Progranulin is a substrate for neutrophil-elastase and proteinase-3 in the airway and its concentration correlates with mediators of airway inflammation in COPD

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Progranulin (PGRN) is a substrate for neutrophil-derived proteinases and can stimulate epithelial cell interleukin (IL)-8. Because dysregulated neutrophilic inflammation is implicated in the pathophysiology of chronic obstructive pulmonary disease (COPD), the possible influence of PGRN and digestion products may be of relevance to understanding and treating inflammation in the disease. PGRN was measured in sputum sol-phase samples from patients with a clinical diagnosis of COPD and chronic sputum production in a clinically stable state; PGRN correlated negatively with bacterial load (colony-forming units/ml) (r = −0.446, P = 0.003, n = 43) and markers of neutrophilic inflammation, including neutrophil elastase (NE, nM) (r = −0.562, P = 0.008, n = 21) and proteinase-3 (PR3, nM) (r = −0.515, P = 0.017, n = 21). Products of PGRN digestion were detected in sputum sol phase, and PGRN conversion activity in sputum sol phase was inhibited with the serine proteinase inhibitor, oracranin) is a glycosylated protein composed of seven and a half cysteine-rich tandem repeats, known as GRN peptides. One of these GRN peptides (GRN-E) has been shown to increase PGRN secretion from normal human bronchial epithelial (NHBE) cells. Infection of NHBE cells with live Haemophilus influenzae significantly increased PGRN secretion compared with untreated cells (P < 0.001). The concentration of PGRN relates negatively to the amplified airway inflammation associated with bacterial colonization in clinically stable COPD. This relationship is driven by the proteolytic action of the neutrophil-derived proteinases NE and PR3; the products released by this action are unlikely to stimulate significant IL-8 secretion from epithelial cells in the airways.

chronic obstructive pulmonary disease

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is characterized by progressive airway obstruction and a decline in exercise capacity and health status and is associated with an abnormal inflammatory response to tobacco smoking and/or air pollution (26). In COPD the inflammatory response is marked by an increase in numbers of inflammatory cells, especially neutrophils (15). Neutrophils are thought to play an integral role in the pathophysiology of COPD (33), and the proteinases they release are implicated in emphysema and mucus hypersecretion (10). The inflammatory response is also marked by an increase in pro-inflammatory cytokine release, including (but not limited to) leukotriene (LT) B4 (24), interleukin (IL)-8, and tumor necrosis factor (TNF)-α (16). This neutrophilic inflammation can be amplified further by bacterial infection in the airways and is often recognized by the production of purulent sputum (13, 34). Sputum appears purulent when it contains a high number of neutrophils (34), since the green color is the result of the presence of myeloperoxidase (MPO) produced and released by the cells.

In addition to causing tissue damage during inflammation, neutrophil-derived proteinases can modulate inflammation by both stimulating cytokine release and altering their function (17). Such a regulatory role has been supported by observations in mice genetically deficient in neutrophil elastase (NE) and proteinase-3 (PR3) (18). The proteinase-deficient mice have reduced immune complex-mediated neutrophil infiltration with the defect directly linked to an accumulation of progranulin (PGRN) (18). PGRN (also referred to as granulin-epithelin precursor, proepithelin, PC cell-derived growth factor, or acrogranin) is a glycosylated protein composed of seven and a half cysteine-rich tandem repeats, known as GRN peptides, and is implicated in a variety of physiological processes and diseases (6). It is expressed in many different mammalian tissues, especially in epithelial and myeloid cells (5, 12). There is a growing body of evidence, from work with murine models of skin wounds (12, 18, 43), inflammatory arthritis (37), contact dermatitis (42), and lipopolysaccharide (LPS)-induced acute lung injury (11), that PGRN is a potent anti-inflammatory molecule (14). This anti-inflammatory action is effected in part by the inhibition of neutrophil degranulation (18, 37, 43) most likely by PGRN binding to the tumor necrosis factor receptors (TNFR) (37). In addition to TNFRs, numerous other interacting partners have been identified for PGRN (6), including other proteins that have roles in infection and inflammation, such as toll-like receptor 9 (TLR9) (22), which is important in antigen presentation, and secretory leukocyte protease inhibitor (SLPI), which binds with PGRN and inhibits PGRN degradation by elastase (43) and MMP-12 in vitro (36).

The anti-inflammatory effect of PGRN can be neutralized by neutrophil proteinases digesting the protein (18, 43), the action of which has the additional effect of releasing 6-kDa GRN peptides. One of these GRN peptides (GRN-B) has been shown to increase the expression of IL-8 from cell lines (43), thereby potentially amplifying neutrophilic inflammation. It is tempting therefore to speculate that PGRN plays a central role in neutrophilic inflammation in the airways. In addition, increased PGRN expression has been identified in a zebra fish model of chronic tuberculosis (20) and alveolar monocytes following
migration to chemoattractants as an anti-inflammatory response (31). However, there have been no relevant studies to date on the involvement of PGRN in the major dysregulated neutrophil response in COPD. The present study was therefore designed to address this by investigating the presence of PGRN in the airways, its breakdown by NE and PR3, the effect of GRN peptides on bronchial epithelial cells, and measuring PGRN and other mediators of inflammation in the sputum sol phase of COPD patients with a range of disease severity who produced either mucoid or purulent sputum. Results were compared with purulence of sputum, bacterial load (a driver of neutrophil influx), and the presence of other neutrophilic inflammatory mediators.

MATERIALS AND METHODS

Subjects. Patients were recruited with a diagnosis of chronic bronchitis and COPD based on clinical grounds. All had a normal α1-antitrypsin (A1AT) phenotype. The study was approved by the local research ethics committee, and all subjects provided written informed consent. The patients were assessed with full postbronchodilator lung function tests according to ATS/ERS guidelines and a high-resolution CT (HRCT) scan of the thorax when clinically stable.

Sample preparation. Spontaneous stable-state sputum samples (at least 8 wk after any exacerbation) were collected by the patients into a sterile container after a mouth wash to reduce saliva contamination during a 4-h period after waking. The samples were graded using a standardized color chart (Bronkotest, Herdilab, UT). Grades 0 to 2 were mucoid (colorless-white), and grades 3 to 8 were increasing degrees of purulence (pale yellow to dark green). The sputum samples were divided, with one aliquot used for quantitative microbiological culture, as described previously (25), and the other ultracentrifuged at 50,000 g for 90 min at 4°C to obtain the particulate free sol phase. All samples were stored at −80°C until analyzed and handled on ice to minimize the postcollection degradation of the various targeted epitopes.

Measurement of inflammatory mediators. LTβ4, IL-8, and TNF-α were measured by ELISA using commercially available kits (R&D Systems) that have been validated for use in sputum previously (32, 40). MPO activity was measured using a chromogenic substrate MeOSuc-AAPV-pNA (Sigma-Aldrich) (13), and PR3 activity was determined using the fluorescent resonance energy transfer substrate Abz-VAD-norV-ADRQ-EDDnp (Alta Biosciences), as described previously (28). PGRN was measured using a commercially available ELISA kit (R&D Systems), which specifically measures PGRN and not the cleavage products generated by elastase (38). The PGRN assay was validated for use with sputum sol-phase samples by spike and recovery and linearity of dilution. All measurements were carried out using dilutions that gave values within an acceptable range for spike recovery (80–120%) and within-batch coefficient of variation <10% for both purulent and mucoid sputum sol-phase samples (lower limit of detection = 0.001 nM). PGRN measurements were made in sputum sol-phase samples where sufficient volumes were available.

Analysis of PGRN digestion in sputum sol phase. To determine the presence of PGRN and GRN peptides in sputum sol phase, samples were separated on reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with a different general anti-PGRN antibody (R&D Systems) that detects both full-length and fragments of digested PGRN.

To evaluate which proteinases are involved in the digestion of PGRN in the airways, sputum sol phase was diluted 20-fold and incubated either alone or with 1 μM recombinant human SLPI (R&D Systems), 1 μM purified human A1AT (Athens Research and Technology), or 5 mM EDTA (Sigma-Alirdich) at 37°C for 1 h. Recombinant human PGRN (R&D Systems) was then added (255 nM) to inhibitor-treated sol phase. In addition, recombinant human (rh) PGRN (255 nM) preincubated with rhSLPI (1 μM) at 37°C for 1 h was also added to an aliquot of the diluted sol phase alone. All mixtures were incubated at 37°C for 1 h, and the products were then separated on reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with the general anti-PGRN antibody.

To determine whether SLPI inhibited the digestion of PGRN by purified human PR3 (Merck), 200 nM rhPGRN was incubated with either 2.5, 1.25, 0.63, 0.31, or 0.16 μM SLPI for 1 h at 37°C and then incubated with 100 nM PR3 for 1 h at 37°C. The products were separated on reducing SDS-PAGE gels and stained with Colloidal Blue (Invitrogen).

To evaluate the digestion of rhPGRN by purified human NE (Athens Research and Technology) and PR3, 640 nM rhPGRN was incubated with either both 100 nM NE and PR3 for 18 h at 37°C, and then the products were separated on reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with the general anti-PGRN antibody.

Cell assays. To determine the effect of digested PGRN on primary bronchial epithelial cells (NHBE) (Lonza Group), cells were plated at 0.5 × 10^5 cells/well in 96-well plates and after an overnight incubation were treated with either pooled and sterile-filtered (0.2 μm) sputum sol-phase samples diluted with bronchial epithelial cell basal medium (BEBM) (Lonza Group) (n = 6), diluted sputum previously

Table 1. Patient baseline characteristics

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>65.8 (1.0)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 (0.7)</td>
</tr>
<tr>
<td>Pack yrs</td>
<td>49.6 (5.1)</td>
</tr>
<tr>
<td>Males, %</td>
<td>60</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>71.9 (3.5)</td>
</tr>
<tr>
<td>FEV₁-to-FVC ratio</td>
<td>0.56 (0.02)</td>
</tr>
<tr>
<td>KCO, %predicted</td>
<td>98.8 (3.8)</td>
</tr>
</tbody>
</table>

BMI, body mass index; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; KCO, carbon monoxide transfer coefficient.

Table 2. Correlations between PGRN and markers of inflammation

<table>
<thead>
<tr>
<th></th>
<th>Correlation (r)</th>
<th>Significance (2 tailed)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8, nM</td>
<td>−0.532</td>
<td>&lt;0.001</td>
<td>42</td>
</tr>
<tr>
<td>MPO, U/ml</td>
<td>−0.610</td>
<td>&lt;0.001</td>
<td>41</td>
</tr>
<tr>
<td>LTβ4, nM</td>
<td>−0.423</td>
<td>0.005</td>
<td>42</td>
</tr>
<tr>
<td>TNF-α, pM</td>
<td>−0.627</td>
<td>0.002</td>
<td>22</td>
</tr>
<tr>
<td>NE, nM</td>
<td>−0.562</td>
<td>0.008</td>
<td>21</td>
</tr>
<tr>
<td>PR3, pM</td>
<td>−0.515</td>
<td>0.017</td>
<td>21</td>
</tr>
<tr>
<td>Bacterial load, cfu/ml</td>
<td>−0.424</td>
<td>0.005</td>
<td>43</td>
</tr>
<tr>
<td>Sputum color</td>
<td>−0.566</td>
<td>&lt;0.001</td>
<td>43</td>
</tr>
</tbody>
</table>

PGRN, progranulin; IL, interleukin; MPO, myeloperoxidase; LTβ4, leukotriene B4; TNF, tumor necrosis factor; NE, total antigenic (active and inhibited) neutrophil elastase; PR3, proteinase-3 not bound by inhibitor; cfu, colony-forming units. The distribution of mucoid and purulent sputum sol phases in the TNF-α measurement dataset and the NE and PR3 measurement dataset was the same as in the larger dataset allowed for the other measurements (due to sample availability) (P = 0.639 and P = 0.887, respectively). There was also no difference between the datasets in distribution of those patients with or without emphysema (P = 0.876 for the TNF-α dataset and P = 0.650 for the proteinase dataset) or those with or without chronic obstructive pulmonary disease (P = 0.255 for the TNF-α dataset and P = 0.661 for the proteinase dataset).
incubated with the anti-PGRN antibody (20 μg/ml) for 1 h at 37°C (n = 6), or products of PGRN digestion generated in vitro (n = 6). The products were generated by the digestion of rhPGRN (2.5 μM) with NE (1 μM) for 18 h at 37°C, and NE was then inhibited with an excess of A1AT (2 μM) for 30 min at 37°C. Reactions were diluted fivefold in BEBM before being used to stimulate the cells. Supernatant was harvested after 24 h and centrifuged, and the IL-8 content was measured by ELISA (R&D Systems).

Coculture of NHBE with Haemophilus influenzae. Brain heart infusion broth (Thermo Scientific) was inoculated with three colonies of a clinically isolated Haemophilus influenzae strain. The culture was incubated for 4 h at 37°C, and bacteria were then harvested by centrifugation and resuspended in BEBM. An aliquot of the bacterial culture was heated at 80°C for 20 min and pelleted by centrifugation, and the pellet was resuspended in BEBM. Aliquots were taken for serial dilution and plated on Chocolate agar/PolYViteX (bioMerieux) plates for determining colony-forming units (CFU) per milliliter. NHBE were plated at 0.5 × 10⁵ cells/well and after an overnight incubation were exposed to the bacteria at 100:1 bacteria-cells (n = 6 for each condition). The supernatants were removed after 24 h and centrifuged, and PGRN was measured by ELISA (R&D Systems).

### Table 3. Inflammatory mediators measured in patients producing purulent or mucoid sputum

<table>
<thead>
<tr>
<th></th>
<th>Purulent Sputum</th>
<th></th>
<th>Mucoid Sputum</th>
<th></th>
<th>Significance (1 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>n</td>
<td>Median (IQR)</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>PGRN, nM</td>
<td>0.49 (0–0.88)</td>
<td>23</td>
<td>0.92 (0.76–1.28)</td>
<td>20</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-8, nM</td>
<td>9.10 (2.77–18.83)</td>
<td>23</td>
<td>1.93 (0.85–5.99)</td>
<td>19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-α, pM</td>
<td>20.03 (3.36–142.90)</td>
<td>11</td>
<td>0.44 (0.13–2.44)</td>
<td>11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MPO, U/ml</td>
<td>0.89 (0.31–3.49)</td>
<td>23</td>
<td>0.38 (0.20–0.48)</td>
<td>18</td>
<td>0.006</td>
</tr>
<tr>
<td>LTB₄, nM</td>
<td>6.5 (4.37–32.62)</td>
<td>23</td>
<td>4.69 (1.85–8.34)</td>
<td>19</td>
<td>0.002</td>
</tr>
<tr>
<td>NE activity, nM</td>
<td>10.04 (0–79.03)</td>
<td>23</td>
<td>0 (0–0)</td>
<td>19</td>
<td>0.001</td>
</tr>
<tr>
<td>PR3 activity, nM</td>
<td>147.85 (0–329.45)</td>
<td>11</td>
<td>5.47 (0–20.23)</td>
<td>10</td>
<td>0.017</td>
</tr>
<tr>
<td>NE, nM</td>
<td>567.24 (336.67–1464.23)</td>
<td>11</td>
<td>85.20 (44.57–199.15)</td>
<td>10</td>
<td>0.003</td>
</tr>
<tr>
<td>PR3, nM</td>
<td>135.60 (14.16–353.12)</td>
<td>11</td>
<td>4.75 (11.18–19.91)</td>
<td>10</td>
<td>0.007</td>
</tr>
</tbody>
</table>

NE and PR3 are given as both enzyme activity and antigenic (for NE this includes active and inhibited forms; for PR3 it includes just free enzyme not bound by inhibitor).

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**Fig. 1.** Recombinant human (rh) progranulin (PGRN, 640 nM) (lane 1) was digested by neutrophil elastase (NE, 100 nM) and proteinase-3 (PR3, 100 nM) (lane 2), PR3 alone (100 nM) (lane 3), or NE alone (100 nM) (lane 4) for 18 h at 37°C. PGRN and digested PGRN were detected in sputum (lanes 5–9). Sample 5 was produced by a patient with emphysema and chronic obstructive pulmonary disease [COPD; Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2], and samples 6, 7, 8, and 9 were produced by patients with COPD (GOLD 2, GOLD 1, GOLD 3, and GOLD 3, respectively). Samples were separated on a reducing SDS-PAGE gel and Western blotted with anti-PGRN antibody.
genic organisms cultured from the sputum samples included Moraxella catarrhalis, Haemophilus influenzae, Haemophilus parainfluenzae, Pseudomonas aeruginosa, and Staphylococcus aureus. In purulent samples the median pathogenic bacterial load was $1.60 \times 10^6$ CFU/ml [interquartile range (IQR) = 0–1.14 $\times 10^5$ CFU/ml], but was lower ($P = 0.035$) in mucoid samples [0 CFU/ml (IQR = 0–1.68 $\times 10^7$ CFU/ml)].

Inflammatory mediators and PGRN in sputum sol-phase samples. None of the measured inflammatory mediators or PGRN differed significantly between the group of patients with emphysema and those without, nor were there significant differences in measurements of inflammatory mediators or PGRN between the groups of patients that did or did not meet the spirometric criteria for COPD. LTB4 and IL-8 correlated negatively with FEV1 (%predicted) ($r = -0.334, P = 0.03, n = 42$ and $r = -0.386, P = 0.012, n = 42$), respectively, and no correlations were seen with carbon monoxide transfer coefficient (%predicted).

PGRN measurements correlated negatively with the numerical score for sputum sample color, the pathogenic bacterial load, and concentrations of other neutrophilic inflammatory mediators measured in the sputum sol-phase samples gathered from the patients (Table 2). PGRN also correlated negatively with NE activity ($r = -0.334, P = 0.03, n = 42$) and PR3 activity ($r = -0.465, P = 0.034, n = 21$). The neutrophilic inflammatory mediators correlated significantly with each other, with correlation coefficients ranging from 0.474 to 0.914 (data not shown). Multivariate analysis using stepwise linear

![Fig. 2. rhPGRN digestion by sol phase from purulent sputum with 3191.5 nM NE and 304.83 nM PR3 measured activity. Sol phase, diluted 20-fold (so that no endogenous PGRN could be detected), was incubated for 30 min at 37°C with inhibitor, then rhPGRN was added [lane 5, SLPI* = rhPGRN was incubated with secretory leukocyte proteinase inhibitor (SLPI) before addition to sol phase], and the reaction was incubated for a further 1 h at 37°C. Reactions were separated on a reducing SDS-PAGE gel and Western blotted with general anti-PGRN antibody. Lane 2, sol phase only control.](http://ajplung.physiology.org/)

![Fig. 3. rhPGRN (200 nM) was incubated with a range of rhSLPI concentrations for 1 h at 37°C, and then 60 nM PR3 was added for a further 1 h at 37°C. Reactions were separated on a reducing SDS-PAGE gel and stained with Colloidal Blue. Lane 1, untreated rhPGRN.](http://ajplung.physiology.org/)
regression and including IL-8, MPO, LTB4, NE activity, bacterial load, and sputum color score indicated that IL-8 and sputum color score were independent predictors of PGRN concentration [unstandardized coefficient (B) = −0.022, SE = 0.009, P = 0.021 and B = −0.155, SE = 0.067, P = 0.026, respectively], which accounted for 44% of the variance. Following the inclusion of PR3 and NE concentration in the analysis in the smaller sample set where these data were available, PR3 concentration was the only significant predictor of PGRN concentration (B = −0.002, SE ≤ 0.001, P = 0.003) accounting for 36% of the variance. The concentration of PGRN was significantly lower, and all the other inflammatory markers were higher, in purulent sputum samples than in mucoid sputum samples (Table 3).

Detection of GRN peptides in sputum sol phase. PGRN breakdown products were detected in sputum sol phase by Western blot (Fig. 1). Intact PGRN migrated at 93 kDa and could be detected in sol phase from mucoid samples and was absent in sol phase from purulent samples in which no PGRN could be detected by the whole protein-specific ELISA. The absence of PGRN was associated with an increase in the amount of fragments migrating to 6 kDa. NE and PR3 digestion of rhPGRN generated detectable fragments similar to the majority of the fragments in the sol-phase samples (Fig. 1). Digestion of rhPGRN with purified enzymes at low concentrations (0.1 µM) did not generate the individual 6-kDa GRN peptides (Fig. 1), although higher concentrations did (Fig. 4, inset).

Inhibition of sputum sol phase-derived PGRN digestion. The serine proteinase inhibitor A1AT (1 µM) inhibited the breakdown of rhPGRN added to sputum sol phase (Fig. 2), whereas the metalloproteinase inhibitor EDTA (5 mM) did not. SLPI, which specifically inhibits NE but not PR3, provided some protective effect, but detectable products of digestion migrating at sizes seen with the digestion of rhPGRN by PR3 (Fig. 1) were still generated, both when the inhibitor was incubated with rhPGRN before sol phase was added and when the inhibitor was incubated with sol phase before rhPGRN was added. Furthermore, incubating rh-PGRN with up to 2.5 µM rhSLPI did not protect it from digestion by purified PR3 (Fig. 3).

![Fig. 4. Interleukin (IL)-8 secretion from normal human bronchial epithelial (NHBE) cells in response to diluted sterile-filtered sol phase from purulent sputum for 24 h (A) IL-8 secretion from NHBE cells in response to anti-PGRN-treated (1 h at 37°C) diluted sol phase or a 5-fold dilution of 2.5 µM rhPGRN digested with 1 µM NE (18 h at 37°C; NE was inhibited with 2 µM α1-antitrypsin before treating cells) (B). Error bars are SE, n = 6. Inset: silver-stained (SilverQuest; Life Technologies) SDS-PAGE representing the rhPGRN digested with NE for 18 h at 37°C that was applied to NHBE cells. Lane 1, rhPGRN digested with 1 µM NE; lane 2, 1 µM NE.](http://ajplung.physiology.org/)

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**Stimulation of NHBE cells with digested PGRN.** GRN peptide B and PGRN digested with NE increase the expression of IL-8 from A549 and SW-13 cells (43). We therefore treated NHBE cells with pooled purulent sputum sol phase, shown by Western blot to contain GRN peptides, with and without the general polyclonal anti-PGRN antibody (Fig. 4). Incubation with the pooled sol-phase sample alone stimulated IL-8 secretion by the NHBE cells in a dose-dependent manner (Fig. 4A), and the pretreatment of the sol phase with PGRN antibody had no effect (Fig. 4B). We also exposed NHBE cells to overnight NE digests of rhPGRN but saw no increase in IL-8 secretion compared with control (Fig. 4B).

**Effect of H. influenzae infection on PGRN secretion by NHBE cells.** To investigate whether the PGRN levels found in sputum sol phase might be the result of expression by epithelial cells in response to infection, NHBE cells were cocultured with live and heat-killed *H. influenza* (Fig. 5). Heat-killed bacteria had no effect on PGRN secretion (140 pM; SE ± 3.27 pM) compared with control (118 pM; SE ± 8.16 pM) (*P* = 0.263), whereas exposure to the live bacteria resulted in a significant increase (*P* ≤ 0.001) in secreted PGRN (220 pM; SE ± 8.16 pM) compared with BEBM control-treated cells and heat-killed bacteria-treated cells.

**DISCUSSION**

These data are the first studying the presence and potential role of PGRN in the airways of patients with COPD. From previous studies (9, 13) it would be expected that the purulence of the sputum is an indicator of bacterial infection/colonization, and enhanced inflammatory burden marked by a significant increase in the concentrations of many inflammatory mediators, including those measured here. We found a significant difference in PGRN concentrations between mucoid and purulent sputum as well as inverse relationships between PGRN, the intensity of sputum color, and neutrophilic inflammatory mediators. Importantly, we found strong inverse relationships between the amount and activity of the neutrophil-derived serine proteinases NE and PR3 and PGRN concentration. The best independent predictor of PGRN concentration was the amount of PR3 in the sample; this is likely a direct consequence of the proteinases action on the protein combined with the greater quantities of PR3 in the neutrophil compared with NE (4), and of PR3 having greater activity in sol phase than NE (Table 3). Previous studies have shown that these proteinases digest PGRN in vitro and in the circulation (18, 43) [cathepsin G, another serine proteinase released by neutrophils in high concentration, does not digest PGRN (43)]. Furthermore, we detected fragments generated by the breakdown of PGRN in sputum sol-phase samples, and comparison with the PGRN digestion profiles of NE and PR3 shows that these serine proteinases are likely responsible for the PGRN digestion in the airways. Furthermore, the serine proteinase inhibitor A1AT inhibited rhPGRN digestion in sol phase, whereas EDTA had very little inhibitory effect, adding further support to this digestion being the result of serine proteinases and that matrix metalloproteinases do not play a significant role in PGRN breakdown in the airways. Although an NE- and PR3-deficient mouse model has impaired PGRN degradation by neutrophils (18), and we have found that NE and PR3 are the predominant sources for PGRN digestion in the airway secretions, this does not discount that other proteinases may also be involved in some PGRN processing. For example, PGRN is cleaved by the macrophage elastase MMP12 produced by microglia although this cleavage was found to occur only intracellularly (36). Interestingly, the digestion profiles from the microglial cell lysates do not show the 6-kDa fragments (36), whereas 6-kDa GRN peptides (A, B, C, and D) have been isolated from neutrophil cell lysates (2), suggesting that cellspecific processing of PGRN occurs, which may account for its pleiotropic properties. Activated macrophages also release cysteine proteases with elastinolytic activity that could potentially play a role in the digestion of PGRN; in particular, the activity of cathepsin S has been shown to be increased in bronchoalveolar lavage fluid (BAL) fluid from COPD patients compared with controls (7). The digestion of PGRN may not even be limited to host proteinases; for example, both *Pseudomonas* (21) and *Staphylococcus* (23) produce proteinases with elastase-like activity that may also digest the protein. Nevertheless, the fragment profile, inhibition effects, and generation of the 6-kDa fragment by NE all suggest that serine proteinases are responsible for the majority of PGRN degradation seen in the sputum sol-phase samples.

No relationship was found between PGRN measurements and disease status or lung function parameters in the limited studies described here to determine a mechanism. It is likely that PGRN levels are reflecting a snapshot of inflammatory burden and proteinase activity; whether the observations play a key role in the pathophysiology of COPD remains to be determined. The findings reflect the nature of the secretions and not the severity of the disease itself. Of the other sol-phase measurements, only LTb4 and IL-8 correlated weakly with FEV1 (%predicted); this association with potent neutrophil chemoattractants is reflective of the association found between sputum neutrophil burden and FEV1 (%predicted) (29).

Previous studies have shown that SLPI protects PGRN from digestion by NE (43) and MMP12 (36) by binding to PGRN and blocking access to cleavage sites between GRN peptides (43). The inhibitor is seen as an important modulator of PGRN digestion and therefore inflammation. However, in our study we confirmed that SLPI does not protect PGRN from PR3 digestion using either the pure proteins or sputum sol-phase samples. This indicates that more than one proteinase inhibitor...
is required to modulate the anti-inflammatory effect of PGRN in neutrophilic inflammation. Fragments of 6 kDa could be detected in the sol-phase samples; individual GRN peptides 6 kDa in size have been isolated from neutrophils (where GRN peptide A is the most abundant) (2) and from human urine (30). Individual GRN peptides have a range of functions depending on cell type (6). The breakdown of PGRN and therefore removal of its anti-inflammatory properties by serine proteinases demonstrates the ability of these enzymes to amplify inflammation and suggests that they might contribute to chronic inflammation by preventing PGRN from exerting its anti-inflammatory effects.

In addition to regulating the anti-inflammatory effects of PGRN, the generation of GRN peptides can potentially stimulate epithelial cell lines to secrete the neutrophil chemoattractant IL-8. This proinflammatory effect has been demonstrated using 10 μM PGRN digested by NE to stimulate the response from A549 cells (43). Having quantified PGRN levels in sputum sol phase and determined the relative band intensities of the products of PGRN digestion by Western blots, we were able to study concentrations more likely to reflect physiological levels in the airways and found that up to 0.5 μM of the digested protein had no effect on IL-8 secretion. To determine whether there was a difference in response due to cell type, experiments were repeated using A549 cells with physiological concentrations, and again no stimulation of IL-8 secretion was seen (results not shown). We also found that PGRN antibody had no detectable effect on the sputum sol phase-induced stimulation of IL-8 secretion by NHBE cells. Sputum sol phase can contain a number of factors that stimulate IL-8 expression from epithelial cells; these include TNF-α (27), bacterial endotoxin (1, 19), and myeloid-related protein-8/14 (1). While it is possible that the polyclonal antibody does not mask the epitope required for the digested PGRN to interact with the epithelial cell and thus would not block its effect, this result combined with the requirement for high concentrations of GRN peptide and digested PGRN compared with the physiological concentrations in the airways suggests that the contribution of any GRN peptide to IL-8 production from epithelial cells in the airways is negligible compared with that stimulated by other factors.

In the lung, PGRN is expressed by epithelial cells (5), and is likely to be expressed by inflammatory cells, including neutrophils and macrophages (12, 31), with human neutrophils exposed to bacteria transiently upregulating expression of the gene (35). The regulation of PGRN during inflammation in the airways has not been studied in detail in humans. However, analysis of the human promoter of GRN reveals that its expression is likely to be regulated by inflammation (3). The results of our study show that NHBE cells increase PGRN secretion in response to exposure to live bacteria in vitro. This increase in PGRN production due to infection has also been shown in response to Helicobacter pylori using human gastric epithelial cells (39); infection activated the p38 and MEK1/2 signal pathway, resulting in upregulated PGRN mRNA and protein production. It is also consistent with a study using a murine model of lipopolysaccharide (derived from Escherichia coli)-induced acute lung injury where PGRN levels increased in BAL at day 1 post-LPS challenge, followed by a decline to below control at day 3. This decrease coincided with an increase in GRN peptide and in inflammatory markers compared with control mice (11). Interestingly, PGRN−/− mice fail to clear bacterial infection efficiently compared with control mice (41), but whether this role in host defense is played by PGRN or GRN peptides has not been established. The increased expression of PGRN associated with infection may relate to the proteins/GRN peptides role as a cofactor in TLR9 signaling (22). PGRN binds to stimulatory CpG-oligonucleotides, fragments of PGRN have been shown to bind TLR9, and CpG-oligonucleotide-stimulated TLR9 signaling was specifically impaired in in vitro experiments with macrophages using an elastase inhibitor, implicating a role for elastase-digested PGRN in the detection of, and response to, microbial DNA (22). However, the negative correlation of PGRN with bacterial load in patient samples indicates that protein levels are more driven by proteolysis resulting from the neutrophilic inflammation than particular local production in response to bacteria, reflecting a production/degradation imbalance.

The current study demonstrates that it is unlikely that GRN peptides generated in inflammatory conditions in the airways stimulate greater IL-8 production, at least from the airway epithelial cells. However, other roles may be played by the products of PGRN digestion. Further investigation of PGRN and delineation of its roles driven by the whole protein or by digested fragments of the protein will provide important data in determining the process of inflammation in the airways and its treatment.

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GRANTS

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Author contributions: M.J.U. and R.A.S. conception and design of research; M.J.U. and R.A.S. performed experiments; M.J.U. and N.J.S. analyzed data; M.J.U. and R.A.S. interpreted results of experiments; M.J.U. prepared figures; M.J.U. drafted manuscript; M.J.U. and R.A.S. edited and revised manuscript; M.J.U., N.J.S., and R.A.S. approved final version of manuscript.

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