



HHS Public Access

Author manuscript

Nanomedicine (Lond). Author manuscript; available in PMC 2016 January 12.

Published in final edited form as:

Nanomedicine (Lond). 2014 May ; 9(6): 895–912. doi:10.2217/nnm.14.42.

The effects of carbon nanotubes on lung and dermal cellular behaviors

Sudjit Luanpitpong^{1,2}, Liying Wang³, and Yon Rojanasakul^{*,1,2}

¹Pharmaceutical & Pharmacological Sciences Program, West Virginia University, WV 26506, USA

²Mary Babb Randolph Cancer Center, West Virginia University, WV 26506, USA

³Pathology & Physiology Research Branch, National Institute for Occupational Safety & Health, Morgantown, WV 26505, USA

Abstract

Carbon nanotubes (CNTs) hold great promise to create new and better products, but their adverse health effect is a major concern. Human exposure to CNTs is primarily through inhalation and dermal contact, especially during the manufacturing and handling processes. Numerous animal studies have demonstrated the potential pulmonary and dermal hazards associated with CNT exposure, while *in vitro* studies have assessed the effects of CNT exposure on various cellular behaviors and have been used to perform mechanistic studies. In this review, we provide an overview of the pathological effects of CNTs and examine the acute and chronic effects of CNT exposure on lung and dermal cellular behaviors, beyond the generally discussed cytotoxicity. We then examine the linkage of cellular behaviors and disease pathogenesis, and discuss the pertinent mechanisms.

Keywords

angiogenesis; carbon nanotubes; carcinogenicity; cellular behavior; cytotoxicity; dermal exposure; genotoxicity; inflammation; lung fibrosis; pulmonary exposure

Nanotechnology presents enormous opportunities to improve and even revolutionize the fields of electronics, energy, waste treatment, biosensors and medicine. Most benefits of nanotechnology result from the ability to engineer the essential structures of nanomaterial to achieve specific properties. Carbon nanotubes (CNTs) are a major class of nanomaterials possessing unique mechanical, electrical and thermal properties [1,2]. They are being produced on a massive scale with the estimated global market of approximately US\$2

* Author for correspondence: Tel.: +1 304 293 1476, yrojan@hsc.wvu.edu.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

One of the first CNT toxicity studies was conducted by Lam *et al.* who investigated the effects of SWCNT with different metal impurities after intratracheal (it.) administration to the lungs of mice at the dosing concentrations of 0.1–0.5 mg [34]. The mice were toxicologically assessed at 7 and 90 days postexposure. All types of SWCNTs studied were found to induce persistent epithelioid granulomas in association with particle aggregates and lung inflammation in a dose- and time-dependent manner. Similar granuloma formation with SWCNT aggregates was observed in the it. study in rats [35]. Progressive interstitial fibrosis and alveolar wall thickening was reported in mice with SWCNT exposure via pharyngeal aspiration at 28 days postexposure onwards, the effect that was dependent on SWCNT dispersion status [36]. Lung inflammation and fibrosis were also observed in mice exposed by pharyngeal aspiration [37] and rats by it. administration to MWCNTs [38,39]. With regard to the effect of surface functionalization, Sager *et al.* recently reported that addition of the carboxylate (COOH) groups to MWCNT significantly reduced the inflammatory and fibrogenic responses after pharyngeal aspiration into mice [23], likely due to the decreased association with target lung cells.

To date, not many inhalation studies have been conducted. Short-term inhalation (nose-only) study of 5 mg/m³ SWCNT (5 h/day for 4 days) with mice revealed acute lung inflammation followed by the development of granulomas and persistent interstitial fibrosis [40]. For MWCNT exposure, lung fibrosis, but not mesothelioma, was observed in an inhalation (nose-only) study with mice exposed to 30 mg/m³ MWCNT for 6 h, but not to lower doses, for example 1 mg/m³ [41]. Mitchell *et al.* reported neither significant lung inflammation nor fibrosis upon 0.3–5 mg/m³ MWCNT inhalation (6 h/day for 14 days), although splenic immunosuppression was observed likely through an activation of cyclooxygenase at 1 mg/m³ dose [42,43]. In mice with allergic asthma sensitized by ovalbumin, 100 mg/m³ MWCNT inhalation for 6 h induced lung fibrosis at 14 days, whereas no fibrosis was observed in mice receiving ovalbumin or MWCNTs alone [44]. A more recent study by Sargent *et al.* using a multistage (initiation-promotion) carcinogenesis model in B6C3F1 mice demonstrated that inhalation of 5 mg/m³ MWCNT (5 h/day, 5 days/week for 15 days) following an intraperitoneal (ip.) injection of DNA damaging agent methylcholanthrene (initiator) led to increased incidence and numbers of bronchioloalveolar adenomas and adenocarcinomas at 17 months postexposure as compared with MCA or MWCNT exposure alone (62 vs 22 or 14%) [45]. This finding indicates MWCNT as a tumor promoter but not tumor initiator in mice.

ip. injection studies exposing fiber particles to the mesothelial linings of the abdominal cavity in mice and rats were used as a surrogate for the mesothelial linings of pleural cavity surrounding the lungs for screening of the mesothelioma pathogenicity in humans [46]. It was first noted by Poland *et al.* that short-term ip. instillation of long MWCNT in wild-type mice caused asbestos-like granuloma, suggesting the potential linkage between CNT exposure and mesothelioma [47]. Further studies by Takagi *et al.* and Kanno *et al.* reported that a single ip. injection of MWCNT in heterozygous p53 mice caused mesothelioma [48,49]. However, it is worthy to note that the animal model used in these two studies was cancer sensitive, since heterozygous p53 mice have some background of spontaneous cancer. The mesothelioma pathogenicity of MWCNT was later observed by Nagai *et al.* in a

noncancer-prone rodent model of Fischer-344/Brown-Norway F₁ hybrid rats [14]. In that study, the pathogenic effect of MWCNT was found to be associated with particle diameter. For example, thin and rigid MWCNTs (diameter: ~50 nm) were most pathogenic, whereas thick (diameter: ~150 nm) and tangled (diameter: ~2–20 nm) MWCNTs were less pathogenic. By contrast, Muller *et al.* and Liang *et al.* reported no mesothelioma formation after an ip. injection of MWCNT in rats and mice [50,51]. It is likely that the difference in animal species and/or exposure conditions used in these studies contributed to the observed discrepancies.

***In vitro* assessment of the CNT effects on lung cellular behaviors**

Various *in vitro* studies using different lung cell types have been performed to evaluate the effects of CNTs on cellular behaviors. The models of these studies that describe the various cell types used and their origin as well as their role in disease pathogenesis are summarized in Table 2.

Acute effects of CNTs on lung cellular behaviors

Cell viability & apoptosis

Assessment of the cellular effects of CNTs has focused mainly on acute toxicity, which is commonly assessed by cell viability and apoptosis. Cell viability can be determined by various means such as cell membrane integrity using propidium iodide and trypan blue assays, and mitochondrial activity using formazan-based 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 4-(3-[4-Iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1) assays. Various CNTs under different treatment conditions have been studied. For example, Manna *et al.* demonstrated a dose-dependent decrease in the viability (MTT assay) of human lung epithelial A549 and H1299 cells after SWCNT treatment (0.1–10 µg/ml) [30]. Magrez *et al.* similarly observed the dose-dependent decrease in cell viability (MTT assay) of a set of lung epithelial cells including H596, H446 and Calu-1 cells after MWCNT exposure (0.002–0.2 µg/ml) for up to 4 days [21]. The same study also reported a further reduction in cell viability upon treatment with MWCNT with surface-modified carbonyl, COOH, and hydroxyl groups. Subsequent studies by Davoren *et al.* and Wick *et al.* showed that aggregation of CNTs determines their cellular toxicity (MTT assay) in A549 lung epithelial cells and MSTO211H mesothelial cells [27,28]. By contrast, Pulskamp *et al.* reported no toxicity (WST-1 assay) in NR8383 rat alveolar macrophages and A459 lung epithelial cells after SWCNT or MWCNT exposure (5–100 µg/ml) for 24 h [25].

Apoptosis is a form of cell death that plays an essential role in the maintenance of tissue homeostasis. An excessive induction of apoptosis, however, could lead to organ dysfunction and failure, such as lung emphysema [54]. Assays of apoptosis are mainly based on the detection of unique morphological changes such as membrane blebbing, cell shrinkage, nuclear condensation and fragmentation. Several apoptotic studies using lung epithelial cells have been conducted. For examples, Ravichandran *et al.* demonstrated the induction of apoptosis in rat lung epithelial cells by MWCNT (0.5–10 µg/ml) after a 24-h exposure [61].

Pacurari *et al.* demonstrated a dose- and time-dependent effect of raw SWCNT (5–600 $\mu\text{g}/\text{cm}^2$) containing nickel and yttrium catalysts on apoptosis of human lung epithelial BEAS-2B cells [62]. In the same study, SWCNT was found to induce MAPK, AP-1 and NF- κ B, all of which recapitulate the key molecular signaling involved in lung carcinogenesis. A similar finding on MAPK, AP-1 and NF- κ B activation by SWCNT (25–50 $\mu\text{g}/\text{cm}^2$) was observed in normal human and malignant mesothelial cells [56]. Notably, pristine graphene sheets (5–80 $\mu\text{g}/\text{ml}$) were also shown to trigger apoptosis of RAW 264.7 macrophages partly through the MAPK pathway [19]. These findings suggest the possible linkage between CNT-induced molecular and cellular changes (e.g., apoptosis) and lung pathogenesis (e.g., cancer and mesothelioma).

Genotoxicity

Cancer is generally accepted as a disease of the genome as significant genomic alterations (e.g., point mutations, large deletions and amplifications) are observed in almost all forms of human cancer [63]. Genotoxicity refers to damage to genomic DNA that could potentially lead to mutations and eventually cancer. Several assays have been used to test the genotoxicity of CNTs in cultured lung cells, for example, comet, chromosomal, micronucleus and phosphorylated H2AX assays [64]. Exposure to SWCNT (25–50 $\mu\text{g}/\text{ml}$) caused a dose-dependent genotoxic effect in normal and malignant mesothelial cells as determined by comet assay and H2AX phosphorylation [56]. SWCNT and MWCNT were also found to be genotoxic in human bronchial BEAS-2B epithelial cells as evaluated by comet and micronucleus assays [65].

Paracellular permeability

Lung epithelium presents a protective barrier against inhaled particles through the formation of tight junction barrier, which limits the paracellular permeability of solutes and toxicants across the epithelium [66]. Alterations in the barrier function are therefore an important determinant of the interaction of xenobiotics with other compartments of the organisms. Tight junction barrier of the lung can be modeled by using Calu-3 lung epithelial cells grown on a permeable filter in culture, and analyzed by transepithelial electrical resistance (TEER) or mannitol flux measurements. Exposure to noncytotoxic MWCNT (100 $\mu\text{g}/\text{ml}$) for 4 days caused a large drop in Calu-3 TEER and a parallel increase in mannitol permeability, while SWCNT (100 $\mu\text{g}/\text{ml}$) produced much smaller effects [67]. The presence of MWCNT and SWCNT in cell culture during the formation of tight junction significantly decreased the TEER. These findings suggest that MWCNT and SWCNT can interfere with the formation and/or maintenance of tight junction barrier of the lung.

Stimulation of inflammatory factors

Pulmonary exposure to CNTs leads to lung inflammation, the pathological condition that has been linked to the development of lung fibrosis and cancer. Animal studies demonstrated the involvement of various inflammatory cytokines and growth factors such as IL-1 β , TGF- β and PDGF in CNT-induced lung fibrosis. For instance, Shvedova *et al.* demonstrated that SWCNT were potent inducer of TGF- β production in association with macrophage recruitment and lung fibrosis [36]. Ryman-Rasmussen *et al.* suggested that MWCNT

induced lung fibrosis through PDGF activation in accordance with TGF- β activation by ovalbumin [44]. PDGF, as a potent mitogen and chemoattractant, is known to stimulate proliferation and migration of lung fibroblasts, while TGF- β is known to stimulate collagen production and deposition [68]. Recently, the critical role of IL-1 signaling in CNT-induced inflammation has been demonstrated using transgenic mice. Knockout of IL-1 receptor (*Il1r1*) gene in C57Bl/6 mice (IL1R $^{-/-}$) abrogated MWCNT-induced lung inflammation [69].

In vitro, the release of IL-1 β , TGF- β and PDGF from THP-1 macrophages and BEAS-2B bronchial epithelial cells has been used to predict the lung fibrotic response to MWCNT [22,52]. Li *et al.* showed that, as compared with pristine MWCNT, anionic functionalized COOH and polyethylene glycol (PEG) MWCNT exhibited a weak stimulating effect on IL-1 β , TGF- β and PDGF, while those with neutral and weak cationic functionalized amine (NH₂) and sidewall NH₂ showed intermediate effect, and the strong cationic functionalized polyetherimide MWCNT showed the most robust effect, the results that were in good agreement with the *in vivo* fibrogenic responses [22]. The production of TGF- β and PDGF from RAW264.7 macrophages by MWCNT was shown to promote the transformation of lung fibroblasts to myofibroblasts, a key step in the initiation and progression of pulmonary fibrosis [70]. Murphy *et al.* studied the effects of MWCNT of various lengths on the release of inflammatory cytokines IL-1 β , TNF- α , IL-6 and chemokine IL-8 from Met5A pleural mesothelial cells and THP-1 macrophages [17]. The results showed that MWCNT (5 $\mu\text{g}/\text{cm}^2$) was able to induce cytokine release from macrophages in a length-dependent manner, which subsequently led to enhanced cytokine release from mesothelial cells, suggesting the mechanism for creating an inflammatory microenvironment in the pleural cavity by MWCNT.

Excessive cell growth & extracellular matrix production

Excessive production and deposition of extracellular matrix (ECM) is a hallmark of fibrosis [55]. Wang *et al.* demonstrated that SWCNT (0.08–0.24 $\mu\text{g}/\text{cm}^2$) dose dependently induced proliferation of human lung fibroblasts and increased their production of ECM proteins including collagen I and III [29]. A similar fibroblast-stimulating effect was observed with MWCNT (0.02–0.06 $\mu\text{g}/\text{cm}^2$) through the induction of FGF-2 [53]. In the same study, MWCNT was also shown to induce collagen production by lung fibroblasts in a dose-dependent manner. These studies suggest that the induction of fibroblast proliferation and collagen production by CNTs might be key determining factors of CNT-induced lung fibrosis.

Angiogenesis

Angiogenesis or formation of new blood vessels has been implicated in the pathogenesis of lung fibrosis as indicated by the increased neovascularization and angiogenic mediators observed in the lungs of patients and animals with pulmonary fibrosis [58]. Azad *et al.* demonstrated that exposure of human lung fibroblasts to SWCNT (5–25 $\mu\text{g}/\text{ml}$) induced the secretion of angiogenic mediator TGF- β 1 and VEGF in a dose-dependent manner [59]. These mediators from the lung fibroblasts stimulated angiogenesis of HUVEC endothelial cells. TGF- β 1 and VEGF additionally have a direct effect on fibrogenesis by inducing

collagen production, suggesting that these two molecules may produce an additive effect through ECM and angiogenesis induction that contributes to CNT fibrosis.

Co-culture of human small airway epithelial cells (SAEC) and HMVEC endothelial cells represents the model for alveolar–capillary interaction of small airways in the lower respiratory tract. Synder-Talkington *et al.* found that exposure of HMVEC cells to MWCNT-stimulated SAEC cells in the Transwell® co-culture system increased the angiogenicity of HMVEC cells [60]. The authors suggest that this might be linked to the pathological angiogenesis in lung fibrosis.

Acute effects of CNTs & their linkage to lung fibrosis

As discussed above, various cellular events, including inflammation, angiogenesis, proliferation and ECM production, contribute to the development of lung fibrosis after CNT exposure. Figure 2 is a schematic representation summarizing the linkage between various cellular events and lung fibrogenesis.

Chronic effects of CNTs on lung cellular behaviors

Because of the similarity between CNTs and asbestos fibers in terms of high aspect ratio, route of exposure and biopersistence, there is a great concern about the potential carcinogenicity of CNTs. Inhaled CNTs have been shown to penetrate the alveolar epithelium, enter the interstitial compartment where the clearance rate is low [36,71–72] and subsequently migrate to the parietal pleural space [73]. As typical developmental period of fiber-induced lung cancer in humans is 30–40 years [74], no human data on CNT-induced cancer are yet available. To mimic the long-term carcinogenic process, our group has developed chronic exposure models in which human lung epithelial cells (e.g., BEAS-2B and SAEC cells) and pleural mesothelial cells (e.g., Met5A cells) were continuously exposed to low-dose ($0.02 \mu\text{g}/\text{cm}^2$), physiologically relevant concentrations of SWCNT and MWCNT for a prolonged period of time (e.g., 4–6 months) [75–77]. These cells were chosen because they are cellular targets of human lung cancer and mesothelioma. The exposed cells were then assessed for various aggressive behaviors known to be cancer hallmarks, such as abnormal cell growth, acquired apoptosis resistance, increased motility and angiogenesis [78]. Figure 3 depicts CNT lung carcinogenesis model and summarizes the chronic effects of CNTs on lung cellular behaviors.

Cell growth & malignant transformation

Tissue homeostasis is the balance between cell death and proliferation. While disturbance in homeostasis that favors cell apoptosis could contribute to organ failure or dysfunction as mentioned above, excessive cell growth could lead to progressive diseases, such as cancer [79]. Chronic exposure to low-dose SWCNT and MWCNT ($0.02 \mu\text{g}/\text{cm}^2$) was reported to induce cell growth of human lung epithelial and mesothelial cells [75–77]. The exposed cells were also shown to grow under anchorage-independent conditions (e.g., on soft agar), an indicator of malignant transformation [75,76]. A similar finding was observed in chronic MWCNT-exposed lung epithelial cells at a higher concentration ($0.16 \mu\text{g}/\text{cm}^2$) [80]. Using whole genome microarray analysis and ingenuity pathway analysis, Wang *et al.* reported the

activation of cancer-related canonical pathways in chronic SWCNT- and MWCNT-exposed lung epithelial cells similar to that observed in asbestos-exposed cells [76]. Western blot analysis further showed the overexpression of proto-oncoproteins (e.g., PPAR γ , cFOS and c-Myc) and downregulation of tumor suppressor proteins (e.g., inhibin- α and p53) in the SWCNT- and MWCNT-exposed cells, supporting the cellular cancer-like behaviors.

Acquired apoptosis resistance

Apoptosis plays an essential role in the removal of mutated or transformed cells, and its disruption contributes to abnormal cell growth and malignancy [81]. Acquired apoptosis resistance promotes cell survival during the carcinogenic process against endogenous antigrowth signals and immune cell killing mechanisms [82]. Chronic exposure of lung epithelial cells to SWCNT (0.02 $\mu\text{g}/\text{cm}^2$) resulted in acquired apoptosis resistance to the CNT itself and to other apoptosis inducers including death ligands such as TNF- α and chemotherapeutic agents such as etoposide [75]. Aberrant p53 signaling was shown to be involved in CNT apoptosis resistance, consistent with the findings that most human cancers have mutated or inactivated p53 [83].

Cell migration & invasion

Cell migration is a general movement of cells from one location to another, while cell invasion is defined as the migration of cells through the 3D tissue structures, for example, ECM or basement membrane [84]. Cell migration and invasion are key determinants of tumor progression and metastasis [85,86]. To enter blood and lymphatic vessels for dissemination into the systemic circulation, tumor cells must migrate or invade through certain barriers, for example, epithelial and vascular basement membrane and stromal ECM [87]. Chronic exposure of human lung epithelial or mesothelial cells to SWCNT and MWCNT (0.02 $\mu\text{g}/\text{cm}^2$) induced migration and invasion of the cells similar to asbestos-exposed cells [75–77]. MMP-2 was identified as the key ECM enzyme responsible for the increased migration and invasion of CNT-transformed mesothelial cells based on microarray data and gene knockdown studies [77].

Angiogenesis

The oxygen and nutrients supplied through blood vessels are crucial for cell functions and survival [88]. With the unlimited proliferative capability of tumor cells, an adequate supply of the nutrients and oxygen is necessary, or else the tumor cells may become apoptotic or necrotic. Tumor angiogenesis must develop in order to keep the tumor cells growing and spreading, and therefore it is an important factor in tumor progression and metastasis [86]. Tumor cells release angiogenic mediators such as VEGF, TGF- β , TNF- α and PDGF to trigger endothelial cell proliferation and capillary formation. Conditioned medium from chronic SWCNT- and MWCNT-exposed lung cells caused a significant increase in endothelial tube formation as indicated by the increased number of branch nodes and more complex pattern of capillary tubes, indicating the increased angiogenicity of chronic CNT-exposed cells [75,76].

Tumor formation

The tumorigenicity of chronic SWCNT and MWCNT-exposed cells was assessed in a xenograft mouse model [75,80]. When the cells were subcutaneously injected into the hind flanks of immunodeficient mice, tumors were observed, whereas a similar injection of control cells gave no tumors [75,80]. Histological analysis of CNT-derived tumors showed classical cancer cell morphology, including the presence of multinucleated cells, an indicator of mitotic dysfunction [89]. Using a high-resolution comparative genomic hybridization technique, Wu *et al.* demonstrated chromosomal aberrations in chronic MWCNT-exposed lung cells particularly in the chromosome 2q31–32 [80]. HOXD9/D13 was further identified as the region contributing to MWCNT carcinogenesis.

Dermal exposure to CNTs

CNT exposure through skin/dermal contact can occur during the manufacturing and handling [8,11]. Dermal exposure to carbon materials has previously been reported to cause carbon fiber dermatitis [90]. However, *in vivo* assessment of the CNT dermal effects is limited. Koyama *et al.* demonstrated that implantation of SWCNT and MWCNT into the subcutaneous tissues of mice induced granuloma with entrapped CNT agglomerates after 3 weeks of exposure [91], consistent with the earlier finding by Sato *et al.* showing granuloma-like structures in the rat skin after MWCNT implantation [92]. Murray *et al.* investigated the topical effects of SWCNT in mice and showed an increase in skin bi-fold thickness, an indicator of edema and inflammation, after topical exposure to SWCNT (40–160 µg/mouse) for 5 days [93]. These studies suggest that there is a dermal hazard associated with CNT exposure and that the dermal response is primarily inflammatory in nature.

In vitro assessment of the CNT effects on dermal cellular behaviors

Assessment of the dermal effects of CNTs has focused on human keratinocytes and dermal fibroblasts. Keratinocytes are the predominant cell type in the skin epidermis, the outer epithelial layer of the skin, while dermal fibroblasts are cells that live within the dermis to generate and maintain skin connective tissue [94]. To date, only the acute effects of CNTs on dermal cellular behaviors have been reported, which are summarized and discussed below.

Acute effects of CNTs on dermal cellular behaviors

Cell viability

An early study by Shvedova *et al.* demonstrated that exposure of human HaCaT keratinocytes to SWCNT (0.06–0.24 mg/ml) for 18 h caused a dose-dependent decrease in cell viability as determined by the Alamar blue assay [26]. Transmission electron micrographs revealed ultrastructural alterations to both mitochondria and nuclei after SWCNT (0.24 mg/ml) exposure. Likewise, exposure of HaCaT cells to MWCNT (0.025–1 mg/ml) for 24 h decreased cell viability; however, no cytotoxicity (MTT assay) of MWCNT (1 mg/ml) was observed in the human skin equivalent model called 3D EpiDerm™

[(Epi-200; Mat-Tek Co., MA, USA)[95]. In addition, MWCNT (0.001–0.1 mg/ml) was found to be toxic (MTT assay) on human dermal fibroblasts after a 24-h exposure [96].

Genotoxicity

The genotoxicity of MWCNT was evaluated by Patlolla *et al.* in normal human dermal fibroblasts using comet and DNA ladder assays [96]. Exposure of MWCNT (40–400 µg/ml) for 48 h caused comet tail formation at all concentrations tested and DNA ladder formation at the highest dose. The genotoxicity of SWCNT (0.5–30 µg/ml) was reported by Cveticanin *et al.* in normal human dermal fibroblasts after 24-hour exposure using phosphorylated H2AX assay [97]. It was suggested that CNTs are efficient in interacting with DNA due to their similarity in dimension [96].

Inflammation

Studies by Witzmann and Monteiro-Riviere, and Zhang *et al.* demonstrated that SWCNT and MWCNT induced proinflammatory cytokines, such as IL-8 in HEK keratinocytes [98,99]. Using human skin equivalent model EpiDerm-FT™, Murray *et al.* showed an increase in IL-12, IL-6 and IFN-γ release in parallel with increased collagen production and dermis thickness after SWCNT (75 µg) exposure for 18 h [93]. These findings strengthen the involvement of inflammation in the dermal effects of CNTs.

Cell adhesion

Cell adhesion maintains contact between neighboring cells and substrata, and is important in proper cell signaling for cellular functions such as cell growth, migration, differentiation and tissue organization. Zhang *et al.* investigated the effects of MWCNT (20–50 µg/ml) on cell adhesion of primary human dermal fibroblasts and NIH 3T3 murine embryonic fibroblasts [100]. They showed that MWCNT exposure for 24 h decreased cell adhesion in both cell types, possibly through a decrease in adhesion-related genes including fibronectin, laminin and focal adhesion kinase.

Cell migration & wound healing

Cell migration contributes to tissue repair and regeneration during wound healing. In the initial state of tissue repair, fibroblasts from surrounding tissues proliferate and migrate into the wound area. Fibroblasts then generate ECM, while keratinocytes at the wound edge multiply and migrate towards the wound bed [101]. Treatment of human dermal fibroblasts and NIH 3T3 fibroblasts with MWCNT (25–50 µg/ml) for 24 h reduced the migratory activity of the cells by 20–40%, as determined by Transwell and scratch wound healing assays [100]. In the same study, MWCNT was shown to disrupt the assembly of F-actin stress fibers, which may contribute to the decreased cell migration.

Conclusions & future perspective

With the rapid development and widespread use of CNTs, increasing human exposure to the nanomaterial is expected. Therefore, it is important to determine their health hazards in a timely manner because of their potential pathogenicity. A detailed understanding of the biological activities and pathogenesis mechanisms is required to aid their toxicological

assessment. Numerous animal studies have demonstrated the overall pulmonary and dermal hazards associated with CNT exposure. *In vitro* studies have been used to assess the specific effects of CNT exposure on cellular behaviors and elucidate the underlying mechanisms. In this review, we examine the acute and chronic effects of CNT exposure on lung and dermal cellular behaviors beyond the generally discussed cytotoxicity, and evaluate their linkage to disease pathogenesis. For instance, acute exposure to CNTs was shown to induce inflammatory cytokine release by macrophages and lung epithelial cells, ECM production by lung fibroblasts, and angiogenesis by endothelial cells, all of which are key characteristics of lung fibrosis (Figure 2). Chronic exposure to CNTs induces neoplastic transformation of human lung epithelial and mesothelial cells with aggressive cancer-like behaviors (Figure 3).

With regards to the carcinogenicity of CNTs, it should be noted that although some animal and cell culture studies pointed out that chronic exposure to CNTs could induce or promote lung cancer and/or mesothelioma, negative findings were also reported. Therefore, more rigorous and systemic studies, especially with human exposure, are needed to ascertain the carcinogenicity of CNTs. As the typical developmental period of fiber-induced lung cancer in humans is 30–40 years, more rapid experimental models or alternate assays that are predictive of human carcinogenic responses are greatly needed. For the time being, prudent adoption of prevention strategies and implementation of exposure control are strongly advised.

Conflicting results regarding the pathologic effects and underlying mechanisms of CNTs could possibly be due to the use of different types of tested CNTs with various variables, experimental models and conditions (Table 1). There is a pressing need to develop a library of CNTs with well-defined physicochemical properties and standardized methodologies to elucidate the structure–activity relationships. Because of the great variety of CNTs with different purity and physicochemical properties, such as length, diameter, functionalization and surface chemistry, high-throughput screening assays that are predictive of the *in vivo* pathological responses are needed. The use of mechanism-based biomarkers might serve as a platform for high-throughput screening of CNT pathogenicity. In addition, it is of significant importance to establish appropriate dosimetry standards for nanotoxicological research since most studies to date have used unrealistically high concentrations of nanomaterials that may not be relevant to real-life exposure conditions.

Acknowledgments

This work was supported by the National Institute for Occupational Safety and Health and by grants from NIH (R01-HL095579 and R01-ES022968), National Science Foundation (EPS-1003907) and Mary Babb Randolph Cancer Center (MBRCC) Sara C Allen Lung and James F Allen Comp Lung Cancer Research Fund.

References

Papers of special note have been highlighted as:

- of interest;
- of considerable interest

1. Shvedova AA, Kisin ER, Porter D, et al. Mechanisms of pulmonary toxicity and medical applications of carbon nanotubes: two faces of janus? *Pharmacol. Ther.* 2009; 121(2):192–204. [PubMed: 19103221]
2. Helland A, Wick P, Koehler A, Schmid K, Som C. Reviewing the environmental and human health knowledge base of carbon nanotubes. *Environ. Health Perspect.* 2007; 115(8):1125–1131. [PubMed: 17687437]
3. Thayer AM. Carbon nanotubes by the metric ton: anticipating new commercial applications, producers increase capacity. *Chem. Eng. News.* 2007; 85(46):29–35.
4. Holman, MW.; Lackner, DI. *The Nanotech Report*. 4th Edition. Lux Research, Inc.; NY, USA: 2006.
5. De Volder MFL, Tawfick SH, Baughman RH, Hart AJ. Carbon nanotubes: present and future commercial applications. *Science.* 2013; 339(6119):535–539. [PubMed: 23372006]
6. Heister E, Brunner EW, Dieckmann GR, Jurewicz I, Dalton AB. Are carbon nanotubes a natural solution? Applications in biology and medicine. *ACS Appl. Mater. Interfaces.* 2013; 5(6):1870–1891. [PubMed: 23427832]
7. Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol. Sci.* 2006; 92(1):5–22. [PubMed: 16484287]
- 8•. Aschberger K, Johnston HJ, Stone V, et al. Review of carbon nanotubes toxicity and exposure – appraisal of human health risk assessment based on open literature. *Crit. Rev. Toxicol.* 2010; 40(9):759–790. [PubMed: 20860524] [Comprehensive review of carbon nanotube (CNT) toxicity upon inhalation and dermal exposure and possible human health risk.]
9. Awasthi K, Srivastava A, Srivastava ON. Synthesis of carbon nanotubes. *J. Nanosci. Nanotechnol.* 2005; 5(10):1616–1636. [PubMed: 16245519]
10. Eitan A, Jiang K, Dukes D, Andrews R, Schadler LS. Surface modification of multiwalled carbon nanotubes: toward the tailoring of the interface in polymer composites. *Chem. Mater.* 2013; 15(16):3198–3201.
- 11•. Johnston HJ, Hutchison GR, Christensen FM, et al. A critical review of the biological mechanisms underlying the *in vivo* and *in vitro* toxicity of carbon nanotubes: the contribution of physico-chemical characteristics. *Nanotoxicology.* 2010; 4(2):207–246. [PubMed: 20795897] [Comprehensive review of the *in vitro* and *in vivo* assessment of CNT toxicity and a summary of physicochemical characteristics that link to CNT biological effects.]
- 12••. Manke A, Wang L, Rojanasakul Y. Pulmonary toxicity and fibrogenic response of carbon nanotubes. *Toxicol. Mech. Methods.* 2013; 23(3):196–206. [PubMed: 23194015] [Discusses the physicochemical properties of CNTs on pulmonary toxicity and molecular mechanisms contributing to lung fibrosis.]
13. Liu Y, Zhao Y, Sun B, Chen C. Understanding the toxicity of carbon nanotubes. *Acc. Chem. Res.* 2013; 46(3):702–713. [PubMed: 22999420]
14. Nagai H, Okazaki Y, Chew S, et al. Diameter and rigidity of multi-walled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. *Proc. Natl Acad. Sci. USA.* 2011; 108(49):E1330–E1338. [PubMed: 22084097]
15. Yamashita K, Yoshioka Y, Higashisaka K, et al. Carbon nanotubes elicit DNA damage and inflammatory response relative to their size and shape. *Inflammation.* 2010; 33(4):276–280. [PubMed: 20174859]
16. Liu D, Wang L, Wang Z, Cuschieri A. Different cellular response mechanisms contribute to the length-dependent cytotoxicity of multi-walled carbon nanotubes. *Nanoscale Res. Lett.* 2012; 7(1): 361. [PubMed: 22748010]
17. Murphy FA, Schinwald A, Poland CA, Donaldson K. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. *Part. Fibre Toxicol.* 2012; 9:8. [PubMed: 22472194]
18. Palomaki J, Valimaki E, Sund J, et al. Long, needle-like carbon nanotubes and asbestos activate the NRLP3 inflammasome through a similar mechanism. *ACS Nano.* 2011; 5(9):6861–6870. [PubMed: 21800904]

19. Li Y, Liu Y, Fu Y, et al. The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF-beta signaling pathways. *Biomaterials*. 2012; 33(2):402–411. [PubMed: 22019121]
20. Hu X, Cook S, Wang P, Hwang HM, Liu X, Williams QL. *In vitro* evaluation of cytotoxicity of engineered carbon nanotubes in selected human cell lines. *Sci. Total Environ*. 2010; 408(8):1812–1817.
21. Magrez A, Kasas S, Salicio V, et al. Cellular toxicity of carbon-based nanomaterials. *Nano Lett*. 2006; 6(6):1121–1125. [PubMed: 16771565]
22. Li R, Wang X, Ji Z, et al. Surface charge and cellular processing of covalently functionalized multiwall carbon nanotubes determine pulmonary toxicity. *ACS Nano*. 2013; 7(3):2352–2368. [PubMed: 23414138]
23. Sager TM, Wolfarth MW, Andrew M, et al. Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model. *Nanotoxicology*. 2014; 8(3):317–327. [PubMed: 23432020]
24. Meng J, Cheng X, Liu J, et al. Effects of long and short carboxylated or aminated multiwalled carbon nanotubes on blood coagulation. *PLoS ONE*. 2012; 7(7):e38995. [PubMed: 22808023]
25. Pulskamp K, Diabate S, Krug HF. Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicol. Lett*. 2007; 168(2): 58–74. [PubMed: 17141434]
26. Shvedova AA, Castranova V, Kisin ER, et al. Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. *J. Toxicol. Environ. Health A*. 2003; 66(20):1909–1926. [PubMed: 14514433]
27. Davoren M, Herzog E, Casey A, et al. *In vitro* toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. *Toxicol. In Vitro*. 2007; 21(3):438–448. [PubMed: 17125965]
28. Wick P, Manser P, Limbach LK, et al. The degree and kind of agglomeration affect carbon nanotube cytotoxicity. *Toxicol. Lett*. 2007; 168(2):121–131. [PubMed: 17169512]
29. Wang L, Mercer RR, Rojanasakul Y, et al. Direct fibrogenic effects of dispersed single-walled carbon nanotubes on human lung fibroblasts. *J. Toxicol. Environ. Health A*. 2010; 73(5–6):410–422. [PubMed: 20155582]
30. Manna SK, Sarkar S, Barr J, et al. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes. *Nano Lett*. 2005; 5(9):1676–1684. [PubMed: 16159204]
31. Dahm M, Chen BT, Birch ME, et al. Carbon nanotube dosimetry: from workplace exposure assessment to inhalation toxicology. *Toxicologist*. 2013; 132:99.
32. Han JH, Lee EJ, Lee JH, et al. Monitoring multiwalled carbon nanotube exposure in carbon nanotube research facility. *Inhal. Toxicol*. 2008; 20:741–749. [PubMed: 18569096]
33. NIOSH. Occupational exposure to carbon nanotubes and nanofibers.; Current Intelligence Bulletin. 2013. p. 2013-145. www.cdc.gov/niosh/docs/2013-145/pdfs/2013-145.pdf [Summarizes the current CNT animal studies assessing potential hazards and risks to provide guidance for protecting workers.]
34. Lam CW, James JT, McCluskey R, Hunter RL. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol. Sci*. 2004; 77(1):126–134. [PubMed: 14514958]
35. Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GA, Webb TR. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. *Toxicol. Sci*. 2004; 77(1): 117–125. [PubMed: 14514968]
36. Shvedova AA, Kisin ER, Mercer R, et al. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 2005; 289(5):L698–L708. [PubMed: 15951334]
37. Mercer RR, Hubbs AF, Scabilloni JF, et al. Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. *Part. Fibre Toxicol*. 2011; 8:21. [PubMed: 21781304]
38. Muller J, Huaux F, Moreau N, et al. Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol. Appl. Pharmacol*. 2005; 207(3):221–231. [PubMed: 16129115]

39. Aiso S, Yamazaki K, Umeda Y, et al. Pulmonary toxicity of intratracheal instilled multiwall carbon nanotubes in male Fischer 344 rats. *Ind. Health.* 2010; 48(6):783–795. [PubMed: 20616469]
40. Shvedova AA, Kisin E, Murray AR, et al. Inhalation versus aspiration of single walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress and mutagenesis. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2008; 295(4):L552–L565. [PubMed: 18658273]
41. Ryman-Rasmussen JP, Cesta MF, Broday AR, et al. Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nat. Nanotechnol.* 2009; 4(11):747–751. [PubMed: 19893520]
42. Mitchell LA, Gao J, Wal RV, Gigliotti A, Burchiel SW, McDonald JD. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol. Sci.* 2007; 100(1):203–214. [PubMed: 17660506]
43. Mitchell LA, Lauer FT, Burchiel SW, McDonald JD. Mechanisms of how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice. *Nat. Nanotechnol.* 2009; 4(7):451–456. [PubMed: 19581899]
44. Ryman-Rasmussen JP, Tewksbury EW, Moss OR, Cesta MF, Wong BA, Bonner JC. Inhaled multiwalled carbon nanotubes potentiates airway fibrosis in murine allergic asthma. *Am. J. Respir. Cell. Mol. Biol.* 2009; 40(3):349–358. [PubMed: 18787175]
45. Sargent LM, Porter DW, Staska LM, et al. Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. *Part. Fibre Toxicol.* 2014; 11(1):3. [PubMed: 24405760]
46. Davis JM, Bolton RE, Miller BG, Niven K. Mesothelioma dose response following intraperitoneal injection of mineral fibres. *Int. J. Exp. Pathol.* 1991; 72(3):263–274. [PubMed: 1843255]
47. Poland CA, Duffin R, Kinloch L, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat. Nanotechnol.* 2008; 3(7):423–428. [PubMed: 18654567]
48. Takagi A, Hirose A, Nishimura T, et al. Induction of mesothelioma in p53^{+/-} mouse by intraperitoneal application of multi-wall carbon nanotube. *J. Toxicol. Sci.* 2008; 33(1):105–116. [PubMed: 18303189]
49. Kanno J, Takagi A, Nishimura T, Hirose A. Mesothelioma induction by micrometer-sized multi-walled carbon nanotube intraperitoneally injected to p53 heterozygous mice. *Toxicologist.* 2010; 114:A1397.
50. Muller J, Delos M, Panin N, Rabolli V, Huaux F, Lison D. Absence of carcinogenic response to multiwall carbon nanotubes in 2-year bioassay in the peritoneal cavity of the rat. *Toxicol. Sci.* 2009; 110(2):442–448. [PubMed: 19429663]
51. Liang G, Yin L, Zhang J, et al. Effects of subchronic exposure to multi-walled carbon nanotubes in mice. *J. Toxicol. Environ. Health A.* 2010; 73(7):463–470. [PubMed: 20391125]
52. Wang X, Xia T, Duch MC, et al. Pluronic F108 coating decreases the lung fibrosis potential of multiwall carbon nanotubes by reducing lysosomal injury. *Nano Lett.* 2012; 12(6):3050–3061. [PubMed: 22546002]
53. Mishra A, Rojanasakul Y, Chen BT, Castranova V, Mercer RR, Wang L. Assessment of pulmonary fibrogenic potential of multiwalled carbon nanotubes in human lung cells. *J. Nanomater.* 2012; 2012:930931.
54. Demedts IK, Demoor T, Bracke KR, Joos GF, Brusselle GG. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. *Respir. Res.* 2006; 7(1):53. [PubMed: 16571143]
55. Todd NW, Luzina IG, Atamas SP. Molecular and cellular mechanisms of pulmonary fibrosis. *Fibrogenesis Tissue Repair.* 2012; 5(1):11. [PubMed: 22824096]
56. Pacurari M, Yin XJ, Zhao J, et al. Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells. *Environ. Health Perspect.* 2008; 116(9):1211–1217. [PubMed: 18795165]
57. Bhattacharya K, Andón FT, El-Sayed R, Fadeel B. Mechanisms of carbon nanotube-induced toxicity: focus on pulmonary inflammation. *Adv. Drug Deliv. Rev.* 2013; 65(15):2087–2097. [PubMed: 23751779]
58. Cosgrove GP, Brown KK, Schiemann WP, et al. Pigment epithelium-derived factor in idiopathic pulmonary fibrosis: a role in aberrant angiogenesis. *Am. J. Respir. Crit. Care Med.* 2004; 170(3):242–251. [PubMed: 15117744]

59. Azad N, Iyer AKV, Wang L, Liu Y, Lu Y, Rojanasakul Y. Reactive oxygen species-mediated p38 MAPK regulates carbon nanotube-induced fibrogenic and angiogenic responses. *Nanotoxicology*. 2013; 7(2):157–168. [PubMed: 22263913]
60. Snyder-Talkington BN, Schwegler-Berry D, Castranova V, Qian Y, Guo NL. Multi-walled carbon nanotubes induce human micro vascular endothelial cellular effects in an alveolar-capillary co-culture with small airway epithelial cells. Part. *Fibre Toxicol*. 2013; 10:35. [PubMed: 23903001]
61. Ravichandran P, Periyakaruppan A, Sadanandan B, et al. Induction of apoptosis in rat lung epithelial cells by multiwalled carbon nanotubes. *J. Biochem. Mol. Toxicol*. 2009; 23(5):333–344. [PubMed: 19827037]
62. Pacurari M, Schwegler-Berry D, Friend S, et al. Raw single-walled carbon nanotube-induced cytotoxic effects in human bronchial epithelial cells: comparison to asbestos. *Toxicol. Environ. Chem*. 2011; 93(5):1045–1072.
63. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature*. 1998; 396(6712):643–649. [PubMed: 9872311]
- 64••. Toyokuni S. Genotoxicity and carcinogenicity risk of carbon nanotubes. *Adv. Drug Deliv. Rev*. 2013; 65(15):2098–2110. [PubMed: 23751780] [Summarizes the recent *in vitro* and *in vivo* studies on the genotoxicity and carcinogenicity of CNTs.]
65. Lindberg HK, Falck GCM, Suhonen S, et al. Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells *in vitro*. *Toxicol. Lett*. 2009; 186(3):166–173. [PubMed: 19114091]
66. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am. J. Physiol. Cell. Physiol*. 2004; 286(6):C1213–C1228. [PubMed: 15151915]
67. Rotolia BM, Bussolatia O, Bianchia MG, et al. Nonfunctionalized multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells. *Toxicol. Lett*. 2008; 178(2): 95–102. [PubMed: 18403140]
68. Bonner JC. Mesenchymal cell survival in airway and interstitial pulmonary fibrosis. *Fibrogenesis Tissue Repair*. 2010; 3:15. [PubMed: 20738867]
69. Girtsman TA, Beamer CA, Wu N, Buford M, Holian A. IL-1R signaling is critical for regulation of multi-walled carbon nanotubes-induced acute lung inflammation in C57Bl/6 mice. *Nanotoxicology*. 2014; 8(1):17–27. [PubMed: 23094697]
70. He X, Young S, Schwegler-Berry D, Chisholm WP, Fernback JE, Ma Q. Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF- κ B signaling, and promoting fibroblast-to-myofibroblast transformation. *Chem. Res. Toxicol*. 2011; 24(12):2237–2248. [PubMed: 22081859]
71. Mercer RR, Scabilloni J, Wang L, et al. Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 2008; 294(1):L87–L97. [PubMed: 18024722]
72. Mercer RR, Hubbs AF, Scabilloni JF, et al. Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes. Part. *Fibre Toxicol*. 2010; 7:28. [PubMed: 20920331]
73. Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part. *Fibre Toxicol*. 2010; 7:5. [PubMed: 20307263]
74. Shukla A, Vacek P, Mossman BT. Dose–response relationships in expression of biomarkers of cell proliferation in *in vitro* assays and inhalation experiments. *Nonlinearity Biol. Toxicol. Med*. 2004; 2(2):117–128. [PubMed: 19330127]
- 75••. Wang L, Luanpitpong S, Castranova V, et al. Carbon nanotubes induce malignant transformation and tumorigenesis of human lung epithelial cells. *Nano Lett*. 2011; 11(7):2796–2803. [PubMed: 21657258] [Describes the chronic exposure model for lung carcinogenesis studies of carbon nanotubes, which could be used as a screening assay for the carcinogenicity testing and for mechanistic studies.]
76. Wang L, Stueckle T, Mishra A, et al. Neoplastic-like transformation effect of single-walled and multi-walled carbon nanotubes compared with asbestos on human lung small airway epithelial cells. *Nanotoxicology*. 2013 doi:10.3109/17435390.2013.801089 (Epub ahead of print).

77. Lohcharoenkal W, Wang L, Stueckle TA, et al. Chronic exposure to carbon nanotubes induces invasion of human mesothelial cells through matrix metalloproteinase-2. *ACS Nano*. 2013; 7(9): 7711–7723. [PubMed: 23924264]
78. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100(1):57–70. [PubMed: 10647931]
79. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. *Cell*. 2007; 130(2): 223–233. [PubMed: 17662938]
80. Wu P, Yuan SS, Ho CC, et al. Focal amplification of HOXD-harboring chromosome region is implicated in multiple-walled carbon nanotubes-induced carcinogenicity. *Nano Lett*. 2013; 13(10): 4632–4641. [PubMed: 23984819]
81. Shivapurkar N, Reddy J, Chaudhary PM, Gazdar AF. Apoptosis and lung cancer: a review. *J. Cell. Biochem*. 2003; 88(5):885–898. [PubMed: 12616528]
82. Stenner-Liewen F, Reed JC. Apoptosis and cancer: basic mechanisms and therapeutic opportunities in the postgenomic era. *Cancer Res*. 2003; 63(1):263–268.
83. Harris CC. p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and cancer risk assessment. *Environ. Health Perspect*. 1996; 104(Suppl. 3):435–439. [PubMed: 8781359]
84. Wolf K, Friedl P. Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. *Trends Cell Biol*. 2011; 21(12):736–744. [PubMed: 22036198]
85. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer*. 2003; 3(5):362–374. [PubMed: 12724734]
86. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell*. 2006; 127(4):679–695. [PubMed: 17110329]
87. Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. *Curr. Opin. Cell Biol*. 2012; 24(2):277–283. [PubMed: 22209238]
88. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. *Vasc. Health Risk Manag*. 2006; 2(3):213–219. [PubMed: 17326328]
89. Ullmann R, Bongiovanni M, Halbwedl I, et al. Bronchiolar columnar cell dysplasia – genetic analysis of a novel preneoplastic lesion of peripheral lung. *Virchows Arch*. 2003; 442(5):429–436. [PubMed: 12684770]
90. Lachapelle, JM. Occupational airborne skin diseases.. In: Kanerva, L.; Elsner, P.; Wahlberg, JE.; Maibach, HI., editors. *Handbook of Occupational Dermatology*. Springer-Verlag; Berlin, Germany: 2002. p. 193-199.
91. Koyama S, Endo M, Kim YA, et al. Role of systemic T-cells and histopathological aspects after subcutaneous implantation of various carbon nanotubes in mice. *Carbon*. 2006; 44(6):1079–1092.
92. Sato Y, Yokoyama A, Shibata K, et al. Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1. *In vitro* and subcutaneous tissue of rats *in vivo*. *Mol. Biosys*. 2005; 1(2):176–182.
93. Murray AR, Kisin E, Leonard SS, et al. Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes. *Toxicology*. 2009; 257(3):161–171. [PubMed: 19150385]
94. Wickett RR. Basics of skin structure. *J. Cosmet. Sci*. 2004; 55(1):132–133. [PubMed: 15065588]
95. Park YH, Jeong SH, Lee EY, et al. Assessment of dermal irritation potential of MWCNT. *Toxicol. Environ. Health. Sci*. 2010; 2(2):115–118.
96. Patlolla A, Knighten B, Tchounwou P. Multi-walled carbon nanotubes induce cytotoxicity, genotoxicity and apoptosis in normal human dermal fibroblast cells. *Ethn. Dis*. 2010; 20(1 Suppl. 1):S1–S72. [PubMed: 20521388]
97. Cveticanin J, Joksic G, Leskovic A, Petrovic S, Sobot AV, Neskovic O. Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells. *Nanotechnology*. 2010; 21(1):015102. [PubMed: 19946169]
98. Witzmann FA, Monteiro-Riviere NA. Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. *Nanomedicine*. 2006; 2(3):158–168. [PubMed: 17292138]

99. Zhang LW, Zeng L, Barron AR, Monteiro-Riviere NA. Biological interactions of functionalized single-wall carbon nanotubes in human epidermal keratinocytes. *Int. J. Toxicol.* 2007; 26(2):103–113. [PubMed: 17454250]
100. Zhang Y, Wang B, Meng X, Sun G, Gao C. Influences of acid-treated multiwalled carbon nanotubes on fibroblasts: proliferation, adhesion, migration, and wound healing. *Ann. Biomed. Eng.* 2011; 39(1):414–426. [PubMed: 20824344]
101. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. *Am. J. Surg.* 1998; 176(2A Suppl.):S26–S38.

Box 1. Notable variable factors affecting carbon nanotube biological activities**Intrinsic physicochemical properties**

- Particle size (diameter) and length
- Particle shape
- Wall number
- Particle surface chemistry
- Surface area
- Surface functionalization
- Presence of metal impurities

Extrinsic factors

- Tested cell types or animals
- Experimental conditions
- Dispersion status (agglomeration)
- Dose (concentration)
- Exposure time
- Test assays and sensitivity

Executive summary

Pulmonary exposure to carbon nanotubes

- Carbon nanotubes (CNTs) can come into contact with the human body mainly through inhalation and dermal exposure. Inhalation exposure can lead to biopersistence and are therefore more potentially pathogenic.
- Animal models of CNT pulmonary exposure revealed adverse health effects, including lung inflammation, granuloma and fibrosis. CNTs have also been shown to induce mesothelioma after an intraperitoneal injection, raising a concern about their potential lung mesotheliogenicity.

In vitro assessment of the CNT effects on lung cellular behaviors

- Various lung cell types, including epithelial, mesothelial, endothelial, fibroblast and immune cells, have been used to investigate the effects of CNTs on lung cell behaviors.
- Acute CNT exposure can induce cytotoxicity, genotoxicity and permeability alterations depending on their physicochemical properties and exposure conditions. CNTs have been shown to induce cytokine release, extracellular matrix production and angiogenesis *in vitro* that have been linked to fibrosis *in vivo*.
- Chronic, low-dose CNT exposure causes neoplastic transformation of human lung epithelial and mesothelial cells exhibiting aggressive cancer-like behaviors.

Dermal exposure to CNTs

- To date, studies of CNT dermal exposure are limited.
- However, existing data have pointed out that the dermal responses to CNT exposure are primarily inflammatory in nature.

In vitro assessment of the CNT effects on dermal cellular behaviors

- The major cell types used to investigate the dermal effects of CNTs are keratinocytes and dermal fibroblasts.
- Acute CNT exposure decreases cell viability and initiates the inflammatory response of keratinocytes. CNTs have been shown to induce genotoxicity and alter cellular behaviors such as cell adhesion, migration, and wound healing of dermal fibroblasts. No studies on the effects of chronic CNT exposure on the dermal system have been reported to date.

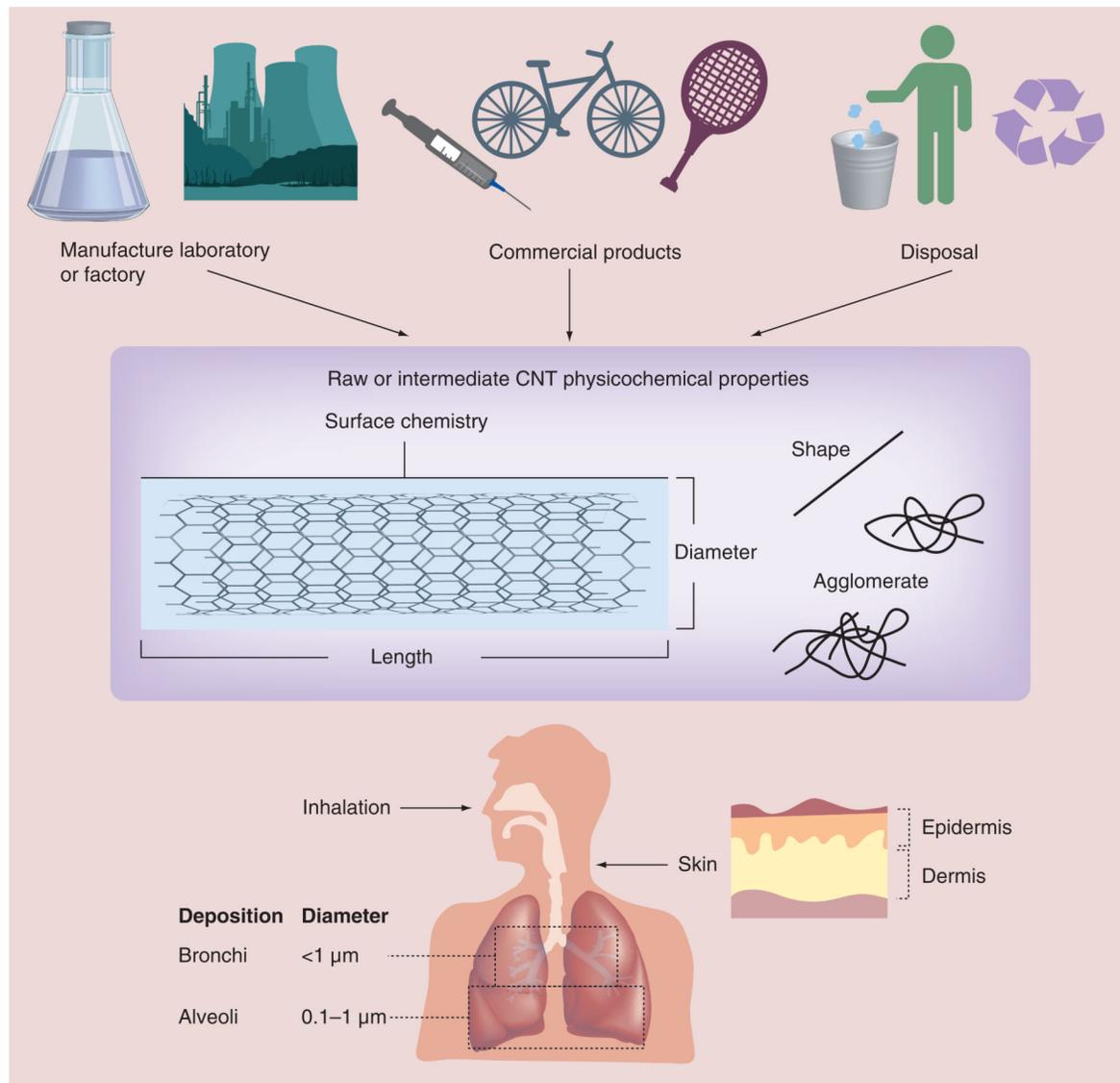


Figure 1. Sources of human carbon nanotube exposure and physicochemical factors influencing carbon nanotube bioactivities.
CNT: Carbon nanotube.

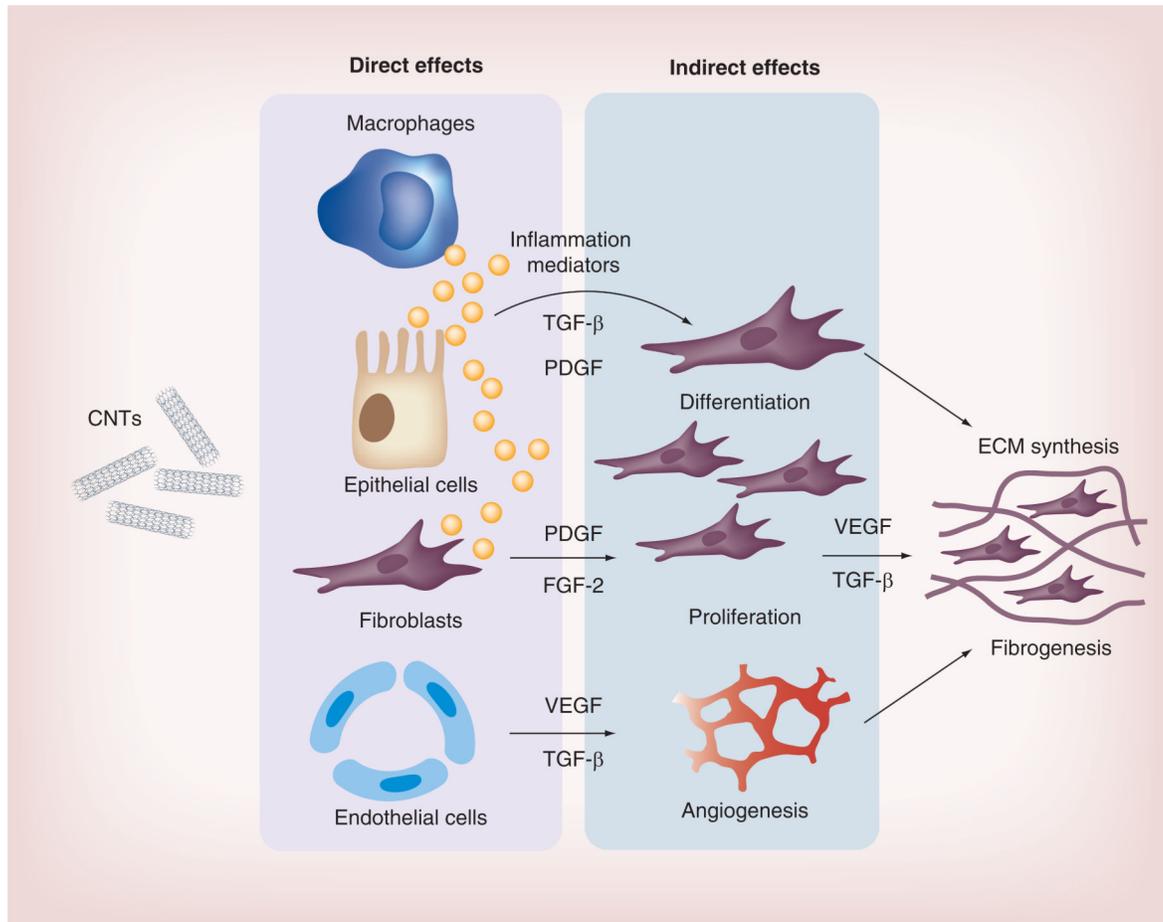


Figure 2. Various cellular behaviors involved in carbon nanotube-induced lung fibrosis. CNT: Carbon nanotube; ECM: Extracellular matrix.

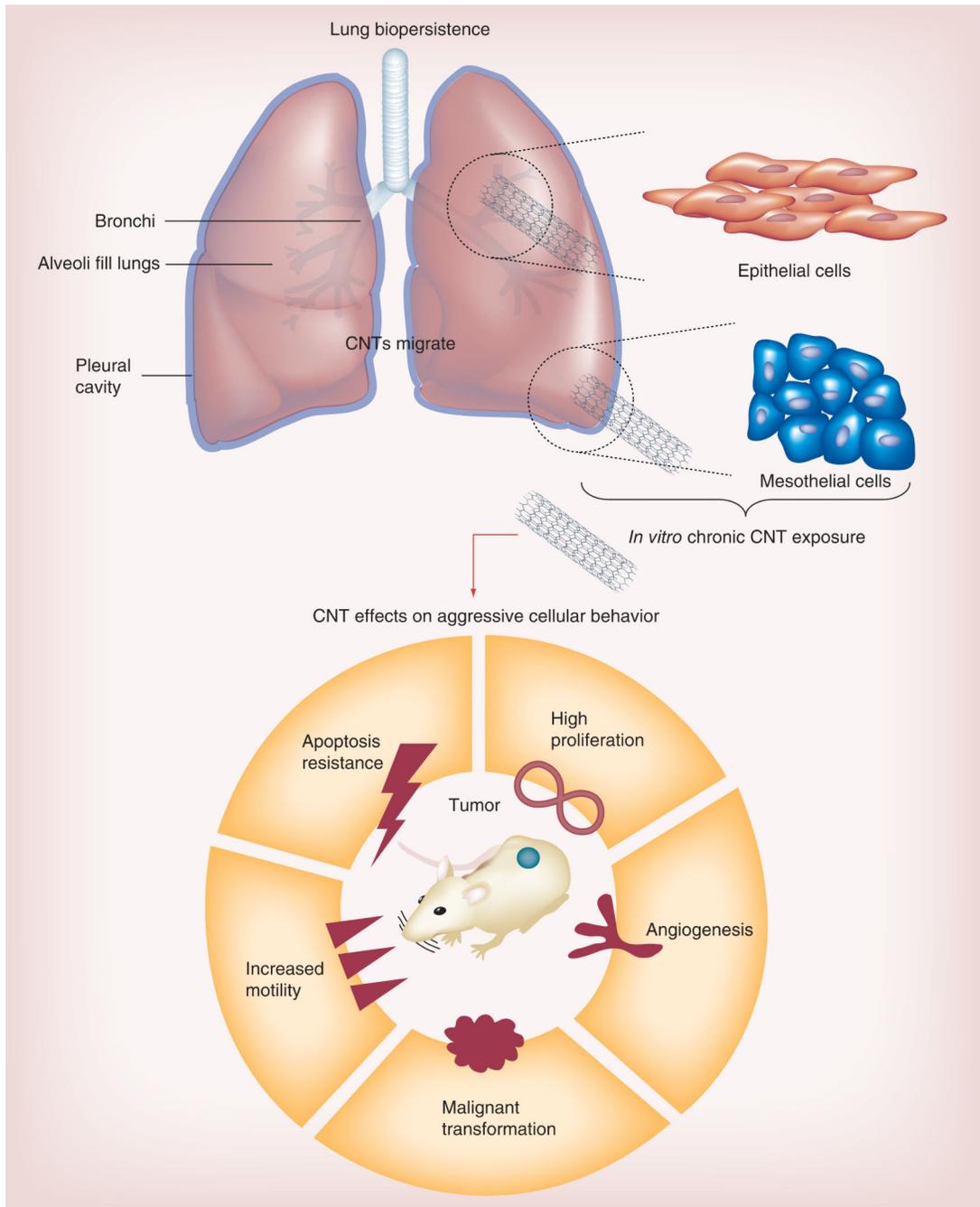


Figure 3. Lung models for carcinogenesis studies and chronic effects of carbon nanotube exposure on aggressive cellular behaviors. CNT: Carbon nanotube.

Table 1

Basis of carbon nanotube biological activities.

Types of CNT	Sources	Cells/animals	Exposure dose/time	Dispersant	Test assays	Conclusions/discussions	Ref.
Intrinsic physicochemical properties: D							
MWCNT: thin (~50 nm); thick (~150 nm); tangled (~2–20 nm)	Mitsui & Co. (Toyko, Japan); Showa Denko (Japan)	Mesothelial cells; F344/BNF1 rats	5 µg/cm ² , 4 days; ip. 1 mg	0.5% BSA in saline solution	ATP; WST-1; live-cell protease	Thin MWCNTs with high crystallinity were most cytotoxic. Inflammatory and carcinogenic, likely due to their high membrane-piercing capability	[14]
Intrinsic physicochemical properties: L							
MWCNT: L 5–15 µm/D 20–60 nm; L 1–2 µm/D 60–100 nm; L 1–2 µm/D <10 nm	Meijo Nano Carbon (Aichi, Japan); SES Research (TX, USA)	A549; C57BL/6 mice	50 µg/ml, 3 h; ip. 50 µg, 24 h	NA	Comet; cell number in lavage fluid	Long and thick MWCNTs induced the strongest DNA damaging and inflammatory effects, likely due to the rigidity of nanotubes and frustrated phagocytosis of the exposed cells	[15]
MWCNT: long, L 3–14 µm; short, L 1.5 µm	Nanothinx S.A. (Platani, Greece)	RAW 264.7; MCF-7	12.5–200 µg/ml, 0–7 days	Pluronic F-127	CellTiter-Blue; Trypan Blue	Long MWCNTs were more toxic	[16]
MWCNT: short, L 1–2 µm; long, L 13 or 36 µm; tangle, L 1–5 or 5–20 µm	Nanostructured & Amorphous Materials, Inc. (TX, USA); NanoLab, Inc. (MA, USA); Mitsui & Co.	Met5A; THP-1	5–50 µg/cm ² , 24 h	0.5% BSA in RPMI media	IL-1β, TNF-α, IL-6, IL-8	MWCNTs directly induced a length-dependent proinflammatory response from THP-1 cells, the effects of which can be amplified by cytokines from Met5A cells	[17]
Intrinsic physicochemical properties: shape							
MW L CNT: short; long tangled; long needle-like; carbon black; asbestos	Bayer Material Science (Leverkrusen, Germany); Cheap Tubes, Inc (VT, USA); Mitsui & Co.	Human primary monocyte-derived macrophages	100 µg/ml, 6 h	2% FBS in PBS	IL-1 family secretion	Long needle-like CNTs and asbestos activated NLRP3 inflammasome (IL-1β) from LPS-induced macrophages, while other types of carbon nanomaterials were weak inducers. Thus, length and rigidity of CNTs are key contributors to their inflammogenicity	[18]

Types of CNT	Sources	Cells/animals	Exposure dose/time	Dispersant	Test assays	Conclusions/discussions	Ref.
Intrinsic physicochemical properties: layer number							
Pristine graphene	Kinik Company (Taiwan)	RAW 264.7	5–100 µg/ml, for 48 h	1% pluronic F108	Cell Counting Kit-8; Annexin V-FITC	Depletion of the mitochondrial membrane potential and increase of apoptosis	[19]
MWCNT: D 20–60 nm; SWCNT: D <2 nm; both L 5–15 µm	SES Research (TX, USA)	A549	50 µg/ml, 3 h	NA	Comet	MWCNT induced strong DNA damaging effect, while SWCNT caused minimal effect	[15]
SWCNT: L 5–30 µm/D 1–2 nm; MWCNT: 10–30 µm/D 20–30 nm	Cheap Tubes, Inc.	MSTO-211H	2–10 ppm, 60 min	Tween 80	US FDA uptake	SWCNT were more cytotoxic than MWCNT	[20]
Intrinsic physicochemical properties: surface chemistry							
MWCNT with C=O, COOH and/or OH surfaces	Own making; chemical modification by acid treatment	H596	0.02 µg/ml, 4 days	Diluted gelatin solution	MTT	The toxicity of MWCNT increased with the surface modifications, likely due to their increased dispersion and interaction with cells	[21]
MWCNT: COOH; PEG; NH ₂ ; sidewall NH ₂ ; PEI-modified	Raw CNTs from Cheap Tubes, Inc.; own modified	BEAS-2B & THP-1 co-culture; C57BL/6 mice	60 µg/ml, 24 h; 2 mg/kg; analyzed after 21 days	Cell culture medium; added BSA and DPPC prior to treatment	IL-1β, TGF-β1, and PDGF-AA	Fibrogenicity ranking: anionic (COOH and PEG) < pristine ~ neutral < strong cationic (PEI), possibly due to decreased interactions of the anionic CNTs with cells	[22]
MWCNT: COOH	Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA); own modified	C57BL/6 mice	40 µg pharyngeal aspiration	DPPC and mouse albumin	Inflammatory cell count; histology	Functionalized COOH significantly reduced inflammation and lung fibrosis	[23]
MWCNT: long and short COOH; long and short NH ₂	Chengdu Organic Chemicals Co. Ltd. (Chengdu, China); own modified	Red blood cells	0.005–0.16 mg/ml	Pure water and NaCl aqueous solution	Blood clot; platelet activation; cell viability	The effects of MWCNT depended on both length and surface chemistry; long COOH and NH ₂ induced more platelet activation than short ones; long COOH and short NH ₂ softened the clots more effectively; only long NH ₂ reduced cell viability	[24]

Types of CNT	Sources	Cells/animals	Exposure dose/time	Dispersant	Test assays	Conclusions/discussions	Ref.
Intrinsic physicochemical properties: metal impurities							
SWCNT: purified; metal trace (0.009% Fe, 2.8% Co, 4.2% Mo)	Own making; Nanostructured & Amorphous Materials, Inc.	NR8383, A549	5–100 µg/ml, 24 h	NA	DCF; TMRE	Dose-dependent increase in intracellular ROS and loss of mitochondrial membrane potential with metal trace SWCNT	[25]
SWCNT: 30% iron	Own making; HIPCO process	HaCaT	0.06–0.24 mg/ml	Phenol-free KGM medium	Alamar Blue	SWCNT induced ROS-dependent toxicity, likely due to the catalytic activity of iron	[26]
Extrinsic factors: agglomeration							
SWCNT with 10 wt% iron	CNI, Inc. (CA, USA)	A549	3.125–800 µg/ml, 24 h	Serum containing (5%); serum-free (0%) medium	MTT	Greater toxicity was observed in the absence of serum (more aggregates)	[27]
SWCNT: rope-like agglomerates; bundles	Yangtze Nanotechnology (Shanghai, China)	MSTO-211H	7.5–30 µg/ml, 3 days	PS80 for suspended CNT bundles	MTT; DNA	Toxicity ranking: CNT agglomerates > asbestos > CNT-bundle	[28]
SWCNT	CNI, Inc.	W138; C57BL/6J mice	0.02 µg/cm ² , 2 days; 10 µg pharyngeal aspiration	Natural lung surfactant Survanta [®] ; acetone/sonication	Hemocytometry; Sircol; Col I	Well-dispersed SWCNT exhibited a growth stimulating effect, whereas non-dispersed SWCNT had no effect; well-dispersed SWCNT induced collagen production <i>in vitro</i> and <i>in vivo</i>	[29]
Extrinsic factors: dose/cell type							
SWCNT	Own making	HaCaT; HeLa; A549; HI299	0.1–10 µg/ml, 72 h	DMF (solvent)	MTT	Dose-dependent decrease of cell viability, similar response in all cell types	[30]
MWCNT	Own making	H596; H446; Calu-1	0.02–0.2 µg/ml, 1–4 days	Diluted gelatin solution	MTT	Dose-dependent decrease of cell viability with H596 cells showing the highest sensitivity; MWCNT were less toxic than carbon black and carbon nanofibers	[21]
SWCNT	Cheap Tubes, Inc	A3; MSTO-211H; HaCaT	2–10 ppm, 60 min	Tween 80	FDA uptake	T4 lymphocyte A3 cells were most sensitive	[20]
Extrinsic factors: test assays							

Types of CNT	Sources	Cells/animals	Exposure dose/time	Dispersant	Test assays	Conclusions/discussions	Ref.
SWCNT	Nanostructured & Amorphous Materials, Inc.	NR8383	5–100 µg/ml, 24 h	NA	MTT, WST-1, PI	Dose-dependent decrease of cell viability, while no toxicity was observed using WST-1 and PI assays	[25]

BSA: Bovine serum albumin; CNT: Carbon nanotube; Col I: Collagen I; D: Diameter; DCF: Dichlorofluorescein; DMF: Dimethylformamide; DPPC: Dipalmitoyl phosphatidylcholine; FBS: Fetal bovine serum; ip.: Intraperitoneal; KGM: Keratinocyte growth medium; L: Length; MTT: 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; MWCNT: Multiwalled carbon nanotube; NA: Not applicable; PBS: Phosphate buffered saline; PEG: Polyethylene glycol; PEI: Polyetherimide; PI: Propidium iodide; ROS: Reactive oxygen species; RPMI: Roswell Park Memorial Institute; S: Shape; SWCNT: Single-walled carbon nanotube; TMRE: Tetramethylrhodamine, ethyl ester.

Table 2Lung cell models for *in vitro* assessment of carbon nanotube pathogenicity.

Cell lines	Origin	Roles in pathogenesis
Lung epithelial cells		
A549	Human alveolar type II epithelial cells (adenocarcinoma)	Lung epithelial cells are the primary targets of CNT lung exposure and carcinogenesis. They are also involved in lung fibrosis by regulating fibroblast activities, for example, proliferation, migration and ECM production, through the release of profibrogenic mediators [22,52–53]. In contrast, apoptosis of lung epithelial cells can lead to emphysema and the breakdown of lung tissue [54]
H1299, H596, H446, Calu-1	Human lung epithelial cells (carcinoma)	
HNBE, BEAS-2B	Human normal and SV40-immortalized bronchial epithelial cells	
SAEC	Human small airway epithelial cells	
Calu-3	Human sub-bronchial gland epithelial cells (adenocarcinoma)	
Lung fibroblasts		
CRL-1490 (WI-38), NHLF	Human normal lung fibroblasts	Lung fibroblasts represent one of the major cell types in lung interstitium. Their excessive proliferation and overproduction of ECM underlie lung fibrosis [29,55]
V79	Chinese hamster lung fibroblasts	
Pleural mesothelial cells		
NM, Met5A	Human normal and SV40-immortalized mesothelial cells	Cellular studies using mesothelial cells are reported to mimic important biological events involved in mesothelioma development [56]
MM, MSTO211H	Human malignant mesothelial cells (mesothelioma)	
Immune cells		
THP-1	Human monocytes, which can differentiate into macrophage-like cells	Immune cells, particularly macrophages, are the front line of body immune defense in response to foreign bodies, e.g. engulfing them by phagocytosis [57]
RAW264.7	Mouse leukemic monocyte macrophages	
NR8383	Rat alveolar macrophages	
Endothelial cells		
HUVEC, HMVEC	Human umbilical vein or microvascular endothelial cells	Angiogenesis, an increasing number of new capillaries, has been linked to lung fibrosis [58,59]. In addition, the co-culture of small airway epithelial cells and HMVEC cells was employed to model the alveolar–capillary interaction in the lower respiratory tract [60]

CNT: Carbon nanotube; ECM: Extracellular matrix; HMVEC: Human dermal microvascular endothelial cells.