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Potential *in vitro* model for testing the effect of exposure to nanoparticles on the lung alveolar epithelial barrier ☆

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ABSTRACT

Pulmonary barrier function plays a pivotal role in protection from inhaled particles. However, some nano-scaled particles, such as carbon nanotubes (CNT), have demonstrated the ability to penetrate this barrier in animal models, resulting in an unusual, rapid interstitial fibrosis. To delineate the underlying mechanism and specific bio-effect of inhaled nanoparticles in respiratory toxicity, models of lung epithelial barriers are required that allow accurate representation of *in vivo* systems; however, there is currently a lack of consistent methods to do so. Thus, this work demonstrates a well-characterized *in vitro* model of pulmonary barrier function using Calu-3 cells, and provides the experimental conditions required for achieving tight junction complexes in cell culture, with trans-epithelial electrical resistance measurement used as a biosensor for proper barrier formation and integrity. The effects of cell number and serum constituents have been examined and we found that changes in each of these parameters can greatly affect barrier formation. Our data demonstrate that use of 5.0×10^4 Calu-3 cells/well in the Transwell cell culture system, with 10% serum concentrations in culture media is optimal for assessing epithelial barrier function. In addition, we have utilized CNT exposure to analyze the dose-, time-, and nanoparticle property-dependent alterations of epithelial barrier permeability as a means to validate this model. Such high throughput *in vitro* cell models of the epithelium could be used to predict the interaction of other nanoparticles with lung epithelial barriers to mimic respiratory behavior *in vivo*, thus providing essential tools and bio-sensing techniques that can be uniformly employed.

1. Introduction

The rapidly growing nanotechnology industry has revolutionized various industrial fields. Engineered nanomaterials (ENM) are of significant relevance owing to their unique physical, electrical and chemical properties, which have been exploited for widespread applications in electronics, aerospace, medicinal drug delivery, engineering, and cosmetics. However, their mass production, and exposures associated with workplace handling, raises serious health concerns, especially in the context of lung hazards, since ENMs are readily aerosolized.

The biological effects of ENMs, including carbon nanotubes (CNTs), remain poorly understood and are an object of debate regardless of several attempts to fully characterize them [7,11,12,20,23]. Despite this, it is commonly accepted that inhalation of ENMs, the primary route of exposure for CNTs, can cause major airway and lung disorders [4,7,12]. Recent research has divulged potential harmful effects of CNTs in the lungs including oxidative stress, inflammatory cytokine production, fibrosis, granuloma formation, and lung cancer promotion [8,16,20,31], while other studies have found similar effects as a result of various alternative ENMs such as cerium dioxide [14,15] and titanium dioxide [3,25].

Airway epithelial cells, present as one of the first lines of defense for inhaled particulate matter, represent a major determinant in the interaction of a foreign body with other major body compartments. In addition, epithelial cells are involved with the

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formation and maintenance of tight junctions between neighboring epithelial cells, only permitting polarized secretory functions (such as ion transport) and routine cellular trafficking, while preventing access to xenobiotics and pathogens [1,28,32,34]. Moreover, these tight junctions assist in keeping cytokines, toxins, and pathogens from infiltrating the epithelial layer. Indeed, these dynamic protein structures govern the paracellular permeability of the epithelium, permitting only that which is necessary under normal circumstances [13,26]. Consequently, pulmonary barrier function plays a pivotal role in controlling penetration of inhaled nanoparticles into the interstitium, which can lead to rapid interstitial fibrosis [19,35]. For example, dispersed single- or multi-walled CNTs rapidly enter the alveolar interstitial space and induce a progressive interstitial fibrotic response with minimal lung inflammation [19,23,35]. However, due to a lack of studies evaluating the effects of CNT exposure on lung epithelial barriers, a key determinant of pulmonary toxicity due to ENMs, the pathogenic mechanisms underlying these effects have not been fully elucidated. This is due, in part, to a lack of effective, consistent methods to reliably predict *in vivo* outcomes in an *in vitro* setting.

Further compounding this issue is the notion that unique physicochemical characteristics of ENMs, such as particle size, shape, and surface modification, may contribute to progressive, toxic responses, including heightened lung epithelial barrier permeability. To date, it remains inadequately understood how each of these fundamental features distinctly affects the airway epithelium. In addition, due to the escalating costs and limitations of evaluating individual cell type-specific outcomes when animals are exposed to ENMs *in vivo*, more *in vitro* approaches are being explored as alternatives.

Epithelial cells in culture form tight junction complexes, thereby making *in vitro* models of lung epithelium a favorable choice to mimic and predict the respiratory behavior *in vivo* upon exposure to ENMs such as CNT materials [30]. Several studies have sought to explore the effects of various ENMs on epithelial function *in vitro* using the Calu-3 small airway epithelial cell line [21,28,38]; however, inconsistent methods to do so limit comparisons and conclusions. Hence, our current study utilized the Calu-3 cell line that demonstrates the characteristics of differentiated, functional human epithelia [9,33] with the primary objective to provide the technical details and characterization of an *in vitro* pulmonary barrier model to consistently screen for the pathogenicity of various nanoparticles on lung epithelium. Trans-epithelial electrical resistance (TEER) is a commonly used endpoint to assess integrity and permeability of epithelial monolayers, as it is an instantaneous measure of ion flux [32] and an indirect measurement of the formation of tight junctions [36]. Thus, we have employed this parameter for use as a biosensor for epithelial monolayer formation and integrity. In addition, the peculiar effects of cell number and serum constituents have been examined in an effort to reliably study the effect of exposed particles on lung epithelial barrier permeability *in vitro*. Providing the results of our various testing strategies to optimize these conditions will not only provide consistent methodologies that can be implemented by others, but will also help to elucidate potential differences in past studies that have used alternate culture conditions with similar model systems, particularly Calu-3 cells.

Additionally, as a means to substantiate this model, we have utilized the optimized conditions outlined in this study with an exposure to CNTs. We hypothesized that the physicochemical properties of CNTs play a key role in determining the effects that these particles have on epithelial barrier integrity, thereby affecting penetration of CNTs into the interstitium. Such high-throughput *in vitro* cell models of the airway epithelium could be advantageous in predicting the interaction of other nanoparticles with lung epithelial barriers to mimic the respiratory behavior *in vivo*.

2. Materials and methods

2.1. Reagents

Eagle's minimum essential medium (EMEM) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Phosphate buffer saline (PBS), trypsin, fetal bovine serum (FBS), and penicillin/streptomycin antibiotic solution were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell monolayer assessment

Human lung epithelial cells (Calu-3, ATCC, Manassas, VA) were seeded onto Transwell® cell culture support dishes (Corning Life Sciences, Tewksbury, MA) at various densities, 1.0×10^4 cells/well, 2.0×10^4 cells/well, and 5.0×10^4 cells/well, with the optimal density determined to be 5.0×10^4 cells/well. Inserts had a diameter of 6.5 mm, with a growth area of 0.33 cm^2 and 3.0 μm pores. Cells were cultured in EMEM media supplemented with L-glutamine (1%), penicillin-streptomycin (1%), and fetal bovine serum (FBS; 10%) at 37 °C in humidified air with 5% carbon dioxide. After 24 h, cells were washed and medium was changed to EMEM media with various FBS concentrations, 2%, 5%, 10%, and 15%, in an effort to determine whether FBS concentration alters monolayer formation.

Monolayer formation was evaluated by measuring electrical resistance of the cultured Calu-3 cells and subsequently confirmed using bright field microscopy (Ziess Axiovert 100 TV inverted microscope, Carl Zeiss Microscopy, LLC, Thornwood, NY), CytoViva hyperspectral microscopy (CytoViva, Auburn, AL), and analysis of the tight junction protein ZO-1 via confocal microscopy. Resistance was measured using an Epithelial Voltohmmeter (EVOM2) with a STX2 electrode (World Precision Instruments, Sarasota, FL) every 24 h after washing. Trans-epithelial electrical resistance (TEER) readings were determined by subtracting the resistance (in ohms) of the blank insert from the recorded resistance of the monolayer, and subsequently multiplying the resulting value by the effective membrane surface area of the insert to yield ohms·cm^2.

2.3. Immunofluorescence of tight junctions of the Calu-3 cell monolayer

Cells were fixed in 3.7% formaldehyde for 10 min at room temperature and then permeabilized and blocked in a solution containing 0.5% saponin, 1% bovine serum, and 1.5% goat serum for 30 min. After primary antibody incubation with ZO-1 antibody (Invitrogen, Carlsbad, California) at 1:50 dilution for 1 h, cells were washed and incubated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, California) for 30 min. The transwell cell culture support membrane was cut and mounted on glass microscope slides using Prolong gold anti-fade reagent containing DAPI (Invitrogen, Carlsbad, California). Images were acquired by confocal laser scanning microscopy using a Zeiss LSM 510 (Carl Zeiss Microscopy, LLC, Thornwood, NY).

2.4. Preparation of carbon nanotubes

Single-walled carbon nanotubes (SWCNT; Carbon Nanotechnologies, Inc., Houston, TX) were produced by the high pressure CO disproportionation (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and Fe $(CO)_5$ as the iron-containing catalyst precursor. These SWCNT were then purified by acid treatment to remove metal contaminants. Elemental analysis of the supplied SWCNT by nitric acid dissolution and inductively coupled plasma–atomic emission spectrometry