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Regulation of human lung epithelial cell numbers by diesel exhaust particles

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ABSTRACT: Particulate air pollution is associated with respiratory morbidity and has cytotoxic and pro-inflammatory effects. The effects of diesel exhaust particles (DEP) on proliferation and apoptosis of A549 lung epithelial cells were examined.

When deprived of serum (serum starvation), epithelial cell numbers fell, but DEP (5–200 μg·mL^{-1}) prevented this. Using flow cytometric analysis of propidium iodide (PI) staining, DEP (10 μg·mL^{-1}) increased cells in the S phase of cell cycle from 12.85 to 18.75% after 48 h, reversing serum starvation-induced $G_{0/1}$ arrest. DEP also reduced the increase in apoptotic cells, as defined by double expression of annexin V/PI, observed after serum starvation (from 28.35 to 15.46%). The antioxidants, *N*-acetylcysteine (NAC; 33 mM) and AEOL10113 (10–100 μM), the N-terminal c-jun kinase inhibitor, SP600125 (33 μM), and nuclear factor-κB inhibitor, SN50 (33 μM), inhibited DEP-induced cell number increase. NAC inhibited DEP-induced reduction of $G_{0/1}$ and increase in cells in the S and G_2/M phases. Expression of p21$^{CIP1/WAF1}$ mRNA and protein seen with serum starvation was reduced by DEP.

In conclusion, diesel exhaust particles prevented serum starvation-led decreases in A549 epithelial cells by inducing cell cycle progression and preventing apoptosis, processes involving oxidative stress, inhibition of p21$^{CIP1/WAF1}$ expression and stimulation of N-terminal c-jun kinase and nuclear factor-κB. Therefore, low-dose diesel exhaust particle exposure may lead to lung epithelial cell hyperplasia.

KEYWORDS: Antioxidants, apoptosis, cell proliferation, diesel exhaust particles, nuclear factor-κB

With the increasing use of diesel-powered engines, particulate air pollution is increasingly being recognised as a major public health hazard and as a contributor to the burden of pulmonary and cardiovascular diseases. Thus, there is a strong association between particulate air pollution and impaired lung function, deficits in lung function growth, worsening of asthmatic symptoms, and increased emergency room visits for asthma and chronic obstructive pulmonary disease [1, 2]. In addition, a relationship between all-cause mortality, cardiopulmonary deaths and lung cancer mortality in adults living in metropolitan areas and the level of particulate air pollution has been reported [3–5].

The mechanisms underlying the deleterious effects of particulates on the lung are unclear. On exposure to particulates, airway epithelial cells, which form the first line of innate immune defence against particles, produce inflammatory mediators, such as interleukin (IL)-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), RANTES and soluble intercellular adhesion molecule (sICAM)-1 [6, 7] and reactive oxygen species, hydrogen peroxide (H_2O_2) and superoxide (O_2^-) [8, 9], that can activate the transcription factors, nuclear factor (NF)-κB and activator protein (AP)-1 pathways [10, 11]. In addition, particulates such as those of 10 μm in diameter (PM10) can also cause cell death by necrosis, and by the process of apoptosis [12, 13]. A dose-dependent increase of murine airway epithelial cell proliferation *in vivo* has also been reported [14]. The process of proliferation and of apoptosis may occur simultaneously [15]. The effects of particulates on regulation of cell numbers may be related to specific cell types, to different concentrations of particulates and to differences between species. They could also result from a balance of effects of particulates on signal transduction pathways that regulate both cell apoptosis and proliferation.

The molecular mechanisms of survival or proliferation or cell death of airway epithelial cells induced by particulates are also unclear. NF-κB activation, which is a cellular effect of particulate exposure, has been associated with cell survival and also with induction of apoptosis [16]. In some models of oxidative stress, activation of the

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mitogen-activated protein kinase (MAPK) pathway *via* the c-jun N-terminal kinase (JNK) has been associated with programmed cell death or apoptosis [17, 18]. Under certain circumstances, JNK may also increase cell survival and play a role in cell proliferation [19, 20]. The final key determinant of cell cycle, growth arrest and differentiation is the cyclin-dependent kinase inhibitor, $p21^{CIP1/WAF1}$ [21], which is highly responsive to oxidative stress [22, 23]; however, the effect of particulates on $p21^{CIP1/WAF1}$ expression in lung epithelial cells is unknown.

The purpose of the current investigation is to improve understanding of the cellular molecular mechanisms by which particulates cause damage to the airway epithelium. In order to address this, the effect of diesel exhaust particulates (DEP) on the viability, proliferation and death of the human epithelial A549 cell line was investigated. In the present *in vitro* model, the survival and proliferative status of these cells are dependent on the presence of serum which keeps the cells in a proliferating state, while its removal leads to cell death [24]. This model may reflect the effect of extravasated plasma during airway inflammation; under normal circumstances, the airway epithelium is not in contact with serum factors. It was hypothesised that the effects of DEP would depend on whether serum was present or not, since the presence of serum determines the basal cell cycle status. It was also hypothesised that the outcome of the cell cycle depended on the activation of JNK and NF-κB and the expression of $p21^{CIP1/WAF1}$ during these processes.

It was found that DEP at a low concentration inhibited cell apoptosis present when serum was removed and caused proliferation of airway epithelial cells, effects that were mediated through the induction of oxidative stress with activation of the JNK and NF-κB. These mechanisms indicate that, in a normal, non-inflamed epithelium, low-dose exposure to DEP causes increased epithelial cell survival by preventing apoptosis.

METHODS

Preparation of diesel exhaust particle suspension

DEP were a gift from H. Takano (National Institute for Environmental Studies, Tsukuba, Japan). They were collected from a 4JB1-type, light-duty, four-cylinder, 2.74-L Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan) operated by using standard diesel fuel at a speed of 1,500 rpm under a load of 10 $kg \cdot m^{-1}$ torque. DEP were collected as previously described [8], and the mean diameter of the particles was 0.4 μm [25]. The purified DEP were suspended in colourless Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Paisley, UK) containing 2 mM L-glutamine and 1 mM sodium pyruvate (serum-free medium (SF); Sigma Chemical Company, Poole, UK) at concentrations of 1–200 $\mu g \cdot mL^{-1}$, as previously described [7].

A549 cell culture and cell viability measurement

A549 cells (ATCC, LGC Promochem, Teddington, UK) were cultured using phenol red (-) DMEM (Gibco, Invitrogen) containing 10% foetal calf serum (FCS) and 2 mM L-glutamine (Sigma Chemical Company) in 12-well plates (Falcon; BD Biosciences, Oxford, UK) for 72 h, until 70–80% confluence has been reached, when the culture medium was replaced with SF. Twenty-four hours later, DEP suspension (0, 5, 10, 50, 100 and 200 $\mu g \cdot mL^{-1}$) was added for 24, 48 and 72 h in the absence or presence of study drugs including an antioxidant, NAC, a catalytic antioxidant and a superoxide mimetic, AEOL10113 [26], an inhibitor of JNK, SP600125 [27], and an inhibitor of the translocation of the p50 subunit of the transcription factor NF-κB, SN50 [28]. As a positive control, A549 cells were left to culture in medium containing 10% FCS. The measurement of alive cell numbers was based upon the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan dye by mitochondrial enzymes associated with metabolic activity, indicating living cells. Therefore, the test can be used to detect cytotoxicity and proliferation of cells [29]. The culture medium was replaced with SF containing 1 $mg \cdot mL^{-1}$ MTT (Sigma Chemical Company) and incubated for 15 min at 37°C. MTT solution was removed and replaced with dimethyl sulphoxide (DMSO), and the change in colour was read at 550 nm using a colorimetric plate reader.

Measurement of cell cycle progression and apoptosis

Cell cycle progression was measured by fluorescence-activated cell sorter analysis using propidium iodide (PI)-stained cells, as described previously [30]. Annexin V and PI binding was determined using an apoptosis detection kit according to the manufacturer's instruction (Becton Dickinson Pharmingen, Oxford, UK). To distinguish between apoptosis and necrosis, cells were double-stained with annexin V (green fluorescence) and PI (red fluorescence). Briefly, cells (100,000 $cells \cdot sample^{-1}$) were washed in cold PBS twice and suspended in binding buffer containing fluorescein isothiocyanate-conjugated annexin V (10 $\mu g \cdot mL^{-1}$) and PI (10 $\mu g \cdot mL^{-1}$). The cell suspension was incubated in the dark for 15 min and then signals acquired using a Becton Dickinson FACScan flow cytometer. A total of 10,000 events was analysed for each sample with Cell Quest software (BD Biosciences, San Jose, CA, USA).

Western blotting

Cells were rinsed with ice-cold PBS containing protease inhibitors (200 μM Na_3VO_4, 2 mM phenylmethylsulphonyl fluoride) and lysed in radioimmunoprecipitation assay buffer (PBS containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Igepal® (Sigma Chemical Company), and complete protease inhibitor cocktail tablet (Roche Diagnostics, Lewes, UK)). Cells were scraped off the flasks and solubilised by sonication followed by centrifugation ($10,000 \times g$, 4°C, 4 min). Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Lysates were boiled for 5 min and total protein extracts (20 $\mu g \cdot lane^{-1}$) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 16% Tris-SDS polyacrylamide precast gel (Novex; Invitrogen, Paisley, UK). The separated proteins were electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Amersham, UK) as previously described [31], and rabbit anti-human $p21^{CIP1/WAF1}$ antibody (Santa Cruz Biotechnology, Calne, UK) was used in a dilution of 1:200. Bands were visualised by enhanced chemiluminescence as recommended by the manufacturer (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified using a densitometer with Grab-It and GelWorks software (UVP, Cambridge, UK). The individual band optical

density values for each lane of $p21^{CIP1/WAF1}$ were expressed as the ratio with the corresponding internal control β-actin optical density values.

Quantitative reverse transcription polymerase chain reaction for $p21^{CIP1/WAF1}$

Cells were harvested for total RNA isolation. Commercially available kits were used to extract total cellular RNA (RNeasy; Qiagen, Crawley, UK) and to perform reverse transcription (Omniscript RT; Qiagen). Gene transcript level of $p21^{CIP1/WAF1}$ and the housekeeping gene GAPDH were quantified by real-time PCR using a Taqman system (Applied Bioscience, Arlington, VA, USA) on a Rotor-Gene 3000 PCR apparatus (Corbett Research, Sydney, Australia). The primer pair of GAPDH was purchased from Applied Bioscience, and the primer pair of $p21^{CIP1/WAF1}$ was designed as described below. (Forward; F) 5′-CAGACCAGCATGACAGATTTC, (Reverse; R) 3′-GGCTTCCTCTTGGAGAAGAT, (Taqman probe) 5′-FAM-TACCACTCCAAACGCCGGCT-TAMRA. Variation in cDNA concentration in different samples was corrected for GAPDH expression in each cDNA sample by dividing the calculated value for the gene of interest by the housekeeping gene value.

Study drugs

NAC (Sigma Chemical Company) and SN50 (an inhibitor of NF-κB; Calbiochem, Nottingham, UK) were dissolved in DMEM and further diluted to the desired working concentrations in the same medium. A catalytic antioxidant, AEOL10113, chemical name manganese(III) *meso*-tetrakis-(*N*-methylpyridinium-2-yl) porphyrin (a gift from J.D. Crapo, National Jewish Medical and Research Centre, Denver, CO, USA) was dissolved in DMEM. SP600125, a JNK inhibitor (a gift from B. Bennett, Celgene, San Diego, CA, USA) was dissolved in a stock concentration of 50 mM DMSO, and then diluted to the desired concentration in SF. The final concentration of DMSO was no more than 0.33% volume/volume.

Statistical analysis

Data were analysed using unpaired t-test or one-way ANOVA/Bonferroni's multiple comparison test. Results are expressed as mean±SEM. p-values of <0.05 were considered to be significant.

RESULTS

Effect of DEP on A549 cell numbers, cell cycle and apoptosis

A549 cells showed time-dependent growth in the presence of 10% FCS, with confluence achieved by 48 h. In the presence of FCS (1, 3.3 and 10%), DEP at 10 μg·mL^{-1} had no effect on cell numbers up to 48 h. In the absence of serum, the number of cells decreased after 48 and 72 h (table 1 and fig. 1). As indicated by MTT staining, incubation of A549 cells with 5–200 μg·mL^{-1} DEP did not affect their viability up to 24 h; at 48 h, DEP prevented the reduction in cells numbers caused by removal of serum with a maximum effect at 10 μg·mL^{-1} ($p<0.0001$; table 1 and fig. 1). Similar results were obtained at 72 h. In order to confirm that DEP (10 μg·mL^{-1}) induces proliferation of A549 cells, the experiments were repeated and A549 cells in the wells were counted directly using a haemocytometer. As shown in figure 1d, there was a reduction in cell numbers in the absence of FCS, but DEP induced an increase in cell numbers at 48 and 72 h of incubation. In the presence of FCS alone, there was an increase in A549 cells but without any further increase in the presence of DEP. These results confirm the data obtained using the MTT assay.

TABLE 1 Effect of diesel exhaust particles (DEP) on A549 epithelial cell numbers at 48 h. Cell numbers were assayed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to an insoluble formazan dye measured at an optical density of 550 nm after 48 h

DEP	Optical density			
	SF	1% FCS	3.3% FCS	10% FCS
0 μg·mL^{-1}	0.41±0.06	0.92±0.03	1.18±0.06	1.19±0.06
10 μg·mL^{-1}	1.04±0.06$^{\#}$	0.90±0.06	1.03±0.07	1.20±0.05

Results are expressed as mean±SEM of at least three experiments. SF: serum-free medium; FCS: foetal calf serum. $^{\#}$: $p<0.0001$ *versus* 0 μg·mL^{-1} DEP.

Next, the effect of DEP (10 μg·mL^{-1}) on cell cycle and apoptosis was examined. When cells were serum-starved, cells in the $G_{0/1}$ phase increased in number and those in the S and G_2/M phases decreased in comparison with cells left in 10% FCS. DEP (10 μg·mL^{-1}) increased the percentage of serum-starved A549 cells in the S phase (18.8±0.46%; $p<0.0001$) as compared with untreated serum-starved cells (12.9±0.66%) after 48 h, with a reduction in the percentage of cells in $G_{0/1}$ (73.2±0.16%; $p<0.0001$) and G_2/M (8.1±0.42%; $p<0.02$) phases as compared with serum-starved cells ($G_{0/1}$ 77.4±0.66% and G_2/M 9.75±0.37%) after 48 h (fig. 2a). Serum starvation of cells for 48 h increased annexin V- and PI-positive apoptotic cells in comparison with cells left in 10% FCS (SF 28.35±2.03 *versus* FCS 13.94±3.95; $p<0.05$), but did not induce any change in PI-positive, annexin V-negative cells, representing necrotic cells. Therefore, DEP prevented the increase in apoptotic cells seen with serum starvation, as indicated by positive staining with annexin V and PI (15.46±2.79% *versus* 28.35±2.03%; $p<0.03$; fig. 2b).

Effect of N*-acetylcysteine*

NAC at 3.3 and 10 mM had no effect on cell numbers in SF, but at 33 mM there was a significant increase in cell numbers, indicating that oxidative mechanisms were important in cell death induced by serum starvation. In contrast, an inhibition of the increase in cells induced by DEP (10 μg·mL^{-1}) with 10 and 33 mM NAC by 27 and 35% respectively, occurred at 48 h (fig. 3a). This indicates that oxidants may mediate in the increase in cell numbers induced by DEP.

NAC (10 mM) inhibited the DEP-induced increase in A549 cell numbers in the S phase ($p<0.0001$) while leading to an increase in cells in $G_{0/1}$ phase ($p<0.001$). The percentage of cells in G_2/M phase was further decreased by 10 mM NAC as compared with DEP alone ($p<0.0001$; see fig. 4a).

Effect of AEOL10113

AEOL10113 caused a dose-dependent decrease in the number of cells in the presence of DEP (10 μg·mL^{-1}), with significant

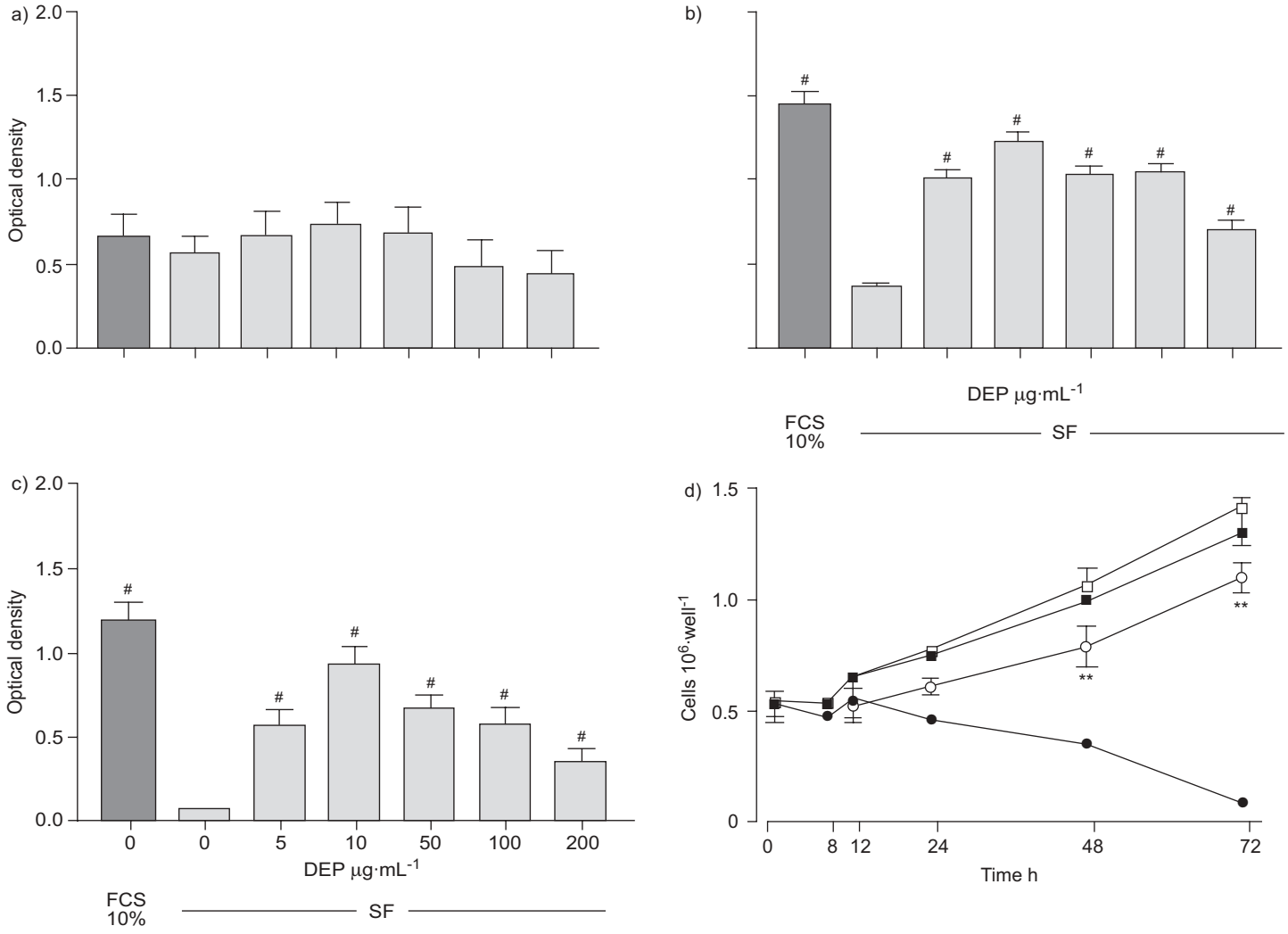

FIGURE 1. Effect of diesel exhaust particles (DEP; 0–200 μg·mL^{-1}) on viability of A549 cells as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after a) 24, b) 48 and c) 72 h in serum-free medium (SF). Cell numbers grown in the presence of 10% foetal calf serum (FCS) are shown for comparison. Results are expressed as mean±SEM of at least five experiments. #: $p<0.0001$ *versus* 0 μg·mL^{-1} DEP. d) shows the effect of DEP (10 μg·mL^{-1}) in the presence or absence of FCS on cell numbers, measured directly by haemocytometry, over a 72-h period. Results are expressed as mean±SEM of at least three experiments. **$p<0.01$ compared with FCS(-). □: FCS(+)+DEP; ■: FCS(+); ○: FCS(-)+DEP; ●: FCS(-).

effects at 10 and 100 μM. At these concentrations, there was also a significant increase in cell numbers when A549 cells were serum-starved alone (fig. 3b). Incubation of A549 cells with AEOL10113 (10 μM) also led to a significant decrease in the percentage of S-phase cells induced by 10 μg·mL^{-1} DEP ($p<0.05$; fig. 4b).

Effect of JNK inhibitor

While the JNK inhibitor (SP600125) had no effect at either 3.3 or 10 μM, at 33 μM it increased the number of cells in SF alone (mean optical density (OD) from 0.033±0.003 to 0.219±0.015; $p<0.0002$) after 48 h (fig. 5a). By contrast, SP600125 (33 μM) caused a decrease in the number of cells induced by 10 μg·mL^{-1} DEP (fig. 5a).

Effect of NF-κB inhibitor

SN50 had no effect on cell numbers in SF, but dose-dependently inhibited the enhancement of A549 cell numbers induced by DEP (10 μg·mL^{-1}), with significant effect at 33 μM (a decrease in mean OD from 1.319±0.102 to 0.595±0.004 was observed; $p<0.01$; fig. 5b).

Effect of DEP on p21$^{CIP1/WAF1}$ expression

p21$^{CIP1/WAF1}$ protein expression increased following 48 h of serum starvation in comparison with cells continuously incubated with 10% FCS ($p<0.01$). DEP added to SF-treated cells dose-dependently decreased p21$^{CIP1/WAF1}$ protein expression ($p<0.05$; fig. 6a and b). Similarly, the mRNA expression of p21$^{CIP1/WAF1}$ mRNA was decreased by DEP at 10 μg·mL^{-1} ($p<0.05$; fig. 6c).

DISCUSSION

Under *in vitro* conditions of cell culture, with withdrawal of serum factors, A549 cells undergo programmed cell death with a reduction in viable cell numbers within 48 h. Under these conditions, it was found that DEP improved cell viability, but

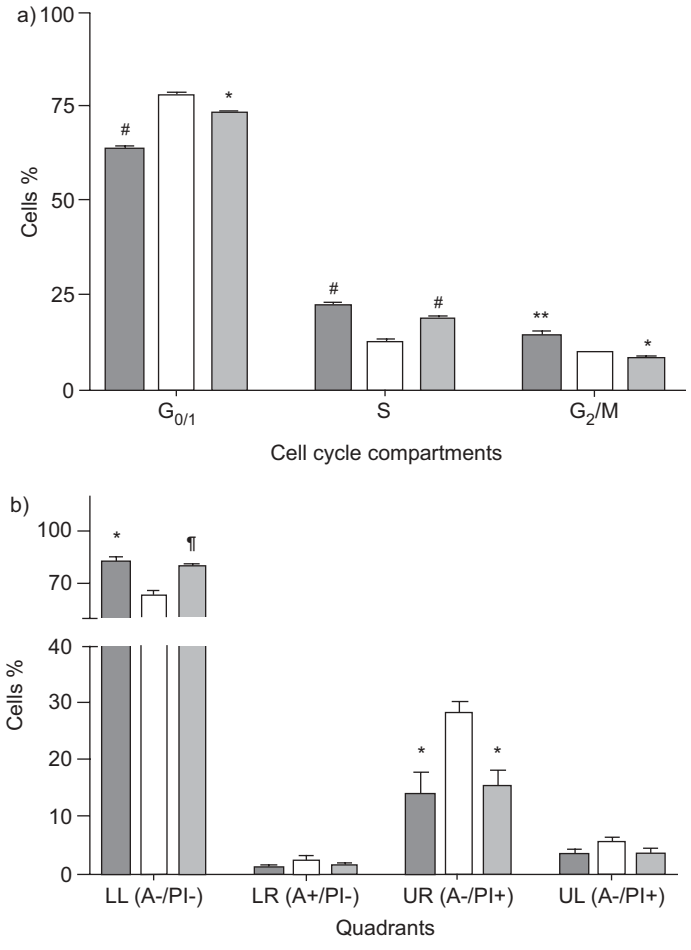

FIGURE 2. a) Effect of 10 μg·mL^{-1} diesel exhaust particles (DEP) on cell cycle of A549 cells following 48 h of incubation. Results are expressed as mean±SEM of at least three experiments. *: $p<0.05$; **: $p<0.01$; #: $p<0.0001$ *versus* serum-free treatment. b) Effect of DEP (10 μg·mL^{-1}) on apoptosis or necrosis of A549 cells following 48-h incubation with serum-free medium (SF), as assessed by flow cytometric analysis of annexin V (A) and propidium iodide (PI). LL: lower left=live cells; LR: lower right=apoptotic cells; UR: upper right=late apoptotic/early necrotic cells; UL: upper left=necrotic cells. Results are expressed as mean±SEM of at least three experiments. *: $p<0.05$; ¶: $p<0.005$ *versus* SF-treated cells.

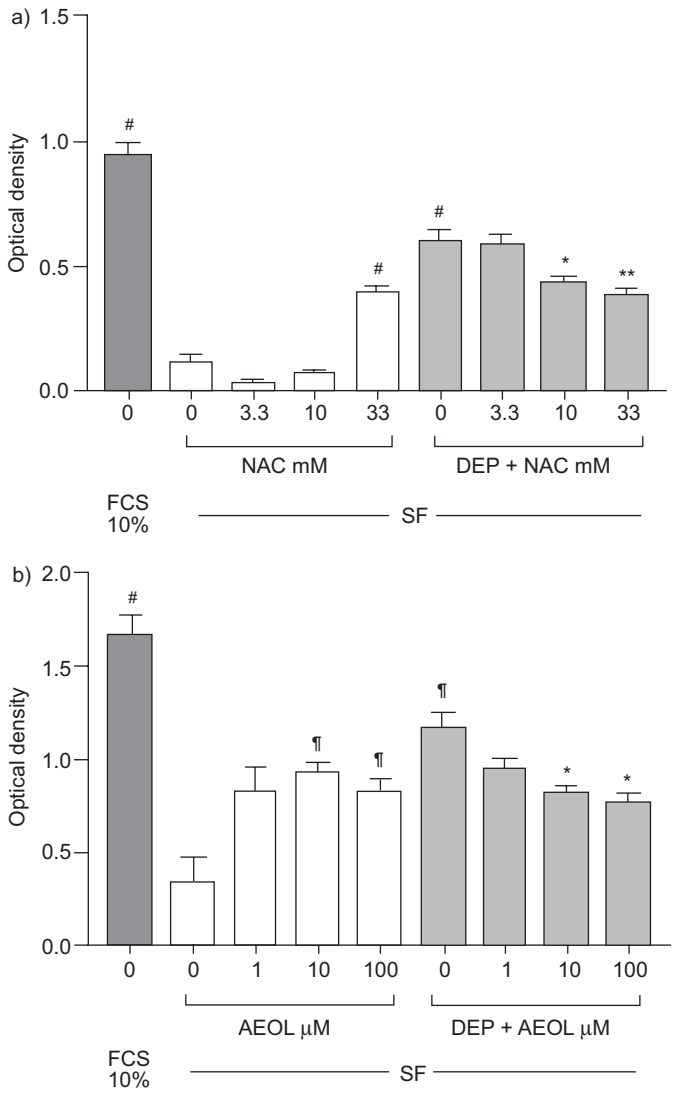

FIGURE 3. Effect of a) *N*-acetylcysteine (NAC) and b) AEOL10113 (AEOL) at 48 h on A549 cell number changes induced by diesel exhaust particles (DEP; 10 μg·mL^{-1}) added to cells grown in serum-free medium (SF). Cell numbers grown in the presence of 10% foetal calf serum (FCS) are shown for comparison. Results are expressed as mean±SEM of at least three experiments. #: $p<0.0001$ *versus* SF; ¶: $p<0.05$ *versus* SF; *: $p<0.05$ and **: $p<0.01$ *versus* DEP.

not in a dose-dependent fashion, with a maximum effect at a concentration of 10 μg·mL^{-1}. The increase in cell death found with serum withdrawal was inhibited by low-dose DEP by at least 50%. DEP also prevented $G_{0/1}$ cell arrest, while elevating the percentage of cells in the S phase of the cell cycle under serum-free conditions. DEP reduced apoptosis of A549 cells, as indicated by a decreased number of cells double-stained with annexin V and PI following 48-h incubation; the percentage of viable cells (annexin V/PI negative) was also increased. Thus, DEP protected against the reduction in cell numbers caused by serum withdrawal; in other words, it induced a proliferative response and inhibited apoptosis that occurred under conditions of serum starvation. Although there are limitations to this data obtained *in vitro* on an alveolar epithelial cell line when extrapolated to the *in vivo* situation, these data would indicate that DEP exposure at a relatively low concentration would cause proliferation of the normal surface epithelium that is not in a state of inflammation. This may in turn lead to an increase in the epithelial layer and possibly to metaplastic changes.

The effect of the antioxidants NAC and AEOL10113 on serum-starved A549 cells was to increase their survival, which was particularly effective with the superoxide dismutase-mimetic AEOL10113. On the other hand, the DEP-induced effects on viability and cell cycle of A549 cells were inhibited to a certain extent by the antioxidants. NAC inhibits activation of JNK, p38-MAPK and redox-sensitive AP-1, and NF-κB transcription factor activities, which regulate the expression of numerous genes [32]. Additionally, NAC prevents apoptosis and promotes survival by activating the extracellular signal-regulated kinase pathway. The effect of these antioxidants was to decrease cells in the S phase, thus arresting DNA replication. Therefore, products of oxidative stress were responsible for the

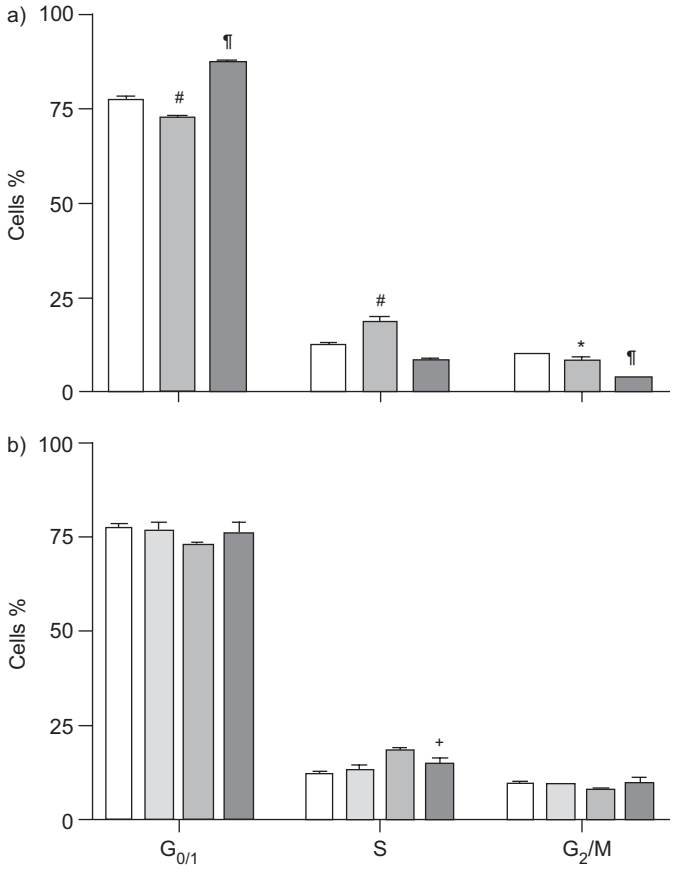

FIGURE 4. Effect of a) *N*-acetylcysteine (NAC) (*: $p<0.05$ and #: $p<0.0001$ *versus* serum-free medium (SF); ¶: $p<0.0001$ *versus* SF+diesel exhaust patients (DEP)) and b) AEOL10113 (10 μM) (+: $p<0.05$ *versus* SF+DEP) on cell cycle progression of A549 cells following 48-h incubation with 10 μg·mL^{-1} DEP. Results are expressed as mean±SEM of at least two experiments (for NAC: three experiments; for AEOL: two experiments). a) □: SF; ■: SF+DEP (10 μg·mL^{-1}); ■: SF+DEP+NAC (10 mM). b) □: SF; ■: SF+10 mM AEOL10113; ■: SF+DEP (10 μg·mL^{-1}); ■: SF+DEP+AEOL10113 (10 μM).

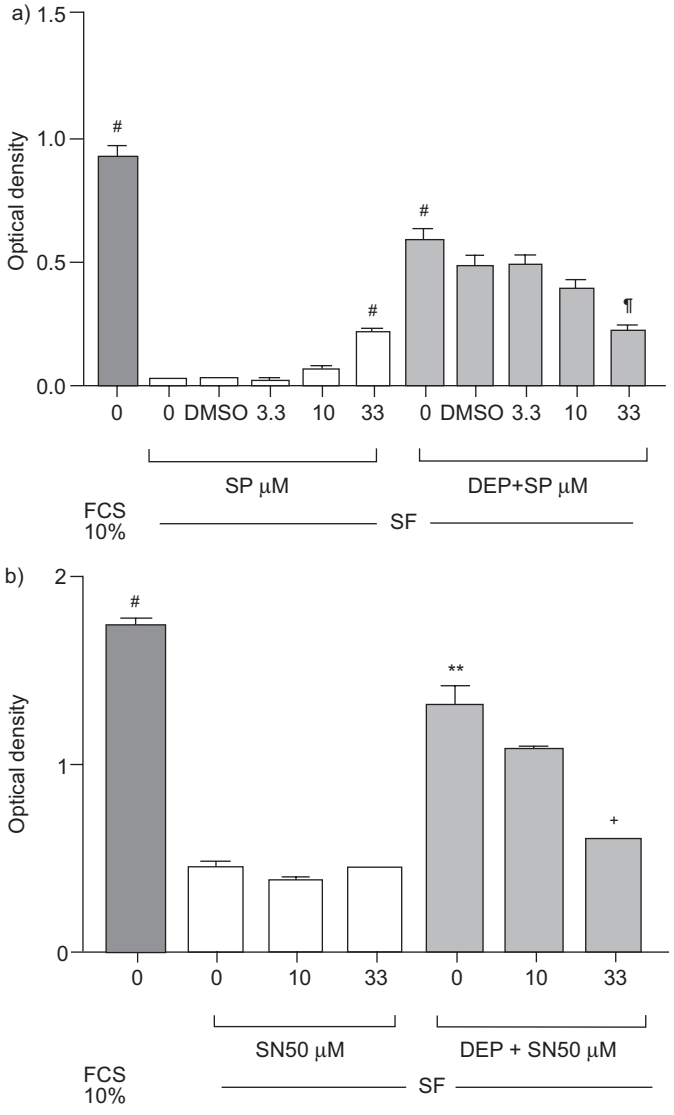

FIGURE 5. Effects of a) SP 600125 (SP), a c-jun N-terminal kinase inhibitor, and b) of SN50, a nuclear factor-κB inhibitor, at 48 h on A549 cell number changes induced by diesel exhaust particles (DEP; 10 μg·mL^{-1}) added to cells grown in serum-free medium (SF). Cell numbers grown in the presence of 10% foetal calf serum (FCS) are shown for comparison. For a), a control dimethyl sulphoxide (DMSO) experiment is shown, as the SP600125 was dissolved in 0.33% DMSO. Results are expressed as mean±SEM of at least three experiments; #: $p<0.0001$ and **: $p<0.01$ *versus* SF; +: $p<0.01$ *versus* DEP; ¶: $p<0.0001$ *versus* 0.33% DMSO+DEP.

effect of DEP on inhibition of apoptosis and on stimulation of cell growth in serum-starved cells. In contrast, on serum-starved cells not exposed to DEP, products of oxidative stress were involved in cell apoptosis and reduced cell growth. These data would indicate that antioxidants may be useful in counteracting the proliferative effect of DEP exposure on the airway epithelium; however, the inhibitory effects of the antioxidants were only partial.

To further elucidate the potential signalling pathways by which DEP effects cell survival, the effects of SP600125, a selective inhibitor of JNK, were examined. SP600125 also inhibited DEP-induced increase in cell viability and, similarly to the effect of the antioxidants, increased cell viability in serum-starved cells. Thus, DEP activation of JNK and of oxidative stress was associated with increases in cell viability, while serum starvation was not only associated with activation of JNK and oxidative stress but with a reduction in cell viability, through the induction of apoptosis. SP600125 has been shown to inhibit proliferation of an adenocarcinoma cell line, KB-3, with a similar dose dependence. Similar effects were obtained when JNK was inhibited with antisense oligonucleotides to JNK2, indicating that the effect of SP600125 was through selective inhibition of JNK activation [19]. JNK activation has been shown in serum-starved A549 cells, coincident with the initiation of apoptosis [24]. The JNK pathway may therefore be implicated in both apoptosis and survival signalling [20].

SN50, which prevents the nuclear translocation of NF-κB, inhibited DEP-induced cell viability but not the effects of serum starvation alone, indicating the importance of NF-κB activation in DEP-induced inhibition of apoptosis. NF-κB

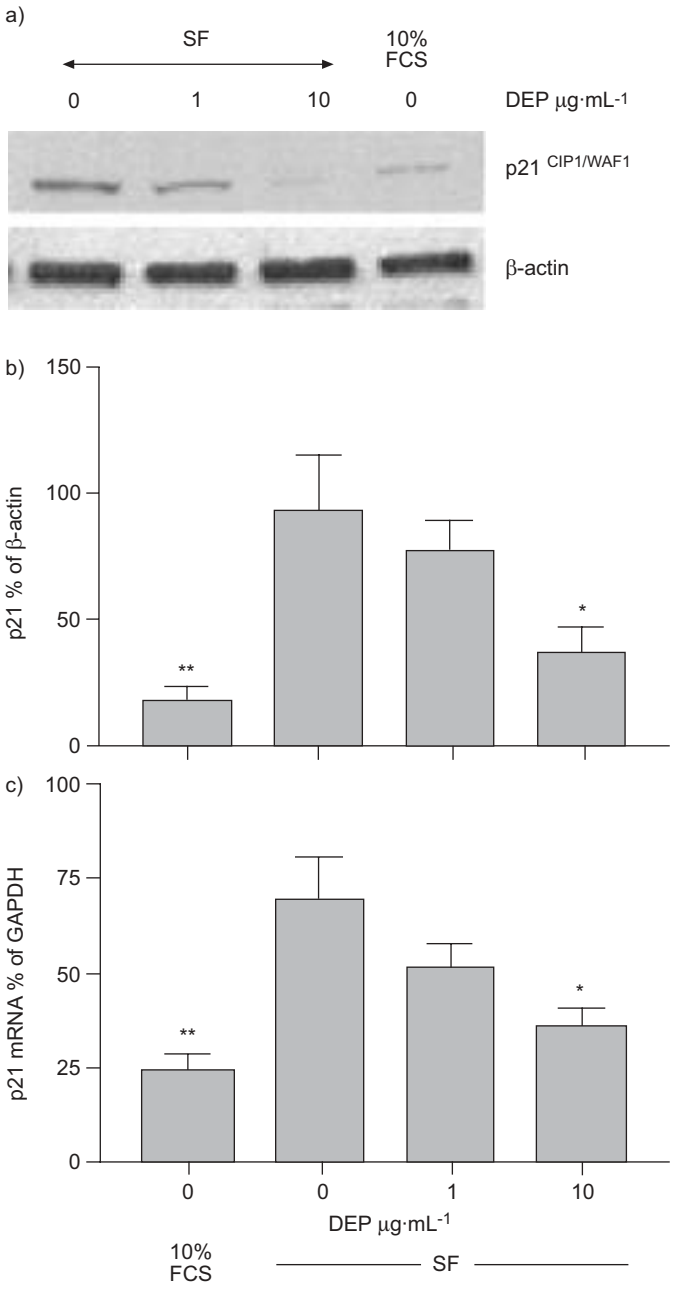

FIGURE 6. Effect of diesel exhaust particles (DEP; 0, 1 and 10 μg·mL^{-1}) on p21$^{CIP1/WAF1}$ protein expression measured by Western blot (a and b) and mRNA expression (c) measured by quantitative PCR of A549 cells following 48-h incubation in serum-free media (SF). a) shows representative Western blots and the effect of culture in 10% foetal calf serum (FCS) alone is shown. Results are shown as mean±SEM of at least three experiments. *: $p<0.05$ and **: $p<0.01$ *versus* cells in serum-free conditions, unexposed to DEP.

transcriptionally activates genes for cytokine, chemokine and adhesion molecules that participate in inflammatory reactions in the lung. The DEP-induced release of cytokines, such as IL-8, GM-CSF and RANTES, and the adhesion molecule sICAM-1 from human bronchial epithelial cells may be secondary to the activation of NF-κB [6, 7]. Exposure of epithelial cells to DEP also leads to the increased expression and secretion of amphiregulin, a ligand of epidermal growth factor receptor, which in turn induces the secretion of GM-CSF [33]. However, it is also possible that amphiregulin directly modulates apoptosis [34].

It has been shown that DEP inhibits serum withdrawal-induced increase in expression of p21$^{CIP1/WAF1}$ mRNA and protein. DEP may exert, at least in part, its preventive effect on apoptosis of A549 cells through inhibition of p21$^{CIP1/WAF1}$, since it is one of the key proteins regulating cell cycle, growth arrest and differentiation [35], and is responsive to oxidative stress [23]. Therefore, DEP inhibition of p21$^{CIP1/WAF1}$ expression may prevent $G_{0/1}$ cell arrest, which was induced by serum removal. A549 cells in the presence of serum starvation expressed high levels of p21$^{CIP1/WAF1}$, while those cultured in 10% FCS had lower levels and, under these conditions, modulation of p21$^{CIP1/WAF1}$ is likely to control the cell cycle since it binds to and inactivates cyclin D/cyclin-dependent kinase complexes.

The adverse effects of DEP on different lung cell types are known to involve oxidative stress pathways [10, 36, 37]. Thus, DEP and their organic extracts induce the generation of reactive oxygen species, such as H_2O_2 and O_2^- in macrophages and human bronchial epithelial cells [13, 38, 39], in turn, activating transcription factors such as NF-κB and AP-1 [10, 11]. Oxidative stress can initiate pro-inflammatory effects in macrophages and bronchial epithelial cells [6, 7, 13, 40, 41] mediated by phosphorylation-dependent cell signalling pathways, including activation of the MAPK pathways. The data indicate that there is a direct effect of DEP, partly through oxidative stress to increase cell numbers by direct inhibition of apoptosis occurring under conditions of serum starvation, with activation of JNK and NF-κB under these conditions.

In conclusion, the current observations may have several implications of clinical relevance. Under the normal situation of an intact epithelium in the absence of serum with no inflammatory response, the epithelial cells are under a balanced turnover of proliferating and apoptotic cells. The data of this study would indicate that, at low levels of exposure, diesel exhaust particles may induce hyperplasia of a normal epithelium by preventing cell apoptosis, perhaps forming the basis for a metaplastic epithelium. However, in the presence of inflammation with serum extravasation, the effects of diesel exhaust particles on cell numbers are masked by those of serum, which itself induces proliferation. Oxidative stress factors may underlie the diesel exhaust particle-induced prevention of apoptosis of epithelial cells and antioxidants can protect against diesel exhaust particle-induced increase in epithelial cells. Inhibitors of nuclear factor-κB and c-jun N-terminal kinase pathways may also be useful.

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