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Carcinogenic Potential of High Aspect Ratio Carbon Nanomaterials

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Abstract

Engineered nanomaterials, including high aspect ratio carbon nanomaterials, are often commercialized without a complete human risk assessment and safety evaluation. A health concern has been raised that high aspect ratio nanomaterials such as carbon nanotubes may cause unintended health consequences, such as asbestos-like lung cancer and mesothelioma, when chronically inhaled. Considering the widespread industrial and clinical applications and the increasing incidence of human exposure to nanomaterials, it is important to address the issue of nanomaterial carcinogenicity in a timely manner. This review summarizes recent advances in nanomaterial genotoxicity and carcinogenicity with a focus on high aspect ratio carbon nanotubes, and discusses current knowledge gaps and future research directions.

Keywords

carbon nanotubes; carcinogenicity; genotoxicity; pulmonary exposure

Introduction

Engineered nanomaterials (ENMs) possess novel and unique physicochemical properties that provide technical advantages over conventional materials in the fields of electronics, energy, waste treatment, biosensors, and medicine. Most benefits of ENMs arise from the ability to engineer the essential structures of nanomaterials to achieve specific properties. High aspect ratio nanomaterials (HARNs), most notably carbon nanotubes (CNTs), represent a major class of ENMs that are being used in diverse fields, including energy storage, sporting goods, textiles, drug delivery, and biomedical imaging. Due to their diverse applications, CNTs are among the fastest growing groups of HARNs.^{1,2} The potential for

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adverse health effects of CNTs and other HARNs, e.g. nanorods, nanowires and nanofibers, have been raised based on their characteristics that conform to the fiber pathogenicity paradigm, which specifies the importance of fiber morphology, durability, and biopersistence on the pathogenicity of HARNs.³ Specifically, CNT health risks are attributable to their high aspect ratio and nanosized structure, as well as their mode of exposure (e.g. inhalation) and graphitic nature that contributes to their high durability and biopersistence.^{3,4} Additionally, synthesis of CNTs, either by evaporation, vapor deposition, or laser ablation, can leave residual metal catalysts, e.g. cobalt, iron, nickel, molybdenum, and contaminated amorphous carbon and fullerenes that may exert toxicity.⁵ In this review, we provide an overview of recent findings on nanomaterial carcinogenicity and associated molecular and cellular mechanisms, and discuss how current knowledge can be applied to future risk assessments of ENMs as well as current knowledge gaps and future research directions.

Pulmonary exposure to CNTs and long-term health risks

Documented and expected CNT exposures include occupational exposure, i.e. in research laboratories and production facilities, and household exposure, i.e. from consumer goods and end-of-life activities including recycling and waste disposal.⁶ Unexpectedly, CNTs were found in the dust samples collected from the tragic World Trade Center incident.⁷ Similar to pathogenic fibers, the major target organ of CNT exposure is the lung via inhalation due to the extremely small size that is respirable to the lung alveolar region. Average airborne CNT level in US research facilities was reported at 10 $\mu\text{g}/\text{m}^3$,⁸ while the high CNT level was at 400 $\mu\text{g}/\text{m}^3$.⁹ Human extrapolation to lifetime cumulative worker exposure was estimated based on an average airborne CNT exposure of 10 $\mu\text{g}/\text{m}^3$ for 8 hours per day with a minute ventilation of 20 L/minute (31% sitting and 69% light exercise) and 4.07 μg per day alveolar deposition.⁸ Animal studies have shown that CNTs could deposit and be retained in the deep alveolar tissue for up to 336 days post-exposure,¹⁰ thus potential long-term health effects such as carcinogenesis might be expected. Although no reports on the direct CNT adverse health effects in humans are available at present, the National Institute for Occupational Safety and Health (NIOSH) issued a recommended exposure limit (REL) of 1 $\mu\text{g}/\text{m}^3$ of respirable elemental carbon for an 8-hour time-weighted average (TWA) based on current acute and chronic toxicological data that was collected from mice and rats using instillation, aspiration, and inhalation techniques to enable CNT exposure.¹¹ These studies have shown consistent, adverse pulmonary effects upon CNT exposure, including lung inflammation, granuloma formation, and fibrosis.

Overview of carcinogenesis

Carcinogenesis is a multiple step process consisting of three major stages for tumor growth: initiation, promotion, and progression.^{12,13} A *carcinogen* is defined as any substance that directly or indirectly affects one or all of these stages, which may be due to the ability to damage the genome or alter cellular metabolic processes. A *genotoxic substance* induces damage to genetic material, e.g. DNA, RNA, or chromosomes, which may cause mutations or aneuploidy that may subsequently lead to cancer. Notably, while all mutagens are genotoxic, not all genotoxic substances are mutagenic and carcinogenic. A *co-carcinogen* is a substance that is not carcinogenic by itself, but has the capability to promote the effect of a

carcinogen, i.e. by enhancing the cellular proliferation or disturbing the immune system.^{14–16} To aid in the risk assessment of ENM carcinogenicity, we provide an overview of the current carcinogenesis paradigm *in vivo* and *in vitro* (Figure 1).

Genotoxicity might eventually lead to carcinogenesis – repeated exposure to a genotoxin might increase mutation frequency, resulting in genome instability that can be one of the initiating events leading to carcinogenesis, i.e. by altering programmed cell death and cell survival signaling.¹⁵ Recent evidence indicates that epigenetics, heritable changes in gene expression that are not accompanied by changes in gene sequence, can also serve as a tumor-initiating event. For example, global hypomethylation of DNA was identified in various human cancers relative to control normal tissues, while DNA hypermethylation was frequently observed in the promoter region of tumor suppressor genes resulting in the silencing of their expression.^{17,18}

Tumor promotion involves the selective clonal expansion of initiated cells that generally exhibit an inability to repair DNA damage, enhanced cell proliferation and resistance to cell death, including those induced by tumor suppression factors.¹⁹ Because the accumulation rate of mutations is proportional to the rate of cell division, a greater proportion of cells that are at risk of further genetic changes and malignant transformation, such as pre-neoplastic or benign daughter cells, are being produced.²⁰ Cells that undergo tumor promotion will either a) advance towards progression and eventual cancer, b) continue to grow with no signs of aggression, c) persist without further phenotypic advancement or reversal, or d) regress.²¹ Substances that are capable of reducing the latency period of tumor formation after exposure to a tumor initiator and/or increasing the number of tumors formed are considered *tumor promoters*, whereas any substances that cause both tumor initiation and promotion are known as *complete carcinogens*.²⁰

Malignant transformation describes the transformation of pre-neoplastic cells into cells that express a malignant phenotype, which is characterized by an acquisition of cancer hallmarks such as accelerated cell growth, resistance to cell death, replicative immortality, angiogenesis capability, and increased cell migration and invasion (*neoplastic transformation*), together with *in vivo* tumorigenic capability.²²

The last stage of carcinogenesis is tumor progression, which involves the acquisition of more aggressive tumor behavior over time that ultimately lead to a formation of secondary tumors at distant sites, also known as metastasis. Malignant tumors possess multiple heritable genetic and epigenetic changes, including activation of proto-oncogenes and functional loss of tumor suppressor genes.^{11,20} Several substances may not cause tumor initiation or promotion but progression, and early disease screening assays or models may not detect this phenomenon. Assessment of ENM carcinogenesis must evaluate each major step of the process using appropriate initiation, promotion, and progression models.

Challenges in ENM carcinogenesis studies

As mentioned above, carcinogenesis is a multistep process that requires long-term exposure, especially to low-dose carcinogens or environmental agents. For example, a typical

developmental period of fiber-induced lung cancer in humans is at least 15 years and even longer, i.e. over 30–40 years, for mesothelioma.²³ Analysis of mesothelioma mortality revealed that occupational workers in the construction industry were at greatest risk due to the presence of massive asbestos in the building materials during the 1950s to 1970s, and although the use of asbestos was banned in the 1980s, mesothelioma incidence would continue to increase perhaps until 2020.²⁴ Establishing methodologies that reliably predict human health risks associated with ENM exposure, i.e. with appropriate dosing, exposure time and route of exposure resembling real-life ENM exposure scenarios, are challenging. Concerns over the great variety of ENMs with different purities and physicochemical properties such as length, diameter, functionalization, and surface chemistry, high-throughput screening assays for evaluating cancer and mesothelioma pathogenicity are greatly needed, as they would aid in the identification of key contributing factors to pathogenicity and the development of safer nanomaterials.

Accumulating evidence indicates that tumors are heterogeneous in cell composition comprising of many different cell types, including normal tumor cells, cancer stem cells (CSCs), and many other cell types in the tumor microenvironment such as cancer-associated fibroblasts, endothelial cells, and immune cells.^{25–27} Thus, studying carcinogenesis and assessing cancer risks can be challenging due to the complexity of tumor biology. Currently, a transition is underway to include different cell types and to evaluate carcinogenesis at the tissue and systemic levels.

Carcinogenic potential of CNTs: *in vivo* assessment

Instillation, aspiration, and inhalation studies

Early CNT toxicity studies employing intratracheal (i.t.) administration and pharyngeal aspiration to enable pulmonary CNT delivery provide initial evidence to support the potential carcinogenicity of CNTs. One of the first studies showed that a single i.t. instillation of single-walled (SW) CNTs induced tumor-associated pathologies including epithelioid granulomas in association with particle aggregates and lung inflammation in a dose- and time-dependent manner in both mice and rats.^{28,29} Later, pharyngeal aspiration of SWCNT was reported to induce an unusual acute inflammatory response followed by an early onset of progressive interstitial fibrosis and alveolar wall thickening at 28 days post-exposure onwards.³⁰ Further studies reported the importance of fiber length on such fibrogenic effects, i.e. long SWCNTs (~12.5 µm in length) were more potent than short SWCNTs (~1 µm).³¹ Similar observations on lung inflammation and fibrosis were made in mice and rats exposed to multi-walled (MW) CNTs via pharyngeal and i.t. administration.^{32–34} In addition, lung inflammation and fibrosis have been associated with an increased risk for lung cancer,^{35,36} thus suggesting a potential early carcinogenic event accompanying CNT exposure.

To avoid the potential artifacts associated with the bolus delivery of nanomaterials to the lung, inhalation studies have been conducted. For example, one short-term inhalation (nose-only) study in mice using 5 mg/m³ SWCNTs at 5 hours/day for 4 days revealed a robust inflammatory response, oxidative stress, collagen deposition, and fibrosis at a greater extent than pharyngeal aspiration at 10 µg/mouse.³⁷ Analysis of CNT genotoxicity by measuring

proto-oncogene *K-ras* mutations, a frequent occurrence in lung tumors, showed a much higher mutation frequency in mice receiving SWCNTs by inhalation (62.5%) compared to inhalation of air (26.7%) or SWCNT aspiration (12.5%). The specific roles of *K-ras* mutations in fibrosis and fibrosis-associated malignancies are unclear and need further elucidation for risk assessment and pathologic intervention. A single inhalation (nose-only) exposure to high-concentration MWCNTs (30 mg/m³) for 6 hours similarly induced lung fibrosis in mice, however, an inhalation (whole-body) exposure of MWCNTs (5 mg/m³) at 6 hours/day for 14 days had no significant effect on lung inflammation or fibrosis.^{38–40} A sub-chronic inhalation (nose-only) exposure of MWCNTs (1.5–6 mg/m³) at 6 hours/day, 5 days/week, for 13 weeks in Wistar rats induced a sustained elevation of neutrophils in bronchoalveolar lavage, suggesting an inflammatory event that would become detectable after sustained exposure.⁴¹ Similar delayed inflammation was reported in another inhalation (nose-only) study in Wistar rats that utilized a single inhalation (nose-only) exposure of MWCNTs, followed by a 3-month post-exposure period to study the effects from long-term sequelae of retained MWCNTs.⁴² Using a mouse model of allergic asthma, Ryman-Rasmussen *et al.* suggested that inhaled MWCNTs require pre-existing inflammation to cause airway fibrosis, and thus extra precautions should be taken on CNT inhalation in individuals with pre-existing allergic inflammation.⁴³

A recent CNT inhalation study was conducted using a classic multistage initiation-promotion carcinogenesis model. Briefly, B6C3F1 mice were intraperitoneally (i.p.) injected with the DNA damaging agent methylcholanthrene (MCA), which served as an initiator, followed by a whole-body inhalation of 5 mg/m³ MWCNT (Mitsui #7) or filtered air (control) at 5 hours/day, 5 days a week, for 15 days.⁴⁴ The results showed that mice that received MCA and MWCNT together exhibited an increased incidence and number of bronchoalveolar adenomas and adenocarcinomas at 17 months post-exposure as compared to air, MCA, or MWCNT treatment alone, i.e. 62% vs. 13%, 22%, or 14%, respectively. Interestingly, 9% of mice exposed to MCA and MWCNT exhibited serosal tumors that were morphologically consistent with sarcomatous mesothelioma. These findings indicate MWCNTs (Mitsui #7) as a lung tumor promoter, but not a tumor initiator, in mice.

Although inhalation of nanomaterials represents physiological deposition of aerosolized materials over an extended period of time, important things to be taken into considerations are interspecies differences between humans and rodents or other experimental animal models with respect to the inhalability, respirability and lung burden of CNTs. While CNTs with an aerodynamic size below 10 µm are inhalable in humans, they may not be inhalable in rodents, making the subsequent delivered doses to their lower respiratory tract highly variable.⁴⁵ Long CNTs and agglomerates that present in the workplace aerosol are less likely to be respirable by rodents, and thus adjustment of the experimental aerosol is needed to achieve comparable lung burdens in rodents. Other complications in the rodent inhalation experiments include its complex settings, high cost of maintenance and laboriousness, especially for long-term studies.

Mesothelioma pathogenicity studies

Mesothelioma pathogenicity screening was typically performed using i.p. injection studies exposing pathogenic fibers to the mesothelial linings of the abdominal cavity in mice or rats in order to resemble the exposure to mesothelial linings of the pleural cavity surrounding the lungs.⁴⁶ In one of the early studies, a single i.p. injection of long (>15 μm) MWCNTs, but not short, tangled MWCNTs, was shown to induce asbestos-like inflammation and granuloma formation at 7 days post-exposure, suggesting the potential mesothelioma pathogenicity of CNTs.⁴⁷ Subsequent studies also showed that a single i.p. injection of MWCNTs (Mitsui #7) caused a dose-dependent induction of mesothelioma in heterozygous p53 knockout mice, which have a genetic background of spontaneous tumors.^{48,49} The observations that the onset of mesothelioma was not dose-dependent and the reactive mesothelial cells were not accompanied by epitheloid cell granulomas or fibrous scars, but by mononuclear inflammatory cells with MWCNT fibers, suggest frustrated phagocytosis as a mechanism of CNT-induced carcinogenesis.⁴⁹ The mesothelioma pathogenicity of MWCNTs was also later observed by Nagai *et al.* in a non-cancer-prone rodent model of Fischer-344/Brown-Norway F hybrid rats.⁵⁰ One month following a single i.p. injection of thin and rigid MWCNTs (diameter ~50 nm), fibrosis was observed around most i.p. exposed organs, while thick (diameter ~150 nm) and tangled (diameter ~2–20 nm) MWCNTs were less pathogenic. A one-year follow-up study found that thin and rigid MWCNTs caused mesothelioma that harbored Cdkn2a/2b homozygous deletions, a common feature in human and rodent mesothelioma, likely due to their ability to pierce and injure mesothelial cells along with macrophage activation. The proposed mechanisms of CNT-induced mesothelioma are summarized in Figure 2 in comparison to asbestos fiber, which shows a distinct cellular entry mechanism into mesothelial cells.

A large, two-year study was conducted in Wistar rats (50 per group) comparing the effects of i.p. injected MWCNTs with different lengths, diameters, and curvatures at the WHO-recommended dimensions for carcinogenicity testing of mineral fibers.⁵¹ Consistent with the earlier studies in p53 heterozygous mice showing the mesothelioma-inducing effect of needle-like MWCNTs (Mitsui #7), this two-year study reported that custom-made, needle-like MWCNTs (diameter ~60–85 nm) induced mesothelioma with more than 80% morbidity within a year. A slower tumor onset and lower morbidity rate were observed in MWCNTs with a smaller diameter (40 nm) and more bent structure (curved and narrow), with 80% morbidity after 16 and 23 months, respectively. This result suggests that in addition to aspect ratio, curvature seems to be an important factor determining the carcinogenicity of CNTs. It should be noted that negative findings for CNT mesothelioma pathogenicity have also been reported.^{52,53} For example, Muller *et al.* observed no apparent mesothelioma induction after an i.p. injection of short MWCNTs (<1 μm) in rats over a period of two years.⁵²

The pathogenicity of CNTs was also evaluated in rats receiving multiple doses of intratracheally instilled MWCNTs over a 9-day period. MWCNTs were mainly found in alveolar macrophages and mediastinal lymph nodes, as well as in macrophages residing in the pleural cavity lavage.⁵⁴ It was further found that MWCNTs, like asbestos, induced mesothelial hyperplasia, which was associated with macrophage infiltration and inflammation-associated fibrosis, and likely linked to mesothelioma development. A follow-

up study by the same group highlighted the importance of size and shape of MWCNTs on mesothelial cell proliferation, i.e. long, needle-like MWCNTs (length 8 μm , diameter 150 nm), but not small, tangled MWCNTs (length 3 μm , diameter 15 nm) can translocate into the pleural mesothelium and induce inflammation and hyperplasia.⁵⁵ This study supports the growing hypothesis that tangled nanotubes behave like granular particles with few free single CNTs available to induce mesothelioma.

For the pleural cavity, stomata are the main portal to the lymphatics, making them the primary route of particle clearance either by flow or after phagocytosis by macrophages.^{56,57} Short, tangled, and agglomerated CNTs are more efficiently cleared by macrophages, and thus, are less likely to be pathogenic. In contrast, long and rigid CNTs ($>5 \mu\text{m}$) are inefficiently cleared by macrophages and are restricted to the stomata, which are about 2-12 μm in diameter in humans, allowing the CNTs to interact with resident mesothelial and immune cells.⁵⁸ Given that the majority of CNTs in occupational and environmental settings possess a tangled and agglomerated morphology, it is still prudent to evaluate their adverse effects in spite of their limited reactivity.⁸

CNTs and the tumor microenvironment

The immune system is a key defense mechanism for the body against cancer, and it plays a vital role in identifying and eliminating transformed or damaged cells.⁵⁹ CNTs can induce oxidative stress and inflammation that can interfere with the immune responses, leading to an enhanced susceptibility to pathogenic agents.⁶⁰ Previous studies showed that a single pharyngeal aspiration of short, pristine SWCNTs ($\sim 230 \text{ nm}$ in length) at a dose of 80 $\mu\text{g}/\text{mouse}$, which was deemed a non-carcinogen,⁵⁰ modulated the lung tumor microenvironment that resulted in an increase in the growth of transplanted metastatic Lewis lung carcinoma (LCC).⁶¹ Specifically, pristine SWCNTs induced a marked acute accumulation of myeloid-derived suppressor cells (MDSCs), which are one of the main cell populations responsible for the regulation of host immune responses to tumors by inhibiting antitumor immunity and stimulating neo-angiogenesis. Depletion of MDSCs by anti-Gr-1 antibody completely blocked SWCNT-induced tumor growth and lung metastasis, thus supporting the importance of MDSCs in CNT-enhanced lung metastasis. It was further found that MDSC-derived TGF- β signaling was critical to SWCNT-induced immunosuppression through the inhibition of T-cell activation and proliferation.⁶² These findings could lead to the development of screening platforms for evaluating the CNT effects on tumor microenvironment.

Carcinogenic potential of CNTs: *in vitro* assessment

In vitro models offer the advantages of genetic or pharmacologic manipulations that provide insights into the disease mechanisms and facilitate the development of rapid high-throughput risk assessment platforms at lower costs. Various cell types have been used to assess the carcinogenic potential of CNTs based on their disease relevance, including (i) lung epithelial cells such as alveolar type II cells, bronchial epithelial cells, and small airway epithelial cells (SAECs), which are key targets of CNT exposure and lung carcinogenesis, (ii) plural mesothelial cells to investigate the cellular events controlling mesothelioma, and (iii)

neighboring cells that comprise the tumor microenvironment including fibroblasts and immune cells such as macrophages and dendritic cells.

Acute pulmonary toxicology studies

Cancer is generally accepted as a disease of the genome, and thus genotoxicity is one of the most common assessment parameters for the CNT effects with the goal of predicting cancer risks from genetic mutation data.⁶³ Remarkably, *in vitro* genotoxicity of pathogenic fibers by means of structural chromosomal aberrations was strongly correlated with their *in vivo* carcinogenicity.^{64,65} Several other assays have been used for CNT genotoxicity testing *in vitro*, including Comet (DNA damage), chromosomal, micronucleus and phosphorylated H2AX assays, which detect DNA damage and repair.⁶³ A wide range of human cell lines, including normal lung epithelial BEAS-2B and SAEC cells, malignant alveolar epithelial A549 cells, normal and malignant mesothelial cells, fibroblasts and RAW264.7 macrophages, all showed positive genotoxicity to high-dose (>50 to 100 µg/mL) SWCNT and MWCNT treatment, mostly within 24 hours. It was reported that CNTs mimic microtubules and disrupt their functions and dynamics. CNTs bind to DNA at G-C rich regions in the chromosomes including telomeric DNA, resulting in conformational change and chromosomal fragmentation.^{66,67}

Recent studies were conducted to evaluate the genotoxic effects of SWCNTs and MWCNTs at relatively low (surface area) doses (0.024 to 24 µg/cm²) to recapitulate real world exposure scenarios. The low dose of 0.024 µg/cm² is equivalent to a 4-week exposure to the Occupational Safety and Health Administration (OSHA) permissible limit of 5 mg/m³ for particles with an aerodynamic diameter of 5 µm or less, or 34 years of exposure at the NIOSH recommended exposure limit of 1 µg/m³. Similar to high dose exposures, low-dose SWCNT and MWCNT exposures were shown to cause centrosome fragmentation, disruption of mitotic spindle, and aneuploidy in lung epithelial BEAS-2B and primary SAEC cells at 24 hours post-exposure, resembling the effects caused by vanadium pentoxide, a known human carcinogen.^{68,69} In mitotic cells, SWCNTs and MWCNTs were shown to associate with microtubules, DNA, and within the centrosome structure, which may lead to failed cytokinesis and a gain of chromosomal material (polyploidy), possibly due to the change in elasticity of the mitotic apparatus. Although the majority of cells were polyploidy, a loss of chromosome was also observed in a significant number of cells, suggesting that the genetic damage was due to more than a failure of cytokinesis. Functionally, a dramatic increase in colony formation, an indicator of clonal growth and neoplastic transformation, was observed in BEAS-2B cells upon SWCNT exposure, and in SAEC cells upon MWCNT exposure. Different mechanisms of SWCNT and MWCNT genotoxicity were suggested, i.e. SWCNTs caused G2 cell cycle arrest and monopolar spindle formation, while MWCNTs caused G1/S blockage and multipolar spindle formation. These microtubule alterations could have an influence on many cellular processes, including cellular transport of organelles, RNA and protein transport, phagocytosis and cell movement, as well as various cellular signaling pathways.⁷⁰

Chronic pulmonary toxicology studies

In an effort to mimic a carcinogenic process, which is a long-term developmental process, several chronic and sub-chronic exposure models have been developed. In these models, target lung cells, e.g. epithelial cells and mesothelial cells, were continuously exposed to low-dose, physiologically relevant concentrations of CNTs for a prolonged period of time, e.g. 4 to 6 months. The exposed cells were then assessed for cancer phenotypes such as abnormal cell growth, apoptosis resistance, and increased cell migration and invasion. Initial studies in human lung epithelial BEAS-2B cells showed that chronic exposure to SWCNTs induced malignant transformation, as evidenced by accelerated cell growth, loss of contact inhibition, colony formation, and increased cell invasion and angiogenic activity.⁷¹ The transformed cells also induced tumorigenesis in mice and exhibited aberrant p53 signaling that contributed to apoptosis resistance and possibly carcinogenesis. Consistently, in an additional study investigating chronic exposure to SWCNTs, analysis of apoptosis pathways revealed that SWCNT-transformed cells exhibited a defective death receptor pathway caused by an upregulation of the anti-apoptotic cellular FLICE-inhibitory protein (c-FLIP).⁷² Whole-genome expression profiling and ontology analysis identified inhibition of apoptosis as a top-ranked cellular dysfunction mechanism ($Z = -1.96$ and -2.2 , respectively). Interestingly, activated apoptotic inhibitors *BCL2* and *BCLXL* and apoptotic activators *CYCS*, *APAF1*, *AIF* and *ENDOG* were found, suggesting that the mitochondrial pathway was still active. Downstream and upstream prediction analysis centered on *CFLAR* (c-FLIP protein) was further conducted to identify upstream regulators and downstream effectors in the SWCNT-transformed cells. Apoptotic canonical pathway analysis revealed that stimulation of FasL/TNF together with c-FLIP activation resulted in an inhibition of caspase-8, BID activity, and downstream caspase-8, resulting in the defective death receptor pathway (Figure 3). Oncogenesis pathway analysis also indicated the involvement of activated pAkt/p53/Bcl-2 signaling axis, increased Ras expression, Dsh-mediated Notch1, overexpressed Bcl-2, and downregulated *BAX* and *Noxa*.⁷³

Similar to SWCNTs, Wu *et al.* reported that chronic exposure to MWCNTs induced oncogenic transformation of lung epithelial BEAS-2B cells that were tumorigenic in mice.⁷⁴ Using a high-resolution comparative genomic hybridization technique, chromosomal aberrations in MWCNT-transformed cells at chromosome 2q31–32 harboring oncogenes *HOXD9* and *HOXD13* were revealed, suggesting that MWCNTs altered genomic stability and oncogenic copy numbers. An extension study comparing the effects of SWCNTs and MWCNTs to asbestos fiber and ultrafine carbon black (UFCB) in human SAEC cells was subsequently reported.⁷⁵ SWCNTs and MWCNTs (Mitsui #7) were found to cause excessive cell growth, neoplastic transformation, increased motility and angiogenesis to a greater extent than asbestos fibers and UFCB. Whole genome profiling and hierarchical cluster analysis demonstrated that both SWCNT- and MWCNT-transformed cells exhibited similar yet unique genome signatures that differed substantially from asbestos- and UFCB-treated cells. Gene ontology analysis revealed cell growth, cell movement, cell death and development signaling as top-ranked altered cellular functions. Cancer was a top-ranked disease and cancer-associated gene signaling networks (GSNs) were centered on upregulated *MYC*, *PPARG*, *COL18A1*, *PDGFRB* and *CASP8* in the CNT-treated cells. In asbestos-treated cells, pro-inflammatory networks under the control of *IL1B*, *CCL2* and *SPI1* were

dominant. Protein and mRNA expression analyses of the indicated cancer- and inflammation-associated genes confirmed the whole genome profiling results and specified clear differences between the CNT- and asbestos-treated cells.

Similar gene expression and functional analyses were conducted in human plural mesothelial cells to assess the mesothelioma pathogenicity of CNTs.⁷⁶ Mesothelial cells were chronically exposed to low-dose SWCNTs, MWCNTs, or asbestos, and subsequently analyzed for cancer phenotypes and altered gene expression. Increased cell proliferation and invasion were observed in SWCNT- and MWCNT-treated cells, as well as asbestos-treated cells. Functional analysis and gene knockdown experiments unveiled MMP-2, an extracellular matrix-degrading enzyme, as a key player responsible for the invasiveness of CNT- and asbestos-treated cells. Whole genome profiling indicated similar gene signatures between SWCNT- and MWCNT-treated cells. Analysis of invasion-associated GSNs confirmed that *MMP2* occupied a focal position of the GSNs in both CNT- and asbestos-treated cells. Other signaling hub genes with a first order connection to *MMP-2* in the CNT GSNs include *PLAU*, *STAT3*, *AKT*, and *VEGFA*, while the hub genes for the asbestos GSNs include overexpressed *CAV1* and *CCL2* and underexpressed *EGFR* and *TIMP2*, suggesting that although CNTs and asbestos exhibited some common gene signatures, these two materials have distinct gene signaling pathways. Further studies showed dose-dependent neoplastic transformation and increased cell invasion caused by chronic exposure to SWCNTs (0.02 to 0.2 $\mu\text{g}/\text{cm}^2$) in plural mesothelial Met5A cells and peritoneal mesothelial LP-9 cells.⁷⁷ Using gene knockdown and pharmacological inhibition strategies, H-Ras-ERK signaling was identified as a key signaling pathway in these aggressive phenotypes. Whole genome profiling indicated that *MMP2*, *PLAU*, *AKT*, *CCND1*, *ITG*, and several inflammatory and EMT-related genes (*TWIST*, *FN1*) were upregulated with first order relationship with ERK in the invasion GSN. Subsequently, integrin alpha V and cortactin were shown to be the downstream targets of H-Ras-ERK signaling in SWCNT-treated mesothelial cells.

CNTs and cancer stem cells

Increasing evidence indicates that cancer stem cells (CSCs), also known as tumor-initiating cells, are a driving force of tumor initiation and progression due to their self-renewal and tumorigenic properties.^{78,79} Our group first reported that chronic SWCNT exposure triggered normal lung epithelial BEAS-2B and SAEC cells to initiate CSC-like cells with tumorigenic properties.⁸⁰ These SWCNT-induced CSC-like cells exhibited self-renewal properties and aggressive cancer phenotypes, including spheroid formation, anchorage-independent cell growth, apoptosis resistance, increased cell invasion and migration, and most importantly, tumor initiating capability *in vivo*. These cells expressed general stem cell markers such as Nanog, SOX2 and SOX17, as well as unique stem cell surface markers such as high CD133 and low CD24, which may be used for risk assessment and early detection of CNT-induced carcinogenesis.⁸¹ GSNs of tumorigenesis and metastasis, which are important features of CSCs, indicated *CAV1* as a potential regulator of CNT-induced CSCs. Further mechanistic studies revealed a functional role of the Cav-1/p53 axis in SWCNT-induced carcinogenesis through the regulation of CSCs that overexpressed Cav-1 (Figure 4). Repression of Cav-1 upregulated p53 expression and inhibited CSC formation as well as

tumor growth in mice. The tumor-promoting effect of Cav-1 and the tumor-inhibitory effect of p53 in the SWCNT-transformed cells are similar to those observed in known human lung cancer cells,⁸¹ indicating a potential shared mechanism between laboratory-generated CNT-induced carcinogenesis and established human lung cancer.

Conclusions and perspectives

Mounting *in vitro* and *in vivo* evidence suggests that various CNTs induce genotoxicity and carcinogenicity, most notably MWCNT Mitsui #7 which has been the subject of numerous investigations. Based on currently available data, the International Agency for Research on Cancer (IARC) classified MWCNT Mitsui #7 as Group 2B – possibly carcinogenic to humans.⁸² Although there are substantial evidence for CNT carcinogenicity in experimental models that could be reasonably extrapolated to humans, the IARC concluded that these data were insufficient at the present time and therefore considered CNTs other than MWCNT Mitsui #7 as Group 3 – not classifiable as to their carcinogenicity in humans. An effective risk assessment of CNTs and other ENMs will depend on high quality data from well-characterized and well-defined nanomaterials, as well as standardized methodologies to address the impact of their physicochemical characteristics and develop quantitative structure-activity relationships (QSARs). It is also important to justify and adequately explain the exposure route, dosimetry, and experimental models employed when investigating these materials in an effort to garner whether they are relevant to human exposures and to aid in further comparisons with other available data.

Currently, there are no specific guidelines or consistent frameworks to assess the carcinogenicity of nanomaterials and the associated health risks, although some useful models and approaches have been described.^{83,84} Nonetheless, there are some general guidelines and considerations for effective carcinogenicity assessment of nanomaterials, which include the following: (i) provide thorough physicochemical characterization data to discuss conflicting results and develop QSARs, (ii) mimic real exposure conditions, especially long-term exposure, (iii) justify chosen doses and avoid particle overload, (iv) use multiple doses to create a dose-response curve, (v) evaluate stability and pharmacokinetics of nanomaterials, e.g. agglomeration and dispersion, before, during, and once internalized within exposed tissues, (vi) justify background of cells and animal models, e.g. p53 status, since they influence the experimental outcomes, (vii) include well-established positive particle controls such as crocidolite asbestos, (viii) assess carcinogenic potential at different developmental stages, e.g. on tumor initiation, promotion and progression, and (ix) validate the findings in more than one experimental model *in vitro* and *in vivo*. Since no single experimental model is likely to recapitulate the entire carcinogenic process, multiple models and tests are needed to provide a complete risk assessment of nanomaterial carcinogenicity.

In recent years, several new technologies have been developed to facilitate risk assessment of nanomaterials. For example, high-throughput screening (HTS) and automation that together with the use of a well-characterized nanomaterial library could provide enormous information on QSARs. An example study attempting to develop QSARs that determined CNT pulmonary toxicity using a small CNT library is given herein. In order to evaluate the impact of surface charge of functionalized CNTs (f-CNTs) on fibrosis, various f-MWCNTs

with anionic, cationic or neutral charge were established from the same starting materials of pristine MWCNTs and their pro-inflammatory and pro-fibrogenic activities were evaluated *in vitro* and validated *in vivo*.⁸⁵ Compared to pristine MWCNTs, strong cationic MWCNTs induced significant lung fibrosis, whereas neutral and weak cationic MWCNTs produced weaker effects. In addition, the omics and bioinformatics technologies including genomic SNPs, transcriptomics, proteomics, and metabolomics, could provide new insights into the mechanisms of nanomaterial pathogenicity. By comparing multiple *in vitro* and *in vivo* data, common mechanisms of nanomaterial carcinogenicity might be identified. For example, the critical role of the TGF- β signaling network in CNT-induced MDSC-driven immunosuppression and subsequent lung cancer promotion were unveiled through the comparative list of cytokines, chemokines, and growth factors that affect MDSC generation, recruitment, and expansion. These cytokines and chemokines, which are upregulated in CNT-exposed lung epithelial cells,³⁵ are known to facilitate lung cancer progression and metastasis. Other new technologies that are being developed to facilitate risk assessment of nanomaterials include single cell analysis, e.g. next-generation sequencing that could help determine the impact of DNA damage on mutation risk assessment, and various new cell culture models that better mimic physiological conditions such as (i) air-liquid interface (ALI) culture of respiratory epithelial cells, (ii) 3D spheroids of tumor cells with various extracellular matrix materials, (iii) microfluidic organ-on-a-chip devices for better control of fluid dynamics, and (iv) co-culture of multiple cell types including lung epithelial cells or mesothelial cells with neighboring cells such as macrophages, cancer-associated fibroblasts, and endothelial cells. With better systemic approaches and subsequent generation of data that are more relevant to human exposures in occupational and environmental settings, exposure limits for CNTs, which are important and essential tools for anticipating, recognizing and controlling CNT health risks⁸⁶ will be issued and updated by regulatory agencies.

In summary, accumulating evidence supports the carcinogenicity of certain types of CNTs, although more long-term animal and human studies are needed. As the number of nanomaterials rapidly expands, the development of fast and robust assessment tools for nanomaterial carcinogenicity testing is a pressing need. Multi-disciplinary and translational approaches must be developed through new technologies to improve the understanding on nanomaterial carcinogenicity, to identify key physicochemical properties influencing their pathogenicity, and to develop effective safe-by-design strategies. In addition, appropriate exposure control and prevention strategies must be developed and implemented to ensure safety from nanomaterial exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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In vivo carcinogenesis

Fig. 1.

Overview of *in vivo* carcinogenesis and *in vitro* cell transformation in comparison. *In vivo* carcinogenesis is a multiple step process consisting of three major stages for tumor growth, namely initiation, promotion, and progression, while *in vitro* cell transformation involves initiation, followed by neoplastic and malignant transformation.

Fig. 2.

Proposed mechanisms of MWCNT-induced mesothelioma in comparison to asbestos fiber. Asbestos and rigid MWCNTs could enter mesothelial cells, although via different mechanisms, and cause cell injury, while tangled MWCNTs could not penetrate the cells, and thus produce no or minimal cell injury. Chronic inflammation represented by frustrated macrophage phagocytosis appears to be a promoter of mesothelioma formation.

Fig. 3. Defective death receptor pathway of apoptosis in chronic SWCNT-exposed lung epithelial cells and the role of c-FLIP in the apoptosis resistance.

Fig. 4.

CNTs induce cancer stem-like cells (CSCs) and its regulation by Cav-1. CSCs are believed to be a key driver of CNT carcinogenesis due to their self-renewal and unlimited replicative capabilities. Mechanistic studies suggest that the regulation of CSCs by CNTs is mediated through p53/Cav-1 axis. Repression of p53 is a common event in human cancers and is observed in chronic CNT-exposed cells.