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Development of a repeated exposure protocol of human bronchial epithelium in vitro to study the long-term effects of atmospheric particles

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ABSTRACT

Chronic exposure to atmospheric particles is suspected of exacerbating chronic inflammatory respiratory diseases but the underlying mechanisms remain poorly understood. An experimental strategy using human bronchial epithelial cells (NHBE) known to be one of the main target cells of particles in the lung was developed to investigate the long term effects of repeated exposure to particles.

Primary cultures of NHBE cells were grown at an air–liquid interface and subjected to repeated treatments to particles. Fate of particles, pro inflammatory response and epithelial differentiation were studied during the 5 weeks following the final treatment.

Ultrastructural observations revealed the biopersistence of particles in the bronchial epithelium. The expression of cytochrome P450 1A1, was transiently induced, suggesting that organic compounds could have been metabolized. The release of GM–CSF and IL-6 (biomarkers of pro-inflammatory response), was induced by particle treatments and was maintained up to 5 weeks after treatments. The release of amphiregulin and TGFα (Growth Factor) was induced after each treatment. The number of cells expressing the mucin MUC5AC, a differentiation marker, was increased in particle-exposed epithelium.

The experimental strategy we developed is suitable for investigating in greater depth the long term effects of particles on bronchial epithelial cells repeatedly exposed to atmospheric particles in vitro.

1. Introduction

Atmospheric pollution periodically raises concern due to its impact on human health. According to the World Health Organization, atmospheric pollution is responsible for two million premature deaths every year and reducing particulate pollution from traffic could contribute to decreasing deaths by 15% in polluted cities (WHO, 2012). In urban areas, particulate pollution results mainly from anthropogenic activities such as combustion processes due to traffic, domestic heating, and industry. Such activities produce significant amounts of fine and ultrafine particles constituted of a solid carbonaceous core associated with ions, organic compounds and metals. The small size of these particles promotes their penetration to the distal part of the respiratory tract. Despite the mucociliary and alveolar clearance making it possible to remove particles from the airways and alveoli respectively, there is evidence of the biopersistence of particles in the lungs, especially for people living in polluted cities (Brauer et al., 2001).

Exposure to $PM_{2.5}$ and PM_{10} (Particulate matter with an aerodynamic diameter below 2.5 and 10 μm, respectively is associated with mortality and morbidity in respiratory and cardiovascular disorders (Analitis et al., 2006; Pope et al., 2002). PM is specifically suspected of exacerbating chronic pulmonary inflammatory diseases such as asthma or chronic obstructive pulmonary disease (COPD) (Sunyer, 2001; Ling and Van Eeden, 2009). Toxicological studies have shown that the main short-term effects of PM are lung and systemic inflammation (Samet et al., 2007) resulting from the induction of an oxidative stress in lung target tissues (Li et al., 2008). PM components (i.e. specific metals and organic compounds) contribute to the generation of reactive oxygen species

Abbreviations: DEP, diesel exhaust particle; TEM, transmission electron microscopy; NHBE, normal human bronchial epithelial; GM–CSF, colony stimulating factor; IL-6, interleukin-6; IL-1β, interleukin-1β; ALI, air liquid interface; PM, particulate matter; EGFR, epidermal growth factor receptor; TGF alpha, transforming growth factor alpha.

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(ROS) (Bonvallot et al., 2001; Baulig et al., 2003) that trigger signaling pathways involved in the expression of inflammatory mediators (Lodovici and Bigagli, 2011). The airway epithelium, as the first tissue encountered by PM, contributes to the development of inflammation by synthesizing and releasing cytokines and chemokines which attract and activate various inflammatory cells. Previous studies carried out by our laboratory have shown that the bronchial epithelium responds to PM exposure by increased release of growth factors from the family of the epidermal growth factor (EGF), such as amphiregulin and TGFα (Blanchet et al., 2004; Rumelhard et al., 2007). These growth factors are known to be involved in the differentiation and proliferation of airway epithelial cells, smooth muscle cells and fibroblasts (Lemjabbar et al., 2003; Shim et al., 2008; Wang et al., 2005). Their specific receptor (EGFR) is overexpressed in the airway epithelium of asthmatic subjects where it mediates tissue repair and airway remodeling (Puddicombe et al., 2000; Burgel and Nadel, 2008; Le Cras et al., 2011) and amphiregulin is found in the sputum of patients with asthma attacks (Enomoto et al., 2009). Remodeling features such as fibrosis, distortion of the small airways and muscle hyperplasia in association with an accumulation of particles in bronchial wall have been identified in the lung of women chronically exposed to PM (Churg et al., 2003). Other studies have shown that chronic exposure to diesel exhaust particles (DEP) triggers an overproduction of mucus (Yoshizaki et al., 2010) and a thickening of airways (Souza et al., 1998). Altogether these data suggest that chronic exposure to PM could favor airway remodeling but up-to-now the underlying mechanisms are far from being understood.

In vitro models are useful tools for investigating the direct effect of toxics on their cellular targets and many toxicological in vitro studies have provided significant advances in the understanding of the mode of action of PM. There is, however, now an urgent need to better understand their long-term effect and more relevant in vitro models have to be developed.

For this purpose we used primary cultures of human bronchial epithelial cells that can differentiate in vitro when grown at the air–liquid interface (ALI) and can be maintained under these conditions for several weeks (Million et al., 2001; Bérubé et al., 2010). We developed an experimental strategy of repeated exposure of the bronchial epithelium to PM at the beginning of the differentiation process and studied the consequences in terms of fate and effects of PM of such treatments during the five following weeks of culture during which the differentiation occurs. The fate of PM was investigated both by morphological studies and by assessing the expression of a xenobiotic metabolizing enzyme involved in the metabolism of one type of organic compound present on PM (polyaromatic hydrocarbons, PAH). The course of the pro-inflammatory response was characterized by measuring the release of different pro-inflammatory cytokines (granulocyte macrophage–colony stimulating factor (GM–CSF), interleukine-6 (IL-6) and (IL-1β) as well as the release of the amphiregulin and TFGα growth factors. Moreover, PM effects on differentiation were investigated studying MUC5AC, one of the predominant mucins produced by the human airway epithelium. DEP as well as coarse, fine and ultra-fine PM sampled at a background urban site in Paris (France) were used to develop our experimental strategy.

2. Materials and methods

2.1. Particles

Diesel exhaust particles (DEP) SRM1650 were purchased from the National Institute of Standards and Technology (Interchim, Montluçon, France). Particles were suspended at 2 mg/mL in DMEM/F12 medium (Invitrogen, Cergy-Pontoise, France) with streptomycin at 100 μg/ml (Sigma–Aldrich, Saint-Quentin Fallavier, France) and fungizone at 1 μg/ml (Sigma–Aldrich) then stored at −20 °C until use.

Ambient particles (PM) were sampled in Paris (site of background pollution in 13th district) with a 13-stage low pressure Dekati impactor (Dekati, Tampere, Finland) in order to collect particles with A.D. between 30 nm and 10 μm on polycarbonate filters. Mass size distribution was obtained using gravimetric (filter weighing) prior and after sampling as reported in details by Sciare et al. (2007). Coarse (C, $PM_{10\text{-}1,02\mu m}$), fine (F, $PM_{1,02\text{-}0,17\mu m}$) and ultra-fine (UF, $PM_{0,17\text{-}0,03\mu m}$) fractions were reconstituted by particle detachment from sampling filters using filter sonication (3 × 30 s, 60 Watts, Ultrasonic Processor, Bioblock scientific, Illkrich, France). They were then placed in DMEM/F12 medium supplemented with streptomycin and fungizone as previously described (Ramgolam et al., 2008). Stock particle suspensions were made at a concentration of 1 mg/mL and stored at −20 °C until use. Before cell treatment, particle suspensions were sonicated 3 × 10 s.

2.2. NHBE cell culture and exposure

Normal human bronchial epithelial (NHBE) cells (Lonza, Levallois–Perret, France) were grown at passage two in 75 cm^2 flasks (Corning, Illkirch, France) with bronchial epithelial basal medium (BEBM, Lonza) supplemented with 5 μg/mL insulin, 0.5 ng/mL hEGF, 0.5 μg/mL hydrocortisone, 0.5 μg/mL epinephrine, 50 μg/mL gentamycin, 50 μg/mL amphotericin B, 10 μg/mL transferrin, 6.5 ng/mL triiodothyronin, and 0.13 mg/mL bovine pituitary extract (all supplied by Lonza: singleQuot Kit Lonza). Cultures were incubated in 95% humidified air with 5% CO_2 at 37 °C. Between 70% and 80% confluency, NHBE cells were trypsinized and seeded at 10,000 cells/cm^2 on polycarbonate or polyester transwells inserts with 0.4 μm pore size diameter (Costar, Illkirch, France). A total number of 150 inserts were necessary to investigate the effects of the three particle size-fractions at three different concentrations and at different exposure times over the 6 weeks in the same experiment but the number was still insufficient to allow replicates for mRNA extraction. Basal and apical media were changed every 2–3 days. Once cells had reached confluency, the air–liquid interface (ALI) was established by removing the apical medium and retinoic acid (10^{-7} M) was added to the basal medium that was changed every 2 days. Four treatments (spaced 48 h: T1, T2, T3 and T4) of 4 h (at 1, 5 and 10 μg/cm^2 for PM and at 2, 5 and 10 μg/cm^2 for DEP) were applied on NHBE cells (Fig. 1) and no cytotoxicity was observed 48 h after the last treatment (data not shown). Basal media were collected 48 h after each treatment in order to measure cytokine release. At the end of the sequence of repeated treatments, basal media were collected every 48 h and aggregated every week to evaluate cytokine release over the five following weeks. Each week, some cultures were used either for RNA or fixed for immunocytochemistry. At day 47, the remaining cultures were prepared for electron microscopy.

2.3. Quantification of cytokines and growth factors release by NHBE cells

The basal media removed 48 h after each of the four treatments and those pooled for one week at two, three, four and five weeks after treatments (w3, w4, w5, w6, Fig. 1) were assessed for their content in GM–CSF, IL-6, IL-1β, amphiregulin and TGF α using a commercially available human enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Europe; Abingdon, United Kingdom) according to the manufacturer's recommendations. Color development was measured at 450 nm with a microplate photometer MRX 5000 (Dynex Technology, Issy les Moulineaux, France).