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Interfering with extracellular matrix degradation to blunt inflammation

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Summary of recent advances

Chemoattractant properties of matrix proteins, like collagen and elastin, for neutrophils and monocytes *in vitro* have long been recognized. This activity often resides in fragments of these proteins. These peptides may play a role in diseases of the lung matrix, such as chronic obstructive pulmonary disease. Recent advances include the elucidation of the structure of chemotactic collagen fragments and the demonstration that their activity may reside in a structural relatedness to CXC chemokines. Collagen and elastin fragments have been demonstrated to have a role in *in vivo* lung pathophysiology and have been quantified in patients with chronic lung diseases where they may activate autoimmune pathways. Elucidation of these pathways may provide novel biomarkers and therapeutic targets for chronic lung diseases.

Introduction

Chronic, neutrophilic lung diseases, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), are responsible for increasing morbidity, mortality and healthcare costs worldwide[1]. Damage to extracellular matrix (ECM) proteins, such as collagen and elastin, is central to the pathology of COPD and other chronic lung diseases. One of the hallmarks of COPD is emphysema, defined as dilation and destruction of lung parenchyma distal to the terminal bronchiole[2]. This is believed to result from the activity of proteolytic enzymes secreted by inflammatory cells, initially recruited to the lung by environmental irritants such as cigarette smoke, leading to ECM turnover and remodeling[3]. This is supported by the fact that patients with alpha 1-antitrypsin deficiency, an inhibitor of serine proteases, develop severe, early-onset emphysema. By contrast, there is increased collagen deposition in the lung parenchyma in chronic fibrosing lung diseases, such as interstitial pulmonary fibrosis (IPF) and sarcoidosis, and subepithelial fibrosis in the airways is the hallmark of asthma in its chronic and irreversible form.

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A major contribution to the morbidity associated with chronic lung disease is the lack of effective treatments that address underlying pathogenic mechanisms and improve outcomes. Although inflammatory cells, such as macrophages and neutrophils, are strongly implicated in the pathogenesis of these disorders, standard anti-inflammatory drugs such as corticosteroids are largely ineffective in altering the natural history of the disease[4]. The implacable progression of diseases such as IPF and the persistence of airway inflammation and continued lung function decline in COPD patients despite smoking cessation remain to be explained[5]. This review will examine the evidence that matrix proteins in the lung are not bystanders but are active participants in novel pro-inflammatory pathways central to progression of these diseases. If fully elucidated, these pathways might provide us with novel biomarkers and therapeutic targets for chronic lung diseases.

Proteases and the lung

The importance of extracellular matrix destruction in the pathology of COPD has led to interest in the enzymes responsible for collagen and elastin turnover in the lung. Human neutrophil elastase (HNE) is a serine protease that has been shown to be a biomarker of pulmonary inflammation and to disrupt the pulmonary architecture through damage to structural proteins in CF and COPD[6–8]. HNE also has potent immunologic effects and has been reported to cause induction of IL-8 release from airway epithelium and cleavage/inactivation of important regulators of immunity such as immunoglobulins and CD14 from monocytes[9;10]. Recently, Hartl et al. have described a novel role for HNE in the cleavage of CXCR1 from neutrophils causing defective oxidative burst in these cells in patients with COPD and CF[11].

Another group of proteases receiving attention in chronic lung diseases are the matrix metalloproteinases (MMP's), a family of zinc-dependent metalloendopeptidases. More than 20 MMP's have been identified and are subdivided into collagenases, gelatinases, stromelysins, matrilysin, macrophage elastase, and membrane-type MMP's, based on structure and substrate specificity[12]. MMP's perform numerous biologic functions, including degradation of matrix components and remodeling of tissues, release of cytokines, growth factors and chemokines, and modulation of cell mobility and migration[13]. Possible sources of MMP's in the lung include neutrophils, alveolar macrophages, and airway epithelial cells. Neutrophils are a particularly rich source of MMP's, expressing MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), and MMP-2 (gelatinase A). Together, these have the capacity to digest multiple extracellular matrix molecules, including types I, II, III and IV collagen in basement membranes, fibronectin, chondroitin sulfate proteoglycans, dermatan sulfate proteoglycans, elastin, and laminin. Through degradation of extracellular matrix components, MMP's can destroy the alveolar epithelium and disrupt reorganization during the repair process.

Multiple lines of evidence implicate HNE and MMP's in the pathogenesis of chronic lung diseases. Recently, our group has described the expression of discrete MMP isoforms (MMP's-8, 9, 11, and 12) in sputum from CF patients[14]. CF alveolar epithelium demonstrates increased MMP-7 expression[15]. Mice deficient in HNE or MMP-12 demonstrate decreased airspace enlargement and inflammatory cell infiltration after long-term exposure to cigarette smoke compared to their wild-type littermates, revealing a possible role for these proteases in COPD[16–18]. COPD patients demonstrate increased activity of a variety of proteases in lung specimens, including HNE and MMP's-8, 9, 2 and 12[19;20]. Both neutrophils and alveolar macrophages are felt to play a role in the secretion of these proteases. MMP's -1, 2, 8 and 9 have also been demonstrated to be elevated in asthmatic airways albeit to a lesser extent than in COPD[20]. Increased expression of MMPs-8, 9 and 7 has been demonstrated in the fibrosing diseases, IPF and pulmonary sarcoidosis[21–24].

Chemotactic properties of collagen and elastin fragments

Critically, it is increasingly being recognized that not only proteases but also their products are important in the pathology of chronic lung diseases. The ability of structural proteins or fragments of structural proteins generated by the action of proteolytic enzymes to induce chemotaxis of monocytes and neutrophils has been recognized for more than 20 years, though only recently has their role in *in vivo* pathophysiology become evident. Postlethwaite et al recognized that intact and degraded collagen, including collagenase-generated peptides with estimated sizes in the range of tri to decapeptides, were chemotactic for monocytes but not neutrophils[25]. Riley et al demonstrated that collagen-derived peptides, when instilled in the trachea, could cause neutrophil influx into the lungs of rats[26]. Different collagen fragments have been described to either augment or suppress IL-1 β production from human peripheral blood monocytes[27]. Similarly, Senior et al have described chemotactic activity of elastase digests of elastin for monocytes but not neutrophils[28]. This chemotactic activity was found principally in peptides of 14,000 to 20,000 mol wt. A repeating hexamer in elastin, Val-Gly-Val-Ala-Pro-Gly, is believed to be an important component of its chemotactic activity[29]. Additionally, fragments of fibronectin may stimulate fibroblast proliferation implicating them in the process of fibrosis and scarring of the lung in diseases such as sarcoidosis and IPF [30]. As with collagen, full-length fibronectin does not show bioactivity.

A recent publication further outlined the potential importance of elastin fragments in pulmonary emphysema. Bronchoalveolar lavage fluid (BALF) from cigarette smoke-exposed wild type mice contained elastin fragments that were chemotactic for monocytes *in vitro* and *in vivo* but not for neutrophils[31]. These fragments were not present in the BALF of cigarette smoke-exposed MMP12^{-/-} mice. Antagonism of elastin fragments reduced porcine pancreatic elastase induced macrophage recruitment and development of emphysema in mouse lungs. Macrophage derived MMP-12 was implicated in the generation of elastin fragments, monocyte recruitment and chronic lung inflammation (See Fig. 1 for an overview of a putative MMP-12/elastin/monocyte pathway). These data suggest that products of collagen and elastin degradation in emphysema may provide signals for the further recruitment of inflammatory cells into the lung. Fragments of fibronectin and laminin have also been shown to be chemotactic for monocytes[32;33].

N-acetyl-Pro-Gly-Pro, a chemotactic collagen fragment

We have recently described a potentially new pathway that signals neutrophil infiltration followed by damage to the airways which may represent a novel etiology as well as diagnostic and therapeutic target for chronic airway diseases[34]. Our elucidation of this pathway has its origins in alkali injury to the eye. Alkali injury to the eye causes a profound influx of neutrophils and it is the neutrophils rather than the alkali per se that precipitates inflammation leading to corneal ulceration and perforation[35]. In 1995, Pfister and colleagues demonstrated that alkali degradation of whole cornea generated two tri-peptides, N-acetyl-Pro-Gly-Pro (N- α -PGP) and N-methyl-PGP that are chemotactic for neutrophils and likely result from hydrolysis of collagen[35]. Injection of these peptides into normal corneas recapitulated the early events in alkali injury to the eye[36]. Previously, Laskin and colleagues demonstrated that not only collagenase digests of collagen but also synthetic peptides representing 1, 5, or 10 repeats of the collagen sequence PPG were potent chemoattractants for PMN[37]. Thus chemotactic peptides for neutrophils can be elicited by either enzymatic or chemical hydrolysis of collagen. Muccio et al further demonstrated that the modified or unmodified PGP sequence was required for activity and PG or GP or PGG was inactive. Acetylation of the N terminus greatly increased activity of PGP as did adding a flanking amino acid on the amino and carboxy termini[38]. Pfister et al., like Laskin and colleagues, also found that PPG was chemotactic for neutrophils, but was not as active as PGP[35].

After delineating the structural requirements for PGP activity on neutrophils, we designed a peptide antagonist to PGP. This peptide, RTR, was tetramerized as a branched structure by synthesis on a multiple antigenic peptide resin to increase the avidity for PGP[39]. The tetramer was found by nuclear magnetic resonance spectroscopy (NMR) techniques to interact with PGP in solution. RTR, but not a control tetramer with the sequence ASA, was a potent inhibitor of *in vitro* PGP, but not leukotriene (LT) B₄, activation and chemotaxis of neutrophils. RTR also markedly inhibited and ameliorated alkali damage to rabbit corneas *in vivo* by blunting neutrophil influx[40].

As a result of the apparent role of PGP in PMN infiltration into the damaged cornea, we evaluated whether it could fulfill a similar function in the airways. N- α -PGP was chemotactic for neutrophils in a dose range between 10 nM and 100 μ M, while the PGG control peptide had no chemotactic activity. Following intratracheal instillation, N- α -PGP caused a marked increase in the total number of cells in lungs of peptide exposed C57Bl/6 mice, while PGG or PBS alone showed no change[34]. The increase in neutrophils completely accounted for the increase in total cells, and other cell types were unaffected by either peptide.

N- α -PGP has structural homology with CXC chemokines

The CXC chemokines active on neutrophils possess a Glu-Leu-Arg motif and are identified as ELR-positive CXC chemokines. In humans, these include IL-8, active on CXCR1 and 2 chemokine receptors, and the GRO- α , β and γ chemokines, which ligate only CXCR2. Several ELR⁺ CXC chemokines contain a conserved PPGPH sequence immediately N-terminal to the third structural cysteine, while IL-8 has the sequence ESGPH in this position. Structure-function studies of IL-8 show the 'GP' motif as a very important requirement for neutrophil cell binding and activation in radioligand and elastase release assays, respectively. To assess structural similarity between PGP and this important domain of these chemokines, we compared the available structures of IL-8 and PGP. The molecular orientation of IL-8's SGP motif is well represented in the solution structure of the predominant 'all trans' isomer of PGP. To determine if this 'SGP' region of IL-8 is alone active as a chemoattractant, we tested human PMN chemotaxis to N-acetyl Ser-Gly-Pro, and found it has similar activity and dosage as that seen with PGP. Thus, N- α -PGP has structural homology to CXC chemokines which possibly underlies its neutrophil chemoattractant properties. We also demonstrated that N- α -PGP competitively binds CXC chemokine receptors and requires the CXCRs for its neutrophil chemoattractant activity[34]. These findings were confirmed *in vivo* by the demonstration that in contrast to wild type Balb/C animals, N- α -PGP-treated CXCR2^{-/-} mice did not accumulate PMN in the airway compared to PBS-treated animals.

N- α -PGP is generated by lung inflammation

We found that N- α -PGP was present in LPS-challenged mouse airways[34]. In the first six hrs after challenge, the neutrophil chemokines KC and MIP-2 were produced at high levels, and neutrophils began to infiltrate the airway. Between 6 and 12 hrs after exposure, PMN cell numbers continued to increase though chemokine concentrations declined significantly. During this time the N- α -PGP peptide became detectable in nanogram amounts, and 12 hrs after LPS exposure, N- α -PGP concentrations had risen to over 700-fold molar excess to KC. From 24 to 48 hrs after exposure, airway neutrophil numbers declined as the N- α -PGP peptide signal disappeared. These results suggest that chemokines such as KC and MIP-2 initiate the influx of neutrophils into the airways after LPS exposure. These neutrophils in turn may degrade collagen to PGP and PGP-containing peptides. N- α -PGP then contributes to the maintenance and extension of PMN influx during the period of declining chemokine levels. Studies using monoclonal antibodies demonstrated that PGP-containing peptides and the chemokines, KC and MIP-2, contribute equally to neutrophil chemotaxis to BAL fluid from

LPS-challenged mouse airways. Finally, C57Bl/6 and Balb/C mice treated with N- α -PGP by direct airway instillation twice weekly for 12 weeks developed emphysematous changes similar to those reported for C57Bl/6 mice exposed to smoke from 2 cigarettes daily 6 days per week for 6 months. This indicates that N- α -PGP can generate chronic lung inflammation leading to COPD-like pathology.

We have detected N- α -PGP in BAL fluid from patients with COPD but not healthy controls or asthmatics indicating that N- α -PGP may be a potential biomarker or therapeutic target in COPD[34]. Inhibition studies have demonstrated that IL-8 and LTB₄ together are responsible for at most 40% of neutrophil chemotaxis to COPD sputum[41]. This indicates that other as yet undiscovered pathways are mostly responsible for neutrophil recruitment to the airways in COPD. Blockade of both N- α -PGP and IL-8 may be required to address airway inflammation in COPD, which may explain the lack of success with anti-IL-8 therapy alone[42]. N- α -PGP represents an attractive therapeutic target as it can be impacted at three levels; the receptor (using CXCR antagonists), the ligand (using PGP antagonists such as RTR) and the generating enzyme.

Targeting matrix degradation in chronic lung disease

We are currently investigating the proteolytic enzymes responsible for generation of N- α -PGP from collagen. MMP's are candidate enzymes due to their known properties as collagenases and their role in chronic lung diseases, including COPD. In fact, a recent paper has demonstrated that MMP-9^{-/-} mice fail to generate N- α -PGP or recruit neutrophils to their lungs in a model of pneumonic tularemia[43]. (See Fig. 2 for an overview of a putative MMP-9/N- α -PGP/neutrophil pathway). Although MMP inhibitors have been demonstrated to have therapeutic potential in mouse models of COPD, their efficacy may be limited due to problems with specificity and toxicity[44]. Furthermore, MMP's may have beneficial effects in resolution of inflammation and reconstitution of normal lung. A drug class of demonstrated efficacy and safety in chronic lung diseases are the macrolide antibiotics. Macrolides have been demonstrated to have numerous anti-inflammatory effects that could be important in COPD, including effects on neutrophils and cytokines[45;46]. Long-term treatment with macrolides has demonstrated efficacy in several chronic, neutrophilic lung diseases, including bronchiectasis, post-transplant bronchiolitis obliterans syndrome (BOS), panbronchiolitis and cystic fibrosis[47–49]. Anti-inflammatory and clinical effects of macrolides may be mediated, in part, by reducing MMP-9 expression by inflammatory cells and, thereby, generation of N- α -PGP or other matrix derived chemoattractants[50;51].

Conclusion

In summary, fragments of matrix proteins generated by proteolytic enzymes provide novel biomarkers and therapeutic targets in chronic lung diseases such as COPD through their effects on neutrophil and monocyte migration. Beyond inflammation and innate immunity, there may be auto-immune consequences to the generation of collagen and elastin fragments which may contribute to the progression of diseases such as COPD and BOS[52;53]. The relevance of these pathways to chronic lung diseases seems clear, particularly as the lung is rich in collagen and elastin. Realizing the full potential of these pathways as therapeutic targets will require additional work to fully describe proteolytic pathways and ligand-receptor interactions and to associate different proteases and their products with phenotypes of emphysematous and fibrotic lung diseases.

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Airway epithelium is damaged, often from infection, leading to the release of monocyte chemoattractants, such as MCP-1, into the interstitium. Increased concentrations of MCP 1 in the interstitium induce chemotaxis of monocytes from the vascular space via CCR2 binding. Activated monocytes release MMP-12 and other MMPs and activated neutrophils release HNE which cleave elastin into fragments containing the motifs GXXPG or XGXP. These fragments act on elastin receptors on monocytes to induce further monocyte recruitment and perpetuate damage to the lung.

Figure 2.

Airway epithelium is damaged, often from infection, leading to the release of IL-8 into the interstitium. Increased concentrations of IL-8 in the interstitium induce chemotaxis of neutrophils from the vascular space via a CXCR 1/2 mechanism. Activated neutrophils release MMP-9 which cleaves collagen into fragments containing PGP. PGP acts on CXCR to induce further neutrophil recruitment and perpetuate damage to the lung.