Chapter 8
Respiratory Toxicity of Carbon Nanotubes

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Abstract Carbon nanotubes (CNT) are emblematic nanomaterials, and have generated a highly competitive international scientific research activity. Since their initial description in 1991, the understanding of their unique physicochemical properties led to a large number of actual applications and uses, as well as future developments. Because of these promising applications, there is an increasing concern regarding the consequences that could result from human exposure to CNT. Analysis of the existing literature shows that respiratory exposure to CNT can lead to the occurrence of pulmonary inflammation, the formation of granuloma, and the development of pulmonary fibrosis. The exact determinants of these effects still remain to be clearly identified, although intrinsic physicochemical characteristics of CNT (i.e. length, dispersion status, and residual catalyst content) seem to be of importance. Several critical issues still remain to be solved, such as the translocation of CNT outside the lungs and the occurrence of their biotransformation, which should open a new understanding to the respiratory effects of CNT.

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8.1 Introduction

Carbon nanotubes (CNT) are emblematic nanomaterials, and have generated a highly competitive international scientific research activity. CNT are cylinders of one or several (up to 20) concentric graphite layer(s) (single- or multi-walled CNTs respectively—SWCNT and MWCNT). Their diameter is in the order of the nanometer, and they can measure up to several micrometers in length. Since their initial description in 1991, the understanding of their unique physicochemical properties, such as mechanical, thermal, or electrical conductivity, led to a large number of actual applications and uses, as well as future developments in aerospace, automobiles, nanoelectronic, or nanomedicine. CNT are currently used in computers, aircraft airframe, and many sporting goods such as tennis rackets, bicycles, golf irons, sport shoes spikes, hockey sticks, or baseball bats (see *Woodrow Wilson International Center for Scholars* web site for inventory). Because of these actual and future applications, there is an increasing concern regarding the consequences that could result from human exposure to CNT.

In this chapter, the context of pulmonary exposure to nanomaterials, and CNT in particular, will be first exposed. Then, what is currently known about respiratory effects of CNT will be presented, along with the proposed underlying mechanisms. Finally, the remaining issues regarding pulmonary toxicity of CNT will be discussed.

8.2 Context of Pulmonary Exposure to CNT

The determinants of an exposure to CNT are multifactorial. They can be characterized by several factors, including: (1) in which environmental compartment are the nanomaterials?, (2) what is the context of exposure (occupational or not)?, and (3) which compartment of the human body is exposed?

8.2.1 Which Compartment of the Environment?

Because of the always increasing production and use of manufactured nanomaterials, and CNT in particular, their release in the environment is a more and more probable phenomenon. Several environmental compartments can be targeted: air, water, soil. Indeed, unintentional release of CNT in the air, during their production process, can happen, as well as the contamination of wastes, water, and/or soil in the vicinity of production sites. Exposure can also result from the particular uses of nanomaterials. CNT are for example proposed to be promising tools in the context of nanomedicine, and therefore, be associated to a direct exposure of the human body to them.

8.2.2 What Context of Exposure?

Exposure to CNT can occur in an occupational or a private context. Workers from nanotechnologies can be directly exposed at the time of production of CNT, as well as during their transport, storage, or during their incorporation in final products. Moreover, because of the life cycle of nanomaterials, and particularly their degradation, the general population can be exposed. Finally, because of the use of CNT in sporting goods for example, the general population can also be exposed when using them, such as tennis rackets or CNT-containing bicycles.

8.2.3 Which Compartment of the Human Body?

Four main portals of entry of nanomaterials in the human body can be considered; respiratory, digestive, cutaneous, and systemic compartments (in the context of nanomedicine particularly). Only the respiratory system will be considered in this chapter, as it represents a unique portal of entry for inhaled nanomaterials, but also because it receives the entire cardiac output. The respiratory system can therefore be exposed secondary to a systemic passage of CNT. Respiratory apparatus can be considered as an assembly of tubing, the airways, which allow the air to flow from the nose and mouth to the pulmonary alveoli. Extrathoracic (or upper) airways are composed of nose, mouth, pharynx, and larynx, while intrathoracic (or lower) airways are represented by conducting airways (trachea, bronchi), and respiratory airways, with pulmonary alveoli. These latter are the site of gaseous exchanges between air and blood. There are approximately 300 million alveoli in the adult lung, representing a very large exchange surface (circa 140 m^2, about the surface of a tennis court).

8.2.4 Deposition of CNT in the Pulmonary System

As for other nanomaterials, CNT can be deposited in the respiratory tract. However, the identification of the determinants ruling the site of deposition is considered as a remaining issue in the actual literature. When in suspension in the air, particles in general, and CNT in particular, form an aerosol. The behavior of this aerosol is largely conditioned by the size of the particles, which further condition the deposition mode of the particles. Moreover, as for other nanomaterials, CNTs have a tendency to form agglomerates and aggregates, which will also influence their site of deposition in the respiratory tract. Mathematical predictive models have been proposed to help understand this phenomenon. In these models, the respiratory tract is arbitrarily divided into three regions: nasopharyngeal (upper airways), tracheobronchial, and alveolar. From these models,

established for particles between 1 and 100 μm aerodynamic diameter, one can conclude that the smaller the particles are, the best they are able to deposit in the respiratory tract. Each region of the respiratory tract is the target for different classes of particles, the alveoli for example being the preferred deposition site of nanoparticles around 20 nm [1]. These differences of deposition site could result in differential effects of nanoparticles. It must be noted that such mathematical models have been proposed for ideal spherical nanoparticles, and may not be well suited for CNT in particular. Moreover, the calculations have been performed considering mouth breathing, at rest. It is easily imaginable that these parameters can be modified while breathing during an effort (higher volume of air breathed, greatest air flux perturbations), or in case of respiratory pathology (asthma, bronchitis). For example, it is predicted that deposition will be higher for nanoparticles in constricted airways. In accordance, a higher retention of nanoparticles has been demonstrated in obstructed or asthmatic airways [2–5].

8.3 Respiratory Effects of CNT

The existing literature regarding respiratory effects of CNT is relatively new, as the first study came out in 2004 [6]. Since then, there have been an always increasing number of studies dealing with respiratory toxicity of CNT, mainly after exposure of mainly mice or rats to either SWCNT or MWCNT. Several administration routes have been used so far; intratracheal or intrapharyngeal administration, and more recently, inhalation (by means of whole-body or nose-only exposure systems). The different studies used a large range of doses; 10–500 μg/mouse for intratracheal or intrapharyngeal administration [7, 8], and 0.3–30 mg/m^3 for inhalation exposure for example [9–11, 12]. The duration of exposure has also been wide; from 24 h up to 6 months (only a very limited number of studies evaluated later time points). Three major endpoints have been studied so far and will be described here. They are focused on specific immediate and/or long-term responses of lung tissue: occurrence of pulmonary inflammation, the formation of granulomas, and the development of lung fibrosis. A few more endpoints have been promptly studied and will be discussed at the end of this chapter.

8.3.1 Pulmonary Inflammation

The induction of an inflammatory response in the lung is the most reported phenomenon after pulmonary exposure to CNT. This inflammation is an early event, occurring as early as 6–24 h after the initial exposure, with the recruitment of neutrophils in the bronchoalveolar lavage (BAL) fluid [8, 13]. This neutrophil-driven infiltration is often reported as being transient, and usually resolved within 15 days after the initial exposure. The release of proinflammatory cytokines,

including tumor necrosis factor (TNF) alpha, interleukin (IL)-1ß, -6, monocyte chemoattractant protein (MCP)-1, or macrophage inflammatory protein (MIP)-2 (or CXCL-2) [8, 14, 15] occurs during this acute-phase inflammatory response. This is observable at the level of the BAL fluid, as well as in whole lung tissue homogenates (RNA and protein levels). This response is consistent with a foreign body response, and is often followed by the formation of multifocal granulomas.

8.3.2 Formation of Granulomas

The granulomas contain macrophages, and are usually surrounding CNT clusters, but can also appear distal, where dispersed CNT are believed to be present (although, because of the difficulty to observe dispersed CNT in biological media, it is hard to be confident on that matter). The presence of granulomas is documented to be a persistent event following CNT administration, as they can be still observable 6 months after the initial exposure [8, 13].

8.3.3 Pulmonary Fibrosis

The development of pulmonary fibrosis is the third biological consequence of CNT exposure that is well documented in the current literature [14, 16]. This pathological event can occur both within granulomas or distal to them, as diffuse interstitial and septal fibrosis. Fibrosis development has been described as soon as 15 days after the initial exposure to CNT. This is also a long-term event, since the fibrotic lesions can still be persistent after 6 months [15, 17]. The histological alteration is accompanied by the expression of markers of extracellular matrix deposition (collagen I and III), increase in hydroxyproline lung content, as well as the production of profibrotic mediators (i.e. transforming growth factor (TGF) beta).

8.3.4 Carcinogenic Effects

Only a limited number of studies have considered the carcinogenic potential of CNT so far. A study performed in mice exposed to SWCNT by inhalation showed the occurrence of mutation in the *k-ras* oncogene locus within the first week of exposure, and until the end of the experiment 4 weeks later [10, 11]. These findings were, however, not confirmed in a study using the pharyngeal aspiration route of exposure, implying that exposure route is a critical parameter to consider in terms of influencing the biological effects of CNT [14]. Because of the physical and chemical durability and fibrous shape of CNT, concerns have early been raised

that they may exhibit potentially significant health hazards similar to asbestos, i.e., development of mesothelioma. To date, no such effects of CNT have been described in response to pulmonary exposure of laboratory animals. However, evidences have been given that intraperitoneal as well as intrascrotal administration of MWCNT in mice or rats can lead to the formation of abdominal and/or thoracic mesothelioma [18, 19]. Moreover, it has been recently described that CNT can rapidly reach the pleura after pulmonary exposure to CNT by either inhalation or pharyngeal aspiration [20]. From these studies, it is clear that more toxicological research is necessary to determine the effective carcinogenic potential of CNT, but that some of the studies suggest proceeding with caution.

8.4 Proposed Underlying Mechanisms

8.4.1 Reactive Oxygen Species

Besides the development of pulmonary inflammation already described in this chapter, the presence of an oxidative stress is considered to be a major mechanism underlying pulmonary response to CNT exposure. Oxidative stress is defined by the existence of an imbalance between oxidants production and antioxidants defense, in favor of oxidants. Several approaches have been used to demonstrate the presence and role of an oxidative stress. First, the detection of biomarkers of oxidative stress such as lung protein thiols or malone dialdehyde (MDA) content is increased as soon as 24 h after the initial exposure to CNT [10, 11, 21]. Moreover, the presence, in the total lung, of a product of lipid peroxidation 4 hydroxynonenal (4-HNE), is rapidly increased after pharyngeal aspiration of SWCNT in mice [15], as well as protein carbonyls [10, 11]. Second, the role of oxidants in pulmonary response to CNT has been assessed using mice presenting low antioxidant defenses, thanks to a vitamin E-deficient diet. Exposure of such mice to CNT induced a higher cellular inflammatory response (total cell count in BAL, as well as total neutrophil content), accompanied by an increased secretion of two major inflammatory cytokines; TNF and IL-6 [10, 11, 21, 22]. Similar mice also developed an exaggerated fibrosis response, as demonstrated by increased TGF beta levels in the lungs, and increased thickness of alveolar walls [21]. Finally, a genetic approach using $gp91^{phox-/-}$ mice confirmed the implication of oxidants in CNT biological effects. Indeed, the absence of a functional NAD(P)H oxidase in these mice was associated to a diminished induction of collagen deposition, footprint of fibrosis development, in response to SWCNT administrated by pharyngeal aspiration 28 days earlier [10, 11].

8.4.2 Systemic Effects of Respiratory Exposure to CNT

As stated before, the occurrence of an inflammatory response, the formation of granuloma, and/or the development of pulmonary fibrosis have been the three major endpoints studied so far to describe pulmonary effects of CNT administration. More recent studies describe the systemic effects of CNT administered via pulmonary route. After inhalation of in whole-body chamber exposure, 0.3, 1 or 5 mg/m^3, 6 h a day, for 7 or 14 days, Mitchell and collaborators [23] have demonstrated that systemic immunity was affected. Interestingly, they observe an absence of pulmonary effects in terms of inflammation, granuloma formation, or fibrosis as well as tissue injury. In this study, the authors demonstrate that spleen-derived T cells show a suppressed T cell-dependent antibody response, a decreased proliferation of T cells following stimulation, as well as an altered natural killer cell's killing activity. This was the first evidence of systemic effects induced by CNT secondary to pulmonary exposure. Some methodological issues can however be raised; because of the experimental setting utilizing a whole-body chamber exposure, an ingestion of CNT because of the cleaning of the animal's fur and skin and the subsequent access of CNT to the systemic circulation cannot be ruled out. Since then, other studies, utilizing less physiologically relevant exposure routes (pharyngeal aspiration, intratracheal administration) allow concluding with better certainty on extrapulmonary effects of CNT. In such studies, as soon as 4 h after the initial exposure, increased systemic levels of inflammatory proteins (IL-1ß, CXCL1, CXCL2, IL-8rß, S100a8, Mac-1) as well as stress response markers [hypoxia inducible factor 3 (HIF-3a), matrix metalloproteinase (MMP)-9, arginase II, osteopontin, colony stimulating factor-1 (CSF-1), and insulin growth factor receptor 1 (IGF-1R)] have been described [24]. These markers were detected in lung as well as total blood and/or in serum, but, interestingly, some of them were detected exclusively in the blood of the exposed animals. In addition, total and active levels of plasminogen activator inhibitor 1 (PAI-1), a procoagulant acute-phase protein which is involved in inhibition of the fibrinolytic cascade, were significantly increased in the lungs as well as in the plasma of CNT-exposed animals. This study gives the evidence that 4 h after CNT deposition in the lungs, local, and systemic inflammatory as well as prothrombotic responses are activated [23–25]. This kind of systemic response, particularly if chronic and persistent, may trigger or exacerbate cardiovascular dysfunction and disease, such as atherosclerosis. Unfortunately, as no assessment of the presence of CNT outside the lungs of the animals was performed, one cannot conclude on the necessity or not for CNT to translocate outside the lung to have systemic effects.

8.4.3 Influence of Existing Pathologies

The literature on respiratory effects of CNT initially focused on healthy animals. However, more recent studies investigated the effect of CNT exposure in the

context of existing pathologies such as asthma or bacterial infections, or along with exposure to environmental contaminants such as ozone. Indeed, exposure to CNT occurring concomitantly with an installed pulmonary disease or pathogenic infections may modify (and potentially enhance) the natural pathological response. Murine models of asthma have been used to evaluate the potential effects of CNT in such pathological condition. SWCNT as well as MWCNT have been described to increase the early inflammation and the susceptibility to develop airway fibrosis compared to CNT alone or ovalbumin sensitization only (ovalbumin sensitization being a common method used to develop asthma in rodents). Moreover, CNT induced an increased expression of immunoglobulins (Ig) related to allergic response in blood [9, 26]. Bacterial infection is a common pathological condition. Effects of an exposure to CNT concomitantly with bacterial infection have been explored using Gram-positive (*Listeria monocytogenes*) and Gram-negative (lipopolysaccharide) bacteria. In both cases, CNT exposure in combination with bacterial infection was able to induce an increased airway fibrosis as compared to bacterial infection alone [27, 28]. A less reproducible event is the occurrence of an increased inflammatory acute and/or chronic response. Interestingly, pharyngeal administration of SWCNT following an exposure to *Listeria monocytogenes* induced a decreased bacterial clearance. Altogether, these studies demonstrate that CNT may interfere with existing allergies and with natural responses against pathogenic infections. However, studies are still needed to deeply understand the underlying mechanisms of these effects.

8.5 Determinants of CNT Biological Effects

When trying to figure out an Ariadne's thread to CNT effects after pulmonary exposure, scientists are quickly confronted with what could be considered as discordant results from the literature. It actually comes from the fact that CNT cannot be considered as a single material; the so-called physicochemical characteristics of CNT are to be taken into account to accurately evaluate their effects. These characteristics are the result of CNT synthesis and post-synthesis processes and will be described hereafter.

8.5.1 Synthesis Processes

Several processes are commonly used for CNT synthesis: arc discharge, chemical vapor deposition (CVD), and laser ablation. These methods share in common that energy (which can be either electrical, thermal, or high intensity light) is supplied to a carbon source (carbon monoxide, alcohols, or hydrocarbons for example), enabling the further production of carbon atoms that recombine together to result in the generation of CNT. The synthesis of CNT is therefore a complex process

resulting in a heterogeneous population of CNT presenting a whole set of various physical and chemical characteristics. Post-manufacturing processes such as (partial) removal of residual catalyst content may also lead to physicochemical characteristics different from that of the initially produced CNT. From the existing literature concerning biological effects of CNT, it clearly appears that several factors mainly related to the physicochemical characteristics of CNT are major determinants of their subsequent biological effects. The exact physicochemical determinant(s) conditioning CNT biological effects is/are unknown for now, but these factors include for example the length, catalytic residues nature and content, surface properties, etc. of CNT.

8.5.2 Length

Length has been accepted to influence fiber clearance, because it dictates the ability of phagocytic cells in general, and macrophages in particular, to completely internalize and further process the fibers. The influence of CNT length on their biological effects has been therefore assessed in a few studies, and from these data, it appears that shorter CNT have less biological effects than long CNT, in terms of reactive oxygen species production, inflammatory mediators release, and inflammatory cell recruitment [29, 30]. Longer CNT (with a cut-off at 5–10 μm) promote frustrated phagocytosis, with a failure of macrophages to entirely engulf CNT [31]. However, several issues must be raised concerning the effect of length in CNT biological effects, apart from those mentioned above. First of all, because of still remaining technical issues, it is difficult to measure accurately CNT length. Indeed, they have a tendency to entangle and aggregate, which complicates a linear measurement. Microscopy techniques (usually transmission electron microscopy, TEM) are often used to demonstrate (but not quantify) the differences of CNT length, but it necessitates an amazingly high amount of measurements from the investigator. Moreover, shortening of CNT is often obtained by grinding raw CNT materials, by the use of an agate ball mill, a process which can introduce structural defects in CNT [32]. This would lead to the modification of not only the length of CNT, but also other physicochemical characteristics of the CNT. In this regard, Wako and collaborators recently demonstrated that grinding CNT could modify inflammatory response in terms of localization of macrophages infiltrates [33]. These authors showed that intratracheal administration of ground MWCNT in rats lead to the infiltration of MWCNT-laden macrophages in the alveoli, whereas unground MWCNT-laden macrophages accumulated in the pulmonary interstitium. This was associated with the formation of smaller aggregates in the case of ground MWCNT, as compared to unground MWCNT.

8.5.3 Dispersion Status

Dispersion is often proposed as an important determinant of biological effects of CNT. Indeed, dispersion can condition the potency for CNT to form aggregates in solution. What is currently believed is that well dispersed CNT seem to preferentially induce the development of fibrosis, whereas less dispersed CNT lead to the formation of granuloma. A better dispersion of CNT can be achieved by the use of dispersing agents (such as surfactants), attachment of functional groups to CNT's surface (functionalization process), use of solvents, or mechanical treatment, such as grinding or sonication. Another way to achieve different dispersion status is the administration mode, as seen in a study by Li and coworkers. These authors demonstrated the formation of small aggregates after inhalation of CNT, as compared to the formation of larger aggregates consequent to intratracheal instillation of similar amounts of CNT [34].

8.5.4 Residual Catalyst Content

Finally, and directly resulting from their production process, the amount of residual catalyst particles present in CNT is believed to play an important role in determining CNT biological effects. Iron, nickel, and cobalt are the most common catalyst particles primarily used during CNT manufacture. Their amount in the final product varies with the manufacturing process, as well as the post-treatments undertaken to purify as produced raw CNT. Several studies suggest that the metal content (and particularly that of iron) of CNT can contribute to their effects probably via the induction of an oxidative stress [14, 35, 36]. However, these studies are not consistent in their conclusions; some show that a high content in catalyst residues is associated to increased biological effects, and some others report opposite findings. The discrepancies between the different studies may relate to the bioavailability of the catalyst residues in the various CNT samples; some catalyst residues are engulfed inside the carbon structure of CNT, although in some other cases, catalyst residues are present at the surface of the carbon structure, being therefore more accessible and prone to play a role in CNT's biological effects. Finally, the purification process (high temperature, strong acidification process) used to get rid of (at least in part) catalyst residues may introduce other physicochemical modifications in the so-called purified CNT, and therefore complicate the interpretation of the results.

As a general comment, care must be taken regarding the experimental design of the studies aimed to determine the importance of specific physicochemical determinant(s), in particular concerning the origin of the CNT utilized to perform the study. Indeed, as exposed earlier, fabrication processes for CNT synthesis are highly complex, and can lead to the production of a large variety of CNT. Trying to compare results obtained with CNT issued from different origins (producers, fabrication process) can be hazardous when it comes to giving straightforward conclusions.

8.6 Remaining Issues Regarding CNT Toxicity

Several specific endpoints have been either not or only poorly considered until now regarding CNT respiratory effects. This essentially concerns two major endpoints: the translocation of CNT as well as their further processing (*devenir*) after pulmonary exposure.

8.6.1 Translocation of CNT After Pulmonary Exposure

It is quite clear, given the actual literature on the subject, that CNT can have extrapulmonary effects after pulmonary administration. However, the underlying mechanisms are quite unclear, particularly regarding the need or absence of need for CNT to be translocated to the systemic circulation or other organs to have an extrapulmonary effect. There are some evidences of biopersistence of CNT after pulmonary exposure; Muller and collaborators early demonstrated that 60 days after the initial intratracheal administration, roughly 80 % of MWCNT was found inside the lungs of the exposed rats [17]. Interestingly, only 36 % of the initial dose of MWCNT was found inside the lungs when the authors considered ground MWCNT. Unfortunately, no indication on the localization (organ, body fluids) of the CNT has been achieved in this study. It is important to have in mind that observation of CNT in a biological context is a complex matter when considering the biodistribution of CNT. Optical and conventional electronic microscopy observations are of limited interest, especially in the case of small diameter SWCNT, because of limitations related to the spatial resolution of these techniques and to the low contrast of these materials [37]. A possibility might reside in the grafting of a functionalized group to the raw CNT, but this could affect the intrinsic physicochemical characteristics of the CNT, therefore modifying their biological effects. Another possibility is to take advantage of the residual catalyst present in the CNT, such as iron. However, it requires highly demanding techniques, such as synchrotron-based X ray fluorescence microscopy (μXRF) [38], which is clearly not convenient to use on a routine basis.

8.6.2 Devenir of CNT

Another important remaining issue to get a comprehensive understanding of CNT's biological effects is the question of their *devenir* and potential biotransformation in a biological context. Indeed, despite the earlier mentioned indications of biopersistance, along with evidences of CNT penetration inside cells, no actual data are available on that matter, although such data are very important to obtain since CNT biotransformation products could have their own biological effects and

therefore participate in CNT's reactivity and further toxicity. A few groups have demonstrated, yet essentially in test tubes, the possible modifications of SWCNT [39, 40]. However, more extensive research is clearly needed regarding this matter.

8.7 Conclusions

Given the existing literature on the subject, it can be concluded that respiratory exposure to CNT lead to the occurrence of pulmonary inflammation, the formation of granuloma, and the development of pulmonary fibrosis. The exact determinants of these effects still remain to be clearly identified, although intrinsic physico-chemical characteristics of CNT such as length, dispersion status, and residual catalyst content seem to be of importance. Several critical issues still remain to be solved, such as the translocation of CNT outside the lungs and the occurrence of their biotransformation. This should open a new understanding to the respiratory effects of CNT.

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