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Direct and indirect air particle cytotoxicity in human alveolar epithelial cells

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ABSTRACT

Air particulate matter has been associated with adverse impact on the respiratory system leading to cytotoxic and proinflammatory effects. The biological mechanisms behind these associations may be initiated by inhaled small size particles, particle components (soluble fraction) and/or mediators released by particle-exposed cells (conditioned media).

The effect of Urban Air Particles from Buenos Aires (UAP-BA) and Residual Oil Fly Ash (ROFA) a surrogate of ambient air pollution, their Soluble Fractions (SF) and Conditioned Media (CM) on A549 lung epithelial cells was examined. After 24 h exposure to TP (10 and 100 μg/ml), SF or CM, several biological parameters were assayed on cultured A549 cells. We tested cell viability by MTT, superoxide anion (O_2^-) generation by NBT and proinflammatory cytokine (TNFα, IL-6 and IL-8) production by ELISA.

UAP-BA particles or its SF (direct effect) did not modify cell viability and generation of O_2^- for any of the doses tested. On the contrary, UAP-BA CM (indirect effect) reduced cell viability and increased both generation of O_2^- and IL-8 production. Exposure to ROFA particles, SF or ROFA CM reduced proliferation and O_2^- but, stimulated IL-8. It is worth to note that UAP-BA and ROFA depicted distinct effects on particle-exposed A549 cells implicating morphochemical dependence. These *in vitro* findings support the hypothesis that particle-induced lung inflammation and disease may involve lung-derived mediators.

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1. Introduction

Increased levels of air particulate matter (PM) have been associated with adverse effects not only on the respiratory system but on other distant systems such as the cardiovascular and nervous systems (Brito et al., 2010; Pope et al., 2009; Zanchi et al., 2010). Particularly, PM-negative effects in one of its main targets, the respiratory system, may be triggered by inhaled small size particles and/or particle components (soluble fraction). Inhaled fine (micro) or ultrafine (nano) particles and/or its Soluble Fraction (SF) may interact directly with lung cells (Nemmar et al., 2002; Oberdörster et al., 2002; Wallenborn et al., 2007), or induce an indirect response through the release of several pathophysiological mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines (Sun et al., 2008; Brook and Rajagopalan, 2009).

Airway epithelial cells play critical roles in homeostasis and host defense. These include acting as a physical barrier, removing particles via mucociliary transport, secreting components of the innate immune system, recognizing and responding to pathogen associated molecular patterns and signaling to leukocytes. Recently, the importance of controlled ROS production by non-phagocytic cells, including lung epithelial cells, in the regulation of physiological functions was suggested (Geiszt et al., 2003; van der Vliet, 2008) Therefore, the use of the human lung adenocarcinoma epithelial cell line A549 *in vitro* to study particle toxicology has been selected as one of the major cell lines able to provide valuable insights into the detailed functions and capabilities of *in vivo* airway epithelial cells from humans.

Previously, we characterized Urban Air Particles from downtown Buenos Aires (UAP-BA), a Latin American megacity, and evaluated its effect on the respiratory tract employing an *in vivo* animal

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model (Martin et al., 2007). We demonstrated that UAP-BA are ultrafine particles with no metallic traces, able to generate lung inflammation and an imbalance of the oxidative metabolism probably due to the high content of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) adsorbed to their carbon core (Martin et al., 2007, 2010). On the contrary, Residual Oil Fly Ash (ROFA), a known surrogate of ambient pollution, widely used to study biological impact in experimental animal models, proved not only to exert an inflammatory response inducing the release of a variety of pro-inflammatory cytokines (Antonini et al., 2004; Gardner et al., 2000; Gavett et al., 1999; Ghio et al., 2002) but, due to its high metal content (V, Al and Si), was able to induce the generation of ROS, which in turn could result in an imbalance of the redox metabolism (Martin et al., 2007, 2010).

In the present study we examined the effect of these two very different ambient air particles: Urban Air Particles from Buenos Aires (UAP-BA) and Residual Oil Fly Ash (ROFA), their Soluble Fraction (SF) and mediators released from particle-exposed cells (Conditioned Media, CM) on the lung epithelia cell line A549.

2. Materials and methods

2.1. Particle characterization

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) were employed to analyze particle morphology and chemical composition. For SEM observations, collected particles were coated with silver by direct current sputtering. Stub preparations were examined in a quanta SEM FEG-S50 (SEI, Oregon, USA). Chemical composition was analyzed with a Phillips SEM 505 SEM (Philips Electron Optics, NL, USA) coupled to a EDX dispersion detection unit 4100 with a silicon–lithium detector (EDAX Inc., NJ, USA) used to collect elemental spectra, specifically at energies corresponding to 1.74 and 3.7 keV, representing spectral signals for silicon and calcium, respectively.

2.2. Particle sampling

Urban Air particles from downtown Buenos Aires (UAP-BA) were collected in an area characterized by its intense vehicle traffic with a high exposure to diesel exhaust. A MiniVol™ Portable Air Sampler with 2.5 μm cut-point impactors was employed using a flow rate of 5 l min^{-1} (Baldauf et al., 2001). Teflon filters (0.8 μm pore) were placed in a clean plastic cassette during transport and storage. The filters were weighed (after moisture equilibration) before and after sampling to determine the net particulate mass gain with a microbalance (Mettler M3, weighing accuracy of 0.01 mg), using an alpha source to remove the electrostatic charge. Particle concentration results from sonicate the filters 5 times for 5 min (Astrason, Misonix) in an appropriate volume of phosphate buffer solution (PBS) 1×. This particle suspension is referred as "stock suspension".

Residual Oil Fly Ash (ROFA) from the Mystic Power Plant, CT, USA was generously provided by J. Godleski (Harvard School of Public Health, Boston, MA, USA).

2.3. Lung epithelial A549 cell culture

Human lung carcinoma cell line A549 (American Type Culture Collection, Manassas, VA) was grown and maintained in Minimal Essential Medium Eagle (MEM, Sigma–Aldrich, USA) supplemented with 10% fetal bovine serum (FCS, Internegocios, Argentina), 2 mM glutamine, and 100 IU/ml penicillin, 100 μg/ml streptomycin. According to ATCC cells were detached by trypsinization, centrifuged at 800*g* for 10 min at 4 °C, resuspended in MEM and seeded at a density of 0.8×10^6 cells/ml in 24-well culture plates at 37 °C in a humidified atmosphere with 5% CO_2. Exposure to either UAP-BA or ROFA was always performed after 24 h in culture.

(a) Total Particle (TP)

To prepare Total Particle (TP) suspensions for either UAP-BA or ROFA, aliquots from our "stock suspension" were resuspended in MEM media to the final concentrations desired (10–100 μg/ml). To disrupt possible particle aggregates or agglomerates, prior to use all suspensions were always sonicated for 10 min.

(b) Soluble Fraction (SF)

Soluble Fraction (SF) was obtained by incubating particles, either UAP-BA or ROFA (10 or 100 μg/ml), in MEM during 24 h on wells free of cells. Supernatant from UAP-BA or ROFA (10 or 100 μg/ml) were centrifuged for 10 min at 12,000*g* in order to get rid of the particles.

(c) Conditioned Media (CM)

Conditioned media were obtained by centrifugation of media from A549 cell cultures exposed to UAP-BA or ROFA (10 or 100 μg/ml) for 24 h. The media were centrifuged for 10 min at 12,000*g* in order to remove particles and cell debris.

2.4. In vitro exposure protocol

A549 cells were exposed to

(a) Total Particle (TP) from UAP-BA or ROFA (10 or 100 μg/ml): briefly, cells were incubated with the particle suspension or free-particle media (controls) for 24 h. Media were centrifuged for 10 min at 12,000*g* and supernatant aliquots were reserved and stored at −20 °C until cytokine production was evaluated. Adherent cells were assayed for MTT and NBT. Results are shown in panel A from Figs. 3–5.

(b) Soluble Fraction (SF): briefly, Total Particle (TP) from UAP-BA or ROFA (10 or 100 μg/ml) was incubated with media in cell-free wells. After incubation for 24 h, the media were collected and centrifuged to obtain the SF that were added to fresh A549 cells for 24 h. Media alone incubated in cell-free wells were used as control under the same experimental conditions. Supernatant aliquots and adherent cells were tested as before for subsequent assays. The results are shown in panel B from Figs. 3–5.

(c) Conditioned Media (CM): briefly, A549 cells were incubated with Total Particle (TP) from UAP-BA or ROFA (10 or 100 μg/ml) for 24 h. After incubation the media were collected and centrifuged to obtain the Conditioned Media (CM) that were added to fresh A549 cells for another 24 h. Conditioned media obtained from A549 cells incubated with particle free media were used as control under the same experimental conditions. The experimental design is illustrated in Fig. 1. Supernatant aliquots and adherent cells were tested as before for subsequent assays. The results are shown in panel C from Figs. 3–5.

Possible microbiological contamination was checked under light microscopy throughout the all experiment. No contaminations were observed neither for TP, SF nor CM cultures.

2.5. Spectrophotometric assays

2.5.1. Cell viability

A549 proliferation was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described elsewhere (Morgan, 1998; Molinari et al., 2003). This assay is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells. Briefly, medium from control or treated with TP, SF or CM, was removed from cell

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