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Role of epithelial-mesenchymal transition (EMT) and fibroblast function in cerium oxide nanoparticles-induced lung fibrosis

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ABSTRACT

The emission of cerium oxide nanoparticles (CeO_2) from diesel engines, using cerium compounds as a catalyst to lower the diesel exhaust particles, is a health concern. We have previously shown that CeO_2 induced pulmonary inflammation and lung fibrosis. The objective of the present study was to investigate the modification of fibroblast function and the role of epithelial-mesenchymal transition (EMT) in CeO_2-induced fibrosis. Male Sprague-Dawley rats were exposed to CeO_2 (0.15 to 7 mg/kg) by a single intratracheal instillation and sacrificed at various times post-exposure. The results show that at 28 days after CeO_2 (3.5 mg/kg) exposure, lung fibrosis was evidenced by increased soluble collagen in bronchoalveolar lavage fluid, elevated hydroxyproline content in lung tissues, and enhanced sirius red staining for collagen in the lung tissue. Lung fibroblasts and alveolar type II (ATII) cells isolated from CeO_2-exposed rats at 28 days post-exposure demonstrated decreasing proliferation rate when compare to the controls. CeO_2 exposure was cytotoxic and altered cell function as demonstrated by fibroblast apoptosis and aggregation, and ATII cell hypertrophy and hyperplasia with increased surfactant. The presence of stress fibers, expressed as α-smooth muscle actin (SMA), in CeO_2-exposed fibroblasts and ATII cells was significantly increased compared to the control. Immunohistofluorescence analysis demonstrated co-localization of TGF-β or α-SMA with prosurfactant protein C (SPC)-stained ATII cells. These results demonstrate that CeO_2 exposure affects fibroblast function and induces EMT in ATII cells that play a role in lung fibrosis. These findings suggest potential adverse health effects in response to CeO_2 nanoparticle exposure.

1. Introduction

Cerium, a member of the lanthanide metals, has wide industrial usage (EPA, 2009), and recently it has been used as a diesel fuel-borne catalyst to reduce the emission of diesel exhaust particles (DEP) (HEI, 2001). Although cerium oxide substantially decreases total particle mass in the diesel exhaust, a small amount of cerium oxide is emitted in the particulate phase of the exhaust, primarily in the oxide form as particles <0.5 μm in diameter (HEI, 2001). Rare earth pneumoconiosis with pathologic conditions, including granulomas and interstitial fibrosis, has been demonstrated in workers exposed to rare earths metals, of which cerium was the major component (McDonald et al., 1995; Sabbioni et al., 1982; Waring and Watling, 1990). A common feature of rare earth pneumoconiosis is the presence of cerium particles in the alveoli and interstitial tissue of the patients long after exposure has ended (Pairon et al., 1995). These findings indicate that cerium oxide is potentially a fibrotic agent that may pose a serious health risk to those exposed to cerium oxide nanoparticles in either occupational or environmental settings.

Studies have shown that exposure of rats to cerium oxide induces both pulmonary and systemic toxicity (EPA, 2009; HEI, 2001), and leads to impaired pulmonary clearance of these particles. Previous studies carried out in our laboratory have demonstrated that exposure of rats to a single intratracheal instillation of cerium oxide nanoparticles induced sustained pulmonary inflammatory and phospholipidotic responses, and modified the balance of mediators involved in tissue repair processes leading to pulmonary fibrosis associated with persistence of cerium oxide nanoparticles in the exposed lungs (Ma et al., 2012). Cerium oxide-induced lung fibrosis has also been reported in a mouse model by Park et al. (2010).

Keogh and Crystal (1982) proposed that pulmonary fibrosis was due to chronic inflammation and is characterized by an excessive deposition of extracellular matrix (ECM) in the interstitium. Regardless of the

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initiating events, a common feature of all fibrotic diseases is the activation of ECM producing myofibroblasts, which are the key mediator of fibrotic tissue remodeling and produce new ECM components (Gabbiani, 2003; Kalluri and Zeisberg, 2006). Myofibroblasts, an activated fibroblast form, are characterized by a spindle or stellate morphology with intracytoplasmic stress fibers, a contractile phenotype, expression of various mesenchymal immunocytochemical markers including α-smooth muscle actin (α-SMA), and collagen production (Zhang et al., 1994). Studies on silica-induced lung fibrosis have demonstrated that the production of fibrogenic mediators, such as transforming growth factor-β (TGF-β)-1 and osteopontin, by resident macrophages and fibroblasts induces ECM gene expression and plays a key role in fibroblast activation (Natoli et al., 1998; Nau et al., 1997). Recently a competing hypothesis proposed that the pathogenesis of idiopathic pulmonary fibrosis (IPF) is the consequence of epithelial injury followed by abnormal wound healing, which can be independent of preceding inflammation (Willis et al., 2006). The possibility that epithelial cells, including alveolar epithelial cells (AEC), may undergo dramatic changes to a mesenchymal phenotype through epithelial-mesenchymal transition (EMT) has been suggested (Kalluri and Neilson, 2003; Willis et al., 2005). EMT is a process through which fully differentiated epithelial cells undergo transition to the mesenchymal phenotype of fibroblasts and myofibroblasts. EMT plays an important role in repair and scar formation following epithelial injury in a number of tissues, including the lung (Kalluri and Neilson, 2003). The cytokine, TGF-β, has been reported to play a major role in the induction of fibrosis in many organs, including the lung, and is a major mediator of EMT in a number of physiological responses, including tissue fibrosis (Willis and Borok, 2007). Studies have demonstrated that *in vitro* exposure of ATII-like cells to TGF-β1 led to phenotype changes including acquisition of fibroblastic morphology and upregulation of the mesenchymal marker, α-SMA, in AEC (Alipio et al., 2011; Gharaee-Kermani et al., 2009). This TGF-β-mediated EMT in AEC would lead to increased fibroblast proliferation, stimulates the synthesis and deposition of connective tissue, and inhibits connective tissue breakdown, resulting in fibrosis. Exposure of mice to single-walled carbon nanotubes has demonstrated that the EMT plays a role in pulmonary fibrosis formation (Chang et al., 2012). Cerium oxide-induced lung fibrosis is associated with retention of this particle in the lungs, the induction of inflammatory and fibrotic cytokines, including TGF-β1, and the imbalance of the mediators involved in ECM remodeling (Ma et al., 2012). However, the effects of cerium oxide on fibroblast function and the involvement of EMT in the resultant particle-induced lung fibrosis have not been investigated. Therefore, the objective of the present study was to investigate cerium oxide exposure-induced functional changes of fibroblasts and the involvement of epithelial cells and EMT in pulmonary fibrosis.

2. Methods

2.1. Animal exposures

Specific pathogen-free male Sprague-Dawley (Hla:SD-CVF) rats (~250 g) were purchased from Hilltop Laboratories (Scottdale, PA). Rats were kept in cages individually ventilated with HEPA-filtered air, housed in an Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC)-approved facility, and provided food and water *ad libitum*. Animals were used after a 1 week acclimation period. Cerium oxide nanoparticles, 10 wt% in water with average primary diameter of ~20 nm, were obtained from Sigma-Aldrich (St. Louis, MO). Cerium oxide samples diluted in saline were used for animal exposures as described previously by Ma et al. (2011). Briefly, rats were anesthetized with sodium methohexital (35 mg/kg, i.p.) and placed on an inclined restraint board. Rats were exposed to 0.3 ml suspensions of cerium oxide (with final concentrations at 0.15, 0.5, 1.0, 3.5 or 7 mg/kg body weight) *via* intratracheal instillation. Saline (0.3 ml) was administered to rats as a control. The treated animals were sacrificed at different time points post-exposure as indicated in different experiments. All rats were exposed and euthanized according to a standardized experimental protocol that complied with the Guide for the Care and Use of Laboratory Animals and was approved by the National Institute for Occupational Safety and Health Animal Care and Use Committee.

2.2. Isolation of AM, collection of bronchoalveolar lavage fluid and AM cultures

Animals were given an overdose of sodium pentobarbital (0.2 g/kg, i.p.) and exsanguinated by cutting the renal artery. AM were obtained by bronchoalveolar lavage (BAL) with a Ca^{2+}, Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4) as described previously (Yang et al., 2001). Briefly, the lungs were lavaged with 6 ml Ca^{2+}, Mg^{2+}-free phosphate-buffered medium in and out twice for the first lavage, and subsequently lavaged with 8 ml of the medium 10 times or when ~ a total of 80 ml BAL fluid (BALF) were collected from each rat.

The acellular supernate from the first lavage was saved for further analysis. Cell pellets from the lavages for each animal were combined, washed, and resuspended in a HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, and 1.0 mM $CaCl_2$; pH 7.4). Cell counts and purity were measured using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer; Beckman Coulter; Fullerton, CA).

AM-enriched cells were obtained by adherence of lavaged cells to a tissue culture plate as described previously (Yang et al., 2001). After removal of nonadherent cells, AM were cultured in fresh Eagle minimum essential medium (MEM; BioWhittaker; Walkersville, MD) for an additional 24 h. AM-conditioned media were collected, centrifuged, and the supernates were saved in aliquots at −80 °C for further analysis of cytokines.

2.3. Isolation of lung fibroblasts

Fibroblasts were isolated from the lung tissue according to the method described by Reist et al. (1993). Briefly, the lung tissue was minced four times with a McIlwain tissue chopper (0.5 mm) and then suspended in 20 ml of HEPES-buffered solution, containing collagenase (0.1%), elastase (40 U/ml), DNase (0.018%), and bovine serum albumin (BSA, 0.5%). This chopped lung suspension was incubated in a shaking water bath for 30 min at 37 °C to digest the lung tissue.

After digestion, the lung tissue suspension was filtered through two layers of sterile gauze that was rinsed with MEM containing Earle's salts, glutamine (2 mM), penicillin (100 U/ml), steptomycin (100 μg/ml), and 10% heat-inactivated FCS. This medium was also used to maintain all stock cultures. The filtrate was centrifuged at 560 ×*g* for 10 min at 4 °C. The supernatant was discarded; the cells were resuspended in 20 ml of MEM plus 10% heat-inactivated FBS; and the pneumocytes plated in two 25 cm2 tissue culture flasks. The medium was changed within 24 h after plating. All fibroblast cultures were maintained in closed flasks in MEM plus 10% heat inactivated FBS. The medium was changed three times weekly. Lung fibroblasts were harvested by treating the attached cells with 0.25% trypsin in phosphate-buffered saline (PBS) at 22 °C, centrifuged and resuspended in medium for further analysis. Purity of this fibroblast preparation was nearly 100% as determined by morphological characteristics.

2.4. Analysis of α-SMA from the whole lungs

Immunoblot analysis was performed as described previously (Zhao et al., 2004). Briefly, approximately 30 mg of lung tissue was homogenized in 250 μl of ice cold RIPA lysis buffer (Santa Cruz; Santa Cruz, CA), incubated on ice for 30 min, and centrifuged at 12,000 RPM for 20 min at 4 °C. The supernate was collected and protein concentration