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REVIEW

Toxicity of Carbon Nanotubes as Anti-Tumor Drug Carriers

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Abstract: Nanoparticle drug formulations have enormous application prospects owing to achievement of targeted and sustained release drug delivery, improvement in drug solubility and reduction of adverse drug reactions. Recently, a variety of efficient drug nanometer carriers have been developed, among which carbon nanotubes (CNT) have been increasingly utilized in the field of cancer therapy. However, these nanotubes exert various toxic effects on the body due to their unique physical and chemical properties. CNT-induced toxicity is related to surface modification, degree of aggregation in vivo, and nanoparticle concentration. This review has focused on the potential toxic effects of CNTs utilized as anti-tumor drug carriers. The main modes by which CNTs enter target sites, the toxicity expressive types and the factors affecting toxicity are discussed.

Keywords: anti-tumor, cancer, CNTs, nanometer preparation, nanometer carrier, toxicity

Introduction

Malignant tumors are one of the leading causes of human disease and death, contributing to increasing mortality rates over the years.[1] All clinically available anti-cancer drugs have several limitations, such as poor stability, low bioavailability,[2] restricted targeting ability, degradation and potential drug resistance.[3–5] Although breakthroughs have been achieved in the clinical field of oncology, various treatment options have been shown to cause damage to normal cells along with eliminating tumor cells, resulting in local or systemic toxicity. Therefore, development of a targeting drug system (TDS) that allows delivery of drugs to tumors while avoiding injury to normal tissue is essential to improve therapeutic efficacy. Nanometer TDS based on the advantages of nanotechnology has developed rapidly in recent years. Nanometer-targeted preparations have successfully achieved improved drug solubility and bioavailability and specific targeting of drugs to organs or cells, allowing sustained or controlled release, prolongation of drug retention times, and more rapid and efficient drug entry through physiological barriers. These preparations have enriched the selection range of pharmaceutical dosage forms and thus attracted considerable research attention. Nanometer materials are ubiquitous in biomedical fields, including in vivo imaging,[6] cancer treatment,[7] targeted transport[8,9] and drug discovery.[10]

CNTs are a type of highly efficient nanometer TDS displaying adequate adsorption activity that have considerable potential as anticancer drugs with high selectivity for tumor sites.[11,12] Recent in vitro studies have shown that CNTs internalize into mammalian cells easily, effectively transporting molecular cargo into the cytoplasm and potentially nucleus.[13–16] CNTs mainly comprise multiple coaxial

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tubes composed of hexagonal carbon atoms. A seamless, hollow tubular novel nanometer material is rolled into a graphite sheet constituting carbon atom bonds (sp^2 hybridization).[17,18] According to the number of sp^2 hybrid carbon atoms, CNTs are subdivided into single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). Due to their unique structures, SWCNTs and MWCNTs display excellent physical, chemical, electrical and thermodynamic properties, such as ultra-high specific surface area, good adsorption ability, unique fluorescence, and Raman spectroscopy in the near-infrared region.[19,20] CNTs can convert infrared light to heat and effectively utilize the property of poor heat resistance of tumor cells. At a tumor site temperature of >42°C, cell killing phenomena are evident, such as destruction of cell membrane, denaturation of proteins and irreversible damage of tumor cells,[21,22] while normal cells remain intact. These anti-tumor effects are greatly enhanced upon coupling with anti-tumor drugs.[23,24] Combination of CNTs with inorganic materials, polymers, can be utilized as a strategy to simultaneously diagnose and treat cancers.[25–27] As a nanometer carrier type displaying high drug loading, strong targeting and easily penetrable cell membranes,[28,29] CNTs are commonly employed in multiple biomedical fields, in particular, drug delivery[30–33] and cancer treatment.[34–36]

On the other hand, the safety of clinical application of CNTs as anti-tumor drug carriers has been a subject of concern in recent years.[37] Toxic effects exerted by CNTs mainly stems from their similarities in structure to asbestos fibers.[38–40] Commonly reported toxicities include inflammatory response,[41,42] malignant mesothelioma[43,44] and biological persistence.[45,46] A recent study by Ursini et al[47] clearly demonstrated the toxicity of original MWCNTs. Moreover, functionalized MWCNTs (MWCNT-OH and MWCNT-COOH) exerted toxicity to specific cell types (e.g., human alveolar (A549) epithelial cells and normal bronchial (BEAS-2B) cells) through multiple mechanisms. However, inconsistent findings on the potential toxicity of CNTs have been obtained to date. A number of other studies have reported no damage or toxicity to normal tissue by CNTs.[48,49] Induction of toxicity by anti-tumor nanometer preparations of carrier CNTs may be attributable to the specific methods used for the experiment and related to surface modification, degree of aggregation, and nanotube concentration.

In an earlier study, Jabr-Milane and co-workers bound doxorubicin (DOX) to the SWCNT complex for targeting WiDr colon cancer cells. Upon separation of the DOX-SWCNT complex, DOX was released into the nucleus while SWCNTs remained in the cytoplasm.[50] Following injection of SWCNT into tumor-bearing mice, transmission electron microscopy (TEM) observation disclosed a large quantity of SWNT in urine of mice after 30 min. After 2 h, SWCNTs were collected from blood-rich tissues, such as liver and heart, and showed accumulation in the tumor area after 20 h.[51] Free CNTs were preferentially distributed in normal tissues, giving rise to the concern that these nanomolecules may be more toxic to normal than tumor cells.[52] This review has focused on the toxicity of CNTs used as anti-tumor carriers in terms of: (1) main routes used by CNTs to enter the target site; (2) toxicity expressive types; and (3) the factors affecting toxicity.

Entry Mechanisms of Anti-Tumor Carriers CNTs into Target Sites

Relative to the low cell permeability of macromolecules and small-molecule anticancer drugs, CNTs are considered a highly efficient novel material carrier for the delivery of anticancer drugs and diagnostic molecules.[53,54] CNTs containing antitumor drugs should be delivered to cancer cells from the site of administration. Subsequently, free CNTs are dispersed from the target site to the excretory organ.[55,56] The ability of CNTs to move forward in vivo may depend on their chemical reactivity, surface characteristics, and ability to combine with body proteins.[57,58] CNTs transport anti-tumor drugs into target cells through two principal pathways: non-energy-dependent diffusion and energy-dependent endocytic pathways[59,60] (Figure 1). The differences in access routes are related to CNT size.[61] Kan and colleagues co-cultured SWNTs of different lengths with Hep G2 cells for 5 h. Confocal imaging and flow cytometry data disclosed that the internalization of L-SWNTs into cells mainly occurred through energy-dependent endocytosis. S-SWNTs partly utilized energy-independent pathways such as diffusion across the cell membranes for cell entry.[62] The group of Imaninezhad further highlighted that integrins promote CNT entry into cells. In their experiments, CNTs were co-cultured with NIH 3T3 fibroblasts and PC12 neuron-like cells and randomly divided into two groups (one treated with an integrin inhibitor CWHM-96). Cells treated with CWHM-96 spread further and showed elongated shape while control cells with no CWHM-96 displayed round morphology. These findings confirmed that integrins promote CNT entry into cells, leading to more significant effects.

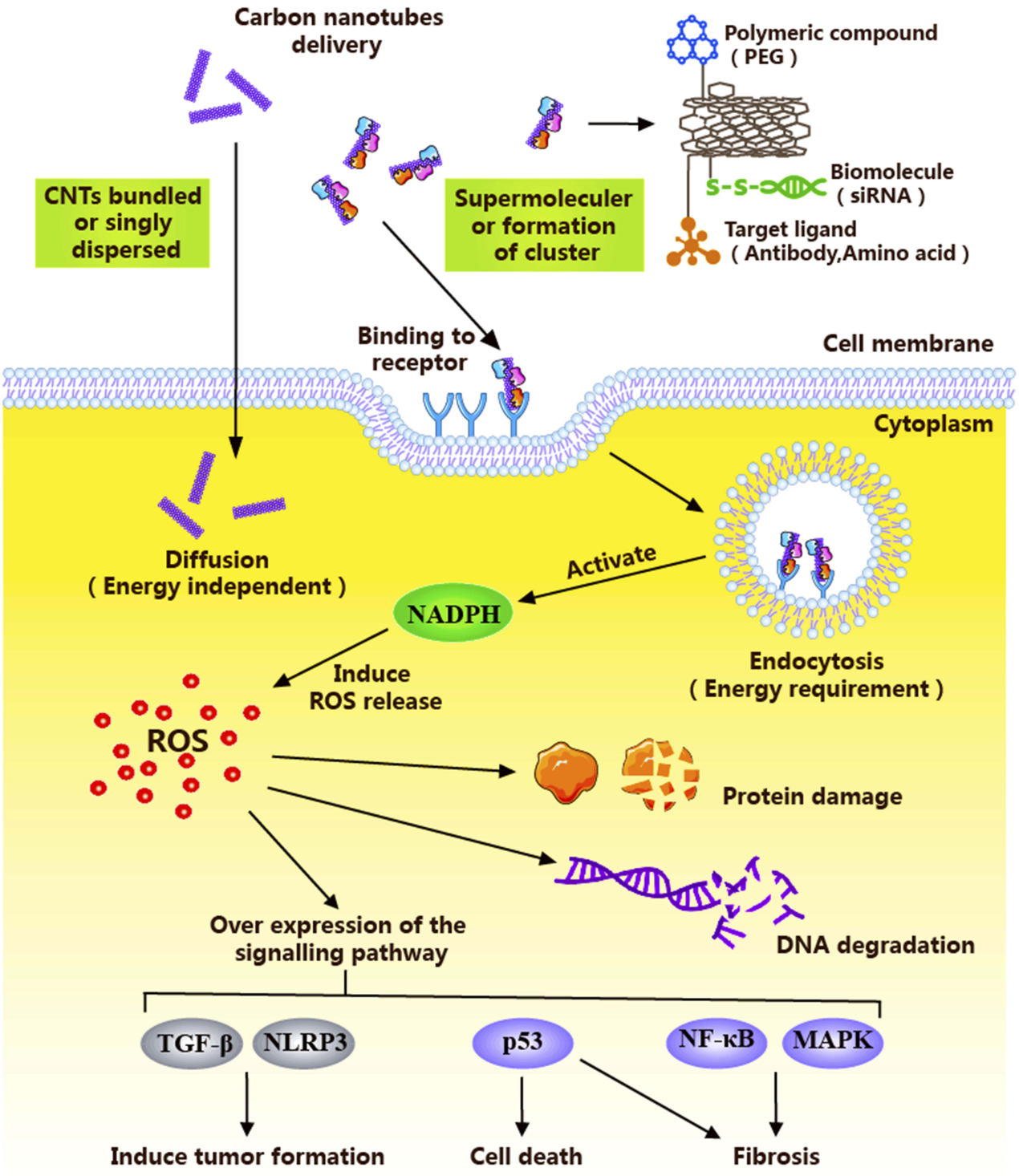

Figure 1 Schematic diagram illustrating the cells entering process of anti-tumor nanometer preparation with CNTs as a carrier.

Non-Energy-Dependent Diffusion Pathways

The length and chemical nature of CNTs are the main factors affecting entry into target cells.[63] Since the cell membrane consists of a phospholipid bilayer, CNTs in the non-energy diffusion pathway enter the cell based on their diminutive sizes (submicron-sized) and hydrophobicity. Pantarotto and co-workers[64] incubated HeLa cells with CNTs containing sodium azide (an inhibitor of energy-dependent cellular

processes). Molecular dynamics simulation revealed that cationic functional groups on the CNT surface bind the HeLa cell membrane surface, allowing spontaneous entry through cell membrane diffusion with no energy dependence. Raffa et al[65] reached a similar conclusion that functionalized CNTs can effectively enter cells through non-energy-dependent internalization pathways.

Energy-Dependent Endocytic Pathways

Supramolecular CNTs complexes enter cells in a manner dependent on energy. The CNT surface is usually loaded with bio-macromolecules, such as antibodies, amino acids and siRNAs, which are often involved in energy-dependent endocytic pathways.[66] Several studies have confirmed that CNTs enter the cell with the aid of endocytosis.[67–71] In a study by Kayo et al, endocytosis was shown to incorporate a combination of three pathways: (1) clathrin-mediated endocytosis, (2) caveolae-mediated endocytosis, and (3) macropinocytosis.[72] Lima and co-workers suggested that the endocytic pathway is divided into three stages: (1) cell membrane contact, (2) penetration of lipid head groups, and (3) entry of lipid tails. Moreover, entry of CNTs into cells depends on physicochemical characteristics, such as size and shape. CNTs with sizes ranging from 100 to 200 nm undergo clathrin-mediated endocytosis while CNTs <50 nm enter the cells through energy-independent passive diffusion.[95] In a study conducted by Kam et al[73] green fluorescence-labeled SWCNT (a) and SWCNT-biotin-green fluorescently streptavidin (b) were incubated with HL60 cells for 1 h at 37°C. Confocal microscopy revealed green fluorescence in group A and yellow fluorescence in group B (SWCNT showed green fluorescence and endosomes of red dots overlapped to produce yellow fluorescence). Their findings indicate that SWCNTs use the endocytotic pathway as a cellular uptake mechanism and accumulate in the cytoplasm after internalization. The group of Ke[74] cultured HeLa cells with AO-SWCNTs (20 mg/mL) for 30 min at 37°C (AO binds DNA to emit green fluorescence and RNA to emit red fluorescence). TEM observations revealed green fluorescence in the cytoplasm of HeLa cells. After treatment with chlorpromazine (an endocytosis inhibitor), green fluorescence was attenuated. The authors concluded that CNTs enter cells through the endocytic pathway after transporting the anticancer drug to the target site.

When CNTs enter cells into the blood circulation, phagocytic cells of the immune system (neutrophils, eosinophils and macrophages) internalize nanoparticles and NADPH oxidase is activated for accumulation on the phagolysosomal membrane. Electrons are transferred to oxygen to form superoxide (ROS).[75,76] The degradation of CNTs by neutrophils,[77] macrophages[78] and primary microglia[79] has been further investigated. CNTs are removed mainly via enzymatic degradation. Hydrogen peroxide is converted to different acids that eliminates CNTs by peroxidases, such as human myeloperoxidase,[80] the horseradish peroxidase system,[81] and catalase.[82,83] In addition, degradation of CNTs is associated with proteins, whereby binding to proteins enhances their ability to enter cells, thus promoting degradation.[84–86] The group of Karimi[87] conjugated actin to CNTs via covalent bonding, which was subsequently incubated with HeLa cells (immunofluorescence labeling) for 4 h. CNTs were indirectly modified by actin, as determined based on fluorescence intensity. Actin can generate mechanical forces to drive CNTs into the nucleus. Therefore, future studies should focus on utilization of the properties of enzymes and proteins in cells to enhance degradation of CNTs.

Oxidative stress is one of the leading causes of cytotoxicity.[88,89] During the process of CNT entry into cells for degradation, high levels of ROS are induced, leading to destruction of cellular structure and enhanced lethality against cancer cells. Homeostasis may be disrupted upon significant elevation of intracellular ROS levels. However, during the process of blood transport, CNTs are also in contact with normal cells. Excessive ROS levels can trigger DNA strand breakage,[90] protein-peptide chain disruption,[91] lipid peroxidation[92] and other macromolecular damage, eventually leading to cell death.[93,94] The main targets of CNT cytotoxicity are cell membrane, lysosome, mitochondria, nucleus and cytoskeleton (such as actin). Damage to these structures induces loss of phagocytic ability, release of ROS, and injury to normal tissues.[95,96] At the molecular level, many of the mechanisms underlying CNT toxicity involve specific cellular signaling pathways and programs. Signaling pathways activated by CNTs include NF-kB, NLRP3 inflammasome, p53, TGF-β, and MAPK.[93] However, these pathways may be involved in the development of fibrosis, leading to cytotoxicity and apoptosis of normal cells. Huaux et al[97] proposed immunosuppression as another mechanism of CNT-induced cytotoxicity. In their experiments, a specific type of CNT, Mitsui-7 CNT, was injected into the peritoneal cavity of Wistar rats and C57BL/6 mice for 12 months, and development of mesothelioma and monocytic myeloid-derived suppressor cells (M-Changes in MDSC) were examined. In the early stages

of CNT-induced mesothelioma formation, M-MDSC rapidly and continuously accumulated in the peritoneal cavity of rats, preventing tumor cell monitoring by immune cells and thereby inducing mesothelioma. The group of Shvedova additionally reported that CNT-induced lung tumor formation is associated with the upregulation of MDSC.[98] Therefore, immunosuppression presents another key mechanism underlying cytotoxicity, mainly through accumulation of MDSC and upregulation of TGF-β toxicity, promoting tumor emergence.[99]

Toxic Manifestations of Anti-Tumor Drug Carrier CNTs

Following delivery of anticancer drugs to target cells, CNTs are transported by blood to the heart, liver, lungs, kidneys,[100] brain,[101] embryo[102] and other organs (Figure 2), producing oxidative stress and causing cellular damage.[103,104] Due to their specific surface properties and small size, even purified CNTs can cause toxicity to tissues or organs. For example, after 48 h co-culture of HeLa cells with 100 μg/mL untreated and purified SWCNTs by Tsuji and colleagues, 70% HeLa cells in the untreated group displayed apoptosis relative to 40% HeLa cells in the purified group, supporting the theory that CNTs are inherently toxic irrespective of the purity of the preparation.[105] Therefore, it is necessary to consider the potential types of toxicity to body organs induced by anti-tumor nanoformulation prepared with CNTs. CNT-induced associated toxicities include hepatotoxicity,[106] lung toxicity,[107] and cardiovascular toxicity.[108]

Hepatotoxicity

Since most chemicals are metabolized by the liver, the problem of potential CNT-triggered liver toxicity should not be underestimated. Recent studies have indicated that CNTs intercepted by the reticuloendothelial system are primarily concentrated in the liver of mice.[109,110] Pathological changes caused by CNTs accumulating in the liver mainly include macrophage damage, cell swelling, non-specific inflammation, spot necrosis and blood coagulation.[111]

An earlier study by Ji et al[112] reported on the hepatotoxicity of MWCNTs. In their experiments, Kunming mice were injected with phosphate buffer saline (PBS) (10 and

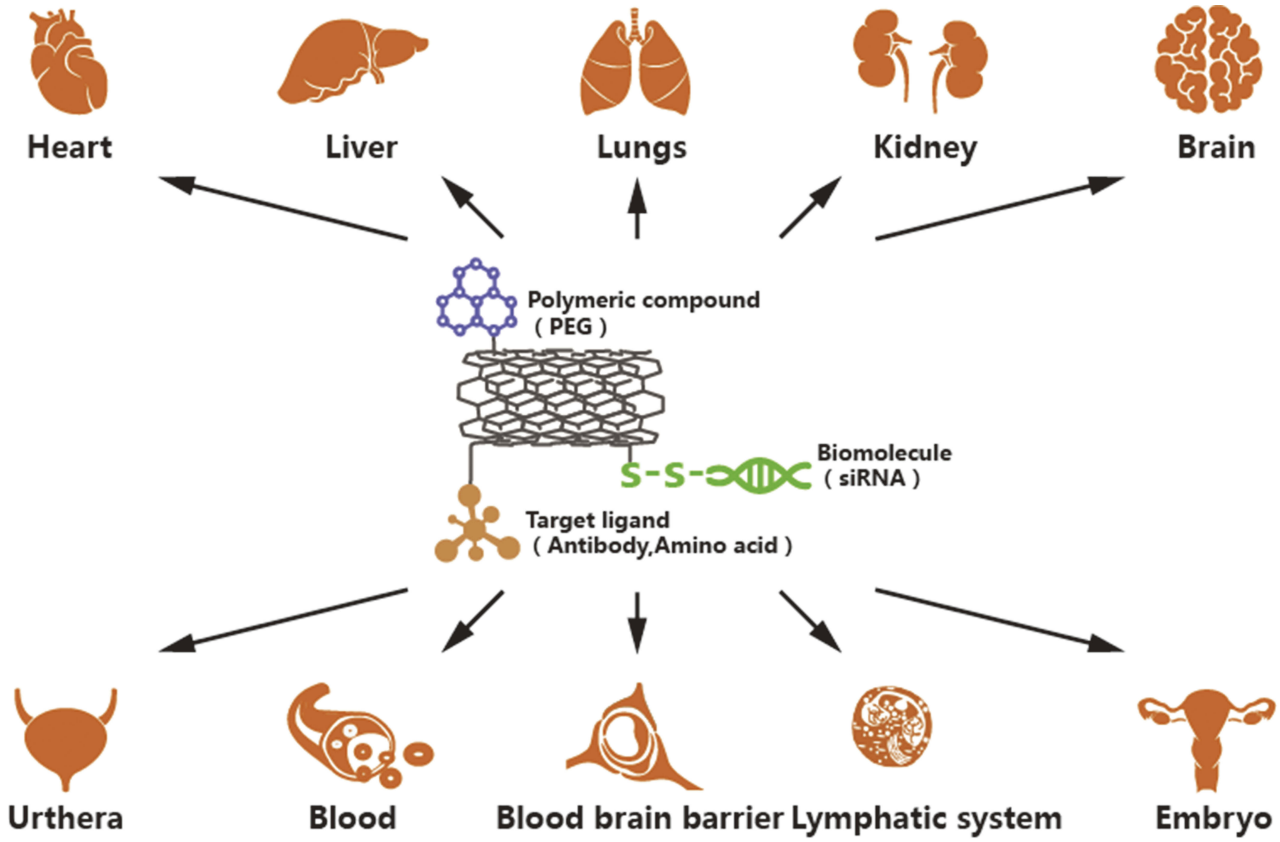

Figure 2 This figure shows the sites that CNTs may accumulate after separation from anticancer drugs, resulting in toxicity to different organs. CNTs mainly cause damage and toxicity to organs such as heart, liver, lung, kidney, brain and embryo.

60 mg/kg) and MWCNT (10 and 60 mg/kg), respectively, and changes evaluated after 15 and 60 days. Compared with the PBS group, total bilirubin and aspartate aminotransferase levels of the MWCNT group were increased in a dose-dependent manner and hepatocyte mitochondria showed swelling and dissolving. Meanwhile, partial gene expression patterns of mouse liver in the MWCNT group changed, including those associated with G protein coupled receptor, cholesterol biosynthesis, cytochrome P450 metabolism, along with Gsta2 downregulation, Cyp2B19 upregulation and Cyp2C50 downregulation. These results clearly indicated that MWCNTs cause hepatotoxicity in mice. Patlolla et al[113] intraperitoneally administered varying doses of functionalized MWCNTs (carboxyl groups) (0.25, 0.5, and 0.75 mg/kg) to mice for 5 days and examined consequent hepatotoxicity produced based on pathological features of the liver. Compared to the control group, mice exposed to functional MWCNTs showed significantly increased liver weight, hepatocyte vacuolization mucus or nuclear cohesion, hepatocyte rupture and atrophy of hepatocytes. Moreover, activities of liver enzymes (ALT/GPT and AST/GOT) in various types of serum were enhanced with functionalized MWCNT concentration, leading to the conclusion that functionalized MWCNTs induce hepatotoxicity and oxidative stress as the main toxicological mechanisms. Isaac and co-workers administered a suspension of carboxylate MWCNTs at concentrations of 0.25, 0.5, 0.75, and 1.0 mg/kg in rats for 5 consecutive days. Rats in the control group were administered normal saline plus 1% Tween-80 in a similar manner to the treatment group. Venous blood was obtained from the iliac crest and the liver function index analyzed. Notably, serum activities of aspartate AST, alanine aminotransferase (ALT), alkaline phosphatase and gamma glutamyltransferase were significantly higher in the treatment than the control group. Conversely, super superoxide dismutase and glutathione S-transferase activities as well as glutathione levels were significantly reduced. The collective results clearly suggest that carboxylate MWCNTs cause damage to the liver by destroying the antioxidant defense system.[113]

Pulmonary Toxicity

The lung is the target organ of nanoparticles and one of the pathways of nanoparticle entry into the body. Some nanoparticles are engulfed by pulmonary macrophages or absorbed by epithelial cells and finally deposited in the lungs.[114,115] A proportion of nanoparticles can also be transferred to liver, embryo, kidney and lymph nodes, causing toxic effects in other organs.[116] Nanoparticles induce significant production of ROS, which have an oxidative stress effect. Pulmonary toxicity of CNTs is mainly attributable to their similar structures to asbestos. Inhalation of asbestos fibers is known to trigger asbestosis, lung cancer and pleural malignant mesothelioma.[40] Epithelioid granulomas and small nodules have been reported in the lungs of rodents as a dose-dependent inflammation. Even purified CNTs are known to induce granuloma in the lung.[114,117–119] At the same time, even purified CNTs are known to induce granuloma in the lung.[41,120] Anti-tumor nanoparticles used as therapy for lung cancer reach the organ and effectively act on cancer cells but can additionally exert toxic effects on normal cells. The group of Chou divided ICR mice into two groups (untreated control and treatment group administered a single dose of 0.5 mg/kg SWCNTs into the trachea). On day 3, the condition of mice was assessed. Foamy macrophages with SWCNTs in the injection group had accumulated in the alveolus. After day 14, granuloma containing multifocal macrophages was produced around the SWCNT aggregation site and chronic lung inflammation observed. In these in vivo experiments, production of granulomas promoted SWCNT cytotoxicity characterized by abnormal lung inflammation.[121] In a study by Ming et al,[122] 0, 0.1 and 0.5 mg CNTs were instilled into trachea of mice (using carbon black as a negative control). Animals were euthanized 7 and 90 days after a single treatment and the lungs isolated for histopathological analysis. In terms of changes, CNT aggregation in alveolar macrophages and concentration-dependent cytotoxicity were observed. Inflammation around bronchi was evident 7 days after treatment, with a more pronounced degree of inflammation at 90 days after administration. In contrast, the lungs of mice remained unaffected in the control group. These experiments clearly demonstrated serious damage induced by CNTs to lung. In another study, Qin and co-workers injected SWCNT into the tail vein of experimental mice, which were assigned to six groups (1, 7, 30, 60, 90 and 120 days). At the end of each time-period, 8 mice were randomly selected and lungs removed for follow-up studies. The results showed that the total amount of carbon in lung was positively correlated with length of time. Pulmonary capillary continuous embolization, granuloma formation, pulmonary fibrosis, and numerous pro-inflammatory factors were stimulated.[123] Therefore, consideration of the chronic toxicity and cumulative toxicity of free CNTs distributed in the body is essential before clinical application of CNTs as anti-tumor drug carriers.

Cardiovascular Toxicity

CNTs released in the body are strongly dependent on the blood vessel wall. These nanomolecules have a significant killing effect on tumor blood vessels and can also cause cardiovascular damage during circulation in the body. CNTs possess high hardness and mechanical strength, which may cause mechanical damage upon contact with the vessel wall.[124] Furthermore, CNTs can trigger substantial release of ROS or inflammatory factors, leading to cellular damage and inhibition of growth. At the same time, these nanotubes could affect the reconstruction of new blood vessel walls and cause myocardial ischemia, leading to cardiovascular toxicity, such as atherosclerosis.[125]

Ge et al[126] administered a solution containing SWCNTs to male spontaneous hypertensive rats once a day for two continuous days, followed by examination of mouse hearts. Compared with the control group, arterial vascular thickening and myocardial fibrosis were evident in treated mice. Capillary congestion and spongy appearance were obvious upon microstructural analysis, along with thrombosis and oozing of blood vessels as well as mitochondrial swelling. The results clearly suggest that that SWCNTs cause damage to the cardiovascular system and are therefore a high risk for patients with cardiovascular disease. Similar findings were reported by Chen et al[127] who highlighted the risk of long-term toxicity of SWCNTs. The effects of CNTs on important monocyte adhesion during atherogenesis and endothelial progenitor cells (EPCs) isolated from human atherosclerotic model ApoE/mouse bone marrow were ascertained by Suzuki and co-workers.[128] To this end, normal human aortic endothelial cells (HAECs) were cultured and exposed to SWCNTs for 16 h. ApoE/mice were exposed to SWCNTs or DWCNTs (10 or 40 μg/mouse) once every other week for 10 weeks via pharyngeal aspiration. As a result, adhesion molecule (ICAM-1) was upregulated and THP-1 monocyte adhesion with HAEC enhanced. Compared with the blank group, the ApoE/mouse plaque area was increased, as observed from aortic oil red O staining, and ICAM-1 expression upregulated. The study concluded that SWCNTs and DWCNTs enhance atherogenesis by promoting adhesion of monocytes to endothelial cells and inducing EPC dysfunction. Cell morphology of human umbilical vein endothelial cells (HUVEC) co-cultured with MWCNTs (0.5, 5 and 20 μg/mL) for 24 h was examined by the group of Guo. Microscopic examination revealed formation of cell solute vacuoles, disordering of cellular orientation and decreased cell densities. TEM analysis showed that compared with the control group, the MWCNT group (20 μg/mL, 24 h) displayed significant vacuolization and internalization of HUVECs, with vacuoles containing several MWCNTs. Moreover, MWCNT-induced cytotoxic effects were dose-dependent. Flow cytometry using Annexin V-FITC and PI staining was used to examine the extent of HUVEC apoptosis in the MWCNT-treated groups. Cells in the MWCNT group showed a greater decrease in viability relative to control cells, indicating that the decrease in HUVEC activity may be at least partially attributed to MWCNT-induced apoptosis.[129]

Other Toxicities

Upon administration of CNT anti-tumor drug carriers, free CNTs not only accumulate in normal cell but are also distributed through the blood to other organs and exert toxic effects. The studies below represent recent investigations on the toxicity of CNTs to various tissues and organs (Table 1).

Factors Influencing CNT-Induced Toxicity

Unlike traditional chemical materials, surface modification, aggregation, concentration, size and shape of CNTs are associated with biological effects. To optimize the therapeutic efficacy of CNTs in the medical field, elucidation of the factors and mechanisms underlying CNT-mediated toxicity is critical.

Surface Modification

Surface modification is performed to improve the dispersion, excretion, and biocompatibility of CNTs.[145,146] Poor water solubility of CNTs carrying anti-tumor drugs is a major contributory factor to their toxic effects on the body, which may be addressed by surface modification.[147–150] The addition of proteins and surfactants on the CNT surface has been shown to not only facilitate effective targeting of cancer cells but also reduce toxicity[83] and improve therapeutic effects.[151–154] Among these, folate receptors are expressed on a variety of tumor cells, and binding of folate to CNTs improves both tumor targeting and toxicity in vivo.[155–157]

Ji and co-workers[158] designed a novel drug delivery system involving modification of chitosan (CHI) on the surface of SWNTs to control the loading and release of the anticancer agent DOX, which led to simultaneous improvement of the water solubility and biocompatibility of SWNTs.

Table 1 Toxicities of CNTs to Different Organs

Carbon Nanotubes	Subject	Types of Toxicity	Result	Reference
MWCNTs	Male Sprague Dawley rats	Nervous system	Inhalation of MWCNTs significantly alters the balance between sympathetic and parasympathetic nervous system.	[130]
MWCNTs	Mice	Nervous System and BBB	Acute pulmonary exposure to MWCNTs induce nerve inflammation responses dependent on the disruption of BBB integrity.	[131]
CNTs	Male NMRI mice	Neurotoxicity	CNTs may cause behavioral toxicity associated with depression or anxiety expression.	[132]
SWCNTs	PC-12 cells	Neurotoxicity	SWCNTs are toxic to PC-12 cells and more toxic to differentiated PC-12 cells.	[133]
SWCNTs	Male C57BL/6 mice	Pulmonary immune system	SWCNTs can increase susceptibility to respiratory viral infections as a novel mechanism of toxicity.	[134]
SWCNTs	Six-week-old specific-pathogen-free ICR mice	Immune toxicity and reproductive toxicity	SWCNTs produce immune toxicity and have an impact on reproduction and development.	[135]
SWCNTs	BALB/c macrophage cell line J774A and Female BALB/c mice	Immune toxicity	SWCNTs are immune toxic to the body, and the dispersion of SWCNTs is negatively correlated with immune toxicity.	[136]
MWCNTs	T lymphocytes	Immune toxicity	MWCNTs are toxic to human T cells in a concentration-dependent manner.	[137]
SWCNTs	Six to eight weeks old females of the CD1 outbred strain. Mouse ES cell line D3 and NIH3T3 cells	Embryo toxicity	SWCNTs can trigger embryo toxicity in mammals.	[138]
CNTs	Kunming mice	Embryo toxicity	CNTs can cause embryo toxicity, damage to the fetus and even miscarriage.	[139]
MWCNTs	Zebrafish embryo	Embryo toxicity	MWCNTs have serious developmental toxicity, which is related to the length of MWCNTs.	[140]
CNTs	Mouse embryonic fibroblasts (MEFs) and p53+/− (C57BL/6J) male and female mice	Embryo toxicity	CNTs may induce embryo toxicity, which is hereditary.	[141]
Oxidized SWCNTs	Artemia salina	Developmental toxicity	O-SWCNTs cause deformity to salina and produce a lot of ROS.	[142]
CNTs	Male BALB/c mice	Genital toxicity	CNTs have toxic effects on the reproductive organs of mice.	[143]
SWCNTs and MWCNTs	MeT-5A cells and BEAS 2B cells	Genotoxicity	MWCNTs and SWCNTs induce DNA damage in MeT-5A cells.	[144]

Loading of folic acid (FA) on SWCNTs was shown to achieve targeted killing of tumor cells. In these experiments, the liver cancer cell line, HCC SMMC-7721, was treated with DOX and DOX/FA/CHI/SWNT (100 μg/mL), and cell viability recorded at 24, 48 and 72 h. The results showed lower cell viability of the DOX/FA/CHI/SWNT than the DOX group, indicative of time-dependent inhibition of liver cancer growth in nude mice.

Wu et al[159] performed surface functionalization of CNTs via enrichment of carboxylic groups with optimized oxidization treatment, followed by covalent linking of hydrophilic diaminotriethylene glycol via amidation reaction. Finally, hydroxylcamptothecin (HCPT) was chemically attached to CNTs through a cleavable ester linkage to successfully generate a novel MWCNT drug delivery system. Subcutaneous liver H22 tumor-bearing mice were used as model animals for injection of MWCNT-HCPT (5 mg/kg). After 15 days, compared with the currently used HCPT preparations, tumors treated with the MWCNT-HCPT complex were extensively damaged while normal tissue sites remained relatively unaffected. Overall, the newly generated MWCNT-HCPT complex showed excellent antitumor activity and low toxicity. Furthermore, the complexity of MWCNT-HCPT led to longer blood circulation times and higher tumor-specific drug accumulation. Therefore, reasonable surface modification of CNTs should enhance the antitumor effect and decrease toxicity to normal tissues of the body. The group of Patlolla additionally evaluated the toxicity of primary and oxidized MWNTs on normal human dermal fibroblasts (NHDF). To this end, NHDFs were cultured with three different concentrations (40, 200, 400 g/mL) of raw and oxidized MWCNTs. The results showed dose-dependent toxicity of both MWNT types. Compared to the control group, 400 g/mL oxidized MWNTs inactivated NHDF via DNA damage.[111] Therefore, for effective utilization of CNTs as drug carriers, both the concentrations of CNTs accumulating in the body and modifying groups on the CNT surface need to be considered.

Degree of Aggregation

Nanoparticles with small particle size and large specific surface area have intense aggregation tendency owing to van der Waals attractions in solution.[160] A number of studies have indicated that SWCNT toxicity in vivo is caused by aggregates rather than individual molecules.[161,162] Highly aggregated CNTs can become bulky and strong,[163] consequently exerting more harmful effect on cells.

To investigate the potential lung toxicity of dispersed SWCNTs, Mutlu et al[107] administered equivalent doses of dispersed and aggregated SWCNTs for 30 days after intratracheal administration to mice. Dispersed SWCNTs were taken up by alveolar macrophages through cilia via mucosal clearance or other mechanisms that gradually cleared over time. Aggregated SWCNTs displayed a granulomatous structure with mild fibrosis in mouse trachea. Accordingly, it was concluded that the toxicity caused by SWCNTs in vivo is mainly attributable to aggregates rather than SWCNTs with a large aspect ratio.[164] A number of studies have highlighted that dispersed MWCNTs with extreme aspect ratios induce higher cytotoxicity than those with low aspect ratios.[165] In a study by Wick et al[166] the mesothelioma cell line, MSTO-211H, was exposed to disperse CNT bundles and three different concentrations of CNT agglomerates (7.5, 15 and 30 g/mL). After three days, significant cellular morphological changes and decreased cell activity were observed in the CNT aggregation groups. Toxicity was increased in a concentration-dependent manner. CNTs exert toxic effects on cancer cells, and therefore, damage to normal cells is not unexpected. Belyanskaya and co-workers studied the effects of SWCNTs with varying degrees of aggregation on chicken embryonic spinal cord and dorsal root ganglia. Two dissimilar degrees of agglomerates were utilized, specifically, SWCNT agglomerates (SWCNT-a) and better dispersed SWCNT bundles (SWCNT-b). The overall DNA content of mixed glial cells in SWCNT-a and SWCNT-b groups at a concentration of 30 μg/mL was determined with the Hoechst assay, which revealed a marked decrease in the DNA content in the SWCNT-a group. SWCNTs induced acute toxicity in the central and peripheral nervous systems after entry into the body.[167] Moreover, the level of toxicity was dependent, in part, on the agglomeration state of SWCNTs. Dispersed SWCNTs showed an increased aspect ratio relative to the resulting aggregates. Phagocytic cells were able to eliminate SWCNTs and reduce toxicity to the body to a greater extent.[168,169]

Concentration

After anti-tumor nanometer preparations with CNTs are separated from target organs and drugs, a proportion is removed from the body while the remainder translocates to different parts of the body via the blood circulation and exerts toxic effects. The magnitude of toxicity is significantly correlated with the concentration of aggregated CNTs.[170] Bottini et al[137] incubated T lymphocyte cells with 40 μg/mL and 400 μg/mL CNTs and collected them for examination at different time-periods. The trypan blue exclusion assay was employed to assess the effects of CNTs on T cell viability and annexinV binding assay used to determine cell apoptosis. Cells lost 80% viability within 5 days in the presence of 400 μg/mL CNTs. Further microscopic examination was performed for chromatin condensation and membrane vesicles, which are markers of apoptosis. At a concentration of 40 μg/mL, CNTs did not appear to have toxic effects on T cells, leading to the

conclusion that CNTs do not cause measurable damage to cells at this concentration and toxicity is positively correlated with dose. Fanizza and co-workers evaluated MWCNT toxicity to human bronchial normal cells (BEAS-2B) by exposing cells to 10, 40 and 100 μg/mL MWCNTs. Cellular microvilli structural changes, microvilli reduction and mild herpes development were observed after 24 h. At the same time, cell DNA damage in the 40 and 100 μg/mL MWCNT-treated groups was evident after 4 h using the comet assay. Data from these experiments confirmed the cytotoxicity of MWCNTs in normal cells.[171]

CNT Size

Different sizes of CNTs may induce various degrees of toxicity.[172–177] Diminutive CNTs have a large specific surface area and strong ability to cross cell membranes.[153] These molecules can damage cellular components and proteins, causing dysfunction or even death of macrophages.[132] In a study by Sohaebuddin et al[178] 3T3 fibroblasts were positioned in the environment of MWCNTs with diameters <8 nm and morphology recorded after 12 h. MWCNTs with small diameters could induce membrane instability of lysosomes and promote release of components while those with large diameters caused little damage to lysosomes. The toxicities of different lengths of SWCNTs on HepG2 cells were further reported by the group of Shen.[180] Measurements of cell viability and oxidative stress revealed that SWCNTs of different lengths induced a decrease in HepG2 viability and increase in intracellular ROS. Martinez et al[179] used a zebrafish model to evaluate the effects of different sizes of MWCNTs on juveniles. The physiological and behavioral responses of juvenile fish indicated that short MWCNTs are neurotoxic and immunotoxic to larvae while long MWCNTs induce developmental malformations, cardiotoxicity and immunotoxicity, indicating that different-sized CNTs of the same material exert different toxicities. However, long SWCNTs exerted stronger cytotoxic effects than their shorter counterparts, supporting the theory that cytotoxicity may be effectively reduced by controlling CNT size.

Shape

CNTs are needle-like structures similar to fibers that form various shapes depending on the number of layers and length, such as single-walled carbon nanotubes (SWCNT), multi-walled carbon nanotubes (MWCNT), high aspect ratio nanotubes, short nanotubes, straight carbon nanotubes, and curved carbon nanotubes. Interestingly, CNTs of different shapes may exert differential toxic effects.[177,181–184] A number of reports suggest that more profound toxicity stems from SWCNTs than MWCNTs[185] and SWCNTs inhibit phagocytosis more intensely than equivalent doses of MWCNTs.[181] In an earlier investigation, El-Gazzar and co-workers divided rats into DWCNT and MWCNT-7 treatment groups. Equivalent doses of DWCNT and MWCNT-7 were administered via intratracheal intrapulmonary spray every other day for 15 days and rats sacrificed after six weeks. Notably, the PCNA index of lung cells of the MWCNT-7 group was increased, compared with the DWCNT group.[186] The results indicate that it is preferable to use CNTs with a small number of layers as carriers for antitumor nanometer preparations. In another study, Fenoglio and colleagues incubated MWCNTs of different thicknesses with murine alveolar macrophages (MH-S) and assessed cytotoxicity based on changes in ROS and glutathione. In these experiments, thin MWCNTs appeared significantly more toxic than the thicker counterparts.[187] MWCNTs with four different shapes were injected into mice by the group of Rittinghausen and the effects on mesothelioma examined. With increased curvature of different MWCNT types, the probability of inducing mesothelioma was decreased. In other words, straight needle-shaped MWCNTs induced the greatest toxicity and carcinogenicity.[188] Luisana et al[189] incubated needle-like and tangled MWCNTs with the mouse macrophage cell line, Raw 264.7, and alveolar macrophages, MH-S. The cell viability value of the needle-like MWCNT-treated group was significantly decreased with high nitrite accumulation in the medium, clearly supporting the theory that MWCNT shape is related to cytotoxicity. Sakamoto and co-workers further compared the carcinogenicity of seven MWCNTs showing differences in size and shape. The carcinogenic rates of four needle-shaped MWCNTs were as high as 100% while tangled MWCNTs did not induce mesothelioma.[190] Comprehensive findings from multiple studies thus suggest that different shapes of CNTs exert differing levels of cytotoxicity, with the greatest toxicity induced by needle-shaped CNTs.

Conclusion

With the extensive development and utilization of nanotechnology in the field of oncology, CNTs have been generated that play an irreplaceable role as anticancer drug carriers. However, these nanomolecules have a number of drawbacks in the clinic. During CNT-mediated delivery of anticancer drugs to target organs, free CNTs are retained in the body after dissociation of

the drug, causing secondary damage. The mechanisms of action of CNTs on normal cell tissues are not fully understood at present, thus limiting their clinical application. To resolve this issue, specific proteins could be loaded on the surface of CNTs, which stimulate MPO release by neutrophils so that CNTs themselves degrade and eventually achieve attenuation effects. Simultaneously, dual drug-loading methods may be effective in protecting normal tissues.

In recent years, the changes and mechanisms of gene expression associated with CNT toxicity have been a considerable focus of research interest. The development of CNTs as anti-tumor nanometer carriers in the future will depend on the consequences of effective treatment. Due to the special nanostructural properties of CNTs, potential toxic effects and improved biocompatibility may be avoided to ensure clinical drug safety. To achieve these positive effects, we need to clarify the mechanisms underlying CNT-induced toxicity to eliminate toxic nanometer formulations. Furthermore, determination of the absorption, distribution, metabolism and excretion properties of CNTs in the body is essential. In summary, comprehensive evaluation of the safety of nanoformulations, optimization of drug payload and reduction of potential toxicity are essential steps to maximize their anti-tumor effects.

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Disclosure

The authors report no conflicts of interest in this work.

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