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# The effects of carbon nanotubes on lung and dermal cellular behaviors

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## 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

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billion by 2014 [3,4]. CNTs are being used in diverse fields, including electronics, textiles, sporting goods, aerospace, drug delivery and bioimaging [5,6]. Such widespread use has led to the unprecedented avenues of human exposure. In terms of structure, CNTs are high aspect ratio nanomaterials comprised of single or concentrically stacked multiwalled graphene sheets rolled into a cylinder, referred to as single-walled (SW) or multiwalled (MW) CNTs, respectively. The potential health risks of CNT exposure have been raised, attributable to the following reasons: their small nanosized structure that makes them more reactive and toxic than larger particles; their high aspect ratio and mode of exposure similar to asbestos fibers, prompting a concern about their potential fiber-like toxicity; and their graphitic structure that is expected to have high durability and biopersistence [7,8]. Synthesis of CNTs by methods such as evaporation, vapor deposition and laser ablation often leaves residual metal catalysts such as cobalt, iron, nickel, molybdenum and contaminated amorphous carbon and fullerenes [9], which may contribute to the observed toxic effects of CNTs. In addition, the surface of CNTs can be modified and functionalized to introduce specific functions [10], increasing the diversity of available CNTs with different surface chemistry, purity, dimension, wall number and functionalization. All of these intrinsic physicochemical factors, as well as extrinsic factors, such as experimental conditions, can influence the observed biological activities of CNTs. Box 1 summarizes key factors influencing CNT bioactivities. More detailed information on the types and sources of CNTs, experimental conditions, and major findings are summarized and discussed in Table 1. Specific discussions of CNT biological activities are provided in subsequent sections (also see [11–13]).

Due to their widespread use and possible human exposure, especially in occupational and environmental settings during manufacturing and disposal (Figure 1), it is important to determine the safety and potential hazards of CNTs. To date, most toxicity studies have focused on CNT exposure via inhalation and dermal contact, and evaluated their effect on general cytotoxicity [7–8,11]. In this review, we examine the various effects of CNT exposure on cellular behaviors as a result of pulmonary and dermal CNT exposure. We discuss the potential linkage of these cellular behaviors to the disease process and examine the underlying mechanisms.

## Pulmonary exposure to CNTs

The major target organ of CNT exposure is the lung via inhalation. Inhalation exposure to CNTs has been evaluated in US research facilities with the report of average airborne CNT level of 10 $\mu g/m^3$ [31] and high CNT level of 400 $\mu g/m^3$ [32]. Although no reports on the adverse health effects of CNTs are available at present, due to the early developmental stage of CNTs and their usage, the National Institute for Occupational Safety and Health (NIOSH) has recently recommended the exposure limit (REL) of 1 $\mu g/m^3$ of respirable element carbon as an 8-h time-weighted average (TWA). This recommendation is based on the current estimation from animal studies, which have been conducted using instillation, aspiration, and inhalation techniques to enable CNT exposure of rats and mice [33]. These studies have shown consistent adverse pulmonary effects, including lung inflammation, granuloma and fibrosis. The important findings of CNT pulmonary effects are described below.

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 $mg/m^3$ 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 $mg/m^3$ MWCNT for 6 h, but not to lower doses, for example 1 $mg/m^3$ [41]. Mitchell *et al.* reported neither significant lung inflammation nor fibrosis upon 0.3–5 $mg/m^3$ MWCNT inhalation (6 h/day for 14 days), although splenic immunosuppression was observed likely through an activation of cyclooxygenase at 1 $mg/m^3$ dose [42,43]. In mice with allergic asthma sensitized by ovalbumin, 100 $mg/m^3$ 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 $mg/m^3$ 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 $F_1$ 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 μg/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-κB, all of which recapitulate the key molecular signaling involved in lung carcinogenesis. A similar finding on MAPK, AP-1 and NF-κB activation by SWCNT (25–50 μg/cm$^2$) was observed in normal human and malignant mesothelial cells [56]. Notably, pristine graphene sheets (5–80 μg/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 μg/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 μg/ml) for 4 days caused a large drop in Calu-3 TEER and a parallel increase in mannitol permeability, while SWCNT (100 μg/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-1β, 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-Rassmussen *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-1β, 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-1β, TGF-β and PDGF, while those with neutral and weak cationic functionalized amine ($NH_2$) and sidewall $NH_2$ 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-1β, TNF-α, IL-6 and chemokine IL-8 from Met5A pleural mesothelial cells and THP-1 macrophages [17]. The results showed that MWCNT (5 μg/$cm^2$) 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 μg/$cm^2$) 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 μg/$cm^2$) 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 μg/ml) induced the secretion of angiogenic mediator TGF-β1 and VEGF in a dose-dependent manner [59]. These mediators from the lung fibroblasts stimulated angiogenesis of HUVEC endothelial cells. TGF-β1 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 μg/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 μg/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 μg/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., PPARγ, 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 μg/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 μg/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.

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**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.

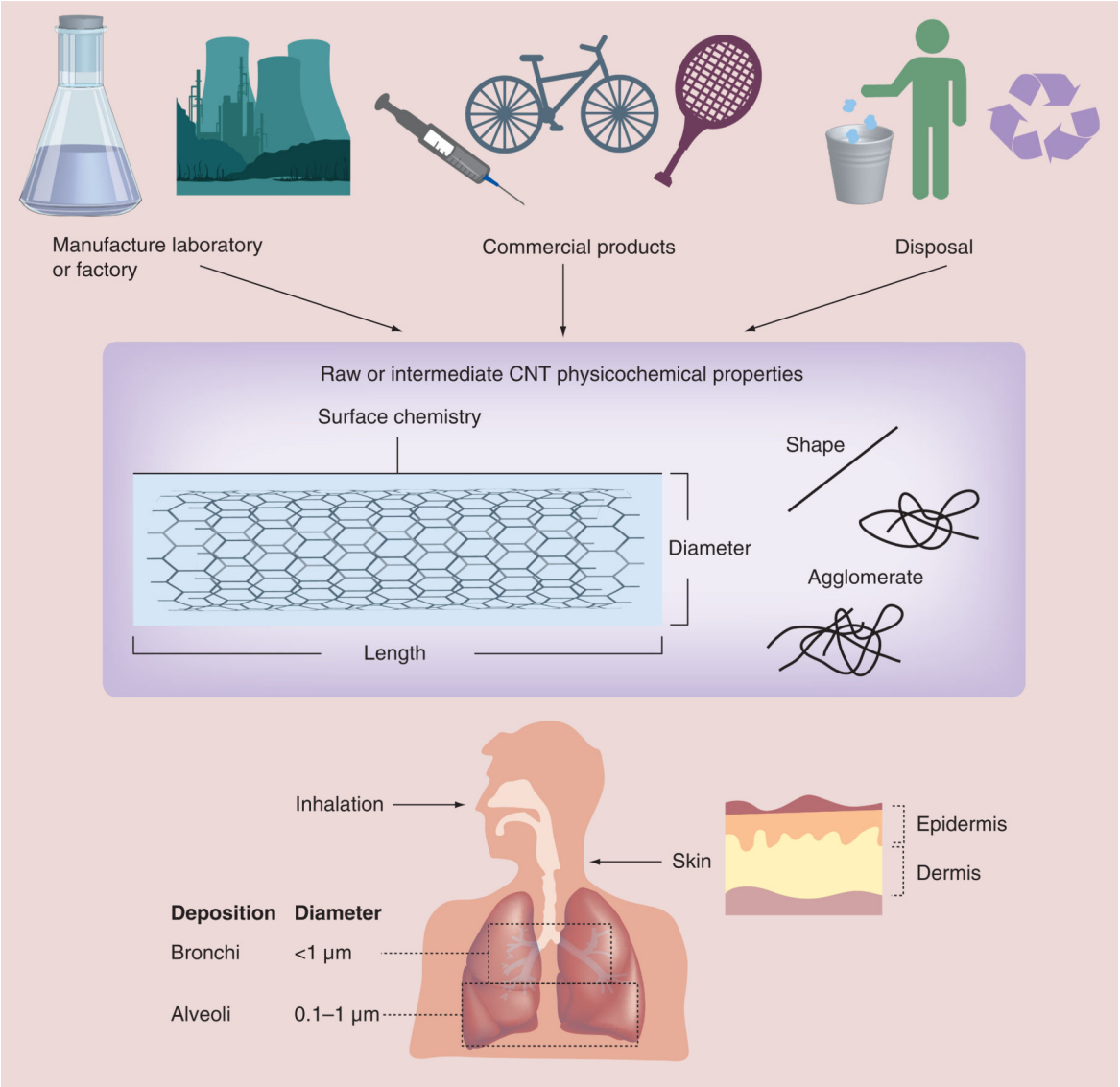


**Figure 1.**

Sources of human carbon nanotubeexposure and physicochemical factors influencingcarbon nanotube bioactivities.

CNT: Carbon nanotube.

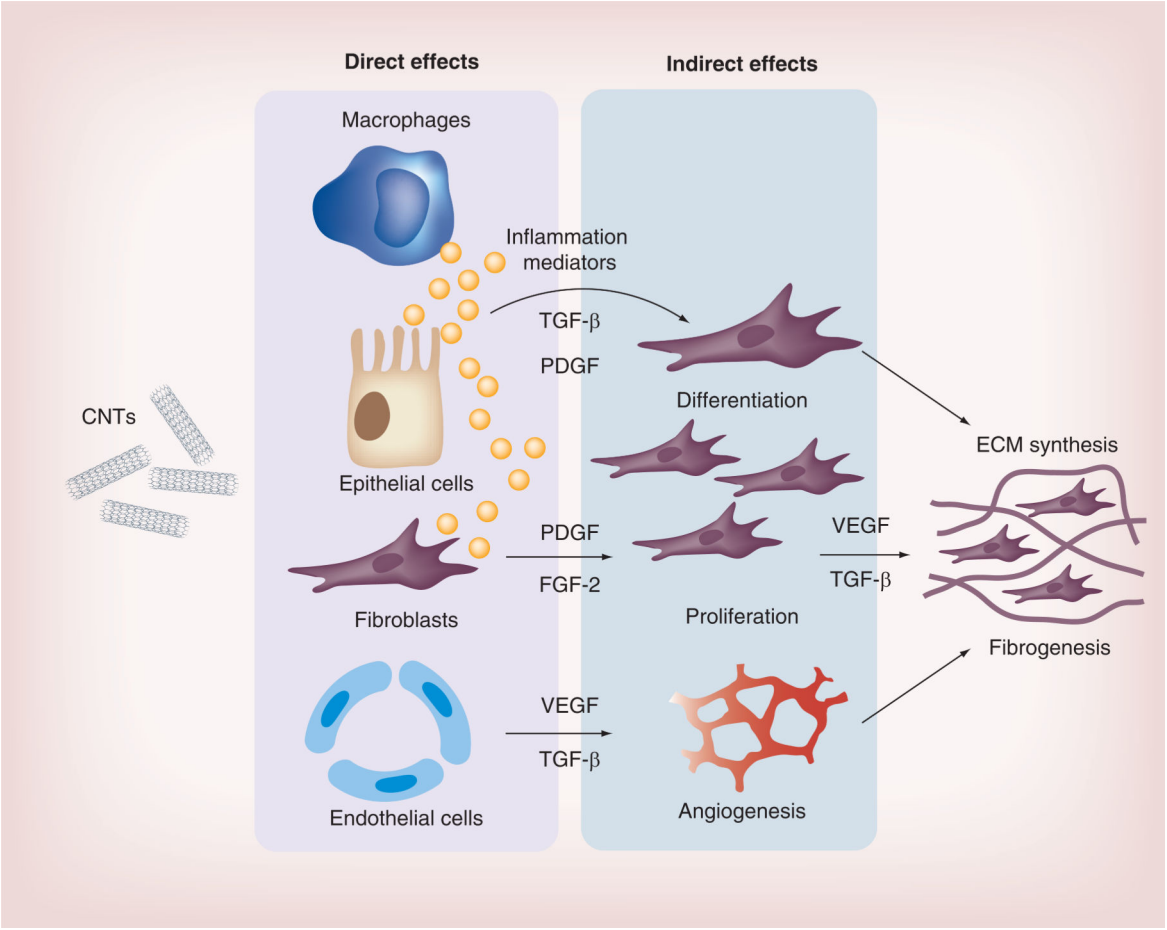


**Figure 2.**

Various cellular behaviors involved in carbon nanotube-induced lung fibrosis.

CNT: Carbon nanotube; ECM: Extracellular matrix.

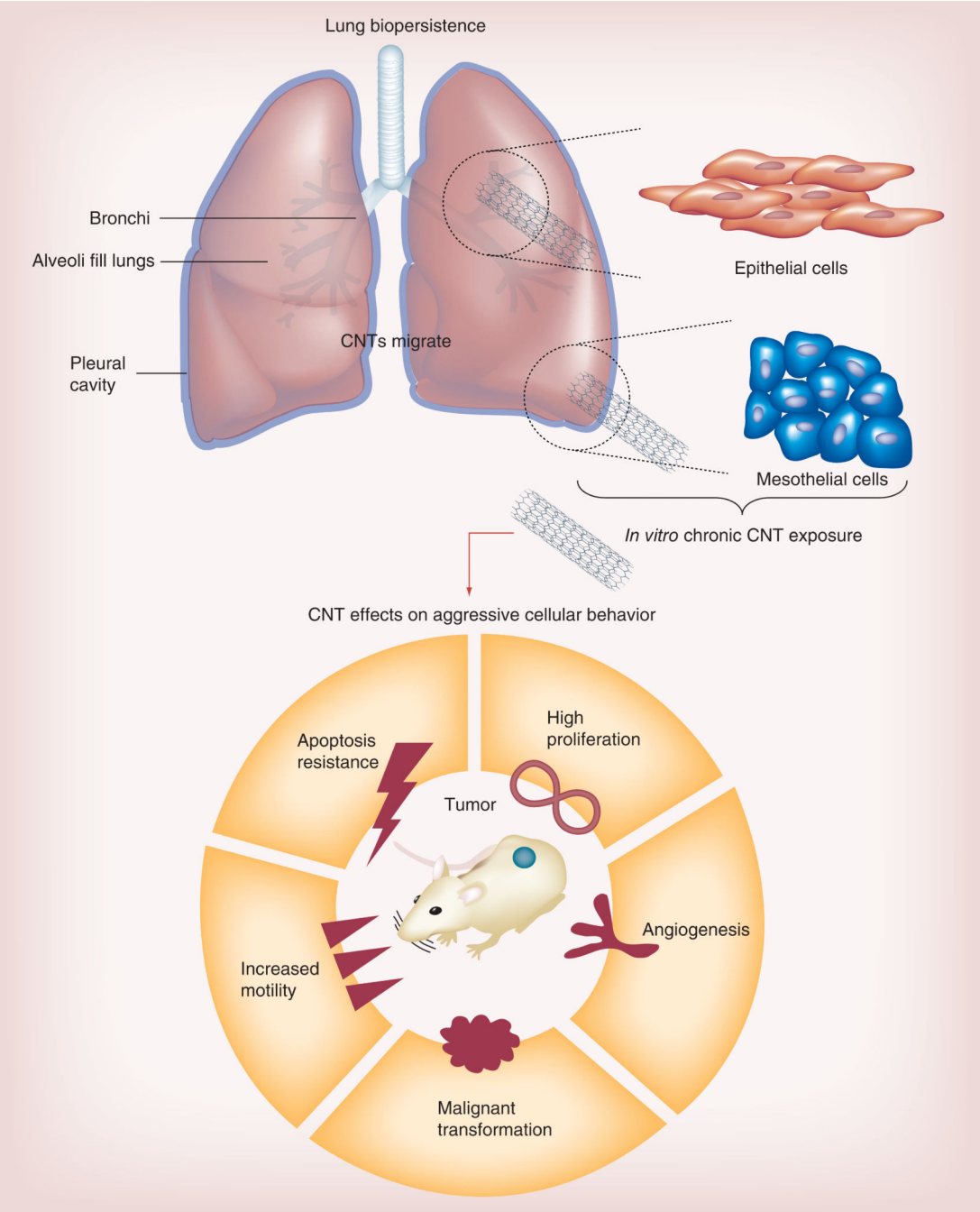


**Figure 3.**

Lung models for carcinogenesis studies and chronic effects of carbon nanotube exposure on aggressive cellular behaviors.

CNT: Carbon nanotube.

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**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, inflammogenic 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 NRLP3 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 mm; 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] |

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| 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 agglome-rates; 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] |
| SWNCT | CNI, Inc. | WI38; 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 *in vitro* and *in vivo* | [29] |
| **Extrinsic factors: dose/cell type** | | | | | | | |
| SWCNT | Own making | HaCaT; HeLa; A549; H1299 | 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 1; Collagen 1; D: Diameter: DCF: Dichlorofluorescin; DMF: Dimetylformamide; 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: Tetrametylrhodamine, ethyl ester.

## Table 2

Lung 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.