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Potent lung tumor promotion by inhaled MWCNT

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Abstract

In the lung, carcinogenesis is a multi-stage process that includes initiation by a genotoxic agent, promotion that expands the population of cells with damaged DNA to form a tumor, and progression from benign to malignant neoplasms. We have previously shown that Mitsui-7, a long and rigid multi-walled carbon nanotube (MWCNT), promotes pulmonary carcinogenesis in a mouse model. To investigate the potential exposure threshold and dose-response for tumor promotion by this MWCNT, 3-methylcholanthrene (MC) initiated (10 μg/g, i.p., once) or vehicle (corn oil) treated B6C3F1 mice were exposed by inhalation to filtered air or MWCNT(5 mg/m^3) for 5 h/day for 0, 2, 5, or 10 days and were followed for 17 months post-exposure for evidence of lung tumors. Pulmonary neoplasia incidence in MC-initiated mice significantly increased with each MWCNT exposure duration. Exposure to either MCor MWCNT alone did not affect pulmonary neoplasia incidence compared with vehicle controls. Lung tumor multiplicity in MC-initiated mice also significantly increased with each MWCNT exposure duration. Thus,

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Disclaimer

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a significantly higher lung tumor multiplicity was observed after a 10-day MWCNT exposure than following a 2-day exposure. Both bronchioloalveolar adenoma and bronchioloalveolar adenocarcinoma multiplicity in MC-initiated mice were significantly increased following 5 -and 10-day MWCNT exposure, while a 2-day MWCNT exposure in MC-initiated mice significantly increased the multiplicity of adenomas but not adenocarcinomas. In this study, even the lowest promoted lung tumors in MC-initiated mice. Our findings indicate that exposure to this promotes pulmonary carcinogenesis.

Keywords

Multi-walled carbon nanotubes; mice; inhalation exposure; lung cancer; tumor promoter

Introduction

Carbon nanotubes have unique chemical and physical properties that have facilitated the development of many innovative consumer, industrial, and medical applications, including compact electronic devices that conduct electrons at the speed of light, lightweight automotive products as well as aerospace engineering materials. The strength and the low density have also made possible the production of strong, light weight sporting equipment and durable concrete additives to reinforce roads. The unusual electrical conductance of carbon nanotubes and their composites have enabled the development of many commercial products, including long-term batteries for green energy (Bloomberg 2020; De Volder et al. 2013). In addition to incredible strength and electrical conductance, some carbon nanotubes integrate well with tissues which have enabled the development of carbon nanotube-based scaffolds for the regeneration of bone (Lalwani et al. 2015), cartilage (Chahine et al. 2014; Zanello et al. 2006), nerves (Hu et al. 2004), and muscle (MacDonald et al. 2005), as well as delivery systems for nanomedicine (De Volder et al. 2013). These tubular-shaped nanomaterials are manufactured from graphene sheets rolled either into a single-walled carbon nanotube (SWCNT) or multi-walled carbon nanotubes (MWCNT). Due to less expensive manufacturing costs, MWCNT have dominated the market (Bloomberg 2020).

Many of the unique properties of carbon nanotubes that have made the development of novel materials possible are also the basis for potential human health hazards. Because the long, thin MWCNT are lightweight, nanotubes are easily aerosolized during the production and processing of the material into end products, making inhalation a potentially significant route of human exposure. Carbon nanotubes resist degradation and can reach the alveolar region of the lung where clearance is low, and as a result may persist in the body for extended periods of time (Donaldson et al. 2010; Mercer et al. 2010; Oberdörster, Oberdörster, and Oberdörster 2005). Previous investigations demonstrated that MWCNT deposited in mouse lungs by pharyngeal aspiration or inhalation produced many histological changes, including inflammation and fibrosis as well as hypertrophied and hyperplastic bronchiolar and alveolar epithelial cells (Donaldson et al. 2010; Fraser et al. 2020; Mercer et al. 2011; Mercer et al. 2010; Porter et al. 2010). Some alveolar epithelial type II cells with aberrant nuclear sizes have been observed following carbon nanotube exposure (Porter et al. 2010). In addition to the alveolar region, MWCNT can reach the interstitium and the pleural

space after both aspiration and inhalation exposures (Mercer et al. 2013; Porter et al. 2010; Ryman-Rasmussen et al. 2009). In vitro studies have demonstrated that exposure of primary lung cells to 15 nm diameter MWCNT disrupted the cell division apparatus resulting in errors in chromosome number (Siegrist et al. 2014). Exposure of primary lung cells to MWCNT in vitro induced not only errors in chromosome number but caused dramatic fragmentation of the center of the chromosome (centromere) as well as chromosomal translocations (Siegrist et al. 2019). The centromere is a chromosomal structure that serves as the foundation for attachment of microtubules and the sister chromatids. Because of the importance of the centromere in proper segregation of the sister chromatids during cell division, centromeric fragmentation leads to errors in division and widespread chromo-some instability (Barra and Fachinetti 2018). Indeed, this type of event is seen predominantly in later stage tumor development in human cancers and is correlated with a shorter survival time as well as resistance to chemotherapy (Beeharry et al. 2013; Gisselsson 2005; Zhang et al. 2016).

Inflammation, cell proliferation, and errors in chromosome number following exposure to MWCNT indicate the potential of this material to induce neoplasia. Indeed, intrascrotal or intraperitoneal injections of MWCNT have been shown to induce malignant mesothelioma in both rats and mice (Sakamoto et al. 2009; Takagi et al. 2012; Takagi et al. 2008); while exposure to the Mitsui-7 MWCNT by inhalation or intratracheal instillation, as well as multiple, thick, rigid MWCNT caused lung tumors (Kasai et al. 2016). Rats administered Mitsui-7 MWCNT by trans-tracheal intrapulmonary spraying developed malignant mesothelioma (Numano et al. 2019; Suzui et al. 2016). Other types of MWCNT, particularly those with physiochemical characteristics like Mitsui-7 MWCNT, also caused lung tumors and mesothelioma in rats (Rittinghausen et al. 2014; Saleh et al. 2020).

Although Mitsui-7 MWCNT exposure in rats is a complete carcinogen (Kasai et al. 2016; Numano et al. 2019; Suzui et al. 2016), previous research from our laboratory indicated that the tumor incidence and multiplicity in B6C3F1 mice exposed to MWCNT (5 mg/m^3) alone for 15 days were not significantly different from air controls, indicating that MWCNT per se is not a complete carcinogen in the mouse (Sargent et al. 2014). However, our findings showed that Mitsui-7 MWCNT was a strong promoter of the growth and progression of mouse lung tumors after initiation by a DNA-damaging agent, MC (Sargent et al. 2014). The development of cancer is a multistep process involving an initial heritable DNA mutation (initiation), proliferation (hyperplasia) of DNA-damaged cells (promotion) and evolving genetic aberrations during the progression of cells from adenoma to adenocarcinoma and eventual metastatic disease (Barrett 1993; Pitot 1993b; Pitot et al. 1989). The observation that inhaled Mitsui-7 MWCNT is a promoter of lung adenocarcinoma is a concern because DNA damage can arise in humans spontaneously. In addition to endogenous mutagens, humans are constantly exposed to a multitude of potential DNA-damaging agents through the environment and diet (Jeffrey et al. 1990).

To investigate the exposure threshold for Mitsui-7 MWCNT-induced promotion of pulmonary carcinogenesis, a 17-month (74 weeks) initiation-promotion carcinogenesis bioassay was employed as previously described (Sargent et al. 2014). MC-initiated mice

were exposed to MWCNT for varying durations via whole body inhalation, and tumor formation was evaluated after 17 months.

Materials and methods

MWCNT

MWCNT used in this study (MWNT-7, lot #061220–31; also termed Mitsui-7 MWCNT) were obtained from Mitsui & Company (Tokyo, Japan). They were manufactured using a floating reactant catalytic chemical vapor deposition method followed by high-temperature thermal treatment under argon at 2500 °C using a continuous furnace (Kim et al. 2005). As previously described, the bulk material was characterized by: (a) high-resolution transmission electron microscopy which showed the particles had a distinctive crystalline structure of MWCNT, and by (b) chemically digesting bulk MWCNT followed by inductively coupled plasma-optical emission spectroscopy, which showed trace metal contamination of 1.32%, with iron being the predominant contaminant (Porter et al. 2013).

MWCNT inhalation exposure

The MWCNT aerosol was generated using an acoustical-based computer controlled whole body inhalation system (McKinney, Chen, and Frazer 2009). The system has previously been tested and validated by comparisons of MWCNT particle size and shape in the aerosols produced by the exposure system and workplace samples (McKinney, Chen, and Frazer 2009). In brief, the inhalation exposure system combines air flow controllers, aerosol particle monitors, data acquisition devices, and custom software with automated feedback control to achieve constant and repeatable exposure chamber temperature, relative humidity, pressure, aerosol concentration, and particle size distributions. The generator produces airborne particles continuously for long periods of time with minimal fluctuations during an exposure period. The uniformity of the test atmosphere in the chamber was evaluated and shown to have a total variation of <5%. In this study, the MWCNT aerosol mass concentration was continuously monitored with a Data RAM (DR-40000 Thermo Electron Co, Franklin, MA), and gravimetric determinations (37 mm cassettes with 0.45 μm pore-size Teflon filters) was used to calibrate and verify the Data RAM readings. A cascade impactor (MOUDI, model 110 R, MSP Co., Shoreview, MN) was used to determine the mass-based particle size distribution by fractionating the particles into 10 size fractions ranging from 50 nm to 18 μm. Particle morphology was assessed from Nucleopore polycarbonate filters (Whatman, Clinton PA) using a Hitachi S4800 field emission scanning electron microscope (Hitachi High-Tech America, Inc, Dallas, TX). Mass concentration was used as the measure MWCNT concentration in this study because mass concentrations are used as the measure of workplace exposures to carbon nanotubes and nanofibers (NIOSH 2013). The target concentration of the mouse exposure was 5 mg/m^3 for 5 h/day.

Initiation promotion protocol

The B6C3F1 mouse was chosen for use in this study for several reasons. First, a previous study from our laboratory reported that Mitsui-7 MWCNT was a strong promoter of the growth and progression of mouse lung tumors after initiation by a DNA-damaging agent using an initiation-promotion protocol in the B6C3F1 hybrid mouse (Sargent et al. 2014).

Second, it has intermediate susceptibility for spontaneous lung tumor formation (Devereux, Anderson, and Belinsky 1991; Malkinson 1989). Third, the B6C3F1 mouse is the strain used by the National Toxicology Program to evaluate chemicals for potential carcinogenicity and there is data available from these studies on spontaneous lung tumor formation from a variety of different exposure methods, including inhalation to clean air (NTP 2006, 2007a, 2007b, 2019a). Fourth, in addition to the data available from the National Toxicology Program studies, there is also information on the spontaneous tumor response and lifespan of the B6C3F1 mouse strain from studies conducted by other investigators (Devereux, Anderson, and Belinsky 1991; Hutt et al. 2005; Pandiri et al. 2012; Wakamatsu et al. 2007). Finally, it is a model being used by the National Toxicology Program for investigating the toxicity of multi-walled carbon nanotubes (NTP 2019b). In the present study, we continue the investigation of Mitsui-7 MWCNT induced lung tumor promotion and progression. To maintain continuity between these two studies, the same mouse strain (B6C3F1) and initiation-promotion protocol were used.

Six-week-old male B6C3F1 mice (Jackson Laboratories, Bar Harbor, ME) were single housed in polycarbonate ventilated cages with HEPA-filtered air. The mice were fed ad libitum with Harlan 7913 irradiated NIH-31 modified 6% rodent chow (Envigo, Indianapolis, IN). All animals in this study were housed in an AAALAC-accredited, specific pathogen-free, environmentally controlled facility. All procedures involving animals were approved by the CDC-Morgantown Animal Care and Use Committee.

After 1-week of acclimation, mice were randomly assigned to a treatment group. Mice were treated following a two-stage (initiation-promotion) protocol previously described (Malkinson et al. 1997). This two-stage initiation-promotion protocol involves the administration of a DNA damaging agent, methylcholanthrene (MC), followed by administration of a suspected carcinogen, MWCNT in this case, that may promote the growth of DNA-damaged cells. All mice received a single dose of either MC (10 μg/g bw, i.p.) or vehicle (corn oil). One week after receiving MC or vehicle, mice were exposed by whole-body inhalation to Mitsui-7 MWCNT (5 mg/m^3, 5 hrs/day) or filtered air (control) for 2, 5, or 10 days. Mice were divided into randomized complete blocks with staggered start and end dates.

Necropsy, histopathology and lung tumor counts

At 17 months post-exposure, mice were euthanized by an intraperitoneal injection of ≥100 mg/kg bw of sodium pentobarbital euthanasia solution followed by exsanguination and bilateral thoracotomy. The lungs were fixed by intratracheal perfusion with 1 mL of 10% neutral buffered formalin (NBF). The mice were then necropsied following standard techniques. Masses and lesions seen grossly were recorded on individual animal necropsy records. All gross lesions and masses were collected and fixed in 10% NBF. Lungs and any lesions were trimmed the same day and processed overnight. Tissues were embedded in paraffin and sectioned at approximately 5 μm. Hematoxylin and eosin (H & E) stained slides were prepared from each of the five separate lung lobes and from any masses seen at necropsy. The tumor counts were based on histopathological analysis.

Because animals developing lung tumors may exhibit general signs of pain and distress, animals were monitored weekly for overt signs of morbidity and changes in body weight. Overt signs of morbidity included skin lesions, ruffled fur, lethargy, shaking, penis or anal prolapse, erratic movements, hunched posture, or paralysis. If an animal had overt signs of morbidity or lost 20% or greater of body weight from the previous measurement, they were euthanized as described above, before the scheduled terminal sacrifice. Lungs and any masses from mice euthanized early were collected for histopathological analysis. The mice that were euthanized early were analyzed separately from animals that were sacrificed 17 months post-exposure.

Histology slides were examined by a board-certified veterinary pathologist using light microscopy. Polarized light microscopy was occasionally used to confirm the presence or absence of foreign material (presumptive test material). The severity of non-neoplastic lesions was graded on a 5-point scale of minimal (1), mild (2), moderate (3), marked (4), or severe (5) consistent with toxicologic pathology guidelines (Mann et al. 2012). Presumptive foreign material (MWCNT in this study) was recorded when present without severity grade. Histologic diagnoses were entered into the Provantis® (Instem, Philadelphia, PA) data collection and management system. A peer review of the tumor data was conducted by two additional veterinary pathologists. The peer review included a review of all bronchioloalveolar adenocarcinomas and all lung slides from 5% of total cases. The reported histopathology findings represent the consensus of all three veterinary pathologists following the peer review.

MWCNT lung burden

MWCNT lung burden determinations were made using a procedure previously described by our laboratory (Porter et al. 2013; Sargent et al. 2014). At one day post-exposure, animals separate from those used for histopathology were euthanized as described above, and lungs removed and frozen at −80 °C until further processing. After thawing, the lung tissue was digested in 25% KOH/methanol (w/v) at 60 °C overnight, followed by centrifugation at 16,000 × g for 10 min. The supernatant was removed; the remaining pellet was mixed with 50% HNO_3/methanol (v/v), and incubated at 60 ° Cover-night, followed by centrifugation (16,000 × g, 10 min). After centrifugation, the supernatant was removed, and the pellet was resuspended in 10% NP-40 (v/v) in dH_2O, followed by 30 s sonication using a cup horn sonicator. MWCNT standards were processed in parallel with the lung samples. The optical densities of the solutions were measured at 700 nm using a UV/visible spectrophotometer. Lung MWCNT content was determined from a standard curve.

Statistical analysis

All analyses were performed using SAS/STAT version 9.4 for Windows, or JMP version 13.2 (SAS Institute, Cary NC). Separate analyses for each tumor type and total tumor counts were performed using mixed models to include block of animals as a random factor. Binary outcomes of tumor incidence (tumor or not) were performed using logistic regression (logit) analyses, and tumor multiplicity was evaluated on tumor counts using Poisson regression analysis. Treatment effects on early death were evaluated using survival analysis. All analyses were considered significant at $p < 0.05$.

Results

MWCNT inhalation exposure and aerosol characterization

Particle morphology of MWCNT aerosol samples from our exposure chamber were analyzed by field emission scanning electron microscopy. The images indicated a diverse configuration of MWCNT particle shapes ranging from single fiber-like nanotubes to tangled agglomerates (Figure 1(A)) which were like those in our previous study (Sargent et al. 2014). It is also important to note that the morphology of MWCNT particles in our previous study (Sargent et al. 2014) and the current study are like those found in the personal breathing zone of workers employed by MWCNT manufacturers and users (Erdely et al. 2013), indicating the MWCNT aerosols the mice were exposed to are representative to those human workers are exposed during MWCNT manufacturer and industries that use them as end-users. The MOUDI cascade impactor was used to determine the mass-based particle size distribution of the MWCNT aerosol in the exposure chamber. When characterized by log normal statistics, the distribution had a mass median aerodynamic diameter (MMAD) of 1.7 μm and geometric standard deviation of (GSD) of 3.0 (Figure 1(B)).

Unscheduled deaths and survival analyses

There were 53 unscheduled deaths in this study. Of the 53 unscheduled deaths, 37 were euthanized prior to the established endpoint of 17 months, based on humane endpoint criteria, and 16 were reported to be found dead. For the 16 mice found dead, tissue analyses were inconclusive due to extensive postmortem autolysis. As shown in Table 1, only 20 of the 53 unscheduled deaths had lung lesions, while the other mice only had abnormalities in extra-pulmonary tissues, or no lesion(s) were identified.

The survival curve (Figure 2) demonstrates the time of unscheduled deaths. For the early deaths that occurred between 18 and 51 weeks, postmortem examinations determined none of these mice had lung tumors, but all had various extra-pulmonary abnormalities. For deaths that occurred beyond 51 weeks after exposure, postmortem examination determined that some mice had gross lung masses suggestive of tumors. Five bronchioloalveolar adenomas and 3 bronchioloalveolar adenocarcinomas were diagnosed microscopically in mice with unscheduled deaths (Table 2).

Foreign material

Foreign material (presumptive MWCNT) was seen as 0.5–8 micron, black, finely granular to elongate material with slight white birefringence under polarized light (Figure 3). It was commonly seen at the junctions of terminal bronchioles and alveolar ducts of mice exposed to MWCNT and was present in the cytoplasm of presumptive macrophages or epithelial cells lining the airways, within connective tissue adjacent to airway epithelium, or extracellularly in connective tissue or between cells. It was also seen in random small clusters of macrophages in airways and alveoli, and in macrophages or rarely free in the tracheobronchial lymph node. The diagnostic terminology foreign material was exclusively used to indicate presumptive test article (MWCNT). It was present in most lung lobes of mice exposed to MWCNT and was not observed in the lungs of any air-exposed controls.

MWCNT lung burden and foreign material in lungs

For each exposure time, comparison of corn oil to MC-treated mice showed no difference in lung burden (data not shown). MWCNT lung burden of the mice exposed to MWCNT is shown in Table 3 and as expected, indicates increasing MWCNT lung burden with duration of exposure.

Hyperplasia

Focal bronchioloalveolar hyperplasia was characterized by increased number of crowded epithelial cells that outlined contiguous alveolar septa. This terminology was used to signify focal hyperplasia, thought to be the precursor lesion to bronchioloalveolar adenomas and adenocarcinomas. Representative photomicrographs of hematoxylin and eosin (H&E) stained sections of the lung from mice exposed to MWCNT showed marked focal bronchioloalveolar alveolar hyperplasia (Figure 4, Panel A) to minimal hyperplasia (Figure 4, Panel B).

Focal bronchioloalveolar hyperplasia incidence is shown in Figure 5. For mice that received corn oil vehicle prior to inhalation exposure, bronchioloalveolar hyperplasia incidence ranged from 0% to 16%, and was significantly higher ($p < 0.05$) in MWCNT-exposed groups in comparison to air-exposed groups. Each of the three MC-initiated MWCNT-exposure groups had significantly higher bronchoalveolar hyperplasia incidence compared to the MC-initiated air-exposure group ($p < 0.05$). For mice that received MC prior to inhalation exposure, bronchioloalveolar hyperplasia incidence ranged from 21% to 65% and demonstrated a statistically significant MWCNT dose dependence ($p < 0.05$).

Neoplastic lung lesions

Bronchioloalveolar adenomas (Figure 6, panels A and B) were moderately cellular masses that distorted and replaced alveolar architecture and obliterated alveolar spaces. The proliferative cells formed irregular papillary structures or solid clusters separated by delicate, fibrovascular stroma. The cells were polygonal, moderately uniform in size, and had small to moderate amounts of eosinophilic, occasionally vacuolated cytoplasm. Nuclei were round to oval, moderately uniform, and mitoses were few to absent. The morphology of bronchioloalveolar adenomas in the groups that received MC and/or MWCNT did not differ appreciably from the spontaneously occurring neoplasms in the air-exposed groups that did not receive MC.

Bronchioloalveolar adenocarcinomas (Figure 6, panels C and D) had increased cellular atypia, slightly increased numbers of mitoses, and/or contained several patterns including ribbons, solid clusters, and (pseudo) acini compared to bronchioloalveolar adenomas. Some adenocarcinomas had small clusters of neoplastic cells adjacent to the main mass, thought to primarily represent cross sections of a single papillary infiltrative mass seen in two dimensions (local extension), although local metastases were possible. Adenocarcinomas were also sometimes associated with histiocytic infiltration.

Histiocytic infiltration

The diagnostic terminology “histiocytic infiltration” was used to indicate an increase in numbers of macrophages. It was typically characterized by occasional, random, small clusters of macrophages in airways or alveoli (Figure 7, Panel A). Histiocytic infiltration was sometimes associated with focal hyperplasia (Figure 7, Panel B). It was also noted as densely cellular accumulations in lung lobes with associated masses (Figure 7, Panel C). In some lung lobes, the incidences were slightly higher in the MWCNT-exposed MC-treated mice (data not shown), but these dense cellular accumulations may have represented association with masses, and not a response to the foreign material (MWCNT).

Tumor incidence

Tumor incidence is shown in Figure 8. For mice that received corn oil vehicle prior to inhalation exposure, tumor incidence ranged from 22% to 36%, and there was no statistical difference between any exposure group ($p > 0.05$). For mice that received MC prior to inhalation exposure, tumor incidence ranged from 38% to 71%. MWCNT-exposed MC-treated mice had tumor incidence rates ranging from 65–71%, and there were no statistically significant differences among exposure doses ($p > 0.05$). However, all three MWCNT-exposed MC-treated groups had significantly higher percent tumor incidence in comparison to the air-exposed MC-treated group ($p < 0.05$) and to the corn oil-treated MWCNT-exposed.

Tumor multiplicity

Overall tumor multiplicity is presented in Figure 9. For mice that received corn oil vehicle prior to inhalation exposure, overall tumor multiplicity ranged from 0.30 to 0.37, and there was no statistical difference between any exposure group ($p > 0.05$). For mice that received MC prior to inhalation exposure, tumor multiplicity ranged from 0.43 to 1.39. For air-exposed controls, comparison of corn oil to MC-treated mice showed no statistically significant difference ($p > 0.05$). In contrast, for MWCNT-exposed mice, comparison of corn oil to MC-treated mice indicated a statistically significant difference ($p < 0.05$) for all exposure times. Comparison of MWCNT-exposed and MC-treated mice showed that mice exposed for 2, 5 and 10 days had significantly higher ($p < 0.05$) multiplicity in comparison to air-exposed MC-treated mice.

Tumor multiplicity was further investigated by examining lung adenoma and adenocarcinoma multiplicity. Adenoma multiplicity is shown in Figure 10. For mice that received corn oil vehicle prior to inhalation exposure, overall adenoma multiplicity ranged from 0.18 to 0.33, and there was no statistical difference between any exposure group ($p > 0.05$). For air-exposed control group, comparison of corn oil to MC-treated mice showed no statistically significant difference ($p > 0.05$). In contrast, for MWCNT-exposed mice, comparison of corn oil to MC-treated mice indicated a statistically significant difference ($p < 0.05$) for all exposure times. For MWCNT-exposed MC-treated mice, comparison of different exposure times indicated no statistically significant difference ($p > 0.05$) with increasing exposure time.

Adenocarcinoma multiplicity is shown in Figure 11. For mice that received corn oil vehicle prior to inhalation exposure, overall adenocarcinoma multiplicity ranged from 0.04 to 0.13, and there was no statistical difference between any exposure group ($p > 0.05$). For air-exposed control, comparison of corn oil to MC-treated mice showed no statistically significant difference ($p > 0.05$). In contrast, for MWCNT-exposed mice, comparison of corn oil to MC-treated mice indicated a statistically significant difference ($p < 0.05$) at 5- and 10-day exposures, but not after 2-day exposure. For MWCNT-exposed MC-treated mice, comparison of different exposure times indicated 5- and 10-day exposure groups had significantly higher adenocarcinoma multiplicity ($p < 0.05$) than the mice exposed for 2 days.

Discussion

The objectives of this study were to confirm tumor promotion by MWCNT and identify whether an exposure threshold for promotion existed. We found that MWCNT caused potent tumor promotion even at the lowest lung burden tested in this study. The lowest MWCNT lung burden tested was achieved following exposure to 5 mg/m^3 for 5 h on 2 consecutive days. The 5 mg/m^3 exposure concentration represents the Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) for particles not otherwise regulated (PNORs). OSHA does not yet have a PEL for carbon nanotubes but recommends that worker exposures must not exceed the NIOSH recommended exposure limit (REL) (NIOSH 2013). The NIOSH REL for carbon nanotubes was established in 2013 and is 1 $\mu g/m^3$ of elemental carbon as an 8-h time-weighted average (TWA) concentration (NIOSH 2013). The MWCNT lung burden following the shortest exposure in our study (2 days) was 4.4 ± 0.2 μg/mouse. While there are uncertainties in interspecies extrapolations of lung deposition, a comparative calculation using the alveolar surface areas of mice and humans (NIOSH 2013), suggest that the human equivalent lung deposition after 2 days exposure would be 8.2 mg. This is less than the ~11 mg cumulative lung deposition estimated for a career worker employed in a MWCNT manufacturing facility for a period of 45-years when daily exposures concentrations were equivalent to the NIOSH REL. This was a way to achieve a lung burden in mice equivalent to the lung burden of workers exposed throughout their career, since the short lifespan of the mouse limits the lung burden achievable in their lifetime when exposed to concentrations closer to the NIOSH REL. Thus, the MWCNT exposure in this study achieves total lung burdens like those that can occur in a working lifetime at the NIOSH REL but necessarily was delivered at higher concentrations over a shorter time than workplace exposures. Another uncertainty in this interspecies extrapolation is that the mice in this study were exposed to MC, which was used as the initiating agent, under controlled conditions, i.e., single i.p dose at 10 μg/g bw, before exposure to MWCNT. The situation with humans is much more complex. Humans are exposed to numerous initiating agents at unknown concentrations, durations, and routes of exposure. Furthermore, these exposures to initiators can occur before, during, or after exposure to MWCNT.

Benign and malignant tumors are both considered relevant in assessing carcinogenicity when evidence suggests progression of benign to malignant tumors, as observed in our current and previous studies (Huff 1999; Huff, Eustis, and Haseman 1989; Laube et al. 2019). In

our study, all three MWCNT exposure durations (2, 5, and 10 days) increased the tumor incidence and multiplicity in MC-initiated mice. Because we saw a promotion at the lowest exposure duration, our data indicate that the threshold for tumor promotion by MWCNT was below the exposures used in our study. Our study used a relatively high concentration, short-term exposure duration and a prolonged 17-month "recovery" time. This paradigm produced MWCNT lung burdens that would be comparable to lung burdens anticipated in workers over a working life-time of exposures at the NIOSH REL. In addition, the inhalation exposures and lung burdens achieved in our study mimics an acute high-dose exposure exceeding the NIOSH REL that workers may or have experienced during an industrial accident.

In our study, MWCNTs strongly promoted pulmonary carcinogenesis initiated by MC, a known genotoxic compound. Carcinogenesis is generally a complex process involving multiple stages (Luebeck and Moolgavkar 2002; Pitot 1993a, 1993b). Pulmonary carcinogenesis is no exception and involves multiple stages (Hubbs, Hahn, and Thomassen 1989; Kishimoto et al. 1995; Rondini, Walters, and Bauer 2010; Soh et al. 2008). Carcinogenesis begins with genotoxicity which initiates the carcinogenic process. MC, the initiator used in our study, is a known initiator of pulmonary carcinogenesis (Bauer and Dwyer-Nield 2021; Witschi, Williamson, and Lock 1977). Inflammation, cell proliferation, and evolving genomic instability are among the best described promoters of pulmonary carcinogenesis (Bauer and Dwyer-Nield 2021). The significant increase in bronchioloalveolar adenocarcinomas in our study suggests the possibility that the MWCNT also played a role in tumor progression.

Genotoxicity plays a central role in carcinogenesis by initiating DNA damage. The genotoxicity of MWCNT-7 has been investigated both *in vitro* and *in vivo*. A study from our research team reported MWCNT-7 caused centrosome fragmentation and dose-dependent aneuploidy in BEAS-2B and SAEC cells (Siegrist et al. 2019). Another *in vitro* study, which used BEAS-2B cells exposed to MWCNT-7, reported an increase in mitotic abnormalities and micronucleus-positive cells and an epithelial-mesenchymal transition (Barthel et al. 2021). In contrast, another study employed numerous genotoxicity assays including bacterial reverse mutation test and in vitro mammalian chromosomal aberration test, reported no genotoxicity (Ema et al. 2012). After intrapleural injection in C57BL/6 mice, MWCNT-7 pleural cells did not have higher levels of DNA damage in comparisons to control animals (Wils et al. 2021). In another study, rats exposed to MWCNT-7 by single bolus dose by intratracheal installation found no genotoxicity based on gpt assay (Horibata et al. 2017).

Inflammation may have contributed to the tumorigenic city seen in the current study. Chronic persistent inflammation is strongly implicated in neoplastic progression in multiple tissues and can be involved in initiation as well (Coussens and Werb 2002; Pollard 2004). Consistent with this, inflammation is associated with pulmonary carcinogenesis in humans (Conway et al. 2016; Engels 2008). In our study, mice were only exposed to MC once, and exposures to MWCNT were limited to 2, 5, or 10 days followed by a 17-month post-exposure recovery phase. Despite the long recovery period, tumor-associated macrophages and low levels of persistent macrophages containing MWCNT were observed. Based on previous studies, inflammation would have been more intense at earlier time periods.

Indeed, the inflammatory and fibrogenic responses of the lung to carbon nanotubes were critical factors that first alerted the toxicology community to the potential toxicity of carbon nanomaterials (Service 2004; Shvedova et al. 2005). A recent study demonstrated that inflammatory responses to a spectrum of nanotubes and nanofibers consistently involved macrophage infiltration (granulomatous inflammation and/or alveolar histiocytosis) that persisted long after cessation of exposure, and the magnitude of the chronic inflammatory response was influenced by the physiochemical properties of the nanotubes and nanofibers (Fraser et al. 2021). Further, they showed that Mitsui-7 MWCNT, the same MWCNT used in our study, produced the highest severity grades for granulomatous inflammation, alveolar histiocytosis, and alveolar interstitial fibrosis 84 days after exposure (Fraser et al. 2021). In another recent study, Mitsui-7 MWCNT pharyngeal aspiration exposure caused an early predominantly neutrophilic, inflammation that evolved to foci of granulomatous inflammation as the neutrophilic component of the inflammatory response resolved (Lim et al. 2020). M1 macrophage polarization predominated one day post-exposure, while M2 polarization predominated 1-week after exposure (Dong and Ma 2018; Lim et al. 2020). This is an important observation because in human lung cancers, tumor-associated macrophages often exhibit a M2 phenotype (Sica et al. 2006). In patients with lung cancer, higher numbers of M2 macrophages in the tumor stroma are known to be associated with increased tumor invasiveness, cellular proliferation, lymph node metastases, as well as poor survival rate (Sumitomo et al. 2019). Recently, human lung tumor-associated macrophages with a predominantly M2 phenotype have been associated with increased TGFβ secretion as well as epithelial-mesenchymal transition, enhanced tumor cell proliferation, invasion and metastasis in lung cancer cells (Sumitomo et al. 2023; Zhang et al. 2017).

To summarize the potential role of inflammation in the tumorigenicity seen in our study, chronic inflammation has been associated with pulmonary carcinogenesis, M2 macrophages have been specifically implicated in neoplastic progression in the lung, and M2 polarization has been reported after Mitsui-7 MWCNT exposure. In addition, both MWCNT exposure and M2 macrophages are implicated in epithelial-mesenchymal transition (Barthel et al. 2021; Sumitomo et al. 2023; Zhang et al. 2017). Our current study demonstrated Mitsui-7 MWCNT-mediated lung tumor promotion in B6C3F1 mice. Thus, further delineation of the roles of chronic inflammation and M2 macrophage polarization that result from MWCNT inhalation exposure may be warranted.

MWCNT are fiber-like particles, and the properties of particles and fibers implicated in toxicity are actively being investigated (Barbarino and Giordano 2021; Riediker et al. 2019). In studies of the classic carcinogenic fiber, asbestos, Merle Stanton and colleagues noted that long, thin fibers were the most effective carcinogens, while short and large-diameter fibers tended to be phagocytized and inactivated (Stanton et al. 1981). In those studies, asbestos fibers less than 0.25 μm in diameter and more than 8 μm in length had the highest carcinogenicity, with fibers up to 1.5 μm in diameter and more than 4 μm in length also correlating with carcinogenicity (Stanton et al. 1981). Mitsui-7 MWCNT has an arithmetic mean diameter of 67 ± 2 nm (mean ± *SE*) and an arithmetic mean length of 5.6 ± 0.27 μm (mean ± *SE*) with a range of 1.2–25.8 μm in length (Fraser et al. 2021). Therefore, the Mitsui-7 MWCNT used in our study are long, thin, durable fibers, with many fibers that meet the criteria for carcinogenicity if the asbestos criteria apply to carbon nanotubes.

Genotoxic exposures initiate a population of genetically altered cells that expand in number through the action of tumor promoters and undergo additional genetic changes during the progression process (Barrett 1993; Pitot et al. 1989). Several studies suggest that both genotoxicity and tumor promotion are associated with the classical carcinogenic high-aspect ratio particle, asbestos. Oxidant generation from inflammation has been shown to damage the DNA and initiate cancer. Mitsui-7 MWCNT is also genotoxic, causing aneuploidy, chromosomal trans-locations, and fragmentation of the mitotic spindle poles and the chromosome centromere, thus disrupting the control cell division and the subsequent segregation of the chromosomes (Siegrist et al. 2014, 2019). When the cell divides, centrosomes play a critical role in the forming bipolar mitotic spindles to allow the accurate segregation of chromosomes into daughter cells. Abnormal centrosomes result in chromosome mis-segregation through multipolar and monopolar mitoses (Gisselsson et al. 2002; Lingle and Salisbury 1999). This is important because numerous studies strongly implicate genomic instability and aneuploidy with neoplastic progression in the lung. For example, mice genetically modified to have chromosomal instability and aneuploidy spontaneously develop pulmonary adenomas as they age (Weaver et al. 2007). In the human lung, centrosome abnormalities are associated with aneuploidy and are seen in non-small cell lung cancer, associated areas of hyperplasia, and preneoplastic lesions (Koutsami et al. 2006; Smith et al. 1996). Centrosome defects and chromosome instability are seen in the earliest stages of human cancers in multiple types of cancer. They are believed to play a role in the progression and development of aggressive tumors (Pihan et al. 2003). Consistent with this, aneuploidy in non-small cell lung cancer decreases the probability of patient survival compared to patients with diploid non-small cell lung tumors (Choma et al. 2001). In addition to the centrosome, the centromere is important in the proper distribution of the chromosome during mitosis because the centromere attaches the chromosome to the mitotic spindle microtubules and is also important in maintaining the proper alignment of the chromosomes during mitosis. When the centromere fragments, numerical and structural chromosome aberrations occur (Harasymiw et al. 2019; Muller, Gil, and Drinnenberg 2019). Many of the resulting genetically aberrant cells die; however, clones of cells can evolve with increased proliferation, growth factor independence, and ability to metastasize to other organs (Barra and Fachinetti 2018; Giam and Rancati 2015). Thus, the genotoxicity and chromosomal instability produced by Mitsui-7 MWCNT may have contributed to the strong tumor promotion seen in our study. The narrower single-walled carbon nanotubes and MWCNTs of 10 nm (Rondini, Walters, and Bauer 2010) and 15 nm (Siegrist et al. 2014) diameter cause centrosome fragmentation and aneuploidy but do not fragment the centromere or cause chromosomal translocations. Because carbon nanotubes other than 1 nm SWCNT, 10 nm MWCNT, 15 nm MWCNT and Mitsui-7 MWCNT materials have not been examined for their ability to disrupt the centrosome and/or the centromere, it is not possible to predict the potency of other carbon nanotubes. However, the results of a recent investigation demonstrated that long, large-diameter, rigid MWCNT caused more chromosome breakage and micronuclei in lung epithelial cells in vitro (Fraser et al. 2020).

In summary, this study confirmed Mitsui-7 MWCNT-mediated lung tumor promotion in B6C3F1 mice, a mouse model that is relatively resistant to spontaneous lung tumor development (Wakamatsu et al. 2007). Because Mitsui-7 MWCNT was a promoter even

at the lowest lung burdens in this study, no threshold was identified for promotion. Indeed, observing significant tumor promotion 17 months after a 2-day inhalation exposure demonstrated that Mitsui-7 MWCNT was a very potent tumor promoter. Tumor promotion is a concern in human populations because humans are unintentionally exposed to numerous occupational and environmental xenobiotics throughout their lifetime, and many of these agents may be potential initiators. The observation of promotion at exposure doses that could be anticipated in humans underscores the need for caution during the production, processing, and use of MWCNT materials.

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Data availability statement

The data from this study is available at the NIOSH Data and Statistics Gateway.

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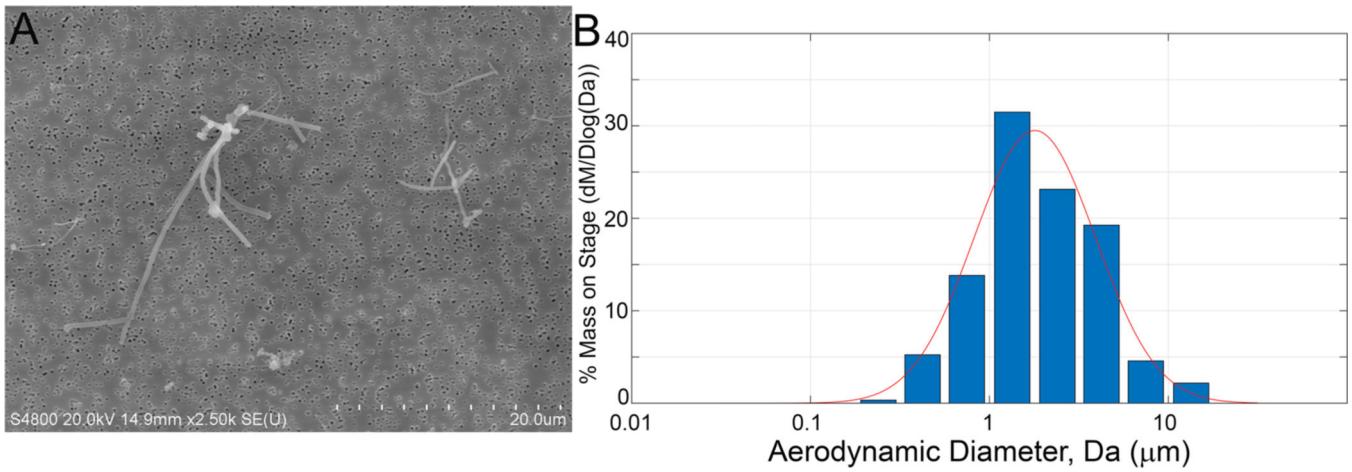

Figure 1.

MWCNT aerosol characterization. Panel A. Field emission scanning electron microscope image showing the morphology of MWCNT aerosol samples from our exposure chamber. Panel B. Mass size distribution of the MWCNT aerosol in the exposure chamber (M = mass concentration and Dae = aerodynamic diameter). The distribution has a MMAD = 1.7 μm and a GSD of 3.0.

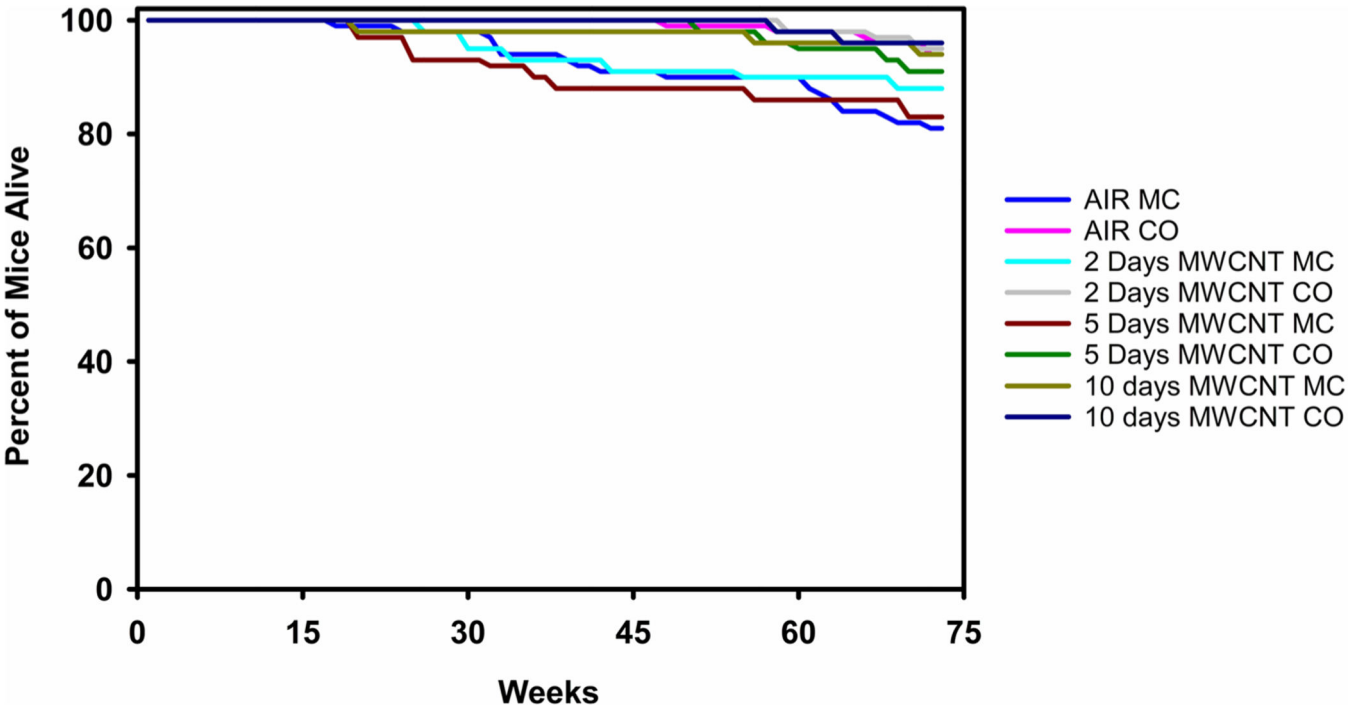

Figure 2.

Survival curve. Depicts the percent of mice alive in each treatment group throughout the duration of the study.

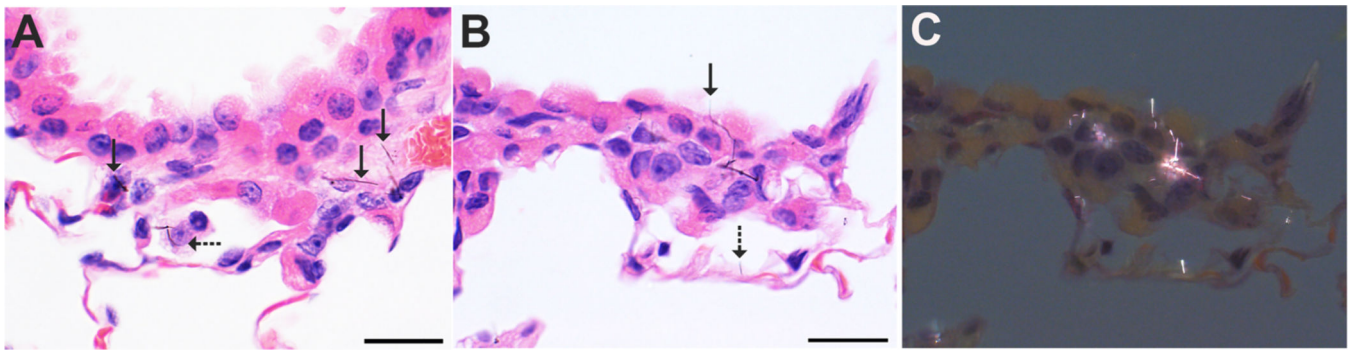

Figure 3.

Panel A. MWCNT persisting in the bronchioloalveolar junction in an H&E section of lung from a representative mouse exposed to MWCNT for 5 days. MWCNT are located within the peribronchiolar interstitium (solid arrows) and in an alveolar macrophage (dashed arrow). Panel B. MWCNT sometimes extend beyond the cytoplasmic margins (solid arrow) as in the bronchiolar epithelium of this mouse and sometimes projected from alveolar septa (dashed arrow). Panel C. This polarized light microscopic image from the same bronchioloalveolar region shown in B demonstrates the numerous MWCNT that are not easily seen with standard light microscopy. Scale bar = 20 microns.

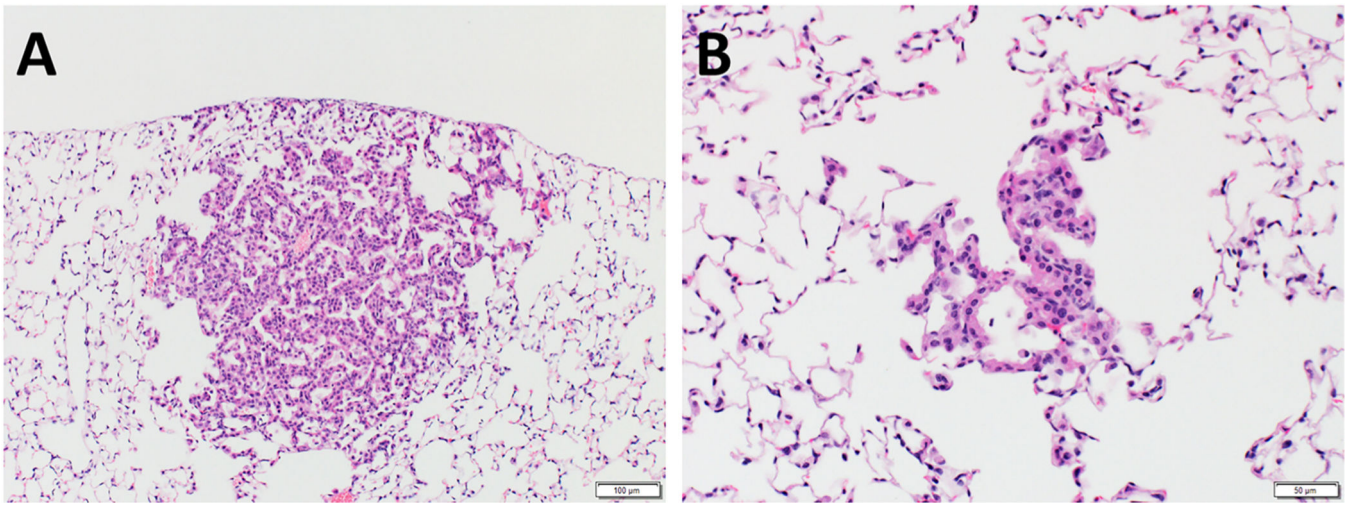

Figure 4.

Hyperplasia. Representative photomicrographs of H&E-stained sections of lung from mice exposed to MWCNT. Focal bronchioloalveolar hyperplasia: Marked hyperplasia (Panel A) is considered a preneoplastic lesion consisting of >20 contiguous alveolar walls being affected. Minimal hyperplasia is seen in Panel B.

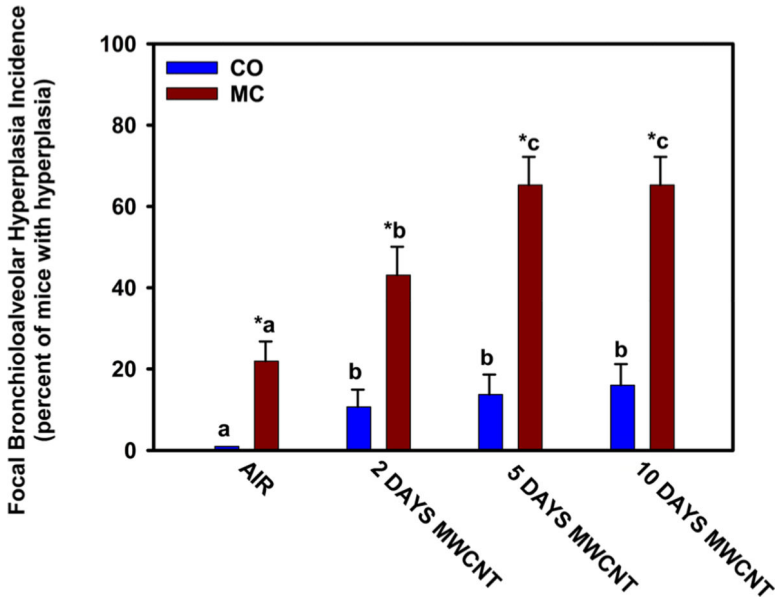

Figure 5.

Incidence of focal bronchioloalveolar hyperplasia. All mice received a single dose of either 3-methylcholanthrene (MC: 10 μg/g bw, i.p.) or corn oil vehicle (CO). One week after receiving MC or CO, mice were exposed by whole-body inhalation to MWCNT (5 mg/m^3, 5 h/day) or filtered air for 2, 5, or 10days. Mice were euthanized 17months after exposure. CO-treated mice exposed to air for 2, 5, or 10days were pooled because statistical analyses indicated no significant difference ($p > 0.05$) between exposure times. Similarly, the number of days of air exposure had no significant effect ($p > 0.05$) on MC-treated mice. Thus, MC-treated mice exposed to air for 2, 5, or 10days were also pooled. At each exposure time, an asterisk (*) indicates that the MC-treated group (MC) was significantly higher ($p < 0.05$) than the corresponding corn oil-treated (CO) group. For MC-exposed mice, bars with different letters are significantly different ($p < 0.05$).

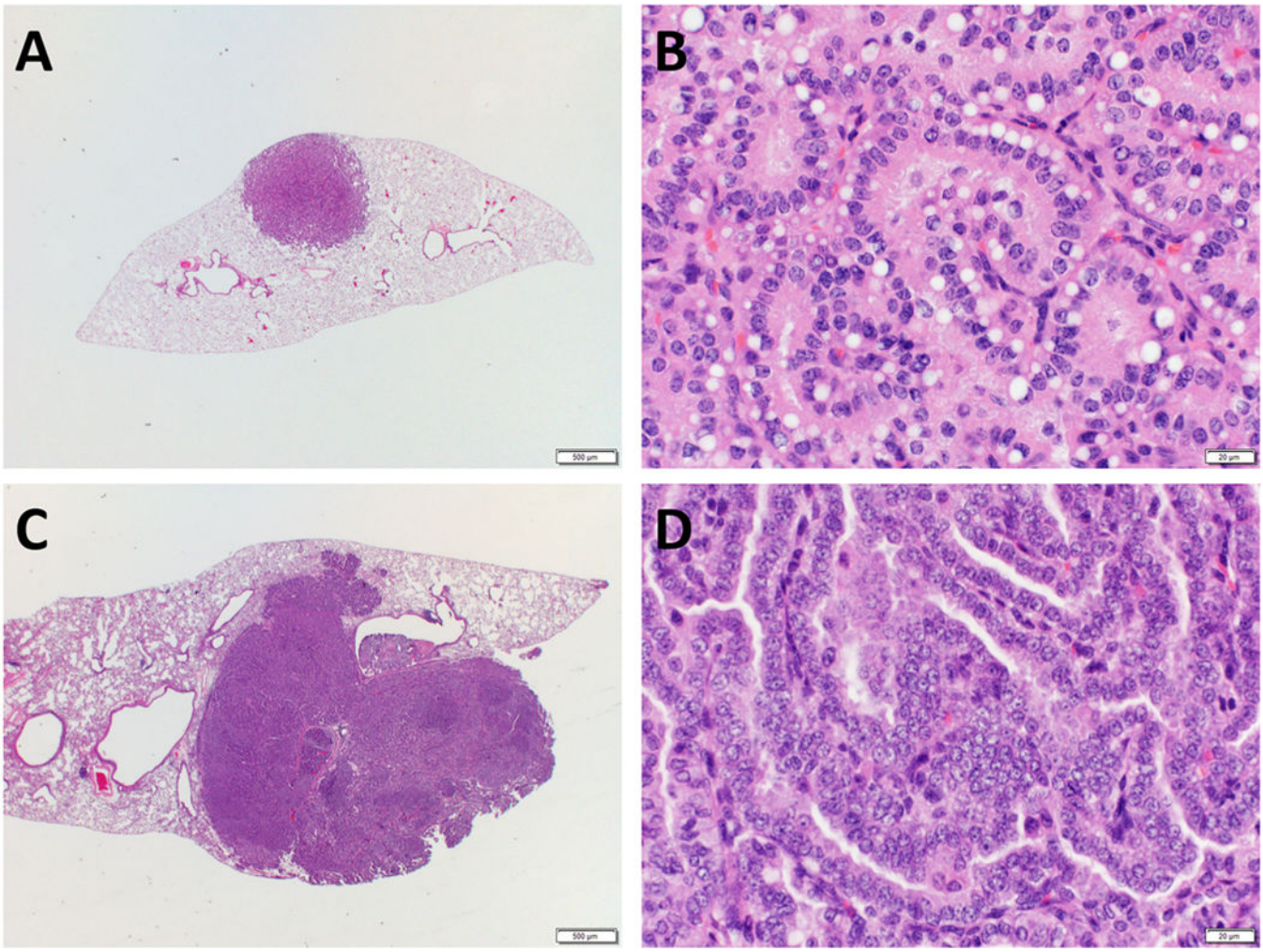

Figure 6.

Photomicrographs of bronchioloalveolar adenoma and bronchioloalveolar adenocarcinoma. Representative photomicrographs of H&E-stained sections of lung tumors from mice exposed to MWCNT. Bronchioloalveolar adenoma (Panels A and B): the adenoma is expansive and well-circumscribed, and the neoplastic cells form irregular papillary structures (Panel B). Bronchioloalveolar adenocarcinoma (Panels C and D): the adenocarcinoma is invasive and extends into the surrounding lung parenchyma. Neoplastic cells show increased cellular atypia with higher nuclear-to-cytoplasmic ratio.

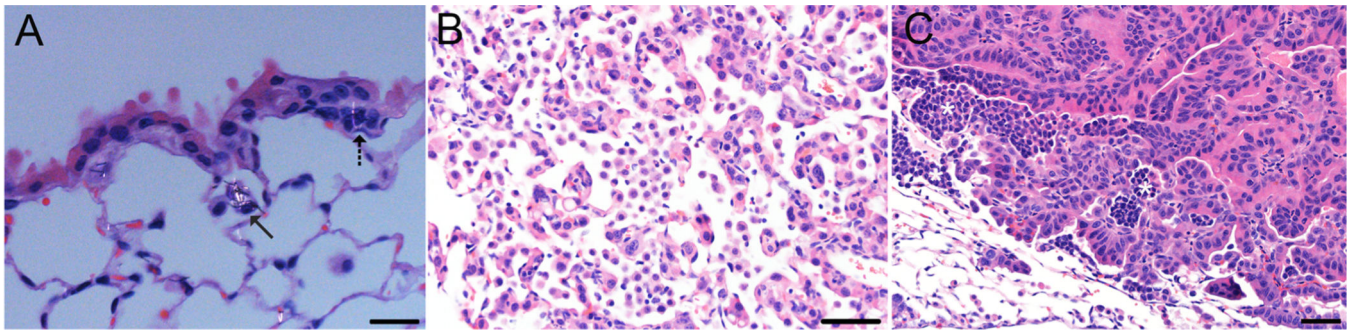

Figure 7.

Persistent Histiocytic Infiltration. In Panel A, a partially polarized image of histiocytic infiltration (alveolar histiocytosis) (solid arrow) and interstitial hypercellularity (dashed arrow) associated with persistent MWCNT (bar = 20 microns) are shown. Panel B is a light microscopic image of diffuse histiocytic infiltration associated with focal hyperplasia (bar = 50 microns). Panel C shows a light microscopic image of tumor-associated predominantly histiocytic infiltrates (asterisks).

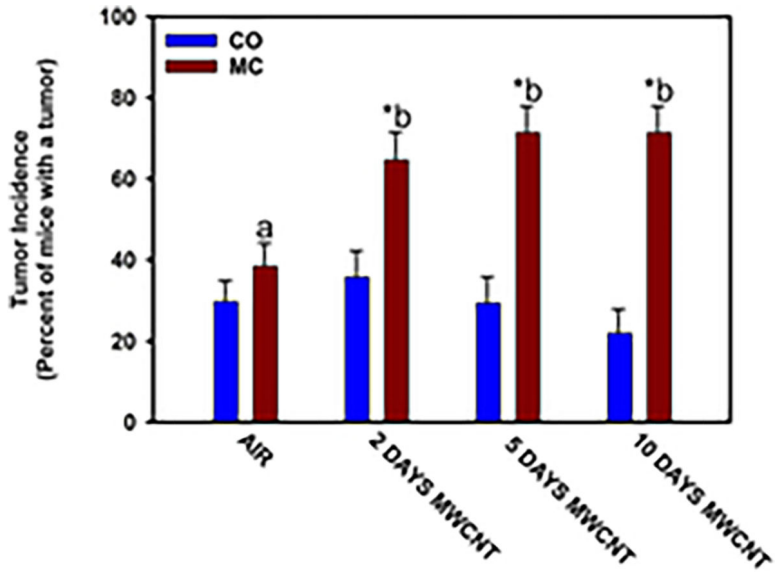

Figure 8.

Percent Tumor Incidence. All mice received a single dose of either 3-methylcholanthrene (MC: 10 μg/g bw, i.p.) or corn oil vehicle (CO). One week after receiving MC or CO, mice were exposed by whole-body inhalation to MWCNT (5 mg/m^3, 5 h/day) or filtered air for 2, 5, or 10 days. Mice were euthanized 17 months after exposure. CO-treated mice exposed to air for 2, 5, or 10 days were pooled because statistical analyses indicated no significant difference ($p > 0.05$) between exposure times. Similarly, the number of days of air exposure had no significant effect ($p > 0.05$) on MC-treated mice. Thus, MC-treated mice exposed to air for 2, 5, or 10 days were also pooled. At each exposure time, an asterisk (*) indicates that the MC-treated group was significantly higher ($p < 0.05$) than the corn oil-treated group. For MC-exposed mice, bars with different letters are significantly different ($p < 0.05$).

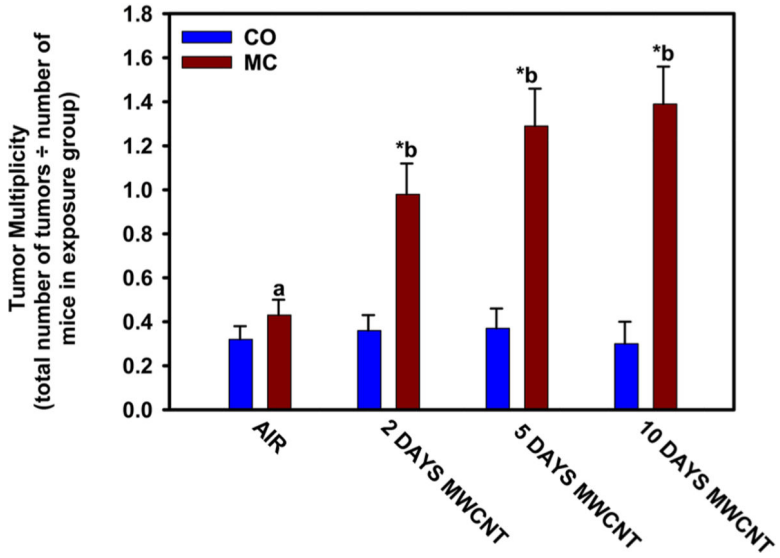

Figure 9.

Tumor Multiplicity. All mice received a single dose of either 3-methylcholanthrene (MC: 10 μg/g bw, i.p.) or corn oil vehicle (CO). One week after receiving MC or CO, mice were exposed by whole-body inhalation to MWCNT (5 mg/m^3, 5 h/day) or filtered air for 2, 5, or 10 days. Mice were euthanized 17 months after exposure. CO-treated mice exposed to air for 2, 5, or 10 days were pooled because statistical analyses indicated no significant difference ($p > 0.05$) between exposure times. Similarly, the number of days of air exposure had no significant effect ($p > 0.05$) on MC-treated mice. Thus, MC-treated mice exposed to air for 2-, 5-, or 10-days were also pooled. At each exposure time, an asterisk (*) indicates that the MC-treated group was significantly higher ($p < 0.05$) than the corn oil-treated group. For MC-exposed mice, bars with different letters are significantly different ($p < 0.05$).

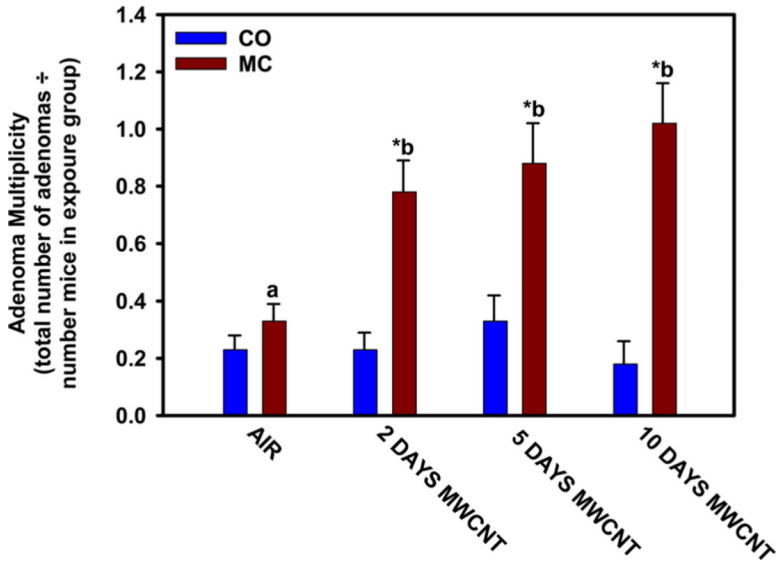

Figure 10.

Adenoma multiplicity. All mice received a single dose of either 3-methylcholanthrene (MC: 10 μg/g bw, i.p.) or corn oil vehicle (CO). One week after receiving MC or CO, mice were exposed by whole-body inhalation to MWCNT (5 mg/m^3, 5 h/day) or filtered air for 2, 5, or 10 days. Mice were euthanized 17 months after exposure. CO-treated mice exposed to air for 2, 5, or 10 days were pooled because statistical analyses indicated no significant difference ($p > 0.05$) between exposure times. Similarly, the number of days of air exposure had no significant effect ($p > 0.05$) on MC-treated mice. Thus, MC-treated mice exposed to air for 2, 5, or 10 days were also pooled. At each exposure time, an asterisk (*) indicates that the MC-treated group was significantly higher ($p < 0.05$) than the corn oil-treated group. For MC-exposed mice, bars with different letters are significantly different ($p < 0.05$).

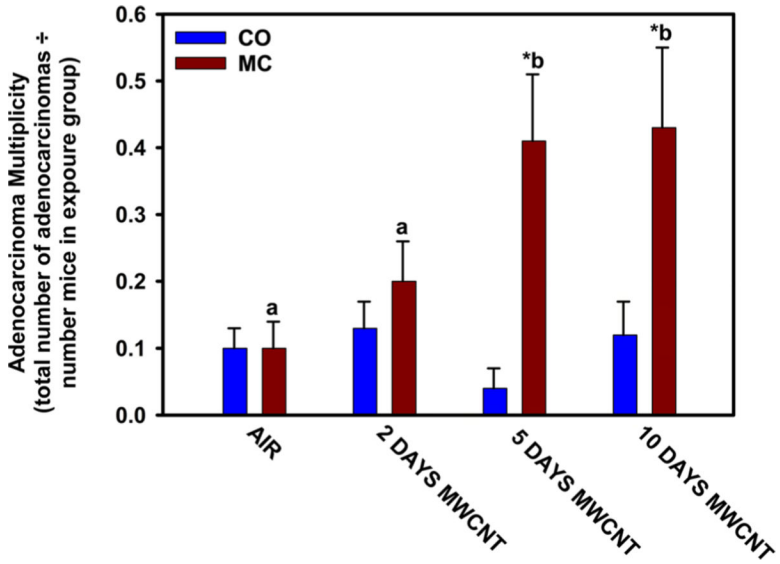

Figure 11.

Adenocarcinoma multiplicity. All mice received a single dose of either 3-methylcholanthrene (MC: 10 μg/g bw, i.p.) or corn oil vehicle (CO). One week after receiving MC or CO, mice were exposed by whole-body inhalation to MWCNT (5 mg/m^3, 5 h/day) or filtered air for 2, 5, or 10 days. Mice were euthanized 17 months after exposure. CO-treated mice exposed to air for 2, 5, or 10 days were pooled because statistical analyses indicated no significant difference ($p > 0.05$) between exposure times. Similarly, the number of days of air exposure had no significant effect ($p > 0.05$) on MC-treated mice. Thus, MC-treated mice exposed to air for 2, 5, or 10 days were also pooled. At each exposure time, an asterisk (*) indicates that the MC-treated group was significantly higher ($p < 0.05$) than the corn oil-treated group. For MC-exposed mice, bars with different letters are significantly different ($p < 0.05$).

Table 1.

Summary of lesions in unscheduled deaths.[a]

			MWCNT					
	Air		2 days		5 days		10 days	
Lesion Location	**Corn Oil**	**MC**	**Corn Oil**	**MC**	**Corn Oil**	**MC**	**Corn Oil**	**MC**
Extra-pulmonary only	1	5	0	3	0	5	0	1
Pulmonary only	0	0	0	1	2	2	0	0
Extra-pulmonary and pulmonary	4	6	0	3	0	1	0	1
No lesion(s) identified	1	1	0	0	0	0	0	0

[a]Number in table indicates number of mice.

Table 2.

Number of mice examined at terminal sacrifice and unscheduled deaths.

	CO						MC					
	Air			MWCNT			Air			MWCNT		
Days of Inhalation Exposure	2	5	10	2	5	10	2	5	10	2	5	10
Number of mice at start of study	32	32	26	57	59	52	32	32	26	57	59	52
Number of mice examined at terminal sacrifice	31	28	25	56	51	50	28	26	19	51	49	49
Number of mice examined with unscheduled deaths	4	1	1	0	2	0	3	5	4	7	8	2
Number of mice with bronchioloalveolar adenoma(s) at terminal sacrifice	7	4	7	13	13	6	8	10	4	30	29	30
Number of mice with bronchioloalveolar adenocarcinoma(s) at terminal sacrifice	5	3	0	7	2	6	2	0	4	10	15	13
Number of mice with bronchioloalveolar adenoma(s) with unscheduled deaths	1	0	0	0	0	0	1	0	0	1	1	1
Number of mice with bronchioloalveolar adenocarcinoma(s) with unscheduled deaths	0	0	0	0	1	0	0	0	0	0	2	0

Table 3.

MWCNT lung burden.[a,b]

Days of exposure	MWCNT lung burden (μg/mouse)
2	4.4 ± 0.2
5	10.2 ± 0.4
10	20.8 ± 0.8

[a] Values represent mean ± *SE*.

[b] For each exposure time, mice used for lung burden determinations were composed of 6 mice which received corn oil (vehicle) and 6 mice which received methylcholanthrene (MC). Statistical analyses indicated no significant difference ($p > 0.05$) between CO -and MC-treated mice at a given exposure time. Thus, the samples at each exposure time were pooled for $N = 12$.