Fine urban atmospheric particulate matter modulates inflammatory gene and protein expression in human bronchial epithelial cells

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1. ABSTRACT

Ambient particulate matter (PM) is known to induce inflammation in the respiratory tract of exposed subjects. The aim of the present study was to detect, in bronchial epithelial cells, candidate inflammatory genes exhibiting transcriptional modifications following urban PM2.5 exposure. Paris urban PM2.5 sampled either at a curbside or a background station in winter and in summer was tested in comparison with diesel exhaust particles (DEP) at 10 microg/cm² on human bronchial epithelial (16-HBE) cells (18 h of exposure). The gene profiling study performed using a 375 cDNA cytokine expression array highlighted the differential expression of certain genes, three of which were selected as genes of interest: the IL-1 alpha cytokine, the GRO-alpha chemokine, and amphiregulin, a ligand of the EGF receptor. Their increased expression was confirmed by RT-PCR and/or Northern blotting in bronchial epithelial cells. In the culture medium of particle-treated cultures, increased release of GRO-alpha and amphiregulin was shown. The particle component responsible for protein release varied for the two genes. The organic extract seemed to be mainly involved in amphiregulin expression and secretion, whereas both the aqueous and organic extracts induced GRO-alpha release. In conclusion, in bronchial epithelial cells, Paris PM2.5 increased mRNA and protein expression of GRO-alpha and AR involved in the chemoattraction process and bronchial remodeling, respectively.

2. INTRODUCTION

Atmospheric particulate pollution has become a major public health problem, particularly in urban areas with high traffic density. Many epidemiological studies have highlighted an increase in cardiopulmonary mortality and lung cancer associated with a high rate of fine particulate matter (PM) (1). In particular, PM collected
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through a 2.5 microm size selective inlet with an efficiency of 50 % (PM$_{2.5}$) has been implicated in increased adverse respiratory effects (2).

The main acute effect induced by PM in the respiratory tract, the PM target organ, is the development of inflammation. In vivo studies have shown that PM induces an inflammatory cell influx (3, 4) and the release of proinflammatory cytokines (5, 6). In addition, in vitro studies have shown that primary cell targets of PM in the respiratory tract, such as macrophages and epithelial cells, respond to PM exposure by the release of inflammatory cytokines such as IL-1β, TNF-α and IL-6 (7, 8), erythropoietic cytokines such as G-CSF and GM-CSF (9, 10) and chemokines such as IL-8 (5, 11).

Much research has been initiated in order to understand how particles induce these biological effects and to determine the particle components involved. Evidence has been obtained with particles rich in metals (residual oil fly ash, ROFA) or organic compounds (diesel exhaust particles, DEP), showing their ability to activate cellular signalling pathways and cytokine release by mechanisms involving reactive oxygen species (ROS). However, these particles differ in many respects from the PM found in the urban atmosphere and for which epidemiological data have been obtained. The mechanisms underlying the proinflammatory response induced by urban PM remain poorly understood. In a previous study, an attempt was made to compare the capacity to induce the release of GM-CSF and TNF-alpha by bronchial epithelial cells of four PM$_{2.5}$ samples collected at two different locations in Paris during winter and summer (12). However, those biomarkers did not succeed in distinguishing the different PM$_{2.5}$ samples, although they did exhibit chemical differences, particularly concerning their polycyclic aromatic hydrocarbons (PAH) and metal content (12). Different reasons might explain this result, such as the bioavailability of the different PM components but also the lack of sensitivity of the chosen biomarkers. Nevertheless, GM-CSF release was previously proven to be a sensitive biomarker for discriminating biological effects induced by DEP according to their organic compound content (13). The complex mixture present on PM probably triggers cross-talk between different cellular signalling pathways that do not converge towards differential GM-CSF or TNF-alpha secretion according to PM composition.

In this context, the aim of the present study was to elucidate, in bronchial epithelial cells, candidate inflammatory genes exhibiting transcriptional modifications upon urban PM$_{2.5}$ exposure. Our objective was to better characterize the inflammatory response and to find new, specific and more sensitive biomarkers of PM exposure. For this purpose, we used a macroarray containing 375 human cDNAs printed in duplicate and specific to inflammation, in order to analyze the response of the 16-HBE (human bronchial epithelial) cell line to non-cytotoxic concentrations of four PM$_{2.5}$ samples collected in Paris either at a curbside or a background station in winter and summer. Differential gene expression was confirmed by RT-PCR or northern blot. In addition, the resulting protein was analyzed by ELISA and the contribution of aqueous and organic fractions of PM$_{2.5}$ to protein secretion was assessed. A recent study highlighted the interest of toxicogenomic techniques for studying biological effects of particles (14). Until now, most of the studies using arrays to screen the effects of particles have been performed on rats (15, 16, 17), mice (18) and in vitro human macrophages with DEP or ROFA (19). But to our knowledge, up until now, no study has investigated the effects of urban PM$_{2.5}$ and their compounds on the expression of inflammatory genes in human bronchial epithelial cells.

3. MATERIALS AND METHODS

3.1. Particle collection

Urban atmospheric particulate matter of 2.5 microm aerodynamic diameter sampled with 50 % efficiency (PM$_{2.5}$), used for biological experiments, was collected at ground level with a high volume sampler machine (DA-80 Digitel, Cugy, Suisse), equipped with a PM$_{2.5}$ selective-inlet head, at two locations in Paris: (1) an urban background station at Vitry-sur-Seine, a suburb of Paris; and (2) a curbside station at Porte d’Auteuil bordering a large highway which is a ring road of Paris. The machine operated at the flow rate of 30 m$^3$/h and particles were recovered on 150 mm diameter nitrocellulose filters (HAWP, Millipore, Saint-Quentin-en-Yvelines, France). Particles were detached from filters as already described (12). Their polyaromatic hydrocarbon and metal content have been previously published (12).

PM$_{2.5}$ were compared with DEP standard reference materiel 1650a purchased from the National Institute of Standards and Technology (Gaithersburg, MD,USA). All particles were used at 10 microg/cm$^2$ (50 microg/mL) from stock solution of 2 mg/mL in 0.04 % dipalmityl phosphatidyl lecithin (DPL, Sigma, Saint-Quentin-Fallavier, France) water solution. DPL is a surfactant component which enables maintaining particles in suspension. Negative controls were done using DPL.

Native PM$_{2.5}$ were also compared to their respective organic and aqueous extracts. Organic extracts were obtained after extraction of PM$_{2.5}$ by dichloromethane with an accelerated solvent extractor ( Dionex ASE 200, Sunnyvale, CA). The obtained extract was dried, then redissolved in dimethyl sulfoxide (DMSO, Sigma). Organic extracts were used at the concentration at which they are found on particles. This value was calculated according to the soluble organic fraction (SOF) determined for each particle sample (12).

The aqueous extract containing soluble components was obtained after washing of PM$_{2.5}$ with sterile ultrapure water. After two centrifugations at 12,000 g, the supernatants were filtered on a 0.2microm filter to eliminate all particles. The aqueous fraction was used at the same volume used to treat cells at 10 microg/ cm$^2$ from the 2 mg/mL stock solution. In some experiments, soluble metals present in PM aqueous extracts were depleted by filtering on chelating ion exchange resin columns according
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to the manufacturer’s procedure (Chelex 100, Bio-Rad, Marnes-La-Coquette, France).

3.2. Chemicals and reagents
All chemicals were purchased from Sigma except when otherwise specified.

3.3. Cell culture and treatment
Dr. D. C. Gruenert (20) (Colchester, VT, USA) kindly provided the human bronchial epithelial cell subclone 16-HBE14o-. This transformed cell line was cultured in DMEM/F12 culture medium supplemented with penicillin (100 U/mL), streptomycin (100 microg/mL), glutamine (1%), fungizone (0.125 microg/mL, Invitrogen, Cergy-Pontoise, France) and UltroserG (UG) (2%, Bio Sepra, Cergy-Saint-Christophe, France). Cells were cultured at 20,000 cells/cm² on collagen (type I, 4 microg/cm²)-coated 75 cm² flasks (Costar, Cambridge, MA, USA) for RNA extraction and coated 6-well plates (Costar, Cambridge, MA, USA) for cytokine release experiments. At the time of treatment, UG was not added to DMEM/F12. Cultures were incubated in humidified 95% air with 5% CO₂ at 37°C.

3.4. RNA isolation
Total RNA were isolated from subconfluent 16-HBE cells cultured in 75 cm² flasks, using Tri Reagent™ (Sigma-Aldrich, Saint-Quentin-Fallavier, France) according to the manufacturer’s instructions. RNA quantitation was realized by measuring the absorbance at 260 nm and 280 nm of an appropriate dilution in an Ultraspec 2100 Pro spectrophotometer, (Amersham Biosciences, Orsay, France); RNA purity was determined by calculating the 260/280 ratio, and integrity was checked by agarose gel electrophoresis. mRNA (poly (A) + RNA) was purified using the Dynabead mRNA purification kit (Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions.

3.5. Reverse transcription and labeling for array hybridization and post-hybridization processes
We used a human cytokine array from R&D Systems (Abingdon, UK) containing 375 different genes specific to inflammation, printed in duplicate on a nylon membrane and using the manufacturer’s instructions. Human cytokine-specific labeling primers were annealed to the manufacturer’s procedure (Chelex 100, Bio-Rad, Marnes-La-Coquette, France) containing 375 different genes specific to inflammation, printed in duplicate on a nylon membrane and using the manufacturer’s instructions. Human cytokine-specific labeling primers were annealed to the manufacturer’s procedure (Chelex 100, Bio-Rad, Marnes-La-Coquette, France) according to the manufacturer’s instructions. RNA quantitation was realized by measuring the absorbance at 260 nm and 280 nm of an appropriate dilution in an Ultraspec 2100 Pro spectrophotometer, (Amersham Biosciences, Orsay, France); RNA purity was determined by calculating the 260/280 ratio, and integrity was checked by agarose gel electrophoresis. mRNA (poly (A) + RNA) was purified using the Dynabead mRNA purification kit (Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions.

3.6. Signal detection
After exposure, the phosphor screen was scanned by the Storm 860 imaging system (Amersham Biosciences, Orsay, France) and image analysis was performed with ArrayVision™ software (Imaging Research Inc., St. Catherine, Ontario, Canada).

3.7. Validation of hybridization signals and data analysis
The average intensity (i) of the duplicate spots of each cDNA was calculated and the hybridization signal was validated if the standard deviation was less than 10%. Values were normalized to the average value of all printed cDNA. The general background of the experiment was the mean of the positions without cDNA. The effect of treatment on gene expression was considered relevant if the ratio (treated)/i(control) was higher than 1.70 (overexpression) or lower than 0.59 (downregulation) and if at least one of the 2 considered signal intensities (treated or control) was stronger than the general background of the experiment. The latter criterium was included in order to avoid considering differentially expressed genes whose signal was below the background. The expression of genes positively or negatively regulated by treatment described in this study was verified by RT-PCR and/or northern blot.

3.8. RT-PCR
2 microg of total RNA were reverse-transcribed into cDNA in the presence of 200 U of M-MuLV reverse transcriptase RNase H⁺(Finnzyme Oy, Espoo, Finland), 500 ng of oligo-dT primer (Roche Applied Sciences, Meylan, France) and 40 U of RNase inhibitor (Promega, Charbonniere, France) for 1 h at 42°C in a 20 microl reaction volume. The RT reaction volume was completed to 50 microl with water. For semiquantitative experiments, 1/20th of the RT reaction was PCR-amplified in a 25 microl reaction containing 1 x PCR buffer, 1.5 mM MgCl₂, 200 microM each dATP, dTTP, dGTP, dCTP (Fermentas Life Science, Burlington, Ontario), 0.5 microM forward and reverse primers (Table 1, Operon Biotechnologies GmbH, Cologne, Germany) and 0.5 U DyNAzyme™II DNA polymerase (Finnzyme Oy, Espoo, Finland) in a PTC-100 thermocycler (MJ Research, Watertown, MA). The PCR reaction conditions were as follows: 94°C, 2 min 1 cycle; 94°C, 30 s ; 58°C, 30 s; 72°C, 1 min n cycles (Table 1); and 72°C, 5 min 1 cycle. Amplified products were then purified using the Dynabead mRNA purification kit (Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions.

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Table 1. primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>N: number of cycles</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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</thead>
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<tr>
<td>AR</td>
<td>25</td>
<td>5'-TGG TGC TGT CGC TCT TGA TA-3'</td>
<td>5'-ACA GCA ACA GCT GTG AGG AT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>21</td>
<td>5'-ACC ACA GTC CAT GCC ATC AC-3'</td>
<td>5'-TCC ACC ACC CTG TGT CTC TA-3'</td>
</tr>
<tr>
<td>GRO-alpha</td>
<td>27</td>
<td>5'-ATG GCC CGG GCC GCT CTC-3'</td>
<td>5'-GTG GGA TTT GTC ACT GTT-3'</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>26</td>
<td>5'-AGA TGA AGA CCA ACC AGT GC-3'</td>
<td>5'-CAT AGT CAG TAC CTC TGG TC-3'</td>
</tr>
</tbody>
</table>

Table 2. Results of macro-array experiment realized with Diesel Exhaust Particles – exposed bronchial epithelial cells versus control cells

<table>
<thead>
<tr>
<th>IDPL</th>
<th>IDEP</th>
<th>IDEP/DPL</th>
<th>Genes</th>
<th>Gene family</th>
<th>Genbank access number</th>
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<tbody>
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<td>15.5</td>
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<td>0.5</td>
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<td>Interleukin</td>
<td>M28983</td>
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<tr>
<td>0.84</td>
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<td>IGF-BP5</td>
<td>Binding protein</td>
<td>M65062</td>
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Table 3. Results of macro-array experiment realized with Winter Urban Background PM2.5 – exposed bronchial epithelial cells versus control cells

<table>
<thead>
<tr>
<th>IDPL</th>
<th>IWUB</th>
<th>IWUB/DPL</th>
<th>Genes</th>
<th>Gene family</th>
<th>Genbank access number</th>
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<tbody>
<tr>
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<td>4.3</td>
<td>Amphiregulin</td>
<td>EGF family</td>
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</tr>
<tr>
<td>0.8</td>
<td>3.4</td>
<td>4</td>
<td>IL-1alpha</td>
<td>Interleukin</td>
<td>M28983</td>
</tr>
<tr>
<td>0.4</td>
<td>0.9</td>
<td>2.5</td>
<td>G-CSF</td>
<td>Cytokine</td>
<td>X03438</td>
</tr>
<tr>
<td>0.3</td>
<td>0.7</td>
<td>2.3</td>
<td>IL-1beta</td>
<td>Interleukin</td>
<td>M15330</td>
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<tr>
<td>0.9</td>
<td>2</td>
<td>2.2</td>
<td>Betacellulin</td>
<td>EGF family</td>
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<tr>
<td>0.4</td>
<td>0.8</td>
<td>1.9</td>
<td>VCAM-1</td>
<td>Adhesion molecule</td>
<td>X59381</td>
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<td>1.2</td>
<td>2.3</td>
<td>1.9</td>
<td>TNF-R1</td>
<td>Cytokine receptor</td>
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<tr>
<td>0.2</td>
<td>0.3</td>
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<td>IL-8</td>
<td>Chemokine</td>
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<tr>
<td>1.70</td>
<td>1.00</td>
<td>0.59</td>
<td>TIMP-2</td>
<td>Protease</td>
<td>M32304</td>
</tr>
</tbody>
</table>

separated by 1 microg/mL ethidium bromide 2% agarose gel electrophoresis and visualized by 312 nm UV exposure.

3.9. Northern blotting

20 microg of total RNA were denatured at 65°C for 10 min in a solution containing 50% formamide, 6% formaldehyde and 1X MOPS at pH 7 and chilled on ice. RNA was separated by size by denaturing gel electrophoresis (1.2% agarose, 1X MOPS, 7% formaldehyde, 0.25 microg/mL ethidium bromide) at 15 mA for 12 h and transferred by capillarity onto an Hybond™-N+ membrane (GE healthcare, Orsay, France) for 18 h in 20X SSC solution (3 M NaCl and 0.3 M sodium citrate at pH 7). Then, RNA was fixed on membranes by heating at 80°C for 2 h.

Radiolabeled probes were generated by Rediprime™ II random priming (GE healthcare) according to the manufacturer’s protocol. Membrane prehybridization, hybridization and washes were performed in roller bottles in a hybridization oven using Rapid-Hyb™ buffer (GE healthcare) recommendations. The membranes were air-dried for 5 min, wrapped in clear plastic wrap, subjected to a phosphor screen for 2 days and development was carried out with a Storm 860 imaging system (Amersham Biosciences, Orsay, France). Signals were quantified with Bio-1D software (Vilber Lourmat, Marne-La-Vallee, France).

3.10. Cytokine assay

Subconfluent cultures were exposed for 24 h to particles. Culture supernatants were recovered and frozen at −80°C until use. The concentrations of amphiregulin (AR) and growth-related oncogen-alpha (GRO-alpha) released into the culture supernatants were measured with human AR and GRO-alpha Duo-set ELISA respectively (R&D Systems). Color development was measured at 450 nm with an MRX 5000 microplate photometer (Dynex Technologies, Issy-les-Moulineaux, France).

3.11. Statistical analysis

All data were expressed as the means ± SD of three cultures for in vitro studies. Means were compared by analysis of variance. The equal variance test was significant with alpha = 0.05 and all pairwise multiple comparisons were made with the Student Newman-Keuls method (SPSS software).

4. RESULTS

4.1. Macroarray results

The exposure of 16-HBE cells for 18 h to 10 microg/cm² of DEP, winter urban background, summer urban background, winter curb side and summer curbside particles versus DPL caused 3, 10, 15, 13 and 3 significant changes in gene expression, respectively, according to the relevant ratios considered (Tables 2-6).

The most striking changes induced by DEP treatment (Table 2) concerned the upregulation of 2 genes: amphiregulin (AR), an EGF receptor ligand that exhibits highly constitutive expression further increased by DEP treatment (2.3-fold induction) and interleukin 1 alpha (IL-1 alpha), a cytokine well known to participate in the proinflammatory process, showing a 2.5-fold induction.

With winter urban background particles (WUB) (Table 3), AR and IL-1 alpha were again the most strongly upregulated genes (4.3- and 4.0-fold induction respectively) in addition to other differentially expressed genes with an induction factor exceeding 2, such as granulocyte colony-stimulating factor (G-CSF), IL-1 beta and betacellulin (BTC), another member of the EGF family.
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Table 4. Results of macro-array experiment realized with Summer Urban Background PM$_{2.5}$ – exposed bronchial epithelial cells versus control cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene family</th>
<th>Genbank access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 alpha</td>
<td>Cytokine</td>
<td>M28983</td>
</tr>
<tr>
<td>IL-10</td>
<td>Cytokine</td>
<td>J03561</td>
</tr>
<tr>
<td>Amphiregulin</td>
<td>Cytokine</td>
<td>M36821</td>
</tr>
<tr>
<td>IGF-BP5</td>
<td>Binding protein</td>
<td>M57627</td>
</tr>
<tr>
<td>GRO- alpha</td>
<td>Chemokine</td>
<td>M25280</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>Chemokine</td>
<td>M06506</td>
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<td>GRO-gamma</td>
<td>Chemokine</td>
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<td>IL-10</td>
<td>Interleukin</td>
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Table 5. Results of macro-array experiment realized with Winter Curbside PM$_{2.5}$ – exposed bronchial epithelial cells versus control cells.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene family</th>
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</thead>
<tbody>
<tr>
<td>IL-1 alpha</td>
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<td>M28983</td>
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<tr>
<td>IL-1 alpha</td>
<td>Chemokine</td>
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<tr>
<td>IGF-BP5</td>
<td>Binding protein</td>
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<td>GRO- alpha</td>
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</tr>
<tr>
<td>IL-1 alpha</td>
<td>Chemokine</td>
<td>M06821</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>Chemokine</td>
<td>M06506</td>
</tr>
</tbody>
</table>

Table 6. Results of macro-array experiment realized with Summer Curbside PM$_{2.5}$ – exposed bronchial epithelial cells versus control cells.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene family</th>
<th>Genbank access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 alpha</td>
<td>Cytokine</td>
<td>M28983</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>Chemokine</td>
<td>J03561</td>
</tr>
<tr>
<td>IGF-BP5</td>
<td>Binding protein</td>
<td>M57627</td>
</tr>
<tr>
<td>GRO- alpha</td>
<td>Chemokine</td>
<td>M25280</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin</td>
<td>M57627</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>Interleukin</td>
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<td>IL-1 alpha</td>
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<td>IL-1 alpha</td>
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<td>IL-1 alpha</td>
<td>Chemokine</td>
<td>M06821</td>
</tr>
</tbody>
</table>

Summer urban background particles (SUB) (Table 4) gave a very different expression profile in which genes were mainly downregulated (10 genes were involved), and upregulated genes differed from those previously identified with DEP and WUB (TIMP-2, CCR-3, SMDF, neuropilin-1 and L-selectin).

With winter curbside particles (WC) (Table 5), the most strongly upregulated genes were IL-1 alpha and GRO-alpha (3.7- and 2.3-fold induction respectively) in addition to other differentially expressed genes, but with an induction factor lower than 2, among them AR and BTC. One gene, BLC/BCA-1, was clearly downregulated.

Finally, with summer curbside particles (SC) (Table 6), only 3 genes appeared to be upregulated: AR, IL-1 alpha and BTC, with AR and IL-1 alpha exhibiting the highest induction factor (2.7 and 2.0 respectively).

4.2. Functional classification

A functional classification has been constituted for genes modulated for at least one kind of PM$_{2.5}$. Six families were created: interleukins and interleukin receptors (Figure 1A), cytokines and cytokine receptors (Figure 1B), chemokines and chemokine receptors (Figure 1C), the EGF family (Figure 1D), adhesion molecules/binding proteins (Figure 1E) and other genes (Figure 1D). Altogether, these data reveal that modulation of inflammatory genes in bronchial epithelial cells in response to particle stress mainly involves overexpression of a subset of genes. AR and IL-1 alpha are among the most frequently and most substantially upregulated genes by the different particle samples.

4.3. Confirmation by RT-PCR and/or northern blot of PM-induced expression of genes of interest

The upregulation of IL-1 alpha and its differential expression according to PM$_{2.5}$ samples observed with the macroarray was confirmed by northern blot with RNA extracted from cells treated for 18 h (Figure 2A). The highest induction effects were again observed with the PM$_{2.5}$ winter samples, but they were also observed with summer urban background particles (SUB), whereas this was not the case with the macroarray. In addition, a kinetic study carried out by RT-PCR and northern blot with DEP and the winter urban background samples showed that increased IL-1 alpha expression occurred from 6 h and was maintained up to 30 h (Figure 2B and 2C). The differential expression revealed by the macroarray was also confirmed by northern blot for AR (Figure 3A). For AR, early (within 6 h) and sustained (to 30 h) overexpression in DEP and winter urban background PM$_{2.5}$-treated epithelial cells was...
Expression profiling of urban particles

Figure 1. Functional classification of particle-modulated genes. Five families were created: A. Interleukin, B. Cytokines and receptors, C. Chemokines and receptors, D. EGF family, E. Adhesion molecules, F. Other genes. 1st black bar: diesel exhaust particles, 2nd white bar: winter urban background PM$_{2.5}$, 3rd high grey bar: summer urban background PM$_{2.5}$, 4th low grey bar: winter curbside PM$_{2.5}$, 5th medium grey bar: summer curbside PM$_{2.5}$.

already shown in our previous study (21). Moreover AR overexpression is observed with native summer curbside particles (SC) and their respective organic extract but not with its respective aqueous extract (Figure 3B). GRO-alpha gene upregulation observed for winter curbside PM$_{2.5}$ was confirmed by northern blot, in addition to its occurrence with other PM$_{2.5}$ samples not identified by the macroarray approach (Figure 4). Surprisingly, the differential expression of some genes (IGF-BP5, TIMP-2, BTC) in response to several PM samples has not been confirmed by RT-PCR and northern blot experiments (data not shown).

4.4. In vitro inflammatory mediator secretion

All native PM$_{2.5}$ induced similar AR secretion by bronchial epithelial cells, but this release was significantly greater (WB: 93%; SB: 78%; WC: 103%; and SC: 89%) than the release induced by DEP (46%) (Figure 5A). Aqueous extracts of PM$_{2.5}$ induced slight AR secretion for winter PM$_{2.5}$, but were without effect for summer PM$_{2.5}$ (Figure 5A). Moreover, this aqueous extract-induced AR secretion was decreased after metal removal from the aqueous extract by Chelex filtration over a Chelex column (Figure 5B). In contrast, organic extracts induced AR release which was similar to that induced by native particles (Figure 5A). The coexposure of epithelial cells to both the aqueous and organic extracts induced a slight but significant potentiation of the organic extract effect.

Native particles induced a similar increase in GRO-alpha chemokine release after 24 h of treatment (Figure 6A). This increase was low but statistically significant. The aqueous extract generally induced more substantial GRO-alpha release than native particles. However, higher GRO-alpha release was induced by the organic extracts (Figure 6A). In addition, metal removal in aqueous extract by Chelex filtration reduced GRO-alpha release (Figure 6B). Finally, DEP had no effect on GRO-alpha release (Figure 6A). The combination of the aqueous extract with the organic extract of WUB induced a reduction in GRO-alpha release compared to each extract, but which was similar to the respective native particles (Figure 6B).

5. DISCUSSION

For all tested PM$_{2.5}$, this gene profiling study carried out with PM$_{2.5}$ pointed to upregulation, in bronchial epithelial cells, of a gene, amphiregulin, a ligand of the EGF receptor that we had already described in the context of particle exposure (21), as well as upregulation of interleukin genes such as IL-1 alpha and chemokine genes such as GRO-alpha. Such increased gene expression also led to an increase in the corresponding AR and GRO-alpha proteins. Interestingly, the particle fraction responsible for protein release varied for both genes. The organic fraction seemed to be mainly involved in amphiregulin release, whereas both the aqueous and organic fractions were implicated in GRO-alpha release.

In order to evaluate the Paris PM$_{2.5}$ ability to elicit inflammation, a preliminary experiment was performed by intratracheal instillation in rats. It was observed an increase in the cell number in the bronchoalveolar lavage fluid, especially that of neutrophil
Expression profiling of urban particles

Figure 2. Expression of interleukin-1-alpha in 16-HBE cells. A- Treated with the four PM$_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm$^2$. Interleukin-1-alpha expression was analyzed by northern blot and correlated with the GAPDH housekeeping gene expression by densitometric analysis. B- and C-. Treated with Winter Urban Background PM$_{2.5}$ at 10 microg/cm$^2$ from 6 h to 30 h: the time course study of interleukin-1-alpha expression was analyzed by RT-PCR (B) and northern blot (C). All treatments were conducted in triplicate and the results were verified twice.

cells and macrophages that release GRO-alpha chemokine (data not shown). In this context, and in addition to the PM$_{2.5}$-induced GM-CSF secretion previously shown (12), it was scientifically relevant to use a macroarray specific to inflammatory genes with these PM$_{2.5}$. PM$_{2.5}$ was compared with DEP, for which the mechanisms of action have been thoroughly investigated, and particularly the role of organic compounds in the induction of pro-inflammatory cytokine release (22, 23, 24, 25). PM$_{2.5}$ and DEP effects were tested on the transformed 16HBE cell line that we had previously shown to respond to DEP exposure similarly to normal cells (13). Furthermore airway epithelial cells are with macrophages, the first cells encountering particles and their proinflammatory response characterized by the release of cytokines, chemokines and growth factors is likely capable to exacerbate inflammatory associated-lung diseases (e.g. asthma, COPD, airway remodeling...).

Initially, the macroarray technique was used to screen the inflammatory genes most frequently and most strongly modulated by the different PM$_{2.5}$ samples. They were further considered as candidate genes depending on confirmation of their modulated expression by RT-PCR or northern blot experiments, and their biological relevance.
Expression profiling of urban particles

Figure 3. Confirmation of AR expression in 16-HBE cells. A- treated with the four PM$_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm$^2$ for 18 h by northern blot. B- treated with native summer curbside particles (SCn) at 10 microg/cm$^2$ and their respective aqueous (SCa) and organic extract (SCo) by RT-PCR. AR mRNA expression was correlated with the GAPDH housekeeping gene expression. All treatments were conducted in triplicate and the results were verified twice.

From these considerations, we focused on 3 upregulated genes (AR, IL-1alpha and GRO-alpha). With the exception of the PM-SUB sample, all PM samples and DEP systematically and drastically induced the expression of both the AR and IL-1 alpha genes that were chosen for further investigations. In addition, GRO-alpha involved in neutrophil chemotraction was selected due to its relevance based on in vivo data (data not shown, 26).

AR belongs to the EGF receptor ligand family. We have described for the first time the transient upregulation of this gene in relation to particle exposure (21), and we now confirm that this upregulation is a common event in PM$_{2.5}$ mechanisms. In addition, it is a specific event, as it does not occur with carbon black or titanium dioxide particles (21). AR is probably involved in regulation of GM-CSF release as previously described (21), but could also be implicated in bronchial remodeling by an autocrine effect on epithelial cells and paracrine effects on surrounding cells, i.e. fibroblasts and airway smooth muscle cells. Indeed, AR has a known mitogenic effect on bronchial epithelial cells (27) as well as on vascular smooth muscle cells (28, 29). Airway remodeling associated with the presence of particles has been described in women living in a highly polluted city (30, 31). AR expression is differential according to PM$_{2.5}$ samples, but these differences are no longer evident when considering the protein secretion level. These results could be explained by post-translation mechanisms, since AR is first expressed as a membrane precursor further matured after cleavage by tumor-necrosis-factor-alpha-converting enzyme (TACE) (32). In addition to an effect on AR gene expression, PM$_{2.5}$ could also act on TACE activity, as already suggested in the mechanism of action of cigarette smoke (33).

IL-1 alpha is a proinflammatory cytokine controlling the expression of chemokines and adhesion molecules. Moreover, it is involved in the stabilization and lengthening of the half-life of GM-CSF mRNA (34), a cytokine we had previously shown to be induced by DEP and PM$_{2.5}$ (12, 24). Northern blot experiments confirmed the macroarray results for 4 out of 5 tested PM$_{2.5}$ samples and revealed that IL-1 alpha upregulation is also induced by SUB, in contrast to macroarray data. IL-1 alpha overexpression is maintained from 6 h to 30 h.

GRO-alpha is a C-X-C chemokine with neutrophil-attractant properties. GRO-alpha levels are increased in sputum from patients with COPD (35). In addition, this chemokine has already been shown to be upregulated in vivo by DEP (26), but not by particulate matter. As for IL-1 alpha, the expression profiling obtained by macroarray was confirmed by northern blot, for which higher induction factors were observed. In contrast, the protein secretion profile according to PM$_{2.5}$ samples does not follow the mRNA expression profile, as we had already observed with AR expression and secretion. A slight but significant increase in GRO-alpha release is observed for all PM$_{2.5}$ samples, but not with DEP. GRO-alpha is known to be regulated by the NF-kappaB transcription factor, and our previous investigations have shown that DEP activates NF-kappaB in 16-HBE cells through its organic component (24). Altogether, these data suggest that different cellular pathways leading to GRO-alpha secretion are triggered by PM$_{2.5}$ and DEP.

Experiments with DEP and ROFA have evidenced the involvement of organic compounds and
Expression profiling of urban particles

Figure 4. Confirmation by northern blot of GRO-alpha expression in 16-HBE cells treated with the four PM$_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm$^2$ for 18 h. GRO-alpha mRNA expression was correlated with the GAPDH housekeeping gene expression. All treatments were conducted in triplicate and the results were verified twice.

Figure 5. Release of AR by 16-HBE cells exposed for 24 h in triplicate to: A- 10 microg/cm$^2$ of the four PM$_{2.5}$ and their respective aqueous and organic extracts or of DEP. B- a combination of the aqueous and organic extracts or the aqueous extract after metal removal. Values are means ± SD (n=3). * : different from control, ^ : different from respective native particles, ° : different from winter urban background aqueous extract. All these experiments were repeated 3 times.
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- Native particles
- Aqueous extracts
- Aqueous + organic extracts
- Organic extracts
- Aqueous extracts - metals

Figure 6. Release of GRO-alpha by 16-HBE cells exposed for 24 h in triplicate to: A- 10 microg/cm² of the four PM$_{2.5}$ and their respective aqueous and organic extracts or of DEP. B- a combination of the aqueous and organic extracts or the aqueous extract after metal removal. Values are means ± SD (n=3). * : different from control, ^ : different from respective native particles, ° : different from winter urban background aqueous extract. All these experiments were repeated 3 times.

metals, respectively, in the proinflammatory response of airway epithelial cells, likely due to their ability to elicit oxidative stress (22, 24, 5). Concerning AR, analysis of results obtained with particle fractions (aqueous and organic fractions) reveals a major effect of organic extract in AR expression and release that was similar to or slightly greater than with native PM. In contrast, both the aqueous extract and the organic extract induced GRO-alpha release. In addition GRO-alpha release induced by PM aqueous extract is abrogated when metals are removed. Interestingly, this is the first time we observe the involvement of metals in the induction of a proinflammatory gene by PM$_{2.5}$ in 16-HBE cells.

As GRO-alpha release induced by aqueous and organic extracts alone is stronger than GRO-alpha release induced by native particles, it could be due to interactions between metals and organic compounds or metals and organic compounds metabolizing enzymes. Indeed, we had previously shown that DEP induces cytochrome P450 1A1 (CYP 1A1) gene expression and enzymatic activity, suggesting that PAH desorbs from particles (22). It is known that metals such as copper inhibit CYP 1A1 activity (36). This hypothesis was investigated by studying the biological effects of a combination of both aqueous and organic extracts. A reduction in the biological effect induced by a single extract was observed at a level similar to that obtained with native particles, suggesting interactions between metallic and organic compounds. The higher effect induced by aqueous and organic extracts in comparison with native particles could be explained by bioavailability differences. Experiments using electron spin resonance have shown that metals are solubilized in the aqueous extracts, since they induce production of hydroxyl radicals in the presence of H$_2$O$_2$ (personal communication). Concerning organic compounds, especially PAH, they exhibit differences in their bioavailability according to PM samples (personal communication).

Finally, macroarray experiments provide useful tools for simultaneously studying a large variety of genes involved in the inflammatory process and in detecting those specifically overexpressed by PM$_{2.5}$ exposure. It provides potential explanations for in vivo observations, particularly
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in the chemotaxation process and bronchial remodeling induced by particulate matter, which warrant further investigations.

6. ACKNOWLEDGMENTS

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