

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 et al. 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 et al. 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 110R, 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/m³ for 5 hrs/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 et al. 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 (National Toxicology Program 2006; National Toxicology Program 2007a; National Toxicology Program 2007b; National Toxicology Program 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 et al. 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 (National Toxicology Program 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 one-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/m³, 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 $16,000 \times g$ for 10 minutes. The supernatant was removed; the remaining pellet was mixed with 50% HNO₃/methanol (v/v), and incubated at 60°C overnight, followed by centrifugation ($16,000 \times g$, 10 minutes). After centrifugation, the supernatant was removed, and the pellet was resuspended in 10% NP-40 (v/v) in dH₂O, followed by 30 second 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 $p < 0.05$.

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