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Multiwalled Carbon Nanotube Functionalization with High Molecular Weight Hyaluronan Significantly Reduces Pulmonary Injury

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Abstract

Commercialization of multiwalled carbon nanotubes (MWCNT)-based applications has been hampered by concerns regarding their lung toxicity potential. Hyaluronic acid (HA) is a ubiquitously found polysaccharide, which is anti-inflammatory in its native high molecular weight form. HA-functionalized smart MWCNTs have shown promise as tumor-targeting drug delivery agents and can enhance bone repair and regeneration. However, it is unclear whether HA functionalization could reduce the pulmonary toxicity potential of MWCNTs. Using *in vivo* and *in vitro* approaches, we investigated the effectiveness of MWCNT functionalization with HA in increasing nanotube biocompatibility and reducing lung inflammatory and fibrotic effects. We utilized three-dimensional cultures of differentiated primary human bronchial epithelia to translate findings from rodent assays to humans. We found that HA functionalization increased stability and

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Supporting Information

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Detailed methods, characterization of MWCNTs, suspension stability, TEM analysis of Uptake, lung carbon contents, role of metal impurities, PDGF-AA assay, primer sequences used for real time RT-PCR assays (PDF)

Video microscopy of cilia beating (AVI)

dispersion of MWCNTs and reduced postexposure lung inflammation, fibrosis, and mucus cell metaplasia compared with nonfunctionalized MWCNTs. Cocultures of fully differentiated bronchial epithelial cells (cultivated at air–liquid interface) and human lung fibroblasts (submerged) displayed significant reduction in injury, oxidative stress, as well as pro-inflammatory gene and protein expression after exposure to HA-functionalized MWCNTs compared with MWCNTs alone. In contrast, neither type of nanotubes stimulated cytokine production in primary human alveolar macrophages. In aggregate, our results demonstrate the effectiveness of HA functionalization as a safer design approach to eliminate MWCNT-induced lung injury and suggest that HA functionalization works by reducing MWCNT-induced epithelial injury.

Graphical Abstract

Keywords

multiwalled carbon nanotubes; hyaluronan; lung; inflammation; fibrosis; mucous metaplasia; differentiated human bronchial epithelia

Multi-walled carbon nanotubes (MWCNTs) are used in composite materials, thin films, and energy storage devices, as well as in emerging applications like structural engineering, optics, aerospace engineering, biosensors, bioimaging and gene/drug delivery systems.^{1–6} For these reasons, the MWCNT global market is anticipated to reach 1 trillion dollars within the next decade.⁷ However, increasing production and utilization of MWCNTs raise the risk of occupational and environmental human exposures. Given that MWCNTs still have a largely undefined safety profile, there is an urgent need to evaluate their health risks. Inhalation is the major route of occupational exposure to MWCNTs, and it has already been shown that MWCNT exposure can lead to inflammation, fibrosis, and granuloma formation in the lungs.^{8–12}

Hyaluronic acid (HA) is a negatively charged linear polysaccharide composed of repeating β ,1–4-linked D-glucuronic acid and β ,1–3-linked N-acetyl-D-glucosamine disaccharide units.¹³ HA is ubiquitously found in vertebrates and is a major component of the extracellular matrix with significant roles in organogenesis, growth, wound healing, and tissue remodeling. HA, in its native high molecular weight form, is nonimmunogenic,

biocompatible, biodegradable, and inherently noninflammatory in nature. Use of HA in nanomedicine as a safe and selective tumor-targeting vector has been proposed¹⁴ and validated using various types of nanomaterial cargo.^{14–19} HA-functionalized smart MWCNTs have shown promise as tumor-targeting drug delivery agents and can enhance bone repair and regeneration.^{20–22} These formulations also have promising potentials in pulmonary chemotherapy and diagnostics, because inhalant chemotherapy is more effective in treating lung cancer with less systemic side effects.²³ However, concerns for potential lung toxicity limit their development and utilization.

Recently, some attempts have been made to pinpoint the key factors that induce pulmonary toxicities. Surface charge, metal impurity, surface defects, and biostability have been demonstrated to be responsible for MWCNT-induced lung inflammation and profibrogenic effects.²⁴ These findings have facilitated the development of safe design approaches for MWCNTs, including surface charge control,²⁵ pluronic F108 coating,²⁶ heavy metal removal,²⁷ *etc.* However, no study explored the use of anti-inflammatory molecules to reduce the inflammation-related hazard effects by MWCNTs.

In this manuscript we functionalized MWCNT surfaces with HA through a noncovalent phospholipid linkage and evaluated pro-inflammatory and profibrotic changes in the lungs of mice *in vivo* and in primary cultures of human airway epithelial cells, alveolar macrophages, and lung fibroblasts *in vitro* after exposure to these HA-grafted MWCNTs (HA-MW) in comparison to purified, nonfunctionalized MWCNTs (MW). We demonstrate that HA-MW have significantly improved suspension stability and have much lower *in vivo* biological activity, *i.e.*, significantly reduced lung injury: HA-MW exposure results in significantly reduced inflammation, fibrosis, and mucous cell metaplasia (MCM) in mouse lungs as compared to otherwise identical but nonfunctionalized MWCNTs (MW). We further demonstrate that profibrotic gene expression in human fibroblasts accurately predicts *in vivo* fibrotic responses after exposure to MW and HA-MW. Using human differentiated primary bronchial epithelial cells cultivated at air–liquid interface and cocultured with fibroblasts, we validated the reduced pro-inflammatory and toxic potentials of HA-MW as compared to MW. In contrast, neither type of nanotubes stimulated cytokine production in primary human alveolar macrophages. Herein, we demonstrated a cost-effective and efficient chemical functionalization strategy that can significantly reduce the lung injury potential of MWCNTs by reducing MWCNT-induced epithelial injury.

RESULTS AND DISCUSSION

HA Functionalization Improves Stability and Reduces Defects on Nanotube Structure

TEM analysis revealed that the MW sample contains large CNT aggregates. Upon HA modification the tubes showed improved dispersion (Figure 1A). Raman spectroscopy confirmed the nanotube structure for both samples. However, some structural changes of MW upon HA modification were also observed. As shown in Figure 1B, both MW and HA-MW showed two characteristic peaks, *i.e.*, G band at 1570 cm^{−1} assigned to the in-plane vibration of C–C bond and D band at 1340 cm^{−1} activated by the presence of disorder in the carbon system, which confirmed the CNT structure of both samples. However, the intensity ratio of the D band to the G band (I_D/I_G) decreased from 1.04 to 0.94 after HA modification,

suggesting a slight decrease of defective structures in the latter sample, which could be due to the coverage by HA and DPPE molecules on the HA-MW surfaces.²⁸ To identify the specific functional groups on nanotube surfaces, FTIR analysis was also performed. Figure 1C shows that both MW and HA-MW have two strong and broad bands in the 3100–3600 cm^{-1} region, which are attributed to the stretching mode of the O–H group, resulting from ambient atmospheric moisture and oxidation during purification of the raw material.²⁹ A weak band at 1630 cm^{-1} can be assigned to O–H stretching in adsorbed water.³⁰ The C–H stretching bands at 2920, 2850, and 1401 cm^{-1} , C–O stretching mode at 1071 cm^{-1} , which are all characteristic bands of MWCNTs, are observed in both samples.³¹ A narrow region of the FTIR spectrum (Figure 1D) also revealed a unique band at 1660 cm^{-1} for HA-MW, which is characteristic of the C–O carboxyl amide I group in HA, therefore confirming the successful HA modification.³² ICP-OES analysis showed that MW contains ~1.65 wt % Ni and a trace amount of Fe. The Ni content decreased to 0.77 wt % for the HA-MW, which is reasonable considering that the HA functionalization involves acid washing steps, which may remove a part of the metal impurities, and also adds nonmetal containing HA to the molecular structure, thus diluting the measure of metal contents by weight. To estimate the HA coating concentration, phosphorus content in the HA-MW sample was first measured by ICP-OES to be ~0.82 wt %, from which the DPPE concentration was then derived. Since each DPPE molecule is conjugated to one HA repeating unit, HA concentration was then calculated to be ~10 wt %.

Nanotube suspensions in various media used to treat mice and cultured cells were analyzed by dynamic light scattering (DLS), and the results are presented in Table 1. Both types of nanotubes showed ζ potential values of ~10–20 mV in all different exposure media, suggesting the high potential of agglomeration. The hydrodynamic diameters for high aspect ratio (AR) materials like MWCNTs studied here are defined as the equivalent spherical diameters, *i.e.*, the diameters of spherical particles with the same translational diffusion coefficient, and therefore are only semiquantitative. However, our previous studies did show that DLS can be used as a valuable technique for estimating the agglomeration state of high AR materials like CeO_2 and MWCNTs.^{25,27,33} However, the hydrodynamic diameters of tubes in most media are in the range of 200–300 nm, much lower than expected. This could be because large agglomerates have already settled down, and only the small particles that remain suspended in the media are detected. One should note that the principle of DLS is based on light scattering by particles that are undergoing random Brownian motion in liquid suspension. In order to assess bioavailability of nanotubes to the cultured cells, we assayed the suspension stability index (a proxy for nanotube sedimentation) in various dispersion media (Supplementary Figure S1). As shown in the Figure S1, HA-MW and MW displayed similar stability indices in MucilAir medium. To minimize the effect of sedimentation on nanotube bioavailability, we used minimal suspension volume that made only a 0.5 mm thin layer on the bronchial epithelial layer resembling *in vivo* conditions. By this approach we avoided disruption of the air–liquid interface that can be caused by the use of larger volumes and may induce nonspecific changes in cellular responses. Similarly, under *in vivo* conditions, sedimentation plays a minor role in terms of nanotube bioavailability because of the very thin layer of lung lining fluid. We observed that in BEGM and RPMI medium

nearly 20–40% of both MW and HA-MW precipitated to the bottom of the dish in the 24 h experimental period.

HA Functionalization Reduces Acute Lung Injury Potential of Nanotubes

MWCNTs are known to induce acute inflammatory responses in the lungs.^{11,34–36} In order to assess the impact of HA-MW in mouse lungs we exposed mice to 1.5 mg/kg of either HA-MW or MW through oropharyngeal aspiration and compared the effect with vehicle (1 mg/mL bovine serum albumin) or HA (1.5 mg/kg) at day 1 postexposure. We calculated the above nanotube dose by employing an established methodology adopted by the National Institute of Occupational Safety and Health (NIOSH) that calculates human relevant dose for *in vivo* exposures.^{7,37,38} Our *in vivo* dose of $\sim 30 \mu\text{g}/\text{mouse}$ corresponds to 60 mg/lung human burden (assuming 100 m^2 lung surface). This experimental dose can be reached in a worker after 2.25 months of exposure at $400 \mu\text{g}/\text{m}^3$ (inhalable concentration reported in a research facility)³⁹ or 7.5 years of exposure at $10 \mu\text{g}/\text{m}^3$ (average inhalable MWCNT level in US facilities),³⁸ assuming lung deposition fraction of 30%, and a workday inhalation ventilation of 10 m^3 for a person working an 8 h shift. These estimates suggest that our tested nanotube concentrations are relevant for workplace exposures in humans. It is important to note that our objective was to evaluate the impact of HA functionalization on nanotube-induced pathology and for that reason we opted to use an effective dose for pulmonary toxicity end points (especially fibrosis).⁴⁰ Moreover, our *in vitro* doses of $25\text{--}50 \mu\text{g}/\text{mL}$ ($5\text{--}10 \mu\text{g}/\text{cm}^2$) are comparable with mouse exposure doses previously reported in the literature.^{10,25,34}

At day 1 postexposure, MW exposure resulted in a significant increase in broncho-alveolar lavage (BAL) fluid counts of macrophages, neutrophils, and eosinophils, while HA-MW exposure resulted in significantly reduced number of macrophages and eosinophils and demonstrated a trend toward less neutrophils as compared to MW ($p = 0.06$) (Figure 2A). Interleukin- 1β (IL- 1β) is a potent inflammatory mediator, is involved in the pathogenesis of asthma, fibrosis, and chronic obstructive pulmonary disease (COPD), and is considered critical for acute inflammation and its resolution.⁴¹ A significant increase in IL- 1β has already been reported after exposure to CNTs.^{9,10,34,42,43} MWCNT exposure, similar to asbestos, can induce inflammasome activation in the lungs, leading to IL- 1β secretion.⁴² We previously reported IL- 1β secretion from primary human bronchial epithelial cells after MWCNT exposure via activation of the inflammasome that contributed to increased profibrotic gene expression in human fibroblasts.^{44,45} Here we demonstrate a significant increase in IL- 1β levels in BAL fluid of mice only after exposure to MW, while HA and HA-MW did not induce IL- 1β secretion (Figure 2B). Keratinocyte chemoattractant (KC), an analogue of human IL-8, is an inflammatory chemokine (especially for neutrophils), acts as mitogen for epithelial cells and is involved in multiple respiratory disorders such as asthma, COPD, and cystic fibrosis.⁴⁵ Similar to IL- 1β , only MW induced a significant increase in KC levels in BAL fluid (Figure 2C). OPN is a multifunctional immune mediator with diverse roles as a Th1 cytokines regulator, promotes cell-mediated immune responses, and plays a key role in chronic inflammatory and autoimmune diseases.⁴⁶ We previously demonstrated that MWCNTs can either directly (through macrophages) or indirectly (through IL- 1β secretion from bronchial epithelia) lead to OPN gene expression and protein

secretion.^{44,47} Significant increases in OPN levels in the BAL fluid were only noted after MW exposure, while HA-MW did not induce any change (Figure 2D). Epithelial damage is a known initiating factor in the pathogenesis of asthma and lung fibrosis.⁴⁸ We determined airway epithelial cell proliferation by Ki-67 immunostaining and observed significant induction of a proliferative response only by MW (Figure 2E,F). Increased amount of KC (a potent mitogen for epithelia) in the BAL fluid potentially explains increased epithelial proliferation by the MW exposure.

Reduction in Lung Inflammation Is Not Due to Lower Uptake of HA-MW

MWCNTs are widely distributed throughout the lungs following inhalation or aspiration exposures. Macrophages are one of the primary mechanisms for nanomaterial uptake and clearance from the lungs.⁴⁹ MWCNT uptake by macrophages and subsequent inflammasome activation and lung damage has also been previously reported.²⁵ In order to assess whether decreased inflammatory potential of HA-MW was linked to differential uptake, we evaluated uptake in BAL fluid macrophages and lung tissue (H&E stained sections) at days 1 and 21 postexposures (Figures S2 and 3). We observed significant uptake of both types of nanotubes in macrophages, with significant differences in the size of engulfed nanotube aggregates between HA-MW and MW. HA-MW formed smaller size aggregates or were detected as individual tubes, while MW formed larger and solid aggregates inside the cell cytoplasm (Figure 3A,B). Transmission electron microscopy (TEM) confirmed tube structures in both preparations, which localized in the phagosomes and phagolysosomes of macrophages (Figure 3A,B). Few nanotubes were also noted floating in the cytoplasm without any membrane structures around them (insets on TEM images). A semiquantitative imaging method was adapted to evaluate the area of carbon aggregates in the lungs and in BAL cytopins.^{50,51} Total lung carbon contents of HA-MW at day 21 indicated 80% decrease (compared with the day 1 level), while less clearance was observed for MW (53%) (Figure 3C). We further quantified the number of BAL fluid macrophages containing visible clumps of internalized carbon material under light microscopy (100×) and observed a significantly higher number of nanotube positive BAL macrophages in the HA-MW group (70–90%) than in the MW group (20–50%) at both days 1 and 21 postexposure (Supplementary Figure S3). Moreover, in MW treated mice, we observed significantly higher amounts of carbon aggregates in the BAL fluid which were either not internalized or were associated with multiple cells forming dumbbell-shaped structures (inset on Supplementary Figure S3). We hypothesized that the lower number of nanotube-positive macrophages in MW treated mice might be associated with persistent toxicity in the lungs. To this end, we measured released lactate dehydrogenase (LDH) in the BAL fluid and observed significant toxicity only in MW-treated mice, indicating a chronic toxic response in the lungs (Figure 3D). In summary, our results demonstrate a significantly reduced toxic response for HA-MW associated with uptake in significantly higher number of macrophages, lower lung carbon contents at day 21 (higher clearance), and reduced cellular toxicity due to improved dispersion.

In an attempt to characterize the role of dispersion and major metal impurities (Ni) in the observed differences between HA-MW and MWCNT, we employed carboxylic acid-functionalized nanotubes (COOH-MW) that have dispersion and physicochemical

characteristics (length, width, and Ni contents) comparable with the HA-MW (Supplementary Figure S4). We observed no difference between MW and COOH-MWs in terms of inflammation as assessed by numbers of PMN in the BAL fluid, OPN levels in the BAL fluid at day 1 postexposure or fibrosis at day 21 postexposure as determined by collagen deposition (Supplementary Figure S4). These findings confirm that the observed protection from nanotube-induced lung injury was specific to HA-MW and not due to a nonspecific reduction in metal impurities or increase in dispersion rate.

HA-MW Exposure Results in Significantly Decreased Fibrosis in Mouse Lungs

Lung fibrosis is among the most worrisome aspects of nanotube exposure as multiple studies have confirmed the ability of MWCNTs to induce fibrotic changes in the lungs of rodents.^{8,11,25,26,36} We evaluated lung fibrosis in mice at 21 days postexposure by quantifying profibrotic mediators in BAL fluid and by quantitative morphometric analysis of collagen deposition (Masson's trichrome staining) around the airways. Representative images from lung sections and quantification of collagen deposition around airways as well as lung soluble collagen are presented in Figure 4A–C indicating significant fibrotic changes only in MW exposed mice. We did not observe significant changes in collagen deposition in the HA-MW treated group, confirming the beneficial effect of HA grafting in terms of reduced fibrogenic potential. Chronic inflammation is a common feature of fibroproliferative diseases such as pulmonary fibrosis.⁵² In order to assess lung inflammation at postexposure day 21, H&E stained lung sections were blindly scored for inflammation.⁵³ Results show significantly higher lung inflammation in mice treated with MW compared to HA-MW (Figure 4D). We further measured levels of tumor-necrosis factor alpha (TNF- α) and OPN. TNF- α is a known mediator of lung inflammation and fibrosis and has been shown to stimulate collagen synthesis by fibroblasts.⁵⁴ OPN is also a known mediator of granulomatous lung disease and fibrosis.⁵⁵ A significant increase in BAL fluid TNF- α levels was only observed in MW-treated mice, while HA-MW treatment was innocuous (Figure 4E). A similar trend was observed for OPN (Figure 4F). As discussed above, significant increases in OPN levels were noted as early as postexposure day 1, indicating a persistent increase of this profibrotic cytokine in MW-treated mice. Next, we evaluated levels of HA in the BAL fluid (Figure 4G). Increased levels of short fragments of HA are associated with injury and have been observed in lung lavage fluid/plasma of patients suffering from various respiratory disorders such as pulmonary fibrosis⁵⁶ and asthma.⁵⁷ On the other hand, high molecular weight HA prevents epithelial injury in experimental lung fibrosis,⁵⁸ and we wanted to evaluate whether high molecular weight HA is being released by HA-MW, thus contributing to the decreased lung injury we observed. However, we found a significant increase in HA levels only in the MW-treated lungs, suggesting that this elevation was a result of injury, rather than released HA from functionalized nanotubes (Figure 4G). Because of the non-covalent nature of HA–nanotube interaction, there is possibility of HA breakdown from nanotubes over the long-term. However, HA-MW are rapidly cleared from the lungs, and no lung injury (toxicity, inflammation, fibrosis, MCM) is observed during the 21 day study period. Thus, negligible biological impacts of such breakdown are anticipated in the longer term.

The initiation and progression of pulmonary fibrosis likely stem from a variety of factors. IL-1 β potentially acts as a master initiator acting early leading to production of TNF- α , KC, platelet derived growth factor (PDGF) and TGF- β .⁵⁹ As presented in a previous figure (Figure 2B), we observed significant increase in IL-1 β release only after MW exposure at day 1 postexposure. Indeed, temporal IL-1 β production has already been shown after MWCNT exposure (increase observed only at day1 postexposure and not at day 21),¹⁰ and we previously reported IL-1 β secretion from primary human bronchial epithelial cells after MWCNT exposure leading to profibrotic gene expression in lung fibroblasts.⁴⁴ PDGF-AA is a known mediator of fibrosis in humans and acts as prosurvival factor in early stages of pulmonary fibrosis.⁶⁰ Increases in PDGF-AA in multiple animal models of lung fibrosis including MWCNT exposure have already been demonstrated.¹⁰ We observed significant increase in PDGF-AA levels only in the BAL fluid of MW treated mice at day 1 postexposure (Figure S5). These data further point toward the initiation events leading to fibrotic changes observed at day 21.

Recently it has been demonstrated that MWCNTs can have a direct impact on lung fibroblasts.^{27,37,61} Previously we demonstrated that TIMP-1 (TIMP metalloproteinase inhibitor 1), OPN, TNC (Tenascin C), and Procollagen 1 are markers of profibrotic changes in lung fibroblasts after exposure to conditioned medium from MWCNT-treated primary human bronchial epithelia.⁴⁴ Here we employed MRC-5 cells to evaluate profibrotic gene expression after direct nanotube exposure (Figure 5). We observed significant increase in gene expression of TIMP1, OPN, TN-C, and Procollagen 1 only by MW, while HA-MW induced significantly less gene expression. These results are in excellent agreement with our *in vivo* findings, suggesting that fibroblast monolayer cultures may be good alternative high-throughput method for the prediction of fibrotic changes in lungs after nanotube exposure.

HA-MW Induces Less MCM Than MW

MCM is a hallmark of chronic airway disorders such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease and contributes to airway obstruction, morbidity, and mortality.⁶² MWCNTs are associated with MCM development in the exposed lungs.^{34,35,43,63,64} In order to evaluate the impact of HA functionalization in MW-induced MCM development, we analyzed mucus-producing goblet cells in lung sections and quantified chemical mediators involved in the MCM phenotype. Scoring criteria were based on published methodology and are detailed in Supporting Information methods. Blinded scoring of Alcian blue/periodic acid Schiff (AB/PAS)-stained lung sections confirmed significant MCM on day 21 in MW exposed lungs, but not in HA-MW exposed lungs (Figure 6A,B). IL-13 is a Th2 cytokine, which promotes MCM in epithelial cells through STAT6 signaling,⁶⁵ acts as an upstream regulator of TGF- β 1 and PDGF-AA, and thus contributes to the airway fibrosis⁶⁶ and mediates eosinophilic lung inflammation and airway epithelial proliferation.⁶⁷ Our results confirmed a significant increase in the levels of IL-13 at day 1 postexposure, but only in MW-exposed lungs (Figure 6C).

Translational Validation of Reduced Inflammatory Abilities of HA-MW in Primary Human Cells

Alveolar macrophages significantly contribute to airway fibrosis through the release of chemical mediators such as TNF- α , IL-1 β , and OPN. To evaluate whether alveolar macrophage uptake of MW and HA-MW plays a major role in the development of lung inflammation and fibrosis, we treated primary human alveolar macrophages with noncytotoxic dose of nanotubes (*i.e.*, 5 $\mu\text{g}/\text{cm}^2/25 \mu\text{g}/\text{mL}$) and evaluated the release of these mediators in the cell culture supernatants. No significant increase in the amount of released TNF- α , IL-1 β , and OPN was observed after MW and HA-MW treatment, indicating a lack of significant pro-inflammatory response in human alveolar macrophages by both types of nanotubes (Supplementary Figure S6) and suggesting that the activation of alveolar macrophages may occur due to signaling by other cells, such as the airway epithelia. The bronchial epithelia plays an important role in lung homeostasis by preserving barrier integrity, providing mucociliary clearance, and contributing to the immune response to injury by secreting pro-inflammatory and profibrotic mediators.⁶⁸ We and others have previously demonstrated that bronchial epithelial cells play a significant role in inducing profibrotic changes after MWCNT exposure by secreting inflammatory mediators (*e.g.*, IL-8, IL-1 β , TGF- β , and TNF- α) that can activate fibroblasts.^{10,44,69–71} Primary human bronchial epithelial cells were exposed to MW and HA-MW at doses stated above. We found significant induction of IL-8 gene expression and protein release only after MW exposure (Figure 7A,B). We also observed significant induction of inflammasome components (NLRP3 and ASC) gene expression and significant activation of caspase-1, indicating NLRP3 inflammasome assembly in BECs only after MW exposure (Figure 7C–E). These results confirm our previous findings of inflammasome activation in the bronchial epithelia after nanotube exposure.⁴⁴

We further employed a physiological three-dimensional (3D) model of cocultured human differentiated (air–liquid interface) primary bronchial epithelia and primary human lung fibroblasts (MucilAir-HF) to translate our *in vivo* findings. Epithelial differentiation was confirmed by transepithelial resistance values of $359 \pm 12 \Omega\cdot\text{cm}^2$ and cilia beating frequencies of $8.5 \pm 0.1 \text{ Hz}$ (Supplementary Video) as well as AB/PAS staining indicating mucin producing goblet cells. We employed a physiological exposure scenario by exposing only the apical surface to a minimal volume (15 μL) of nanotube suspension (allowing 100% gas exchange) without damaging the integrity of air–liquid interface. A representative image of our culture is presented as Figure 8A, which clearly demonstrates pseudostratified ciliated epithelium similar to *in vivo* conditions. We exposed these cells to 10 $\mu\text{g}/\text{cm}^2$ MW or HA-MW for 24 h and analyzed cells and basolateral media for different inflammatory mediators gene expression (real time quantitative RT-PCR assay) and protein secretion (ELISA assay). We observed a significant increase in gene expression of TNF- α , IL-1 β , IL-8, and granulocyte-macrophage colony stimulating factor (GM-CSF) only after MW exposure (Figure 8B–E). IL-8 is a known inflammatory mediator that can induce pro-inflammatory cytokines such as (TN-C) as well as participate in inflammatory and profibrotic processes.⁴⁴ Indeed, higher levels of IL-8, TNF- α , and IL-1 β were noted in sputum and serum of human workers exposed to MWCNTs during the production process.⁷² Reactive oxygen species (ROS) are known mediators of inflammation, fibrosis, and MCM.^{73,74} We and others have

previously demonstrated role of MWCNT-induced ROS in bronchial epithelial injury.⁴⁴ We observed a protective impact of HA functionalization on ROS production, as only MW significantly increased ROS production, while HA-MW did not (Figure 8F). We further evaluated proliferation of MucilAir-HF cultures after nanotube exposures and observed significant increases in basal epithelial cell proliferation only after MW exposures (Figure 8G,H). Our primary human cell model data confirm the beneficial impact of HA functionalization on nanotube-induced toxicity and demonstrate excellent prediction of the *in vivo* lung inflammatory as well as fibrogenic potentials by providing critical information about the initial mechanistic events that lead to these adverse phenotypes.

CONCLUSIONS

In summary, this study provides assessment of the beneficial impacts of HA functionalization on nanotube-induced lung injury using rodents as well as translational relevant primary human cell models. We demonstrate the ability of HA-MW to widely disseminate in the lungs without causing significant inflammatory or fibrotic changes. We further demonstrate that primary human lung cell cultures (both epithelial and fibroblasts) have a great value in predicting lung inflammatory and fibrotic changes after MWCNT exposures. This study provides essential information that may pave the way for the development of HA-MW-based nanotherapeutic advances for pulmonary chemotherapy. Further research is underway to evaluate the contribution of HA recognition receptors in the beneficial impacts of HA-functionalized MWCNTs.

EXPERIMENTAL METHODS

Carbon Nanotubes and Functionalization

Detailed methodology for nanotube functionalization is provided in the Supporting Information. Briefly, MWs were purchased from Cheap Tubes, Inc. (Brattleboro, VT), and high molecular weight ($>10^6$ D), low-endotoxin (<0.07 EU/mg) HA was purchased from Life Core Biomedical (Chaska, MN). MWs were purified by acid washing as described previously²⁷ and acted as the backbone for noncovalent hyaluronan functionalization. These functionalized nanotubes were referred to as HA-MWs. Both nanotubes (HA-MW and MW) were thoroughly characterized by TEM, inductively coupled plasma atomic emission spectroscopy, Raman spectroscopy, and by Fourier transform infrared spectroscopy (see Results and Discussion section).

In Vivo Mouse Pulmonary Exposures and Assays

C57Bl/6J female mice were given 50 μ L of either vehicle (1 mg/mL BSA in PBS) or 1.5 mg/kg of CNTs (MW or HA-MW) or HA by a single oropharyngeal aspiration. Detailed description of aspiration methodology can be found in Supporting Information. Mice (6–9 per group for each time point) were sacrificed on days 1 and 21 postexposure, BAL fluid collected and analyzed for total as well as differential cell counts, lactate dehydrogenase release (marker of lung injury), and release of inflammatory and profibrogenic mediators. Nanotube uptake and persistence in BAL fluid macrophages were evaluated by light and

TEM. Histopathological analyses and semiquantitative measurements of lung damage, proliferation, collagen deposition, and MCM were made in a blinded manner.

Study Subjects and Isolation of Cells

This study was approved by the NIEHS Institutional Review Board. Adult human never smoker volunteers without a history of lung or systemic inflammatory disease were recruited to the NIEHS Clinical Research Unit. Whole lung lavage and bronchial brushings were performed to collect alveolar macrophages and bronchial epithelial cells.

Cell Culture and CNT Exposure

MucilAir-HF cultures (a 3D primary human airway epithelium model consisting of differentiated primary human bronchial epithelial cells and primary human lung fibroblasts) were procured from Epithelix Sàrl (Geneva, Switzerland) and maintained in chemically defined, serum free MucilAir medium. Differentiated bronchial epithelium is composed of basal cells, ciliated cells, and mucous producing goblet cells in a similar ratio to what is observed *in vivo*.⁷⁵ Differentiation into pseudostratified epithelium containing goblet cells, basal cells, and ciliated cells was assessed by histology, and cilia beating was analyzed by video microscopy according to the method described previously.⁷⁶ Cells cultivated in 24-well inserts (6.5 mm diameter, 0.4 μ m pore size) were exposed to vehicle or 10 μ g/cm² MW, HA-MW or HA alone for 24 h in a minimal volume forming only 0.5 mm liquid layer on top of the cells (to avoid disruption of air-liquid interface). Gene expression and protein expression (basolateral compartment) of inflammatory mediators was evaluated by real time quantitative RT-PCR or ELISA. Oxidative stress and proliferation in epithelial cells was quantified by DCFH-DA and Ki-67 assays, respectively. We also employed monocultures of primary human alveolar macrophages and bronchial epithelial cells, which were collected from healthy human volunteer's in house, and human lung fibroblasts (MRC-5), which were purchased from ATCC (Manassas, VA). Gene expression of inflammatory and profibrotic mediators was evaluated by real-time quantitative RT-PCR analyses. Details on the culture conditions and methods can be found in Supporting Information.

Histopathology and Lung Injury Scoring

During necropsy left lung lobe was fully inflated with 10% neutral buffered formalin and placed in formalin solution for at least 48 h to complete fixation process. Lung tissue was paraffin embedded, and 5 μ m thickness sections were cut. Slides were subjected to hematoxylin and eosin (H&E) stain. Lung injury was scored by an experienced pathologist in a blinded manner following already published criteria.⁵³

Cell Proliferation Assays (Immunohistochemistry for Ki-67)

An immunohistochemical staining for Ki-67 was performed using the HRP-polymer technique, using the ImmPRESS Anti-Rabbit kit (Vector Laboratories, Burlingame, CA.) Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. After heat-induced epitope retrieval and endogenous peroxidase blocking nonspecific sites were blocked by incubating lung slides for 30 min with rodent block M (Biocare Medical, Concord, CA) and human Mucil-Air HF sections

with 2.5% normal horse serum. The lung sections were then incubated with rabbit Ki-67 antibody (CRM325C, Biocare Medical, Concord, CA), and Mucil-Air HF sections were incubated with rabbit polyclonal anti-Ki-67 (SP6) antibody at a 1:100 dilution for 30 min at room temperature. Normal rabbit IgG (Calbiochem, Billerica, MA) at the equivalent dilution was applied to the negative control in lieu of antibody. The antigen–antibody complex was detected using either rabbit on rodent HRP polymer (Biocare Medical, Concord, CA) and 3,3-diaminobenzidine (DAB) chromogen (Dako North America, Inc., Carpinteria, CA). Slides were then counterstained with hematoxylin.

Quantitative Morphometry of Airway Fibrosis

Quantification of the thickness of collagen surrounding airways was performed according to an established method.^{43,63} Briefly, photomicrographs of trichrome-stained sections of lung tissue containing circular to oval-shaped small or medium-sized airways were captured using a 10× objective on an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA) and digitized. The area/perimeter ratio was quantified by measuring the area of airway collagen corrected for the length of the basement membrane. The lasso tool in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) was used to surround the trichrome-positive collagen around an airway (outer area). A second measurement was made by surrounding the basement membrane of the same airway (inner area), and the length of the airway circumference (*i.e.*, perimeter) was also derived from this measurement. The difference between the outer and inner area was defined as the “area” and divided by the “perimeter” to derive area/perimeter measurements. This method was performed in a blinded manner, where the treatment group was unknown to the observer measuring the sections. Airways that fit our criteria were measured (5–20 airways per animal).

MCM Scoring

Changes in the mucous secreting goblet cells were evaluated in a blinded manner using Alcian Blue/Periodic Acid Schiff (AB/PAS) stained lung sections.^{77,78} Histological mucus index (HMI) was calculated which represents the linear percent of bronchial epithelium positive for mucus. Scoring was performed for 5–15 airways per mouse lung, and then mean HMI was calculated for each experimental group ($n = 6–9$ mice).

ROS Production

ROS production was determined using 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO) as described by us previously.⁷⁹ The analysis was performed using BioTek Synergy HT fluorescence plate reader (BioTek Instruments Inc., Winooski, VT USA) at 488 nm excitation and 610 nm emission wavelengths.

ELISA Assays

DuoSet ELISA kits (R&D Systems, Inc.) were utilized to measure TNF- α , IL-1 β , IL-13, PDGF-AA, OPN, HA, and KC in the mouse BAL fluid as well as IL-8, OPN, and TNF- α levels in cell culture supernatants. All the assays were performed following the manufacturer's protocol and previously published methods to avoid assay interferences from nanomaterials. Absorbance was measured at 450 nm by the Multiskan EX microplate

spectrophotometer (Thermo Fisher Scientific) with a correction wavelength of 540 nm. Concentration was determined using standard curve and values were expressed as mean $\pm$ SEM.

LDH Assay

LDH assay on BAL fluid and cell culture supernatants was performed as described previously.¹¹ This assay is a measure of cell death and was performed using commercially available kit (Roch Diagnostics, Montclair, NJ). Absorbance at 340 nm was monitored using BioTek Synergy HT plate reader (BioTek Instruments Inc., Winooski, VT).

Gene Expression Analyses by Real-Time Quantitative RT-PCR

Expression of inflammation and fibrosis pathway genes was evaluated by real-time quantitative RT-PCR analyses as described previously.⁴⁴ Briefly, total RNA extraction and purification was performed using RNeasy Midi Kit (Qiagen, Germantown, MD) according to manufacturer's recommendations. RNA quantification was performed using NanoDrop (Thermo Fisher Scientific, USA). SuperScript III kit with oligo dTs (Invitrogen) was used to synthesize cDNA (reverse transcription reaction) according to the manufacturer's recommendations. PCR reaction was performed in 25 μ L final volume with 1 μ g of cDNA and 500 nM of each specific forward and reverse primer (Supplementary Table 1) in Power SYBR Green Master Mix (Applied Biosciences) using Stratagene Mx3005P real-time PCR instrument (Agilent, CA). Expression of the gene of interest was normalized to the expression of the gene for ribosomal protein (18 s) using Δ Ct analysis and are represented as fold change from vehicle control.

Statistical Analyses

Data were imported into Graphpad Prism 6 (Graphpad Software Inc., San Diego, CA) for conversion into graphs and statistical analysis. Results are expressed as mean $\pm$ SEM. Data were analyzed by two-sided Student's *t* test or by analysis of variance (ANOVA) followed by the Tuckey's posthoc test. A level of $p < 0.05$ (two-tailed) was considered significant for a given comparison.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Physicochemical characterization of nanotubes. (A) TEM, (B) Raman, (C, D) FTIR analysis of purified and hyaluronan-modified nanotubes.

Figure 2.

Hyaluronan functionalization reduces lung injury potential of nanotubes. (A) BAL fluid cell differentials in C57Bl/6J female mice (6–9 animals per group) at day 1 postexposure after single oropharyngeal aspiration of 1.5 mg/kg nanotubes (MW and HA-MW), 1.5 mg/kg HA or vehicle (1 mg/mL BSA in PBS). At least 500 cells per condition were counted to determine the percentage of different cell types (macrophages, neutrophils, eosinophils) and multiplied by total number of cells to get absolute numbers; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B–D) BAL fluid cytokines (IL-1 β , KC, and OPN) from the above-mentioned mice were determined by ELISA kits; * $p < 0.05$, ** $p < 0.01$. (E) Representative images of nanotube induced bronchial epithelial cell proliferation (Ki-67 immunohistochemistry) in the above-mentioned mice. (F) Number of proliferating cells per high-power field (40 $\times$) after 1 day nanotube exposure. Stained lung sections (5–15 small to medium airways per mice) were scored by an observer who was blinded to the group identities and average number of proliferating cells per group (6–9 mice) is presented; * $p < 0.05$, *** $p < 0.001$.

Figure 3.

Nanotube uptake and persistence/clearance from the lungs. Experimental design details are same as described in Figure 2. (A) Representative images of nanotube uptake in BAL fluid macrophages (light as well as TEM) at day 1 postexposure. Figure insets are higher zoom images showing nanotube-induced mitochondrial cristae swelling and individual nanotubes in cytoplasm with MW exposure and less damaged mitochondria and predominately lysosomal localization in HA-MW treated BAL fluid macrophages. (B) Representative images of nanotube uptake in BAL macrophages (light as well as TEM) at day 21 postexposure. Figure insets are higher zoom images showing nanotubes fairly preserve their tube morphology even at day 21 and are seen in lysosomes, having much less clumping in HA-MW as compared to MW group. (C) Area of nanotube aggregates in the lungs and lung lavage cytopspins at various time points (days 1 and 21) after single oropharyngeal aspiration of 50 μ L nanotube suspension containing 1.5 mg/kg nanotubes (MW and HA-MW). (D) Release of LDH (an indicator of cytotoxicity) in BAL fluid at day 21 postexposure. LDH was quantified from BAL fluid using commercially available kit. Data are presented as mean $\pm$ SEM and were analyzed by ANOVA followed by Tukey's posthoc test ** $p < 0.01$.

Figure 4.

Hyaluronan functionalization reduces fibrotic capacity of nanotubes. Experiment described in Figure 2 was performed again, and lung samples from 9 mice per group were collected at day 21 postexposure. (A) Representative images of collagen deposition in the lungs as determined by Masson's trichrome staining. Left lung lobe was fixed and embedded in paraffin, and 5 μ m thick sections were cut. These sections were stained with Masson's trichrome stain, and images were digitized. Blue color represents collagen fibrils. (B) Morphometric quantification of the areas of peri-bronchial collagen deposition was done on digitized images (5–20 small to medium bronchi per lung) using ImageJ software by an observer without knowing the identity of the groups; * $p < 0.05$, ** $p < 0.01$ and NS = nonsignificant. (C) Soluble lung collagen measurement in frozen lungs by sircol assay. (D) Semiquantitative scoring of chronic inflammation in the mice lungs at day 21 postexposure was performed by an experienced pathologist in a blinded manner and inflammation scores (0 indicates no inflammation, 1 indicates mild mononuclear cell infiltration, and 2 indicates moderate mononuclear as well as polymorph nuclear cell infiltration). (E–G) BAL fluid profibrotic cytokines (TNF- α , OPN) and HA at day 21 postexposure as determined by ELISA kits; * $p < 0.05$, *** $p < 0.001$.

Figure 5.

Gene expression analysis of profibrotic markers in MRC-5 cells. MRC-5 fibroblasts were treated with noncytotoxic doses (5–10 $\mu\text{g}/\text{cm}^2$) of MWCNTs and HA-MW for 24 h. mRNA expression of TIMP-1, OPN, TN-C, and Procollagen 1 was performed by real-time quantitative RT-PCR analyses. Data are presented as mean $\pm$ SEM and are analyzed by ANOVA followed by Tukey's posthoc test; ** $p < 0.01$.

Figure 6. Hyaluronan functionalization reduces mucus metaplasia inducing capacity of the nanotubes. (A) Representative images of MCM (AB/PAS stained lung sections) in nanotube treated lungs at day 21 postexposure (same experimental design as described in Figure 3). (B) Histological scoring of MCM represented as histological mucous index (%); *** $p < 0.001$ and NS = nonsignificant. (C) Quantification of IL-13 in the BAL fluid of mice by ELISA assay; ** $p < 0.01$.

Figure 7. Nanotube-induced toxicity and inflammation in primary human bronchial epithelial cells. Primary human bronchial epithelial cells were treated with vehicle (1 mg/mL BSA), HA-MW, MW (5 $\mu\text{g}/\text{cm}^2$), or HA (1 mg/mL) exposures for 24 h. (A) IL-8 gene expression, (B) IL-8 protein release, (C) NLRP3 gene expression, (D) ASC gene expression, and (E) caspase-1 activation in data are presented as mean $\pm$ SEM and were analyzed by ANOVA followed by Tukey's posthoc test; * $p < 0.05$, ** $p < 0.01$, $n = 4$ subjects.

Figure 8.

Translational validation of *in vivo* findings using primary human cell model. (A) Representative image (H&E stained section) of air–liquid interface culture of primary human organotypic cell model (MucilAir-HF) consisting of differentiated human bronchial epithelia and human lung fibroblasts. Only apical surface of the differentiated bronchial epithelia was exposed to either vehicle (1 mg/mL BSA in PBS) or nanotubes (MW and HA-MW) for 24 h. (B–E) mRNA expression of pro-inflammatory and profibrotic markers (TNF- α , IL- β , IL-8, GM-CSF) in the primary human coculture model using real time quantitative RT-PCR analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (F) ROS production in primary human cell model as estimated by DCFH-DA staining. H₂O₂ 800 nM was used as positive control ** $p < 0.01$, *** $p < 0.001$. (G). (H) Proliferation of bronchial epithelia in primary human coculture model quantified using Ki-67 immunostaining and counting at least 600 cells to estimate the percentage of Ki-67 positive cells. * $p < 0.05$.

Table 1
Suspension Characteristics of Various Nanotubes in Different Exposure Media Measured by DLS Analyses

	hydrodynamic diameter (nm)				pDi				ζ potential (mV)			
	BSA/PBS ^a	ALI ^b	RPMI	BEGM	BSA/PBS	ALI	RPMI	BEGM	BSA/PBS	ALI	RPMI	BEGM
HA-MW	180 (30)	150 (25)	1112 (250)	144 (45)	0.483	0.25	0.83	0.64	-13.2	-12.1	-19.2	-14.3
MW	312 (57)	262 (45)	1262 (387)	198 (18)	0.40	0.71	1	0.57	-12.7	-11.8	-9.8	-12.9

^aBSA/PBS = 1 mg/mL BSA in PBS.

^bALI = MucilAir medium.