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Eosinophil-Derived Cationic Proteins Activate the Synthesis of Remodeling Factors by Airway Epithelial Cells

Sophie Pégorier,* Lori A. Wagner,† Gerald J. Gleich,‡ and Marina Pretolani2*

Eosinophil cationic proteins influence several biological functions of the respiratory epithelium, yet their direct contribution to airway remodeling has not been established. We show that incubation of the human bronchial epithelial cell line, BEAS-2B, or primary cultured human bronchial epithelial cells, normal human bronchial epithelial cells, with subcytotoxic concentrations (0.1, 0.3, and 1 μM) of major basic protein (MBP), or eosinophil peroxidase (EPO), augmented the transcripts of endothelin-1, TGF-α, TGF-β1, platelet-derived growth factor (PDGF)-β, epidermal growth factor receptor, metalloproteinase (MMP)-9, fibronectin, and tenasin. A down-regulation of MMP-1 gene expression was observed exclusively in BEAS-2B cells. Cationic protein-induced transcriptional effects were followed by the release of endothelin-1, PDGF-AB in the supernatants by ELISA, and by a down- and up-regulation, respectively, in the levels of MMP-1 and MMP-9 in cell lysates, by Western blot. Cell stimulation with the synthetic polycation, poly-L-arginine, reproduced some but not all effects of MBP and EPO. Finally, simultaneous cell incubation with the polyanion molecules, poly-L-glutamic acid or heparin, restored MMP-1 gene expression but incompletely inhibited MBP- and EPO-induced transcriptional effects as well as endothelin-1 and PDGF-AB release, suggesting that cationic proteins act partially through their cationic charge. We conclude that eosinophil-derived cationic proteins are able to stimulate bronchial epithelium to synthesize factors that influence the number and behavior of structural cells and modify extracellular matrix composition and turnover. The Journal of Immunology, 2006, 177: 4861–4869.

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remodeling factor gene expression and protein release in cultured human airway epithelial cells and that these effects involve partly their cationic nature. Collectively, these findings establish a tangible link between cationic degranulation and airway remodeling, and identify a novel therapeutic target for eosinophil-mediated diseases.

Materials and Methods

Eosinophil-derived cationic protein purification

Eosinophils from patients with marked blood eosinophilia (up to 84%) were collected by cytapheresis and eosinophil granules and granule proteins were purified, as previously described (28–30). Briefly, after cell lysis and granule isolation, granules were solubilized in 0.01 M/L HCl (pH 2.0) by vigorous suspension with a Pasteur pipette. After centrifugation (13,600 × g, 5 min), the supernatant was fractionated on a Sephadex G-50 column equilibrated with 0.025 M/L sodium acetate, 0.15 M/L NaCl (pH 4.3). Individual protein peaks were pooled, and protein concentrations were determined by absorbance at 280 nm with the following extinction coefficients at 280 nM: E1%1cm 14.5 for EPO (29) and 36.7 for MBP (30). All eosinophil granule protein preparations were pure as assessed by Coomassie blue staining after SDS-PAGE.

Epithelial cell culture and stimulation

The human bronchial epithelial cell line, BEAS-2B (American Type Culture Collection-LGC Promochem) (31) and primary cultured normal human bronchial epithelial cells (NHBE; Cambrex BioScience) were seeded in 6-well plastic plates (TPP-AG) previously coated with 2.5 mg/mL collagen type I (Sigma-Aldrich) in 0.016 mM acetic acid. Cells were grown at 37°C in a humidified 5% CO2 atmosphere in bronchial epithelium growth medium (Cambrex) supplemented with 0.5 ng/mL recombinant human epidermal growth factor (EGF), 500 ng/mL hydrocortisone, 0.005 mg/mL salicyclic acid, 0.035 mg/mL bovine pituitary extract, 500 nM ethanolamine, 500 nM phosphoethanolamin, 0.01 mg/mL transferrin, 6.5 ng/mL 3,3',5-triiodothyronine, 500 mg/mL adrenaline, and 0.1 mg/mL retinoic acid. Once they reached ~80% confluence, epithelial cells were stimulated for 1, 3, 6, 24, and 48 h with 1 μM MBP, or EPO, or with their i.e., 0.025 M sodium acetate buffer with 0.15 M NaCl (pH 4.3). The addition of the acetate buffer failed to alter appreciably the pH of the vehicle, i.e., 0.025 M sodium acetate buffer with 0.15 M NaCl (pH 4.3). The levels of PDGF-AB and ET-1 were assessed in supernatants from epithelial cells stimulated for 6, 24, and 48 h with 1 μM MBP, or EPO in the presence of 1 μM poly-L-arginine or 100 U/mL heparin, were recovered in RA1 buffer contained in the NucleoSpin RNA II kit (Macherey-Nagel) supplemented with 3.5 M/L sodium acetate, 0.15 M/L NaCl (pH 4.3). Individual protein peaks were pooled, and protein concentrations were determined by absorbance at 280 nm with the following extinction coefficients at 280 nM: E1%1cm 14.5 for EPO (29) and 36.7 for MBP (30). All eosinophil granule protein preparations were pure as assessed by Coomassie blue staining after SDS-PAGE.

Assessment of epithelial cell viability

The effect of cationic proteins on epithelial cell viability was determined by quantifying lactate dehydrogenase (LDH) activity using a commercially available kit (Cytotoxicity Detection kit, LDH; Roche) according to the manufacturer’s instructions. Epithelial cells were seeded in 24-well plates (TPP) and stimulated with 1 μM MBP, EPO, or poly-L-arginine for 1, 3, 6, 24, and 48 h. Cells were harvested by a 15-min incubation in cell disso- ciation buffer (Invitrogen Life Technologies) at 37°C and pelleted by cen trifugation (150 × g, 5 min, 4°C). Supernatants were further centrifuged before use. Three independent experiments were conducted. Results were calculated as a percentage of LDH activity from supernatants, in relation to cell pellets. The plates were then read on a Microplate reader photometer (Multiskan Ascent; Thermo) at 570-nm wavelength.

RNA isolation and reverse transcription

Confluent epithelial cells that had been cultured for 1–48 h in medium supplemented with 0.1, 0.3, or 1 μM MBP, or with 1 μM EPO, or poly-L-arginine, in the absence or the presence of 1 μM poly-L-glutamic acid or 100 U/mL heparin, were recovered in RA1 buffer contained in the NucleoSpin RNA II kit (Macherey-Nagel) supplemented with 3.5 μL of 2-ME (Sigma-Aldrich) and then stored at ~8°C. Total RNAs were isolated using this same kit according to manufacturer’s instructions and quantified by measuring the OD at 260 nm. Reverse transcription was performed for 2 h at 37°C using Moloney murine leukemia virus reverse transcriptase (In vitro gen Life Technologies) and 2.5 μg/50 μl total RNA were used.

Real-time quantitative PCR

Real-time quantitative PCR was performed using the SYBRGreen Jump Start Taq Ready Mix detection kit (Sigma-Aldrich). In all assays, cDNA was amplified using a standardized program (2 min JumpStart Taq Pol ymerase activation step at 94°C; 40 cycles of 30 s at 94°C and 1 min at 60°C). All assays were performed in a volume of 20 μL, and primers were used at a final concentration of 0.33 μM. Reactions were conducted using the PCR ABI 7700 apparatus (Applied Biosystems). Transcripts showing cycle threshold (Ct) values ≥35 were considered to have minimal expres- sion and were excluded from further analyses. For a more accurate and reliable normalization of the results, the intensity of gene expression was calculated using Ct <35 and it was normalized to the geometrical mean of the levels of transcripts encoding the most stable 3 housekeeping genes hypoxanthine-guanine-phosphoribosyl transferase 1 (HPRT1), succinate dehydrogenase (SDHA), and ribosomal protein 13a (RPL13a; Ref. 32).

Table 1. Real-time primer sequences and basal levels of transcript expression in human bronchial epithelial cells

<table>
<thead>
<tr>
<th>GenBank Identifier</th>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>BEAS-2B</th>
<th>NHBE</th>
</tr>
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<tr>
<td>NM_001955</td>
<td>ET-1</td>
<td>TGGATCTGAGGACGAGGACGT</td>
<td>AGATGAAAGGAGGACGAGCATTAC</td>
<td>24.1 ± 0.1</td>
<td>25.9 ± 0.4</td>
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<tr>
<td>NM_003236</td>
<td>TGF-α</td>
<td>GACAGACCCAGCTCCTGACT</td>
<td>GGCGTGCGGGTTTTCCACT</td>
<td>30.0 ± 2.2</td>
<td>30.0 ± 2.0</td>
</tr>
<tr>
<td>NM_000660</td>
<td>TGF-β1</td>
<td>ACTCATGCTGAGGACGAGGAGCT</td>
<td>CCCTGCGGAGCCACTTCT</td>
<td>27.1 ± 0.7</td>
<td>30.4 ± 0.2</td>
</tr>
<tr>
<td>NM_005228</td>
<td>EGFR</td>
<td>GGGCCGACAGCTGATGAGTT</td>
<td>CCGAGGCTGAGGAGCAGT</td>
<td>21.8 ± 0.4</td>
<td>20.0 ± 1.1</td>
</tr>
<tr>
<td>X09761</td>
<td>PDGF-B</td>
<td>AGCCCATAGGCCGCACCAAC</td>
<td>AAGAACCTGCTGACCCACACT</td>
<td>23.8 ± 0.5</td>
<td>26.6 ± 0.4</td>
</tr>
<tr>
<td>NM_002421</td>
<td>MMP-1</td>
<td>CATCTTACCGGACGACCCTT</td>
<td>TGGTGGTGACACCGAGTACT</td>
<td>30.0 ± 0.5</td>
<td>29.0 ± 2.2</td>
</tr>
<tr>
<td>NM_004994</td>
<td>MMP-9</td>
<td>CGACATTGAGGCCAGGACAG</td>
<td>TGGCCCAAGCAGCCACTT</td>
<td>27.1 ± 0.7</td>
<td>30.4 ± 0.2</td>
</tr>
<tr>
<td>X07671</td>
<td>Fibromodulin</td>
<td>AGTGGACGAGA</td>
<td>CCGAGGCTGAGGAGCAGT</td>
<td>21.8 ± 0.4</td>
<td>20.0 ± 1.1</td>
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<tr>
<td>NM_004168</td>
<td>SDHA</td>
<td>GGAGGAGGACGAGGACGAGGAAGGAAGGA</td>
<td>TGTTTCTGTGCTGCTTCCCTCATA</td>
<td>17.4 ± 0.3</td>
<td>23.4 ± 0.5</td>
</tr>
</tbody>
</table>

* EGFR, epidermal growth factor receptor; TGF, transforming growth factor; HPRT, guanine-phosphoribosyl transferase; RPL13a, ribosomal protein 13a; SDHA, succinate dehydrogenase.
* Basal Ct corresponds to the Ct measured in vehicle (sodium acetate buffer)-treated cells.

Determination of platelet-derived growth factor (PDGF)-AB and endothelin-1 (ET-1) in cell supernatant

The levels of PDGF-AB and ET-1 were assessed in supernatants from epithelial cells stimulated for 6, 24, and 48 h with 1 μM MBP, EPO, or poly-L-arginine, in the absence or presence of 1 μM poly-L-glutamic acid or 100 U/mL heparin by ELISAs (human PDGF-AB Quantikine Immuno assay and human ET-1 QuantiGlo Immunoassay all obtained from R&D Systems Europe), respectively. The threshold of sensitivities was 31.2 pg/mL for PDGF-AB and 0.32 pg/mL for ET-1.

Assessment of MMP-1 and MMP-9 in cell lysates

Unstimulated and MBP-, EPO-, and poly-L-arginine (1 μM)-stimulated epithelial cells, in the absence or presence of 1 μM poly-L-glutamic acid or...
100 U/mL heparin were harvested by a 15-min incubation in cell dissociation buffer at 37°C, then lysed and sonicated with a buffer containing NaCl (150 mM), HEPES (10 mM, pH 8), saccharose (500 mM), Na2 EDTA (1 mM), Nonidet P40 (40%), Igepal; Sigma-Aldrich). Protein contents in clarified supernatants were determined by comparison with an OVA standard curve (ICN Biomedical), using the Bio-Rad protein assay.

The expression of MMP-1 and of MMP-9 were assessed by Western blot after protein fractionation (50 μg) by 10% SDS-PAGE, transfer to polyvinylidene difluoride membranes (Bio-Rad) and sequential reaction with a chicken anti-human MMP-1 Ab (clone 3.4.24.7, 1/2000 dilution; R&D Systems) and with a mouse anti-human MMP-9 Ab (clone AB911, 1/1000 dilution; R&D Systems) and with a mouse anti-β-actin mAb (AC-74, AC-107, AC-74, 1/4000 dilution; Sigma-Aldrich). Immunoblots were then incubated with peroxidase-conjugated goat anti-chicken, donkey anti-goat, or donkey anti-mouse Abs at a 1/3000 dilution and developed using the ECL Western blotting detection system (all from Amersham). The intensities of the expression of MMP-1, MMP-9, and β-actin were quantified using a densitometer (Gel COHU) and Gel Analyst software (ClariVision). Results are expressed as a ratio, defined as the OD values of the MMP-1 or MMP-9 bands/OD values of the corresponding β-actin bands.

Statistical analysis

Data were analyzed statistically using the StatView SE+Graphics program for Macintosh (Abacus Concepts). Comparability between the means was assessed by Wilcoxon test for the MBP dose-response experiments, and ANOVA test for the experiments with MBP, EPO, and poly-γ-arginine in the absence or the presence of heparin or poly-L-glutamic acid. The results are expressed as means ± SEM for the indicated number of experiments.

Results

Effect of cationic proteins on epithelial cell viability

Changes in the viability of BEAS-2B and NHBE cells upon 1–48 h of stimulation with the highest concentration of cationic proteins and poly-γ-arginine i.e., 1 μM, were determined by assessing LDH release. MBP, EPO, and poly-γ-arginine failed to induce the release of amounts of LDH above those detected in vehicle-treated cells (between 15 and 20% release, differences not statistically significant, data not shown).

Effect of cationic proteins and poly-γ-arginine on the expression of transcripts encoding remodeling factors

The ability of MBP and EPO and of the surrogate cationic molecule, poly-γ-arginine, to influence the expression of factors involved in airway remodeling was investigated by real-time quantitative PCR (Figs. 1–4 and Table II). We found detectable levels of the transcripts encoding ET-1, TGF-α, TGF-β1, EGFR, PDGF-β, MMP-1, MMP-9, fibronectin, and tenascin in vehicle (acetate buffer)-stimulated human BEAS-2B cells and in NHBE cells isolated from three distinct healthy donors (C1 < 35, Table I). The highest expressed transcripts in both cell types were those encoding EGRF and the two ECM proteins, fibronectin and tenascin (Table I). However, the basal expression level of MMP-1 mRNA was higher in NHBE, as compared with BEAS-2B cells (mean C1 values of 20.9 and 30.0, respectively, Table I).

Incubation of BEAS-2B cells over 3–24 h in the presence of 0.1, 0.3, and 1 μM MBP significantly up-regulated the expression of mRNA for ET-1, TGF-α, EGRF, TGF-β1, PDGF-β, MMP-9, tenascin, and fibronectin (Table I). Under these conditions, MMP-1 transcripts were significantly reduced (Fig. 2A). No changes in the levels of any of the transcripts examined were noted after 1 h of stimulation (data not shown). Similar induction of ET-1, TGF-α, TGF-β1, EGRF, PDGF-β, and tenascin gene expression and down-regulation of MMP-1 transcription was observed in EPO (1 μM)-stimulated cells (Fig. 3 and Fig. 4, A and C), but contrary to MBP, this cationic protein failed to alter MMP-9 and fibronectin mRNA levels (Fig. 4, B and D).

Cell stimulation with 1 μM poly-γ-arginine up-regulated the levels of the transcripts encoding ET-1, TGF-α, EGRF, and PDGF-β to a similar extent as MBP (Fig. 1, A–C and E). However, poly-γ-arginine failed to augment TGF-β1, MMP-9, tenascin, and fibronectin gene expression (Figs. 1D and 2B–D) and, contrary to MBP and EPO, it augmented the levels of the transcripts encoding MMP-1 (Fig. 2A).

Most of the above findings were confirmed in NHBE cells, where 1 μM MBP and EPO augmented ET-1, TGF-α, TGF-β1, EGRF, PDGF-β, and tenascin mRNA, without altering the levels of MMP-1 transcripts (Table II). In addition, MBP, but not EPO, increased significantly tenascin gene expression, as compared with vehicle-treated cells. Under these conditions, cell stimulation with 1 μM poly-γ-arginine up-regulated exclusively ET-1, TGF-β1, EGRF, and MMP-9 mRNAs (Table II).

FIGURE 1. MBP and poly-γ-arginine modulate the levels of mRNA encoding remodeling factors in the human bronchial epithelial cell line, BEAS-2B. BEAS-2B cells were stimulated for 3, 6, and 24 h with 0.1, 0.3, and 1 μM MBP, with 1 μM poly-γ-arginine, or with the vehicle of MBP, i.e., sodium acetate buffer. Cells were harvested, RNA was extracted, reverse transcribed, and a real-time quantitative PCR for ET-1 (A), TGF-α (B), EGRF (C), TGF-β1 (D), and PDGF-β (E) was performed. Results are expressed as the ratio of each transcript relative to the geometrical average of mRNA expression of the housekeeping genes HPRT1, SDHA, and RPL13a. Data are means ± SEM of five independent experiments. *, p < 0.05, as compared with vehicle-treated cells.
Poly-L-glutamic acid and heparin partially reverse cationic protein-induced effects

To further establish whether the cationic charge of MBP and EPO was involved in their modulatory activities, we examined the effect of 1 μM poly-L-glutamic acid and of 100 U/ml heparin on MBP- and EPO-induced changes in the expression of the different transcripts. For these experiments, we selected the time points corresponding to the highest MBP- and EPO-mediated stimulation or inhibition of gene expression, i.e., 3 h for ET-1, TGF-β1, and tenascin and 24 h for TGF-α, EGFR, PDGF-β, MMP-1, MMP-9, and fibronectin (Figs. 1 and 2) in BEAS-2B cells, and 3 h for ET-1, 6 h for TGF-β1 and 24 h for TGF-α, EGFR, PDGF-β, MMP-1, MMP-9, fibronectin, and tenascin in NHBE cells (Table II). Simultaneous incubation of poly-L-glutamic acid with MBP or EPO reduced significantly the levels of the transcripts encoding ET-1, TGF-α, EGFR, PDGF-β, fibronectin, tenascin, and MMP-9 in BEAS-2B and NHBE cells (Fig. 3 and Table II). In addition, poly-L-glutamic acid partially reversed MBP- and EPO-induced decrease in MMP-1 mRNA in BEAS-2B cells (Fig. 4). Poly-L-glutamic acid by itself, significantly down-regulated MMP-1 gene expression in BEAS-2B cells (Fig. 4A), without altering the basal levels of the other transcripts analyzed. Similar results were obtained using 100 U/ml heparin instead of poly-L-glutamic acid (data not shown).

**Eosinophil-derived cationic proteins and poly-L-arginine generate remodeling factors**

To determine whether the transcriptional effects of cationic proteins and of poly-L-arginine resulted in proportionate changes in the elaboration of the corresponding proteins, we measured the expression of the MMPs and extracellular matrix proteins in BEAS-2B cells. BEAS-2B cells were stimulated for 3, 6, and 24 h with 0.1, 0.3, and 1 μM MBP, with 1 μM poly-L-arginine, or with the vehicle of MBP, i.e., sodium acetate buffer, and the expression of mRNA for MMP-1 (A), MMP-9 (B), tenascin (C), and fibronectin (D) was assessed by real-time quantitative PCR and results are expressed as described in the legend of Fig. 1. Data are means ± SEM of five independent experiments, *p < 0.05, as compared with vehicle-treated cells.

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**Table II. Effect of poly-L-arginine, MBP, and EPO on the levels of the transcripts encoding remodeling factors in primary cultured human bronchial epithelial cells (NHBE) and modulation by poly-L-glutamic acid**

<table>
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<tbody>
<tr>
<td><strong>ET-1</strong></td>
<td></td>
<td>0.36 ± 0.02</td>
<td>1.0</td>
<td>5.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;d&lt;/sup,e</td>
<td>8.5&lt;sup&gt;c&lt;/sup,d,e</td>
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<tr>
<td><strong>TGF-α</strong></td>
<td></td>
<td>0.88 ± 0.36</td>
<td>1.4</td>
<td>1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;c&lt;/sup,d,e</td>
<td>10.2&lt;sup&gt;c&lt;/sup,d,e</td>
</tr>
<tr>
<td><strong>TGF-β1</strong></td>
<td></td>
<td>0.83 ± 0.15</td>
<td>1.1</td>
<td>1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.9&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>EGF R</strong></td>
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<td>0.80 ± 0.26</td>
<td>0.8</td>
<td>1.7&lt;sup&gt;c&lt;/sup,d,e</td>
<td>1.0</td>
<td>2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>PDGF-β</strong></td>
<td></td>
<td>0.49 ± 0.17</td>
<td>1.8</td>
<td>1.6</td>
<td>0.8</td>
<td>7.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.1&lt;sup&gt;c&lt;/sup,d,e</td>
<td>5.8&lt;sup&gt;c&lt;/sup,d,e</td>
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<tr>
<td><strong>MMP-1</strong></td>
<td></td>
<td>1.10 ± 0.19</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
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<td><strong>MMP-9</strong></td>
<td></td>
<td>0.54 ± 0.39</td>
<td>1.6</td>
<td>3.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.2&lt;sup&gt;c&lt;/sup,d,e</td>
<td>4.5&lt;sup&gt;c&lt;/sup,d,e</td>
<td>3.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>Fibronectin</strong></td>
<td></td>
<td>0.61 ± 0.36</td>
<td>0.8</td>
<td>0.6</td>
<td>0.6</td>
<td>5.9&lt;sup&gt;c&lt;/sup,d,e</td>
<td>1.3&lt;sup&gt;c&lt;/sup,d,e</td>
<td>3.8&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td><strong>Tenascin</strong></td>
<td></td>
<td>0.67 ± 0.40</td>
<td>1.3</td>
<td>1.8</td>
<td>0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;c&lt;/sup,e</td>
<td>0.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* NHBE cells were stimulated over 3 h (for ET-1), 6 h (for TGF-β1), and 24 h (for TGF-α, EGF R, PDGF-β, MMP-1, MMP-9, tenascin, and fibronectin) with 1 μM poly-L-arginine (poly-L-arg), MBP, or EPO, or its vehicle, i.e., sodium acetate buffer, or with 1 μM poly-L-glutamic acid (poly-L-glu) alone, or in association with 1 μM poly-L-arginine or MBP.

*<sup>1</sup> The expression of the indicated genes was assessed by real-time quantitative PCR, as described in the legend of Fig. 1. Data (means ± SEM of three independent experiments performed with cells from three distinct donors) are expressed as the ratio of mRNA expression of each transcript relative to the geometrical average of mRNA expression of the three housekeeping genes, RPL13a, HPRT, and SDHA.

*<sup>2</sup> p < 0.05, as compared to vehicle-treated cells.

*<sup>3</sup> p < 0.05, as compared to poly-L-glutamic acid-treated cells.

*<sup>4</sup> p < 0.05, as compared to MBP- or poly-L-arginine-stimulated cells.

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**FIGURE 2.** MBP and poly-L-arginine induce changes in the levels of mRNA encoding MMPs and extracellular matrix proteins in BEAS-2B cells. BEAS-2B cells were stimulated for 3, 6, and 24 h with 0.1, 0.3, and 1 μM MBP, with 1 μM poly-L-arginine, or with the vehicle of MBP, i.e., sodium acetate buffer, and the expression of mRNA for MMP-1 (A), MMP-9 (B), tenascin (C), and fibronectin (D) was assessed by real-time quantitative PCR and results are expressed as described in the legend of Fig. 1. Data are means ± SEM of five independent experiments, *p < 0.05, as compared with vehicle-treated cells.
FIGURE 3. Poly-L-glutamic acid modulates MBP- and EPO-induced changes in the levels of transcripts encoding remodeling factors in BEAS-2B cells. BEAS-2B cells were stimulated with 1 μM MBP, EPO, or their vehicle, i.e., sodium acetate buffer, or with 1 μM poly-L-glutamic acid (poly-L-glu) alone, or in association with cationic proteins. Cells were collected at 3 h, for ET-1 (A) and TGF-β1 (D), or at 24 h, for TGF-α (B), EGFR (C), and PDGF-β (E) and real-time quantitative PCR for these factors was performed, as described in the legend of Fig. 1. Data are means ± SEM of three independent experiments. *, p < 0.05, as compared with vehicle-treated cells; †, p < 0.05, as compared with poly-L-glutamic acid-treated cells; and ‡, p < 0.05, as compared with MBP- or EPO-stimulated cells.

FIGURE 4. Poly-L-glutamic acid modulates MBP- and EPO-induced changes in the levels of transcripts encoding MMPs and ECM proteins in BEAS-2B cells. BEAS-2B cells were stimulated as described in the legend of Fig. 3 and real-time quantitative PCR was assessed at 3 h, for tenascin (C), and at 24 h, for MMP-1 (A), MMP-9 (B), and fibronectin (D). Data are means ± SEM of three independent experiments. *, p < 0.05, as compared with vehicle-treated cells; †, p < 0.05, as compared with poly-L-glutamic acid-treated cells, and ‡, p < 0.05, as compared with MBP- or EPO-stimulated cells.
that had been used in other studies of their effector functions and that failed to induce epithelial cell cytotoxicity (34–37). Importantly, these concentrations of cationic proteins were even lower than those measured in peripheral blood of atopic individuals and in the airways of asthmatics (38, 39).

We found that both MBP and EPO increased the levels of the transcripts encoding ET-1, TGF-β1, EGFR, PDGF-β, and tenascin in both BEAS-2B and NHBE cells. Under these conditions, cationic proteins down-regulated MMP-1 gene expression in BEAS-2B cells, without altering its levels in NHBE cells. In addition, MBP, but not EPO, up-regulated MMP-9 and fibronectin mRNAs in BEAS-2B cells, whereas both MBP and EPO increased these genes in NHBE cells.

To establish whether cationic protein-induced remodeling factor gene expression was associated with proportionate changes in the synthesis of the corresponding proteins, we measured the concentrations of PDGF-AB (A) and ET-1 (B) were measured in the cell-free supernatants by specific ELISAs. The levels of cell-associated MMP-1 (C) and MMP-9 (D) were assessed by immunoblot in protein extracts from BEAS-2B cells stimulated for 24 h. The intensity of the expression of MMP-1 (54 kDa) and MMP-9 (67 kDa) were quantified by densitometry. Values are expressed as a ratio, defined as the OD values MMP-1 or MMP-9 bands/OD value of the corresponding β-actin bands. A representative Western blot profile of MMP-1 and of MMP-9 expression is shown under the corresponding graph. Results are the means ± SEM of three distinct experiments. *, p < 0.05, as compared with vehicle-treated cells; †, p < 0.05, as compared with poly-L-glutamic acid-treated cells, and ‡, p < 0.05, as compared with MBP-, EPO-, or poly-L-arginine-stimulated cells.

0.3, and 1 μM) that had been used in other studies of their effector functions and that failed to induce epithelial cell cytotoxicity (34–37). Importantly, these concentrations of cationic proteins were even lower than those measured in peripheral blood of atopic individuals and in the airways of asthmatics (38, 39).

We found that both MBP and EPO increased the levels of the transcripts encoding ET-1, TGF-α, TGF-β1, EGFR, PDGF-β, and tenascin in both BEAS-2B and NHBE cells. Under these conditions, cationic proteins down-regulated MMP-1 gene expression in BEAS-2B cells, without altering its levels in NHBE cells. In addition, MBP, but not EPO, up-regulated MMP-9 and fibronectin mRNAs in BEAS-2B cells, whereas both MBP and EPO increased these genes in NHBE cells.

To establish whether cationic protein-induced remodeling factor gene expression was associated with proportionate changes in the synthesis of the corresponding proteins, we measured the concentrations of ET-1 and PDGF-AB in the cell supernatants by specific ELISAs and the expression of cell-associated MMP-1 and MMP-9 by Western blot. Cell stimulation with MBP promoted both ET-1 and PDGF-AB release, whereas EPO increased the levels of PDGF-AB, but not of ET-1. In addition, Western blot analyses demonstrated lower amounts of MMP-1 and higher levels of MMP-9 in whole protein extracts from MBP- and EPO- as compared with vehicle-stimulated cells. Collectively, these results indicate that the transcriptional effects of MBP and EPO result in proportionate changes in the synthesis of some, but not all proteins and that these two cationic proteins do not necessarily share all activity-related effects, suggesting that they may use different pathways to activate gene transcription and protein synthesis in airway epithelial cells.

The expression of most of the remodeling factors that we have analyzed was increased in asthmatic airways, including in association with the bronchial epithelium, and it was frequently correlated with disease severity (6, 7, 40–47). These factors modulate several aspects of airway remodeling, as suggested by in vitro studies. Thus, EGFR is likely to play an important role in bronchial epithelial repair in asthma, and its excessive expression and abnormal function contribute to subepithelial fibrosis (26, 40). TGF-α, one of the ligands of EGFR that regulates lung remodeling and morphogenesis (47), is induced in patients with chronic inflammatory lung diseases characterized by tissue remodeling, including asthma (46). TGF-β1 elicits differentiation of fibroblasts into myofibroblasts, which, in turn, secrete interstitial collagen and other fibrogenic growth factors (48). PDGF-β regulates airway smooth muscle cell proliferation, chemotaxis, and activation (49–51), and contributes to lung fibroblast growth (52). ET-1 is a 21-aa peptide that plays an important role in the pathogenesis of airway remodeling by inducing collagen secretion by lung fibroblasts and fibronectin synthesis by bronchial epithelial cells, and by amplifying EGF-induced airway smooth muscle cell proliferation (53–55). Finally, MMPs govern ECM turnover and degradation, and
Regulated ET-1 Cells were stimulated for 6, 24, and/or 48 h with 1 μM MBP, EPO, poly-L-arginine (poly-L-arg), or with the vehicle of cationic proteins, i.e., sodium acetate buffer, or with 1 μM poly-L-glutamic acid (poly-L-glu), alone, or in association with cationic proteins. The concentrations of PDGF-AB (A) and ET-1 (B) were measured in the cell-free supernatants by specific ELISAs. The levels of cell-associated MMP-1 and MMP-9 (C) were assessed by immunoblot in protein extracts from NHBE cells stimulated for 24 h. The intensity of the expression of MMP-1 (25 kDa) and MMP-9 (67 kDa) were quantified by densitometry as explained in the legend of Fig. 5. Results are the means ± SEM of three independent experiments performed with cells from three distinct donors. * p < 0.05, as compared with vehicle-treated cells; †, p < 0.05, as compared with poly-L-glutamic acid-treated cells, and ‡, p < 0.05, as compared with MBP-, EPO-, or poly-L-arginine-stimulated cells.

Among the remodeling factors examined, MMP-1 was unique in showing a marked down-regulation in response to cationic proteins. MMP-1 degrades fibrillary collagen (56) and temporal and spatial expression studies revealed its early increase in migrating corneal epithelial and stromal cells at the wound edge (57). In addition, recent data demonstrated that defective wound closure resulting from the exposure of human bronchial epithelial cells to diesel exhaust particles was accompanied by a selective decrease in MMP-1 expression (58), indicating that MMP-1 plays an important role in the early phases of epithelial cell migration and repair (59). Together, these findings and our present observations suggest that, by inhibiting MMP-1 synthesis, eosinophil granule proteins may contribute to collagen accumulation on the one hand, and delay epithelial regeneration after damage, on the other.

Many of the in vitro and in vivo biological properties of MBP and EPO are mediated by their cationic charge, as determined by the inhibitory activity of anionic molecules, such as heparin and polyanions (18–20, 25, 60). Here we demonstrated that mixing MBP or EPO with poly-L-glutamic acid or with heparin down-regulated ET-1, TGF-α, EGFR, TGF-β1, PDGF-β, MMP-9, tenascin, and fibronectin gene expression and restored the levels of MMP-1 mRNA, suggesting that the charge of these cationic proteins plays an important role in their stimulatory potential. However, poly-L-glutamic acid, by itself, reduced the levels of MMP-1 mRNA (but not the expression of the corresponding protein) and promoted PDGF-AB release. These observations underline the difficulty to firmly conclude that the effects of poly-L-glutamic acid or of heparin on the synthesis and release of remodeling factors in response to MBP and EPO that we presently describe, are exclusively the result of the contrasting charge of these molecules. Nevertheless, we showed that the surrogate cationic molecule, poly-L-arginine, reproduced most of the effects of MBP and EPO in both BEAS-2B and NHBE cells. These include the increase in the levels of ET-1, TGF-β1, EGFR, and MMP-9 mRNAs, the release of ET-1 and PDGF-AB in cell supernatants and the production of cell-associated MMP-9. Overall, these observations support a dominant role for the cationic charge in mediating some, but not all transcriptional and posttranscriptional effects of MBP and EPO on airway epithelial cells. Several hypotheses may explain the disparity in the effects observed between poly-L-arginine, MBP and EPO. These include, for example, their marked differences in tertiary conformation and in the proportion of arginine and other amino acid residues, which may influence their charge and interactions with cell environment (61, 62). In addition and contrary to MBP, EPO belongs to the peroxidase superfamily, which possesses catalytic properties that confer a wide panel of potential activations (62, 63). Finally, Fuchs et al. (64) demonstrated that arginine residues of poly-L-arginine facilitate its transduction in endocytic vesicles of living cells, whereas MBP has a poor solubility and a tendency to polymerize with itself and with other proteins. In contrast, although remaining as a monomeric molecule, EPO may interact with other molecules of its environment, leading to altered biological activities (64).

To our knowledge, few reports have investigated links between eosinophil-derived cationic proteins and airway remodeling in vitro. An early study demonstrated that eosinophil-cationic protein...
inhibited proteoglycan degradation in human lung fibroblasts suggesting that eosinophil degranulation may participate in the generation of pulmonary fibrosis (65). More recently, Rochester et al. (25) showed that MBP synergized with IL-1α and TGF-β1 to increase IL-6-type cytokine mRNA and protein synthesis by human lung fibroblasts, largely by acting through its cationic charge. However, under these conditions, type I collagen production and cell proliferation were unaffected, indicating that MBP regulates certain biological functions in these cells, it does not increase collagen output. Finally, Zagai et al. (66) reported the ability of eosinophil-cationic protein to augment fibroblast-mediated collagen gel contraction, supporting the hypothesis that eosinophil-derived cationic proteins contribute to ECM remodeling by interacting directly with mesenchymal cells.

Persistent airway eosinophilia has been reported in severe asthmatics despite long-lasting, high-dose steroid therapy (67, 68). In these patients, irreversible structural changes of the bronchial wall characterized by subepithelial fibrosis, mucus gland, and airway smooth muscle hypertrophy and/or hyperplasia have been observed (7, 8, 69). Interestingly, a recent report demonstrated that a single allergen challenge in patients with mild asthma induced acute airway remodeling, with activation of epithelial cells and fibroblasts and increased ECM protein deposition within the reticular basement membrane (70). The temporal association between these structural alterations and eosinophil accumulation in the bronchial wall led to the hypothesis that eosinophil-derived products may contribute to a rapid onset of airway remodeling (70).

In conclusion, this study demonstrates that subcytotoxic concentrations of eosinophil cationic proteins influence the synthesis of bioactive molecules by epithelial cells that may disturb in vivo the function and behavior of structural cells and alter the composition of ECM. Hence, these observations argue for a causal relationship between eosinophil degranulation and airway remodeling in asthma and suggest that interventions targeting eosinophil cationic proteins may have a promising therapeutic application. Lastly, these findings have implications for other syndromes associated with fibrosis, such as retropitoneal fibrosis and sclerosing cholangitis, where eosinophil infiltration and degranulation are prominent (71).

Disclosures

The authors have no financial conflict of interest.

References


