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Effects of lipopolysaccharide, multiwalled carbon nanotubes, and the combination on lung alveolar epithelial cells

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Abstract

Multi-walled carbon nanotubes (MWCNT) have been shown to induce lung fibrosis in animal models, however the underlying molecular factors/mechanisms are still unclear. In this study, we investigated the effects of lipopolysaccharide (LPS), MWCNT, and the combination of LPS and MWCNT on the expression of matrix metalloproteinase-9 and metalloproteinase-12 (MMP-9, MMP-12), collagen 3A1 (Col3A1), and transforming growth factor beta (TGF β) in alveolar epithelial A549 cells. MMPs are proteinases that degrade extracellular matrix and play a role in lung fibrosis. A549 cells were exposed to LPS (1 ng/ml), MWCNT (20 μ g/ml), and the combination and analyzed for paracellular permeability, TGF β , Col3A1, MMP-9, MMP-12, NF- κ B activation, and cell migration by real-time PCR and immunofluorescence. LPS, the combination of LPS and MWCNT, and MWCNT only at the highest tested dose induced blue dextran extravation. LPS and MWCNT increased the expression of TGF β and its downstream target gene Col3A, and MMP-9 and MMP-12 mRNA. MWCNT potently induced cell migration toward wound healing, whereas LPS slightly induced cell migration. Both, LPS and MWCNT, induced NF- κ B nuclear translocation. Our results indicate that MWCNT activated alveolar epithelial cells to promote fibrogenesis, and that LPS differentially primes molecular factors involved in lung remodeling. These findings suggest a role of alveolar epithelial cells in fibrogenesis and also may aid in the design and development of tests for screening of fibrogenic agents.

Keywords

Alveolar epithelial cells; Cell migration; Collagen; ECM; LPS; MWCNT; MMP-9; MMP-12; NF- κ B; TGF β

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Conflicts of Interest

The authors declare no conflict of interest.

Introduction

Matrix metalloproteinases (MMPs) are a large gene family of zinc and calcium-dependent endopeptidases that play an important role in the remodeling of extracellular matrix (ECM) during normal lung development but also in many lung pathologies including, chronic obstructive pulmonary disease (COPD), lung cancer, acute lung injury (ALI), acute distress syndrome (ARDS), and lung fibrosis (Lavigne et al., 2004; Lagente and Boichot, 2010; Ricou et al., 2014; Hayashi et al., 1996). In patients with ARDS or pulmonary fibrosis, MMP levels are elevated, and in animal models of lung injury, MMPs facilitate inflammation-driven leukocyte extravasation and endothelial permeability (Li et al., 2002; Warner et al., 2001). Of all MMPs, MMP-9 and MMP-12 are the major proteinases that have been shown to play a role in acute and chronic pulmonary disease (Chaudhuri et al., 2012; Abdel-Fatah et al., 2015; Kim et al., 2009). Lipopolysaccharide (LPS), a Gram-negative bacterial endotoxin, is a classical inducer of acute lung inflammation. Multi-walled carbon nanotubes (MWCNT) are fiber-shaped carbon nanomaterial with wide applications in industry and nanomedicine (Karimi et al., 2015; De Volder et al., 2013). Despite unique and unprecedented advantages as nanomaterial, there are raising concerns over potential adverse health effects of carbon nanotubes, particularly due to their nano size, fiber-like shape, and light weight, properties which make them respirable along the all steps of MWCNT starting with manufacturing, storage, application, and disposition process. Numerous *in vivo* studies showed that MWCNT, regardless of mode to administration, rapidly induce pulmonary fibrosis and inflammation (Castranova et al., 2013). Porter et al. (2010) showed that MWCNT induce pulmonary fibrotic lesions and granulomatous inflammation as early as at 7-days post aspiration. Dong et al. (2015) showed the presence of fibrotic foci after 14 days post exposure with collagen deposits in the alveolar septa. Both of these studies also showed increased levels of inflammatory cytokines TNF α , IL-1 β , IL-6 in the lungs and bronchoalveolar lavage fluid, and these changes were time- and dose-dependent. *In vitro* studies using lung cells showed that MWCNT upregulated α -smooth muscle actin (α -SMA) and induced transition of epithelial cells toward fibroblasts via the TGF β /Smad pathway (Chen et al., 2014), promoted surface morphological changes (Cavallo et al., 2012), and altered the expression of 106 proteins involved in cell proliferation, stress, and cellular skeleton organization (Ju et al., 2014). Fibrotic lesions in the interstitium of lungs is a typical response following inhalation of particles or fibers, however the exact cellular and molecular mechanism of MWCNT-induced pulmonary fibrosis still largely remains unknown. Although, activated fibroblasts are considered the primary cells in the development of fibrosis (Strieter and Mehrad, 2009), there is growing evidence supporting that continuous injury to epithelial cells potentiates transition of these cells toward a fibrogenic cell type (Chang et al., 2012).

In the present study, we investigated the effect of MWCNT alone or in combination with LPS on activating alveolar epithelial cells toward producing extracellular matrix proteins.

Materials and Methods

Cell Culture

Alveolar epithelial cells, A549, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell culture was maintained according to manufacturer's recommendations. Briefly, the cells were grown in Frank's Modified Medium (F-12K, ATTC) supplemented with 10 % fetal bovine serum (FBS), and Streptomycin/Neomycin (10,000/100 units, ATCC) in an incubator at 37 °C, 5% CO₂.

Characterization of MWCNT

MWCNT were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Physical characteristics of MWCNT according to manufacturer were as follow: length 5–9 µm, width 110–170 nm, and purity >95%. We also carried out elemental analysis of MWCNT using ECS 4010 Analytical Platform (Costech Instrument) according to the method previously described (Basaran et al., 2014).

MWCNT preparation

For cell culture studies, MWCNT stock (2 mg/ml) were prepared in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS), pH 7.4, according to methods previously described (Pacurari et al., 2008). MWCNT were prepared in PBS followed by indirect sonication at 4°C for 5 min (Hielscher ultrasonic processor, UIS259L) at amplitude 100% and cycle 1. After indirect sonication, the suspension was directly sonicated at 4°C for 1 min at 5W output and 10% duty cycle (Branson Sonifier 450). The stock solution (2 mg/ml) of MWCNT was kept at 4°C and used within 2–3 weeks. Prior to cell culture experiments, the MWCNT stock solution was directly sonicated for 1 min at the setting indicated above. Samples that were used for cellular studies were freshly prepared from the stock on the day of cell treatments to desired concentrations of MWCNT in RPMI containing 0.1% FBS and resonicated by a direct ultrasonication for 1 min before use.

Transmission Electron Microscopy

For transmission electron microscopy, (TEM), the MWCNT samples prepared as described above in deionized water were diluted and then deposited on a formvar-coated copper grid, dried, and photographed using a JEOL-1011 transmission electron microscope. A large number of discrete nanotubes and few agglomerates of MWCNT were present.

Lipopolysaccharide (LPS)

LPS was obtained from *E. coli* serotype 055:B5 (Sigma-Aldrich, St Louis, MO). A stock solution of 1 mg/ml was prepared and stored at –20°C. For cell culture experiments, the LPS stock solution was further diluted at the time of treatments.

Paracellular permeability assay

Alveolar permeability was assessed using blue dextran (Sigma-Aldrich) extravasation assay and Boyden chamber assay. The assay was performed according to manufacturer's recommendations (BD Biosciences, Franklin Lakes, NJ). Briefly, A549 cells were seeded in

the top insert (0.3 ml of 1×10^6 cells/ml in serum-free media, LPS (1 or 10 ng/ml) MWCNT (20 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$), or combination of LPS (1 ng/ml) and MWCNT (20 $\mu\text{g/ml}$), and the bottom chamber was loaded with 0.5 ml of 0.1% FBS growth medium, followed by incubation at 37°C in 5% CO_2 for 48 h. After 48 h, the cell monolayer received 0.3 ml of basal media containing blue dextran (10 $\mu\text{g/ml}$) and incubated for 30 min at 37°C in 5% CO_2 . Blue dextran extravasation was quantified using a microplate reader to measure the optical density (OD) of the eluted blue dextran to determine alveolar paracellular permeability values. The mean values were obtained from five individual experiments and were subjected to a *t*-test assuming unequal variance. Significance was determined by a *p* 0.05.

Wound scratch assay

Wound healing assay was performed according to the methods previously described (Pacurari et al. 2012). A549 cells were grown on cover slips to a confluent monolayer and then scratched to form a 100- μm “wound” using sterile pipette tips. The cells were treated with LPS (1 ng/ml) MWCNT (20 $\mu\text{g/ml}$) the combination of LPS (1 ng/ml) and MWCNT (20 $\mu\text{g/ml}$) in serum-free media for 24 h and then fixed in 4% paraformaldehyde. Samples were imaged using an Olympus IX70 microscope (Olympus Optical Co., Ltd, Japan) equipped with a Retiga 2000R FAST camera (Qimaging, Canada). Images were acquired using SimplePCI software (Compix Inc., Sewickley, PA).

In vitro cell migration assays

The migration assay was performed in a modified 24-well (8 μm) Boyden chamber and according to manufacturer’s protocol (BD Biosciences, Franklin Lakes, NJ) with a slight modification. The top insert chamber of the transwell was loaded with 0.3 ml of 1×10^6 cells/ml in serum-free media LPS (1 or 10 ng/ml), MWCNT (20 $\mu\text{g/ml}$), and combination of LPS (1 ng/ml) and MWCNT (20 $\mu\text{g/ml}$) and the bottom chamber was loaded with 0.5ml of 0.1% growth medium as a chemoattractant, followed by incubation at 37°C in 5% CO_2 for 48h. Cell migration assay results were analyzed according to manufacturer’s protocol. After 48h, the upper surface of the top insert was swabbed with cotton-tipped applicators to remove non-migratory cells. The migrated cells were stained with 0.1% crystal violet followed by dye elution in 10% acetic acid. A microplate reader was used to measure the optical density (OD) of the eluted solutions to determine the migration values. The mean values were obtained from four individual experiments.

RNA isolation

Total RNA was extracted using Trizol[®] (Invitrogen, CA) according to the manufacturer’s protocol. To ensure a good RNA quality, the quality and integrity of the total RNA was evaluated using 28S/18S ratio and a visual image of the 28S and 18S bands were evaluated on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Concentration of the total RNA was assessed using the NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Germany).

Quantitative real-time PCR

Real-time PCR for target genes was determined using total RNA and cDNA was generated using a High-Capacity cDNA Reverse Transcription kit and TaqMan gene expression assays (Applied Biosystems Inc., CA). The level of Col3A1, TGF β , Col3A1, and 18S mRNA was measured using SYBR-Green Master mix and gene specific primers according to manufacturer's protocol (Applied Biosystems Inc.). All qRT-PCR reactions were performed on 7500 instrument (Applied Biosystems Inc.). In the qRT-PCR analysis of genes, the dissociation curve showed the absence of a secondary peak, indicating no presence of primer dimer. The expression level of each gene was determined by following formulas: fold change = 2^{-Ct} , where Ct (cycle threshold) = $Ct_{\text{target gene}} - Ct_{\text{endogenous control gene}}$, and $Ct = Ct_{\text{treated sample}} - Ct_{\text{control sample}}$. 18S was used as an endogenous control gene (Pacurari et al., 2011).

NF- κ B immunofluorescence

Immunofluorescence assay were used to measure NF- κ B p65 subunit nuclear translocation according to previously published methods (He et al., 2014, Pacurari et al., 2012). Briefly, the cells were grown on cover slips and treated with LPS, MWCNT, or combination for 24 h. After the treatments, the cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% TritonX-100/PBS. Cells were incubated with 5% bovine serum albumin (BSA)/PBS/0.1% TritonX-100 for 2 h at room temperature and then incubated with anti-NF- κ B p65 antibody (Abcam, Cambridge, MA) in 5% BSA/PBS/0.1% TritonX-100 at 1:1000 dilution overnight at 4°C. After primary incubation, the cells were washed three times with PBS and incubated with an Alexa-Fluor[®]-488-conjugated secondary antibody (Molecular Probes, Life Technologies, MA)/5% BSA/PBS/0.1% TritonX-100 at a dilution of 1:1000 for an hour in the dark. After secondary antibody incubation, the cells were washed with PBS three times for 5 min each time, and then mounted with a mounting antifade solution containing DAPI (Molecular Probes,). Images were taken with an Olympus epifluorescence microscope.

Statistical analysis

Data are presented as mean $\pm$ SEM from three experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Student's paired *t*-test with a significance level of $p < 0.05$ versus non-treated control samples.

Results

MWCNT characterization

Elemental analysis of MWCNT using ECS 4010 platform showed that MWCNT samples displayed three peaks with the highest peak corresponding to carbon, a second peak for hydrogen, and a peak for nitrogen (Fig. 1). MWCNT contained 94.2% carbon, 0.6 % nitrogen, and 0.32% hydrogen (Table 1). Transmission electron microscopic micrograph showed the MWCNT samples contained predominately loosely associated long fibers (Fig. 2). The insert shows the diameter of on MWCNT of ~ 11.2 nm. From electron microscopic images, MWNCT length and diameters was measured using digitized images measurements

and showed an average length of $2.24 \pm 1.56 \mu\text{m}$ and an average diameter of $13.22 \pm 2.94 \text{ nm}$.

The effect of MWCNT and LPS on cell layer paracellular permeability

Paracellular permeability of A549 cell monolayer was assessed by measuring the extravasation of blue dextran. Exposure of alveolar epithelial cells to MWCNT $20 \mu\text{g/ml}$ did not induce alveolar epithelial cells monolayer paracellular permeability, whereas exposure to a higher dose of $100 \mu\text{g/ml}$, MWCNT significantly increased blue dextran extravasation across alveolar monolayer barrier (Fig. 3). LPS significantly increased blue dextran extravasation across alveolar barrier regardless of the dose (1 or 10 ng/ml), a 27% and 53% increase above control, respectively (Fig. 3). The addition of LPS (1 ng/ml) to MWCNT ($20 \mu\text{g/ml}$) slightly potentiated MWCNT effect on alveolar epithelial cells paracellular permeability by inducing blue dextran extravasation by 17% increase above control (Fig. 3). Since LPS significantly induced cellular permeability at the tested doses, thus 1 ng/ml LPS was used for the subsequent experiments. MWCNT concentration was selected based on cell viability assays which indicated that cell viability was not affected at the dose of $20 \mu\text{g/ml}$ MWCNT (data not shown).

The effect of LPS and MWCNT on TGF β mRNA

The effect of LPS, MWCNT, and combination on fibrosis markers TGF β and Col3A1 mRNA in A549 cells was analyzed using qPCR (Fig. 4). Exposure of A549 cells to LPS (1 ng/ml) significantly induced the expression of TGF β mRNA a 3.35-fold increase compared to control (Fig. 3A). Exposure of A549 cells to MWCNT ($20 \mu\text{g/ml}$) resulted in a significant increase of TGF β mRNA, a 2.62-fold increase compared to control (Fig. 4A). A higher dose of MWCNT further increased TGF β mRNA by 5.58-fold compared to control. Exposure of A549 cells to the combination of LPS (1 ng/ml) and MWCNT ($20 \mu\text{g/ml}$) resulted in a 2.34-fold increase compared to control (Fig. 4A). LPS significantly increased Col3A1 mRNA by 5.61-fold compared to control (Fig. 4B). Exposure of A549 to MWCNT resulted in an increase of Col3A1 mRNA by 2.34-fold (Fig. 4B). Exposure of A549 to higher dose of MWCNT ($50 \mu\text{g/ml}$) increased Col3A1 mRNA by 12.04-fold compared to control (data not shown).

The effect of MWCNT and LPS on MMP-9 and MMP-12

In the present study, the exposure of A549 to LPS (1 ng/ml), MWCNT ($20 \mu\text{g/ml}$), or combination differentially regulated MMP-9 and MMP-12 mRNA (Fig. 5). LPS significantly increased MMP-9 mRNA by 1.9-fold compared to control (Fig. 5A). Exposure of A549 cells to MWCNT resulted in a significant increase of MMP-9 mRNA, a 1.5-fold increase compared to control. Exposure of A549 cells to the combination of LPS (1 ng/ml) and MWCNT ($20 \mu\text{g/ml}$) resulted in a tremendous increase of MMP-9 mRNA, a 7.97-fold increase compared to control (Fig. 5A). LPS significantly increased MMP-12 mRNA, a 3.44-fold increase versus control (Fig. 5B). MWCNT had a profound effect on MMP-12 by increasing mRNA level by 13.59-fold compared to control. However, the combination of LPS and MWCNT attenuated MWCNT effect on MMP-12 mRNA thus increasing MMP-12 mRNA only by 4.54-fold compared to control (Fig. 5B).

The effect of LPS and MWCNT on alveolar epithelial cells wound healing

The effect of LPS and MWCNT on alveolar epithelial cell wound healing and migration was analyzed using wound scratch assay and Boyden chamber assay. LPS (1 ng/ml) slightly facilitated wound healing as fewer cells migrated toward the wound closure compared to control (Fig. 5A). MWCNT (20 µg/ml) greatly potentiated alveolar epithelial cells migration toward wound healing closure as a large number of cells migrated toward the wound compared to control (Fig. 6A). The addition of LPS to MWCNT limited MWCNT-induced cell migration toward wound healing (Fig. 6A). To further examine the effects of LPS, MWCNT, and the combination on alveolar epithelial cells migration, we used Boyden chamber assay. A549 cells were treated with LPS (1 or 10 ng/ml), MWCNT (20 µg/ml), or combination of LPS (1 ng/ml) and MWCNT (20 µg/ml) and seeded in the Boyden chamber inserts for 48 h. As chemoattractant, we used 0.5% FBS growth media in the lower chamber of Boyden assay. LPS at 1 ng/ml slightly stimulated cell migration toward the chemoattractant thus increasing cell migration by 19% compared to control, whereas 10 ng/ml LPS stimulated cell migration by 79% compared to control (Fig. 6B). MWCNT stimulated cell migration by 39% compared to control (Fig. 6B). Addition of LPS (1 ng/ml) to MWCNT (20 µg/ml) did not stimulate cell migration, but indeed limited MWCNT-stimulated cell migration. The combination induced only a 15% increase in cell migration compared to control, thus a decrease of 24% compared to MWCNT alone (Fig. 6B).

The effect of LPS and MWCNT NF-κB activation

The transcription factor NF-κB plays an important role in regulating inflammatory genes. In the present study, we analyzed NF-κB nuclear translocation in response to LPS, MWCNT, and the combination of LPS and MWCNT as a mechanism for the transcription of MMP-9 and MMP-12 expression. The activation of NF-κB was analyzed using immunofluorescence against NF-κB p65 subunit translocation into the nucleus. LPS (1 ng/ml) activated NF-κB by inducing p65 nuclear translocation (Fig. 7). MWCNT alone or in combination with LPS also induced p65 nuclear translocation, however no synergistic effect was found for NF-κB p65 trans-activation (Fig. 7).

Discussion

The alveolar epithelium acts as a barrier that protects the lungs from inhaled substances. The epithelial integrity and permeability are tightly maintained via tight connections between the cell layer which allows cell-to-cell communication but blocks the entry of inhaled injurious substances (Roche et al., 1993). In the present study, exposure of alveolar epithelial cells to MWCNT low dose (20 µg/ml) did not induce paracellular permeability as assessed by blue dextran extravasation. However, a higher dose of MWCNT (100 µg/ml) significantly increased alveolar paracellular permeability. LPS significantly increased alveolar epithelial paracellular permeability. LPS is a known signaling molecule that increases cellular permeability and induces lung acute injury. LPS weakly potentiated MWCNT-induced paracellular permeability. These results suggest that different mechanism(s) of signaling by MWCNT and LPS are involved in alveolar cell barrier disruption. In the present study, we also investigated the effect of MWCNT on extracellular matrix remodeling factors, TGFβ, Col3A1, MMP-9, and MMP-12. MMPs are proteinases that degrade the components of the

extracellular matrix and numerous non-matrix proteins. Numerous studies have shown that carbon nanotubes induce interstitial fibrosis (Mercer et al., 2011; 2013; Poulsen et al., 2015), however the myriad of contributing factors to fibrogenic response still remains unclear. Typical pulmonary fibrosis results from a dysfunctional wound healing process. In normal tissue repair, extracellular matrix remodeling is a coordinated process such as the degradation and the deposition of new ECM is in balance, whereas in fibrosis, ECM production is excessive and uncontrolled and leads to the formation of fibrotic lesions in the lungs. The main component of the fibrotic lesions is collagen type I and III. In the present study, we show that MWCNT significantly induce the expression of Col3A1 mRNA. MWCNT have been shown to lodge in the lung interstitial tissue and to stimulate residing fibroblast to secrete collagen, however in this study, we show that MWCNT also stimulate alveolar epithelial A549 to produce Col3A1. Wang et al. (2015) showed that MWCNT induce epithelial-mesenchymal transition (EMT) via activation of the TGF β pathway. In the present study, we also report the activation of TGF β in alveolar epithelial cells. TGF β signaling is critical in tissue homeostasis and morphology not only during embryonic development and adulthood, but also during tissue injury. The results of the present study show that MWCNT are injurious to alveolar cell and stimulate the cells to produce key signaling molecules such as TGF β a well known key signaling molecule in lung remodeling. Previous studies have shown that TGF β downregulates E-cadherin and up-regulates α -smooth muscle actin (SMA), both of which are considered markers for the epithelial to mesenchymal characteristic of cells (Willis and Borok, 2007). Chen et al. (2014) showed the up-regulation of E-cadherin and α -SMA by long MWCNT. Although in the present study we did not analyze EMT markers, we show the up-regulation of TGF β and its target, Col 3A1, results that suggest a critical role of TGF β in MWCNT-induced lung fibrosis and modulation of epithelial cells transition to fibroproliferative mode. Recently, Mishra et al. (2015) showed a critical role of TGF β receptor-1 (R1) signaling in MWCNT-induced fibrosis. Many studies have demonstrated that one of the mechanisms by which MWCNT activates TGF β signaling is via the production of reactive oxygen species (ROS), and reciprocally TGF β enhance ROS formation (Chen et al., 2015; Pacurari et al., 2008). We also studied the effects of MWCNT on the expression of key factors of lung remodeling, MMP-9 and MMP-12. We show here a significant increase in the expression of ECM remodeling markers after the exposure to MWCNT. MMP-9 is a proteinase present at low level in healthy lung but abundant in pathological lung diseases including pulmonary fibrosis and chronic obstructive pulmonary disease (COPD) (Atkinson and Senior, 2003). LPS effect on MMP-9 was similar to that of MWCNT, however the combination of LPS and MWCNT had a synergistic effect on the expression of MMP-9. In addition to the role of MMP-9 in lung remodeling, MMP-9 also plays a role in inflammatory reparative responses. Here we show the regulation of MMP-9 by LPS and MWCNT both of which are potent inducers of inflammatory cytokines IL-1 β and TNF α (He et al., 2011; Zhang et al., 2015).

In the present study, we also examined the effect of MWCNT and LPS and combination on cell migration. MMPs are required for cell migration (Kim et al., 2012; Murray et al., 2013), and MMP-9 has been shown to induce macrophages migration (Kim et al., 2012). In the present study, MWCNT potently induced alveolar cell migration, while LPS at low concentration minimally stimulated cell migration and at the highest tested concentration of

10 ng/ml, LPS potently stimulated alveolar cell migration. Previous studies have shown a role of MMP-12 in lung fibrosis. Matute-Bello et al. (2007) demonstrated that deletion of MMP-12 prevented collagen deposition and decreased the expression of profibrotic genes *egr1* and *cyr61* in the lungs of mice treated with Fas ligand (FasL). CYR61 is a matrix-associated cysteine-rich secretory protein expressed in human fibroblast in response to growth factors including TGF β , and modulates fibroblast adhesion and migration and remodeling of ECM (Kireeva et al., 1996). In the present study, we show that MWCNT potently up-regulate MMP-12, whereas LPS modestly up-regulated MMP-12. The addition of LPS to MWCNT limited stimulatory effect of MWCNT on MMP-12. MMP-12 has been shown to be up-regulated by cytokines, growth factors, and other components of ECM (Lavigne et al., 2004). In the present study, we show the regulation of MMP-9 and MMP-12 by LPS and MWCNT; both of which are known to induce pro-inflammatory and pro-fibrotic signaling. MMP-9 gene expression is primarily regulated transcriptionally by NF- κ B and STAT3. The presence of NF- κ B binding sites in the MMP-9 promoter sequence, and inhibition of STAT3 suppressed MMP-9 activation has been previously shown (Kim et al., 2008; Kojima et al., 2014; Senft et al., 2011). In the present study, we show translocation of NF- κ B in response to LPS and MWCNT. Activation of NF- κ B and STAT3 are known to induce activation of genes associated with cell proliferation, transformation, and cell migration (Rahman and Fazal, 2011). NF- κ B is a known master transcription factor that regulates inflammation in response to lung injury (Gao and Ward, 2007; Rahman and Fazal, 2011). Increased expression of MMP-9 and MMP-12 following alveolar epithelial cells treatment with LPS, MWCNT or combination suggests that cellular mediators from alveolar epithelial cells following the treatment are able to elicit remodeling of extracellular matrix and favor deposition of collagen. MMP-9 and MMP-12 have been shown to be expressed by bronchial and alveolar epithelial cells as well as alveolar macrophages, and fibroblasts (Kobayashi et al., 2014; Lavigne et al., 2004; Xie et al., 2005). Moreover, MMP-9 has been shown to release or activate several cytokines including TGF β (Perng et al., 2011), whereas MMP-12 is activated by TGF β . In the present study, we show up-regulation of MMP-9 and MMP-12 in response to LPS and MWCNT exposure of alveolar A549 cells. Previously, CNT have been shown to induce rapid interstitial lung fibrosis (Mercer et al., 2011; Shveodova et al., 2014), however the underlying molecular factors contributing to MWCNT mechanisms are still unclear. Studies have shown that CNT penetrate alveolar epithelium into interstitial tissue and stimulate fibroblast to produce collagen (Mercer et al., 2013; Mishra et al., 2015). In the present study, we show that MWCNT activate alveolar epithelial cells to produce TGF β and collagen, both of which are key players in lung fibrosis. Moreover, we also show that alveolar epithelial cells in response to LPS and MWCNT activation produce MMP-9 and MMP-12, key players in lung ECM remodeling.

In conclusion, our results indicate that MWCNT alone or in combination with LPS can activate alveolar epithelial cells toward fibrogenic status, thus, in addition to fibroblasts, alveolar epithelial cells may contribute to MWCNT-induced fibrogenesis. These findings may aid in the design and development of rapid tests for fibrogenic potential of nanomaterials or other signaling molecules.

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FIG. 1. Elemental analysis of MWCNT using ECS 4010 platform. As shown in the figure, ECS analysis shows three peaks for carbon (95%), nitrogen (0.59%), and hydrogen (0.32%).

FIG. 2.

Transmission electron micrograph of MWCNT. The MWCNT sample was prepared as described in Materials and Methods. As shown in the figure, single MWCNT as well as few agglomerates of MWCNT and few impurities are visible.

FIG. 3. Paracellular permeability of A549 cell monolayer after exposure to LPS (1 or 10 ng/ml), MWCNT (20 or 100 µg/ml), or the combination of LPS (1 ng/ml) and MWCNT (20 µg/ml) for 48 h. Cell layer paracellular permeability was assed using blue dextran extravasation (10 nM). The data represents the mean $\pm$ SEM, n=4, relative to control. *, Statistically significant versus control, ANOVA, $p < 0.05$.

FIG. 4. Regulation of TGF β (**A**) and Col3A1 (**B**) genes in A549 cells treated LPS (1 ng/ml), MWCNT (20 μ g/ml), or the combination of LPS (1 ng/ml) and MWCNT (20 μ g/ml) for 24 h. The data is presented as mRNA fold change relative to 18S. The data represents the mean $\pm$ SEM, n=4. *, Statistically significant versus control, ANOVA, p <0.05.

FIG. 5. Regulation of MMP-9 (**A**) and MMP-12 (**B**) genes in A549 cells treated with LPS (1 ng/ml), MWCNT (20 μ g/ml), or the combination of LPS (1 ng/ml) and MWCNT (20 μ g/ml) for 24 h. The data is presented as mRNA fold change relative to 18S. The data represents the mean $\pm$ SEM, n=4. *, Statistically significant versus control, ANOVA, $p < 0.05$.

FIG. 6.

Cell migration in wound healing of A549 cell layer after exposure to LPS (1 or 10 ng/ml), MWCNT (20 µg/ml), or the combination of LPS (1 ng/ml) and MWCNT (20 µg/ml) after 48 h. Cell wound healing was assed using wound scratch assay (A), and cell migration assay (B). One representative micrograph is shown. The data is mean $\pm$ SEM, n=4. *, Statistically different vs control, ANOVA, $p < 0.05$.

FIG. 7.

NF- κ B p65 subunit nuclear translocation in A549 cells following treatment with LPS (1 ng/ml), MWCNT (20 μ g/ml), and the combination of LPS (1 ng/ml) and MWCNT (20 μ g/ml) after 24 h. Nuclear translocation of p65 subunit was detected using immunofluorescence (green) and DAPI (nucleus). The arrow points to immunofluorescence of p65 in the nucleus. One representative micrograph is shown, n=3.

Table 1

Elemental analysis of MWCNT using ECS 4010

	Reten. Time [min]	Response	Weight [mg]	Weight [%]	Peak Type	Elemental Name	Carbon Response Ration
1	1.409	5.232	0.007	0.59	Refer	Nitrogen	0.001
2	1.996	5243.849	1.102	94.24	Refer	Carbon	1.000
3	5.520	10.978	0.004	0.32	Refer	Hydrogen	0.002
	Total		1.169	95.15			