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# Activation of different pathways of apoptosis by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture

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**Abstract**

Epidemiological studies have associated the increase of respiratory and cardiovascular mortality and morbidity with high levels of air pollution particulate matter (PM). However, the underlying mechanisms of actions by which PM induce adverse health effects are still unclear. We have recently undertaken an extensive investigation of the adverse health effects of air pollution $PM_{2.5}$, and shown that in vitro short-term exposure to $PM_{2.5}$ induced oxidative stress and inflammation in human lung epithelial cells (L132). Hence, it was convenient to complete the physical and chemical characterization of PM and to investigate whether in vitro short-term exposure to PM could be imply in the activation of apoptosis. Accordingly, we found that 92.15% of PM were equal or smaller than 2.5 μm and their specific surface area was 1 $m^2$/g. Inorganic (i.e. Fe, Al, Ca, Na, K, Mg, Pb, etc.) and organic (i.e. polycyclic aromatic hydrocarbons) chemicals were found in PM, suggesting that much of them derived from wind-borne dust from the industrial complex and the heavy motor vehicle traffic. In other respects, we showed that PM exposure induced apoptosis by activating not only the tumor necrosis factor-alpha (TNF-α)-induced pathway (i.e. TNF-α secretion, caspase-8 and -3 activation), but also the mitochondrial pathway (i.e. 8-hydroxy-2′-desoxyguanosine formation, cytochrome *c* release from mitochondria, caspase-9 and -3 activation). Moreover, changes in the transcription rates of p53, bcl-2, and bax genes, on the one hand, and DNA fragmentation, on the other hand, were reported in PM-exposed proliferating L132 cells, revealing the occurrence of apoptotic events. Taken together, these findings suggested that in vitro short-term exposure to $PM_{2.5}$ induced apoptosis in L132 cells.


*Keywords:* Human lung epithelial cells (L132); Particulate matter; Physical and chemical characteristics; Apoptosis

## 1. Introduction

Nowadays air pollution is a real problem of public health for the general population in urban areas since epidemiological studies have shown that fine particulate atmospheric pollution is associated with the increase

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of respiratory and cardiovascular mortality and morbidity (for review see Brunekreef and Holgate, 2002). The majority of research articles published during the last decade on air pollution particulate matter (PM) relate to $PM_{2.5}$, which have been identified as potential mediators of some of the toxicity of $PM_{10}$ (Diociaiuti et al., 2001; Monn and Becker, 1999; Schwartz and Neas, 2000). Because of their relatively small size and large surface area, $PM_{2.5}$ may also gain access to biological sites, which large particles would not reach, and may lead to functional changes of these critical targets in lungs (Donaldson et al., 2001; Hetland et al., 2004; Oberdorster and Utell, 2002). The underlying mechanisms of actions by which air pollution PM induce adverse health effects are, therefore, of intense scientific interest. This includes a call for research on the physical and chemical properties that promote PM toxicity (Pope et al., 2002; Englert, 2004; Spurny, 1998).

At present, it is well known that, depending upon their natural and/or anthropogenic emission sources, air pollution PM is a complex mixture of chemical and/or biological elements, such as metals, salts, carbonaceous material, volatile organic compounds (VOC), polycyclic aromatic hydrocarbons (PAH), endotoxins, which can in fact interact and adsorb on condensation nuclei (Alfaro-Moreno et al., 2002; Elder et al., 2000; Englert, 2004; Knaapen et al., 2002; Soukup and Becker, 2001). Hence, PM mass, PM number, PM specific surface area, qualitative and/or quantitative determinations of bioavailable transition metals, PAH, VOC, and other particle-bound chemicals are suspected to be important in determining the adverse health effects of air pollution (Carter et al., 1997; Frampton et al., 1999; Kennedy et al., 1998; Monn and Becker, 1999; Nel et al., 2006; Patterson and Eatough, 2000). Despite their availability at low-dose or ultra-low-dose, some constituents (i.e. transition metals, VOC, PAH, etc.) could be greatly involved in the global toxicity of PM (Baulig et al., 2004; Diociaiuti et al., 2001; Osornio-Vargas et al., 2003). However, the majority of published studies on PM toxicity have been conducted using complex mixtures, yet their physical and chemical characteristics are usually not well established, and the possible influence of the elements coated-onto the particles are often neglected (Hetland et al., 2004).

Recently, we have undertaken an extensive investigation of the adverse health effects of air pollution $PM_{2.5}$, collected in Dunkerque, a French seaside city located on the southern coast of the North-Sea, characterized by the proximity of industrial activities and heavy motor vehicle traffic. We have already shown that in vitro short-term exposure to $PM_{2.5}$ induced dose-dependent and time-dependent oxidative damage and inflammatory response in human lung epithelial cells (Dagher et al., 2005; Garçon et al., 2006).

There is now increasing evidence that both the production of reactive oxygen species (ROS) and the secretion of inflammatory cytokines could interact by inducing cell death by apoptosis (Haddad, 2004; Hetland et al., 2004; Shukla et al., 2000). In addition to morphological features, apoptosis is defined by complex biochemical processes involving mitochondria, activation of a family of cysteine-ASPartate proteASES (caspases), and endonuclease-induced nuclear fragmentation (Ganju and Eastman, 2003; Kowaltowski et al., 2004; Micheau and Tschopp, 2003). Aggarwal (2004) has proposed that the differential expression of signaling factors involved in apoptosis in human lung exposed to ROS is redox-sensitive and is mediated, at least in part, by a negative feedback mechanism transduced by the nuclear factor-kappa B (NF-κB), and, therefore, by the tumor necrosis factor-alpha (TNF-α).

Within the last few years, a series of biochemical steps has been identified that results in apoptosis, and two main pathways have been delineated in human cells (Aggarwal, 2000). For instance, the extrinsic pathway of apoptosis is represented by TNF-α-induced apoptosis, which involves the activation of the TNF family receptors, TNFRI and TNFRII, and the activation of the initiator caspase-8, which initiate a cascade of additional processing and activation of downstream effector caspase-3 (Nunez et al., 1998). In the mitochondrial pathway, apoptosis is triggered by the release of cytochrome *c* (Cyt. *c*), normally confined to the mitochondrial intermembrane space, into the cytosol, which, in the presence of the apoptosis protease-activating factor-1 (Apaf-1), recruits and converts the latent apoptosis-promoting pro-caspase 9 to its active form, and activates also an overlapping set of effector caspases including caspase 3 (Skulachev, 1998). Active effector caspases mediate the cleavage of apoptosis regulator, the cleavage of housekeeping proteins, and DNA fragmentation, resulting in morphological features of apoptosis (Nunez et al., 1998). The induction of downstream caspase activity can be regulated by the tumor suppressor protein p53 and the Bcl-2 family members (Geske and Gerschenson, 2001). The role of P53 as a nuclear transcription factor is well characterized; it induces apoptosis by upregulating the transcription of many pro-apoptotic genes and by down regulating anti-apoptotic genes (Oren et al., 2002). The Bcl-2 family proteins play pivotal roles in regulation of apoptosis and the balance between pro- and anti-apoptotic Bcl-2 family members determines the mitochondrial response to apoptotic stimuli (Upadhyay et al., 2003). After DNA-damaging events, the multidomain pro-apoptotic pro-

teins (e.g. Bax, Bak, and Bid) translocate from the cytosol to the mitochondrial membrane and induce therefore the release of a variety of apoptogenic factors, including Cyt. *c* into the cytosol (Upadhyay et al., 2003).

Hence, it will be of great interest to study the possible involvement of air pollution $PM_{2.5}$ in the induction of apoptosis in human lungs. This work was, therefore, undertaken to determine the ability of the air pollution $PM_{2.5}$ collected in Dunkerque, to induce some of the events firmly involved in the apoptotic pathways in human lung epithelial cells in culture. Firstly, the two main pathways of apoptosis were studied through the determination of the concentrations of TNF-α, the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), the release of Cyt. *c* from mitochondria into cytosol, and the enzymatic activities of caspases 8, 9 and 3. Secondly, mRNA expressions of p53, bcl-2 and bax genes were carried out. Thirdly, DNA fragmentation was assessed by terminal deoxynucleotidyl transferase mediated deoxyUridine-5′-triphosphate-biotin Nick-End Labeling (TUNEL), DNA laddering, and poly(ADP-ribose) polymerase (PARP) activity.

## 2. Materials and methods

### 2.1. Chemicals

Minimum essential medium (MEM) with Earle's salts, fetal bovine serum (FBS), L-glutamin, penicillin/streptomycin solution, and sterile phosphate-buffered solution (PBS) were from InVitrogen-Life Technologies (Cergy Pontoise, France). Quantikine human TNF-α immunoassays, quantikine human cytochrome *c* immunoassays, caspase-8, caspase-9, and caspase-3 fluorometric activity kits, and PARP inhibition assays were from R&D Systems Europe (Abingdon, United Kingdom). Mitochondria/cytosol fractionation kits were from Biosources Europe SA (Nivelles, Belgium). Qiagen (Courtaboeuf, France) supplied DNeasy tissue kits and RNeasy mini kits. 8-OHdG ELISA kits were from the Japan Institute for the Control of Aging (Haruoka, Japan). Proligo France SAS (Paris, France) synthesized primer pairs for polymerase chain reaction (PCR). All other reagents for reverse transcription (RT) and PCR, and in situ cell death detection kits were from Roche Diagnostics (Meylan, France). BCA protein reagents were from Pierce (IL, USA).

### 2.2. PM sampling and physical and chemical characteristics

#### 2.2.1. PM sampling

PM was collected in Dunkerque (51°04′N; 2°38′E), a French sea-side City located on the southern coast of the North-Sea, using high volume Sierra model 235 cascade impactor (Sierra Anderson, Smyna, GA, USA) (Garçon et al., 2002). The PM we used in the present work provided from the 5 μm-stage of the cascade impactor. Plates were mounted without any filters and no back up filter was used to maintain a constant aspiration flow rate (68 $m^3$/h) for a period of 2 weeks. No back up filter was used and the lowest stage was doubled to increase the efficiency of smallest particle (PM0.33) sampling. Collection was done continuously during 9 months, from January to September, and impacting system was changed every 15 days; two identical systems were used alternatively. Meteorological data (i.e. wind speed, wind direction, temperature) were obtained from Meteo France.

#### 2.2.2. Physical and chemical characteristics

Scanning electronic microscopy coupled with energy dispersive X-ray analysis (SEM-EDX; LeoTM 438 VP microscope and IXRF analysis system; Zeiss SAS, Le Pecq, France) was used to assess PM composition and size distribution. Prior to the analysis, PM were suspended in a *n*-hexane solution using ultrasonic treatment, and then filtered through a nucleopore filter to obtain well-distributed and dispersed PM, without agglomerates, as suitable for the SEM-EDX analysis. Adsorption data from the Brunauer Emmett Teller (BET) method using a Sorptomatic apparatus (Thermo Electron Corporation, Courtaboeuf, France) provided the specific surface area. Inorganic and organic element compositions of PM were carried out by inductively coupled plasma-atomic emission spectrometry (ICP-AES) using a Perkin-Elmer AAnalyst 600 (Perkin-Elmer Life Sciences France, Courtaboeuf, France), and by gas chromatography-mass spectrometry (GC-MS; Brüker EM40, Bruker, Wissembourg, France; Varian Saturn, Varian France, Coutaboeuf, France).

### 2.3. Cells and culture conditions

The cell line we used originates from the normal lung tissue of a human embryo, and is deposited under the designation L132 in the American Type Culture Collection (ATCC; ATCC number: CCL-5). The morphology of these cells is epithelial, and they exhibit typical features of pneumocytes. L132 cells were cultured in sterile plastic flasks (Corning; Fisher Scientific Labosi SAS, Elancourt, France), in MEM with Earle's salts, containing: 5% (v/v) FBS, 1% (v/v) L-glutamin (200 mM), 1% (v/v) penicillin (10000 IU/ml) and 1% (v/v) streptomycin (10000 UG/ml) (In Vitrogen-Life Technologies). Exponentially growing cells were maintained at 37 °C, in a humidified atmosphere containing 5% $CO_2$. All the L132 cells we used in this study derived from the same initial cell culture.

### 2.4. Sampling of culture supernatants and cells for the study of apoptosis

#### 2.4.1. Cell exposure

Depending on the incubation time, L132 cells were seeded at different density (i.e. $3 \times 10^6$, $1.5 \times 10^6$ or $0.75 \times 10^6$ cells/20 mL of culture media, respectively) in sterile

plastic flasks and incubated at 37 °C, in a humidified atmosphere containing 5% $CO_2$, for 24 h. Culture supernatants were removed to eliminate non-adherent cells. Only living cells were also incubated in the continuous presence of PM at their lethal concentration at 10% (LC10; 18.84 μg PM/mL) or at 50% (LC50; 75.36 μg PM/mL) for 24, 48 or 72 h of incubation, without renewing the culture media. Garçon et al. (2006) have calculated recently LC values. Non-exposed cells were used as negative controls. Accordingly, for each incubation time, 10 culture flasks were chosen at random as control cells, and 5 culture flasks per PM concentration were designated at random as exposed cells.

*2.4.2. Sampling of culture supernatants and cells*

After 24, 48 or 72 h of incubation, 1 ml-aliquots of culture supernatants were collected and frozen at −80 °C for the determination of TNF-α concentration or total protein content. Adherent cells were removed and centrifuged (500 × *g*, 10 min, 4 °C). Cell pellets were washed twice with 10 ml-aliquots of sterile PBS (In Vitrogen-Life Technologies) and cell-aliquots were collected and quickly frozen at −80 °C until the further determination of 8-OHdG formation, Cyt. *c* concentration, caspase-8, -9, and -3 activities, mRNA expression of p53, bcl-2 and bax genes, DNA laddering, PARP activity, and total protein content. The latest cell-aliquot was fixed with 2% (v/v) paraformaldehyde in sterile PBS, washed twice with 10 ml-aliquots of sterile PBS, resuspended with 70% (v/v) pre-cold ethanol, centrifuged (500 × *g*, 10 min, 4 °C), and stored at −20 °C until the TUNEL.

*2.4.3. Study of the two main pathways of apoptosis*

TNF-α concentrations in cell culture supernatants were determined using Quantikine Human TNF-α Immunoassay (R&D Systems Europe, France). 8-OHdG formations in cells were determined using the method originally described by Toyokuni et al. (1997) and modified by Garçon et al. (2001a). Briefly, after DNA extraction by DNeasy tissue kits (Qiagen) and digestion, oxidative DNA adduct 8-OHdG were determined using the 8-OHdG ELISA kit (Japan Institute for the Control of Aging). After the isolation of a highly enriched mitochondrial fraction (mitochondria/cytosol fractionation kits, Biosources Europe SA), Cyt. *c* concentrations in both the mitochondrial and the cytosolic fractions were determined using the quantikine human cytochrome *c* Immunoassay (R&D Systems Europe). Caspase-8, 9, and 3 activities in cells were studied using commercially available fluorometric assays (caspase-8, caspase-9, and caspase-3 fluorometric activity kits, respectively, R&D Systems Europe). Total protein contents in culture supernatants, cell lysates, and mitochondria and cytosolic fractions were determined using the BCA protein reagent (Pierce) (Smith et al., 1985).

*2.4.4. Study of mRNA expression of p53, bcl-2 and bax genes*

Total RNA was extracted from cell-aliquots using RNeasy mini kits (Qiagen). Conditions for the RT were as described previously (Garçon et al., 2001b). Specific primer pairs and PCR thermocycling conditions were designed as that published previously by Willey et al. (1998). After gel electrophoresis and ethidium bromide intercalation, PCR-amplified products were visualized under UV light and analyzed by a computerized video/densitometry system (Software: GelAnalyst™ v3.1FR; Vasse Industries, Lille, France).

*2.4.5. Study of DNA fragmentation*

PM-induced DNA fragmentation was carried out using a TUNEL kit (in situ cell death detection kit, Roche Diagnostics, Meylan, France), following the manufacturer's instructions, and measured by an EPICS XLTM Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). The DNA fragmentation in PM-exposed cells was carried out by agarose gel electrophoresis. Briefly, after protein digestion, RNA-free DNA was extracted from cells by DNeasy tissue kits (Qiagen), and 5 μg of DNA were suspended in 20 μL DNase-free loading buffer. After gel electrophoresis and ethidium bromide intercalation, DNA fragments were visualized under UV light and analyzed by a computerized video/densitometry system (Software: GelAnalyst™ v3.1FR; Vasse Industries, Lille, France). The PARP activity was studied in cell lysates using the PARP Inhibition Assay (R&D Systems Europe).

*2.5. Statistical analysis*

Results were expressed as mean values and ranges (minimum value–maximum value). For the different incubation times (i.e. 24, 48, or 72 h), data from cell cultures exposed to increasing concentrations of PM (i.e. CL10 and CL50) were compared with those from non-exposed cell cultures. Thereafter, we looked for correlation between the different markers under study. Statistical analyses were performed by the Mann–Whitney *U*-test and the non-parametric Spearman test (Software: SPSS for Windows, v10.05, 2000; Paris, France). Statistically significant differences were reported with $p$-values $< 0.05$.

## 3. Results

*3.1. PM physical and chemical characteristics*

Table 1 shows the size distribution results of collected PM as depicted as cumulative frequencies (%) and relative frequencies (%). Accordingly, 64.24%, 92.15%, and 98.49% of PM total number were equal or smaller than 1 μm, 2.5 μm, and 5 μm, respectively. The highest number of collected PM were also detected in size classes included in PM with a geometric diameter inferior or equal to 2.5 μm: 0.33–0.5 μm (33.63%), 0.5–1.0 μm (30.61%), 1.0–1.5 μm (14.33%), 1.5–2.0 μm (8.69%), and 2.0–2.5 μm (4.89%). The specific surface area of PM was 1 $m^2$/g. Table 2 shows the inorganic elements found in collected PM. Among them, Fe, Al, Ca, Na, K, Mg,

Table 1
Size distribution of PM

| | PM size ranges (μm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.3–0.5 | 0.5–1 | 1–1.5 | 1.5–2 | 2–2.5 | 2.5–5 | 5–10 |
| Relative frequency (%) | 33.64 | 30.60 | 14.33 | 8.68 | 4.89 | 6.33 | 1.52 |
| | PM size (μm) | | | | | | |
| | 0.5 | 1 | 1.5 | 2 | 2.5 | 5 | 10 |
| Cumulative frequency (%) | 33.64 | 64.24 | 78.57 | 87.26 | 92.15 | 98.48 | 100 |

Table shows the size distribution of the particle matter (PM) collected in Dunkerque, a French seaside city located on the southern coast of the North-Sea, as depicted as relative frequency (%) and cumulative frequency (%).

Pb, and Ti were the most abundantly present. In collected PM, there were not only inorganic elements usually associated with natural environment (i.e. Ca, Na, Mg, Ti, Sr, etc.), but also so-called anthropogenic elements (i.e. Fe, Al, Pb, Mn, Zn, Ba, Cu, Cr, Ni, Cd, etc.). Table 3 shows the list of the polycyclic aromatic hydrocarbons (PAH) coated-onto collected PM.

### 3.2. Effects of PM on the activation of the TNF-α-induced pathway of apoptosis

Table 4 shows that Dunkerque City's $PM_{2.5}$ induced TNF-α secretion by L132 cells in a concentration- and time-dependent manner. Statistically significant increases of TNF-α concentration were determined in the culture supernatants of L132 cells 24 h after their exposure to PM at their LC50 (6.05-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (2.16-fold, $p < 0.01$) and LC50 (10.03-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (8.72-fold, $p < 0.01$) and LC50 (19.10-fold, $p < 0.01$), as compared to control. Table 4 shows increases in caspase-8 and -3 activities in control or PM-exposed proliferating L132 cells. There were also increases of caspase-8 activity in L132 cells, 24 h after their exposure to PM at their LC10 (1.3-fold, $p < 0.05$) and LC50 (1.8-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (1.1-fold, $p < 0.05$) and LC50 (1.6-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.5-fold, $p < 0.05$) and LC50 (1.9-fold, $p < 0.01$), versus control cells. Statistically significant increases of caspase-3 activity were observed in L132 cells, 24 h after their exposure to PM at their LC10 (1.6-fold, $p < 0.01$) and LC50 (3.0-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (1.6-fold, $p < 0.01$) and LC50 (3.1-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (2.1-fold, $p < 0.01$) and LC50 (3.0-fold, $p < 0.01$), as compared to control cells. There were significant correlation between TNF-α concentration and caspase-8 activity, on the one hand, and caspase-8 activity and caspase-3 activity, on the other hand (Spearman's rho = 0.683; $p < 0.05$, and Spearman's rho = 0.802, $p < 0.01$, respectively).

Table 2
Inorganic compounds detected in PM

| Inorganic compounds | Concentrations (% w/w) |
|---|---|
| Fe | 7.84 |
| Al | 5.83 |
| Ca | 4.95 |
| Na | 1.88 |
| K | 0.97 |
| Mg | 0.81 |
| Pb | 0.80 |
| Ti | 0.51 |
| Mn | 0.352 |
| Zn | 0.184 |
| Ba | 0.064 |
| Cu | 0.037 |
| Cr | 0.032 |
| V | 0.030 |
| Ni | 0.029 |
| Sr | 0.022 |
| Li | 0.011 |
| Mo | 0.004 |
| Cd | <0.001 |
| Co | <0.0006 |

Table shows the concentrations (% w/w) of the inorganic compounds detected in the PM.

Table 3
Polycyclic aromatic hydrocarbons detected in PM

| PAH | Concentrations (μg/g) |
|---|---|
| Chrysene | 9.54 |
| Fluoranthene | 1.45 |
| Benzo($b+k$)fluoranthene | 6.55 |
| Benzo($a$)pyrene | 7.90 |
| Phenanthrene | 9.94 |
| Pyrene | 4.03 |

Table shows the polycyclic aromatic hydrocarbons (PAH; μg/g) detected in the PM.

Table 4
Effects of PM on the TNFα-induced apoptotic pathway

| | Incubation time and exposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 24 h | | | 48 h | | | 72 h | | |
| | C | LC10 | LC50 | C | LC10 | LC50 | C | LC10 | LC50 |
| TNF-α (pg/mg protein) | 2.55 | 2.49[a] | 15.47[c] | 2.70 | 5.85[c] | 27.15[c] | 2.40 | 20.90[c] | 45.80[c] |
| | 2.09–3.09 | 2.23–2.72 | 13.30–18.28 | 2.32–3.28 | 4.29–7.11 | 25.21–29–55 | 2.19–2.64 | 19.66–22.95 | 41.29–49.15 |
| Caspase 8 (RFU/mg protein) | 3.63 | 4.76[b] | 6.38[c] | 3.63 | 4.17[b] | 5.93[c] | 2.90 | 4.33[b] | 5.43[c] |
| | 2.53–5.53 | 3.96–5.82 | 5.34–7.69 | 2.73–4.80 | 3.59–4.88 | 4.15–7.42 | 2.06–3.95 | 2.93–5.33 | 4.04–6.78 |
| Caspase 3 (RFU/mg protein) | 0.69 | 1.08[c] | 2.05[c] | 0.71 | 1.15[c] | 2.21[c] | 0.65 | 1.34[c] | 1.95[c] |
| | 0.58–0.75 | 0.91–1.29 | 1.73–2.37 | 0.56–0.91 | 0.89–1.31 | 1.72–3.02 | 0.42–0.92 | 1.05–1.80 | 1.73–2.09 |

Table shows tumor necrosis factor-alpha (TNF-α) concentrations (pg/mg protein) in culture supernatants, and caspase-8 and -3 activities (RFU/mg protein) in L132 cells after their incubation in the continuous presence of particle matter (PM) at their at their lethal concentration at 10% (LC10; 18.84 μg PM/mL) or at 50% (LC50; 75.36 μg PM/mL) for 24, 48 or 72 h of incubation, without renewing the culture media. Non-exposed cells were used as negative controls (C). These values are depicted as mean values and ranges (minimum value–maximum value) of 10 replicates for negative controls and 5 replicates for every PM concentrations (Mann–Whitney *U*-test).

[a] Not significant.
[b] $p < 0.05$.
[c] $p < 0.01$.

### 3.3. Effects of PM on the activation of the mitochondrial pathway of apoptosis

Table 5 shows that Dunkerque City's $PM_{2.5}$ induced the mitochondrial pathway of apoptosis. PM induced concentration and time-dependent formation of 8-OHdG in PM-exposed proliferating L132 cells. Statistically significant increases of 8-OHdG concentration were reported in L132 cells 24 h after their exposure to PM at their LC10 or LC50 (3.37-fold, $p < 0.01$, and 12.91-fold, $p < 0.01$, respectively), 48 h after their exposure to PM at their LC10 or LC50 (6.91-fold, $p < 0.01$, and 16.84-fold, $p < 0.01$, respectively), and 72 h after their exposure to PM at their LC10 or LC50 (11.90-fold, $p < 0.01$, and 27.79-fold, $p < 0.01$, respectively), versus control cells (Table 5). Concentration and time-dependent Cyt. *c* releases from the mitochondria into the cytosol were seen in PM-exposed proliferating L132 cells. Indeed, changes in the ratio between Cyt. *c* concentrations in the cytosolic and the mitochondrial fractions were observed in L132 cells, 24 h after their exposure to PM at their LC10 (2.0-fold, $p < 0.01$) and LC50 (3.0-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (1.6-fold, $p < 0.01$) and LC50 (2.6-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (2.6-fold, $p < 0.01$) and LC50 (3.8-fold, $p < 0.01$), versus control cells (Table 5). Statistically significant increases of caspase-9 activity were reported in L132 cells, 24 h after their exposure to PM at their LC10 (1.2-fold, $p < 0.01$) and LC50 (1.8-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (1.4-fold, $p < 0.01$) and LC50 (1.7-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.5-fold, $p < 0.01$) and LC50 (2.1-fold, $p < 0.01$), versus control cells (Table 5). Statistically significant correlations were reported between 8-OHdG formation and Cyt. *c* release (Spearman's rho = 0.817, $p < 0.01$), Cyt. *c* release and caspase-9 activity (Spearman's rho = 0.662, $p < 0.01$), and caspase-9 activity and caspase-3 activity (Spearman's rho = 0.833, $p < 0.01$).

### 3.4. Effects of PM on the mRNA expression of P53, Bcl-2, and Bax genes

The mRNA expression of p53, bcl-2 and bax genes in Dunkerque City's PM-exposed L132 cells are summarized in Table 6. Statistically significant induction of p53 transcript were observed in L132 cells, 24 h after their exposure to PM at their LC10 (1.45-fold, $p < 0.05$) and LC50 (1.51-fold, $p < 0.05$), 48 h after their exposure to PM at their LC10 (1.32-fold, $p < 0.01$) and LC50 (1.29-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.16-fold, $p < 0.05$) and

Table 5
Effects of PM on the mitochondrial apoptotic pathway

| | Incubation time and exposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 24 h | | | 48 h | | | 72 h | | |
| | C | LC10 | LC50 | C | LC10 | LC50 | C | LC10 | LC50 |
| 8-OHdG (pg/μg DNA) | 10.32<br>7.44–15.22 | 34.83[c]<br>31.35–46.36 | 133.4[c]<br>125.8–138.7 | 9.67<br>7.27–13.51 | 66.89[c]<br>46.50–78.11 | 162.3[c]<br>148.5–182.6 | 7.56<br>5.76–9.18 | 89.94[c]<br>66.38–103.1 | 210.1[c]<br>183.9–239.2 |
| Cyt. $c_M$ (ng/mg protein) | 27.10<br>20.49–39.03 | 20.60[b]<br>15.82–28.69 | 16.15[c]<br>14.19–19.41 | 16.60<br>13.02–19.31 | 13.72[b]<br>10.91–16.90 | 11.07[c]<br>9.21–12.95 | 16.05<br>11.99–22.08 | 7.55[c]<br>6.56–8.54 | 6.82[c]<br>5.98–8.16 |
| Cyt. $c_C$ (ng/mg protein) | 6.77<br>4.08–9.42 | 10.12[b]<br>8.77–12.66 | 12.35[c]<br>10.19–13.42 | 5.64<br>3.06–7.49 | 7.38[b]<br>5.44–8.77 | 9.77[c]<br>8.45–12.26 | 6.49<br>5.09–8.27 | 7.89[b]<br>6.31–9.26 | 10.47[c]<br>9.52–11.76 |
| Cyt. $c_{C/M}$ | 0.26<br>0.18–0.45 | 0.51[c]<br>0.35–0.63 | 0.77[c]<br>0.65–0.95 | 0.34<br>0.18–0.50 | 0.56[c]<br>0.32–0.80 | 0.90[c]<br>0.72–1.33 | 0.41<br>0.34–0.50 | 1.05[c]<br>0.92–1.35 | 1.54[c]<br>1.27–1.69 |
| Caspase 9 (RFU/mg protein) | 2.27<br>1.75–3.26 | 2.74[c]<br>2.23–3.36 | 3.99[c]<br>3.24–4.78 | 2.44<br>1.82–4.45 | 3.32[c]<br>2.11–4.71 | 4.27[c]<br>3.37–4.89 | 2.02<br>1.55–2.85 | 3.00[c]<br>2.41–4.02 | 4.30[c]<br>3.75–5.03 |
| Caspase 3 (RFU/mg protein) | 0.69<br>0.58–0.75 | 1.08[c]<br>0.91–1.29 | 2.05[c]<br>1.73–2.37 | 0.71<br>0.56–0.91 | 1.15[c]<br>0.89–1.31 | 2.21[c]<br>1.72–3.02 | 0.65<br>0.42–0.92 | 1.34[c]<br>1.05–1.80 | 1.95[c]<br>1.73–2.09 |

Table shows 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations (pg/μg DNA), cytochrome *c* (Cyt. *c*) concentrations (ng/mg protein) in mitochondrial and cytosolic fractions (Cyt. $c_M$ and Cyt. $c_C$, respectively), and caspase-9 and -3 activities (RFU/mg protein) in L132 cells after their incubation in the continuous presence of particle matter (PM) at their lethal concentration at 10% (LC10; 18.84 μg PM/mL) or at 50% (LC50; 75.36 μg PM/mL) for 24, 48 or 72 h of incubation, without renewing the culture media. Non-exposed cells were used as negative controls (C). These values are depicted as mean values and ranges (minimum value–maximum value) of 10 replicates for negative controls and 5 replicates for every PM concentrations. (Mann–Whitney *U*-test).
[a]Not significant.
[b] $p < 0.05$
[c] $p < 0.01$.

Table 6
Effects of PM on mRNA expressions of P53, Bcl-2, and Bax genes

| | Incubation time and exposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 24 h | | | 48 h | | | 72 h | | |
| | C | LC10 | LC50 | C | LC10 | LC50 | C | LC10 | LC50 |
| p53/β-actine | 0.585 | 0.850[b] | 0.883[b] | 0.657 | 0.864[c] | 0.845[c] | 0.849 | 0.982[b] | 1.045[b] |
| | 0.507–0.835 | 0.723–1.197 | 0.698–1.118 | 0.502–0.888 | 0.715–1.015 | 0.748–0.932 | 0.725–0.956 | 0.963–1.012 | 0.912–1.230 |
| bcl-2/β-actine | 1.346 | 0.855[b] | 0.858[b] | 1.175 | 0.804[b] | 0.862[b] | 1.190 | 0.977[b] | 0.900[b] |
| | 0.972–1.802 | 0.621–1.018 | 0.667–1.012 | 0.883–1.536 | 0.525–1.009 | 0.711–1.049 | 0.981–1.556 | 0.875–1.071 | 0.645–1.022 |
| bax/β-actine | 0.820 | 0.868[a] | 0.979[a] | 0.815 | 1.329[b] | 1.333[c] | 0.836 | 1.349[c] | 1.354[c] |
| | 0.534–0.971 | 0.607–1.423 | 0.707–1.311 | 0.555–0.989 | 0.947–1.673 | 1.054–1.708 | 0.628–1.158 | 1.238–1.602 | 1.041–1.546 |
| bax/bcl-2 | 0.636 | 1.032[b] | 1.131[c] | 0.602 | 1.706[c] | 1.552[c] | 0.707 | 1.351[c] | 1.546[c] |
| | 0.510–0.954 | 0.655–1.468 | 0.898–1.296 | 0.415–0.766 | 1.126–2.185 | 1.322–1.850 | 0.576–0.996 | 1.198–1.662 | 1.024–2.006 |

Table shows mRNA expressions of p53, bcl-2, and bax genes in L132 cells after their incubation in the continuous presence of particle matter (PM) at their at their lethal concentration at 10% (LC10; 18.84 μg PM/mL) or at 50% (LC50; 75.36 μg PM/mL) for 24, 48 or 72 h of incubation, without renewing the culture media. Non-exposed cells were used as negative controls (C). The ratio between p53, bcl-2, or bax DNA band, on the one hand, and the β-actine DNA band are shown as mean values and ranges (minimum value–maximum value) of 10 replicates for negative controls and 5 replicates for every PM concentrations. (Mann–Whitney *U*-test).

[a] Not significant.
[b] $p < 0.05$.
[c] $p < 0.01$.

LC50 (1.23-fold, $p < 0.05$), compared to control cells. In contrast, decreases in bcl-2 mRNA expressions were seen in L132 cells, 24 h after their exposure to PM at their LC10 (0.64-fold, $p < 0.05$) and LC50 (0.64-fold, $p < 0.05$), 48 h after their exposure to PM at their LC10 (0.68-fold, $p < 0.05$) and LC50 (0.73-fold, $p < 0.05$), and 72 h after their exposure to PM at their LC10 (0.82-fold, $p < 0.05$) and LC50 (0.76-fold, $p < 0.05$), versus control cells. In return, increases of bax transcript were determined in L132 cells, 48 h after their exposure to PM at their LC10 (1.63-fold, $p < 0.05$) and LC50 (1.64-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.61-fold, $p < 0.01$) and LC50 (1.62-fold, $p < 0.01$), versus control cells. Statistically significant increases of the ratio between the transcripts of bax and bcl-2 were reported in L132 cells, 24 h after their exposure to PM at their LC10 (1.62-fold, $p < 0.01$) and LC50 (1.78-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (2.83-fold, $p < 0.01$) and LC50 (2.58-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.91-fold, $p < 0.01$) and LC50 (2.19-fold, $p < 0.01$), compared to control cells. Moreover, statistically significant correlations were observed between the mRNA expressions of p53 and bcl-2 (Spearman's rho = −0.438, $p < 0.01$),the transcripts of p53 and bax (Spearman's rho = 0.474, $p < 0.01$), and the mRNA expression of p53 and the ratio between the transcripts of bax and bcl-2 (Spearman's rho = 0.620, $p < 0.01$).

### 3.5. Effects of PM on DNA fragmentation

Fig. 1 shows Dunkerque city's PM-induced L132 caspase-dependent apoptosis as studied by TUNEL. Accordingly, flow cytometry study indicated increases of fluorescent intensity related to fluorescein labels of genomic DNA strand breaks in L132 cells exposed to PM at their LC10 and LC50. DNA laddering showed, in a concentration- and time-dependent manner, the formation of a high molecular weight fragments in L132 cells (Fig. 2). The presence of a low molecular weight fragments was reported in L132 cells 48 h and 72 h after their exposure to PM at their LC50. Fig. 3 shows PARP activities in control or PM-exposed proliferating L132 cells. Statistically significant increases of PARP activity were attained in L132 cells 24 h after their exposure to PM at their LC10 (1.41-fold, $p < 0.01$) or LC50 (1.45-fold, $p < 0.01$), 48 h after their exposure to PM at their LC10 (1.53-fold, $p < 0.01$) or LC50 (1.68-fold, $p < 0.01$), and 72 h after their exposure to PM at their LC10 (1.28-fold, $p < 0.01$) or LC50 (1.50-fold, $p < 0.01$), versus control.

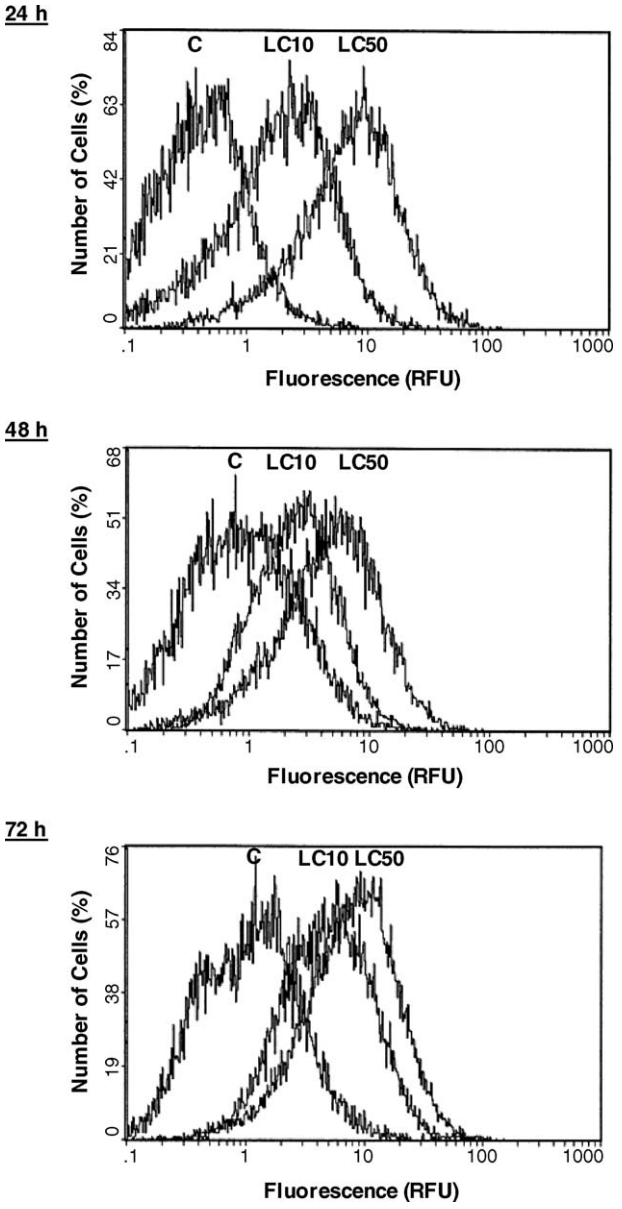


Fig. 1. Terminal deoxynucleotidyl transferase-mediated deoxyuridine-5′-triphosphate-biotin Nick-End Labeling (TUNEL) of L132 cells after their incubation in the continuous presence of particle matter (PM) at their LC10 (i.e. 18.84 μg PM/ml) or LC50 (i.e. 75.36 μg PM/ml) for 24, 48 or 72 h, without renewing the culture media. Non-exposed cells were used as negative controls (C).

## 4. Discussion

Despite intensive investigation, the underlying mechanisms of actions induced by air pollution $PM_{2.5}$, and the components of PM mainly involved in their occurrence in lungs are still poorly understood. For these reasons, we have undertaken an extensive investigation of the adverse

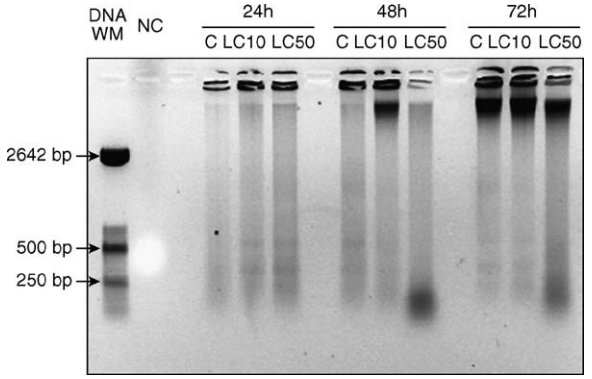


Fig. 2. DNA laddering of L132 cells after their incubation in the continuous presence of particle matter (PM) at their LC10 (i.e. 18.84 μg PM/ml) or LC50 (i.e. 75.36 μg PM/ml) for 24, 48 or 72 h, without renewing the culture media. Non-exposed cells were used as negative controls (C). High molecular weight DNA remained trapped in wells, while DNA that had been digested during apoptosis migrated through the gel and was resolved into multiples of 180–200 base pair fragments (DNA weight marker, DNA WM; base pair, bp).

health effects of Dunkerque City's $PM_{2.5}$, and we have already shown that in vitro short-term exposure to such PM caused dose-dependent and time-dependent oxidative damage and inflammatory response in human lung epithelial cells (Dagher et al., 2005; Garçon et al., 2006). Hence, in the course of these findings, it was convenient to complete the physical and chemical characterization of collected PM and to determine their possible involvement in the activation of apoptosis in these human lung target cells.

The physical and chemical characteristics of air pollution PM were also determined. The size distribution

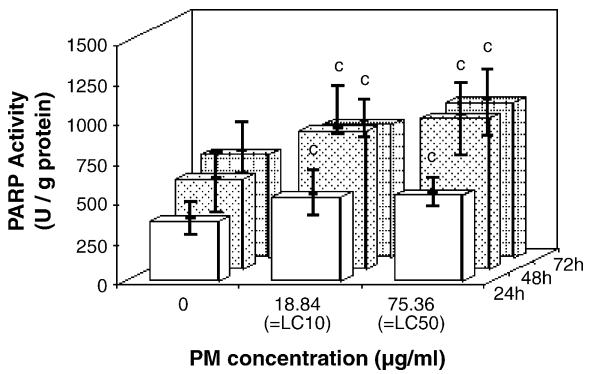


Fig. 3. Poly(ADP-ribose) polymerase (PARP) activity (U/g protein) in L132 cells after their incubation in the continuous presence of particle matter (PM) at their LC10 (i.e. 18.84 μg PM/ml) or LC50 (i.e. 75.36 μg PM/ml) for 24, 48 or 72 h, without renewing the culture media. Non-exposed cells were used as negative controls (C). These values are depicted as mean values and ranges (minimum value–maximum value) of 10 replicates for negative controls and 5 replicates for every PM concentrations. ($c = p < 0.01$; Mann–Whitney $U$-test).

results showed that 92.15% of PM total number were equal or smaller than 2.5 μm, thereby indicating that they are human respirable. According to epidemiological findings from several cities around the world, the greatest health risks correlate with smaller PM which have the ability to reach the distal regions of the lung after inhalation (Levy et al., 2000). Components such as transition metals, VOC, and PAH are known to be associated with PM, either through adherence to the core particle or as an integral component of the particle (Dreher et al., 1997; Monn and Becker, 1999; Nel et al., 2001). A total of 20 inorganic elements were found in Dunkerque City's $PM_{2.5}$. Among them, Fe, Al, Ca, Na, Pb, Mg, and Mn were the most abundantly present and constitute not only inorganic elements usually associated with natural environment (i.e. Ca, Mg, Na), but also so-called anthropogenic elements (i.e. Al, Mn, Pb, Zn). The specific surface area of the PM under study (i.e. 1 $m^2$/g) allowed the adsorption of various chemicals. Accordingly, PAH-coated onto PM were detected. It is also interesting to report that PAH concentrations in Dunkerque City's $PM_{2.5}$ were equal or smaller than those measured in PM samples collected in two locations in Paris (Baulig et al., 2004). Taken together, the physical and chemical characteristics of Dunkerque City's $PM_{2.5}$ suggest that much of PM derived from wind-borne dust from the industrial complex and the heavy diesel motor vehicle traffic. However, Baulig et al. (2004) reported that the physical and chemical properties of particles revealed to be unsatisfactory to predict their levels of biological reactivity. Hence, to better evaluate the adverse health effects of PM exposure, it will be of great interest to consider not only the physical and chemical characteristics of PM but also to determine their toxicological reactivity in target cells.

Although many pathways of apoptosis may exist, only two main caspase cascades have been elucidated in detail in mammalian cells (Baker and Reddy, 1998; Cohen, 1997; Nunez et al., 1998). Hence, with regards to previous findings on oxidative stress and inflammation, we focused our attention on the activation of these two main caspase cascades by Dunkerque City's PM (Dagher et al., 2005; Garçon et al., 2006). We showed that in vitro short-term exposure to air pollution PM activated the TNF-α-induced pathway of apoptosis. Dunkerque City's PM also induced TNF-α secretion by L132 cells, and the subsequent activation of caspase-8 and -3 activities. There were also significant correlation between TNF-α concentration and caspase-8 activity, on the one hand, and caspase-8 activity and caspase-3 activity, on the other hand. The results regarding the TNF-α-induced pathway of apoptosis by air pollution PM are in agreement with other observations previously reported using in vitro systems by Nel et al. (2006). In other respects, we found that air pollution PM activates the mitochondrial pathway of apoptosis. Indeed, concentration and time-dependent formation of 8-OHdG was reported in PM-exposed proliferating L132 cells, thereby indicating the occurrence of oxidative stress conditions. Several authors have reported that oxidant-induced DNA damage is a potent stimulus of mitochondrial dysfunction as detected by the release of agents normally confined to the mitochondrial intermembrane space, such as Cyt. *c* and is critical to caspase activation and, therefore, apoptosis propagation (Finkel, 2000; Higuchi, 2003; Upadhyay et al., 2003). We also showed that in vitro short-term exposure to Dunkerque City's PM induced the release of Cyt. *c*, a necessary component of non-receptor-mediated-caspase activation, from the mitochondria into the cytosol, and the subsequent activation of caspase-9 and -3. Statistically significant correlations were seen between 8-OHdG formation, Cyt. *c* release, and caspase-9 and -8 activities. One of the novel findings of the present work was, therefore, that Dunkerque City's PM could at least induce apoptotic events in human lung epithelial cells through the activation of both the extrinsic and the intrinsic pathways. In agreement with other results, in vitro short term exposure to some of the metals (i.e. Fe, Al, Pb, Mn, Zn) or PAH detected in Dunkerque City's PM have already been demonstrated as inducing oxidative stress conditions, inflammatory response, and/or apoptosis in lung target systems (Alfaro-Moreno et al., 2002; Bayram et al., 1998; Becker et al., 2002; Chin et al., 1998; Dagher et al., 2005; Dye et al., 1999; Garçon et al., 2006; Liden et al., 2003; Monn and Becker, 1999; Nel et al., 2006; Osornio-Vargas et al., 2003; Pozzi et al., 2003; Shukla et al., 2000; Takizawa et al., 1999).

In other respects, we observed not only a transcriptional induction of pro-apoptotic p53 gene, but also an imbalance between the respective transcription rates of bax and bcl-2 genes in PM-exposed cells. Accordingly, several lines of evidence, supportive of a key role for P53, Bcl-2, and Bax in the control of cell cycle, the regulation of downstream caspase activity, and the subsequent induction of apoptosis, have arisen (Geske and Gerschenson, 2001; Oren et al., 2002; Upadhyay et al., 2003). Reisman and Loging (1997) have described that TNF-α enhanced p53 mRNA expression. In addition, in vitro and developing lung ex vivo studies have reported that the reduced transcription of bcl-2 gene, coding for a mitochondrial targeted anti-apoptotic protein, could not yet inhibit Cyt. *c* release from mitochondria and protect against oxidative stress-induced apoptosis (Haddad, 2004).

Evidence presented herein extended these observations by showing that the TNF-α and mitochondria-regulated apoptotic pathways mediated DNA fragmentation in PM-exposed proliferating L132 cells. Results arising from TUNEL, DNA laddering, and PARP activity firmly supported the findings that in vitro short-term exposure to Dunkerque City's PM induced DNA fragmentation. It is now well known that the activation of downstream effector caspase -3 resulted in the cleavage of DNA (Curtin et al., 2002). With other respects, DNA laddering showed the presence of both the low and the high molecular weight fragments that indicates the activation of random caspase-dependent and independent DNA fragmentation in PM-exposed human lung epithelial cells. Nevertheless, the relative contributions of caspase-dependent and caspase-independent post-mitochondrial events to mediate cell death by apoptosis remain a matter of debate.

However, before concluding on the above-presented results, caution is necessary when tackling the question of the helpfulness of in vitro models to improve the extrapolation to the experiments in animals and/or the situations in humans. Further consideration of this key question indicated a general lack of information about the potential uses of data generated from in vitro systems in the risk assessment process. Scientific statements have notably highlight the potential discrepancies between the mechanisms of action occurring in in vitro systems and those appearing in animal systems, and whether the in vitro observations have any real relevance to the mechanisms of PM-induced health affects. Moreover, there are other limitations of such in vitro approaches in toxicology. For example, it is often very difficult to extrapolate from the dose applied to individual cells in culture to the inhaled doses that reach the epithelial cells in animals and/or humans. Indeed, it is likely that the one time application of PM to a target cell is remarkably different than short-term or long-term inhalation exposures of animals and/or humans. The concerns of extrapolating from cells also should be kept in mind prior to claiming that these approaches explain the mechanisms of PM toxicity. However, despite these limitations, in vitro experiments continue to be a prerequisite for a preliminary understanding of mechanism of action that, in general, is currently obtainable only through animal and/or human studies. The observations reported in in vitro approaches require also further validation through experiments in animals and studies in humans before to be validated.

In conclusion, the physical and chemical properties of Dunkerque City's PM allow us to suggest that much of the collected PM derived from wind-borne dust from the industrial complex, and the heavy diesel motor vehicle traffic. The relatively high concentrations of transition metals (i.e. Fe, Al, Pb, Mn, Zn) and the presence of organic compounds (i.e. PAH) found in the PM under study might be firmly involved in the generation of oxidative stress conditions, in the development of an inflammatory response, and therefore, in the induction of cell death by apoptosis in the in vitro lung target model we used.

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