOR), the Occupational Safety and Health Administration distinguishes particles less than 5 micron from total PNOR. For PNOR, for an 8 h average, the permissible exposure limit (PEL) for total particles is 15 mg/m^3 and for particles less than 5 micron the PEL is 5 mg/m^3. Traditionally, occupational and environmental exposure limits for particulates are based upon mass and have been used for particles in a size range of 1–5 microns or greater. NPs are ***much*** smaller, with at least one dimension less than 0.1 micron (100 nm). With most studies of ambient workplace particulates, the percentage of the particles which were NPs is not reported. In the NP size range, collecting the particles to measure them by mass, surface area, particle number, or any other measure becomes a challenge requiring specialized instrumentation. Recent technical improvements enable measurements of aerosolized nanoparticles using area and personal samplers. These technical improvements greatly facilitate studies of occupational nanoparticle exposures.

Carbon nanotubes (CNTs) and carbon nanofibers (CNFs) can be released into the air (aerosolized) within workplaces if sufficiently agitated. Activities causing aerosolization of MWCNT include oven opening, preparation, weighing, transferring, blending, spraying, and sonication. Diameters of MWCNT aerosolized into workplace air depend on the manufacturing process, with mode diameters ranging from 20 to 30 nm for catalyst preparation and 120–300 nm for ultrasonic dispersion. CNF can be aerosolized during weighing, mixing, handling, transfer, and bagging of dry CNF as well as during wet sawing or grinding of CNF composites. Additional NPs have also recently been demonstrated to produce aerosols under workplace conditions. In studies of workers exposed to NPs, recent data suggest that some NPs may cause lung function alterations, modify resting heart rate, and alter biomarkers, suggesting potential adverse effects. However, current data are limited to studies of a few types of NPs. In response to the rapid expansion of nanotechnology products in workplaces, exposure

banding and other categorical approaches to risk assessment are being used to evaluate potential worker risks. Local exhaust ventilation and HEPA filters have been reported to be effective in controlling nanoparticle exposures in at least one MWCNT laboratory (Han et al., 2008).

2. EXPERIMENTAL TOXICOLOGIC PATHOLOGY OF NPS

In 2005, the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group identified the key elements for toxicity screening of NPs as "Physicochemical Characteristics, In Vitro Assays (cellular and noncellular), and In Vivo Assays" (Oberdorster et al., 2005). Determining the physicochemical characteristics of NPs is essential if the study is to produce data that can be interpreted for risk assessment purposes. The chemical composition, size and size distribution, shape, agglomeration, surface properties, porosity, and a biologically relevant measure of exposure dose are each important to understanding the relevance of NP toxicology studies. Some common features of NPs that can influence toxicologic pathology are surface area, solubility, quantum chemistry, and size.

2.1. Enhanced Toxicity of Nanoscale Particulates

Surface Area

Several studies have compared the bioactivity of fine versus ultrafine carbon black or fine versus ultrafine TiO_2 after pulmonary exposure. On an equal mass basis, ultrafine carbon black or TiO_2 were found to be more inflammatory than fine particles of the same chemical composition. However, when dose was converted to total particulate surface area delivered to the lung, the bioactivities of fine versus ultrafine carbon black or TiO_2 were similar. NPs have the tendency to agglomerate. If particulate surface area influences pulmonary response, then the agglomeration state of NPs should have a significant effect on bioactivity. Indeed, Shvedova et al. (2007) have shown that well-dispersed nano-carbon black (dispersed in diluted lung lining fluid) was more inflammatory after intratracheal instillation in rats than an equal mass of poorly dispersed nano-carbon black (suspended in phosphate-buffered saline) (Shvedova et al., 2007). The influence of dispersion was confirmed in a more extensive study which reported that on an equal mass basis, well-dispersed ultrafine carbon black was 65-fold more inflammatory and cytotoxic in the lung than fine carbon black. Similarly, Sager et al. reported that well-dispersed ultrafine TiO_2 was 42-fold more inflammatory and cytotoxic than fine TiO_2 (Sager et al., 2008). When exposure doses were equalized on a basis of total particulate surface area instilled into the lung, no significant difference in potency of fine versus nano-carbon black or TiO_2 was noted.

Solubility

For metals, particulate surface area is also a major determination of solubility. As noted above, for a given mass of particulates, the surface area goes up as particle size goes down. Thus, the solubility of dilute suspensions of organic-coated silver NPs increases on a mass basis as particle diameter decreases (Ma et al., 2012). This is important because dissolution of certain nano-metallic particles and the formation of toxic metal ions have been proposed as an important mechanism determining bioactivity. For example, nano-ZnO has been shown to exert toxicity in a cell culture system via the formation of Zn^{2+} ions and the resultant generation of reactive oxygen species (George et al., 2010). Doping of ZnO with iron (10%) has been shown to decrease dissolution by 93% (Xia et al., 2011). This decrease in Zn^{2+} formation was associated with a striking reduction in pulmonary inflammation and lung damage in a rat model after intratracheal instillation of nano-ZnO. Dissolution of nanoparticles would also affect the translocation of metals from the lung to systemic organs. Indeed, the lung burden of Zn rapidly declines 24 h after exposure to ZnO with a concomitant rise in Zn levels in systemic organs over this time (Sager et al., 2010).

Quantum Chemistry

NPs can have different chemical and physical properties than larger particles with the same chemical properties. This difference is attributed

to changes that occur in the nanoscale where quantum phenomena predominate, particularly in the size range of 10–50 nm (Hakkinen et al., 2003; Roco, 2011). These effects are described in quantum theory in physics and occur when particle size becomes similar in size to physical and chemical phenomena such as wavelengths (Roco, 2011). Even thermodynamic properties can be different in the nanoscale as opposed to bulk materials of the same composition. Further, different nanoparticles with the same chemical composition can differ in their thermodynamic properties because particle volume within the nanoscale size range influences quantum mechanical behavior (Volokitin et al., 1996). Thus, quantum phenomena are very important in the nanoscale and can markedly alter the properties of compounds that are relatively inert when larger.

Fortunately, pathologists do not need to understand quantum theory to understand nanotoxicology. However, it is important that toxicologic pathologists understand that fundamental properties of compounds can change in the nanoscale and that these altered properties can change the toxicity profile. For an in depth understanding of the quantum realm and altered properties in NPs, collaborators in other scientific fields are particularly important members of many nanotoxicology research teams.

Size

Size affects the properties of surface area, solubility, and quantum chemistry. However, size also influences the ability of a particulate to translocate within the body, enter cells, and interact with subcellular structures. Even the ability of a pathologist to find the particulate in a tissue section is dependent upon particulate size.

2.2. Visualizing NPs in Tissue

Some NPs can be very similar in appearance to normal subcellular components (Figure 13.4). The general principles of identifying NPs in light microscopic and ultrastructural tissue sections include familiarity with (1) the appearance of the NP, (2) the appearance of normal and diseased tissue, and (3) a means for clearly distinguishing between the NPs and changes that may be associated with the NPs. For example, eosinophil granules containing tubular structures within their granules and eosinophils can be a component of an inflammatory response. However, the eosinophil granules are not engineered nanoparticles (Figure 13.4A). Thus, structures in NP-exposed animals will ***not necessarily*** be the NP, even when similar in appearance to the test article and absent in controls. It is essential that the evaluation of tissues from NP-exposed animals for intracellular distribution (1) be conducted by someone familiar with the spectrum of pathologic responses in the exposed tissues, (2) include a means for clear distinction between the NP and cellular responses to the NP, and (3) be conducted with an understanding of the dilution effect and detection limits for NPs in tissue. Failure to identify NPs in an organelle is most likely evidence that the technique is not sensitive enough to detect them rather than evidence that they are not there.

In cultured cells and in tissue sections, NPs may be imaged by using the intrinsic optical properties of the nanoparticle or by labeling. For example, many nanoparticles block light, which allows them to be seen in standard H&E-stained sections (Figure 13.5).

Factors which Limit the Ability to Identify NPs in Tissue Sections

When preparing to examine NPs in tissue, it is critical that the NPs occur frequently enough for the sampling strategy. The frequency of occurrence of nanoparticles within the tissue must be sufficiently high that NPs are likely to be in each field of view. Given the high number of NPs present in even a microgram of NP material, visualization of NPs might, at first, appear to be a routine microscopy exercise. For instance, a 1 μg lung burden of well-dispersed MWCNTs in the mouse lung could easily distribute into 200 million or more nanotubes throughout the lungs. Given that the mouse lung has on the order of 4 million alveoli (Mercer et al., 1994), this would, on average, yield approximately 50 or more nanotubes per alveolus. For light microscopy of paraffin sections this would yield 10 nanotubes in a typical alveolar profile (a 5-micron section would be approximately one-fifth of the 25 micron alveolar diameter in the mouse). Thus, the frequency of occurrence is sufficiently high that NPs would likely be in the field of view. However, given the limited resolution of a conventional light microscope (0.2 microns), NPs may not be

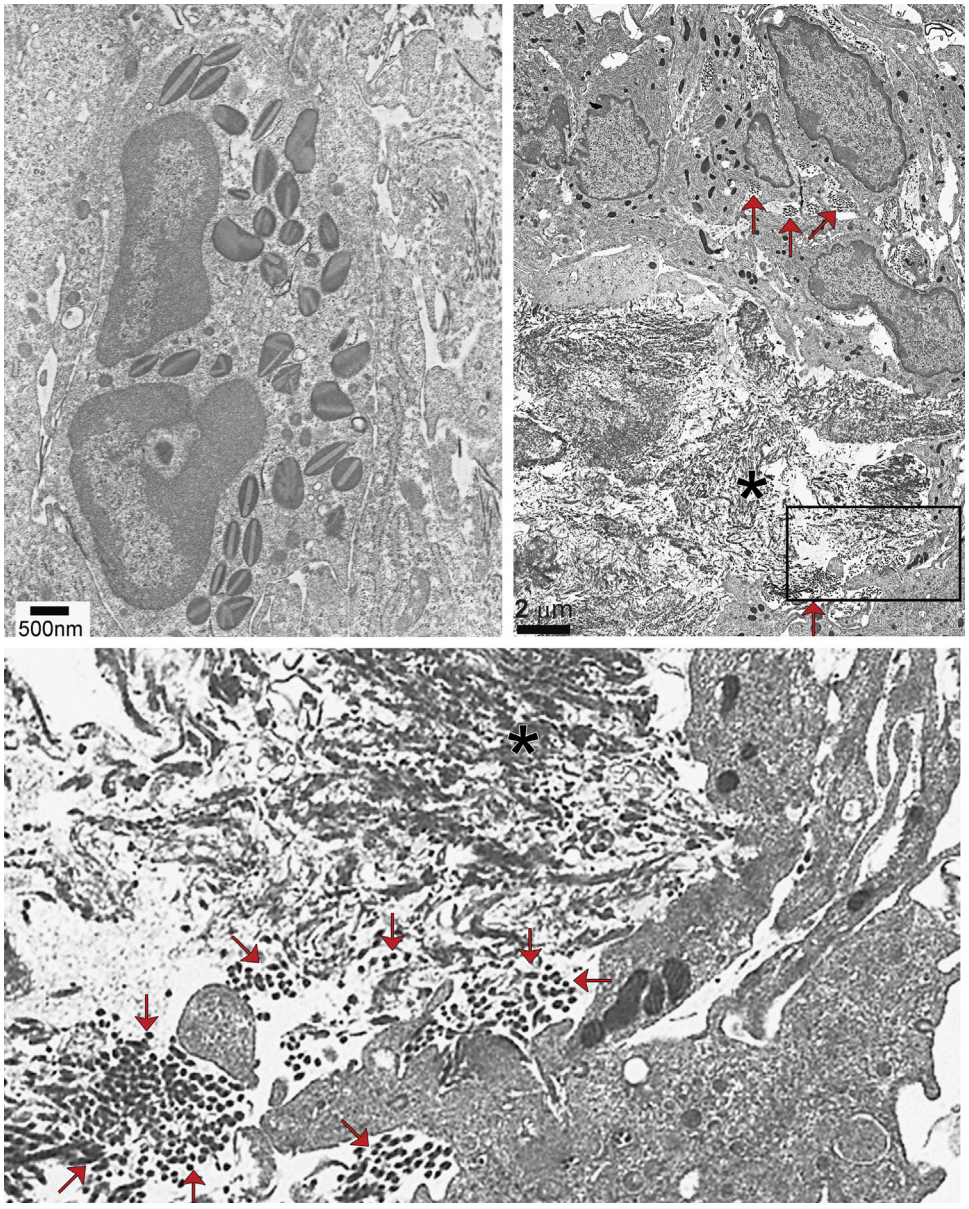

FIGURE 13.4 The image in the upper left is a normal eosinophil in the lung of an SWCNT-exposed mouse. The eosinophil granules contain normal variations in staining intensity that can give the appearance of fiberlike particles, but these are normal structures that should not be confused with SWCNTs even when absent in controls. The image on the *upper right* is a granuloma containing SWCNTs (*asterisk*) that are being walled off by epithelioid macrophages. The SWCNTs are very similar in size and shape to the collagen fibers (*solid red arrows*) within the granuloma. The *rectangle* is a region that was photographed at higher resolution as shown in the lower panel. (*Bottom*) At this higher magnification, the SWCNTs (*asterisk*) can be distinguished from the collagen fibers, which have a more distinct fiber shape. At even higher magnification, the gold label that had been attached to the SWCNTs confirmed that these were the SWCNTs. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.4, p. 1382, with permission.*

detectable in conventional light microscopy. An alternative would be to consider examination with TEM. However, examining the same tissues/burden under TEM would be prohibitively difficult at this dose. A typical TEM section might contain 20 alveolar profiles but would be

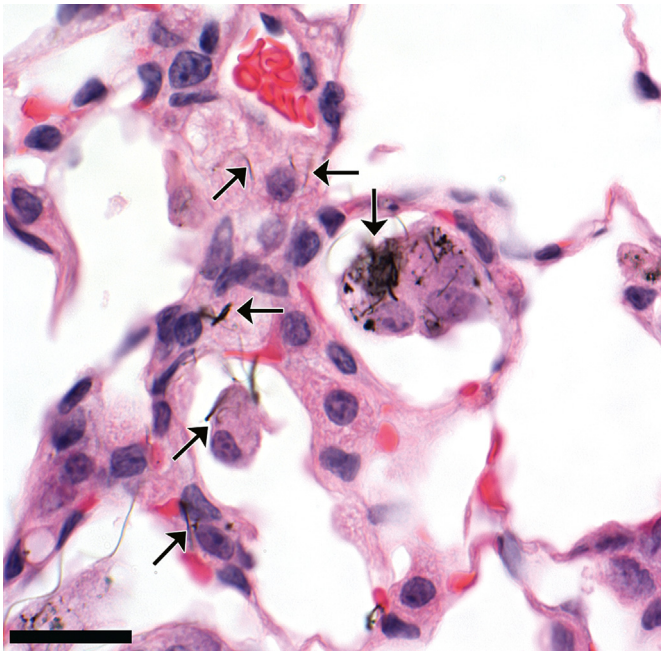

FIGURE 13.5 In this H&E-stained section from an MWCNT-exposed mouse, transmitted light is blocked by the MWCNTs which show up as black structures (*arrows*) in macrophages, giant cells, and alveolar epithelial cells. Bar is 20 microns. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology*, ed 3, Academic Press, 2013, Fig. 43.5, p. 1383, with permission.*

only 60 nm thick. Therefore, on average one would need to examine the entire TEM section to find one or two NPs.

The above example illustrates that both the inherent visibility and the frequency of occurrence of NPs must be considered to successfully evaluate NPs in tissue sections. Numerous other factors work in concert to reduce the possibility of detecting NPs in section. These include: (1) Lack of adequate dispersion. (2) Limitations in visualization due to narrow depth of field. (3) The small fraction of the section area covered by the NPs. (4) The lack of contrast between the biologic tissue and the nanomaterial.

1. *Lack of adequate dispersion*. Although NPs are characterized by the dimensions of single particles, the individual NPs often agglomerate to form functionally larger particulates, particularly when suspended in aqueous solutions. Failure to adequately disperse NPs is one of the major problems in preparation, administration, and detection of NPs. Many of the NPs which are of interest for health risk evaluations have a high self-affinity and require treatment with special dispersants to prevent agglomeration into micrometer dimensions (Sager et al., 2007; Mercer et al., 2008; Porter et al., 2010). In the absence of such treatment, as much as 80% of SWCNTs may remain agglomerated into micrometer-sized clumps.
2. *Limitations in visualization due to narrow depth of field*. Due to their small dimensions, examination of well-dispersed NPs requires high magnification objectives or electron microscopy which typically also have a limited depth of field. Because of the limitations of the depth of field at high numerical aperture only a fraction of the particles in the section will be in focus at one time. For example, a 100× high numerical aperture lens may have a depth of field of only 0.2 micron. Thus only 1/25 of the thickness of a 5-micron section would be in optimal focus at one time. For larger fibers such as asbestos this does not pose a significant problem as the out-of-focus regions of the large fibers are still detectable.
3. *The small fraction of the section's area covered by the NPs*. To be detectable in a microscopic section the nanomaterial must cover a sufficient area of the section to alter the light path. In a ½ cm^2 tissue section of a mouse lung exposed to 50 μg of MWCNTs, there may be as many as 2 million MWCNTs each being 50 nm in diameter by approximately 5 micron long. Even if these fibers were maximally aligned side to side into a sheet parallel to the section, the fibers would cover less than 1% of the ½ cm^2 tissue section. This combined with the lack of contrast makes individual and small clumps of NPs difficult to detect in microscopic sections.
4. *Lack of contrast between the biologic tissue and the nanomaterial*. Many NPs, such as carbon nanotubes, were developed as structural materials and as such are relatively unreactive to conventional biologic stains. Furthermore, the dimensions of the NPs are frequently less than the visible wavelengths of light which further diminishes the likelihood of detection. Carbon nanotubes only give the appearance of being differentiated in the sections because there is sufficient mass of nanomaterial in the light path to block light. Detection of these difficult nanomaterials requires one or more of

the special techniques for detection described in subsequent sections.

These and other factors frequently limit the ability to detect and identify NPs in tissue sections. Specialized instruments and techniques have been developed to overcome these problems. The techniques include labeling of the NPs, use of Field Emission Scanning Electron Microscope (FESEM) in thick sections, and enhanced darkfield microscopy.

Labeled NPs

Labeling of NPs with a fluorescent indicator or some conveniently detected particle such as colloidal gold is one possible solution to make NPs easily visible in sections. Functionalization such as the oxidation of the carbon–carbon bonds may be used to label the CNTs with colloidal gold. Labeling with colloidal gold allows the application of a variety of established techniques developed for immunohistochemistry and other fields. These techniques can be used to allow detection in microscopic sections by silver enhancement and to aid in identification in TEM/FESEM observation (Mercer et al., 2008).

High-Resolution FESEM

Conventional scanning electron microscopes (SEMs) that are used for biologic specimens have been applied with great success to imaging of micrometer-dimensioned inhaled particles which were studied prior to the advent of nanomaterials. For NPs, the conventional SEM does not have the submicrometer resolution necessary to resolve or identify NPs. Difficulties in imaging NPs with an SEM are further complicated by the fact that many NPs, such as carbon nanotubes, have no significant difference in secondary electron or backscatter emissions from the organic carbon in which they are immersed.

Introduction of the FESEM has significantly improved the resolution and applicability of the SEM to examination of NPs in tissue. The unique "cold" cathode design of the FESEM produces high-quality, low-voltage images with significantly lower electrical charging that can be used to identify NPs in tissues at levels of resolution not previously available with the conventional SEM. The high-resolution capability of the FESEM greatly facilitates the imaging of MWCNT interactions with cells and tissues of the lung. The FESEM images in Figure 13.6 show the penetrating nature of MWCNT 28 days after exposure.

To image NPs at high magnification with the FESEM, some consideration of the methods of specimen preparation are necessary to obtain a stable image. Use of thin sections from paraffin embedded tissue has been found to be preferable to large, unevenly cut blocks because it provided a uniform thickness of organic material on a conductive carbon planchet. At 5–8 micron of thickness, paraffin sections are thick enough to convey three-dimensional information and less likely to charge or undergo physical shifts when examined at the high magnifications necessary to study nanomaterials.

Enhanced Darkfield Microscopy

Traditionally, darkfield has been used to examine larger fine-sized particles in tissue sections. Darkfield microscopy suffers from lack of resolution, in part, because the transmitted light is not blocked from the image path. Newer, enhanced darkfield systems have modified optics that virtually eliminate transmitted light from the image. This greatly enhances the contrast between sectioned tissue (a poor source of scattered light) and NPs as can be seen in the enhanced darkfield image of Figure 13.6.

The value of enhanced darkfield is that the vast majority of nanomaterials efficiently scatter light while normal tissue sections do not. Nanomaterials, such as carbon nanotubes, have many of the characteristics which produce Rayleigh scattering of light. These include dimensions less than the wavelength of light, a close and ordered alignment of atoms, and typically have a refractive index significantly different from that of biologic tissues and/or mounting medium. Normal preparation of mounted tissue is designed to minimize scattered light. For instance, the refractive index of glass, mounting medium, and tissue are all closely matched (1.47–1.55). The refractive indices of nanomaterials are much higher being 2.2 for cerium oxide (nanometer-sized diesel fuel catalyst), 2.6 for zinc sulfide (component of quantum dot), and 3.6 for crystalline silica (nanosilica).

Together these characteristics produce significantly greater scattering of light by nanoparticles than by the surrounding tissues. The enhanced

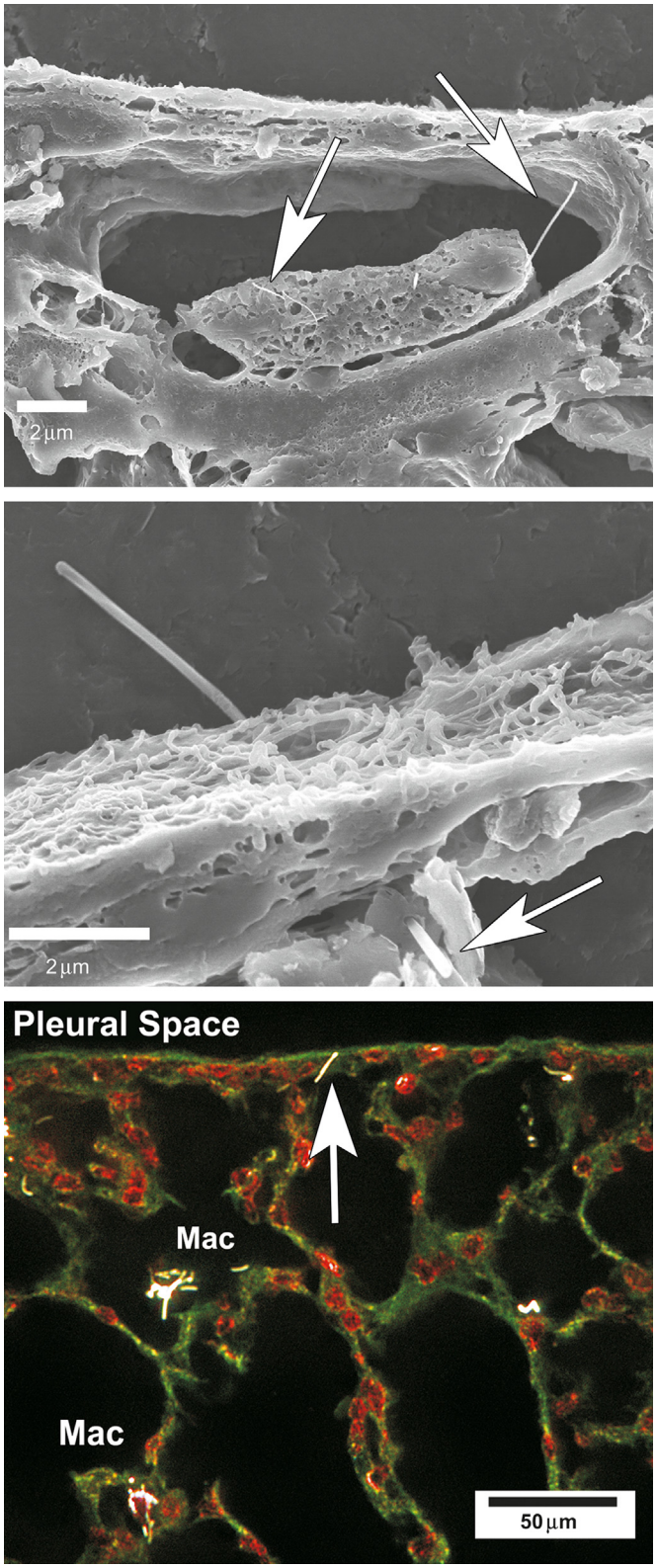

FIGURE 13.6 The *top* image demonstrates MWCNTs within a subpleural lymphatic using FESEM. In the *middle* image, FESEM is used to demonstrate penetration of the visceral pleura by MWCNT. In the *lower* image, enhanced darkfield imaging demonstrates MWCNTs within macrophages, the interstitium and pleura (*arrow*). *Reprinted with permission from Mercer RR, Hubbs AF, Scabilloni JF, Wang L, Battelli LA, Schwegler-Berry D, Castranova V, Porter DW: Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes.* Part Fibre Toxicol *7: 28, 2010. Mercer RR, Hubbs AF, Scabilloni JF, Wang L, Battelli LA, Friend S, Castranova V, Porter DW: Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes.* Part Fibre Toxicol *8:21, 2011.*

darkfield optical system images light scattered in the section and, thus, nanomaterials in the section stand-out from the surrounding tissues with high contrast. Using this method of imaging, large areas can be easily scanned at relatively low magnification to identify NPs that would not be detected by other means. Although the enhanced darkfield technology is relatively new, in our laboratory we have found the technique useful to detect a wide variety of NPs in tissue sections. These have included cerium oxide, titanium oxide, diesel exhaust, welding fumes, SWCNT, MWCNT, silver nanowires, silicon nanowires, nanosilica, quantum dots, colloidal gold, and others (Mercer et al., 2018).

2.3. Cytopathology

Cytoplasmic Membrane Damage

NPs can be produced in almost any shape. The *aspect ratio* of a particle is the ratio of the longest dimension to the shortest dimension of a particle (Figure 13.7A) (Carter and Yan, 2005). Fibers are the classic particulates with a high aspect ratio. Asbestos fibers are naturally occurring carcinogenic mineral fibers, and some asbestos fibers have diameters in nanoscale dimensions. In 1981, Mearl Stanton and colleagues noted that experimental pathology studies indicated: "The probability of pleural sarcoma correlated best with the number of fibers that measured 0.25 µm or less in diameter and more than 8 µm in length …" (Stanton et al., 1981). Some asbestos fibers, and, by definition, all nanotubes, have diameters less than 0.25 µm. Therefore, a great deal of concern has been expressed regarding the similarities between asbestos fibers and

some of the nanotubes (Pacurari et al., 2010). The similarities in some cases include a high aspect ratio, durability, surface reactivity, the inflammatory reaction in the exposed lung, an ability to translocate through the pleura, and incomplete phagocytosis (Shvedova et al., 2005, 2008; Mercer et al., 2010; Porter et al., 2010). It is not just nanotubes that can have these properties; NPs now include nanofibers, nanowires, nanobelts, and many other high aspect ratio particulates with nanoscale dimensions.

Discussions of the potential carcinogenicity of biologically persistent particles with high aspect ratios often focus on incomplete phagocytosis. In normal particle phagocytosis, macrophages and neutrophils phagocytize particles and are then carried out of the lung by mucociliary clearance or lymphatics (Harmsen et al., 1985, 1987; Moller et al., 2004). In some cases, high aspect ratio particles appear to undergo phagocytosis and fusion with the lysosome to form a phagolysosome (Figure 13.7B). Incomplete phagocytosis is the failure to completely internalize a fibrous particulate within the cytoplasm of a phagocytic cell. Classically, phagocytosis is incomplete when the length of the fibrous particle exceeds the dimensions of the phagocytic cells (Archer, 1979; Donaldson et al., 2010). When phagocytosis is incomplete, the phagocytic vacuole may still be open to the exterior of the cell when it fuses with the lysosome to form the phagolysosome (Figure 13.7C). Lysosomal enzymes include enzymes that produce free radicals for microbial killing and enzymes capable of digesting cells. Release of those enzymes outside of the phagolysosome causes cell injury through damage to the cytoplasmic membrane and inflammation (Donaldson et al., 2010).

Human macrophages are larger than rodent macrophages (Krombach et al., 1997). Human macrophages often show less fiber-induced incomplete phagocytosis and less cytotoxicity than rodent macrophages, consistent with a role for incomplete phagocytosis in the pathogenesis of fiber-induced lung disease (Zeidler-Erdely et al., 2006). However, the asbestos fibers most associated with pleural sarcomas are longer than 8 micron, while 8 micron is less than the length of an average macrophage and certainly less than the length of a giant cell (Haley et al., 1991; Krombach et al., 1997). This suggests that incomplete phagocytosis is not the only pathogenic mechanism for fiber-induced cell injury. Studies with NPs are providing data that may help pathologists understand the pathogenesis of diseases such as mesothelioma and asbestosis that have long been associated with durable particles with a high aspect ratio. Some of these NP-induced changes are also manifested as cytoplasmic membrane damage.

MWCNTs are NPs with a high aspect ratio and can be engineered within narrow dimensional

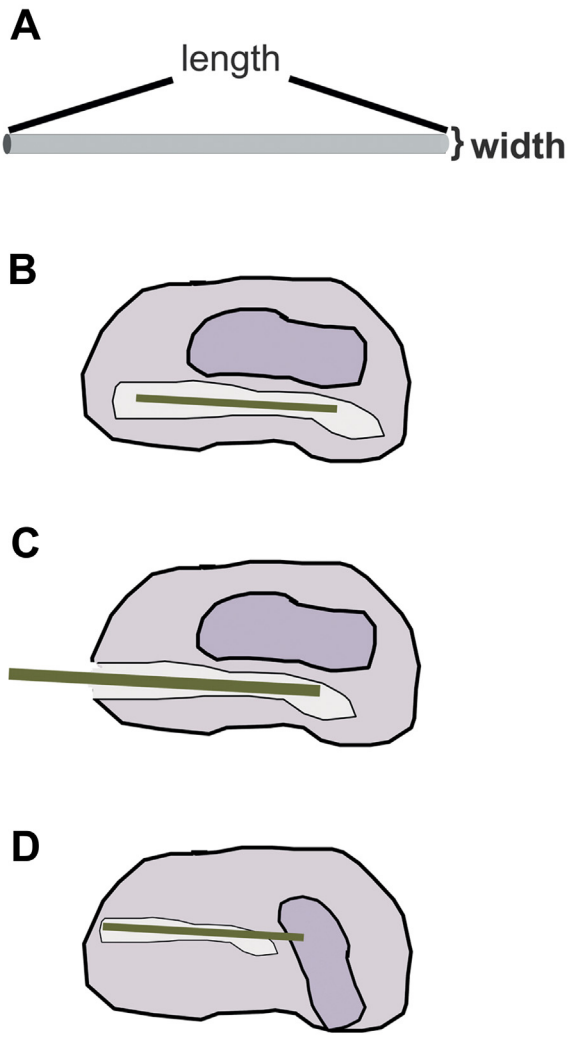

FIGURE 13.7 High aspect ratio particles: (A) A high aspect ratio particle is a particle with a much greater length than width; (B) complete phagocytosis; (C) incomplete phagocytosis; (D) membrane penetration after phagocytosis. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.7, p. 1386, with permission.*

ranges that may permit a greater understanding of fiber toxicology. MWCNTs block transmitted light which makes them relatively easily identified by light microscopy where they are seen as black rodlike structures within the light-transmitting cytoplasm. Even better visualization of MWCNTs is possible with enhanced darkfield microscopy. In the lungs of mice exposed to MWCNTs (median length of 3.86 micron and a median width of 49 nm), incomplete phagocytosis is observed and would be anticipated to release lysosomal enzymes and cause cell membrane damage. Importantly, incomplete phagocytosis or partial engulfment is seen with tangled mats of MWCNTs as well as with long MWCNTs (Porter et al., 2010). In addition, MWCNTs appear to migrate within the lung much like a nanoscale version of a splinter might migrate through tissue. Thus, MWCNTs are seen penetrating the visceral pleura of the lung, extending from alveolar septa and within lymphatics (Figure 13.6) (Mercer et al., 2010, 2011; Porter et al., 2010). MWCNTs can also sometimes penetrate the nuclear envelope of macrophages and other cells (Figure 13.7D) (Porter et al., 2013). Within the cytoplasm, MWCNTs are frequently outside of vacuoles, suggesting that they either enter cells by means other than phagocytosis or do not stay in the phagolysosome (Mercer et al., 2011). Functionalized MWCNTs have been demonstrated to enter cells without phagocytic capabilities and to escape the phagolysosomes of phagocytic cells, suggesting that both mechanisms play a role in the location of MWCNTs within cells (Al-Jamal et al., 2011).

Since the cytoplasmic membrane is basically a protein-containing lipid bilayer, the ability of a thin tube with high tensile strength to migrate through the membrane(s) of mobile cells such as macrophages and into additional cells is not surprising. Nor is it surprising that the migration may continue through additional cells in a tissue such as the lung, which moves and undergoes pressure changes with every breath. In the past decade several endocytic processes have been identified as being able to bring NPs into cells. Those processes are described late in the chapter and are not limited to phagocytic cells and can be influenced by the physiochemical properties of the NPs. Thus, data support penetration of the cytoplasmic and nuclear membranes by NPs with high aspect ratios, and this may be explained by multiple potential processes including: (1) classic incomplete phagocytosis of a high aspect ratio particle that exceeds the length of the cell, (2) incomplete endocytosis that is not part of the phagocytic process, and (3) migration of the NP out of the phagolysome and/or through other cell membranes. In addition, recent studies indicate that high aspect ratio and spherical particles can fundamentally differ in how they enter and traffic through cells, and how they interact with subcellular structures (Zhao and Stenzel, 2018).

Mitotic Spindle Interactions

Toxicologic anatomic pathologists rarely evaluate changes involving the mitotic spindle. However, it is very important that toxicologic pathologists recognize changes in histopathology that are outside of the spectrum of possible changes in normal tissue sections. For example, during the evaluation of histopathology in the lungs of mice inhaling SWCNTs, the toxicologic pathologist noted in a single dividing cell SWCNTs that were gathered at the site of the spindle pole, chromatin streaming from the one chromatin bundle toward the other, SWCNTs attached to the streaming chromatin, and the other chromatin bundle unusually condensed (Figure 13.8). This strongly suggested that SWCNTs could interfere with the specialized system responsible for sending the correct genetic material to the daughter cells during cell division, the mitotic spindle (Shvedova et al., 2008). A summary of some of the most critical information on the mitotic spindle and the genotoxicity of NPs is included below.

OVERVIEW OF THE MITOTIC SPINDLE

The mitotic spindle is a structure that forms during cell division and separates duplicated chromosomes. In eukaryotic cells, the mitotic apparatus is composed of two centrosomes and spindle microtubules (Figure 13.9). The centrosome as well as the microtubules determine the shape of the cell as well as the mitotic spindle apparatus. In eukaryotic cells, the polymerization of microtubules from alpha and beta tubulin is initiated at the centrosome to form the mitotic spindle and the structure for cytokinesis (Yeates and Padilla, 2002; Doxsey et al., 2005). During cell division the

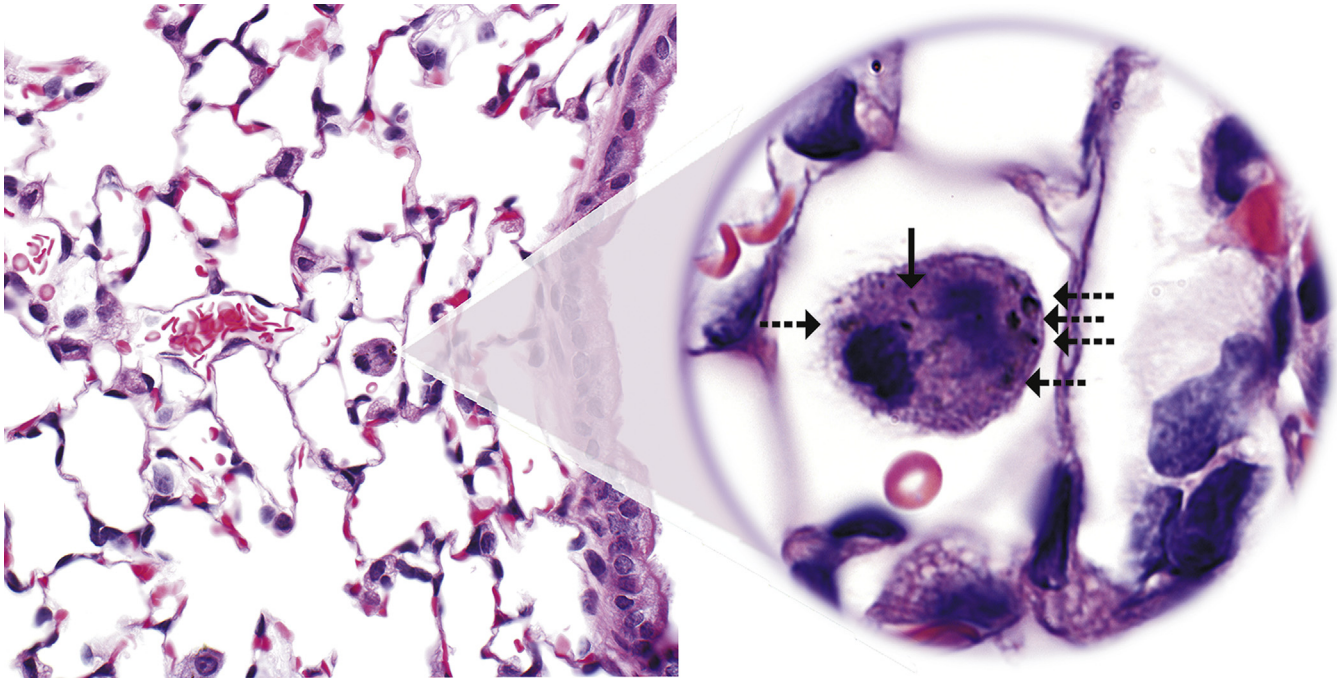

FIGURE 13.8 On the left is a 60× oil field examined by the pathologist and on the right is a high resolution enlarged digital image from a 100× objective showing material consistent with SWCNTs near the spindle poles (*dashed arrows*) and attached to streaming chromatin (*solid arrow*). Identifying this unanticipated change required a detailed histopathologic evaluation and the use of high magnification. Photographing the interaction took a high-resolution digital camera and cropping of the image. Changes such as these are at the limits of resolution of light microscopy. Subsequent studies of SWCNT-exposed cells detected high levels of aneuploidy as well as interactions between SWCNTs and the mitotic spindle. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.8, p. 1388, with permission.*

microtubules continue to polymerize, and the mitotic apparatus elongates until metaphase when the chromosomes line up at the center of the cell at the metaphase plate (Figure 13.10A). As the cell cycle progresses, the chromosomes are separated by the mitotic apparatus as the microtubules of the mitotic apparatus shorten through depolymerization (Figure 13.10B). When mitosis is completed, a furrow is formed between the two dividing daughter cells (Figure 13.10C). The furrow between the dividing cells (midbody) contains microtubules from each pole of the mitosis (Mullins and McIntosh, 1982). Disruption of centrosome number or structure or of the microtubule assembly is common in most cancers and results in aberrant mitotic spindles, failure of cell separation, and errors in chromosome number (aneuploidy) (Pihan et al., 1998; Salisbury et al., 2004; Lingle et al., 2005; Hornick et al., 2008; Salisbury, 2008).

CENTROSOMAL INTERACTIONS

SWCNTs have been shown to fragment the centrosome resulting in multipolar mitotic spindles and dramatic aneuploidy while exposure to some MWCNTs results in monopolar mitotic spindles as well as aneuploidy (Sargent et al., 2009; Siegrist et al., 2014). In both cases, the SWCNT and MWCNT materials were strongly associated with the centrosome. Three-dimensional reconstructions of mitotic figures from SWCNT-dosed respiratory epithelial cells have shown SWCNTs located inside the centrosome structure (Sargent et al., 2012). SWCNTs have also been shown incorporated into the microtubules of mitotic cells (Sargent et al., 2009).

MICROTUBULE INTERACTIONS

SWCNTs have been observed within the nucleus and in association with cellular and

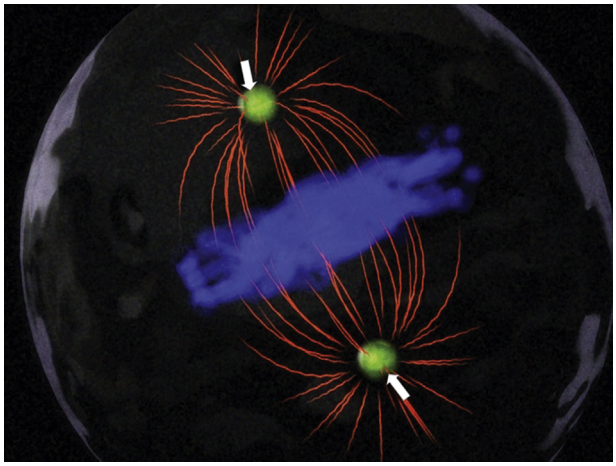

FIGURE 13.9 Drawing of a normal mitotic spindle apparatus. The green stained centrosomes are indicated by *white arrows*, the microtubules are in *red*, and the DNA is in *blue*. The cell is in metaphase stage of cell division with the chromosomes lined up in the middle of the mitotic spindle. *The figure was reproduced with permission from Sargent LM, Reynolds SH, Castranova V: Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. Nanotoxicology 2010, 4:396–408.*

mitotic tubulin, in the bridge separating dividing daughter cells (midbody) as well as in the DNA, potentially disrupting the normal mitotic process as shown in Figure 13.11 (Sargent et al., 2009). The basis of the incorporation of carbon nanotubes into the mitotic apparatus may be due to several mechanisms. In laboratory studies, carbon nanotubes have been shown to form functional hybrid molecules with tubulin (Dinu et al., 2009). The carbon nanotube/microtubule hybrid molecules are transported by the spindle motor kinesin that is essential for normal cell division; however, the hybrids are transported with less efficiency than the cellular microtubules. In addition, spherical nanoparticles less than 40 nm in diameter inhibit the activity of the kinesin motor, further indicating the potential for the disruption of mitosis by nanomaterials (Bachand et al., 2005). Inhibition of kinesin motor activity has been shown to result in mitotic spindle disruption (Ochi, 2002). Carbon nanotubes and microtubules have many physical properties in common including high tensile strength (Pampaloni and Florin, 2008). Carbon nanotubes are five times stronger than steel, and microtubules are 100 times stronger than any other cellular cytoskeletal fibers; however, their strength is 100 times less than that of carbon nanotubes (Dalton et al., 2003; Pampaloni and Florin, 2008). Although there are many physical properties in common, there are also some distinct chemical differences between microtubules and carbon nanotubes. Carbon nanotubes are composed of covalently bound carbon molecules rolled into a tube while microtubules are polymers of alpha and beta tubulin subunits that are bound by noncovalent hydrogen bonds. The microtubules are dynamic structures that polymerize and depolymerize within the cell during cell division (Yeates and Padilla, 2002). Once they are synthesized, individual carbon nanotubes are static in size. The similar size and shape of the carbon nanotubes to the microtubules may make it possible for the nanotubes to displace microtubules at critical cellular targets including the centrosome (Figure 13.12). Alternatively, the nanotubes have also been shown to be incorporated into the microtubules as well as the centrosome. The incorporation of the strong carbon nanotubes into the cellular structures may be responsible for the fragmenting of the centrosome as well as cytokinesis failure during cell division. Fragmented centrosomes and cytokinesis failure have been shown in other systems to result in multipolar mitotic spindles.

CHROMOSOMAL INTERACTIONS

Carbon nanotubes have a high affinity for DNA. SWCNTs have the highest affinity for DNA of GC-rich DNA sequences in the chromosomes and have been shown to bind to the GC-rich regions of the chromosome ends (telomeric DNA) (Li et al., 2006a,b). The DNA intercalation of the nanotubes results in a conformational change which can be stabilized by carboxyl modification of the SWCNT by acid treatment (Li et al., 2006a,b). Intercalating agents can induce chromosome breakage and instability. The damaging effects of carbon nanotubes may be induced by a variety of mechanisms linked in part to the physical and chemical properties of nanotubes. DNA damage and increases in multinucleated cells have been observed following in vitro exposure to SWCNT and MWCNT (Kisin et al., 2007; Lindberg et al., 2009; Yang et al., 2009; Pacurari et al., 2010). In addition, in some, but not all studies of SWCNT-and MWCNT-exposed cells, there is

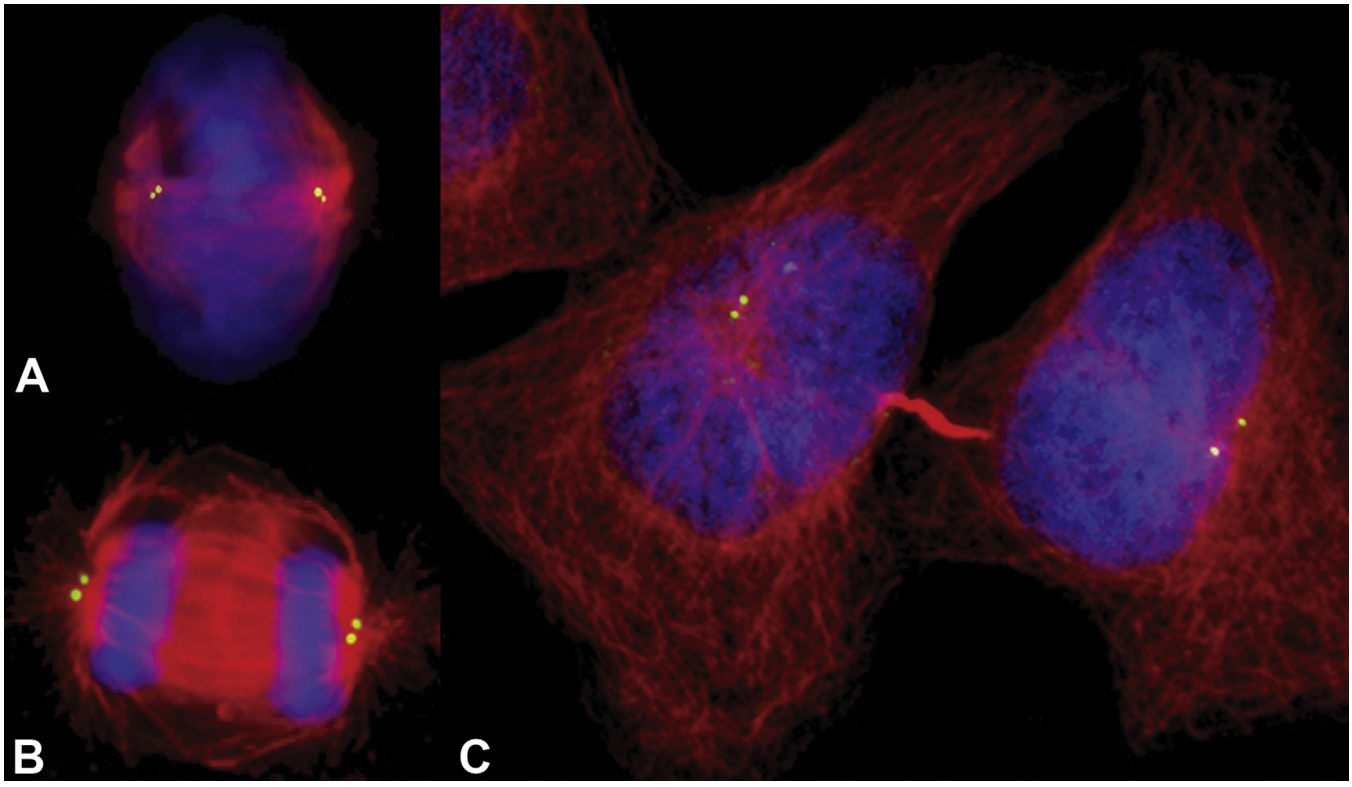

FIGURE 13.10 Composite of mitotic figures. (A) Cell in metaphase. The duplicated centrosomes have formed two mitotic spindle poles. (B) The mitotic spindle apparatus has elongated separating the chromosomes. (C) The cell has progressed through mitosis and a bridge of cytokinesis or midbody separates the cells. The high-resolution confocal images of dividing cells are courtesy of Jeffrey L Salisbury, Department of Biochemistry and Molecular Biology, Tumor Biology Program, Mayo Clinic, Rochester, Minnesota. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013.*

evidence of lactate dehydrogenase leakage from cells as well as depletion of the oxidant protective enzymes (glutathione and superoxide dismutase) indicating reactive oxygen species generation. The generation of reactive oxygen species can damage cell membranes, proteins, and DNA. Oxidant-induced DNA damage has been reported in vivo in both mice and rats exposed to iron-contaminated MWCNT and SWCNT (Folkmann et al., 2009; Jacobsen et al., 2009).

In recent investigations, the more rigid 49 nm Mitsui-7 MWNCT was shown to fragment the center of the chromosome, the centromere. The fragmenting of the chromosome (centromere) resulted in chromosomal translocations and more dramatic aneuploidy than was observed following exposure to SWCNT or a narrower (10–15 nm) MWCNT. The extreme chromosomal damage was not prevented by either heat treatment or nitrogen doping of the Mitsui-7 material (Siegrist et al., 2014, 2019). Additionally, the Mitsui-7 materials were observed in the nucleus and associated with the centrosome, which controls the mitotic spindle, as well as with the microtubules of the mitotic apparatus. Genomic instability can result from damage to the DNA or damage to the mitotic spindle apparatus. The loss of whole chromosomes has been reported in established cancer cell lines indicating a disruption of the mitotic spindle (Muller et al., 2008; Doak et al., 2009; Lindberg et al., 2009). Exposures of rodents to MWCNT have demonstrated micronuclei in primary mouse type II epithelial cells 3 days following intratracheal administration of 1 mg/kg body weight (Muller et al., 2008). Micronuclei indicate either a high level of chromosomal breakage or mitotic spindle disruption. Two in vitro investigations have shown dramatic errors in chromosome number after treatment of primary small airway epithelial cells and immortalized bronchial

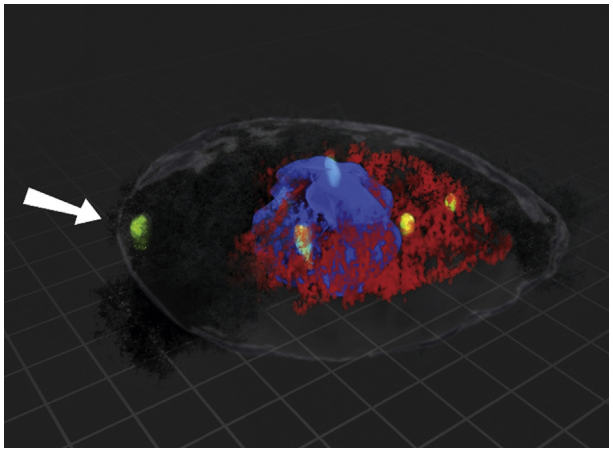

FIGURE 13.11 Three-dimensional reconstruction of a mitotic spindle with three mitotic spindle poles (tripolar mitosis). The mitosis was isolated from a cell exposed to SWCNT for 24 h. The DNA was detected with DAPI and is blue. The tubulin and centrosomes were detected using immunohistochemical methods. The tubulin is *red*, and the centrosomes are *green*. Differential interference contrast imaging images the nanotubes. The nanotubes block the light and produce a *black* image. The cell was imaged using confocal microscopy. Serial optical sections of 0.1 micron in depth were used to construct a three-dimensional reconstructed image of the tripolar mitosis. The reconstructed image shows nanotubes inside the cell in association with each centrosome fragment. The *white* arrow indicates association with one of the centrosome fragments. Nanotubes are also integrated with the microtubules and the DNA. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.11, p. 1391, with permission.*

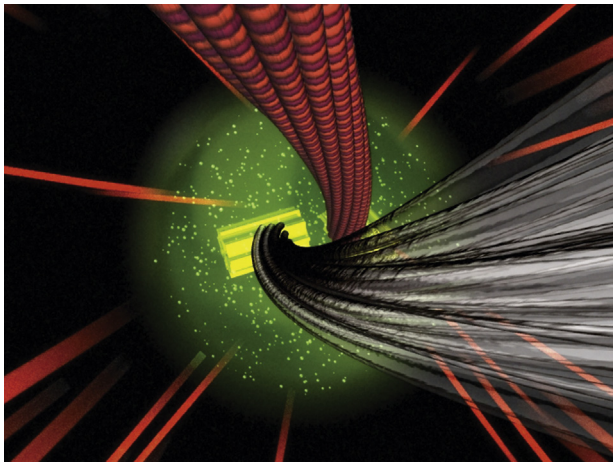

FIGURE 13.12 The drawing demonstrates a proposed model for the interaction of carbon nanotubes with subcellular structures. The carbon nanotubes in this drawing are attached to the centrosome and displace microtubules at the centrosome. *The figure was reproduced with permission from Sargent LM, Reynolds SH, Castranova V: Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. Nanotoxicology 2010, 4:396–408.*

epithelial cells with 0.024–96 μg/cm^2 SWCNT (Sargent et al., 2009, 2012). A more recent investigation demonstrated even greater chromosomal damage following exposure of immortalized and primary small airway cells epithelial cells to 0.0024–24 μg/cm^2 MWCNT (Siegrist et al., 2014). As indicated previously, the chromosome errors were attributed to disruption of the mitotic spindle. The SWCNTs were observed within the nucleus, the DNA, in association with cellular and mitotic tubulin, and in the bridge separating dividing daughter cells (midbody). The association of the nanotubes disrupted the normal mitotic process as shown in Figure 13.11. Additionally, the more rigid Mitsui-7 MWCNT caused further damage by fragmenting the center of the chromosome (Siegrist et al., 2019).

ADDITIONAL CYTOPATHOLOGIC INTERACTIONS

The findings noted above demonstrate that some NPs cause important alterations in cytoplasmic membranes and the mitotic spindle. These are among the best described cytopathologic effects of NP exposure. However, the size range of NPs (<100 nm in at least one dimension) is similar in size to many subcellular organelles and structures, making interactions with any of these a potential concern. As a general group, NPs can have an almost infinite spectrum of one or more chemical constituents, so the designation of something as an NP only refers to a size range—not chemical composition.

There is similar diversity in the intracellular environments that may interact with NPs. The in vivo environment contains an enormous spectrum of different cell types. The diversity of those cells, their ability to internalize NPs, their ability to respond to injury, and their ability to metabolize or detoxify NPs and their products must be considered in evaluating the cytopathology of NPs. NPs can be modified to permit entry into

specific tissues to target drug delivery, with the classic example being NPs designed to cross the BBB (Lockman et al., 2004; De Jong and Borm, 2008; Bhaskar et al., 2010; Brambilla et al., 2011). Some NPs, particularly cationic NPs, that can cross the BBB, can increase the permeability of the barrier (Lockman et al., 2004). Obviously, cytopathologic effects in the BBB are a concern and these are discussed in more detail later in this chapter in the section Neurotoxicity/neuropathology of NPs.

Another concern is that exogenous chemicals in the body are usually metabolized into compounds that are more easily eliminated. This process, known as xenobiotic metabolism, uses enzymes. Many of these enzymes come in many different forms, known as isoforms. The different isoforms have different capabilities for metabolizing different compounds. The foreign compounds, which are often drugs, are the substrate for the enzymatic reactions. Exposure to substrates can markedly increase (induce) some types of xenobiotic metabolism. The products of xenobiotic metabolism are sometimes less toxic but also may be more toxic than the substrate itself depending upon the substrate and the metabolic pathway.

Cytochrome P450 is essential to many xenobiotic-metabolizing reactions. There are numerous isoforms of cytochrome P450 and each tends to metabolize different substrates. The expression of the different cytochrome P450 isoforms is tissue specific and within tissues can be highly cell specific (Dinsdale, 1995; Battelli et al., 2008). In addition, expression of some cytochrome P450 isoforms is affected by particle exposure, inflammation, and/or disruption of microtubules (Ghanem et al., 2004, 2006; Battelli et al., 2008; Vondracek et al., 2011). In the brain, the cytochrome P450s are often regulated differently than in other tissues and some of the brain cytochrome P450s have a role in regulating critical endogenous hormones and signaling molecules. Within cells, the cytochrome P450s tend to be expressed in specific intracellular sites such as the endoplasmic reticulum, mitochondrial membranes, and the cytoplasmic membrane (Miksys and Tyndale, 2004; Haining and Nichols-Haining, 2007; Meyer et al., 2007).

Cell-specific and organelle-specific NP-targeted drug delivery may deliver drugs to cells and to subcellular structures that have not previously metabolized the drug. The xenobiotic-metabolizing systems of the target sites may be unable to metabolize the drug, may produce unanticipated metabolites, or may be induced to greater activity. In short, cytopathologic effects of NPs or their absence could potentially result from NP-mediated passage of drugs to tissues such as the brain or subcellular structures that are not traditionally exposed to those substances. For example, drugs that are easily detoxified by cells of traditional exposure portals in the liver and lung may not necessarily be detoxified in the brain. Xenobiotic-metabolizing pathways may also be modulated by pro-inflammatory effects of NP exposure.

A major technical challenge is localizing NPs within subcellular compartments. This technical challenge affects the evaluation of NP cytopathology in many different cell types. In evaluating NP-induced cytopathologic damage in tissue sections, it is extremely important to recognize the limitations of techniques that detect subcellular distribution of NPs. Thus, colocalizing a fluorescent NP with a mitochondrial marker could mean that the NP is in the mitochondria, or it could mean that it is located on the surface of the mitochondria.

NPs enter cells in several potentially different ways that are affected by the normal function of the cells. For example, NPs can use pathways for entering the cell and trafficking mediators within the cell that use endocytic pathways that are not believed to operate for larger particulates. However, some NPs can also use cell entry pathways that are used for micron-sized particulates. For example, the phagocytic cells that respond to micron-sized particles can sometimes recognize NPs. As mentioned earlier in this chapter, when recognized by a phagocytic cell, the NPs are first engulfed into a phagosome which then fuses with a lysosome to form a phagolysosome, a system generally designed to degrade potential pathogens (Parham, 2009). In addition, some pinocytotic pathways, particularly clathrin-mediated endocytosis, can also deliver NPs to the lysosome. The lysosome is an acidic environment with abundant enzymes which digest pathogens and other complex biological material. Within the lysosome, the fate of the NP is determined by the composition of the NP, which may or may not be digested. In some phagocytic

cells, the digested contents of the lysosome may translocate to the cell surface and be presented to the immune system. Therefore, the potential to stimulate an immune response is a consideration when NPs are recognized by phagocytic cells. NPs that cannot be digested in the phagolysosome can also adversely affect some cells.

Some phagocytized NPs may be cytotoxic because they cause lysosomal rupture. For example, unmodified and negatively charged polystyrene particles are much less inflammatory in the lung than positively charged (cationic) polystyrene NPs (Nemmar et al., 2003). Polystyrene NPs with surface NH_2 molecules and a positive charge have been used as a model of cationic NPs in phagocytic cells. When labeled with FITC to create green fluorescence that allows intracellular tracking, the cationic NPs are seen first in the phagolysosome and are then released into the cytosol due to lysosomal rupture that is believed to be mediated by pumping of chloride ions (Xia et al., 2008). As with incomplete phagocytosis, lysosomal rupture following phagocytosis of cationic NPs is associated with cytotoxicity.

NPs can also enter cells by pinocytosis or macropinocytosis utilizing endocytosis pathways that are distinct from phagocytosis and are particularly effective for intracellular delivery of NPs (Conner and Schmid, 2003; Perez-Martinez et al., 2011; Wang et al., 2011). Endocytic pathways exist in all cell types, including neurons. Macropinocytosis creates relatively large endocytic vacuoles, incorporates extracellular material into the cell and can internalize particulates that are less than a micron in diameter (Conner and Schmid, 2003). The endocytic processes which function to transport particles in the100 nm or smaller size range can be highly selective (Conner and Schmid, 2003; Perez-Martinez et al., 2011; Reider and Wendland, 2011). They provide a pathway to enter cells that are unlikely to be targets of larger particles. In addition, the intracellular pathways can be different when an NP enters a cell by pinocytosis than when the NP enters the cell by phagocytosis. Trafficking for endolysosomal degradation is only one of many potential fates for the cargo of endosomes (Cullen and Korswagen, 2012).

Endocytosis that principally operates in the nanoscale can broadly be divided into three groups: (1) caveolin-mediated endocytosis in caveolae, (2) clathrin-mediated endocytosis, or (3) through processes that are independent of these mediators (Conner and Schmid, 2003; Perez-Martinez et al., 2011). Entry into the cell through these endocytic pathways is dependent upon the cell type and the NP composition and plays a critical role in cytopathology. Caveolae were mentioned earlier in this chapter for their role in shuttling NPs and proteins across the endothelium. However, caveolae also play an important role in endocytosis in cells like the bronchiolar epithelium where cationic NP entry and cytotoxicity is dependent upon caveolae (Conner and Schmid, 2003; Xia et al., 2008). Similarly, cerium oxide NP uptake by keratinocytes is dependent upon caveolae (Singh et al., 2010). In the previous example of cytotoxicity of cationic polystyrene NPs, the cationic NP cytotoxicity is dependent upon the pathway of internalization and is higher in cells with caveolin-mediated or phagocytic pathways of NP internalization than in cells without those pathways (Xia et al., 2008).

Clathrin-mediated endocytosis involves binding of molecules on the surface of the transported protein or NP (the cargo) to cellular receptors. These receptors are sequestered by adapter proteins that interact with clathrin at the cell membrane to form a clathrin-coated pit (Kaksonen et al., 2006; Reider and Wendland, 2011). These endocytic adapter proteins determine whether a given cell will endocytose an NP or protein on the cell membrane. The clathrin-coated pit matures and eventually forms an intracellular vesicle (Kaksonen et al., 2006; Reider and Wendland, 2011). The clathrin coated vesicle then delivers the cargo into the cell, usually through fusion with early endosomes. The cargo is then sorted within the endosomal network and trafficked to designated sites (Cullen and Korswagen, 2012).

Clathrin- and caveolin-independent endocytosis (CIE) is the least understood form of pinocytosis (Conner and Schmid, 2003). Studies suggest that there are multiple types of CIE. At least one of these types of CIE is the major

endocytosis pathway in migrating fibroblasts (Howes et al., 2010). In neural cells, CEI has been shown to be the method for endocytosis of polyethylenimine (PEI)-decorated polymer nanospheres designed for nanomedical applications (Evans et al., 2011). PEI is used as a cationic NP carrier of gene therapy and like the cationic NPs mentioned earlier causes swelling of the enclosing membrane-bound structure. In the case of an endosome carrying PEI-DNA vectors, this leads to endosomal rupture and content release into the cytosol, and improved gene transfection but also cytotoxicity (Sonawane et al., 2003).

Recent studies indicate that the processes affecting cell entry can vary by the cell type and by the physiochemical properties of the nanoparticle (Behzadi et al., 2017; Zhao and Stenzel, 2018). Pinocytotic processes can also be specific or nonspecific, and the NP agglomeration state can affect cell entry processes. For example, the smallest nanoparticles may simply translocate through cell membranes whereas some uptake processes have minimum size limits so that agglomeration facilitates uptake (Zhang et al., 2015). Some NPs can enter the nucleus of a cell through passive diffusion or active pathways. Very small NPs less than the diameter of the pores in the nuclear membrane can diffuse into the nucleus if located free in the cytosol (Lovric et al., 2005). However, the nuclear membrane dissolves during cytokinesis, allowing nuclear access for larger NPs that are free in the cytosol. An example of an active pathway is the use of nucleolin to transport NPs. Nucleolin is a shuttling protein, microtubule dependent, and able to transport DNA NPs, hyperbranched polylysine NPs, and F3-peptide conjugated NPs from the cell membrane or cytoplasm into the nucleus (Chen et al., 2008, 2011; Zhang et al., 2009, 2011).

Since NPs can be similar in size to subcellular organelles or even smaller, interactions with these structures can be produced intentionally rather than as a toxic side effect. For example, surface functionalized NPs can be conjugated with antibodies and transfected into living cells for fluorescent visualization of cellular organelles such as the mitochondria. As mentioned earlier, important NPs include imaging agents, pharmaceuticals, and transporters of gene therapy. Understanding the routes NPs use to enter and exit cells is critical to targeting these new products (Wang et al., 2006; Zhang et al., 2009, 2011).

Currently, interactions between NPs and cell membranes and mechanisms of NP internalization are areas of intense scientific investigation. Our understanding of these interactions is increasing rapidly but is still incomplete. However, we now know that NPs may initially interact with cells through membrane wrapping, a process that can occur in many different cell types, may be complete or incomplete, and can initiate biological responses (Bahrami et al., 2014; Dasgupta et al., 2014; Urbancic et al., 2018). NP shape, size, flexibility, and chemical composition influence the interaction and subsequent internalization (Fraser et al., 2020; de Almeida et al., 2021). NPs can also interact with and damage the cell membrane directly. The fascinating study of particle interactions with cells reveals critical interactions that are well beyond simple cytotoxicity or incomplete phagocytosis. Increased understanding of NP membrane interactions, internalization, and subcellular trafficking may be key to harnessing of NPs for nanomedicine and understanding mechanisms of potential NP toxicity.

2.4. Target Organ and Tissue Toxicity

Near the start of this century, the scientific community realized that the nanoscale manipulation of matter could produce products with adverse health effects not anticipated for larger particles of similar chemical composition (Service, 2004; Shvedova et al., 2005). The earliest in vivo nanotoxicology studies included studies demonstrating the neurotoxic effects of buckyballs in fish and pulmonary inflammation and fibrosis in mice aspirating SWCNTs (Oberdorster, 2004; Shvedova et al., 2005). The lung and the brain are among the most investigated of the target tissues for nanotoxicology studies today. Inhaled respirable particles have long been associated with increased cardiovascular mortality, and studies of NP effects on the cardiovascular system are providing insights into the cardiovascular effects of the smallest particles. The lymphatics are also increasingly recognized both as a means for transporting nanoparticles and as a potential target tissue.

Pulmonary Pathology

Because many NPs are easily aerosolized (Methner et al., 2007; Johnson et al., 2010; Lee et al., 2010), the lung is potentially exposed to NPs through inhalation which is an important consideration in occupational and environmental toxicology. While particles from 1 to 5 micron in diameter are classically thought to have optimal alveolar deposition, recent inhalation studies clearly demonstrate significant pulmonary deposition of the much smaller NPs (Nurkiewicz et al., 2008; Shvedova et al., 2008; Pauluhn, 2010; Ho et al., 2011). In addition, particles less than 1 micron in diameter are more likely to cross epithelial and endothelial barriers (Foster et al., 2001; Dombu et al., 2010; Vllasaliu et al., 2011; Lin et al., 2012). In the pharmaceutical industry, the inhalation of NPs is a highly effective route for drug delivery (Bailey and Berkland, 2009). The lung can also be exposed to NPs that are not inhaled and surface modification of NPs can affect lung delivery both via inhalation and through the blood. Surface modification of NPs is one strategy used to alter toxicity, increase transport across cell barriers, and prolong half-life of nanopharmaceuticals (Knop et al., 2010; Blanco et al., 2011; Vllasaliu et al., 2011). Coating NPs with poly(ethylene glycol) is a common modification for pharmaceuticals because this modification can improve features such as dissolution and can decrease uptake by phagocytes with resulting increases of half-life in circulation (Knop et al., 2010; D'Souza A and Shegokar 2016). However, a study of mesoporous silica NP biodistribution demonstrates increased lung deposition in NPs coated with poly(ethylene glycol) (Huang et al., 2011). Thus, the lung is an important potential target for evaluation by toxicologic pathologists in the pharmaceutical industry as well as those concerned with environmental and occupational exposures (see *Respiratory System*, Vol 4, Chap 4).

Hypertrophy and hyperplasia of the bronchiolar epithelium can be observed following inhalation or aspiration of SWCNT or MWCNT (Shvedova et al., 2008; Porter et al., 2010). Hypertrophy and hyperplasia were also observed by histopathologic evaluation of bronchi and bronchioles in high-dose (50 mg/m^3) rats after short-term inhalation of TiO_2 with a mean primary diameter of 25.1 nm (Ma-Hock et al., 2009). Using BrdU labeling, a directly quantitative and potentially more sensitive measure of cell proliferation identified hyperplasia in bronchi and bronchioles of rats inhaling much lower doses (2 mg/m^3) of nanoscale TiO_2 (Ma-Hock et al., 2009).

Inflammation with rapid development of fibrosis was a sentinel finding in one of the earliest studies of the pulmonary toxicity of NPs, a study of aspirated SWCNTs in the mouse lung (Shvedova et al., 2005). Inflammation is frequently described following acute or subchronic inhalation exposure to several different NPs including metallic gold (Sung et al., 2011), ZnO (Ho et al., 2011), TiO_2 (Ma-Hock et al., 2009), SWCNT (Shvedova et al., 2008), and MWCNT (Pauluhn, 2010). Different mechanisms may trigger inflammation with different NPs. Nano-TiO_2 and nano-SiO_2, but not nano-ZnO, trigger IL-1α and IL-1β release from macrophages in an Nlrp3 inflammasome-dependent process (Yazdi et al., 2010). In contrast, an acute inhalation study using amorphous silica nanoparticles (exposures as high as 86 mg/m^3, 6 h/day for up to 3 days) did not detect significant pulmonary inflammation, suggesting that not all insoluble nanoparticles cause acute inflammation (Sayes et al., 2010).

Fibrosis can develop rapidly in the alveolar septa and within granulomas following a single exposure to aspirated SWCNTs (10 μg) or MWCNTs (80 μg) (Mercer et al., 2008, 2011). Pulmonary fibrosis was a component of the lung disease seen in young women exposed to nanoparticulate polyacrylate (Song et al., 2009). In addition, fibrosis was observed in lung biopsies from responders to the World Trade Center events of September 2011 who subsequently developed severe interstitial lung disease. Although the exposures of World Trade Center responders were clearly complex, SWCNTs were observed in the biopsies from three of the four patients with the most severe interstitial disease and are presumed to have been produced as a combustion product (Wu et al., 2010).

Lymphatic dilation can also occur in the pulmonary lymphatics following nanoparticle exposure (Porter et al., 2010). Lymphatic toxicity

and the role of the lymphatics in translocation of NPs are discussed later in this chapter.

Pleural penetration has been described in the lungs of mice exposed to MWCNTs by aspiration (Mercer et al., 2010; Porter et al., 2010). This is a concern because MWCNTs have a high aspect ratio. Carcinogenic asbestos fibers also have a high aspect ratio, and mesothelial cells lining the pleura are the target cells for the development of mesothelioma.

Carcinogenicity studies indicate that at least one of the first generation of engineered NPs is carcinogenic. Neoplasia of the lung and mesothelium have been reported in multiple carbon nanotube carcinogenicity studies. These include studies in mice and in rats using inhalation, instillation, injection, and/or an induction/promotion model (Nagai et al., 2011; Rittinghausen et al., 2014; Sargent et al., 2014; Kasai et al., 2016; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017) (Figure 13.13). One type of MWCNT, MWCNT-7, was investigated in multiple studies. In 2017, the International Agency for Research on Cancer (IARC) reviewed the data on the carcinogenicity of carbon nanotubes and noted that data gaps existed. In particular, carbon nanotubes are heterogenous and few long-term studies had been conducted. However, IARC concluded there was "*sufficient evidence* in experimental animals for the carcinogenicity of MWCNT-7 multiwalled carbon nanotubes." IARC also concluded that "MWCNT-7 multiwalled carbon nanotubes are *possibly carcinogenic* to humans (Group 2B)." At the time of the IARC review, other single- and multi-walled carbon nanotubes were "*not classifiable* as to their carcinogenicity to humans (Group 3)," due to the lack of sufficient animal studies (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017).

Neurotoxicity/Neuropathology of NPs

Interest in the neurotoxicity of NPs is based on studies that demonstrated extrapulmonary translocation and deposition of ultrafine particles in the brain (Kreyling et al., 2002, 2006; Oberdorster et al., 2002; Oberdorster and Utell, 2002). Subsequent studies demonstrated (1) ultrastructural nasal pathology in children exposed to air pollutants (Calderón-Garcidueñas et al., 2001); (2) DNA damage, neuroinflammation, and neurodegeneration in nasal and brain tissues of canines exposed to air pollutants (Calderón-Garciduenas et al., 2003), (3) chronic brain inflammation, BBB disruption, and Alzheimer's disease (AD)-like pathological changes in humans exposed to fine and ultrafine particulate matter from air pollution (Calderón-Garciduenas et al., 2004, 2007), and (4) titanium-rich nanoparticles in enteric neurons and substantia nigra from heavily polluted metropolitan areas in Mexico City (Calderón-Garcidueñas et al., 2020). High levels of cyclooxygenase 2 (COX2) mRNA, an index of inflammation, were detected in the frontal cortex and hippocampus areas of autopsied brains from human subjects who had prolonged exposure to severe air pollution. Concurrent accumulation of β-amyloid$_{(1\text{-}42)}$ was also observed in these brain regions. Increased production of β-amyloid is thought to precede the formation of plaques and tangles and is known to cause microglial activation and neuroinflammation in the brain. Elevated β-amyloid has also been linked to cognitive decline (Calderón-Garcidueñas et al., 2001). Based on these findings, it is likely that inhalation of fine and/or ultrafine particulates present in the polluted air may be the underpinnings for such abnormal AD-like pathology. As extracellular deposition of aggregated β-amyloid into senile plaques and intracellular accumulation of hyperphosphorylated aggregated Tau as neurofibrillary tangles, respectively, are pathological hallmarks of AD, there is emerging concern to determine if similar exposures to engineered NPs can cause neurotoxicity and neurodegeneration-like changes (see Boyes and van Thriel (2020) for additional information).

In the workplace environment, workers may be at risk for exposure via inhalation of fine and/or ultrafine NPs, and, to a lesser extent through, ingestion or dermal penetration. Inhalation of NPs can result in their deposition in olfactory and/or pulmonary regions. Gastrointestinal exposure to NPs can occur through ingestion or through respiratory clearance and subsequent swallowing of inhaled NPs. Translocation of NPs to the brain (Figure 13.14), either directly by neuronal transmission from the olfactory, pulmonary, and potentially enteric regions, or indirectly via systemic circulation, can potentially cause damage to the brain. The limited regenerative capability of the nervous system, its cellular

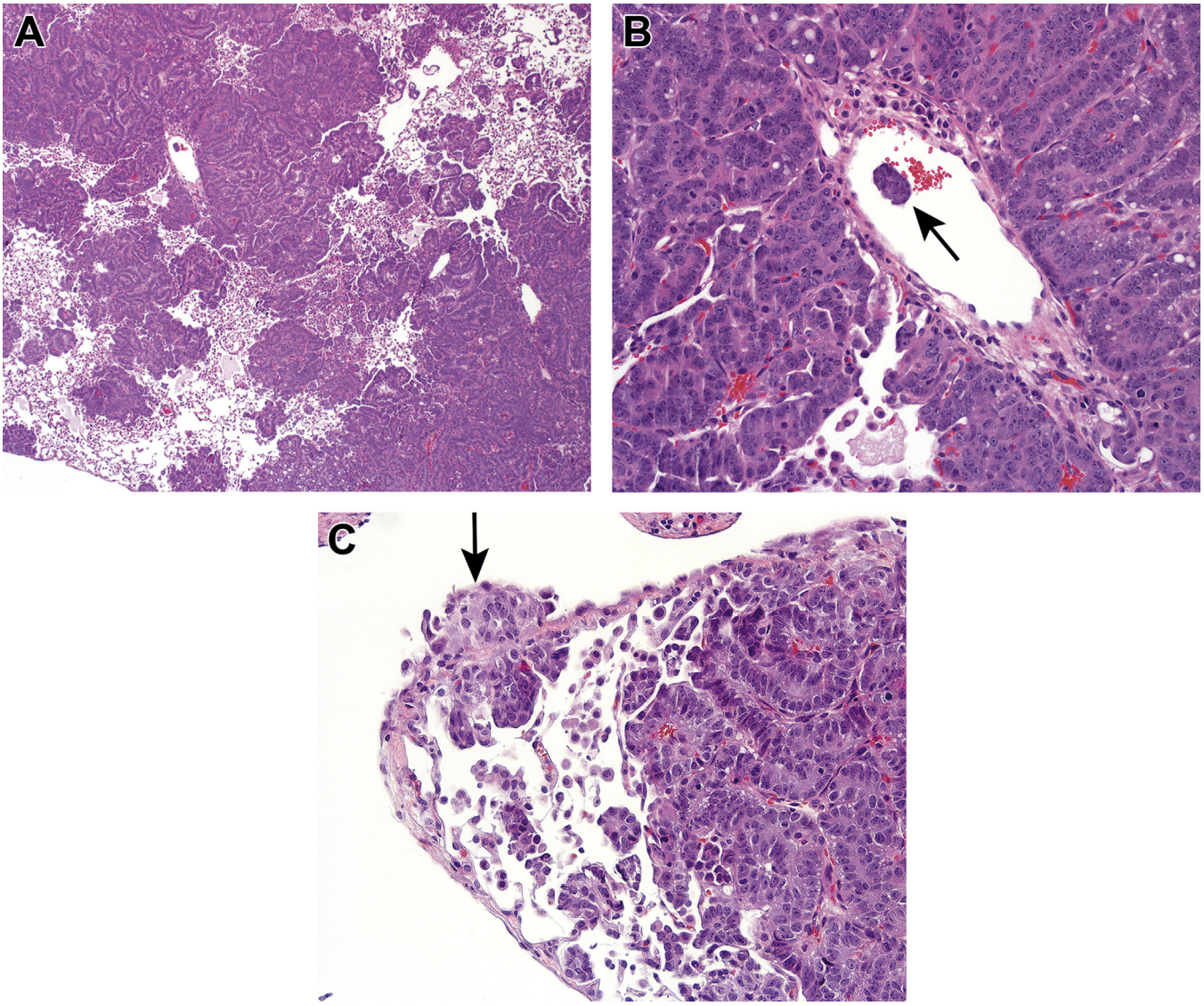

FIGURE 13.13 Infiltrative bronchioloalveolar carcinoma in a mouse after 3-methylcholanthrene initiation by injection followed by MWCNT inhalation as a promoter (Sargent et al., 2014). (A) The infiltrative features of this bronchioloalveolar carcinoma. (B) A higher magnification showing a tumor embolus (*arrow*). (C) Another field showing pleural invasion by this aggressive neoplasm (*arrow*).

heterogeneity, and high lipid content may further render it vulnerable to toxic insults.

The olfactory sensory neurons occupy nearly two-thirds of the sensory or olfactory epithelium. Because of their widespread distribution in the olfactory epithelia, they experience direct access to odorant molecules, as well as allergens, airborne pollutants, toxic chemicals, and microorganisms (see *Respiratory Tract*, Vol 4, Chap 4). NPs inhaled through the nose and air passages have been shown to translocate to the brain via transport across olfactory neurons and accumulate in deeper brain areas (Hunter and Undem, 1999; Oberdörster et al., 2004; Elder et al., 2006). Similarly, the enteric nervous system of the gastrointestinal tract can be exposed to NPs ingested in food or swallowed after pulmonary clearance. The olfactory and enteric nervous systems are potentially important targets because transsynaptic transport of NPs in these systems can bypass the BBB. Indeed, transsynaptic transport of nanoscale material in these systems is well documented since this transmission route is used by neurotropic viruses and recent studies report transsynaptic transport of aggregated α-synuclein protein implicated in the pathogenesis of sporadic Parkinson's disease (Luk et al., 2012; Del Tredici and Braak, 2016;

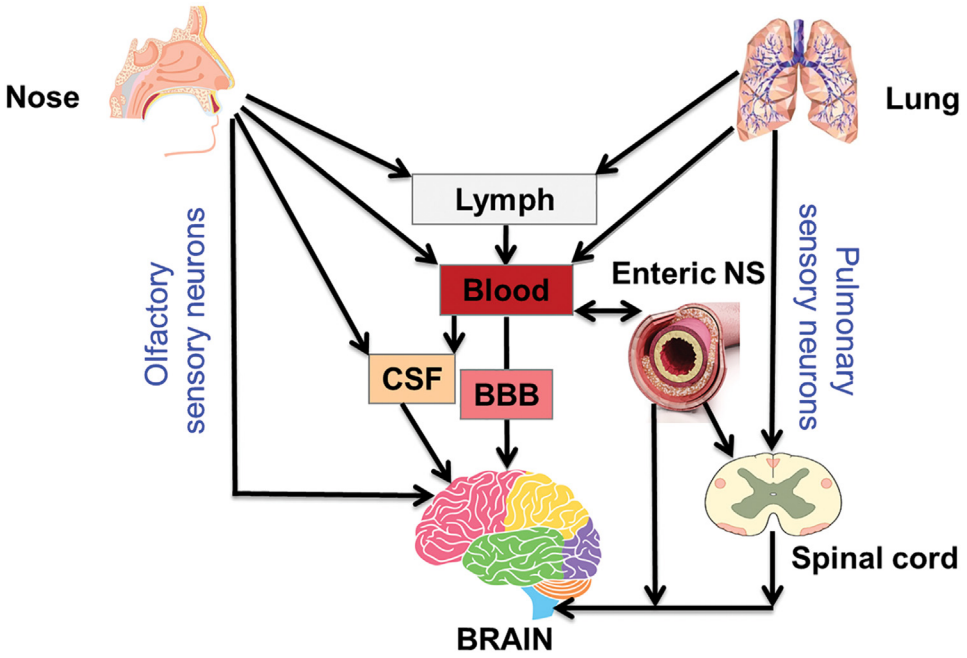

FIGURE 13.14 Potential routes of NP translocation to the brain or NP-mediated stimulation of the brain. Direct translocation of NPs can occur from the nose, enteric system, and lung via neural pathways. Indirect translocation can occur following nose, skin, enteric, or lung deposition of NPs through systemic circulation of the particle or of its soluble fraction (likely in the case of metal-based NPs) in blood. In some cases, NPs may first circulate in the lymphatics and then enter the blood. In the absence of particle translocation, either through direct or indirect mechanisms as illustrated here, inflammatory mediators released into systemic circulation from affected organs (primarily lung) can cause disruption of the blood–brain barrier (BBB) and activation of glial cells and/or neuronal population in discrete brain areas. In addition, perturbation of the olfactory, enteric, or pulmonary sensory neurons can potentially elicit neurogenic inflammation and subsequent activation of specific brain areas. These may include neuronal populations associated with or controlling olfactory, enteric, or respiratory function, and which are in the synaptic pathway of the sensory neurons. Repeated or sustained activation of such pathways may eventually contribute to neuronal dysfunction and glial activation.

Menozzi et al., 2021). Transsynaptic transport of nanoparticles can also occur from the lung and may also play a role in transport from other tissues as well (Oberdorster et al., 2009).

Indirect translocation of NPs to the brain can occur if NPs enter the blood. Following pulmonary deposition, ultrafine particles can permeate the lung–blood barrier by endocytosis, transcytosis, or stochastic transport and enter systemic circulation (Oberdorster et al., 2005). NPs deposited in the lungs have been shown to translocate to the circulatory system (Nemmar et al., 2001; Shimada et al., 2006) and eventually to organs like lung, liver, kidneys, and brain. Translocation mechanisms which have been demonstrated include rapid diffusion of ^{99m}Technetium-labeled colloidal albumin NPs from lung into systemic circulation (Nemmar et al., 2001) and passage of ultrafine carbon black across the air–blood barrier through large gaps formed between the cytoplasmic processes of alveolar epithelial cells (Shimada et al., 2006). In addition, alveolar deposited nanoparticles can initially enter the lymphatics where they may subsequently be delivered to the blood through lymph ducts such as the thoracic duct (Choi et al., 2010). These findings indicate that translocation of NPs from the lung into systemic circulation and potentially into the brain is possible. Translocation from the nose to the systemic circulation is also indicated in a recent study of ^{192}Ir radiolabeled iridium NP-exposed rats that demonstrated higher circulating NP concentrations from nose-only inhalation than from inhalation in intubated rats (Kreyling, 2016). When administered orally, water-miscible ^{14}C-fullerene (60) has been shown to penetrate the BBB (Yamago et al., 1995). Similarly, oral administration of ultrafine and fine titanium dioxide to mice caused hippocampal neuronal damage (Wang

et al., 2007). Systemic (intraperitoneal) administration of 50 nm silica-overcoated magnetic NPs results in migration of these particles across the BBB and subsequent deposition in the brain (Kim et al., 2006). Intravenous administration of gold NPs (10–50 nm) to mice or rats resulted in biodistribution of these particles in the brain (De Jong et al., 2008; Sonavane et al., 2008; Lasagna-Reeves et al., 2010). Regardless of the route of exposure, once in systemic circulation, NPs may interact with the BBB or can translocate to specific neuronal or glial cell populations and elicit a series of intracellular events including neuroinflammation, oxidative stress, mitochondrial dysfunction, glial activation, and disruption of neuronal function. Even in the absence of translocation of NPs to the brain, the release of inflammatory mediators into systemic circulation due to pulmonary exposure may suffice to stimulate BBB changes and elicit neurotoxicity (Figure 13.14). Additionally, stimulation of sensory neurons may likely cause perturbations in specific brain nuclei that can elicit neuroinflammation and abnormal neural changes (Figure 13.14). Indeed, olfactory signals have been shown to be relayed from the olfactory bulb to olfactory cortical areas (including piriform cortex) to the entorhinal cortex, which is a major afferent to the hippocampal dentate gyrus (Hjorth-Simonsen and Jeune, 1972; Kosel et al., 1981). The close anatomical association between the olfactory and hippocampal regions and the critical role played by hippocampus in odor memory (Kosel et al., 1981; Staubli et al., 1984; Eichenbaum et al., 1988) suggest that stimulation and/or disruption of olfactory sensory neurons may contribute, at least in part, to abnormal hippocampal function. Regardless of how NPs stimulate neuronal responses (direct by translocation or indirect via systemic or neurogenic effects), the nervous system appears to be a potential target for NP toxicity. Finally, a variety of underlying health conditions (e.g., hypertension, diabetes, infection) can predispose an individual to NP exposure by altering the permeability of the BBB and thus compromising its normal functioning. Such a breach in the BBB will facilitate rapid translocation of these particles or inflammatory mediators into the brain, thereby increasing the risk of a neurotoxic exposure. For these reasons, it is critical to evaluate the effects of NP exposure on BBB integrity and CNS function.

Translocation of NPs can be highly influenced by their physicochemical characteristics. Particle size, shape, and surface chemistry may further influence their translocation across olfactory sensory neurons, transport across the lung–blood barrier, or circulation in blood. Gold NPs of 10–50 nm in diameter accumulated in the brain following intravenous administration; however, administration of larger (100–250 nm) gold NPs did not result in biodistribution to the brain (De Jong et al., 2008; Sonavane et al., 2008). Similarly, subcutaneous injections of silver NPs resulted in their translocation to brain, whereas silver microparticles failed to reach the brain (Tang et al., 2009). These findings suggest that particle size is a critical factor for brain delivery of NPs. Besides particle size, the surface chemistry of the NP can also influence its translocation and/or permeability across the BBB. Cationic and anionic emulsified wax NPs have been shown to be taken up into the brain more efficiently than neutral NPs (Lockman et al., 2004). Further, cationic emulsified wax NPs have been shown to disrupt BBB and exert toxicity. Thus, understanding the toxicological influence of the physicochemical aspects of NPs may have significant impact on nanomaterial development considering their projected huge market potential in biomedicine, particularly for CNS drug delivery, diagnostics, and therapeutics.

While there is tremendous enthusiasm in developing and manufacturing metal or metal oxide–based NPs like Au, Ag, copper (Cu), and titanium dioxide (TiO_2) for various industrial or biomedical uses, evaluation of their toxicological potential is necessary to avert any adverse health effects. To this end, investigations on the toxicological potential of NP have gradually garnered active research interest. Neuronal damage can be mediated by several mechanisms that include inflammation, mitochondrial dysfunction, oxidative damage, and excitotoxicity. Microglia, the macrophages of the brain, play a crucial role in brain inflammatory responses. Pro-inflammatory cytokines and chemokines elicited by microglia can initiate mitochondrial impairment and oxidative stress. The presence of high levels of polyunsaturated fatty acids in the brain renders this organ vulnerable to reactive oxygen and nitrogen radical attack, due to the presence of double bonds within the membrane (Simonian and Coyle, 1996). In

addition, the presence of high levels of iron in the brain and its accumulation in specific areas following neuronal injury suggests loss of homeostatic mechanisms responsible for its regulation. As a result, the brain becomes susceptible to oxidative stress (Qian and Shen, 2001; Thompson et al., 2001). Thus, neuroinflammatory and oxidative stress responses following NP exposure may serve as the basis for neurotoxicity. Both neuroinflammation and oxidative stress can cause mitochondrial dysfunction and elicit neuronal injury (Figure 13.15).

A few studies have investigated the neurotoxic effects of carbon-based NPs, indicating the involvement of neuroinflammatory and oxidative stress events. Intranasal instillation of ultrafine carbon black induced the expression of proinflammatory cytokines and chemokines in the olfactory bulb (Tin Tin Win et al., 2006). In our laboratories, we have observed brain-region-specific mRNA expression of several proinflammatory chemokines, cytokines, selectins, and markers of cellular stress in mice exposed to a single dose of MWCNTs (10–80 μg) by pharyngeal aspiration. MWCNTs also decreased the expression of certain BBB-related markers suggestive of altered BBB integrity (Sriram et al., 2007, 2009). These observations suggest that NPs can pose a significant neurological risk following exposure.

Intraperitoneal administration of 25 nm Ag NPs caused alterations in the expression of genes associated with oxidative stress in specific brain areas that resulted in apoptosis and neurotoxicity (Rahman et al., 2009). Subcutaneous administration of Ag NPs was shown to cause BBB changes, neuronal injury, and astrocytic swelling (Tang et al., 2009). Immature rats (postnatal day 14) exposed to repeated low doses of 10 nm AgNP by oral gavage exhibited molecular and ultrastructural changes in the postsynaptic neurons of the forebrain. Reduced expression of N-methyl-D-aspartate receptor (NMDAR) associated proteins (GluN1 and GluN2B), synaptic scaffolding proteins (PSD95, SynGAP), and neuronal nitric oxide synthase (nNOS) was

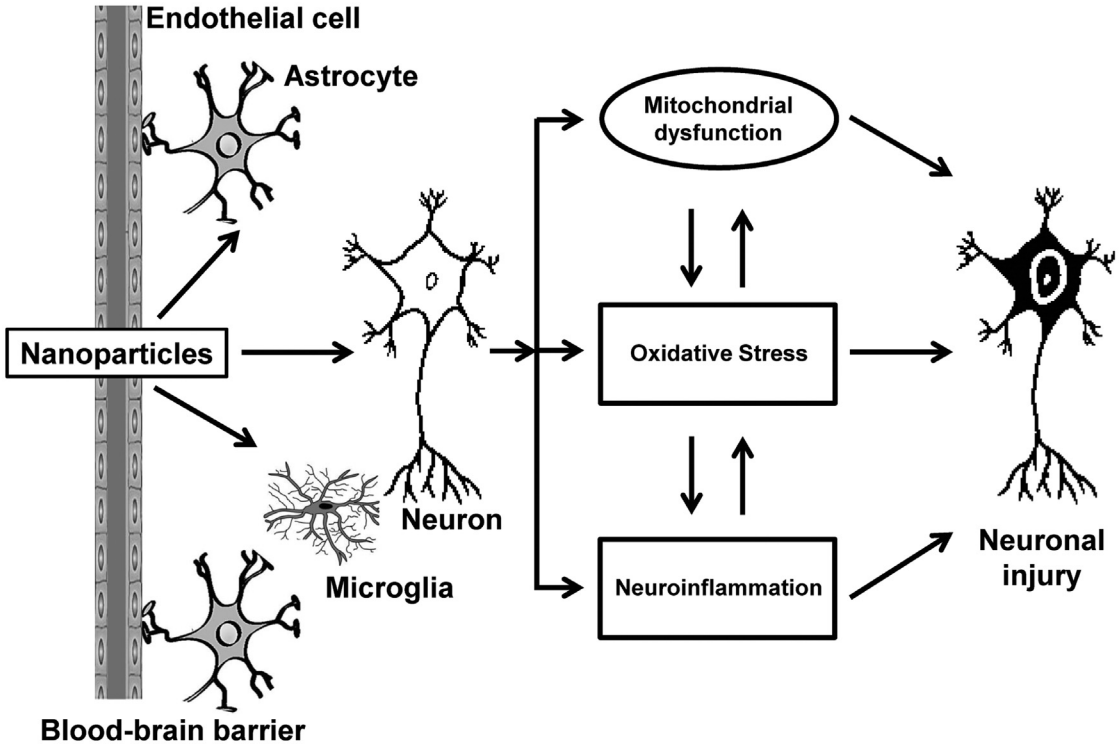

FIGURE 13.15 Potential mechanisms of NP-mediated neuronal injury. Direct translocation of NPs into neural cells (neurons, astrocytes, or microglia) can elicit oxidative stress, neuroinflammation, or mitochondrial dysfunction. Indirectly, inflammatory mediators released into the systemic circulation can potentially activate neural cells. Particularly, activation of microglia, the macrophages of the brain, can result in inflammatory and oxidative burst that can contribute to neuronal injury and astrogliosis. *Figure reproduced from Haschek WM, Rousseaux CG, Wallig MA, editors:* Haschek and Rousseaux's handbook of toxicologic pathology, *ed 3, Academic Press, 2013, Fig. 43.14, p. 1400, with permission.*

observed in these young rats. The NMDAR/PSD95/nNOS/cGMP signaling pathway was downregulated suggesting alteration of LTP/LTD processes that underlie developmental learning and memory (Dabrowska-Bouta et al., 2021). Ag and Cu NPs (50–60 nm) administered by intravenous, intraperitoneal, or intracerebral routes caused disruption of BBB function and induced brain edema in rats (Sharma et al., 2010). Inhalation of ultrafine manganese oxide resulted in translocation of these particles to the CNS (Elder et al., 2006). Nanosized TiO_2 (Degussa P25) has been shown to trigger oxidative stress response and interfere with mitochondrial function in brain BV2 microglial cells (Long et al., 2006). Administration of 80 nm TiO_2 NPs to mice produced subtle brain lesions and vacuolation in hippocampal neurons (Wang et al., 2007). Neuroinflammation in discrete brain areas was observed 24 h following pharyngeal aspiration of titanium dioxide nanowires but not titanium dioxide nanospheres (Porter et al., 2008).

Emerging evidence suggests that humans can take in persistent plastic materials from the environment either through inhalation or by ingestion. Brain uptake of micro- and nanoplastics in aquatic animals and mammals has been reported (Mattsson et al., 2017; Prust et al., 2020). While these plastic particles can reach the brain, there is limited information about particle count and neurotoxic potential of such materials. Existing data suggest that micro- and nanoplastics can elicit oxidative stress, inhibit acetylcholinesterase (AChE) activity, and alter neurotransmitter levels (Prust et al., 2020). Although studies on the neurotoxic potential of NPs are limited, there is sufficient evidence to suggest that the brain is a vulnerable target for NPs. Therefore, it warrants more extensive and detailed investigations to determine if persistent adverse brain changes, reminiscent of neurodegenerative disorders, can result from chronic inhalation exposures to NPs. Such efforts are critical for neurological risk assessment of NPs and for determining the safety efficacy of NPs generated either at the workplace or following attrition into the environment.

Injection (systemic administration) of NP-based drug delivery vectors, an area of intense research in drug delivery, may result in distribution of NPs into specific brain targets. Human risk is well controlled because pharmaceutical agents undergo extensive safety testing prior to clinical testing and registration. Exposure to NP-based vectors, in general, is limited to select populations and less of a concern than for individuals that may be exposed to via inhalation at the workplace. Continued research is necessary because there is limited understanding of the neurotoxicological potential of occupational and environmental exposure to NPs, particularly engineered nanomaterials. Many of the existing studies on NP neurotoxicity are summarized above.

Cardiovascular Pathology

Inhalation of nano-TiO_2 results in significant inhibition of the ability of systemic or coronary arterioles to respond to dilators (Nurkiewicz et al., 2008; LeBlanc et al., 2010). This microvascular dysfunction has been associated with generation of reactive oxygen species and the scavenging of dilator-induced endothelial nitric oxide (Nurkiewicz et al., 2009; LeBlanc et al., 2010). This cardiovascular response to pulmonary exposure to particles appears mediated in part by potentiation of blood neutrophils, adherence to microvessel walls, and release of reactive oxygen species (Nurkiewicz et al., 2006). There also may be a neurogenic mechanism, involving stimulation of pulmonary sensory neurons by particle exposure and resultant activation of sympathetic input to microvessels (Kan et al., 2011; Knuckles et al., 2011).

Inhalation of MWCNTs resulted in a depression of the responsiveness of coronary arterioles to dilators 24 h postexposure (Stapleton et al., 2012). This MWCNT-induced microvascular dysfunction may involve a neurogenic mechanism, like that described above with nano-TiO_2, since pulmonary exposure to CNTs increases baroreflex function by 2-fold (Legramante et al., 2009). In addition, multiple aspirations of SWCNTs (20 μg/mouse, every 2 weeks, for 2 months) in Apo E−/- mice caused an increase in aortic plaques. Recent studies in rodents also indicate that inhalation of some nanoparticles during pregnancy may alter placental blood flow and affect cardiovascular function in the offspring (Stapleton et al., 2015; Hathaway et al., 2017; Abukabda et al., 2019).

Lymphatic Pathology

Lymphatics are difficult to see in standard histopathologic sections. In conducting NP studies, it is important to remember that the lymphatic vasculature is a circulatory system

which plays a key role in fluid homeostasis, particle clearance, cellular transport, metastasis, and the immune system (Mayerson, 1963). The lymphatics are increasingly recognized as being dynamic structures with unique molecular signaling essential for their role in maintaining interstitial and blood capillary homeostasis (Pepper and Skobe, 2003). The endothelium that lines the lymphatic capillaries plays an active role in movement of cells into the lymphatics (Johnson et al., 2006, 2007; Johnson and Jackson, 2008). Physically, the lymphatic endothelium is attenuated; the intercellular spaces between lymphatic endothelial cells are sufficient for movement of cells; and the basement membrane of the lymphatic capillaries is discontinuous (Lauweryns and Baert, 1974; Leak, 1980). These features provide NPs with remarkable access to the lymphatic circulation. Colloid carbon and ferritin particles have been reported within vesicles in the lymphatic endothelium and alveolar type I cells (Lauweryns and Baert, 1974; Leak, 1980). This implies that transcellular transport may occur through the lymphatics and alveolar type I cells of the lung as well as the vascular endothelium and does not always require phagocytosis. Instilled carbon and ferritin particles have been observed in the gaps between lymphatic endothelial cells in the pulmonary lymphatics, suggesting intercellular movement of particulates (Lauweryns and Baert, 1974). Once within the lymphatic lumen, the lymphatic contents filter through lymph nodes and eventually empty into the vasculature at the thoracic duct. This means that the lymphatics are a potential route for delivery of NPs and inflammatory mediators to the blood. For example, lung-deposited NPs less than 30 nm in diameter were demonstrated to first reach the draining lymph nodes and then reach the blood after a time lag not observed with low molecular weight molecules (Choi et al., 2010). This suggests that lymphatic drainage into the vasculature may indeed be important in vascular dissemination of some NPs.

Particulates can enter the lymphatics when carried by phagocytic cells and this appears to be the major route for transport of fine particulates in the micron size range (Harmsen et al., 1985, 1987; Manolova et al., 2008). Fine particulates injected into the footpad or deposited in the alveolar region do not reach the draining lymph node as free particulates (Harmsen et al., 1985, 1987; Manolova et al., 2008). Instead, they are phagocytized by macrophages and neutrophils which can enter the lymphatics and are transported to the draining lymph nodes within phagocytic cells. However, extracellular routes of particulate transport may be important with some NPs. Small NPs (20 nm) that are injected into the footpad rapidly translocate to the draining lymph node and can be seen in the subcapsular sinuses and in the antigen-presenting cells of the lymph node, including dendritic cells and plasmacytoid dendritic cells (Manolova et al., 2008). Multiple studies demonstrate that many NPs are rapidly and widely transported through the lymphatics. For lung deposited NPs, rapid translocation by the lymphatics is highly influenced by surface coatings and charge but importantly is size-limited, with a threshold estimated to be at a functional diameter of between 34 and 48 nm (Choi et al., 2010). A previous study of radiolabeled iridium and carbon NPs indicated that 20 nm diameter NPs, which are below this threshold, translocated from the lung to secondary target tissues more effectively than 80 nm diameter NPs, which are above this threshold (Kreyling et al., 2009). Extracellular lymphatic transport of small NPs is also suggested by recent studies of NPs used in lymphangiography (Kobayashi et al., 2003, 2004; Elias and Tsourkas, 2009).

Lymphangiography using these small NPs can help to map the lymphatics and lymph nodes draining critical sites, such as tumors (Kobayashi et al., 2003, 2004; Elias and Tsourkas, 2009; Ravizzini et al., 2009). In addition, extracellular transport of NPs has implications for possible therapies. Chemotherapeutic agents can potentially be adsorbed onto the NP surface, carried into the lymphatics and from there distributed to the lymph node (Yang et al., 2009). Because of the critical role lymphatics play in the spread of carcinomas, the ability for some NPs to circulate in the lymphatics may be important in targeting the metastatic spread of cancer (Yang et al., 2011).

One important implication of the tropism of NPs for the lymphatics is that the lymphatics are a vascular system separate from the blood vascular system that can transport NPs from an exposed tissue to distant tissues and organs (Aiso et al., 2011). While this feature can be used for therapeutic purpose in some situations,

it is a feature that needs to be considered when toxicologic pathologists evaluate studies. In addition, the lymph nodes play an enormous role in immune function and receive the contents from the lymphatics (Morrow, 1972; Ohtani and Ohtani, 2008). From a toxicologic standpoint, the transport function of the lymphatics makes them a potential route for inadvertently delivering immunotoxic and/or antigenic NPs to the lymph nodes and, therefore, the immune system. In addition, alterations in the lymphatics themselves can play an important role in disease pathogenesis (El-Chemaly et al., 2008, 2009). Lymphangiectasia (lymphatic dilation) was noted in the lymphatics of mice aspirating MWCNTs (Porter et al., 2010). This suggests that the lymphatics may not just transport NPs; they may be damaged by them, resulting in potential adverse effects on lymphatic functions. Pathologists need to recognize that the lymphatics are potential targets in NP studies and that this can have potential functional consequences. The lymphatics should be considered both as a potential target and as a potential route for translocation of NPs, features which distinguish NPs from traditional organic pharmaceuticals (Riviere, 2009). Because lymphatics are not easily visualized in standard H&E sections but can be critical in interpreting NP studies, lymphatic endothelial markers can be helpful for pathologists and improve visualization of the lymphatics in tissue sections (Figure 13.16).

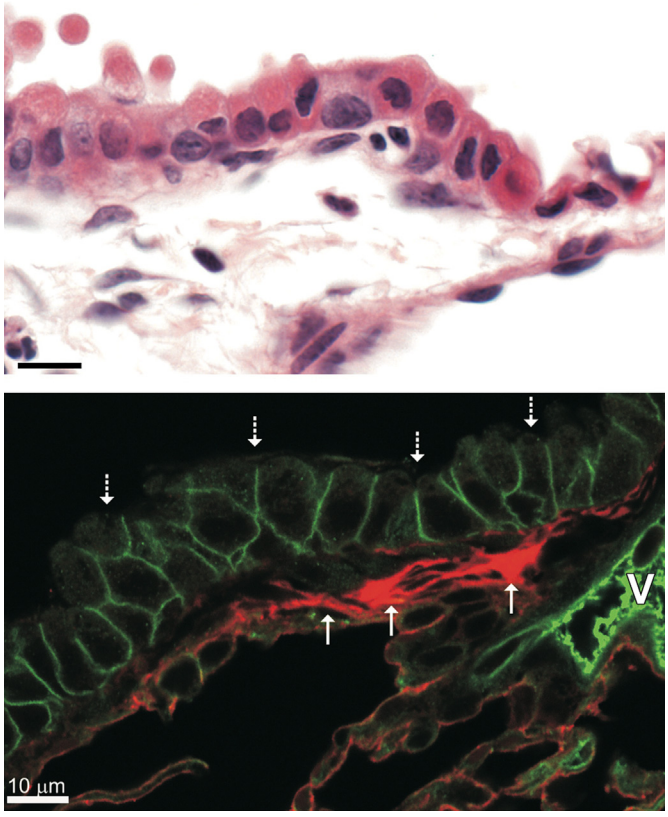

FIGURE 13.16 Lymphatics are difficult to identify in H&E sections (*upper panel*). In the lower panel, indirect immunofluorescence for podoplanin allows the clear demonstration of peribronchiolar lymphatics in *red* (*solid arrows*) while indirect immunofluorescence for e-cadherin permits visualization of the airway epithelium (*dashed arrows*) so that the tissue location of the lymphatics is also demonstrated. Low-level staining for both e-cadherin and podoplanin causes the alveolar type I cells to stain orange. Serum retained in the blood vasculature (V) stains green because the green secondary antibody recognizes IgG in normal mouse serum.

2.5. Human Relevance of Experimental Studies in Animals

Dose considerations are important in designing NP toxicologic pathology studies. For risk assessment purposes, the exposures should include a range of exposure which at a minimum includes those producing no or little effect to exposures comparable to those anticipated in the people who receive the highest exposures (Hubbs et al., 2005). Exposures in workers or in patients receiving intentionally administered NPs are potentially quite high relative to exposures received by the general public. For example, in a study of coal miners, the lung-deposited dust burden ranged from 2.6 to 36 g/lung (Kuempel et al., 2001). An additional consideration in toxicologic pathology studies is obtaining a tissue deposited dose that will allow study of particle interactions with critical target cells in vivo. As has previously been noted, a 50 μg dose of 50 nm × 5 micron MWCNTs in a mouse lung would affect less than 1% of a tissue section evaluated by a pathologist (Hubbs et al., 2011). Thus, the highest exposure dose should be sufficient to allow detection of critical interactions that may occur in people, such as pleural penetration by high aspect ratio NPs (Mercer et al., 2010). However, exposures should not be so high as to produce effects that are due to processes that are not relevant to humans. For example, pathologic changes

associated with exposures exceeding the maximal tolerated dose can be extremely difficult to interpret (Bucher, 2002).

Species differences have not been described much in the existing literature on engineered NPs. However, many ultrafine particulates are NPs that are not products of nanotechnology and species differences in their effects have been investigated (Oberdorster, 2000; Oberdorster et al., 2002, 2004, 2005, 2005, 2007; Oberdorster and Utell, 2002). Studies of the effect of fine particulates, which includes larger but respirable particulates, may also be relevant to studies of NPs. In inhalation studies at high exposure concentration, rodents often sequester micron-sized particulates in alveolar macrophages and develop overload of clearance pathways, inflammation, and an alveolar epithelial proliferative response (Nikula et al., 2001). This response is believed to cause some of the lung tumors that are specific to high dose particle exposures in the rat. Careful interpretation of findings from inhalation studies of high doses of fine particulate matter is important, and tumors produced through chronic inflammation pathways may not be relevant to low dose exposures that do not cause chronic inflammation (International Life Sciences Institute, 2000). In contrast, lung fibrosis is a response that is seen in both rodents and humans. Workers sometimes develop very high lung burdens of inhaled particulates and can respond with fatal interstitial fibrosis, a process that is also seen in rodent models (Oberdorster, 1995; Kuempel et al., 2001).

Existing studies of ultrafine particle inhalation have demonstrated increased interstitial deposition of inhaled NPs relative to fine particles (Ferin et al., 1992). The interstitial dose of NPs appears to be a major determinant of pulmonary fibrosis (Mercer et al., 2011). What studies of NPs have also provided is an understanding that endocytic pathways of intracellular trafficking and lymphatic transport pathways between tissues are not necessarily the same for NPs as for fine particulates (Nikula et al., 2001; Conner and Schmid, 2003; Choi et al., 2010; Dombu et al., 2010; Howes et al., 2010; Chen et al., 2011). This suggests that conclusions based upon the effects of fine particulates will not necessarily be true for NPs, although the effects of fine particulates can certainly generate hypotheses regarding the effects of NPs.

The accumulating human data indeed suggest that at least some inhaled NPs can deposit in the human lung and cause inflammation and fibrosis (Song et al., 2009; Phillips et al., 2010; Cheng et al., 2012). A worker who inhaled nickel NPs with an estimated diameter <25 nm died less than 2 weeks after exposure with severe pulmonary inflammation and damage consistent with adult respiratory distress syndrome (Phillips et al., 2010). Seven young female workers in a print plant exposed to nanoparticulate polyacrylate ($\sim$30 nm in diameter) developed pulmonary inflammation and fibrosis with pleural effusions (Song et al., 2009). A worker exposed to nanoscale TiO_2 in polyester paint powder developed bronchiolitis obliterans organizing pneumonia (Cheng et al., 2012). Since some NPs can enter cells through endocytic pathways that exclude micrometer-sized particulates, NPs have increased interstitial deposition relative to micrometer-sized particulates, NPs appear to be transported extracellularly in the lymphatics, and at least some NPs are associated with fatal pulmonary inflammation and fibrosis in workers; these smallest of particulates may not have the same interspecies toxicity differences that have been described for micron-sized particulates in the lung (International Life Sciences Institute, 2000).

3. FUTURE TRENDS IN NANOPATHOLOGY AND NANOTOXICOLOGY

For workers, as the body of knowledge in nanopathology and nanotoxicology grows, the prudent implementation of engineering controls (containment, local exhaust ventilation, filtration), personal protective equipment (respirators), and administrative controls (training in safe handling practices) is advisable. Current evidence indicates that filtration and ventilation are highly effective in limiting worker exposure (Methner et al., 2007; Han et al., 2008; Rengasamy et al., 2008).

A current emphasis area in nanotoxicology is the determination of which physiochemical properties influence bioactivity. Such information will

drive "safety by design," i.e., the modification of NPs to reduce toxicity while maintaining novel properties desired in commercial applications. Therefore, the goal is that information obtained by nanopathology and nanotoxicology will lead to the safe production and application of nanomaterials and allow the economic growth of the nanotechnology industry.

Recent technical improvements in measuring nanoparticle exposures may enable additional workplace monitoring (Stebounova et al., 2018). Supervised machine learning approaches to nanotoxicology may help identify relevant biomarkers of exposure (Yanamala et al., 2018). In addition, risk assessment and risk management strategies have responded to nanotechnology, and continued evolution in these areas is likely (Schulte et al., 2018, 2019).

Toxicologic pathologists will play an essential role in identifying changes which can occur in the in vivo environment and may not necessarily be predictable through existing knowledge of particle toxicology. Such opportunities for discovery have already been demonstrated by the interactions between SWCNTs and the mitotic spindle and the interaction between MWCNTs and the pulmonary lymphatics (Shvedova et al., 2008; Sargent et al., 2009, 2010, 2012; Porter et al., 2010). Toxicologic pathologists, with their multisystem training, are also critical members of the research teams that will investigate the potential to target nanomedicine to tissues, such as the central nervous system, and to cells, such as tumor stem cells, that are difficult to target with current pharmaceuticals. An essential part of those discoveries and opportunities will be recognition that NPs or their breakdown products can potentially be delivered to cells and subcellular compartments that are not the traditional sites of exposure for xenobiotics. The in-depth understanding of the toxicologic pathology of NPs may also reveal mechanisms of toxicity associated with well-known particulate hazards, such as asbestos. Most importantly, toxicologic pathology studies of NPs will be essential to safely harnessing the enormous promise of nanotechnology.

4. CONCLUSIONS

Nanotechnology is still rapidly growing and a major economic force. Nanomedicine is also rapidly growing. The first nanomedical products included products with improved imaging and chemotherapy capabilities compared with previous products. Nanotechnology is just one of the innovative technologies that comprise advanced manufacturing and NPs are among many potential products of these technologies. Toxicologic pathologists evaluating the safety of new nanotechnology products need important skills.

One important skill is an ability to interact within a multidisciplinary environment. Interactions with engineers, chemists, physicists, cytogeneticists, toxicologists, pharmacologists, and molecular biologists and some understanding of the literature in those fields can be particularly helpful. The nanotechnology and nanomedicine scientific literature is expanding at such a rate that it is a huge challenge to find the studies in those fields which are relevant to toxicologic pathology. In many cases, the papers are written by scientists who have not considered the potential hazard of the NP, and key words may not help in the literature search. Many publications are outside of the standard medical literature and may not be recovered using common search engines such as PubMed.

A second important skill is understanding the similarities and differences between the NPs creating adverse effects and those that do not. When size, shape, and composition are considered, an extensive variety of NPs are possible. Increasingly, the products of nanotechnology will include designer NPs, able to inform our understanding of medicine, disease pathogenesis, and particle toxicology. Toxicologic pathologists will play a critical role in identifying NP features which predict toxicity or safety.

Another important skill is the ability to follow the pathway of the NP, even if it has gone where it cannot be seen in a standard H&E slide at a standard magnification. In this chapter, we have provided examples of some of the tools,

such as FESEM and enhanced darkfield imaging which have helped us to see NPs in tissue sections. We have also given examples of tools that have allowed us to see damage in targets that are not traditionally evaluated by pathologists, including the lymphatics and the mitotic spindle. Following the pathway of the NP also means understanding the limits of light and electron microscopy so that the dose will be sufficient to demonstrate the NP in tissue sections.

A final critical skill of the toxicologic pathologists evaluating NP studies will be their understanding of diverse cells, tissues, and organs. The medical background, pathology training, and toxicology training of the toxicologic pathologist can facilitate an understanding of how these smallest of particulates may be a key to new medical breakthroughs or to adverse consequences.

Nanotechnology and nanomedicine are changing our world. Through understanding the toxicologic pathology of NPs, toxicologic pathologists will play a major role in allowing the safe use of these revolutionary new products.

Acknowledgments

We gratefully acknowledge the assistance of Kimberly Clough-Thomas in figure preparation. We thank Jeffrey L Salisbury for generously supplying the high-resolution confocal images of dividing cells.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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FOURTH EDITION

Volume III: Environmental Toxicologic Pathology and Selected Toxicant Classes

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ISBN: 978-0-443-16153-7

Publisher: Stacy Masucci
Acquisitions Editor: Kattie Washington
Editorial Project Manager: Billie Jean Fernandez
Production Project Manager: Sreejith Viswanathan
Cover Designer: Matthew Limbert

Typeset by TNQ Technologies