in vivo *28*: 557-562 (2014)

Exposure to Submicron Particles ($PM_{1.0}$) from Diesel Exhaust and Pollen Allergens of Human Lung Epithelial Cells Induces Morphological Changes of Mitochondria Tonifilaments and Rough Endoplasmic Reticulum

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Abstract. *In recent literature, little has been said regarding the morphological changes that occur in lung cells after treatment with particles and nanoparticles. Using an in vitro model of type-II lung epithelium (A549), we studied the effects of submicron particles ($PM_{1.0}$), Parietaria officinalis (ALL), and $PM_{1.0}$ + ALL together. To date several biochemical effects have been described, instead few data exist in literature regarding morphological events following these treatments, in particular we focused on the morphological changes and distribution of mitochondria, tonifilaments and rough endoplasmic reticulum, using a transmission electron microscopic (TEM) approach. After exposure to $PM_{1.0}$ particles ($PM_{1.0}$), Parietaria officinalis as allergen, and $PM_{1.0}$ with P. officinalis, changes in the cytoplasmic area were observed, such as damage to mitochondria and morphological alterations of the tonifilaments and rough endoplasmic reticulum. The data obtained strongly support the hypothesis that cells in contact with submicron particles ($PM_{1.0}$), or P. officinalis, undergo alteration of their metabolism.*

At the basis of functional changes occuring in the cells, there are often structural and morphological changes. In the present study, drawing from our previous research experience, we treated A549 cells, derived from human alveolar cell carcinoma, with fine particulates in order to understand the morphological changes of cytoplasmic organelles involved in the control of metabolic functions of the cells (1).

Due to the presence of particulates and other components of air pollution, cities are considered areas with the major incidence of respiratory diseases, such as rhinosinusitis and bronchial asthma (2-5). The most abundant components of air pollution are nitrogen dioxide, ozone, particulate matter (PM) and aeroallergens from fungal spores or other plant-derived particles (6). In cities, diesel exhaust particles represent the greatest proportion of PM (up 90%) (7, 8). PM is a mixture of organic and inorganic solid and liquid particles of different origin, size, and composition. Particulates can be classified upon their diameter. It has been observed that submicron PM1.0 particles are usually mixed with aeroallergens, such as those derived from pollen grains; this leads to allergic sensitization of the airways and to bronchial obstruction in predisposed individuals (9-11). It has been hypothesized that urban fine particulate matter ($PM_{1.0}$) can penetrate deep into airways, inducing alveolar inflammation (6).

In the present study, we used particles with a size of 1.0 μm or less at a concentration of 0.1 mg/ml derived from filters of diesel engine exhausts. The concentration used is one-tenth of that which causes chronic inflammation of the airways.

Several biochemical effects of PM have been described, but few data are present in the literature about morphological events associated with them. We focused on the effects of diesel and allergenic PM on morphological changes and distribution of mitochondria, tonifilaments and rough endoplasmic reticulum (RER) using transmission electron microscopy (TEM).

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Key Words: Human epithelial airway model, A549 cell line, submicron PM, particles, TEM.

Materials and Methods

Particulate sampling. The preparation of the particulate samples was performed as previously described (12). Briefly, diesel PM was 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 unit (AM Wasserwerk, Schwarzenberg, Germany) was used to produce the air/diesel combustion gas mixture; this configuration cleverly resembles conditions under 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 for 24 h at constant temperature and humidity, then weighed; after sampling, filters were weighed again, and 5.5 mg of diesel exhaust PM were placed in a beaker containing HPLC-grade ethanol CHROMASOLV® (Sigma-Aldrich, Milan Italy). The suspension so obtained was sonicated for 30 min on a Misonix XL-2020 device (Misonix Incorporated, NY, USA), at a frequency of 20 kHz. Most ethanol was subsequently removed from the sample using a vacuum rotary evaporator, in order to obtain stock samples with PM concentrated to 900 mg/ml. Aliquots so prepared were stored at 2˚C before usage.

Epithelial cell culture. Treatments were performed using adenocarcinomic human alveolar basal epithelial cells (A549 cell line) cultured as described previously (12). These cells have been described to exhibit a closely matched type-II alveolar cell phenotype, and to share many properties with human primary alveolar ECs, including cytokeratin expression and growth parameters. A549s were grown in RPMI-1640 medium (Sigma, Poole, UK) supplemented with 10% fetal bovine serum and 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml amphotericin B (all Sigma), in a humidified atmosphere of 5% CO_2 and 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 then resuspended in serum-free RPMI-1640 medium only supplemented with antimicrobial agents and plated onto Multi-Glass chambers (Iwaki Asahi Techno Glass,Shizuoka, Japan); incubation took place at 36.5˚C in a humidified atmosphere containing 5% CO_2 for 48 h, where cells normally reached semiconfluence.

At this time (our 'starting time'), the prepared PM1.0 (0.1 mg/ml) and pollen extract *Parietaria officinalis* [0.5 diagnostic biological units (DBU)/ml) (Lofarma, Milan Italy)] were added, separately and in combination, to cell cultures. One DBU is one hundredth of the potency of an extract inducing a wheal equal to that induced by 10 mg/ml histamine chloride.

TEM. The experiment under TEM was performed as previously described (12). Briefly, for the fine ultrastructural investigation, confluent cell monolayers were directly fixed onto dishes with 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2), and post-fixed with 1% OsO_4 in the same buffer for 2 h. After repeated washings, they were dehydrated in a graded ethanol series and embedded in EPONÂ® 812. (Electron Microscopy Sciences, Hatfield, USA). Frontal 60 nm ultrathin sections of the cell layers were cut on a Leica ultracut UCT (Lieca Microsystems GmbH, Wetzlar, Germany) and were routinely stained with 4% uranyl acetate for 15 min and 3% lead citrate for 20 s. The grids were observed under a LEO 912 AB TEM (Carl Zeiss spa Milan, Italy).

Results

A549 cell line was treated with $PM_{1.0}$, *Parietaria officinalis*, and $PM_{1.0}$ *P. officinalis* together. Cells following this treatment were examined at 24, 48, and 72 h and compared to untreated control cells.

As previously described, untreated A549 cells exhibited a rounded shape, with several distributed microvilli on the cell surface. Within the cytoplasm, the nucleus had an irregular shape. Granules with a low electro-density, probably of a lipidic type, were present. There were also numerous multilamellar bodies with a low electro-density, that, at a higher magnification, showed the presence of characteristic thin parallel lamellae (12). Moreover, several mitochondria were visible in untreated cells and were uniformly distributed in the cytoplasm. They exhibited some cristae perpendicular to the long or short axis and an irregular or polymorphic shape. The RER and tonifilaments were neatly arranged around the nucleus (12).

In the present study, we focused on the structural and morphological changes of cytoplasmic components, such as mitochondria, tonifilaments and RER. After exposure to $PM_{1.0}$, *P. officinalis*, and PM1.0 and *P. officinalis* together, changes in the cytoplasmic area were observed, such as damage to mitochondria and morphological alterations of the tonifilaments and RER.

In A549 cells treated with $PM_{1.0}$ at TEM observation after the first 24 h, mitochondria were irregular in shape but the cristae were still visible. The RER and tonifilaments were regularly arranged close to the nucleus. At 48 h, and even more so at 72 h, mitochondria were swollen with a visible disappearance of cristae. RERs were dilated and disintegrated over time, leading to several ribosomes scattered in the cytoplasm. The tonifilaments moved over time towards the periphery of the cell, forming unshaped aggregates or scattering themselves in the cytoplasm (Figure 1).

A549 cells treated with *P. officinalis* at TEM observation after 24 h did not exhibit any significant changes in the structure and organization of mitochondria, RER and tonifilaments. At 48 h, the RER formed very long chains near the cell periphery. The tonifilaments formed large bundles and they too moved to the periphery of the cell. At 72 h, mitochondria were swollen, with a visible disappearance of cristae, and RER were dilated and disintegrated, leading to several scattered ribosomes in the cytoplasm (Figure 2).

At 24 h in A549 cells treated with $PM_{1.0}$ and *P. officinalis*, some mitochondria were irregular in shape and the cristae were not well-visible. The RER and tonifilaments were regularly arranged close to the nucleus. At 48 h, mitochondria were swollen and tended to disappear, while RER began to dilate, tending to break away from the nucleus. The tonifilaments were already not close to the

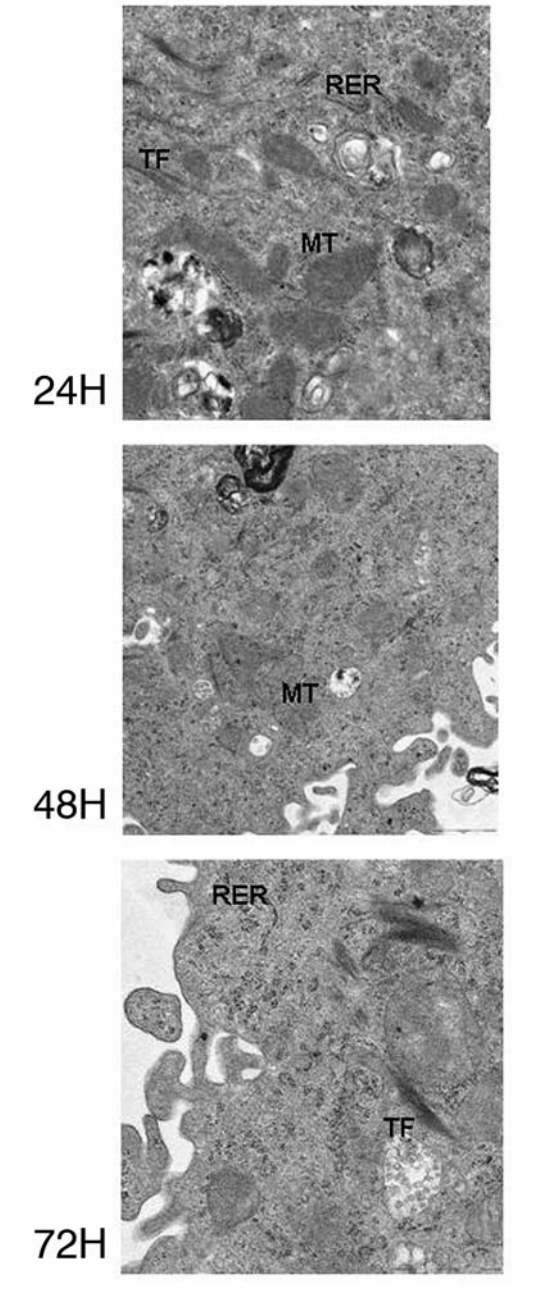

Figure 1. *A549 cells treated with $PM_{1.0}$ particles at 24, 48 and 72 h. TF: Tonifilaments; MT: mitochondria; RER: rough endoplasmic reticulum.*

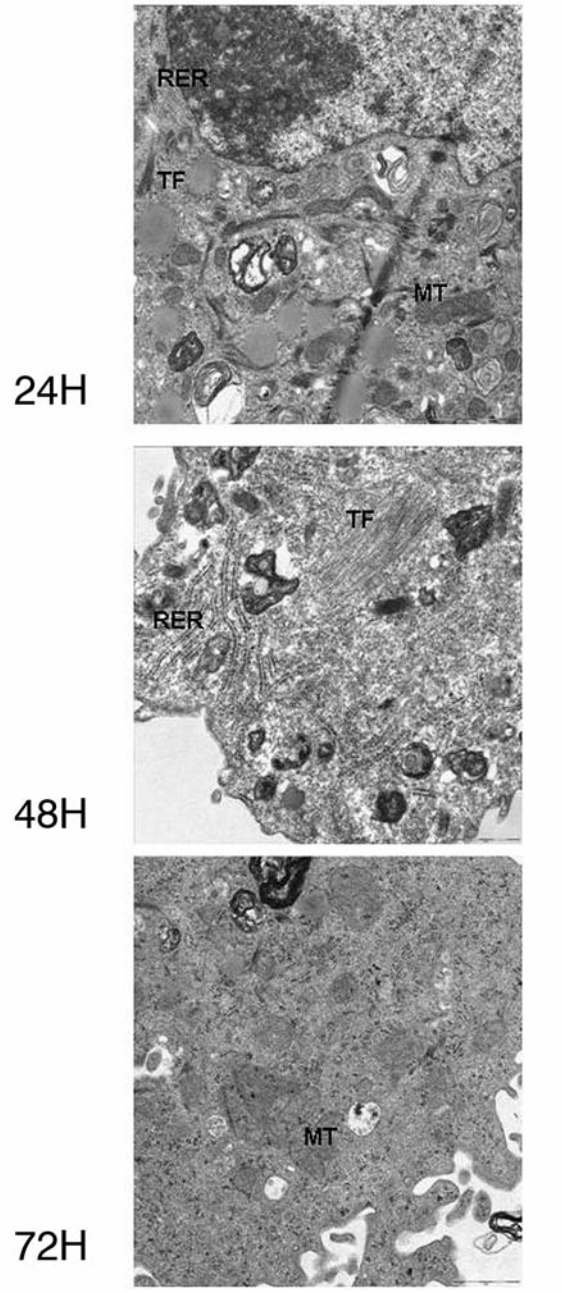

Figure 2. *A549 cells treated with Parietaria officinalis as allergen at 24, 48 and 72 h. TF: Tonifilaments; MT: mitochondria; RER: rough endoplasmic reticulum.*

nucleus and at 72 h, we recognized them toward the cell periphery in an almost spiral shape. The mitochondria were almost completely untraceable and most of the RER already at the cell periphery were dilated and disrupted, with several scattered ribosomes in the cytoplasm, although some normal ones were still distinguishable (Figure 3).

Discussion

Several articles in recent years have focused not only on the biological effects of the treatment of lung cells with particles and nanoparticles, but also on their morphological changes.

Fine urban air particulate interferes with epithelial cell physiology by reducing the proliferation rate and increasing the release of proinflammatory cytokines, tumor necrosis factor-α, interleukin (IL)-6 and -8, with a consequence of immunoregulatory and inflammatory effects on cells (13-15).

Recently, we have also described the release of inflammatory cytokines (IL4, IL5 and IL13) in response to challenge with $PM_{1.0}$ and *P. officinalis*, supporting the potential of lung epithelial cells participation in inflammation and disease (16).

In contrast regarding the morphological changes in lung cells after treatment with particles and nanoparticles, little has been described. Our research group, in a recent article, has described some changes at the morphological level in A549 cells treated with $PM_{1.0}$, *P. officinalis* and their combination under TEM observation (12). We highlighted a significant increase in multilamellar bodies compared to control cells, and we also observed an increase of lysosomal enzymes, which probably are involved in digestion of foreign bodies.

In this study, we focused on the structural and morphological changes of other cytoplasmic components such as mitochondria, tonifilaments and RER. After the exposure to $PM_{1.0}$, *P. officinalis*, and their combination, changes in the cytoplasmic area were observed, such as damage to mitochondria and morphological alterations of the tonifilaments and RER.

In detail, especially in the A549 cells exposed to both $PM_{1.0}$ and *P. officinalis*, we observed that the mitochondria were swollen and tended to disappear and at the end of treatment were almost completely untraceable. The RER began to dilate tending to break away from the nucleus. At the end of the treatment, the RER were at the cell periphery, dilated and disrupted, there were several scattered ribosomes in the cytoplasm, although some normal ones were still distinguishable. The tonifilaments formed large bundles and moved to the periphery of the cell. At the end of the treatment they were localized towards the cell periphery in an almost spiral shape.

These morphological features taken together indicate an alteration of metabolism following treatment. The presence of damaged mitochondria prevents the cell from efficiently synthesizing ATP and lipids that are both related to mitochondrial function. The same abnormal distribution of the RER indicates non-physiological protein synthesis, and disorganization of ribosomes indicates that the cell cannot synthesize secreted proteins and transmembrane proteins that are synthesized by ribosomes associated with the reticulum. The disorganization of the tonifilaments indicates a complete structural disorganization of the cell, since these participate in maintaining cellular integrity, in cell–cell adhesion and in transmitting signals.

In conclusion, the disorganization of these three structures compared to the untreated control cells indicates a state of

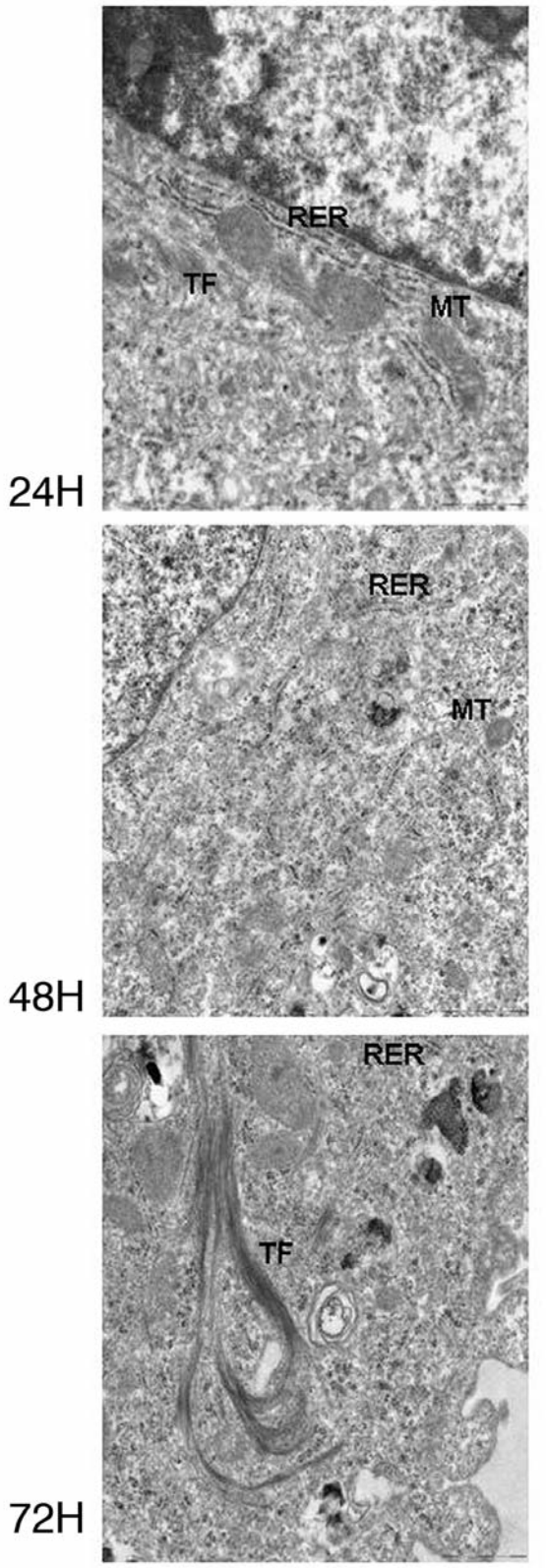

Figure 3. *A549 cells treated in combination with $PM_{1.0}$ particles and Parietaria officinalis as allergen at 24, 48 and 72 h. TF: Tonifilaments; MT: mitochondria; RER: rough endoplasmic reticulum.*

metabolic and functional suffering in treated cells. This suffering is enhanced by the treatments when given in combination and it is also time-dependent.

The data presented herein strongly support the hypothesis that cells in contact with submicron $PM_{1.0}$ particles, allergens such a *P. officinalis* (ALL), and their combination, are slowly, but definitely damaged in both structure and function. This fact would clearly be likely to have consequences on those who live in cities with air pollution.

Acknowledgements

The study was partially supported by Second University of Naples. The Authors would like to thank Dr Pia Furno and Dr. Rosalina Perna for editorial assistance.

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Received December 12, 2013
Revised April 7, 2014
Accepted April 8, 2014