Dose- and Time-dependent Effects of Sub-chronic Functionalized MWCNT Exposure on Human Mesothelial Cell Neoplastic Transformation Potential

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ABSTRACT

Comparative studies evaluating how MWCNT physicochemical properties influence pleural mesothelioma potential are critically needed. We hypothesized that MWCNT surface functionalization would affect human mesothelial cell neoplastic transformation potential in a dose/time dependent manner. Cells were continuously exposed to fully characterized parent(p), two different functionalized (f) MWCNTs, or asbestos (ASB) for 6 months at *in vivo*-relevant doses. Low dose fMWCNT caused significant cell proliferation increase compared to controls throughout the exposure. pMWCNT, one fMWCNT and ASB cells exhibited significantly greater numbers of soft agar colonies compared to controls, with low dose eliciting a more potent effect. Cells exposed to only one fMWCNT exhibited a significant increase in cell invasion ability. Subchronic *in vitro* exposure models can assist in screening and prioritizing engineered nanomaterials for *in vivo* risk to aid occupational-associated disease detection strategies.

Disclaimer: The findings and conclusions in this abstract are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

Keywords: carbon nanotubes, cell transformation, functionalization, *in vitro* model, mesothelium

1 INTRODUCTION

Multi-walled carbon nanotubes (MWCNTs) are characterized by asbestos-like high aspect ratio (HAR) fiber morphology, large surface area, and surface chemistries. Exposure at occupationally relevant concentrations results in transient pulmonary inflammation, biopersistence, onset of fibrosis, extrapulmonary transport and promotion of adenocarcinoma and sarcomatous mesothelioma [1,2]. As MWCNTs become widely used, elevated risk of cancer in pleural mesothelium following inhalation exposure is a concern [3,4]. Physicochemical properties of MWCNTs that influence pleural mesothelioma potential are largely unknown, but critically needed to understand toxicological mechanisms and safe-by-design strategies.

Our previous studies have shown that sub-chronic *in vitro* exposure of single-walled carbon nanotubes (SWCNT) and MWCNT to lung epithelial cells imparts a neoplastic-like and malignant transformation effect [5,6]. Alternative testing strategies (ATS) for engineered nanomaterial (ENM) exposure-associated disease employs sensitive *in vitro* screening models to assess and prioritize ENMs for further testing [7]. We hypothesized that the effect of surface functionalization of MWCNT would affect human mesothelial cell neoplastic transformation potential in a dose and time dependent manner.

2 METHODS

2.1 Nanoparticle Preparation and Dispersion

MWCNT, functionalized during plasma gas synthesis, were acquired (CheapTubes, Inc.) with equal lengths containing less than 1 wt % of contaminants. MWCNT suspensions in water were used to prepare 1.5% bovine serum albumin (BSA)-dispersed 0.1 mg/ml stock solutions. To expose cells, each stock in a sterile glass vial was sonicated for 1 min/ml at 40% intensity using a VibraCell large cup horn sonicator within encloser (Sonics Materials, Inc). All materials were characterized for zeta potential (NanoSeries ZetaSizer, Malvern Instruments), BET surface area, FTIR, XPS and FESEM.

2.2 Cell Culture and Sub-Chronic Exposure

Low passage, immortalized human pleural mesothelial cells (MET5A; ATCC) were seeded ($5x10^4$/well) in triplicate to 6-well plates holding M199 medium supplemented with 10% FBS, EGF, hydrocortisone, insulin, selenious acid, and micronutrients. Following seeding, cells were continuously exposed to 0.002 or 0.02 μg/cm^2 of sonicated, dispersed pMWCNT, fMWCNT (MWCNT-COOH and MWCNT-NHx) or suspended crocidolite asbestos fibers over 6 months. Doses modeled 25- and 250x higher dose expected for MWCNT (0.0008 μg/cm^2) at the pleural mesothelium *in vivo* at 56d following 80 μg/lung [6,8]. Saline- and BSA-only exposed cells were passaged throughout exposure as a controls. Cells were re-exposed to BSA-dispersed MWCNT or saline-dispersed ASB in fresh medium every two days and were passaged every 4-5 days.

2.3 Cancer Hallmark Phenotype Assessment

At pre-selected time points (Figure 1), each treatment group was assessed for four major cancer hallmark phenotypes. First, seeded cells ($5x10^3$ or $5x10^4$/well) in a

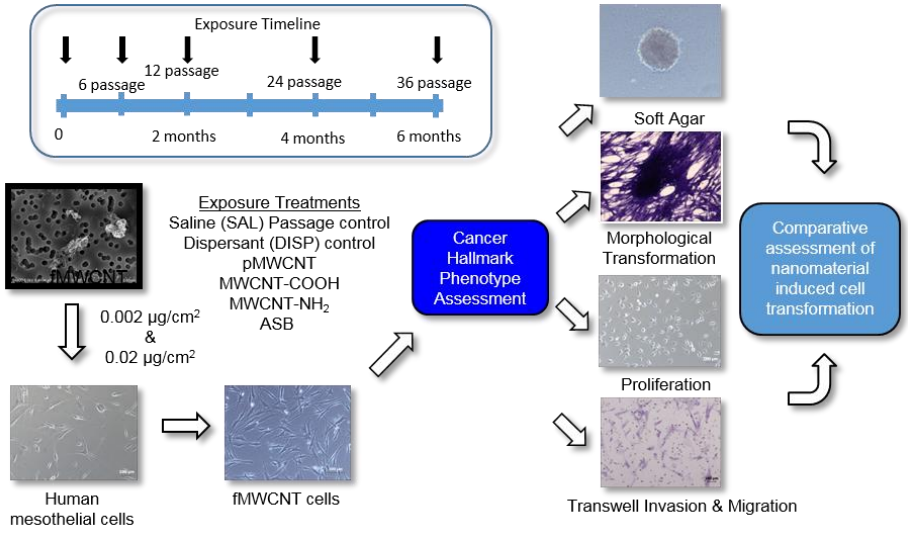

Figure 1. *In vitro* exposure and cancer hallmark screening model to assess dose- and time-dependent sub-chronic nanomaterial exposure potential to promote neoplastic transformation in human pleural mesothelial cells.

96-well or 6-well plate were incubated in a particle-free culture medium for 24 – 144 hours. Cell proliferation was determined by WST-1 spectrophotometric and trypan blue exclusion assays, respectively. Growth rate curves based on live cell counts were plotted.

Cancer cells exhibit enhanced mobility and the ability to invade neighboring tissue, usually in low nutrient microenvironments. To test this ability, exposed cells were suspended above 10% FBS M199 growth media and held for 24 or 48 hours, respectively, to transwell migration or Matrigel® invasion inserts (BD Biosciences) in serum-free medium. Inserts were fixed, DiffQuik stained, dried, and photographed using bright field microscopy.

Neoplastic epithelial cells with tumor-fomring capability typically exhibit attachment-indendent growth characteristics in nutrient rich soft agar. Exposed MET5A cells ($4.3x10^4$) were mixed 1:2 in agar/media containing Difco agar, 15% FBS, 2x minimal MEM medium, and 1% gentamicin. Suspended cells were slowly layered onto cooled agar/media in triplicate in 6-well plates and allowed to solidify at a final $1x10^4$ cell density. Colonies were photographed using dark field inverted microscopy at 14 and 21 d.

Neoplastic epithelial cells typically experience morphological transformation characterized by escape from cell-cell contact inhibition of cell growth, cell mounding, survival in low nutrient conditions, dark cytoplasmic staining, and invasive margins along colony edges. Therefore, cells were seeded at $5x10^4$ cells in 60 mm plates in triplicate per treatment group and cultured for 21 d to determine morphological transformation. At 14 d, confluent layers of cells were held in serum-free medium for the last 7 d. No passaging of cells occurred. Cells were fixed in 10% buffered formalin, crystal violet stained, and manually scored under bright field microscopy.

All experiments were independently replicated 3 – 5 times. Treatments were compared using two-way ANOVA analyses followed by Dunnett's or Tukey-Kramer HSD post-hoc tests ($\alpha = 0.05$).

3 RESULTS

3.1 fMWCNT displayed differences in oxygen functionalization

All MWCNTs and crocidolite asbestos were characterized and combined with manufactorer information (Table 1). All MWCNTs exhibited the same lengths and widths while the primary difference was surface functionalization (CheapTubes Inc). FTIR and XPS spectra of fMWCNT indicated that all MWCNT possessed similar C-O-C and C-OH functionalization in differing amounts. Reduced peak at 1200 cm-1 and peak at 285.7 eV on MW-NHx samples (not shown) suggested presence of nitrogen functionalization [9]. FESEM analysis found that all MWCNT displayed single fiber morphology and tangled agglomerates.

3.2 Low dose MW-COOH and MW-NHx display enhanced proliferation ability

The effect of surface functionalization on proliferation was dose and time dependent. After 3 weeks exposure, low dose of both fMWCNT and ASB caused a significant increase in live cell number, but not mitochondrial activity compared to controls (not shown). At 2 and 4 months, both MW-COOH doses and low dose MW-NHx exhibited proliferation rate while no differences occurred in WST assay (not shown). Lastly at 6 month, low dose MW-COOH and MW-NHx exhibited significantly greater cell count and WST assay activity while ASB showed significantly lower proliferation compared to controls (Figure 2A).

Particle	Parent MWCNT	MW-COOH	MW-NHx	Asbestos
Name/Source	CheapTubes, Inc.	CheapTubes, Inc.	CheapTubes, Inc.	NIEHS, Kalahari Desert
Synthesis	Argon plasma	Proprietary Oxygen Blend plasma Functionalized	Nitrogen plasma Functionalized	Natural
Primary Functionality	Non-functionalized	COOH	N=H	n/a
Mean Length (μm)/Width (nm)	1 to 12 / 13-18	1 to 12 / 13-18	1 to 12 / 13-18	10 / 210
Surface Area (m^2/g)	214	214	214	9.8
Zeta Potential (water/medium)	-24.1 / -9.6	-30.1 / -8.7	-32.5 / -10.1	-35.2 / -8.0

3.3 fMWCNT- and ASB-exposed cells show enhanced attachment-independent colony growth, but limited invasion ability

Similar to proliferation, soft agar colony formation ability in MWCNT-exposed cells was particle functionalization, dose- and time-dependent. At 2 months, high dose MW-NHx and ASB showed significant increase above respective controls. At 4 and 6 months, both doses of most MWCNT and ASB showed a significant increase in soft agar colony number (Figure 2B). Low dose MW-NHx at 4 months showed no difference compared to control. Ranked severity, irrespective of dose and time, for colony formation was ASB > pMWCNT > MW-COOH > MW-NH2>>>passage controls. MW-COOH-exposed cells exhibited significantly greater invasion ability at low dose/6month and high dose at 4 and 6 month (Figure 2C). All other treatments were not different from each other. At 6 months, ASB-exposed cells, irrespective of dose, showed the ability to form Type II foci (i.e. multi-layered cell mounding and dark staining, Figure 2D). All MWCNT-exposed cells exhibited Type I foci, with occasional Type II formation ability. No Type III foci were observed.

4 DISCUSSION

In conclusion, dose, time and differences in surface functionalization of MWCNT impacted several well-established cancer hallmark phenotypes in human pleural mesothelial cells following sub-chronic exposure at occupationally relevant doses. Numerous SWCNT, MWCNT and graphene nanomaterial toxicology studies report that different surface properties and/or modifications to graphene surface is a major factor in determining toxicity and early onset of disease [10-12]. HAR fiber exposures, including MWCNT and asbestos, to mesothelial tissue typically results in mesothelial lesions, inflammation and localized hyperplasia typically associated with biopersistant fibers or phagocytic leukocyte infiltrat [1, 4, 13, 14]. Formation of hyperplastic lesions is considered a pre-neoplastic occurrence in HAR tumorigenesis. A recent i.p. injection studies showed that onset of MWCNT-induced mesothelial cancer was time-independent while incidence rate was largely dose-dependent [4]. Since a low percentage of inhaled CNT is expected to reach mesothelial tissue [3], late onset of HAR fiber-associated carcinogenesis contributes to the difficulty in understanding both mechanism and comparative studies between fibers. This *in vitro* model helps address that challenge.

Low dose MW-COOH–exposed cells exhibited enhanced proliferation throughout the 6 month exposure. This, coupled with enhanced soft agar colony and invasion ability starting at 4 months, suggests that MW-COOH potentially harbors neoplastic transformation potential at occupationally relevant doses. Recent studies with phagocytic cells indicated that negatively charged functional groups (e.g.–COOH) on CNT results in fibers that are more hydrophilic and less reactive with cell plasma membrane, resulting in reduced membrance damage and inflammatory potential. Positively-charged surfaces (e.g. amine groups) can exhibit greater reactivity with negatively-charged phospholipids, thus causing greater damage and inflammation [10,11]. Mesothelial cells, however, do not normally exhibit CNT phagocytosis [15], and it is assumed that hyperplasia is due to pro-inflammatory mediators from pleural macrophages [4, 13, 14]. Recent studies with graphene oxides (GO) and other oxidized/reduced graphene particles indicate that they are more genotoxic than the pristine parent particle, in part due to improved dispersability, enhanced cell uptake, reactivity of oxygen groups and genotoxicity below ROS generation dose-thresholds [16-19]. Previously, we have shown that low doses of CNTs, below thresholds for reduced viability, imparts stimulation of exposed lung cells, resulting in proliferation, collagen production and malignant transformation *in vitro* and is predictive of *in vivo* response [5,6, 20]. Our data indicate that the potential exists for an oxygen-functionalized MWCNT at low dose to impart pre-neoplastic effect, although the mechanism has yet to be determined. Our previous studies with SWCNT and MWCNT sub-chronic exposure to MET5A cells showed an aggressive invasion ability associated with known lung cancer signaling [21]. Asbestos-exposed cells, although slow growing and low invasion ability compared to fMWCNT, showed the largest ability for soft agar colony and *in vitro* morphological transformation. Our *in vitro* data correlates with late onset of asbestos-induced tumorigenesis in both animal and human studies [1].

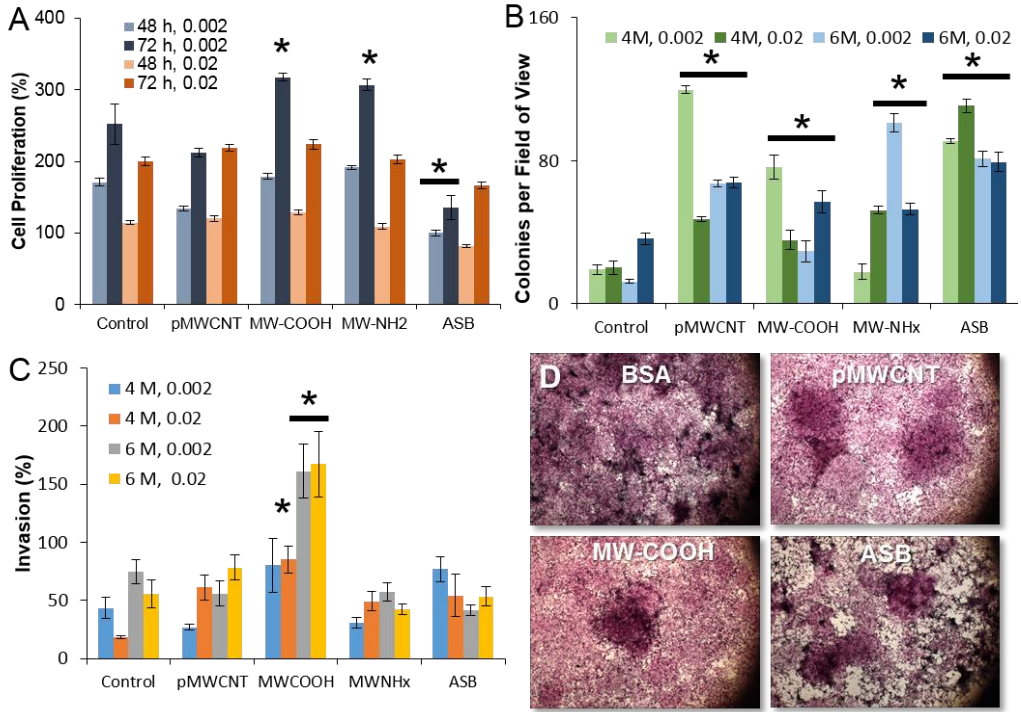

Figure 2. fMWCNT exposure impacts mesothelial cell A) proliferation, B) soft agar colony formation, C) invasion and D) morphological transformation. Error bars are ±SE. * $p < 0.05$ compared to unexposed controls.

Systematic evaluation of carbon nanomaterials and other ENMs with different physicochemical properties, with relevant positive control particles, is a useful *in vitro* ATS screening model (Tier I) for neoplastic transformation potential and can prioritize these nanomaterials for Tier II *in vivo* tumorigenesis studies. Continued efforts in developing this and similar models addresses the pressing need for high-throughput and robust disease detection strategies to assess disease risk in workers during nanomaterial manufacturing, promote 'prevention-through-design,' and protect a promising nanotechonology industry.

REFERENCES

[1] K. Donaldson, C.A. Poland, F.A. Murphy, M. MacFarlane, T. Chernova, A. Schinwald. Adv Drug Deliv Rev., 65, 2078-86, 2013.

[2] L. Sargent, D.W. Porter, L. Staska, A.F. Hubbs, D.T. Lowry, et al. Part Fibre Toxicol., 11, 3, 2014.

[3] R.R. Mercer, J.F. Scabilloni, A.F. Hubbs, L. Wang, L.A. Battelli, et al. Part. Fib. Toxicol. 10, 38, 2013.

[4] A. Takagi, A. Hirose, M. Futakuchi, H. Tsuda and J. Kanno. Cancer Sci, 103, 1440-44, 2012.

[5] L. Wang, S. Luanpitpong, V. Castranova, W. Tse, Y. Lu, V. Pongrakhannon and Y. Rojanasakul. NanoLetters, 11, 2796-803, 2011.

[6] L. Wang, T.A. Stueckle, A. Mishra, R. Derk, V. Castranova and Y. Rojanasakul. Nanotoxicology, 8, 485-507, 2014.

[7] A.E. Nel, E. Nasser, H. Godwin, D. Avery, T. Bahadori, et al. 2013. ACS Nano, 7,6422-33, 2013.

[8] R.R. Mercer, J.F. Scabilloni, A.F. Hubbs, L. Batelli, et al. Part Fibre Toxicol, 10,33, 2013.

[9] W. Chidawanyika, T. Nyokong. Carbon 48, 2831-8, 2010.

[10] X. Wang, T. Xia, S.A. Ntim, Z. Ji, S. Lin, et al. ACS Nano, 5, 9772-87, 2011.

[11] R. Li, X. Wang, Z. Ji, B. Sun, H. Zhang, et al. ACS Nano, 7, 2352-68, 2013.

[12] T.M. Sager, M.W. Wolfarth, M. Andrew, A. Hubbs, et al. Nanotoxicol. 8,317-27, 2014.

[13] F. A. Murphy, A. Schinwald, C.A. Poland, K. Donaldson. Part. Fibre Toxicol. 9, 8, 2012.

[14] J. Xu, D.B. Alexander, M. Futakuchi, T. Numano, K. Fukamachi, et al. Cancer Sci, 105,763-9, 2014.

[15] H. Nagai, Y. Okazaki, S.H. Chew, N. Misawa, Y. Yamashita, et al. PNAS, 108, E1330-38, 2011

[16] O. Akhavan, E. Ghaderi, A. Akhavan. Biomaterials, 33:8017-25, 2012.

[17] N. Chatterjee, H.J. Eom, J. Choi. Biomaterials 35,1109-1127, 2014.

[18] M. Hinzmann, S. Jaworski, M. Kutwin, J. Jagiełło, et al. Int J Nanomedicine, 9, 2409–17, 2014.

[19] S. Tsuruoka, H. Matsumoto, K. Koyama, E. Akiba, T. Yanagisawa, et al. Carbon, 83, 232-239, 2015.

[20] L. Wang, V. Castranova, A. Mishra, B. Chen, R.R. Mercer, et al. Part Fibre Toxicol, 7, 31, 2010.

[21]W. Lohcharoenkal, L. Wang, T.A. Stueckle, CZ Dinu, Y Liu, et al. ACS Nano, 7, 7711-7723, 2013.