Lung Remodeling After Pulmonary Exposure of Mice to Cerium oxide Nanoparticles - Role of Autophagy

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10th Nov. 2016
Introduction


NPs can cause lung fibrosis

• Carbon nanotubes (CNTs) could cause progressive fibrotic response in the alveolar tissues of mice lungs (Shvedova et al. 2008, Mercer et al. 2011)
• Nickel NPs are implicated in exaggerated lung and airway remodeling in mice (Glista-Baker et al. 2014)
• Crystalline silica NPs could cause silicotic nodules with collagen fibers and dust-laden macrophages surrounding the mature collagen (Fujimura, 2000)
• CeO₂ NPs would induce inflammation, air/blood barrier damage, and phospholipidosis with enlarged alveolar macrophages leading to lung fibrosis (Ma et al. 2011, 2012, 2014)

Unanswered questions:

• Where does fibrotic lung remodelling occur? (Bronchial and/or Alveolar)
• What are the underlying mechanisms?

Defective Autophagy has a role to play in idiopathic pulmonary fibrosis

(Mi et al. 2011, Patel et al. 2012, Araya et al. 2013)
Autophagy: potential mechanism for fibrosis?

**Autophagy:** Turnover of unnecessary or dysfunctional cellular components

- **Induction, Autophagosome formation, Fusion and Degradation**

- Cohignac et al. 2014

**Autophagy in fibrosis**

- Several factors (environmental agents, CS, ROS, ER stress) (Monick et al. 2012, Araya 2013)

- Defective or insufficient autophagy

- In lung cells

- **Macrophages**
  - Secrete higher levels of ROS-induced IL1A and IL1B implicating in fibrosis development (Lodder, et al. 2015)

- **Epithelial cells**
  - Increase apoptosis and accelerate senescence – could lead to abnormal epithelial-mesenchymal interactions (Mi et al. 2011, Araya et al. 2013)

- **Fibroblasts (bronchial and parenchymal)**
  - Excess production of extracellular matrix in fibroblasts, myofibroblasts differentiation (Del Principe et al. 2011)

- **Lung fibrotic development** (Patel et al. 2012, Mi et al. 2011, Araya et al. 2013a,b, Del Principe et al. 2011)
Hypothesis

To characterize the pulmonary fibrosis induced by exposure of mice to CeO$_2$NPs

To evaluate the role of autophagy in the fibrotic response to CeO$_2$NPs

Objectives
**Methods**

**Nanoparticles used:** CeO$_2$NPs, (99.9% purity, Size range 15-30nm, spherical)

Diesel fuel catalysts to reduce the emission of particulate matter in diesel

**Exposure Protocol:**

Non surgical intratracheal instillation of mice Saline or 5, 50 µg of CeO$_2$ NPs

- **Day 0**
- **1 day post treatment**
- **7 days post treatment**
- **28 days post treatment**
- **90 days post treatment**

Lung (Histopathology, BAL, RNA/Protein)
Results:

**CeO$_2$NPs induce lung fibrosis in mice**

Alveolar and broncholar thickening or inflammation observed in mice exposed to nanoceria after 1 week and 90 days of exposure

(n=6)
CeO$_2$NPs induce lung fibrosis in mice

α-SMA and expression of TGF-β1 in lung sections of mice exposed to CeO$_2$NPs

- An increase in α-SMA and TGF-β1 expression expression observed

90 days exposure

IHC

(n=6)
Induction of autophagy in GFP-LC3 mice exposed to CeO$_2$NPs

LC3 seems to be accumulated in macrophages *in vivo*

CeO$_2$NPs activate autophagy in macrophages a evidenced by co-localisation of LC3 and LAMP1

Role of autophagy in macrophages?
Atg5: an early marker of autophagy

What if Atg5 is floxed in macrophages?

- Conditional knockout of Atg5 gene in myeloid lineage
- Lacks Atg5 activity in Macrophages
- Defective autophagy in Macrophages
- Implicated in CeO$_2$NPs-induced lung fibrosis?
**Mice exposed to CeO$_2$NPs**

- Alveolar thickening or diffused inflammation in Wild type mice exposed to CeO$_2$NPs
- Atg5$^{-/-}$ mice are protected from CeO$_2$NPs induced alveolar thickening
- Bronchial thickening in both wild type and atg5$^{-/-}$ mice exposed to CeO$_2$NPs
- Bronchial inflammation characterized by macrophages infiltration in atg5$^{-/-}$ mice

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28 days exposure

HE staining

(n=5)
Mice exposed to CeO$_2$NPs

- Type 1 collagen deposition in alveoli of wild type mice exposed to CeO$_2$NPs
- No Type 1 collagen deposition in alveoli occurred in atg5$^{-/-}$ mice exposed to CeO$_2$ NPs
- Type 1 collagen deposition in bronchi of wild type mice treated with CeO$_2$NPs
- Type 1 collagen bundles in bronchi of atg5$^{-/-}$ treated with CeO$_2$NPs

28 days exposure
Picro sirius red staining
(n=5)
α-SMA expression in wild type and atg5⁻/⁻ mice exposed to CeO₂NPs

- Increased α-SMA in alveloli of wild type but not in alveloli of in atg5⁻/⁻ mice
  - Similar increase in α-SMA in bronchi of wild type and atg5⁻/⁻ mice

28 days exposure

IHC: α-SMA

(n=5)
TGF-β1 expression in Wild type and atg5⁻/⁻ mice exposed to CeO₂NPs

- Expression of TGF-β1 in alveoli and bronchi in wild type mice noticed
- Atg5⁻/⁻ mice are protected from CeO₂NPs-induced accumulation of TGF-β1 in alveoli but no protective effect in bronchi

28 days exposure
IHC:TGF-β1
(n=5)
**Summary**

**Alveoli**

<table>
<thead>
<tr>
<th>Fibrotic markers</th>
<th>Mice exposed to CeO₂NPs</th>
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<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td><strong>atg5⁻/⁻</strong></td>
</tr>
<tr>
<td>Thickening/Inflammation</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>Typel collagen</td>
<td>↑↑↑</td>
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<tr>
<td>TGFβ1</td>
<td>↑↑↑</td>
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**Bronchiole**

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*Lack of ATG5 gene in myeloid lineage seems to be protective in alveoli but not in bronchi of atg5⁻/⁻ over wild type mice*

*Autophagy may possibly play a dual role in CeO₂NPs-induced lung fibrosis*
Thank you for your attention

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Future studies

1. Characterization of alveolar modifications:
   • Quantification of histological modification and markers like Type collagen1, alpha SMA, TGF beta1, elastin,
   • To study inflammatory infiltration by macrophages markers

2. Characterization of bronchial modifications:
   • Quantification of histological modifications and expression of fibrotic markers

3. Luminex will be done on BALF samples of 24h, 1 week and 90 days exposures

4. Mechanisms of pulmonary fibrosis in vitro:
   • Isolation of bronchial and parenchymal fibroblasts from mice lungs (in progress)
   • Exposure to NPs
   • Myofibroblasts analysis: α- Sma, collagen, migration and proliferation

5. Characterization and role of autophagy: In vitro
   • Expression of LC3, p62 and LAMP1 in fibroblasts treated with nanoceria
   • Exposing the fibroblasts with supernatants of macrophages treated with nanoceria
   • Co-culture of the fibroblasts with marcopahges, exposing to nanoceria

6. Analyses of lung sections from WT and atg5-/- mice exposed to nanoceria for 90 days (sections are ready)
   • HES, IHC for alphaSMA, TGF beta1, collagen Type III, IV etc, Picro Sirius Red staining for Type 1 collagen etc
p62 is still subject to autophagy in cells experiencing cellular stress.

Autophagy-defective cells and tissues, the autophagy substrate p62 is not degraded.
REOs $\rightarrow$ RE$^{3+}$ $\rightarrow$ REPO$_4$ $\rightarrow$ Inactive enzyme

Active enzyme $\rightarrow$ PO$_4$ $\rightarrow$ Neutral

Fusion & degradation in autolysosome

IL-1β

Inflammasome accumulation

Autophagosome accumulation

REOs $\rightarrow$ Macrophage $\rightarrow$ Lysosome $\rightarrow$ NLRP3 inflammasome $\rightarrow$ ASC

p62, Ub $\rightarrow$ LC-3II $\rightarrow$ Autophagosome
Autophagy-defective (ATG5 gene knockout) in cells and tissues, the autophagy substrate p62 is not degraded.

High levels of p62 Binds to Keap1 releasing NRF2

constitutive activation of NRF2 and antioxidant defence

Could counter NP induced oxidative stress
Thank you for your attention

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Results:

CeO₂ NPs are not cytotoxic in peritoneal macrophages

![Graph: Cytoxicity of CeO₂ NPs after 24 h](image)

- 24h treatment
- Control
- Nano ceria (µg/mL)
- % cytotoxicity
- N=3

Induction of autophagy by CeO₂ NPs in GFP-LC3 peritoneal macrophages

![Images: GFP-LC3 and lysosomal associated membrane protein 1 (LAMP1)](image)

- 6h treatment
- Control
- Rapamycin (100 nM)
- CQ 100 µM
- Rapa + CQ
- 20 µg/mL
- 40 µg/mL
- 80 µg/mL

CeO₂ NPs did induce autophagy in GFP-LC3 macrophages

Our next idea was to see the co-localisation of GFP-LC3 and lysosomal associated membrane protein 1 (LAMP1)
CeO$_2$ NPs

Control

6h treatment

LC3 (green)  LAMP1 (Red)  Merged (green, red)

5 µg/mL

10 µg/mL
Increased expression of P62 in macrophages (RAW 264.7) due to CeO$_2$NPs

24h treatment

Increased expression of P62 in macrophages could possibly indicate autophagy blockade due to CeO$_2$NPs

CeO$_2$NPs could possibly be involved in defective autophagy
Fig. 2. Auto-regenerative red-ox cycle over CeO$_2$ NPs surface aids in scavenging oxygen free radicals.
NPs interact and inhibit chaperones activity?
The projected human pulmonary dose for inhalation of CeO2 in diesel exhaust from engines using a CeO2 fuel additive is 0.09 mg/kg body weight for 8 h (Health Effects Institute [HEI] 2001). CeO2 is insoluble particle, and studies have shown that the clearance of CeO2 from the lung may take 20 years or more (Pairon et al. 1994). As a diesel exhaust product, it is likely that the potential exposure (occupational or environmental) to CeO2 is continuous and the lung burden is cumulative. Assuming a person has been exposed to the projected dose for 40 years with 8 h working day, the total lung burden of CeO2 will be 936 mg/kg (0.09 mg/kg.d 5 d/week 52 week/year 40 years = 936 mg/kg).

Usually, conversion from rodents to humans includes a safety factor of 10-fold.

Therefore, to assess the potential toxicological consequence of CeO2 NPs we used 50µg well with the range  .