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Morphology changes in human lung epithelial cells after exposure to diesel exhaust micron sub particles ($PM_{1.0}$) and pollen allergens

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ABSTRACT

In the recent literature there has been an increased interest in the effects of particulate matter on the respiratory tract. The objective of this study was to use an in vitro model of type II lung epithelium (A549) to evaluate the cell ability to take up sub-micron $PM_{1.0}$ particles ($PM_{1.0}$), *Parietaria officinalis* (ALL), and $PM_{1.0}$ + ALL together.

Morphological analysis performed by Transmission Electron Microscope (TEM) showed that PM and ALL interacted with the cell surface, then penetrating into the cytoplasm. Each single treatment was able to point out a specific change in the morphology. The cells treated appear healthy and not apoptotic. The main effect was the increase of: multilamellar bodies, lysosomal enzymes, microvilli, and presence of vesicle/vacuoles containing particles.

These observations demonstrate morphological and functional alterations related to the $PM_{1.0}$ and *P. officinalis* and confirm the induction of the inflammatory response in lung cells exposed to the inhalable particles.

1. Introduction

In the recent literature there has been an increased interest in the effects of particulate matter on the respiratory tract (Anderson et al., 2011; Donaldson et al., 2005; Gehr et al., 2006; Kreyling et al., 2006; Mühlfeld et al., 2008). In particular epidemiological data outline a strict correlation between the exposure to particulate matter and several forms of pulmonary diseases (BruneKreef and Holgate, 2002; Knaapen et al., 2004; Turner et al., 2011).

Particulate matter is a mixture of organic and inorganic solid and liquid particles of different origin, size, and composition. Penetration of the tracheobronchial tract is related to particle size and the efficiency of airway defense mechanisms (Mazzarella et al., 2012).

Particulates can be classified according to their composition and size. Epidemiological study has demonstrated that only smaller size of particulate, commonly indicated as “fine particulate”, can reach the lower airways and can play a role as major contributor to adverse health effects.

It has been demonstrated that urban fine particulate can penetrate deep into the airways and induce alveolar inflammation, which is responsible for variation in blood coagulability and release of mediators favoring acute episodes of respiratory and cardiovascular diseases (Mazzarella et al., 2012, 1998; Catena et al., 1993).

Particulate matter, according to the criteria of particle size, is traditionally classified as coarse, fine and ultrafine (Gualtieri et al., 2009). Particles with a diameter ranging from 2.5 μm to 10 μm belong to coarse PM, commonly known as $PM_{10-2.5}$; particles with a diameter less than 2.5 μm belong to the fine fraction, named $PM_{2.5}$; instead particles with a diameter less than 100 nm belong to the ultrafine fraction indicated as $PM_{0.1}$ (Annesi-Maesano et al., 2007; Gualtieri et al., 2009; Mazzarella et al., 2007).

Diesel exhaust particles (DEPs) represent the greatest part of particulate (up 90%) given the increase in the number of new cars with diesel engines in industrialized countries (Diaz Sanchez et al., 1997; Mazzarella et al., 2007).

People that live in big cities are usually exposed to higher levels of vehicle emissions and several other forms of pollutants (due to

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the economic and industrial growth) that mixed with aero-allergens, such as those derived from pollen grains, help to facilitate allergic sensitization of the airways and lead to bronchial obstruction in predisposed individuals (D'Amato et al., 2010; Gilmour et al., 2006; Cazzola et al., 1999).

Pollen particles, in high environmental humidity conditions, can be subjected to osmotic shock, resulting in the release of micro-particles or paucimicronic particles that may contain allergenic proteins (Suphioglu et al., 1992; Taketomi et al., 2006).

In the same way, through physical contact with the pollen particles, diesel exhaust particles can disrupt the former, leading to the release of paucimicronic particles and transporting them by air thus facilitating their penetration of the human airways (Churg and Brauer, 1997).

The interaction among cells, pollen particles and particulate has been deeply studied and several biochemical effects have been described upon size and quantity used. However few informations are present about morphological events following these treatments.

The objective of this study was to use an in vitro model of type II lung epithelium (A549) to evaluate the cell ability to take up sub-micron $PM_{1.0}$ particles ($PM_{1.0}$), *Parietaria officinalis* (ALL), and $PM_{1.0}$ + ALL together.

In the present study we used particles with a size of the 1.0 μm or less at a concentration of 0.1 mg/mL derived from filters of diesel engine exhausts. The concentration used is 1/10 compared to that which causes chronic inflammation of the airways.

The principal interest was to investigate if type II lung epithelium cells undergo morphological changes after treatment; in particular we analyzed the morphological distribution and the quantitative incorporation using a transmission electron microscopic (TEM).

2. Materials and methods

2.1. Particulate sampling

Particulate matter was collected at the exhaust of a primary brand automobile equipped with a 2000 cm^3 turbo-diesel engine, compliant to Euro IV emission regulations. Particulate matter was obtained from Diesel exhaust particles as described previously (Mazzarella et al., 2007). Briefly, Diesel PM is sampled by filtering air-diluted diesel car exhaust fumes at temperatures not higher than 52 °C. A dilution tunnel connected with a NOVA-MMB Messtechnik PDP-CVS unit has been used to produce the air/diesel combustion gases mixture; this configuration cleverly resembles conditions at which diesel exhaust PM is released from vehicles into the atmosphere.

Furthermore, in order to obtain $PM_{1.0}$ the particulate sampling filters were conditioned, then weighted; after sampling, filters were weighed again, and 5.5 mg of DEP-PM were placed in a beaker containing HPLC-grade Et–OH (Aldrich CHROMASOLV®). The suspension so obtained was sonication-treated for 30 min on a Misonix XL-2020 device, at a frequency of 20 kHz. Most Et–OH was subsequently removed from sample using a vacuum rotary evaporator, in order to get stock samples with PM concentrated to 900 mg/mL. Aliquots so prepared were stored at 2 °C upon usage.

2.2. Epithelial cell culture

We used the A549 cell line and it was cultured as described previously (Mazzarella et al., 2007). Briefly, A549 cells were cultured in RPMI-1640 (Sigma, Poole, UK) medium supplemented with 10% fetal bovine serum (Sigma) and 10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B (Sigma), in humidified atmosphere of 5% CO_2, 95% air at 37.5 °C to confluence. At this stage, A549 monolayers were gently detached using ready prepared Trypsin solution (Sigma), cells were rinsed and pelleted; pellets were resuspended in serum-free RPMI-1640 medium only supplemented with antimicrobial agents and plated onto "Multi-G-Lass" chambers (Iwaki Asahi Techno Glass); incubation took place at 36.5 °C in humidified atmosphere containing 5% $C0_2$ for 48 h, where cells normally reached semiconfluence.

At this time (our "starting time") $PM_{1.0}$ (0.1 mg/mL) and pollen extract *P. officinalis* (0.5 DBU/mL) (Diagnostic Biological Units by Lofarma). One DBU is one hundredth of the potency of one extract inducing a wheal equal to that induced by histamine chloride 10 mg/ml, were added, separately and in combination, to cell cultures.

2.3. Transmission Electron Microscope – TEM

For the fine ultrastructural investigation, confluent cell monolayers were directly fixed on dishes with 2.5% glutaraldehyde in 0.2 M in sodium phosphate buffer (PH 7.2), and post- fixed with 1% OsO4 in the same buffer for 2 h. After repeated washings, they were dehydrated in a graded ethanol series and embedded in EPONÂ® 812. Frontal 60 nm ultrathin sections of the cell layers were cut on a LAIKA Ultracut UCT and they were routinely stained with 4% uranyl acetate for 15 min and 3% lead citrate for 20 s. The grids were observed under a Transmission Electron Microscope (TEM) LEO 912 AB.

3. Results

A549 cell line was treated with sub-micron $PM_{1.0}$ particles ($PM_{1.0}$), *P. officinalis* (ALL), and $PM_{1.0}$ + ALL together. Cells following this treatment were examined at 24, 48, and 72 h and compared with control cells.

Cells, after treatment, in culture flask were observed using an inverted microscope. A morphological comparison among the different points shows that untreated cells appeared moderately large and exhibited different kinds of shapes. Most of the cells appear separated from one another nicely and neatly with a shape often hexagonal or spindle-like. Instead treated cells appear with clearly atypical nuclei and moderately big. The cytoplasmic compartment showed granular inclusions. Furthermore we investigated the different cell treatment with Transmission Electron Microscope (TEM).

A549 cells untreated show a rounded shape, with several distributed microvilli on the cell surface. Within the cytoplasm, the nucleus has an irregular shape. Several mitochondria are visible: they have a roundish/oval shape and are uniformly distributed in the cytoplasm. Granules with a low electrodensity, probably of a lipidic type, are present. There are also numerous multilamellar bodies with a low electrodensity, but that, at higher magnification, show the presence of characteristic thin parallel lamellae (Fig. 1).

A549 cells treated with $PM_{1.0}$ at TEM observation showed a round shape. The microvilli, distributed on the cell surface are increased compared to control cells, and these allow the increase of the cell surface. In the cytoplasm, the nucleus is irregularly shaped. Mitochondria, of a round/oval shape, are numerous and equally distributed in the cytoplasm. Low electrodensity granules, probably of a lipidic nature are present in numbers similar to control cells. The cells are characterized by a cytoplasm particularly rich in multilamellar bodies compared to control cells. In addition, these multilamellar bodies show a higher electrodensity probably due to the presence of lysosomal enzymes. In the cytoplasm of cells at 72 h after treatment it is possible to highlight the presence of some vesicles containing small particles. These probably are particles that have been phagocytized/endocytosed by epithelial cells (Fig. 2).

A549 cells treated with ALL show a rounded shape, with a greater number of microvilli distributed on the cell surface compared to control cells. In the cytoplasm, the nucleus is irregularly shaped. Mitochondria are numerous of round/oval shape, equally distributed in the cytoplasm. Granules of low electrodensity, probably of lipidic kind, are present in numbers similar to control cells and to those treated with the particulate.

In these cells, it is possible to highlight a significant increase in multilamellar bodies, as compared with both the control cells and those treated with the particulate only. Even multilamellar bodies with higher electrodensity appear to be increased in number compared both to control cells and to cells treated with the particulate. There are also numerous vesicles containing small particles (probably allergen particles that have been phagocytized). These vesicles are markedly increased compared to cells treated with the particulate (Fig. 3).