Negative impact of DEP exposure on human airway epithelial cell adhesion, stiffness, and repair

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Doornaert, Blandine, Valerie Leblond, Stephane Galiacy, Gabriel Gras, Emmanuelle Planus, Valerie Laurent, Daniel Isabey, and Chantal Lafuma. Negative impact of DEP exposure on human airway epithelial cell adhesion, stiffness, and repair. Am J Physiol Lung Cell Mol Physiol 284: L119–L132, 2003. First published August 23, 2002; 10.1152/ajplung.00039.2002.—Epidemiological and experimental studies suggest that diesel exhaust particles (DEPs) may be associated with increased respiratory mortality and morbidity. Several recent studies have also shown that DEPs increase the production of inflammatory cytokines by human bronchial epithelium (HBE) cells in vitro. The present study investigates the effects of DEPs on the interaction of HBE cells (16HBE14o) with the cell and matrix microenvironment based on evaluation of integrin-type cell/matrix ligand expression, cytoskeleton (CSK) stiffness, and matrix remodeling via matrix metalloproteinase (MMP)-1, MMP-2, and MMP-9 expression. The results showed that DEP exposure induced: 1) a net dose-dependent decrease in CSK stiffness through actin fibers, 2) a concomitant specific reduction of both α5- and β1-integrin subunits extensively expressed on the HBE cell surface, 3) a decrease in the level of CD44, which is a major HBE cell-cell and HBE cell-matrix adhesion molecule; and 4) an isolated decrease in MMP-1 expression without any change in tissue inhibitor of matrix metalloproteinase (TIMP)-1 or TIMP-2 tissue inhibitors. Restrictive modulation of cell-matrix interaction, cell-cell connection, CSK stiffness, and fibrillary collagen remodeling results in a decreased wound closure capacity and an increased deadhesion capacity. In conclusion, on the basis of these results, we can propose that, in addition to their ability to increase the production of inflammatory cytokines, DEPs could also alter the links between actin CSK and the extracellular matrix, suggesting that they might facilitate HBE cell detachment in vivo.

A PROGRESSIVE CHANGE IN air pollution has been observed over the last 15 years, characterized by high concentrations of atmospheric hydrocarbons, nitrogen oxides, ozone, and especially respirable particulate matter (PM 10, PM 2.5, and now PM 0.1; see Ref. 16). These particles are mainly derived from diesel combustion, which is particularly high in France because of the extensive development of diesel engines (30% of all cars in France in 2000; see Ref. 38). Diesel exhaust particles (DEPs) are composed of a carbonaceous core with adsorbed traces of heavy metals and a vast number of organic compounds, such as polyaromatic hydrocarbons (45). DEPs tend to form aggregates 0.1–0.5 μm in diameter, placing these particles within the respirable range (20).

Airway epithelial cells are the primary target for air pollution and may play a key role in the pathophysiology of airway diseases. Recent studies have emphasized the role of DEPs in the development of an inflammatory response of bronchial epithelial cells. In vitro studies have shown that exposure of human bronchial epithelial (HBE) cells to DEPs significantly increased cell electrical resistance, decreased ciliary beat frequency (4), and induced release of interleukin (IL)-1β, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) inflammatory cytokines (4, 6, 7). However, although the proinflammatory impact of DEPs on bronchial epithelium is starting to be elucidated, little or no information about the other possible biological effects of DEPs is available at the present time. In particular, bronchial epithelial cells establish intercellular tight junctions and adhere to underlying basement membranes, which in turn may regulate bronchial epithelial cell shape, cell proliferation, and differentiation. In pulmonary diseases such as asthma, damage to bronchial epithelium is often associated with disruption of the underlying basement membrane and cell-cell interactions (34). On the basis of these data, it appeared important to determine whether DEPs may impair the interaction of bronchial epithelial cells with the matrix and cellular microenvironment.

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The present study investigates modulation of cell-cell interactions between bronchial epithelial cells in response to DEP exposure, in terms of E-cadherin and CD44 (Cluster of Differentiation) adhesion molecule expression, since E-cadherin is responsible for homotypic adhesive interactions with E-cadherin on the surface of opposing cells (19), whereas hyaluronic acid receptor CD44 has a variety of functions, including participation in cell-cell adhesion by heterotypic and homotypic adhesions as well as cell-matrix interactions (11, 36).

Cell-matrix interactions between bronchial epithelial cells and underlying basement membrane were also investigated by evaluating integrin expression in response to DEPs. Each transmembrane integrin is composed of α- and β-subunit heterodimers, and the combination of a particular β-subunit with a given α-subunit imparts a defined matrix ligand specificity to each integrin (8, 23). In addition, because integrins link the extracellular matrix (ECM) components to the cytoskeleton (CSK) via actin filaments (F-actin), and since the focal adhesion points mediated by these integrins are involved in cell shape, stiffness, and migration, actin CSK stiffness in response to DEP exposure was also investigated.

Adhesive contacts may be broken by extracellular proteolytic matrix metalloproteinases (MMPs). MMPs degrade all components of the ECM and play key roles in normal physiological processes involving ECM remodeling, such as wound healing, angiogenesis, or development. More particularly, MMP-1 cleaves fibrillar collagen at a unique site in the triple helix of the protein, whereas MMP-2 and MMP-9 (gelatinases A and B, respectively) specifically degrade gelatin and type IV collagen, and to a lesser extent, laminin, entactin, fibronectin, fibrillin, and elastin. Our previous studies reported that these two gelatinases were expressed by HBE cells (9, 30, 46). MMP activity is also regulated by specific tissue inhibitors of matrix metalloproteinases (TIMPs; see Ref. 35), and our previous studies have demonstrated that the two tissue inhibitors (TIMP-1 and TIMP-2) were also expressed by HBE cells (47, 48). The balance between these MMPs and TIMPs present in these cells in response to DEP exposure was therefore examined.

Overall, our results consistently showed that DEPs are able to alter bronchial epithelial cell-matrix interactions and epithelial cell-cell connections and ECM remodeling, in association with increased deadhesion cell capacity and a decreased rate of cell repair in response to mechanical injury.

MATERIALS AND METHODS

Cell Cultures

All parameters were investigated in cultures of a HBE cell line (16HBE14o-), allowing us to perform a large number of reproducible experiments by using methods as different as flow cytometry, magnetic twisting cytometry (MTC), or gel zymography. Some primary HBE cell cultures were also carried out to compare the data obtained with the 16HBE14o- cell line.

Isolation and culture of primary HBE cells. HBE biopsies were obtained by fibroscopy in several patients investigated for lung cancer. Biopsies were taken away from the tumor. Pathological examination confirmed the presence of normal bronchial mucosa on each specimen. All the patients whose biopsies were used for collection of epithelial cells gave their informed written consent, and the experimental design was approved by a relevant ethical committee (Comité Consultatif pour la Protection des Personnes dans la Recherche Médicale) in accordance with good clinical practice and French bioethical laws.

Primary (p) HBE cells were cultured according to the modified method of Baeea-Squiban et al. (3). Two or three explants (0.5 × 0.5 mm) were placed on sterile 35-mm plastic dishes (Nunc, Napperville, IL) coated with a collagen G matrix (bovine type I and III collagens; Biochrom, Berlin, Germany). The explants were covered with 600 μl of culture medium and incubated for 24 h. Culture medium (2 ml) was then added to each dish. The culture medium consisted of serum-free DMEM-Ham’s F-12 (1:1; Life Technologies, Cergy-Pontoise, France) supplemented with 2% Ultroser G (UG; Life Technologies), 1% antibiotics (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate), 25 μg/ml amphotericin B (Life Technologies), and 2 mM glutamine (Life Technologies) at a final concentration. Explants were placed in a humidified incubator at 37°C in 5% CO2-95% air. The culture medium was changed every 2 days. Cells were cultured for 2 wk until confluence of p-HBE cells. The same explants were also successively transferred to new sterile coated plastic dishes, at 5- to 8-day intervals, to initiate new p-HBE cell cultures.

HBE cell line culture. The 16HBE14o- cell line (a gift from Dr. D. C. Gruenert, University of Vermont, to B. Housset, Centre Hospitalo-Universitaire Henri Mondor, Créteil, France) was cultured on collagen G- and matrix-coated dishes. Different dish types (Nunc) were used depending on the experiment: 48-well plates for cytotoxicity assays, 24-well plates for wound healing assays, 8 Lab-Tek wells for actin structure investigation and for proliferating cell localization plates for wound healing, 32-mm2 dishes for MTC, and 25-cm2 flasks for flow cytometry assays. The culture medium was the same as that described above. Experiments were performed at passages 25 to 40, and all cultures were incubated in a humidified incubator at 37°C in 5% CO2-95% air. The culture medium was changed every 2 days.

Cell treatment by DEPs or carbon black. Carbon black, which represents the carbonaceous core of DEPs, was obtained from Sigma (L’Ile d’Abeau Chêne, France) and was used to test the involvement of the carboneous core in comparison with entire DEPs. Diesel PM SRM 1650 was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). Stock solutions of particles were dispersed in 0.04% dipalmitoyl phosphatidylcholine (DPC; Sigma) in distilled water and then sonicated in ice for 5 min at maximum power (8 kilocycles). As previously described (6), DPC was used to mimic the lung surfactant lipid, and entire DEPs or carbon black were used at the final concentration of 1–100 μg/ml in the cell culture medium. Equivalent volumes of DPC alone were also used as DPC controls for each particle concentration. Subconfluent cultures of 1-HBE cells were treated in the presence of particles for 24 h and compared with nontreated cells (controls) or DPC-treated cells. Concerning p-HBE cells, they were first incubated in culture medium for 24 h (control cells) and then treated with DPC.
saponin (25 min. Glutaraldehyde was removed, and samples were rinsed in PBS supplemented with 1% glutaraldehyde (Sigma) for 15 min. Cells were then fixed with 4% paraformaldehyde, and the suspension was centrifuged and incubated for 30 min at 4°C. CSK and Actin CSK Stiffness Evaluation by MTC

Control or treated hBE cells were incubated in UGM-free medium supplemented with 1% BSA (Sigma) for 30 min at 37°C to block nonspecific binding. Carboxyfluorescein diacetate (CFDA) was added to the culture. The CFDA was allowed to enter the cell for 30 min at 37°C, and the cells were then washed with PBS. The CFDA was then reduced with 10% DMSO for 5 min. The excess DMSO was then removed, and the cells were incubated with 1% BSA for 30 min before use. Beads were then incubated with the cells (30 µg/dish) for 30 min at 37°C in 5% CO₂/95% air, and unbound beads were washed away with serum-free medium supplemented with 1% BSA. Dishes were then placed in the magnetocytometer. A brief 1,500-G magnetic pulse was then applied to magnetize all surface-bound beads in a unique horizontal direction, and a magnetic torque was then generated by applying an orthogonal uniform magnetic field (42 G). Associated changes in angular strain of the beads were measured by an on-line magnetometer and subsequently transformed to analog data with an acquisition system (AcqKnowledge III; BIOPAC). Stiffness was defined as the ratio of stress to angular strain (43). This ratio measures the cell's ability to resist localized deformation, corresponding to CSK stiffness (Pa). After a first measurement of CSK stiffness, cytochalasin D (cyto D; Sigma), an agent that depolymerizes actin microfilaments, was added (10 µg/ml) for 25 min, and CSK stiffness was measured again. The difference of CSK stiffness before and after cyto D was calculated (Pa) and was representative of actin CSK stiffness or cell tone.

Quantification of HBE Cell Adhesion Molecule Level by Flow Cytometry Assays

Expression of cell surface adhesion molecules. All washings were performed in PBS supplemented with 0.01% NaN₃ (Sigma). All centrifugations (500 g) were performed for 10 min at 4°C. hBE or p-HBE cells were detached by trypsinization and incubated with 1% BSA for 30 min at 4°C in PBS supplemented with 0.01% NaN₃ and 10% AB human serum (ABHys, Paris, France) to avoid nonspecific binding to Fc fragments. Cells (10⁶ to 2 × 10⁶) were then incubated with saturating concentrations of either adhesion molecule-specific antibodies, purified mouse or rat isotype-matched controls (IgG1, IgG2a, or IgG2b; Immunotech, Marseille, France), or preimmune goat serum (Life Technologies) for 30 min at 4°C.
4°C. We used monoclonal antibodies for α1, α3, α5, α6, αv, α1, α2, α3, α4, α5, α6, αv, β1, β2, β3, CD44, E-cadherin antigens (Immunotech), or for βa (Novocastra Laboratories, Newcastle, UK) and polyclonal antibodies for βγ and βα-antigens (Santa Cruz Biotechnology). After incubation with specific antibodies or isotype-matched controls, cells were washed two times with PBS supplemented with 0.01% NaN3 and labeled with 50 μl appropriate secondary antibody for 30 min (either FITC-conjugated F(ab)2 rabbit anti-mouse immunoglobulins (DakoPatts, Glostrup, Denmark), FITC-conjugated F(ab)2 goat anti-rat immunoglobulins, or FITC-conjugated F(ab)2 rabbit anti-goat immunoglobulins (Jackson Immunoresearch Laboratories)). These secondary antibodies were diluted in rabbit anti-goat immunoglobulins (Jackson Immunoresearch Laboratories). The activity of gelatin-degrading enzymes (MMP-2 and MMP-9) were identified by semiautomated image analysis (Scion image), which evaluates both the surface and intensity of lysis bands after gel scanning.

**ELISA assays**

The levels of MMP-1, TIMP-1, and TIMP-2 released in cell culture media were measured by human MMP-1, TIMP-1, and TIMP-2 ELISA kits (Amersham), according to the manufacturer's recommendations.

**Cell Deadsorption**

After reaching confluency, 1-HBE cells were dissociated with enzyme-free dissociation buffer and the number of detached cells was counted with an hemocytometer (Calibro device) every 5 min for 40 min.

**In Vitro Wound Healing Assay**

The in vitro wound healing assay was carried out as previously described (32, 37). A linear wound was made by gently scratching a subconfluent culture of 1-HBE cells with a pipette tip followed by extensive rinsing with culture medium to remove all cellular debris. The area of denuded surface was quantified for 24 h. The plate was placed on an inverted microscope (Zeiss, Rueil-Malmaison, France), and an image was obtained by using a charge-coupled device camera (CCD-IRIS, Sony) connected to the microscope. The image was subsequently captured by an image-analyzing frame-grabber (Video enhancer FA320; FUTEK) and analyzed by image analysis software (Scion Image 1.3b). Wound closure was evaluated as the average surface covered by the cells after 3.5, 7, and 24 h of incubation and expressed as a percentage of exposed surface compared with the covered surface in the DPC control.

**Assessment of Cell Proliferation by Bromodeoxyuridine Incorporation**

Localisation of cell proliferation. 1-HBE cells were plated on Lab-Tek 8 wells (Nunc) dedicated for immunofluorescence assay. At subconfluence, cells were either treated or not treated by particles or DPC for 24 h. A linear wound was then made as described above. Cultures were incubated for another 7 h at 37°C in humidified incubators under 5% CO2 in air, and 5-bromo-2′-deoxyuridine (BrDU) incorporation and detection were performed as previously described (17) using a BrDU Labeling and Detection Kit according to the manufacturer's recommendations (Boehringer-Mannheim). Samples were stored at 4°C overnight, and localization of cell proliferation was analyzed by laser confocal microscopy using an LSM 410 inverted microscope.

**Gel Zymography**

Culture supernatants were centrifuged, and samples were frozen at −20°C until use. Collected media were resolved by 8% SDS-PAGE containing 1 mg/ml copolymerized porcine skin gelatin according to the method of Heussen and Dodwell (21), excluding any reducing agents or boiling procedures. After electrophoresis, the gel was washed for 30 min in 2.5% Triton X-100 at room temperature to remove SDS. The gel was then incubated overnight at 37°C in reaction buffer (100 mM Tris-HCl and 10 mM CaCl2 pH 7.4). After being stained with Coomassie Brilliant blue G-250 (ICN Biomedicals, Aurora, OH), gelatin-degrading enzymes (MMP-2 and MMP-9) were identified as clear zones of lysis against a blue background. Molecular masses of gelatinolytic bands were estimated using prestained molecular mass markers (Amersham, Buckinghamshire, UK). Activities in gel slabs can be quantified using semiautomated image analysis (Scion image), which evaluates both the surface and intensity of lysis bands after gel scanning.
assessed by the Kolmogorov-Smirnov test, using CellQuest 3.1 software (Becton-Dickinson).

RESULTS

Cytotoxicity of DEPs

DEPs, carbon black, and DPC cytotoxicity was evaluated on the basis of l-HBE cell staining by Neutral Red dye after 24, 48, and 72 h of treatment. A net significant cytotoxicity was found for the highest concentration of DEPs (100 μg/ml) and was time dependent since 31 ± 4 and 83 ± 8% of cells had died at 48 and 72 h, respectively. Lower levels of cytotoxicity were observed with carbon black (100 μg/ml) or equivalent DPC exposure for 48 and 72 h. Indeed, significant toxicity was observed in 20 ± 4 and 36 ± 5% of cells in response to 72 h of exposure to carbon black and DPC, respectively. The reliability of the method was attested by the high level of cytotoxicity (98%) induced by 5% DMSO at all times and by the lack of staining interference with collagen G matrix alone. No cytotoxicity was found after 24 h of treatment, whatever the treatment and the dose. This absence of any cytotoxicity was corroborated after 24 h of treatment, using MTT assay (data not shown). Because our results for longer culture times showed a time- and dose-dependent cytotoxicity induced by DEP exposure, as previously described (6, 40), subsequent investigations of DEP effects were evaluated on l-HBE cells cultured for 24 h.

DEPs Are in Contact with the Cell Membrane and May Be Phagocytosed by HBE Cells

The number of l-HBE cells that were in contact with DEPs and carbon black particles at different concentrations (5, 20, and 100 μg/ml) and/or that have phagocytosed these particles was approximately evaluated by optical microscopy by counting the cells with an hemocytometer (Malassez device). Carbon black and DEP cell phagocytosis and/or cell contact was a dose-dependent process, and DEPs appeared to be more intensely in contact with the cell membrane and more phagocytosed than carbon black (Table 1).

Table 1. Evaluation by optical microscopy of cell percentage in association with DEP or carbon black particles

<table>
<thead>
<tr>
<th>Concentration, μg/ml</th>
<th>Carbon black</th>
<th>DEPs</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>10.6 ± 4.3</td>
<td>12.7 ± 4.6</td>
</tr>
<tr>
<td>20</td>
<td>20.0 ± 7.0</td>
<td>39.1 ± 8.8*</td>
</tr>
<tr>
<td>100</td>
<td>37.4 ± 10.0</td>
<td>57.6 ± 13.0*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE; n = 4 experiments. After exposure of 1-human bronchial epithelial (HBE) cells to diesel exhaust particles (DEPs) or carbon black for 24 h, cells were dissociated with enzyme-free dissociation buffer. Cells remaining in contact with particles and cells having phagocytosed them were counted with an hemocytometer. A significant dose-dependent increase in the percentage of cells with associated particles was observed in response to DEP exposure (*P < 0.01) compared with carbon black exposure.

Flow cytometry assays were also performed for quantitative determination of DEP phagocytosis and/or DEP cell contact. The DPC solution used to disperse the particles had no effect on the SSC parameter compared with control l-HBE cells. Subconfluent l-HBE cells were exposed to carbon black (CB) or DEPs at a concentration of 5, 20, or 100 μg/ml or with equivalent volumes of dipalmitoyl phosphatidylcholine (DPC) for 24 h. After treatment, cells were dissociated in free-enzyme dissociation buffer and fixed in CellFix. A: side scatter (SSC) parameter corresponding to DPC and particle exposure (100 μg/ml). B: amplification of l-HBE autofluorescence in response to particle exposure (100 μg/ml).

Fig. 1. Evaluation by flow cytometry of diesel exhaust particle (DEP) phagocytosis and l-human bronchial epithelium (HBE) cell contact. Subconfluent l-HBE cells were exposed to carbon black (CB) or DEPs at a concentration of 5, 20, or 100 μg/ml or with equivalent volumes of dipalmitoyl phosphatidylcholine (DPC) for 24 h. After treatment, cells were dissociated in free-enzyme dissociation buffer and fixed in CellFix. A: side scatter (SSC) parameter corresponding to DPC and particle exposure (100 μg/ml). B: amplification of l-HBE autofluorescence in response to particle exposure (100 μg/ml).
DEPs Are Engulfed by F-actin

Localization and distribution of F-actin staining were observed by confocal microscopy by performing optical cell sections every 1 μm from the apical to basal cell surface and after image reconstruction (Fig. 2). In the middle section of the confluent control and DPC-treated l-HBE cells (Fig. 2, A and B, respectively), intense and dense actin staining was observed at the cell periphery, whereas weak and scattered staining was observed in the cytosol. In carbon black-exposed l-HBE cells, internalized particles were clearly observed in the absence of any fluorescence (Fig. 2Cr), whereas little or no organized F-actin staining was observed around these internalized particles on adjacent serial sections (Fig. 2Cl). In DEP-exposed l-HBE cells, phagocytosed particles and particles located close to the cell membrane were observed in the absence of fluorescence (Fig. 2Dr), whereas colocalized and well-organized F-actin staining was observed on adjacent serial sections and generally engulfed these particles (Fig. 2Dl). Figure 2E presents a higher-power view of F-actin engulfing DEPs.

DEP Exposure Induces a Dose-Dependent Decrease in Actin CSK Stiffness

Results for CSK stiffness and actin CSK stiffness (corresponding to the difference between CSK stiffness before and after cyto D) are shown in Fig. 3. The presence of DPC in the culture medium did not affect CSK stiffness or actin CSK stiffness (data not shown) of l-HBE cells. In contrast, DEP cell exposure induced a similar significant decrease in CSK stiffness, i.e., 24.4, 21, and 20% for the three levels of DEP concentration used (5, 20, and 100 μg/ml) and a dose-dependent decrease in actin CSK stiffness (34.4, 50, and 55%, respectively). No change in CSK stiffness or actin CSK stiffness was detected in response to DEP exposure at the lower concentration of 1 μg/ml (data not shown), confirming the dose-dependent response. Effects induced by carbon black exposure were usually associated with no change or a nonsignificant decrease in CSK stiffness and actin CSK stiffness (data not shown).

DEPs Induce a Decrease in α₃ (CD49)- and β₁ (CD29)-Integrin Subunits and CD44 Receptor Expression at the l-HBE Cell Surface

In the absence of DEPs, our results showed a similar pattern of adhesion molecule expression both by pc...
DEP exposure induced a decrease in intracellular distribution of \( \alpha_3 \)- and \( \beta_1 \)-integrin subunits and CD44 expression.

To test whether cell internalization or intracytoplasmic retention of the \( \alpha_3 \), \( \beta_1 \), and CD44 receptors was a possible mechanism to explain decreased expression of these molecules at the cell surface, double cell membrane and internal pool immunostaining of both integrins and CD44 receptor were performed. This double immunostaining showed the presence of a relatively large internal pool (~20%) comprising \( \alpha_3 \)- and \( \beta_1 \)-subunits and a smaller internal pool (8%) for CD44 (Table 2). The results both confirmed the dose-dependent decrease in \( \alpha_3 \), \( \beta_1 \), and CD44 expression on l-HBE cell surface in response to 20 and 100 \( \mu \)g/ml DEP exposure and clearly demonstrated a similar decrease in their intracellular counterparts. These results therefore supported that decreased expression of integrins and CD44 on the cell surface was not because of the increased cellular internalization of these molecules but was rather because of their global decreased expression by cells in response to DEP exposure.

DEP Phagocytosis Is Not Required to Induce a Reduction of \( \alpha_3 \), \( \beta_1 \), and CD44 Expression at the HBE Cell Surface

To assess whether or not DEP phagocytosis is required to induce a decrease in \( \alpha_3 \), \( \beta_1 \), and CD44 expression at the cell surface, l-HBE cells were exposed for...
24 h to DEPs (100 μg/ml) in the presence of cyto D (10 μg/ml). As reported above, cyto D is an agent that disrupts actin microfilament polymerization, which is necessary for particle phagocytosis (1), since the presence of cyto D for 24 h actively inhibited DEP phagocytosis (50–70%) but inhibited carbon black phagocytosis to a lesser extent (10%). The results summarized in Fig. 5 show that 1) cyto D treatment alone induced a reproducible and significant increase in the expression of β1, α5, and CD44 adhesion molecules, by 18, 26, and 39%, respectively; 2) cyto D treatment in the presence of DEP did not prevent the decreased expression of β1, α5, and CD44 adhesion molecules; and 3) the decreased expression of β1, α5, and CD44 adhesion molecule in the presence of cyto D (39, 47, and 37%, respectively) was therefore clearly higher than the decrease observed in the absence of cyto D (29, 35, and 22%, respectively).

**DEP Exposure Induces a Specific Decrease in MMP-1 Protein Expression with No Change in TIMP-1 and TIMP-2 Expression by p-HBE and l-HBE Cells**

To assess whether DEP exposure was associated with a change in ECM remodeling, the balance of MMPs/TIMPs was investigated. MMP-2, MMP-9, MMP-1, TIMP-1, and TIMP-2 protein expression was investigated in p-HBE and l-HBE cell supernatants. Gelatin gel zymography results clearly showed that MMP-2 and MMP-9 gelatinase protein expression, released for 24 h in p-HBE and l-HBE cell supernatants, in proactivated and activated forms (72 and 68 kDa for MMP-2 and 92 and 88 kDa for MMP-9), was unchanged in response to DPC, DEP, or carbon black exposure, regardless of the dose and regardless of the type of cell culture. Figure 6 shows gel zymography results obtained for the highest dose of particles. Concomitant TIMP-1 and TIMP-2 expression evaluated by ELISA assays also remained constant (Fig. 7). In contrast, exposure to the highest DEP dose (100 μg/ml) induced a significant net decrease in MMP-1 protein expression and a discrete nonsignificant decrease at the lower DEP dose (20 μg/ml), whereas DPC or carbon black exposure did not alter this expression (Fig. 7). However, this MMP-1 decrease was only observed in p-HBE cells.

**Table 2. Inhibitory effect of DEP exposure (100 μg/ml) on 1-HBE cell membrane and intracellular expression of CD29 (β1), CD49 (α5), and CD44**

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Untreated Cells</th>
<th>DPC-treated Cells</th>
<th>DEP-treated Cells (100 μg/ml)</th>
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</thead>
<tbody>
<tr>
<td>CD29 n</td>
<td>534</td>
<td>539</td>
<td>368*</td>
</tr>
<tr>
<td>i</td>
<td>190</td>
<td>219</td>
<td>126*</td>
</tr>
<tr>
<td>CD44 n</td>
<td>805</td>
<td>851</td>
<td>578*</td>
</tr>
<tr>
<td>i</td>
<td>71</td>
<td>121</td>
<td>83*</td>
</tr>
<tr>
<td>CD49c n</td>
<td>310</td>
<td>303</td>
<td>164*</td>
</tr>
<tr>
<td>i</td>
<td>73</td>
<td>76</td>
<td>51*</td>
</tr>
</tbody>
</table>

After exposure of 1-HBE cells to DEPs or carbon black or equivalent volumes of dipalmitoyl phosphatidyl choline (DPC) for 24 h, cells were studied as described in MATERIALS AND METHODS. Adhesion molecule expression was evaluated by flow cytometry, and data were expressed as mean fluorescence intensity/cell, based on 10⁴ cells. Expression of both intracellular (i) and cell membrane (m) pools of each adhesion molecule differed in response to DEP exposure compared with DPC treatment or with controls (*P < 0.001).
DEP Exposure Is Associated with an Increase in l-HBE Cell Deadhesion Capacity

HBE cells were left untreated or were treated with DPC, carbon black, or DEP for 24 h and then incubated for 40 min in enzyme-free cell dissociation buffer. Kinetic evaluation of cell detachment was performed every 5 min for 40 min. The results presented in Fig. 8 indicate that DEP exposure induced a dose-dependent amplification of cell detachment at the early times, since, after 5 min of incubation, 4% of l-HBE cells were still detached in response to 5 μg/ml DEP, whereas only 0.5% was detached in response to an equivalent dose of DPC. After 10 min of incubation, 60% of cells were detached, whereas only 22% of cells were detached in the presence of DPC alone. For a concentration of 20 μg/ml DEPs, the percentage of detached cells was 3% at 5 min and 82% at 10 min vs. 0.8 and 32%, respectively in the presence of DPC alone. Previous exposure to carbon black induced no change or a nonsignificant increase in the percentage of detached cells (data not shown).

DEP Exposure Reduces l-HBE Cell Wound Repair Capacity

Kinetic studies of cell wound repair capacity were performed 3.5, 7, and 24 h after a linear mechanical wound performed on l-HBE cell confluent cultures. Wound closure, expressed as the average percentage of surface exposed compared with the surface covered in DPC controls, was not significantly changed in response to carbon black exposure. In contrast, DEPs inhibited wound closure in a dose-dependent fashion (Fig. 9). At 24 h, the wound was totally closed regardless of the type of treatment.

Alteration of l-HBE Cell Repair Capacity Induced by DEP Exposure Is Partially Due to Decreased Cell Proliferation

At 7 h postwound, l-HBE cell proliferation was localized by immunofluorescence (BrDU incorporation and detection) and was similar at the edge of the wound and any other site of the cell culture (data not shown). To more precisely quantify a possible discrete change in HBE cell proliferation in response to DEP exposure, BrDU-specific fluorescence was analyzed by flow cytometry on subconfluent l-HBE cells. A weak and nonsignificant increase in cell proliferation was observed in response to DPC volume used to disperse DEPs at a concentration ≥20 μg/ml. Com-

Fig. 5. Maintenance of decrease in CD29 (β1), CD49 (α3), and CD44 expression induced by DEP exposure in response to concomitant cyto D treatment (A, B, and C, respectively). Subconfluent l-HBE cells were treated for 24 h with either 100 μg/ml DEPs or CB or DPC alone, with 10 μg/ml cytochalasin D alone, or with particles in the presence of cytochalasin D. Cells were then dissociated with enzyme-free dissociation buffer, incubated with adhesion molecule-specific monoclonal antibodies or isotype-matched controls, and were then treated as described in MATERIALS AND METHODS. Data obtained by flow cytometry were expressed as MFI/cell, taking into account 10^4 cells, and are representative of 3 independent experiments. Expression of each adhesion molecule at the cell surface significantly differed in response to cytochalasin treatment alone and to DEP exposure with or without concomitant cytochalasin D treatment (**P < 0.001).

Fig. 6. Effect of DEP exposure on matrix metalloproteinase (MMP)-2 and MMP-9 protein expression (gel zymography). l-HBE cells were exposed to 5, 20, or 100 μg/ml DEPs or CB or equivalent volumes of DPC for 24 h. Collected media were resolved by 8% SDS-PAGE in the presence of 1 mg/ml porcine skin gelatin as described in MATERIALS AND METHODS. Molecular weights of gelatinolytic bands were estimated using prestained molecular mass markers. MMP-2 and MMP-9 protein expression appeared unchanged in response to 100 μg/ml DEP or CB or DPC exposure.
pared with an equivalent amount of DPC, DEP exposure induced a dose-dependent decrease in cell proliferation (Fig. 10) that was decreased by 26 and 35% in response to DEP exposure at the concentrations of 20 and 100 μg/ml, respectively. The effects of carbon black were not reproducible and were associated with no significant effect on cell proliferation (data not shown).

**DISCUSSION**

The main objective of the present study was to determine whether DEP exposure could alter the balanced interaction between HBE cells and their matrix microenvironment. Our data clearly demonstrate that DEP exposure induces a dose-dependent reduction of l-HBE cell actin CSK stiffness and matrix adhesion molecule expression, associated with impaired wound repair capacities and increased cell deadhesion potential. These results therefore suggest that L-HBE cells exposed to DEP pollution might lose their capacity to interact with the environmental ECM, thereby accentuating their shedding susceptibility.

**Fig. 8.** Stimulatory effect of DEP exposure on cell deadhesion capacity. Subconfluent l-HBE cells were exposed to 5 or 20 μg/ml DEPs or CB or to equivalent volumes of DPC for 24 h. After exposure, cells were incubated in enzyme-free dissociation buffer, and the number of detached cells was evaluated every 5 min with an hemocytometer (Malassez device). Data are cell detachment kinetics representative of 3 independent experiments carried out in the presence of 5 or 20 μg/ml DEPs. The percentage of detached cells differed in response to DEP exposure at the early times.

**Fig. 9.** Inhibitory effect of DEP exposure on cell repair capacity after in vitro mechanical wound healing. l-HBE cells were seeded on 24-well plates. After confluence, the cells were exposed to 1, 5, 20, or 100 μg/ml DEPs or CB or equivalent volumes of DPC for 24 h. Cell monolayers were then scratched with a pipette tip and extensively rinsed with medium to remove all cellular debris. Wound closure was observed at 3.5, 7, and 24 h, as described in MATERIALS AND METHODS. Wound closure was measured as the average surface covered by the cells (mm²). Results are expressed as the mean percentage of uncovered surface compared with the covered surface in the presence of DPC. Data illustrate 1 representative experiment (sexuplicates) among 3 independent experiments. The rate of wound healing in response to DEP exposure significantly differed compared with CB.
DEPs Are in Contact with the Cell Membrane and May Be Phagocytosed by l-HBE Cells

First, to more clearly define our cell culture model, we analyzed contacts between l-HBE cells and DEPs by using three different approaches. Our optical microscopy analysis (Table 1), SSC determination by flow cytometry (Fig. 1A), and PAH-induced autofluorescence determination by flow cytometry (Fig. 1B) showed that DEPs were in contact with the cell membrane and/or phagocytosed by l-HBE cells in a dose-dependent fashion. However, neither of the three approaches was able to clearly distinguish between cell-ingested or cell-bound particles; evaluation of phagocytosis was therefore only approximate. The obvious more intense phagocytosis of DEPs compared with carbon black may be because of their different size. The particle size of Sigma carbon black was slightly greater than that of DEPs, and it has been proposed that small particles are more readily phagocytosed by epithelial cells than larger particles (12, 13). Previous studies (22) have also reported phagocytosis of DEPs by cat bronchial epithelial cells in vivo.

DEP phagocytosis is shown by the pattern of distribution of F-actin fluorescent phallotoxin staining in l-HBE-cells (Fig. 2). In control cells, cortical actin filaments are mainly organized into stress fibers involved in adhesion plaques at the basal surface and in circumferential belts, whereas scattered cytosolic actin represents small and slightly polymerized actin. In l-HBE cells exposed to DEPs, organized F-actin often engulfed the phagocytosed DEPs and also clearly extended as lamellipodia around the DEPs close to the cell membrane. This process appeared to be much more intense for DEPs than for carbon black particles and may be because of the presence of various organic compounds and metals adsorbed on the DEP surface.

DEP Exposure Induces Alteration of Cell-Matrix and Cell-Cell Interactions

In the present study, we used MTC to evaluate possible changes in HBE cell CSK stiffness and more particularly changes in actin CSK stiffness in response to DEP exposure. MTC is a relatively new technique used to measure CSK stiffness by application of controlled mechanical stress applied directly to cell-surface integrins, using RGD-coated microbeads (43). This type of CSK stiffness measurement is directly proportional to the strength of cell-matrix interactions (29, 44), and the difference between stiffness before and after brief exposure (25 min) to cyto D reflects actin CSK stiffness. Our results demonstrated that CSK stiffness of l-HBE cells was significantly reduced by 20% in response to DEP exposure and that actin CSK stiffness was reduced in a dose-dependent fashion by 35, 50, and 55% for 5, 20, and 100 μg/ml DEPs, respectively (Fig. 3). Compared with the variable effect of carbon black, these results suggest that DEPs are specifically able to induce loss of CSK stiffness.

Also, DEPs are specifically able to induce a loss of interactive cell-ECM properties, since flow cytometry quantification of the expression of all integrins at the l-HBE cell surface showed a significant dose-dependent decrease between 20 and 40% in major α3- and β1-integrin subunits and CD44 receptor in response to DEP exposure (Fig. 4). This decrease in both α3- and β1-integrin subunits strongly suggests a concomitant decrease in α3β1-integrin heterodimer. The α3β1-integrins and CD44 hyaluronic acid receptor have already been described in HBE cells (5, 33), and the marked decrease in these two major molecules in response to DEP exposure indicates that they play a key role in the loss of l-HBE cell-matrix interactions. Together, these data strongly suggest that the DEP-induced decrease in actin CSK stiffness may be at least partly because of the DEP-induced loss of adhesion molecules, in addition to the different F-actin distribution pattern. There is now increasing evidence that integrins regulate CSK assembly via a signaling pathway. More particularly, in hepatic stellate cells, Kojima et al. (25) observed that cell surface integrin binding to interstitial collagen induced a change in their CSK via a signal transduction system. Nevertheless, some studies have shown that the CSK itself may actively regulate integrin expression and binding conformation as well their mobility in the membrane (2).

Moreover, because α3β1-integrins and CD44 receptor are also known to be involved in cell-cell interactions (28, 41), their downregulation induced by DEP exposure may result in loss of cell-cell interconnections, predisposing to cell detachment. Our results confirm this predisposition, since the percentage of detached l-HBE cells in response to dissociation buffer was higher in the presence of DEPs (Fig. 8). Finally, we propose that this higher deadhesion capacity probably reflects an alteration of structural links between CSK actin and the ECM via α3β1-integrin and CD44 downregulation. Reduced expres-
sion of α3β1-integrins induced by puromycin amino-
nucleoside was also recently proposed to contribute to
glomerular epithelial cell detachment from the
glomerular basement membrane (26).

Decrease in β1, α3, and CD44 Expression by l-HBE
Cells in Response to DEP Exposure Is Not Due to Intracellular Internalization or Cytoplasmic Sequestration

The DEP-induced reduction of α3β1-integrin and CD44 expression at the l-HBE cell surface raises the question of the possible intracellular internalization of these matrix adhesion molecules. Our data demonstrated that the intracellular pool related to each adhesion molecule exhibited a similar reduction to that observed for the cell membrane pool (Table 2). These data support that DEP exposure actually induces a negative global modulation of α3β1-integrin and CD44 receptor expression without specific modification of intracellular internalization or cytoplasmic sequestration of cell adhesion molecules.

Decrease in β1, α3, and CD44 Expression by l-HBE
Cells in Response to DEP Exposure Is Not Due to DEP Phagocytosis

We also raised the question about the possible in-
volve ment of the actin-mediated DEP phagocytosis
process in the decrease in α3, β1, and CD44 expression. Curiously, blockade of actin-mediated DEP phagocy-
tosis by treatment with 10 μg/ml cyto D for 24 h did not
prevent the decrease in α3, β1, and CD44 expression by
l-HBE cells after DEP exposure (Fig. 5). It is interest-
ing to note that cyto D treatment alone induced a
significant increase by ~20% in α3, β1, and CD44 adhesion molecule expression so that the specific inhibitory effect of DEP appeared to be largely amplified in the presence of cyto D. Overall, these data support the hypothesis that 1) the loss of actin CSK stiffness might result from a decrease in integrin expression, but the latter would not be secondary to loss of actin CSK stiffness; and 2) the negative modulation of α3, β1, and CD44 expression induced by DEPs might be because of mechanisms other than phagocytosis. Recent studies by Boland et al. (7) showed that cyto D, used at the same concentration (10 μg/ml) and for the same incubation time (24 h), was able to reduce DEP-in-
duced GM-CSF secretion by l-HBE cells. DEP phago-
cytosis therefore appears to be a prerequisite for induc-
tion of cytokine release but not for negative modulation of α3, β1, and CD44 expression, suggesting that the respective transduction signaling pathway underlying transcriptional regulation may be somewhat different.

DEP Exposure Is Associated with a Reduction
of the HBE Cell Repair Process

In agreement with our postulate that DEP exposure
can alter the link between actin CSK and the ECM via
a reduction in adhesion molecule expression, we also
observed that, at 3.5 and 7 h, in vitro wound closure
was dose dependent and decreased in the presence of
DEP (Fig. 9) but seemed to occur without any apparent
signs of cell migration or cell spreading. In general, cell locomotion is a dynamic interplay between cell-ECM adhesion, extension of the leading edge of the cell, and retraction of the trailing edge. To move along the ECM, cells, and more particularly p-HBE cells, first adhere to the matrix by establishing primordial cell-ECM con-
tacts distributed at the cell front heading in the direc-
tion of migration (30, 39). In contrast with p-HBE and
type II alveolar pneumocyte repair models (37), no
migrating or spreading cell morphology was identified at the leading edge of the wound during the repair
process of our l-HBE cell repair model. In the latter,
cell proliferation appeared to be the key factor during
wound healing and was decreased in response to DEP
exposure, as stated by quantitative BrDU incorpora-
tion data (Fig. 10).

Taken together, these results, demonstrating a re-
duction of the cell repair process, decreased cell prolif-
eration, and decreased α3β1-integrin and CD44 recep-
tor expression, are in accordance with previous studies indicating a possible relationship between the level of integrin or CD44 expression and cell functions, such as cell repair or cell proliferation. High expression of CD44 was found during in vitro repair of bronchial epithelial cells after a mechanical injury (31). CD44 can also stimulate cell proliferation (14), and CD44 upregulation has been demonstrated in areas of dam-
aged bronchial epithelium both in normal and asth-
matic subjects, strongly suggesting that CD44 may be
directly or indirectly associated with repair processes
occurring after damage (27). As for integrins, a new
concept is emerging to suggest that integrins are mul-
tifunctional and may contribute not only to epithelial
cell adhesion but also to regulation of cell growth and proliferation via a signaling pathway involving mito-
gen-activated protein kinase (18). More particularly,
proliferation of human epithelial cells was signifi-
cantly inhibited by a function-altering α3-integrin antibody (18).

DEP Exposure Appears to Weaken Matrix Remodeling
by Interstitial Collagenase MMP-1 Expressed
by l-HBE Cells

Our results concerning ECM remodeling by subcon-
fluent l-HBE or p-HBE cells did not demonstrate any
change in MMP-2 or MMP-9 protein expression (Fig. 6)
or TIMP-1 and TIMP-2 levels (Fig. 7) in response to
DEP exposure. This result emphasizes the persistence of a harmonious balance between matrix gelatinases and their tissue inhibitors. Because MMP-9 expressed by p-HBE cells during wound repair has been recently proposed to play a key role in remodeling primordial contacts via specific degradation of type IV collagen (9, 30), unchanged MMP-9 expression appears relevant to the absence of any migrating event associated with our cell model in response to DEP exposure. De-Bentz-
mann et al. (15) recently suggested that MMP-2 over-
activation associated with a limited increase in TIMP-2
was responsible for inhibition of Pseudomonas aerugi-
nosa wound closure in vitro. In contrast, our results suggest that DEP wound closure inhibition would not depend on MMP-2 activation, probably because of different proteolytic mechanisms. Interestingly, interstitial collagenase MMP-1 production by l-HBE cells appeared to be markedly decreased in response to the highest dose of DEPs (Fig. 7), supporting the hypothesis that DEP exposure may reduce interstitial matrix turnover. Conversely, but not in opposition to the present study, recent studies by our group have demonstrated that the addition of exogenous MMP-1 collagenase to cultured alveolar epithelial cells could enhance wound healing by promoting cell migration on type I collagen (37).

In conclusion, the present study proposes that, besides inducing a well-known inflammatory reaction, DEP exposure of l-HBE cells could alter cell-matrix interactions and cell cohesion via a concomitant decrease in α3β1-integrin subunits and CD44 adhesion molecule and a reduction of actin CSK stiffness. Alteration of cell-matrix interactions and cell cohesion results in a dose-dependent reduction of the wound closure capacity and enhancement of the cell deadhesion capacity. A reduction in wound closure also appears to result from reduced cell proliferation and probably from alteration of matrix remodeling via an imbalance between MMP-1 and TIMP-1 and -2 in favor of inhibitors. Overall, these results support the concept that DEP exposure tends to break the link between actin CSK and the ECM, suggesting an increased potential for cell detachment from the underlying basement membrane in vivo. Finally, the fact that there was nonsignificant or no effect of carbon black particle exposure on actin CSK stiffness, cell adhesion molecule expression, wound repair capacity, the cell death process, or cell proliferation supports a key role for polycyclic aromatic hydrocarbons adsorbed on the surface of DEP.

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