Adenoviral E1A modulates inflammatory mediator expression by lung epithelial cells exposed to PM$_{10}$

TAKESHI FUJIIT, JAMES C. HOGG, NAOTO KEICHO, RENAUD VINCENT, STEPHAN F. VAN EEDEN, AND SHIZU HAYASHI. Adenoviral E1A modulates inflammatory mediator expression by lung epithelial cells exposed to PM$_{10}$. Am J Physiol Lung Cell Mol Physiol 284: L290–L297, 2003. First published October 18, 2002; 10.1152/ajplung.00197.2002.—We examined the hypothesis that ambient particulate matter with a diameter of $<10 \mu m$ (PM$_{10}$)-induced lung inflammation is amplified by latent adenovirus infection. Inflammatory mediator expression in response to PM$_{10}$ exposure was compared between adenovirus E1A-transfected A549 alveolar epithelial cells and cells transfected with control plasmid. Messenger RNA was measured by the RNase protection assay and protein by ELISA or immunocytochemistry. Intercellular adhesion molecule-1 and IL-8 mRNA and protein were increased in E1A-positive cells exposed to 500 $\mu g/ml$ PM$_{10}$. Monocyte chemoattractant protein-1 mRNA and protein were unchanged in E1A-positive cells but increased in E1A-negative cells after 100 and 500 $\mu g/ml$ PM$_{10}$ exposure. Electrophoretic mobility shift assays showed increased NF-$\kappa$B and decreased specificity protein 1 nuclear binding in E1A-positive cells exposed to PM$_{10}$. These results indicate that E1A modulates cytokine and adhesion molecule expression in epithelial cells in a manner that could amplify PM$_{10}$-induced lung inflammation. We suggest that this amplified inflammatory response may contribute to the pathogenesis of exacerbations of chronic obstructive pulmonary disease associated with exposure to particulate matter air pollution.

interleukin-8; monocyte chemoattractant protein-1; intercellular adhesion molecule-1; nuclear factor-$\kappa$B; transcription factor specificity protein 1; particulate matter

RESIDENTS OF COMMUNITIES exposed to high, compared with low, levels of air pollution, especially ambient particulate matter with a diameter of $<10 \mu m$ (PM$_{10}$), are at a greater risk of developing lung disease. Several epidemiological studies have shown that they develop faster rates of decline in lung function and have increased hospital admissions for pneumonia and more chronic obstructive pulmonary disease (COPD) even after adjusting for individual risk factors such as cigarette smoking (10, 30). Although the precise biological mechanisms responsible for this are unclear, PM$_{10}$ are known to stimulate the production of reactive oxygen species and inflammatory mediators by alveolar macrophages (3, 28, 37), airway epithelial (6, 8, 13, 27), and other lung cells (14, 24).

Cigarette smoking is the main risk factor for COPD, because it causes peripheral lung inflammation in everyone; but only 10–20% of chronic heavy smokers develop emphysema and airway obstruction (12). Childhood respiratory infections primarily caused by viruses (reviewed in Ref. 25) are an independent risk factor for the development of COPD (15), and previous work from our laboratory has implicated the E1A gene of adenovirus in the amplification of cigarette smoke-induced lung inflammation in both human and animal studies (11, 25, 26, 31). Investigations into the mechanism of this response in a human tumor cell line with type II cell characteristics (A549 cells) have shown that transfection with the adenovirus E1A gene increased intercellular adhesion molecule-1 (ICAM-1) and interleukin (IL)-8 expression in response to lipopolysaccharide stimulation (21, 22). This upregulation correlated with increased activity of the transcription factor nuclear factor-$\kappa$B (NF-$\kappa$B) in these E1A-positive cells (23).

Gilmour and coworkers (14) have shown that adenovirus E1A increased IL-8 production when A549 cells are exposed to particles. The aim of the current study was to test the hypothesis that the presence of E1A in lung epithelial cells modulates the expression of several inflammatory mediators in response to exposure to ambient particulate matter. The mRNAs for 17 candidate inflammatory mediators were monitored by an RNase protection assay that simultaneously quantifies several mRNA species in a single sample of total RNA. ELISA and immunocytochemistry were used to detect the proteins specified by these mRNAs, and an electrophoretic mobility assay (EMSA) was used to identify transcription factors that were modulated by PM$_{10}$ stimulation of the E1A expressing A549 cells.

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MATERIALS AND METHODS

Particles (PM\textsubscript{10}). PM\textsubscript{10} particles (EHC-93) were collected by the Environmental Health Directorate, Health Canada, Ottawa. A detailed analysis of the EHC-93 has been presented elsewhere (13). Particles were suspended at a concentration of 1 mg/ml in MEM containing 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD), antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Sigma, St. Louis, MO), and Fungizone (1 μg/ml; GIBCO-BRL, Gaithersburg, MD) and were sonicated three times for 1 min each at maximal power on a Vibra Cell VC-50 sonicator (Sonic & Materials, Danbury, CT) before being added to the cells. The endotoxin content of this suspension was measured and reported to be very low (<0.015 EU/ml) (13).

Cell culture. A549 cells (American Type Culture Collection, Manassas, VA), a human lung epithelial cell line, had been stably transfected with the plasmid pE1Aneo, a generous gift from Dr. F. L. Graham (McMaster University, Hamilton, Canada), using polycationic liposomes as previously reported (21). This plasmid carries the promoter and entire coding region of the E1A gene of adenovirus 5. A plasmid generated from pE1Aneo by deleting a 1.8-kb BamHI-SacI fragment containing the E1A DNA was used as a transfection control (21). E1A transfectants (E1A-positive cells) analyzed in this study, E4 and E11, are two independent clones of these transfected cells. The control transfectants (E1A-negative cells) tested here, C4 and C8, are two independent clones of A549 cells transfected with the control plasmid lacking the E1A gene (21). Northern and Western blotting and immunocytochemistry showed that all E1A transfectants produced relatively high levels of E1A mRNA and E1A proteins (21). These transfectants were grown in MEM supplemented with 10% heat-inactivated FBS in 5% CO\textsubscript{2} at 37°C.

E1A-positive cells and E1A-negative cells grown to confluence in MEM plus 10% FBS in 60-mm cell culture dishes (Falcon; Becton Dickinson, Ontario, Canada) were exposed to culture medium or PM\textsubscript{10} suspension to give a final concentration of 100 or 500 μg/ml PM\textsubscript{10} for 1 and 2 h. After being washed twice with PBS, the nuclear proteins were extracted as previously described (23) except for the use of 1,500 di- luted proteinase inhibitor cocktail (Sigma, St. Louis, MO) in the place of phenylmethylsulfonyl fluoride and leupeptin. EMSA of specific nuclear proteins was carried out as previously described (23). Briefly, 10 μg of the nuclear protein extract were incubated at 4°C for 30 min with four volumes of binding buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 4% glycerol) and 1.5 μg of poly[dI- dC] (Boehringer Mannheim, Mannheim, Germany) followed by the addition of [γ\textsuperscript{32}P]ATP end-labeled double-strand consensus oligonucleotides for NFκB: 5'-AGT TGA GGC GAC TTT CCC AGG-3’, AP-1: 5’-CGG TTC ATG AGT CAG CCG GAA-3’, or specificity protein 1 (Sp1): 5’-ATT CGA TCG GGG CGG GCC GAG-3’ (Promega, Madison, WI). Further incubation for 20 min allowed the formation of protein-DNA complexes. For competition assays, excess unlabeled double-stranded oligonucleotides (3.5 pmol) of sequence identical with or unrelated to that of the labeled probe were added to the nuclear extract before addition of the labeled probe. The protein-DNA complexes were separated on a nondenaturing 6% polyacrylamide gel. Gels were dried under vacuum and autoradiographed.

Statistical analysis. Data are expressed as the means ± SE. The minimum number of replicates for all measurement was at least four. Differences between multiple groups were compared by one-way analysis of variance (ANOVA). The
post hoc test for multiple comparison was the Fisher’s protected least significant difference test. Because of the small sample size of the RPA (n = 4), the densitometric results from this assay were also analyzed by the nonparametric Kruskal-Wallis one-way ANOVA. Significance was assumed at P < 0.05.

RESULTS

Effect of PM₁₀ on expression of cytokine and ICAM-1 mRNAs. Representative autoradiographs of cytokine mRNA expression by E1A-negative cells and -positive cells after incubation in medium alone (control) or with 100 or 500 μg/ml of PM₁₀ suspension are shown in Fig. 1. In the absence of stimulation, ICAM-1 and IL-8 mRNA levels were greater in E1A-positive cells than in -negative cells (Fig. 1A). After 6 h of incubation, E1A-positive cells increased expression of both mRNAs in a PM₁₀-dose-dependent manner. In E1A-negative cells, IL-8 mRNA levels increased with dose of PM₁₀, whereas ICAM-1 mRNA was difficult to assess because of very low levels of expression. These IL-8 and ICAM-1 mRNA levels were all lower than those of the E1A-positive cells. TGF-β1 mRNA was constitutively expressed in a dose-dependent manner after 3 h of incubation. Basal levels of MCP-1 mRNA were greater in E1A-negative cells than in E1A-positive cells (Fig. 1B). In E1A-negative cells this mRNA expression was increased by PM₁₀ stimulation in a dose-dependent manner after 3 h of incubation, while E1A-positive cells did not show this effect. LIF and VEGF mRNAs were constitutively expressed by both cell types with a small increase in LIF and little or no difference in VEGF expression with PM₁₀ stimulation (Fig. 1B). Similar results as those reported above for all the mRNAs were also found after 3 or 6 h and after 9 h of incubation (data not shown). In both E1A-negative and E1A-positive cells, PM₁₀ at either concentration did not induce mRNA expression of IL-12, IL-10, MIP-1α, G-CSF, IFN-γ, RANTES, TNF-α, GM-CSF, IL-1β, IL-6, and OSM after 3, 6, and 9 h of incubation (Fig. 1, A and B, and data not shown).

ANOVA of the densitometric results of the four separate RPA confirmed that after stimulation with 500 μg/ml PM₁₀ for 6 h, the levels of ICAM-1 and IL-8 mRNA in E1A-positive cells were significantly higher than those in unstimulated cells or in E1A-negative cells with or without stimulation (Fig. 2, A and B). There was no significant difference between unstimulated E1A-negative cells and E1A-positive cells. E1A-negative cells stimulated with 100 or 500 μg/ml PM₁₀ for 3 h expressed significantly higher levels of MCP-1 mRNA compared with either unstimulated E1A-negative cells or E1A-positive cells with or without stimulation (Fig. 2C). Control values of MCP-1 mRNA were also significantly higher in E1A-negative cells than E1A-positive cells. Similar results were obtained when the data were analyzed by the Kruskal-Wallis test. The levels of LIF mRNA in either E1A-negative or -positive cells were not significantly increased by PM₁₀ stimulation (data not shown).

Effect of PM₁₀ on expression of surface ICAM-1. Figure 3 shows E1A-negative and E1A-positive cells immunocytochemically stained with CD54 to identify ICAM-1 expression. In the absence of stimulation E1A-negative and E1A-positive cells rarely expressed ICAM-1 on the cell surface (Fig. 3, A and C). After
stimulation with 500 μg/ml PM10 for 24 h, E1A-positive cells stained positively for ICAM-1 (Fig. 3D), while E1A-negative cells were unaffected (Fig. 3B).

Effect of PM10 on release of cytokines. The numbers of E1A-negative cells and E1A-positive cells at the time the supernatants were collected for ELISA were 3.0 ± 0.2 × 10^6 cells/well (n = 6) and 4.9 ± 0.2 × 10^6 cells/well (n = 6), respectively. The cytokine concentrations measured by ELISA were corrected for these cell numbers. Figure 4 shows IL-8 and MCP-1 protein levels in supernatants of E1A-negative and E1A-positive cells incubated for 24 h with medium alone (control) or with 100 or 500 μg/ml PM10 suspension. Basal IL-8 protein levels were similar in both cell types. IL-8 production in response to 500 μg/ml PM10 by E1A-positive cells was significantly higher than that of the corresponding unstimulated or 100 μg/ml PM10 stimulated cells and of the E1A-negative cells with or without stimulation (Fig. 4A). In contrast, basal MCP-1 production was significantly higher in E1A-negative cells than positive cells. MCP-1 production by E1A-negative cells increased significantly after 500 μg/ml PM10 stimulation, and this value was higher than those of the E1A-positive cells with or without stimulation (Fig. 4B).

Effect of PM10 on NF-κB, activator protein-1, and Sp1 binding activity. Representative autoradiographs of EMSA to detect nuclear NF-κB, activator protein (AP)-1, and Sp1 binding activity in E1A-negative and -positive cells after 2 h of incubation in medium alone or 500 μg/ml PM10 suspension are shown in Fig. 5. The basal level of NF-κB binding activity in E1A-positive cells was higher than in E1A-negative cells. After PM10 exposure, the binding activity of NF-κB was weakly increased in the nucleus of E1A-negative cells but, in contrast, very strongly increased in E1A-positive cells (Fig. 5A). Basal AP-1 binding activity in the two cell types was similar, and this binding was weakly increased after PM10 stimulation of both cells (Fig. 5B). In contrast to NF-κB, E1A-negative cells showed a higher basal level of Sp1 binding activity than E1A-positive cells. After PM10 stimulation, binding of this transcription factor was increased in E1A-negative cells as well as in E1A-positive cells (Fig. 5C). Specific binding was abolished by competition with excess unlabeled oligonucleotide specific for each transcription factor, whereas the nonspecific competitor was ineffective (Fig. 5, A–C, two lanes at right). Similar results...
were obtained when these experiments were repeated twice for NF-κB and AP-1 binding and three times for Sp1 after 3 h of PM10 stimulation and once for each of these transcription factors after 1 h of stimulation (data not presented).

DISCUSSION

In the present study we showed that exposure of lung epithelial cells to ambient particulate matter stimulates these cells to produce and release a variety of proinflammatory mediators and that this response is further modified by adenovirus E1A. Our data confirmed an earlier report by Gilmour and coworkers (14), who found that E1A increased IL-8 expression by these cells. We also showed that E1A upregulated ICAM-1 expression and that the increased ICAM-1 and IL-8 expression was associated with increased activation of the transcription factor NF-κB. In contrast, the increased MCP-1 expression in response to particulate matter was attenuated by E1A, and this attenuation was associated with decreased activation of the transcription factor Sp1. We suspect that E1A modulation of the inflammatory response of epithelial cells to ambient particles compromises particle processing and increases the particle burden in the lung.

ICAM-1 and IL-8 are major inflammatory mediators important for the site-specific recruitment of neutrophils and have been implicated in neutrophil-mediated injury (32). They coordinate neutrophil migration by promoting firm adhesion of these cells to the endothelium and epithelium and by providing a chemotactic gradient, respectively. The recruitment and activation of neutrophils to the lung are thought to play an important role in the pathogenesis of COPD by creating an imbalance between neutrophil proteases and the antiproteases that results in proteolytic lung tissue damage (2). ICAM-1 has been reported to be upregulated in the epithelium of patients with chronic bronchitis (reviewed in Ref. 21), and this adhesion molecule is overexpressed as emphysematous lung destruction
progresses (31). Studies have shown that particulate matter, such as diesel exhaust particle and silica, stimulates airway epithelial cells to increase ICAM-1 expression (8, 17). Excessive amounts of IL-8 are also present in the airways of patients with COPD (7, 20), and IL-8 production by airway epithelial cells is increased by PM$_{10}$ exposure (6, 8, 13). This suggests that ICAM-1 and IL-8 might be crucial in regulating the local inflammatory response induced by PM$_{10}$ exposure, and our results suggest that latent adenoviral infection might amplify the lung inflammation by increasing the expression of these mediators that promote excess neutrophil recruitment.

The expression of ICAM-1 and IL-8 is regulated by redox-sensitive transcription factors such as NF-$\kappa$B and AP-1 (reviewed in Ref. 23), which are activated in lung cells in response to particles (18). The results presented here show that the levels of binding of nuclear NF-$\kappa$B are enhanced in E1A-positive cells following PM$_{10}$ stimulation. This result is consistent with previous reports using different stimuli, including particles (14, 23), suggesting that NF-$\kappa$B activation is responsible for the upregulation of ICAM-1 and IL-8 genes in E1A-positive cells. Our results also show that AP-1 binding activity was relatively high in both E1A-positive as well as negative cells before stimulation with a small increase in both cell types after PM$_{10}$ stimulation. In contrast Gilmour and colleagues (14) showed a significant elevation of AP-1 binding activity in response to PM$_{10}$ stimulation in E1A-positive cells compared with negative cells. This discrepancy could be due to differences in dose (500 vs. 100 $\mu$g/ml PM$_{10}$) or treatment time (2 vs. 18 h) between our studies and Gilmour’s, respectively. Another variable that could influence the biological response and thus explain this discrepancy is the composition of the particles (37). Gilmour and colleagues (14) used ambient particles with unspecified physical and biochemical properties, whereas the current study used well-characterized ambient particles collected over a major North American city (3, 28, 37).

MCP-1 is a potent monocyte/macrophage chemoattractant that also attracts T lymphocytes and basophils to inflammatory sites (1). Several studies have shown that the conducting airways and lung parenchyma of patients with severe COPD are infiltrated with macrophages and T lymphocytes, as well as neutrophils and B cells (31). Macrophages appear to play a critical role in the pathogenesis of COPD by releasing multiple proteinases, especially matrix metalloproteinases, which by breaking down connective tissue in the lung parenchyma produce emphysema (2, 33). By drawing macrophages into the lung, MCP-1 is implicated in disease progression in COPD, and studies on human lung tissue (7) and in a mouse model (16) support this notion. Boitelle and colleagues (4) showed that increased concentrations of MCP-1 are found in bronchoalveolar lavage fluid from patients with coal workers’ pneumoconiosis and that macrophages, fibroblasts, and type II cells are involved in the enhanced production of MCP-1. This suggests that MCP-1 production in the lung following particle exposure results in the recruitment of monocytes from the circulating blood into the peripheral airways. These monocytes become activated macrophages that generate multiple proteinases and proinflammatory cytokines involved in the development of lesions associated with COPD (33).

Interestingly, our results show that adenovirus E1A represses MCP-1 expression induced by PM$_{10}$ stimulation. This observation seems to contradict the hypothesis that E1A contributes to the pathogenesis of COPD, because an increase in the numbers of macrophages is one of the features of this disease (9, 29, 31). However, localized expression of E1A in widely separated epithelial cells could increase the particle burden in these regions of the lung. Inhaled particles deposited deep in the lung are cleared primarily as intracellular particles within macrophages via the tracheo-bronchial route with minor clearance by transport to the regional lymph nodes (5). Therefore, localized repression of macrophage mobilization could interfere with particle clearance in areas where the adenoviral E1A decreased MCP-1 expression. The overstimulation of the alveolar epithelium by this increased particle burden could result in excess production of IL-8 and increase neutrophil traffic. Furthermore, MCP-1 has been shown to protect mice from lethal endotoxemia possibly by decreasing the production of proinflammatory cytokines such as IL-12 and TNF and by promoting anti-inflammatory cytokine IL-10 production (39). Repression of MCP-1 by E1A could amplify the lung inflammation induced by PM$_{10}$ exposure by removing these protective mechanisms. Therefore, the apparent discrepancy between the well-documented macrophage accumulation in COPD and the results of our studies showing E1A repression of MCP-1 expression in lung epithelial cells could be reconciled by the widespread distribution of E1A-positive cells and might help explain why the disease is localized to certain areas of the lung.

In our studies, adenovirus E1A repression of MCP-1 expression in response to PM$_{10}$ stimulation was preceded by reduced Sp1 binding activity. Sp1 is one of the first sequence-specific eukaryotic transcription factors to be purified and cloned (19), and Sp1 binding sites or GC boxes are found in numerous genes including that of MCP-1. Ueda and colleagues (36) reported that removal of the GC box resulted in complete loss of basal MCP-1 transcription, whereas overexpression of Sp1 increased its transcription, suggesting that Sp1 controls MCP-1 transcription via the GC box. Other studies have shown that the expression of the murine JE gene that is highly homologous to the human MCP-1 was severely reduced in cells expressing adenovirus E1A (35), that E1A binds the transcriptional coactivator p300, and that this coactivator collaborates with Sp1 in activating gene expression (38). Suzuki and colleagues (34) showed that E1A can induce expression of G10BP, which competes with Sp1 for binding to the G-rich DNA sequences and can therefore inhibit binding of Sp1 to its DNA response element. Collectively, these data suggest that the downregulation of MCP-1 induced by adenovirus in the studies reported here
may result from competition for coactivator by E1A and the consequent repression of Sp1 activity.

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