In vitro toxicity assessment of pre- and post-incinerated organomodified nanoclays on airway epithelial and lung fibroblast cells

Introduction

Use of two-dimensional organomodified nanoclays (ONCs) in nanocomposites continues to grow, however, may pose an inhalation hazard and risk for fixed airway disease which partially relies on epithelial and fibroblast cell responses. We hypothesized that physicochemical property differences associated with coating presence/absence and incineration status of nanoclays will elicit changes in key events in exposed human small airway epithelial (SAECs) and normal lung fibroblast (NHLF) cells that contribute to pulmonary lung fibrosis. Using multiplex high-throughput screening strategies, SAEC and NHLF cells were acutely exposed (0-20 μg/cm^2) to pristine nanoclay (CloisNa), an ONC (Clois30B), their incinerated byproducts (I-CloisNa and I-Clois30B), and crystalline silica (CS), to evaluate how ONC characteristics influence several key events in the pulmonary fibrosis adverse outcome pathway. In vitro exposure to pre-incinerated nanoclay induced organic coating-dependent cytotoxicity in SAECs. CloisNa caused disruption of mitochondrial membrane potential which coincided with loss in viability in both cell types. Clois30B exposure caused dose-dependent SAEC cytotoxicity, micronuclei formation, and mitochondrial hyperpolarization in SAECs and NHLFs. Incinerated nanoclays were non-cytotoxic but elicited a SAEC mitochondrial radical and pro-inflammatory response. Direct in vitro exposure to NHLFs exhibited particle-dependent increased live cell count, reactive oxygen species production, and α-smooth muscle actin expression. Nanoclay-exposed NHLFs possessed elevated collagen I levels while the same mass dose in vivo favored elevated fibronectin and collagen III deposition for CloisNa and CS. In conclusion, organic coating presence and incineration status influenced nanoclays' effects on cellular interaction, membrane integrity, inflammation, fibroblast activation, and collagen accumulation in exposed cell models. Although pre-incinerated nanoclay exposure promoted collagen accumulation in vitro, it was a poor predictor of in vivo model reticular fiber deposition.

Methods Collection

Particle preparation and characterization

- Cloisite Na+ (CloisNa) and an organomodified nanoclay (Cloisite 30B; Clois30B) were purchased from Southern Clay Products (Gonzalez, TX).
- Incinerated CloisNa and Clois30B nanoclay particles were produced by incineration during thermogravimetric analysis (900 °C for 100 minutes).
- All particles were previously characterized in other publications.
- Stock particle suspensions were made by adding sterile MilliQ water to known particle mass in sterile glass test tubes. Discrete sonication was used to disperse stock particle suspensions. Briefly, particle suspensions were lightly vortexed and sonicated three separate times to reach relatively stable suspensions in different cell culture growth mediums.
- Each media's density, viscosity, and refractive index were determined.
- Effective density (ρ_{EV}) of each particle (0.1 mg/ml) for each culture medium was measured using particle density, packed pellet volume, medium density, and theoretical stacking factor (SF=0.634) as previously described.

- Soluble endotoxin in sonicated stock suspensions were determined with the Pierce LAL chromogenic method.
- Dynamic light scattering (DLS), particle dispersity index (PdI), and zeta potential analyses using a Zetasizer Nano ZS were performed for each particle in water and three culture mediums.

FESEM analyses

- Each sonicated particle stock suspension was diluted into each cell culture medium at 25 – 100 µg/mL, filtered onto polycarbonate filter, gold/palladium sputter coated, and imaged with a Hitachi S-4800 FESEM. 20-30 representative images were taken to compare to dynamic light scattering analyses.
- Length and maximum width for each located particle in digital FESEM images were measured in ImageJ.

Cell culture

- Human small airway epithelial cells (SAEC) immortalized with human telomerase reverse transcriptase (hTERT; Lonza) were cultured in a Corning® tissue culture-treated flask containing SAGM supplemented with growth factor bullet kit previously described. Epithelial cells were used between passages 5 and 20.
- Cells were seeded at 5 or 10 x103 cells per well in a 96-well plate overnight prior to exposure. Primary normal human lung fibroblasts (NHLF) were acquired from Lonza and cultured in a Corning® tissue culture-treated flask from passages 2-8 in FGM-2 medium with growth supplements, including 2% FBS, fibroblast growth factor, and 1% penicillin/streptomycin.
- NHLFs were seeded overnight at $5x10^3$ and $3x10^5$ in 96-well and 6-well plates, respectively, prior to exposure.
- Both SAEC and NHLF were passaged using trypsin and trypsin inhibitor and reseeded in culture flasks according to manufacturer's instructions.
- THP-1 monocytes (ATCC, Gaithersburg, MD) were cultured in RPMI medium with 10% FBS and differentiated into attached macrophages as previously described. Briefly, THP-1 monocytes were seeded at 1.5×10^5 cells per well in 6 well plates then differentiated with Vitamin D3 and PMA for 48 hrs.
- All cell cultures were maintained in a humidified incubator at 37 °C with 5% CO2 air.

Particle Uptake via TEM Analysis

- SAECs were seeded at 7×10^5 in 6-well plates overnight, then exposed to test particles at 0.6 µg/cm^2 for 24 hours.
- Cells were rinsed with saline wash, collected, and centrifuged to collect a pellet of exposed cells. The pellet was resuspended in fixative, processed, blocked, sectioned, mounted, and imaged with a TEM JEOL 1400.
- 20-30 images were taken to evaluate cellular uptake potential for each particle. Two independent experiments were performed for each particle.
- Enhanced darkfield microscopy (CytoViva) was used to help identify particle co-localization within different cell compartments. Sterile glass coverslips stored in 70%

ethanol were placed in 24-well plate, rinsed, and incubated for 24 h in SAGM to condition glass surface.
- SAECs were seeded at 3 x10^4 per well in fresh warm SAGM for 24 h, then exposed to 0.6 µg/cm^2 for another 24 h.
- Cells were washed in HEPES saline, fixed 4% paraformaldehyde, and stained with HEMA3 (Fisher Scientific) according to manufacturer's protocol to maximize the visualization of Clois30B.
- Images at 60X and 100X were taken on a CytoViva-enabled Olympus scope with a DP73 camera. Four independent experimental runs were conducted.

WST-1 and LDH assays

- SAECs and NHLFs were seeded at 1x 10^4 cells per well in quadruplicate overnight. Next, cells were exposed to each freshly sonicated particle at 0.02 - 20 µg/cm^2 in 200 µl (0.032 - 32 µg/ml) for 24 h.
- 1% Triton-X applied to cells for 2 hours served as a positive 100% cytotoxicity control.
- Following exposure, plates were centrifuged at 1000 rpm for 5 minutes and the top 100 µl of supernatant was collected for LDH assay to measure cytotoxicity according to manufacturer's instructions (Roche). Next, 10 µL of WST-1 reagent (Roche) was added to the remaining medium with cells and incubated for 2 h to measure cell viability.
- LDH interference assay was conducted by incubating 2.5 mU/well of purified LDH-A in each medium type in the presence of serially diluted particle for 24h.
- Absorbance of each well was read on a microplate spectrophotometer at 490 and 450 nm for LDH and WST-1 assays, respectively.

High-throughput screening analyses

- SAECs were seeded in 96- or 384-well black plates at 1x10^4 cells/well while NHLFs were seed in 96-well black plates at 5x10^3 cells/well.
- Both cell types were exposed to 0 – 20 µg/cm^2 applied dose for 0-48 hrs. A mass per unit alveolar surface area dose metric was used to directly compare in vitro responses to in vivo pulmonary exposure responses. The low (30 µg/mouse) and high (300 µg/mouse) dose in vivo exposures in a 500 cm^2 alveolar epithelial surface area in a mouse lung would equal an applied in vitro dose of 0.06 and 0.6 µg/cm^2, respectively.
- A two-way (5 particle x 8 dose) design for each time point was used with ≥ quadruplicate replicates per treatment group.
- Cells were assayed using multiplex fluorescent staining panels and imaged at 4x to 40x magnification with the ImageXpress Micro XLS system (Molecular Devices).
- Assays were conducted following either dye manufacturers' recommendations or validated, standardized methods within our laboratory. Multiplex fluorescent dye cocktails were prepared at 10X working concentrations along with 1 µM of Hoescht 33342 as a nuclear stain.
- Following exposure, live cells were exposed to dye cocktails and incubated for 30 – 60 min then subjected to live cell imaging on the ImageXpress system. If high background signal was observed, cells were washed with fresh warm culture medium and imaged in warm, clear Fluorobrite DMEM (ThermoFisher, Pittsburgh, PA).
- One, four, nine, or sixteen replicate sites were imaged per well at 4X, 10X, 20X, and 40X magnification, respectively.

- MetaXpress application modules (v6.2) or Custom Module Editor were used to quantitively measure cell parameter endpoints (e.g. dye intensity) and correct for background interference, non-cell specific staining, and particle-only controls as previously described.

ELISA and Western Blot analysis

- SAECs were plated at 5 x 10^5 cells per well in 6-well plates and allowed to culture for 24 hours. Cells were exposed to each particle (0 – 6 μg/cm^2) for 24 hours (n=3).
- Plates were centrifuged at 220 x g for 5 minutes to remove cell debris followed by collection of conditioned medium in microcentrifuge tubes which were stored at -80 °C until assayed.
- Samples were thawed on ice and assayed neat in duplicate for release of inflammatory cytokines using a Luminex magnetic bead 27-plex kit containing MIP-1α, MIP-1β, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-7, IL-9, IL-12p70, IL-13, IL-15, IL-17α, IFN-y, IL-1ra, GM-CSF, Eotaxin, FGFβ, IP-10, G-CSF, MCP-1, PDGF-ββ, RANTES, TNFα, and VEGF.
- Mean fluorescent intensities were calculated off a 7-point standard curve.
- Separate PDGF-ββ and TGFβ ELISAs (R and D Systems) were conducted on conditioned medium from nanoclay-exposed NHLFs using the above-mentioned cell seeding (3x10^5 in a 6-well plate) and exposure procedures (n=3).
- NHLF were exposed to particulate (0 – 6 μg/cm^2) for 48 h (n=3).
- Supernatants of conditioned medium were collected, stored, and then assayed for human PDGF-ββ using R and D Biosystems Duoset kit in duplicate.
- TGFβ samples were activated with HCl solution, neutralized, and assessed for TGFβ following the manufacturer's procedure in duplicate.
- Human THP-1 monocytes were seeded at 1.5x10^5 cell per well and differentiated into macrophages using Vitamin D and PMA in 6-well plates as previously described.
- Macrophages were exposed to particulate (0 – 6 μg/cm^2) for 24 h (n=3) in the absence or presence of lipopolysaccharide (LPS). The same PDGF-ββ procedure above was used for macrophage supernatants. Results were compared to in vivo levels.
- Correlation analysis of secreted PDGF-ββ levels comparing in vitro macrophage to in vivo BAL response was conducted on equal (0, 0.06, and 0.6 μg/cm^2) and 10x (0, 0.6, and 6 μg/cm^2) dose equivalents. Only CloisNa and Clois30B responses were evaluated since both incinerated nanoclays and CS did not produce significantly different responses in each model.

Animals

- Analyzed animal tissues presented in this study were collected as part of a previously conducted study evaluating the effects of oropharyngeal aspiration exposure of these same particles at 30 μg and 300 μg/mouse to the lungs of male C57Bl/6J mice.
- The study was conducted in an AAALAC-accredited facility. The CDC-Morgantown Institutional Animal Care and Use Committee reviewed and approved all procedures for the animal study.

Fibroblast activation and ECM synthesis comparison between in vitro and in vivo models

- NHLFs, seeded into 6-well plates overnight, were exposed to 0 – 0.6 μg/cm^2 to each particle for 48 hrs to evaluate changes in fibroblast activation and extracellular matrix protein expression.

- Exposed cells were held on ice, washed in cold PBS, and lysed on ice for 10 minutes with Invitrogen cell lysis buffer supplemented with PMSF and protease inhibitor cocktail (ThermoFisher) as previously described (16). Cell lysates were collected via rubber policeman and micropipette, placed into an Eppendorf tube, and immediately frozen at -80 °C until assayed.
- To evaluate both nanoclay-exposed in vivo lung tissue and in vitro NHLF ECM protein expression, total protein in Day 28 lung lysates (n=4) and NHLF lysates (n=4) was determined using the BCA assay following manufacturer's instructions (ThermoFisher Scientific).
- 30 µg of protein for each sample was loaded onto a 5% and 10% bis-acrylamide gel for SDS-PAGE analysis.
- Proteins were separated via electrophoresis in Tris glycine buffer and transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Fisher Scientific) in tris buffer containing 20% methanol.
- A SNAP id 2.0 (MilliporeSigma, Burlington, MA) system was then used for all antibody incubations. Membranes were placed onto midi blot membranes and blocked with 0.5% non-fat dry milk in tris-buffered saline containing 0.1% Tween 20 (TBS-T) under suction.
- Each primary antibody, including collagen I (Fitzgerald, Acton, MA), collagen III, fibronectin, α-SMA (Abcam, Cambridge, MA), and β-actin (Sigma-Aldrich), was diluted and incubated twice for 10 minutes.
- Membranes were thrice washed with TBS-T and incubated with rabbit or mouse secondary HRP antibody for 10 minutes followed by suction.
- Membranes were again TBS-T rinsed, incubated with HRP chemiluminescent substrate (MilliporeSigma), exposed to X-ray film, and developed.
- Visible bands on films were digitally scanned and quantified using densitometry using ImageJ v1.52.

Fibronectin and Collagen III Immunohistochemistry

- 5 µm fixed left lung tissue sections were placed on glass slides and subjected to immunohistochemistry staining for collagen III and fibronectin based on previously published methods.
- Tissue sections for immunohistochemical analysis was deparaffinized and rehydrated xylene and graded alcohol respectively.
- Antigen was retrieved by three microwave exposures (20% power for 5 minutes each) in citrate buffer (1mM, pH 6.0) and then endogenous peroxidases quenched by incubation with 1% hydrogen peroxide for 10 minutes.
- Sections were blocked with 5% horse serum for 45 minutes, then a rabbit polyclonal antibody against fibronectin (1:50 dilution, ab2413, Abcam) and a rabbit polyclonal antibody against collagen III (1mg/mL, 1:50 dilution, ab7778, Abcam, Cambridge, MA) were applied separately on the samples and incubated overnight at 4ºC.
- Samples was then incubated in a biotinylated horse anti-rabbit secondary for 45 minutes at room temperature (1:200 dilution, BA-1100, Vector Laboratories Inc., Burlingame, CA).
- Samples were incubated with the Vectastain Elite ABC reagent for 30 minutes according to manufacturer's instructions (PK-6100). DAB (brown) was used as the substrate according to manufacturer's instructions (SK-4100). Sections were counterstained with hematoxylin.
- The primary antibody was replaced with 4% BSA in 1X PBS for the negative control slides.
- Images were taken using Olympus AX70 microscope and DP73 camera. On average, nine random fields of view from three or four slides per treatment group were analyzed.
- Lung fibronectin and collagen III were quantified by binary color threshold pixel analysis using Nikon Elements analysis software (AR 5.02.00).

Citations

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