A DUAL PROTEASE INHIBITOR/RECEPTOR ANTAGONIST WITH THERAPEUTIC IMPLICATIONS FOR CHRONIC INFLAMMATORY LUNG DISEASES

by

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A DISSERTATION

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Chronic neutrophilic inflammation is a hallmark of numerous pulmonary diseases. It is commonly associated with declining lung function, collagen turnover, and increased protease activity. Our laboratory has recently published several articles describing a biologically active collagen breakdown product, proline-glycine-proline (PGP) and its more active amino-terminus acetylated form, N-α-PGP. PGP acts via classical chemokine receptors CXCR1 and 2 to draw neutrophils (PMNs) into sites of inflammation in what is potentially a feed-forward mechanism of disease. The tri-peptide appears to be a bio-marker in certain clinical diseases like cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). The present work first details PMN proteases and their mechanism of release, with an emphasis on their role in inflammation. A closer examination is taken of a serine protease, prolyl endopeptidase (PE), that performs the final proteolytic cleavage of PGP from collagen fragments. These experiments detail for the first time that PE is present and active in PMNs. In turn demonstrating that neutrophils contain all of the necessary enzymes to take intact collagen and produce PGP, indicating a possible feed-forward mechanism of PMN inflammation. Additionally, another model of chronic neutrophilic inflammation in pulmonary disease, bronchiolitis obliterans syndrome (BOS) is examined to determine if PGP is a potential mediator of aspects of this condition. Indeed, it appears that at the
time of diagnosis of BOS, or chronic rejection of lung transplant, there is an increase in
the both the proteases responsible for its production, and PGP itself. Moreover, there is a
shift from the traditional PMN chemokine, interleukin-8 (IL-8) to PGP playing the more
prominent role in neutrophil migration. Finally, we describe an extremely novel concept
of a dual PE inhibitor and CXCR antagonist activity residing in a single compound,
benzyloxycarbonyl-proline-prolinal (ZPP). We show that ZPP is capable of blocking
both PE activity and generation of PGP, but also CXCR mediated PMN recruitment and
directly competes with IL-8 for binding. This work advances the concept of a self
sustaining mechanism of chronic neutrophilic inflammation and introduces a novel
concept for potential therapeutics directed at blunting the condition.
DEDICATION

This dissertation is dedicated to my family. To my beautiful loving wife who I am eternally grateful for, and who has supported me throughout this work and so much else. Also to my parents, who have supported me in every way possible throughout my life and whom I thank for everything they have done.
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I extend my heartfelt thanks to all of my family, friends and colleagues without whom this work would not have been accomplished. I thank my wife Andrea for her support and patience with my during my work on this project, she has been a true encourager and blessing to me. I also thank my parents who supported and aided me in anyway asked. I would be remiss to not include my extended family; my grandparents, my brothers Benjamin and Jonathan, along with his wife Kristen, and Geoff Moeck who helped me to enjoy life away from this project. I also thank my in-laws, the Smiths for being supportive and encouraging to their Son-In-Law. I especially want to acknowledge the guidance and mentorship of Ed Blalock. He has truly been a wonderful example of a scientist, mentor and friend. I would like to thank my committee members Amit Gaggar, Patricia L. Jackson, Suzanne Oparil, Lisa M. Schwiebert, and Eric Sorscher for their advice and assistance in the completion of this work. Additionally, this work would not have been possible without the collaboration of F. Shawn Galin, Robert Snelgrove, Xin Xu, Steven Rowe, Brett Noerager, and Doug and Diane Weigent.
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## INTRODUCTION

1 Model of PMNs and their proteases in chronic inflammation

## PROTEASE RELEASE FROM NEUTROPHILS IN INFLAMMATION: IMPACT ON INNATE IMMUNITY SEEN IN CHRONIC PULMONARY DISEASE

1 Protease Modulation of Innate Immunity in Lungs

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A1AT  alpha-1 anti trypsin deficiency
Aa amino acid
Ab antibody
Ac acetyl
APMA aminophenylmercuric acetate
AR acute rejection
ARDS acute respiratory distress syndrome
BAL/BALF broncho alveolar lavage fluid
BOS bronchioloitis obliterans syndrome
BSA bovine serum albumin
C-terminal carboxy-terminal
Ca$^+$ calcium
CF cystic fibrosis
Cl$^-$ chloride
CMV cytomegalovirus
COOH carboxyl
COPD chronic obstructive pulmonary disease
CXC CXC chemokine
CXCL CXC chemokine ligand
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<tr>
<td>Deg</td>
<td>degree</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELR</td>
<td>glutamate-leucine-arginine</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>forced expiratory volume in 1 second</td>
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<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GPCR</td>
<td>g protein-coupled receptor</td>
</tr>
<tr>
<td>GRO</td>
<td>growth related oncogene</td>
</tr>
<tr>
<td>HNE</td>
<td>human neutrophil elastase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitor concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>interleukin</td>
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<td>IV</td>
<td>intravenous</td>
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<td>LC</td>
<td>liquid chromatography</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>TBB</td>
<td>transbronchiale biopsy</td>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteases</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>UAB</td>
<td>University of Alabama-Birmingham</td>
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<tr>
<td>ZGP-pNA</td>
<td>benzyloxy carbonyl-glycine-proline-paranitroaniline</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>zinc</td>
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<tr>
<td>ZPP</td>
<td>benzyloxy carbonyl-proline-proline-prolinal</td>
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INTRODUCTION

Neutrophilic Inflammation

Polymorphonuclear leukocytes (PMNs), or neutrophils, play an important role in the body’s innate immune system’s response to invading pathogens. PMNs originate in the bone marrow and traffic through the blood until such time as they are recruited to a site of infection or inflammation in the tissues (1). A family of molecules called chemokines is responsible for neutrophil migration out of the blood stream into the periphery (2). There are several sub-groups of chemokines, but neutrophil specific chemokines are glutamate-leucine-arginine positive CXC chemokines (ELR+CXC). Interleukin-8 (IL-8) or CXCL8, growth related oncogene (GRO)-α (CXCL1), GRO-β (CXCL2), and GRO–γ (CXCL3) are the primary chemokines in humans. In mice, they are cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein 2 (MIP2) (3, 4). In humans these ELR+CXC chemokines bind to CXC receptors 1 and 2 (CXCR1 and CXCR2), although there is only CXCR2 in mice (5). Neutrophils follow a concentration gradient of these chemokines from the circulation into the tissues to sites of inflammation (6). The CXC receptors are G-protein coupled receptors (GPCRs), which signal rearrangement of the cytoskeleton via a Gβγ subunit. CXCR activation leads to polymerization of the actin-myosin network in a subcellular localization with actin-myosin depolymerization away from the site of receptor ligation, leading to directional movement of the cell (7). Upon reaching the tissue the PMNs
release their anti-microbial enzymes through a process of degranulation, and can perform oxidative burst by which the cells can destroy the invading pathogen (1, 8, 9).

**Neutrophil-Derived Proteases**

While neutrophils contain large numbers of enzymes, some of the more important types of enzymes pertinent to this work are proteases. Several of the protease families contained in neutrophils, like matrix-metalloproteases (MMPs) and serine proteases, have been implicated in inflammation (10-12). MMPs are a family of zinc dependent endopeptidases that are capable of degrading almost all types of basement membrane proteins. To date, over 25 types of MMPs have been identified in humans, and are classified based on structure and selection of substrate (13). Due to MMPs’ capability to hydrolyze components of the extra-cellular matrix (ECM), these enzymes are kept under tight control. MMPs undergo transcriptional and translational regulation, along with post-translational modification by a number of cytokines and conversion from a pro to active form via enzymatic cleavage (14). MMPs are different than other proteases however, in that they are naturally inhibited by a group of molecules known as tissue inhibitors of metalloproteinases (TIMPs) (15). There are numerous studies to indicate that a dysregulation or imbalance of MMPs with their naturally occurring anti-proteases (TIMPs) can lead to progressive destruction of the underlying matrix and increasing fibrosis (16, 17). MMP-8 and MMP-9, a collagenase and gelatinase respectively, are two of the primary MMPs contained in PMNs (18, 19). These two proteases, capable of degrading all types of collagen, have been implicated in multiple chronic inflammatory
conditions in the lung, where unremitting airway remodeling and inflammation occur (20, 21).

Human neutrophil elastase (HNE), proteinase 3 (PR3), along with cathepsins D and G are all serine proteases that are also contained in neutrophil granules (22, 23). HNE activity has been shown to be upregulated in a number of chronic inflammatory conditions, specifically lung diseases such as α₁-antitrypsin deficiency (A1AT) and cystic fibrosis (CF) (24, 25). HNE is able to break down several matrix proteins and is a potent activator of other proteases such as MMPs, cleaving them from the pro to active form (20, 26). It was only in work recently published by our group that prolyl endopeptidase (PE) was discovered in PMNs. Prolyl endopeptidase is a protease only recently described in inflammation. PE is unique in that it is one of the few enzymes capable of hydrolyzing the peptide bond on the carboxy side of a proline (27). It is composed of two structural domains, a catalytic domain composed of αβ hydrolase folds, and a propeller domain thought to be important in regulation of substrate availability to the catalytic site (28). Prior to the work of our group the prevailing opinion was that its role was restricted to the processing of neuropeptides and an alternative pathway in the renin-angiotensin pathway (29, 30). Work done by our laboratory has shown that PE, in conjunction with MMP-8 or MMP-9, is integral in producing a chemotactic tripeptide (proline-glycine-proline (PGP)) from collagen (5, 31).

**PGP in Chronic Neutrophilic Disorders**

The majority of the background material presented so far has dealt with the role of neutrophils and their proteases in normal homeostatic processes and in chronic
inflammation. Nowhere is tight regulation of PMNs and their potent proteases more important than in the lung, where the various components of the extracellular matrix (ECM) provide ample substrate and opportunity for dysregulated inflammation to damage normal lung architecture. These proteases are important not only for the part they play in the pathogenesis of disease but also in the maintenance and progressive nature of many of the chronic conditions discussed. MMP-8 and -9 are capable of digesting all four types of collagen, including type-I collagen which is the major collagen present in the lung (32, 33).

It has been known for several years that collagen fragments are chemotactic to neutrophils. What was not clear was exactly how and which specific fragments are causing the PMN migration (34-36). In 1995, Pfister et al. were able to determine that the sequence, proline-glycine-proline (Pro-Gly-Pro, PGP) confers chemotactic potential to collagen breakdown products. The group also noted an N-terminal acetylation present on the peptide which increases chemotactic activity (37). In a 2006 Nature Medicine paper, our group demonstrated the mechanism by which PGP is able to attract neutrophils into tissue. PGP shares sequence and structural homology with almost all ELR+CXC chemokines, which act via CXC receptors 1 and 2 in human (CXCR1, CXCR2). It was shown that PGP competes with the classical CXCR ligand, IL-8, for binding, is able to cause chemotaxis in CXCR transfected cells and produces a similar oxidative burst to IL-8 stimulation (5). Through a series of studies it appears that PGP’s effectiveness is based on its structural relatedness to IL-8. Additionally, when instilled into the tracheas of mice PGP caused neutrophil influx into the airways, alveolar enlargement, and right ventricular hypertrophy (38). Thus, this peptide alone recapitulates the features of
COPD. More recently we have also elucidated the putative pathway for its production. Gaggar, et. al. demonstrated that there is a step-wise breakdown of whole collagen to the tripeptide.

MMP-8 and/or -9 is/are capable of initially digesting collagen but is/are not capable of performing the final cleavage to PGP. Thus, a second step is necessary for the tripeptide’s liberation. Our lab was able to demonstrate that the serine protease PE performs the necessary proteolysis (31). PE cleaves after a proline in a peptide substrate of about 100 amino acids or less in length. MMPs perform the initial degradation of collagen, followed by a subsequent cleavage by PE to release the potent neutrophil chemoattractant, PGP from the often repeated PPGP sequence in collagen (38).

PGP and its acetylated form have been identified in several chronic inflammatory lung conditions, most notably cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD) (20, 21, 39). A hallmark of all of these conditions is a destruction, remodeling, and fibrosis of the airways coincident with an increase in protease activity. In a recent study, sputum collected from CF patients demonstrated both a dramatic increase in the amount of the more potent form of PGP, its acetylated form, N-α-PGP, and the sputum’s ability to cause PMN chemotaxis compared to that of controls (31). Multiple studies have demonstrated an increase in general proteolytic activity, and more specifically MMP activity in CF. The CF study performed by Gaggar, et al also indicated an increase in not just MMPs but PE activity as well. The chemotactic collagen peptide and the proteases responsible for its production were both elevated in CF compared to that of normal controls (31). COPD has proven to be similar in its proteolytic profile. Similar to CF, PGP and N-α-PGP are both increased in COPD, as are the proteases
responsible for their production, MMP-8, MMP-9, and PE. Additionally, when sputum or bronchoalveolar lavage fluid from these patients is incubated with collagen, PGP is produced. When the appropriate inhibitors of the proteases (MMP, PE) are added to these samples this ablates their generative capacity. Unfortunately all of these conditions have very poor outcomes, with current therapeutics only able to address the symptoms, and unable to reverse the course of disease (40, 41). The optimal and final recourse for many physicians is in fact lung transplantation.

Lung transplantation is utilized in approximately 1500 patients per year in the United States to treat end stage lung disease. The most common conditions resulting in transplantation are chronic obstructive pulmonary disease (COPD), α₁-antitrypsin deficiency (A1AT), cystic fibrosis (CF), and pulmonary fibrosis (42). Lung transplant currently has the worst outcome of any whole organ transplant (43, 44). Unfortunately, lung transplantation is the only remaining option for patients with chronic progressive lung disorders. The relative lack of donors and difficulty of transplant have contributed to a five year survival rate of less than 50%.

Rejection continues to serve as a major problem for post-transplant individuals. There are two primary types of allograft rejection; acute and chronic. Acute rejection (AR) is predominantly a lymphocytic rejection that occurs in the majority of patients. Most patients will have at least a single episode of AR within the first year post-transplant (45). The diagnosis of acute rejection is made by a pathologic judgment of a transbronchial biopsy (TBB). Histologically, AR is characterized by a preponderance of lymphocytes centered around the vasculature and interstitium (46). While multiple studies have indicated that multiple occurrences of AR increase the risk for development
of chronic rejection or BOS, AR is much more easily treatable (47, 48). A normal course of treatment for AR is a three day course of intravenous (i.v.) steroids followed by a three day oral taper (this varies slightly from center to center) (49). AR will usually resolve quickly and, if treated properly, is not life-threatening to the patient.

The primary reason for such poor long term survival of the transplant recipient is chronic allograft rejection, or clinically, bronchiolitis obliterans syndrome (BOS) (50). With only 15% of all multiple organ donors’ lungs considered suitable for transplant there is a lack of opportunity for a large multi-center study to produce a consensus opinion as to the reason for BOS development (51, 52). Diagnosis of chronic rejection (BOS) in a lung transplant recipient is a much more serious occurrence, with BOS responsible for approximately 30% of late mortality (53). Chronic rejection is a progressive, debilitating disease that dramatically impairs quality of life in patients and leads to eventual pulmonary failure. Almost 50% of patients surviving five years will be diagnosed with BOS, creating a dire need for effective treatments for this progressive condition (54). Differential diagnosis of BOS is difficult due to its similarities with other post-transplant maladies. There is no specific test to determine chronic rejection, rather, it is a diagnosis of exclusion. A persistent, unexplainable drop in forced expiratory volume in one second (FEV$_1$) (~80% of baseline) with accompanying decline in FEV$_{25-75}$ (less than or equal to 75% of baseline) is defined as BOS stage 0 (48, 55). BOS has been categorized into progressive stages (Stage 0 - Stage 4) based on percent of FEV$_1$ and FEV$_{25-75}$ with BOS Stage 3 being an FEV$_1$ of less than 50% of baseline (55, 56).

Histologically, BOS has a significant neutrophilic component with an observable increase in fibrosis and collagen turnover (57, 58). Chronic neutrophilia in
bronchoalveolar lavage fluid (BAL) is a hallmark of BOS and is seen in the majority of patients. Indeed, in a recent study, when neutrophilia (greater than 20% of cells) was seen in BAL those patients did not survive past 7 years as opposed to non-neutrophilic BAL patients whose survival extended beyond 10 years (59). Whereas in AR the cellular infiltrate is centered around the vasculature, in BOS the cells are found mainly in and around the airways with a dramatic increase in total cell number and activation level of leukocytes (60). In addition to the increase in immune cell number there is a distinct uptick in fibrosis and collagen turnover seen in BOS (21). The causes of BOS are not well known, although there is some evidence that the risk of incidence increases with bouts of acute rejection and that cytomegalovirus (CMV) may play a role, although there is not a unified viewpoint held at this time (61). Most likely, in the initial stages of BOS, there is damage to the pulmonary epithelium (possibly from ischemia/reperfusion injury associated with the transplant itself) these cells release pro-inflammatory chemokines, recruiting neutrophils into the airway and initiating the events which may trigger the dysregulated immune response and eventual ongoing airway damage (58).

Possible Therapeutic Targets in the PGP Pathway

Central to the three main medical conditions discussed herein is the IL-8/PGP mediated pathway of neutrophilic inflammation. To date, multiple attempts at blocking the IL-8 pathway of inflammation have been largely unsuccessful. This presumably is due to the parallel PGP pathway. To be truly effective in staving off chronic neutrophilia, an effective treatment regimen would involve addressing both pathways of PMN recruitment. As multiple studies have shown, there are several points along the
pathway of PGP production that would be possible targets for novel therapeutics aimed at ablating chronic neutrophilia. One site is blunting the activity or presence of proteases that are so prevalent in CF, COPD, and BOS. Doxycycline, a member of the tetracycline class of antibiotics, is also a potent inhibitor of MMP activity, specifically MMP-2, -8, and -9 (62). A study in which doxycycline was prophylactically given to pigs prior to administration of LPS showed that it prevented acute respiratory distress syndrome (ARDS). ARDS is characterized by high levels of cytokines and chemokines, vascular permeability and neutrophil recruitment resulting in increased levels of MMPs, HNE, etc. (63). A clinical trial currently underway in the Netherlands is investigating the use of doxycycline as a possible MMP inhibitor, with outcomes based on myeloperoxidase and MMP levels in induced sputum from patients with COPD (ClinicalTrials.gov identifier: NCT00857038). In addition, Gaggar, et al are currently examining the potential therapeutics impact of doxycycline in CF lung disease in a Phase I/II trial in the US.

Another possible target for alleviating or even preventing the burden of chronic neutrophilic inflammation is prolyl endopeptidase. There are several specific inhibitors of the enzyme with a half maximal inhibitor concentration (IC\textsubscript{50}) in the nanomolar range. Benzyloxycarbonyl (ZPP), a potent inhibitor of PE acts by slow-tight binding in the active site of the enzyme (64). An orally active small molecule inhibitor of the enzyme would be quite effective in shutting down PGP production before it is able to tilt the scales from acute to chronic inflammation. S 17092 is one such recently developed compound. Fortunately, due to PE’s perceived role in schizophrenia and other neurological conditions, multiple clinical trials with S 17092 have already taken place which indicates that it is indeed safe for treatment in humans and provides measureable
benefits in neurodegenerative diseases (65, 66). Currently no clinical trials are underway investigating S 17092’s possible anti-inflammatory effects but due to PE’s apparent role in chronic inflammation this seems the next logical step.

What would be most effective in neutralizing the IL-8/PGP pathway of neutrophilic inflammation would be blocking the action of both chemokines simultaneously, either through binding the ligands or blocking the receptor. Neutralizing antibodies to both CXCR1 and CXCR2 are currently available in the laboratory setting but have not been adequately tested in humans. Recent work done in Switzerland has determined two peptides based on the probable binding sites of monoclonal antibodies (mAb) to CXCR1 and CXCR2 that are potent inhibitors of CXCR activity. In \textit{in vitro} and \textit{in vivo} experiments in rabbits this group has demonstrated an ablation of PMN chemotaxis, IL-8 binding, and skin inflammation (67). Likely to be more useful than mAbs against CXCR1 and 2 would be small molecule inhibitors of the receptors. Repertaxin/reperixin is such a compound that is a non-competitive, allosteric inhibitor of CXCR1 and 2. Again, due to the known role of the chemokine receptors in disease, a Phase I clinical trial has already been performed with repertaxin/reparixin in acute, lipopolysaccharide induced inflammation in healthy volunteers, ostensibly to prevent any resulting IL-8 induced PMN inflammation. Unfortunately there were no observable differences in reparixin treated patients vs. placebo in this study (68). However, pertinent to this work, a study investigating repertaxin’s effects on recipients of lung transplant concluded in May of 2008 but the data has not been released yet [ClinicalTrials.gov Identifier: NCT00224406]. Another possible tool for negating CXCR1 and 2 functions may lie in a molecule developed in our laboratory. A formulation of PGP in which both
prolines exist as the “d” rather than the “l” isomer functions as a competitive inhibitor. In a series of yet unpublished experiments dP-G-dP binds to the receptors but does not cause any of the potent downstream effects instigated by IL-8, PGP and other ELR+CXC chemokines. Thus dP-G-dP could possibly be used as a competitive inhibitor of the naturally occurring pro-inflammatory molecules.

The final family of molecules susceptible to therapeutic intervention would be the chemokine ligands themselves. Since IL-8 has long been known as a modulator of neutrophilic inflammation, there have been two clinical trials investigating the potential efficacy of a neutralizing monoclonal antibody (mAb) against IL-8. In 1998, Abgenix began a study assessing its anti-IL-8 mAb in rheumatoid arthritis (RA), COPD, psoriasis (PS), and metastatic melanoma (69). The study was discontinued in 2002 due to a failure to meet the primary endpoints in Phase II for RA and PS but there was data to suggest that an improvement in COPD occurred. There was slight improvement in dyspnea but no measurable differences in lung function or frequency of pulmonary exacerbation (70). It could be argued that this may be because the authors only addressed the IL-8 pathway but were unaware of the role that PGP may be playing in COPD. An interesting study in the future would be to employ mAbs against both IL-8 and PGP in a pulmonary disease characterized by chronic neutrophilia and matrix turnover. Perhaps a more intriguing idea would be that of a single molecule inhibitor of both IL-8 and PGP. Blalock and colleagues, using an algorithm of inverted hydropathy, identified a PGP antagonist, Arginine-Threonine-Arginine (RTR). RTR was tetramerized on a dilysine core to increase its binding avidity for PGP (38, 71, 72). In both a rabbit cornea injury model, and mouse models of COPD, RTR was more potent than expected in blunting
PMN migration into the tissue and ablating pathology. In fact, due to the structural/sequence similarities between PGP and IL-8, it appears that RTR is capable of binding both chemokines and negating their effects (38). Thus the IL-8/PGP pathway of PMN inflammation is rife with opportunities for creating novel, potent therapeutics aimed at blunting the devastating effects of unchecked, aggressive neutrophilia.

**Brief Overview of Dissertation**

The initial paper contained in this thesis presents an in depth look into the release of protease from neutrophilic granules. This review provides analysis of current opinions and knowledge of the intracellular location of specific proteases and the mechanism by which PMNs undergo degranulation. The manuscript discusses these matters in the context of innate immunity and chronic pulmonary disease. As mentioned previously in the introduction, it is when the innate immune system becomes dysregulated that severe pathology can occur as is evidenced in multiple chronic pulmonary conditions. The matrix metalloprotease family is discussed, along with serine proteases HNE, PE and the cathepsins. A detailed description of the circumstances leading to their release and regulation is included in this book chapter, along with a brief discourse on current attempts to modulate and control the action of the granular contents.

The presence and function of prolyl endopeptidase in neutrophils is addressed in the second manuscript. Using a variety of techniques, this paper demonstrates that PE is both present and active in PMNs. PE’s role in the pathway of PGP production has already been elucidated, but the cellular source of the enzyme was previously unknown. This paper demonstrates that neutrophils contain PE and therefore are capable of
producing PGP and furthering their own recruitment and activation in inflammation. PE was detected using immunoblotting and activity was measured using a colorimetric substrate. Additionally, mRNA for PE was detected using PCR. To demonstrate a possible biological role, the cells were stimulated with lipopolysaccharide (LPS), and incubated with Type-I collagen. After analysis by electrospray-ionization liquid chromatography mass spectrometry/mass spectrometry (ESI-LC-MS/MS), we confirmed neutrophils produce easily measurable amounts of both PGP and N-α-PGP ex vivo. This paper demonstrates that isolated neutrophils are indeed capable of participating in a self-sustaining system, themselves producing a neutrophil chemokine from collagen, thus recruiting more PMNs to a site of inflammation, creating a feed forward pathological process.

The third paper included in this dissertation investigates the role of PE and its product, PGP, in clinical disease. Chronic rejection of lung transplant, or bronchiolitis obliterans syndrome (BOS) is associated with high PMN counts in bronchoalveolar lavage (BAL), increased protease activity and elevated levels of IL-8. Using matched samples of patients collected three months prior to, and at the time of diagnosis of BOS, we were able to define a shift in the chemokine profile seen in this cohort of patients. The activities of MMP-8, -9 and PE were all elevated in the samples collected at diagnosis of BOS compared to control transplant recipients and matched samples collected before confirmation of disease. Utilizing a previously published mass spectrometry technique, we observed that while PGP was present in both sets of samples, there was a dramatic increase in the levels seen in BAL fluid collected at the time of diagnosis (5). By employing neutralizing antibodies to IL-8 and PGP, first individually
and then in conjunction, we showed a shift in the relative importance of the classical PMN chemoattractant, IL-8, to the more novel molecule, PGP. It appears that between three months prior to and the time of diagnosis a change occurs from IL-8 being the predominant chemokine to PGP playing the more important role in neutrophil recruitment.

The final manuscript contained herein investigates the possibility of a dual PE inhibitor and CXCR1 and 2 antagonist as a therapeutic for pulmonary inflammation. This paper demonstrates that through structural similarities between PGP and IL-8, it may be possible that the catalytic site of PE and a binding pocket of CXCR1 and 2 are similar. A commercially available PE substrate, benzyloxcarbonyl-glycine-proline-paranitroaniline (ZGP-pNA) was proven to be chemotactic to both human and mouse neutrophils, specifically via CXCR1 and 2. Furthermore, a specific PE inhibitor, ZPP potently, and dose dependently blocked human and murine neutrophil chemotaxis to a variety of ELR+CXC chemokines, including PGP, that act via CXCR1 and 2. ZPP, as expected blocks neutrophil generation of PGP from intact collagen and blunts neutrophil recruitment into the airways following administration of LPS. Finally, ZPP competes with radio-labeled IL-8 for binding to CXC receptors. To our knowledge, the idea of a single compound which acts to inhibit ligand generation and block the site of that ligand’s action is unprecedented.

In total, this dissertation investigates the role of the serine protease, prolyl endopeptidase, and its chemokine product PGP in inflammation using chronic pulmonary conditions as models for disease. By using a theory of rational drug design, we were able to create a novel drug concept in which a single compound is capable of preventing the
production of a neutrophil chemokine, and blocking its site of action. Due to the apparent role that PGP plays, both as a biomarker of disease and as a mediator of chronic neutrophilic inflammation, it is important to develop therapeutics that are capable of addressing this new pathway of PMN inflammation. ZPP not only inhibits that PGP pathway of inflammation, but it also appears that it could also be effective against the more traditional IL-8 route of neutrophil influx and activation (Fig.1). This work represents a good example of translational research in utilizing well established laboratory techniques to predict and test a potential type of therapeutic for multiple clinical diseases. By using patient samples from chronic conditions such as CF, COPD, BOS, we show that ZPP is active in a biological system and represents an intriguing new concept for rational design of therapeutic agents for treatments aimed at neutrophilic inflammation.
Figure 1. Model of PMNs and their proteases in chronic inflammation.

Neutrophil proteases MMP-8/9 and PE participate in a proteolytic cascade generating PGP from collagen. PGP and IL-8 are both neutrophil chemoattractants involved in chronic pulmonary inflammation capable of binding to CXCR1 and CXCR2.
PROTEASE RELEASE FROM NEUTROPHILS IN INFLAMMATION: IMPACT ON INNATE IMMUNITY SEEN IN CHRONIC PULMONARY DISEASE

by

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Handbook of Granulocytes: Classification, Toxic Materials Produced and Pathology
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Abbreviations Used

A1AT (alpha-1 antitrypsin)
BAL (bronchoalveolar lavage)
CF (cystic fibrosis)
CFTR (cystic fibrosis transmembrane conductance regulator)
cGMP (cyclic guanosine monophosphate)
COPD (chronic obstructive pulmonary disease)
CXC (cysteine-X-cysteine)
ECM (extracellular matrix)
ENA-78 (epithelial neutrophil-activating peptide 78)
GCP (granulocyte chemotactic factor)
GRO (growth related oncogene)
HNE (human neutrophil elastase)
HPLC (high performance liquid chromatography)
IL (interleukin)
MMP (matrix metalloprotease)
NF-kappa B (nuclear factor kappa beta)
P2X/P2Y (purinergic receptors)
PGP (proline-glycine-proline)
PMN (polymorphonuclear leukocyte)
RTR (arginine-threonine-arginine)
SLPI (secretory leukocyte protease inhibitor)
SP (surfactant associated protein)
TGF (transforming growth factor)
TIMP (tissue inhibitor of metalloproteases)
TLR (toll-like receptors)
Abstract.

Proteases are a group of enzymes with the capacity to degrade proteins to either smaller-sized proteins or to peptides. These enzymes are found in abundant concentrations in immune cells, most notably polymorphonuclear leukocytes (PMNs or neutrophils), and have been implicated in chronic inflammatory pulmonary diseases. In non-activated neutrophils, most proteases are maintained in discrete granules. However, upon stimulation, there is degranulation and the proteases are released to impact the extracellular environment.

Evidence suggests that the imbalance of proteases and their regulatory antiproteases may lead to ongoing inflammation and changes in the immune response. Proteases seem to play an important role in recently described pathways of extracellular matrix (ECM) remodeling associated with persistent pulmonary inflammation. Protease activity has also been found to affect several soluble components of the innate immune response. These include such integral proteins as interleukin-8 (IL-8) and other chemokines, components of the complement cascade, lung surfactant associated proteins, and other regulatory proteases/antiproteases. In addition, PMN-derived proteases also affect cellular components of the innate immune system including changes in toll-like receptor (TLR) activation, CXC chemokine receptors (CXCR), integrins, and CD14 pattern recognition receptors.
This chapter will more specifically examine the role of neutrophil-derived proteases in the control of the innate immune response in the lung. This chapter will also address antiprotease therapies in chronic pulmonary diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). A better understanding of the pathways of protease dysregulation may lead to improved outcomes in these and other conditions.
**Introduction:**

The pulmonary microenvironment serves as an important location for the interactions of external antigens and host response. With the large volume (15,000 liters) of air inhaled daily, the increased surface area of the lung (approx 160 square meters), and the close proximity of terminal airways/alveoli with capillary networks, the lung is a difficult environment in which to coordinate host defense. As such, innate immune mechanisms have developed to work via distinct pathways to maintain lung homeostasis. These mechanisms include, but are not limited to, pattern-recognition receptors (toll-like receptors, dectin-1, and CD14), surfactant proteins A and D, TGF beta activation, chemokine production, and extracellular matrix remodeling.

During acute and non-resolving chronic inflammatory responses, a variety of cell types are recruited to the lung. Perhaps the most notable cell populations observed in these situations are polymorphonuclear cells (PMNs), or neutrophils, which are recruited via specific chemoattractants to sites of injury. These cells contain various products in discrete granules which may be utilized, upon release, to assist in the clearance of microbial organisms. However, some of these products may also modulate the innate immune response, leading often to a dysregulated host response.

This review will focus on mechanisms by which neutrophil-derived proteases may modulate innate immune response in the lung. This is not intended to serve as an exhaustive review of innate immune mechanisms which may be modulated by proteases but instead focuses on important pathways which the authors suspect may play an
important role in pulmonary disease. Finally, this review will discuss specific therapeutics directed at proteases in two pulmonary conditions, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), highlighting important clinical studies and the current state of antiprotease therapy in these conditions.

**Neutrophils and Granules**

*Granule Production and Classification*

Neutrophils are the most numerous leukocytes in adult peripheral blood. Neutrophils represent more than 95% of the granulocytes, which also includes eosinophils and basophils. They are produced from stem cells in the bone marrow and over a period of about two weeks undergo a maturation process. During the maturation process the various granules and their constituent proteins are formed [1]. The granules are classified in subsets: primary or azurophilic, secondary or specific granules, tertiary granules and secretory vesicles. The primary (first emerging) or azurophilic granules are formed in the promyelocyte stage of neutrophil development. Azurophilic granules were characterized and so named by early hematologists by their reactions with the azure dyes used in blood stains. By the myelocyte stage, the development of azurophilic granules is mostly complete and the production of secondary granules has begun. In the past, these were known as specific granules because they were colored by both acid and base dyes and could thus be used to differentiate neutrophils from the eosinophils and basophils. Tertiary granules are formed at the metamyelocyte phase while secretory vesicles are formed while the mature neutrophils are circulating [2, 3, 4].
Granule Content

Neutrophil granules contain a variety of proteins including enzymes, host defense proteins, adhesion molecules and signaling proteins. For a long time it was thought that each granule type contained a specific set of proteins. Primary granules contain the majority of lysosomal enzymes, including myeloperoxidase, and are thus characterized as peroxidase positive. The typical protein markers of azurophilic granules are myeloperoxidase, beta-glucuronidase, and lysozyme [3]. Secondary granules are peroxidase negative and contain a large number of extracellular matrix receptors. Secondary granules also contain such proteins as lactoferrin. Tertiary granules are peroxidase negative and were first identified in the 1980s using subcellular fractionation. Both secondary and tertiary granules also contain proteins involved in adhesion and signaling [5]. Secretory vesicles are more recently described and have alternately been named as alkaline phosphatase-containing granules. They are characterized using albumin as the matrix marker and alkaline phosphatase as a membrane marker [3, 6].

Currently, granules are viewed not as containing distinct subsets of proteins but as a heterogenic continuum of proteins, based upon the timing of granule protein synthesis during maturation [1]. A recent proteomic study defined a set of specific proteins found in each granule subset along with a variety of multiple granule subsets. Granules were separated and subtyped by individual protein markers. Two-dimensional SDS PAGE with in-gel trypsin digestion followed by mass spectrometry was used to identify the soluble proteins. Membrane proteins were identified by 2-dimensional microcapillary chromatography using both strong cation exchange and reverse phase high performance
liquid chromatography (HPLC) and subsequently analyzed with electrospray ionization tandem mass spectroscopy. They were able to identify 26 unique proteins in primary granules, 28 unique proteins in secondary granules and 86 unique proteins in tertiary granules [7].

**Degranulation**

Degranulation involves the directed movement of the granules to the cell membrane followed by exocytosis. The mechanism of this process is still very poorly understood. It appears to be a multistep process that may be unique for each subtype of granule. It begins when a neutrophil is stimulated by secretagogue binding to an extracellular receptor. Then the granules are recruited from the cytoplasm and translocate to the plasma membrane. There they dock and begin to fuse with the membrane. At this point the neutrophils are “primed”. A primed neutrophil is “fixed” in position and no longer undergoes chemotaxis or apoptosis. Segretagogues can only cause degranulation once the neutrophil has been primed [8]. Finally there is complete fusion of the granule with the plasma membrane a rapid formation of a fusion pore followed by rapid release of the granule contents to the extracellular space.

Granules differ in their tendency to undergo exocytosis. When a neutrophil comes into contact with the endothelium, secretory granules most readily undergo exocytosis. Gelatinase granules exocytosis occurs during neutrophil transmigration; one specific mechanism of tertiary granule release involves the binding of CXC receptors on neutrophils by activating ligands [9]. Both specific granules and azurophilic’s exocytosis
are released later at the site of inflammation. Typically, azurophilic granules fuse with phagosomes containing processed microorganisms before exocytosing [3].

An unregulated exocytosis of the proteins in the granules could lead to undesired tissue damage. It is known that an increase in the intracellular $\text{Ca}^{2+}$ concentration, as well as ATP hydrolysis, is required for granule translocation [1, 10]. One set of compounds known to be necessary for degranulation are extracellular nucleotides such as cGMP. These compounds can elicit their response through the P2 receptors, including the P2X receptors which are intrinsic ion channels and P2Y receptors which are metabolic and coupled to heteromeric G proteins [11, 12].

Greater mechanistic understanding of exocytosis is increasingly being delineated by various groups. Eitzen et al have recently suggested that Rac-mediated F-actin formation is necessary for primary granule translocation to the cell membrane, whereas actin depolymerization at the cell cortex stimulates granule exocytosis in general [13]. Mollinedo and colleagues have shown Rab27 to be highly concentrated in secondary and tertiary granules and suggest it helps regulate their secretion [13]. The mechanism of fusion with the cellular membrane is unknown but recent work indicates the involvement of the SNAP-23 and syntaxin 6 proteins [5].

One group of neutrophil-related products which are capable of modulating immune responses is proteases. The release and activation of these enzymes must be
highly coordinated and regulated in vivo. The following section will discuss these enzymes in detail.

**Neutrophil-derived proteases**

**Matrix Metalloproteases**

Matrix metalloproteases (MMPs) are a family of zinc-dependant enzymes generally thought to be responsible for normal matrix turnover and the degradation of some soluble factors. Moreover, there are several types of MMPs such as collagenases, gelatinases, matrilysins, stromelysins, and membrane-bound isoforms [14]. MMPs exist as proenzymes and are activated after release [15]. Neutrophils are a well known source of MMPs, having been shown to produce MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) [16].

These potent enzymes are normally held in check by a family of antiproteases known as tissue inhibitors of metalloproteases (TIMPs). These are proteins which decrease endogenous MMP activity by binding to the metalloprotease and inhibiting its enzymatic activity. Both MMP-8 and MMP-9 have naturally occurring TIMPs that are responsible for continued regulation of MMP activity in vivo [17]. The imbalance of these proteases with their respective antiproteases is thought play several roles in the development of chronic inflammatory diseases.
Human Neutrophil Elastase

Human neutrophil elastase (HNE) is a member of the serine protease family, indicating that it has a serine as part of its catalytic triad along with histidine and aspartic acid [18]. HNE is a prominent protease prevalent in a variety of pulmonary conditions. One of the primary mechanisms of HNE regulation is by alpha-1-antitrypsin (A1AT). A1AT, an inhibitor of a wide range of proteases is loosely bound to HNE under normal circumstances but is neutralized under oxidating conditions and allows HNE to act upon substrate [18, 19]. HNE has recently been shown to be involved in several pulmonary diseases including A1AT deficiency, COPD and CF. The current hypothesis is that, like with MMPs, there is an imbalance that occurs between HNE and its endogenous inhibitors, leading to dysregulated HNE activity in vivo and ongoing tissue remodeling [20, 21].

Cathepsins B, D, G and Proteinase 3

Cathepsins B (a cysteine protease) and D (an aspartyl protease) are activated by neutrophils upon reaching sites of inflammation and have a diverse range of functions. Cathepsins B, and D are both among the most abundant proteins in the lysosome and appear to play roles in both the mitochondrial and caspase dependant pathways of apoptosis [22]. Cathepsin G, a serine protease, is a potent agent of activated neutrophils at sites of inflammation and has various effects including cleavage of thrombin receptors and clotting factors [23]. Proteinase 3, like cathepsin G is another neutrophil serine protease. Both of these enzymes, along with HNE, are integral in bacterial killing through fusion of the primary (azurophilic) granules with the phagolysosome [24].
While there is emerging evidence of the role of these proteases on the modulation of innate immunity in the lung, due to the focus of this chapter, further discussion of these enzymes’ immune effects will be deferred.

**Proteases in Granules**

The azurophilic granules contain members of the serine protease family of enzymes (such as cathepsin G, neutrophil elastase, and proteinase 3), although both cathepsin B and D are also present in these granules as well. The serine protease mRNA is most highly expressed in neutrophils in their promyelocytic stage of differentiation and is then subsequently down-regulated [3]. This explains why serine proteases, especially neutrophil elastase, are found almost exclusively in azurophilic granules- since granule content is dependent upon timing of protein synthesis. However, recent proteomics work showed the presence of cathepsin G in three granule subtypes- tertiary, secondary and azurophilic [7]. It is not clear from this study if the amount of cathepsin G in each type of granule differs drastically or is nearly the same.

Matrix metalloproteases such as MMP-9 were traditionally thought to exist only in the tertiary granules. However recent proteomics work has shown the presence of MMP-9 in both the tertiary granules and specific granules. Neutrophil collagenase or MMP-8 was found to be in specific granules only [7]. This seems in agreement with earlier work which indicates a different kinetics for the release of MMP-8 and MMP-9 from granules. While all of these proteases have regulated activity inside granules, the release of these proteases leads to diverse immunologic effects.
Innate Immune Regulation of Neutrophil-Derived Proteases- Soluble Mediators

Chemokines

One potential area of interest in protease activity has centered on the modulation of preformed mediators of cellular recruitment known as chemokines. Extensive examination of these interactions has yielded increasing understanding of important mechanisms of both transcriptional and post-translational modifications leading to changes in innate immune response.

MMPs and ELR+ CXC chemokines

Interleukin-8 (IL-8) is a prominent chemokine involved in neutrophil recruitment to sites of injury and inflammation. It is a member of the family of glutamate-leucine-arginine (ELR+) containing cysteine-x-cysteine (CXC) chemokines which also include GRO-α, -β, and -γ, granulocyte chemotactic factor (GCP) and epithelial neutrophil activating peptide 78 (ENA-78) all of which are specific for neutrophil recruitment via binding to CXC receptors on neutrophils [25]. IL-8 is secreted as either as a 77 aa isoform from endothelial cells or a 72 aa isoform from leukocytes, although other isoforms have been identified [26]. While the 72 aa isoform is bioactive, the 77 amino acid isoform is not as active. Van den steen and colleagues demonstrated that, in vitro, MMP-9 can cleave IL-8 to a 70 aa isoform with 10-25 fold increase in potency relative to its native form [27]. While intriguing, the presence of this isoform of IL-8 has not been documented in significant amounts in clinical disease specimens to date. It has also been shown that 72 aa IL-8 binding to CXC receptors of neutrophils can induce release of
MMP-9 from tertiary granules [9]; it is unknown, however, if different IL-8 isoforms release MMP-9 variably from neutrophils. Recently, processing of ENA-78 and GCP with modulation of chemotactic function has been reported for both MMP-8 and MMP-9 [28].

**HNE and IL-8**

In addition to post-translational changes observed in preformed chemokines, HNE has also been shown to induce IL-8 release from airway epithelia through a MyD-88/NFkappaB-mediated pathway [29], suggesting the possibility of TLR-4 modulation on the extracellular surface of airway epithelia [30].

**Proteases and Extracellular Matrix Fragmentation**

It is increasingly recognized that the remodeling of the extracellular matrix (ECM) leads not only to increased pathology but that some of these ECM-derived fragments are bioactive and have tremendous impact on innate immunity and inflammation.

**Elastin Fragments**

Elastin is an important component of the ECM which aids in elasticity of a variety of organs including the lung [31]. Interestingly, elastin fragments have been implicated in the recruitment of monocytes in a variety of conditions [32-34]. The repeating hexamer of valine-glycine-valine-alanine-proline-glycine seems to be an important component of the fragments ability to cause chemotaxis of both fibroblasts and monocytes [35].
Whereas macrophage-derived metalloelastase (MMP-12) is thought to play an important role in cleavage of elastin [36], HNE is another possible elastase which may contribute to the liberation of these bioactive fragments.

**Collagen Fragments**

Digested collagen causes increased injury and inflammation in various in vitro and animal models of disease, with recruitment of fibroblasts, monocytes, and neutrophils [37-41]. However, specific mechanisms of cellular recruitment have not been well described until recently when the collagen fragment proline-glycine-proline (PGP) was found to induce neutrophil chemotaxis via binding to CXC receptors (CXCR1 and CXCR2), inducing activation of the neutrophil [42]. The liberation of this peptide from collagen involves the action of both MMP-8 and MMP-9 [43], suggesting that neutrophils may play an important feed-forward role in their own recruitment during ongoing injury and inflammation.

**Laminin Fragments**

Another component of the ECM is laminins, which are heterotrimeric proteins essential for basement membrane integrity. Laminin-5, a specific laminin isoform, plays an important component in hemidesmosomes of basement membranes [44]. HNE has the capacity to cleave laminin-5 and the resulting fragment is chemotactic for neutrophils [45], although the specific mechanism for this chemotaxis is currently not known.
Protease Activation/ Antiprotease inactivation

Another potential mechanism by which neutrophil-derived proteases may modulate immune function is by the activation of protease or deactivation of antiproteases [46]. These protease cascades lead to a pro-proteolytic environment with multiple implications to innate immune response.

HNE activation of MMP-9

HNE has been shown to activate various proteases. Perhaps one of the most important functions is the cleavage of the prodomain of MMP-9, another major neutrophil-derived protease, thereby inducing activation. While this mechanism has been shown in vitro [47], recent evidence suggests that this relationship may exist in vivo [48]. In addition to activation of MMP-9, HNE also has the capability of inactivating important antiproteases. TIMP-1, a naturally occurring antiprotease for various MMPs including MMP-9, is cleaved and inactivated by HNE [48, 49]. This activation of MMP-9 and inactivation of TIMP-1 lead to a MMP-9-rich environment and have widespread implications to innate immune response.

HNE activation of MMP-2 and cathepsins

HNE (along with cathepsin G) has the capability of activating MMP-2 [50], which then has the capability of cleaving important innate immune molecules. HNE has recently been shown to increase gene transcription of cathepsin B and MMP-2 from macrophage via a TLR-4/NF-kappa B mechanism [51], suggesting the possibility of “protease hierarchies” in vivo.
**Other soluble targets**

*Surfactant-associated proteins*

An important mechanism of innate immunity observed in the lungs is the activation of surfactant-associated proteins (SP), specifically SP-A and SP-D [52]. These proteins are involved in the binding and presentation of microbial organisms inhaled during respiration and presenting these to resident alveolar macrophages for opsonization and clearance [53]. HNE has been shown to cleave both SP-A and SP-D, impairing agglutination and opsonization of bacteria [54, 55]. To date, it is unknown if metalloproteases may cleave surfactant associated proteins, thereby changing their functionality.

*TGF-beta*

TGF-beta is an important molecule in epithelial cell biology and cancer progression. However, increasing evidence suggests its role in immunity is diverse, including activation/deactivation of macrophages/monocytes response, inhibition of B cell proliferation, and augment production of CD8+ T-cells. It is secreted in a latent form which is then cleaved to be activated [56]. MMP-9 activity has been found to be a potent inducer of this cleavage and may, in part, explain increased TGF-beta activity in a variety of clinical pulmonary conditions [57].
Complement cascade

The complement cascade serves an important role in humoral immunity; it is a series of proteins which act as a pathway to help opsonize and phagocytize bacteria [58]. One of the components necessary for the opsonization of the membrane attack complex (MAC), C3bi, can be cleaved from the surfaces of Pseudomonas via HNE. In addition, HNE can also cleave complement receptor 1 (CR1) from the surface of neutrophils, leading to a functional opsonization deficiency via this pathway [59].

Innate Immune Regulation of Neutrophil-Derived Proteases- Cellular Targets

In addition to all of the external targets of neutrophil-derived proteases there are several cellular-associated proteins that also provide a substrate, leading to changes in innate immune response.

CD14 and HNE

Macrophages are intricately involved in the inflammatory process, particularly in the resolution of the insult and removal of cellular debris and apoptotic cells. Henriksen et al have shown that CD14 functions as a recognition molecule for apoptotic cells, and is important in the resolution of inflammation [60]. Human neutrophil elastase (HNE) is capable of CD14 inactivation/cleavage on primary human monocytes and fibroblasts [61]. Henriksen and colleagues were later able to demonstrate that through overexpression of r-elafin, a potent inhibitor of HNE, in peripheral blood-derived macrophages they were able to blunt the proteolytic cleavage of the surface receptor [20].
**CXCR1 and HNE**

As previously mentioned, in chronic inflammatory pulmonary conditions such as CF and COPD, there is an ongoing and persistent neutrophil inflammation [62]. However, it is thought that some of the effector functions of the cells (i.e. superoxide production) are impaired [63]. One proposed mechanism for this is the inactivation of neutrophils through CXCR1 cleavage by HNE. Neutrophils collected from these patients have both increased HNE expression and decreased levels of CXCR1 on PMN surface. CXCR1 is specific for IL-8 and necessary for priming the oxidative burst so important in bactericidal effects, leading to neutrophils which will travel to areas of inflammation but have a reduced ability to clear invading microbes [64].

**MMP-9 and β2 integrins**

Integrins are cell surface receptors which interact with ECM components, modulating both intracellular and cell-to-cell signaling. These receptors are obligate heterodimers composed of an alpha (α) and beta (β) chain [65]. Integrins with the β2 chain are found on various immune cells and modulate inflammatory cell activation [66]. β2 integrins on alveolar macrophages have recently been found to serve as a substrate for MMP-9 [67]; the implications of these findings are yet to be completely determined.

**Antiprotease Therapeutics in Inflammatory Lung Disease**

Because of the diverse immunologic actions of proteases, there has been increasing interest in inhibiting their actions in clinical disease to modulate both natural history of the condition and disease-specific outcomes. While the protease/antiprotease
imbalance has been examined in a variety of pulmonary disorders, this review will examine therapeutics in chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).

**COPD**

COPD is a condition characterized by a fixed airflow obstruction (as measured by pulmonary function testing), often related to repeated exposure of cigarette smoke. To date, COPD is one of the leading causes of death worldwide and its incidence is expected to increase over the next 10 years. Over time, this condition leads to permanent airway remodeling, alveolar space destruction, increased mucous production, and chronic neutrophilic inflammation. To date, the only therapies known to change the natural history of the condition is smoking cessation and, for those with severe hypoxemia, supplemental oxygen [68]. It is, therefore, imperative to find novel therapeutic avenues for the treatment of this condition.

Due to the degree of airway remodeling seen in this condition, proteases have been implicated in the pathology observed in COPD. A variety of studies have characterized the imbalance of various neutrophil-derived proteases such as HNE, specific MMPs, proteinase-3, and cathepsin G (although for the latter 2 proteases, no specific therapeutics have been attempted in COPD patients) [69]. To date, various strategies have been attempted to re-establish protease/antiprotease imbalance in these conditions.
HNE

HNE is the most prevalent protease found in COPD lung disease- indeed, the genetic condition alpha-1 antitrypsin (A1AT) deficiency which causes early-onset emphysema is directly related to an imbalance of HNE with A1AT [70]. Similarly, prolonged cigarette smoke exposure can reduce A1AT levels in vivo. One potential strategy is to give back A1AT to patients with COPD, similar to treatment strategies for A1AT deficiency. While an inhalation study in a smoke-exposure mouse model demonstrated benefit in airway enlargement and neutrophil recruitment [71], a human study (in which 250 mg/kg of A1AT was infused every 4 weeks for 3 years) yielded no change in lung function [72]. Other small molecule blockers for HNE have been developed including sivelestat (ONO-5456) and ICI-200, 800 [73]. While these blockers have been used in other inflammatory lung diseases with success they have not been examined in COPD. One small molecule blocker, MR-8899, has been given to individuals with COPD (500 mg twice a day for 4 weeks) and demonstrated no significant change in urine elastin fragment levels when compared to placebo-treated controls [74].

MMPs

Various MMPs have been implicated in the pathology observed in COPD lung disease, including MMP-1, MMP-8, MMP-9, and MMP-12. The neutrophil-derived MMPs (MMP-8 and MMP-9) have received increasing attention due to their ubiquitous presence in a variety of chronic inflammatory lung disease- in general, therapies for MMPs have centered around broad MMP inhibitors rather than targeting specific MMP
isoforms [69]. Tissue inhibitors of metalloproteases (TIMPs) are naturally occurring inhibitors for MMPs. Despite obvious appeal on the use of these inhibitors in COPD lung disease, the limitations of the delivery of large amounts of TIMP protein necessary for inhibition of MMP activity remain inherent hurdles for this treatment [75].

To date, several small molecule blockers for MMPs (i.e. GM-6001, marimastat, RS113,45) have been studied in various animal models of COPD lung disease with favorable benefits [73]. However, most of these inhibitors have demonstrated a class-wide side effect of severe arthralgias (“Musculoskeletal syndrome”) in treatment of other conditions and these side effects may limit studies and usage in COPD patients [76]. Finally, some antibiotics have broad-spectrum MMP inhibitory effects and may be very interesting as well-tolerated anti-inflammatory therapeutics in COPD. For example, macrolide antibiotics have been shown to serve both as a direct inhibitor of MMP-9 activity and also to reduce MMP-9 transcription from airway epithelia [77, 78]. The fact that macrolide antibiotics now serve as a mainstay anti-inflammatory therapy in conditions (cystic fibrosis, panbronchiolitis, and chronic allograft rejection post-lung transplantation) with increased MMP-9 and neutrophil activity makes it an appealing potential therapy for COPD. The role of macrolide therapy as a long-term anti-inflammatory therapy in COPD is currently being examined in a multi-center randomized control trial in the United States.
Cystic Fibrosis

Cystic fibrosis is a condition characterized by decreased chloride transport in the airway epithelia of individuals due to either deficient or abnormal functioning of the cystic fibrosis transmembrane conductance regulator (CFTR) [80]. This leads to desiccated airway surface liquid, bacterial overgrowth, and an exuberant neutrophilic inflammatory response. This inflammation, in turn, leads to airway remodeling, bronchiectasis, and eventual respiratory failure leading to death [80]. While the scope of the problem is not as broad world-wide as COPD, the degree of airway remodeling seen leads to a median life expectancy of only 37 years [81].

Due to the increased burden of bronchiectasis and neutrophilic inflammatory response seen in this disease, protease imbalance has been thought to play an important role in this condition as well. Indeed, many of the same neutrophilic proteases-HNE, MMPs, proteinase-3- found in COPD lung disease are also found to have increased activity in CF lung disease. However, unlike COPD, only HNE has been significantly examined as a therapeutic avenue in CF lung disease [82].

HNE

HNE is the prominent protease found in CF lung disease; to date, it is also the most robust biomarker for inflammatory response in CF [83]. Various strategies have been employed to reduce the HNE protease activity seen in CF lung disease. Similar to A1AT deficiency syndrome and trials in COPD, A1AT was initially tried in individuals with CF. Unfortunately, due to increased quantity and frequency of dosing to obtain
significant HNE inhibition, this approach has been abandoned. Instead, delivery of an inhaled A1AT has been the primary mechanism of therapy examined in CF clinical trials, albeit most of these studies have small numbers. One phase 1 study of 12 adult patients, with mild-moderate CF lung disease, who were given 100-200 mg of A1AT twice a day for 1 week demonstrated a significant reduction in BAL HNE activity [84]. Another small study has shown similar results of inhibition of HNE activity in BAL using 100 mg of A1AT twice a day for 8 weeks [85]. While these reductions in HNE activity are seen in studies examining CF BAL, these results are not as consistent in the sputum of CF individuals [86, 87]. These results suggest that either optimal dosing to inhibit HNE activity in sputum has not been determined or inactivators of A1AT are present at higher concentrations in sputum versus BAL.

In addition to A1AT, another naturally occurring HNE inhibitor examined for possible therapy in CF lung disease is the secretory leukocyte protease inhibitor (SLPI) [88]. This serpin is not specific for HNE but is produced in high quantities in the airways and, therefore, may play an important role in HNE regulation in vivo. A study which examined the delivery of inhaled SLPI to 16 adult CF patients showed that at 100 mg twice a day, HNE activity was decreased only temporarily and that at lower dose of 50 mg twice a day, this inhibition was not noted [89]. Despite these results, to the authors knowledge, no significant studies have been conducted in CF patients with this therapeutic although new generations of mixed A1AT and SLPI formulations are currently being developed for clinical consideration [90].
Small molecule inhibitors of HNE have currently not been aggressively examined in CF lung disease. DMP-777 was begun in a phase 2 clinical trial for CF lung disease but its production was discontinued by its parent company. A semisynthetic small molecule HNE inhibitor, EPI-hNE-4, is currently in phase 2 clinical trial for CF lung disease [82].

Antimicrobials

As previously mentioned, the current standard of long term clinical care of patients with CF lung disease often involve 3 times per week dosing of a macrolide antibiotic. While these antibiotics have multiple biological effects, a potent effect is the inhibition of MMP activities. Currently, tetracycline antibiotics have not been systematically examined for similar anti-protease or anti-inflammatory activities in CF. Recent ex vivo data suggests that this class of antibiotic may effectively reduce MMP activity in CF sputum [91]. A single center randomized-controlled clinical trial is currently planned to examine these effects in an adult inpatient CF population in the United States.

Conclusion

Despite the increasingly prominent role of proteases in systems of immune disarmament and persistent inflammation, therapeutics targeting these proteins has led to a limited utility at this time. One potential reason for this is that optimal formulations of antiproteases (those with long half-lives and well-tolerated by patients) have yet to be determined. It is also possible that for a given condition (or even individual), a tailored
A cocktail of specific antiproteases must be administered. Another consideration for the lack of significant success with antiprotease therapy in lung disease is that optimal mechanisms of delivery have not been developed for targeting the correct location in the lungs (i.e. central airways for CF and distal airways/parenchyma for COPD).

Despite these limitations, it is exciting to observe a renewed and increased interest in neutrophil targeting in lung conditions. Various neutrophil and chemokine antagonists are currently in clinical studies or are in development which may limit the persistence of inflammation observed in these diseases. Recently, van Houwelingen et al reported a novel antagonist, arginine-threonine-arginine (RTR), which binds both PGP and CXC chemokines in vitro and causes a decrease in both neutrophil recruitment and development of emphysema in an animal model of COPD [93].

In summary, the dysregulated activation and release of proteases from these cells impacts the innate immune response in the lung (Figure 1) and have widespread effects systemically. The specific therapies outlined in this chapter may provide novel approaches for disease-modifying agents not only in the conditions discussed but in other chronic inflammatory disorders in which neutrophils and proteases play a prominent role.
Figure 1: Protease Modulation of Innate Immunity in Lungs
In the pulmonary microenvironment, proteases may have a myriad of effects in modulating immunity. Human neutrophil elastase (HNE) has the capacity to augment protease cascades, cleaves cellular receptors, and disarms surfactant proteins. Matrix metalloprotease (MMP)-8 and -9 are able to cleave various chemokines and extracellular matrix proteins to modulate inflammatory response. These proteases may serve as future therapeutic targets in lung disease.
References


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82. CF Foundation Website: www.cff.org


NEUTROPHILS CONTAIN PROLYL ENDOPEPTIDASE AND GENERATE THE CHEMOTACTIC PEPTIDE, PGP FROM COLLAGEN

by

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* These Authors Contributed Equally to this Work
Abstract

Prolyl endopeptidase (PE), a protease that cleaves after proline residues in oligopeptides, is highly active in brain and degrades neuropeptides in vitro. We have recently demonstrated that PE, in concert with MMPs, can generate PGP (proline–glycine–proline), a novel, neutrophil chemoattractant, from collagen. In this study, we demonstrate that human peripheral blood neutrophils contain PE, which is constitutively active, and can generate PGP de novo from collagen after activation with LPS. This novel, pro-inflammatory role for PE raises the possibility of a self-sustaining pathway of neutrophilic inflammation and may provide biomarkers and therapeutic targets for diseases caused by chronic, neutrophilic inflammation.
Introduction

Prolyl endopeptidase (PE) is an endopeptidase, which cleaves at the carboxyl side of proline residues in oligopeptides. PE is the only proline specific endopeptidase currently known in mammals and belongs to a group of serine proteases, which also includes dipeptidyl peptidase IV, oligopeptidase B and acylaminoacyl peptidase (Polgar, 2002). These peptidases differ significantly from classical serine proteases, such as trypsin or subtilisin, in their structure and selectivity for small peptide substrates. In PE, the catalytic triad is covered by the central tunnel of a β propeller domain, which excludes peptides larger than 30–100 amino acids from the active site (Fulop et al., 1998).

Peptide bonds involving proline residues are seldom cleaved by classical serine proteases since they do not fit into the catalytic site. Many biologically active peptides contain prolines within their amino acid sequence and enzymes that cleave peptides at a proline may consequently have important biological effects (Cunningham and O'Connor, 1997; Mentlein, 1988). PE is highly active in brain tissue (Kalwant and Porter, 1991), degrades neuropeptides in vitro (Knisatschek and Bauer, 1979; Taylor and Dixon, 1980; Wilk et al., 1979) and may play a role in the pathogenesis of depression and Alzheimer's disease (Maes et al., 1994; Rossner et al., 2005). PE inactivates bradykinin, a vasodilator, and converts angiotensin I and II to angiotensin (residues 1–7), which liberates vasopressin from the hypothalamus, and may play a role in hypertension (Welches et al., 1993). However, the physiologic function of PE remains obscure despite its ubiquitous presence in human tissues as well as serum (Goossens et al., 1996).
We have recently identified a novel pathway signaling neutrophil influx to the lung in which PE plays a major role. Chemical or enzymatic breakdown of collagen releases a tripeptide, proline–glycine–proline (PGP) that is chemotactic for neutrophils in vitro and in vivo (Weathington et al., 2006). The neutrophil chemotactic activity of PGP may be due to a marked structural relatedness to a receptor-binding domain of CXC chemokines, such as interleukin-8, which contain this collagen sequence or a close analog. PGP production from collagen is dependent on initial digestion of collagen by MMP-8 and MMP-9 with PE catalyzing the final reaction (Gaggar et al., 2008). PGP and PE are elevated in lung diseases characterized by chronic, neutrophilic, airway inflammation. Sputum from patients with chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) contains increased amounts of PGP and generates PGP from collagen in a PE-dependent fashion (Gaggar et al., 2008; O'Reilly et al., 2009). PE activity is elevated in sputum from CF patients and bronchoalveolar lavage fluid from lung transplant patients with chronic allograft rejection (Gaggar et al., 2008; Hardison et al., 2009). To our knowledge, this is the first time PE has been implicated in inflammation or in disorders of the respiratory system.

Given the detection of PE in neutrophilic lung diseases, we hypothesized that neutrophils might be a source of PE. We demonstrate herein, using a variety of molecular and biochemical techniques, that human peripheral blood neutrophils contain constitutively active PE and can generate PGP from collagen. The presence in neutrophils of all the enzymes necessary for generation of PGP raises the possibility of a self-perpetuating cycle of neutrophilic inflammation.
Materials and Methods

Materials

Neutrophils were isolated from peripheral blood of healthy volunteers as previously described (Hardison et al., 2009) by separation on a Ficoll gradient (Sigma-Aldrich, St. Louis, MO). Neutrophil lysate was obtained by three freeze–thaw cycles and brief sonication with aprotinin (Sigma-Aldrich), followed by centrifugation at 13000 rpm. Research using human samples was approved by the Institutional Review Board at the University of Alabama at Birmingham. Informed consent of all participating subjects was obtained. A monospecific, polyclonal, anti-PE antibody was raised against a synthetic peptide representing residues 190–219 of mouse PE as described (Hardison et al., 2009). This antibody detects human PE whose sequence differs from mouse by one residue between residues 190 and 219. Recombinant human PE (rhPE) was cloned using a human PE cDNA, kindly provided by Drs. Anne Mudge and Michael Lumb (University College London). PE cDNA was cloned into the pTrcHisB vector (Invitrogen, Carlsbad, CA), which contains a His tag. PE expression in E.coli was induced with 1 mM IPTG and purified on a 6-His column.

PE Activity Assay

PE activity was determined as previously described (Gaggar et al., 2008; Hardison et al., 2009) by incubating samples with a PE-specific substrate, 2 mM ZGP-pNA (benzylcarboxy-glycine-proline-p-nitroaniline, ChemImpex International, WoodDale, IL) at 37 °C and 5%CO₂. Cleavage of p-nitroaniline by PE was detected using a spectrophotometer at 405 nm.
Immunofluorescence microscopy

Cytospin preparations of neutrophils on glass slides were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. After blocking with 3% BSA in PBS, neutrophils were incubated with anti-PE antibody (45 μg/ml in PBS/1% BSA), pre-immune rabbit antibody or anti-PE antibody which had been pre-adsorbed with rhPE (200 μg/ml) for 2 h at room temperature. After a second blocking step with 3% BSA, neutrophils were incubated with FITC-labeled goat anti-rabbit secondary antibody (1:12,000 in PBS/1% BSA, Southern Biotechnology, Birmingham, AL) for 1 h. Nuclei were stained with Hoechst (1:2000, Sigma-Aldrich) and neutrophils examined by immunofluorescence microscopy.

Western blotting

Neutrophil lysate was separated by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked in 5% dry non-fat milk for 1 h at room temperature and incubated with polyclonal, rabbit, anti-PE antibody (22.4 μg/ml) for 1 h at room temperature. After incubation, membranes were washed and incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Southern Biotechnology, Birmingham, AL) for 1 h. Immunoblots were developed by chemiluminescence (Pierce, Rockford, IL).
**RT-PCR**

Total RNA from human peripheral blood neutrophils was isolated and first strand cDNA synthesized using a kit according to manufacturer's specifications (Superarray, Frederick, MD). PCR for human PE was performed using specific primers (Superarray) and continued for 45 cycles.

**PGP generation assay**

Human peripheral blood neutrophils (1×10^6) were incubated with 15 μl of a 1 mg/ml solution of Type 1 collagen (Sigma-Aldrich) in PBS containing bestatin (Cayman Chemical, Ann Arbor, MI) with or without LPS (100 μg/ml, Sigma-Aldrich) for 1.5 h at 37 °C and 5% CO₂. The collagen was extensively dialyzed beforehand to remove PGP. After incubation, samples were 10 kDa filtered, washed with 40 μl of 1 N HCl, and analyzed by ESI-LC-MS/MS for levels of PGP and N-α-PGP.

**ESI-LC-MS/MS protocol**

PGP and N-α-PGP were measured simultaneously in samples as previously described (Hardison et al., 2009) using a MDS Sciex API-4000 spectrometer (Applied Biosystems, Foster City, CA) equipped with HPLC (Shimadzu, Kyoto, Japan). HPLC was performed using a 2.1×150 mm Develosi C30 column (buffer A: 0.1% formic acid, buffer B: acetonitrile plus 0.1% formic acid; 80% buffer A/20% buffer B from 0 to 0.6 min, 0% buffer A/100% buffer B from 0.6 to 5 min). Background was removed by flushing with 100% isopropanol/0.1% formic acid. Positive electrospray mass transitions were at 270–70 and 270–116 (PGP) and 312–140 and 312–112 (N-α-PGP).
**Results**

*PE protein and activity are detected in human peripheral blood neutrophils*

Cell lysates from unstimulated, human, peripheral blood neutrophils were able to cleave the PE substrate, ZGP-pNA. Increased cleavage of ZGP-pNA was detected with increasing amount of neutrophil lysate (Fig. 1A). This suggested that human neutrophils contain a PE-like enzymatic activity, which is constitutively active.

As there are additional enzymes with PE-like activity that can cleave ZGP-pNA and may be present in neutrophils (Shariat-Madar et al., 2002), we developed specific reagents to ensure we were detecting PE and not another enzyme. Using a monospecific, polyclonal anti-PE antibody, PE was clearly detected in human peripheral blood neutrophils by immunofluorescence microscopy (Fig. 1B). PE appeared to have a diffuse cytoplasmic distribution in neutrophils with increased concentration in granule-like structures.

In further confirmation of these findings, we detected PE in human neutrophil lysates by Western blotting, using the same anti-PE antibody. PE migrated as a 75 kDa monomer, similar to recombinant human PE (rhPE) used as a positive control (Fig. 2A). Total RNA was isolated from human peripheral blood neutrophils and examined for PE mRNA by RT-PCR. A PCR product of the appropriate size of 95 bp was found in human neutrophils and in a pTrcHisB vector containing rhPE, used as a positive control (Fig. 2B).
Fig. 1 Indicated amounts of human neutrophil lysate ($10^6$ cells) were assayed for PE enzymatic activity (expressed as mU/ml). Results are presented as mean ± SEM (A) and compared using the two sample $t$ test. Increased PE activity was observed with greater amounts of neutrophil lysate (*$p<0.05$ compared with 0 μl lysate, $n=3$ per group). Results are representative of several experiments. Cytospins of human peripheral blood neutrophils on glass slides were incubated with anti-PE antibody (B), anti-PE antibody pre-adsorbed with PE (C) or pre-immune rabbit antibody (D), followed by FITC-labeled goat anti-rabbit IgG, and examined by immunofluorescence microscopy. PE in human neutrophils was located in the cytoplasm in a granular pattern (B).
Human neutrophils generate PGP de novo from collagen

Since neutrophils are known to contain MMP-8 and MMP-9 and, having demonstrated that neutrophils contain PE, we hypothesized that they would contain all the enzymes necessary to generate PGP from intact collagen. We tested this idea by incubating human neutrophils with collagen and measuring PGP generation. When incubated alone with collagen, human neutrophils did not generate any PGP in excess of that found in collagen alone. However, when incubated with LPS and collagen, human neutrophils generated large amounts of PGP and N-α-PGP (Fig. 3).
Fig. 2 Human neutrophil lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed for PE (A). Lanes 1, 2 and 3 are a coomassie blue stained gel of MW markers, human PMN lysate (18 μg protein per lane) and rhPE respectively. Lanes 4, 5 and 6 are a chemiluminescence developed Western using our rabbit anti-PE antibody corresponding to Lanes 1, 2 and 3. PE in human neutrophils was a monomer and migrated at 75 kDa, similar to rhPE. PCR for PE was performed on total RNA isolated from human peripheral blood neutrophils and the product run on a 2% agarose gel (B). Human PE primers should amplify a 95 bp product. PCR for actin was performed concurrently as a positive control. Lanes: Invitrogen 1Kb+ladder (1), negative control (2), 95 bp PCR product from human PMN (3 and 4), 95 bp PCR product from pTrcHisB vector containing rhPE (5), negative control (6), 200 bp PCR product for actin (7 and 8).
Discussion

These results demonstrate that PE is present and constitutively active in human neutrophils, where it had a cytoplasmic distribution and was a monomer of 75 kDa in size. These results concur with previous studies in other human cell types and tissues, where PE was generally found in the cytoplasm and was a monomer with a molecular mass between 65 and 80 kDa (Goossens et al., 1995; Hasebe et al., 2001; Kalwant and Porter, 1991; Mizutani et al., 1984; Pratt et al., 1989), although nuclear and membrane-bound isoforms have also been described (Dresdner et al., 1982; Ishino et al., 1998; O'Leary and O'Connor, 1995). Although PE appears to be a single copy gene, different forms of PE, arising from post-translational modification and/or different gene products, may account for the variety of functions and locations of the enzyme (Kimura et al., 1999).

The action of PE along with MMP-8 (neutrophil collagenase) and/or MMP-9 (neutrophil gelatinase) is sufficient to generate the neutrophil chemoattractant, PGP, from collagen (Gaggar et al., 2008). In PGP generation, PE likely acts on oligopeptides generated by the prior digestion of collagen by MMP's. As human neutrophils are known to express MMP-8 and MMP-9 (Gaggar et al., 2007; Lin et al., 2008) and are now shown to contain PE, we hypothesized that they would be capable, by themselves, of generating PGP from collagen. Consistent with this idea, LPS-stimulated human neutrophils, but not unstimulated neutrophils, generated PGP from collagen (Fig. 3). This provides further evidence that human neutrophils contain PE, which, to our knowledge, is the only protease capable of releasing PGP from the often-repeated PPGP sequence in collagen. PE appeared to be concentrated in granule-like structures in neutrophils (Fig. 1B) and

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Previously dialyzed Type 1 collagen was incubated with human peripheral blood neutrophils ($1 \times 10^6$) with and without LPS (100 μg/ml) for 1.5 h. Supernatants were passed through a 10 kDa filter and analyzed by ESI-LC-MS/MS for PGP and N-α-PGP. Levels of PGP and N-α-PGP generated are presented as mean ± SEM and compared using the two group t test. LPS-stimulated neutrophils generated significantly greater amounts of PGP and N-α-PGP (*p<0.01 compared with collagen alone or collagen incubated with neutrophils without LPS, n=6 per group).
may be released from them through degranulation in response to pro-inflammatory stimuli along with other neutrophil products, such as elastase, myeloperoxidase and MMP's. Human neutrophils also appear to contain an enzymatic activity, which acetylates PGP to N-α-PGP (Fig.3). N-α-PGP is a more potent neutrophil chemoattractant than PGP (Haddox et al., 1999) and, like PGP, is a biomarker for COPD and CF in sputum (Gaggar et al., 2008; O'Reilly et al., 2009). Precise localization of PE within neutrophils, the mechanism of its release and the stimuli that cause PE release are the subject of ongoing studies.

Although PE is widely distributed in tissues, its precise physiological role is unknown. It has been suggested to play a role in neuropeptide processing and secretion (Schulz et al., 2005) and in the pathogenesis of hypertension, through effects on the renin–angiotensin system. A role for PE in inflammation has been suggested by a small number of studies. For example, higher amounts of PE were detected in the synovial fluid of patients with rheumatoid arthritis than of patients with osteoarthritis (Kamori et al., 1991). However, no specific mechanisms have been elicited for this pro-inflammatory role. Our data provide a novel mechanism where PE may contribute to neutrophilic inflammation by generating the neutrophil chemoattractant, PGP, from collagen. Generation of PGP by PE from neutrophils provides a pathway for neutrophilic inflammation to feed forward in a self-perpetuating manner after an initial pro-inflammatory insult and provides a link between neutrophilic inflammation and the matrix destruction and remodeling seen in a wide variety of chronic inflammatory diseases. Our previous studies have implicated PE in PGP generation in patients with COPD and CF (Gaggar et al., 2008; O'Reilly et al., 2009). As we have previously shown
for PGP (van Houwelingen et al., 2008), PE may be a biomarker and therapeutic target for these and other chronic inflammatory disorders.
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References


THE PRESENCE OF A MATRIX-DERIVED NEUTROPHIL CHEMOATTRACTANT IN BRONCHIOLITIS OBLITERANS SYNDROME AFTER LUNG TRANSPLANTATION

by

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Abstract

Lung transplantation is a therapeutic modality frequently used in end-stage lung disease. Unfortunately, lung transplant recipients have poor clinical outcomes, often due to the development of bronchiolitis obliterans syndrome (BOS). This process is often characterized by the pathologic findings of obliterative bronchiolitis: neutrophil influx and extracellular matrix remodeling leading to luminal obstruction and airway inflammation. The molecular mechanisms underlying BOS are poorly understood and disease-specific biomarkers are lacking. We report that in addition to increased levels of IL-8, the level of the neutrophil chemoattractant proline-glycine-proline (PGP) is elevated in BOS patient bronchoalveolar lavage (BAL) fluid. The enzymes responsible for generating PGP, matrix metalloproteases 8 and -9 and prolyl endopeptidase, are also elevated in these samples. Together, IL-8 and PGP account for most of the neutrophil chemoattractant capacity seen in BOS BAL fluid. Using specific neutralizing Abs to both IL-8 and PGP, we demonstrate that PGP is a prominent neutrophil chemoattractant found in BAL fluid from individuals at the time of diagnosis of BOS. These findings highlight the influence of a matrix-derived neutrophil chemoattractant in posttransplantation BOS and provide opportunities for the development of unique diagnostics and therapeutics to potentially improve disease outcomes.
Introduction

With the increasing prevalence of end-stage chronic pulmonary conditions and lack of disease-modifying medications, lung transplantation is often the only therapeutic modality available for affected individuals (1). Unfortunately, complications are frequent and result in reduced long-term preservation of graft function and patient survival (1, 2). Chronic allograft rejection accounts for poor rates of patient survival. More than 50% of all lung transplant recipients will eventually develop this condition (2). The clinical correlate of this condition is known as bronchiolitis obliterans syndrome (BOS). It is a clinical diagnosis of exclusion made with decline in lung function (3–5). It is manifested histologically as obliterative bronchiolitis (OB), a fibroproliferative process which targets airways. Five-year survival after chronic rejection is 30% (6).

The specific pathogenic mechanism of OB is poorly understood but there is damage to both epithelial cells and subepithelial structures (7). Neutrophils are a prominent cell type found in the bronchoalveolar lavage (BAL) of BOS patients (8), which may have the capacity of inducing ongoing airway remodeling and inflammation. Chemokines are thought to be important effectors in cellular recruitment in the development of chronic rejection. Specifically, glutamate-leucine-arginine (ELR) positive CXC chemokines, important in neutrophil recruitment (9), may play an important role in the pathogenesis of this condition. Patients with BOS demonstrate increased IL-8 levels in BAL fluid (10, 11). CXCR2 ligands have been shown to be important in early neutrophil recruitment and ongoing vascular remodeling in OB (12).
Due to the degree of remodeling seen in OB, interest has turned to the role of proteases in the development of this condition. Several chronic inflammatory conditions are characterized by an imbalance of proteases with their naturally occurring antiproteases (13). We have recently reported a proteolytic pathway for the generation of a collagen-derived neutrophil chemoattractant (proline-glycine-proline (PGP)) (14), which involves the efforts of matrix metalloproteases (MMP-8 and MMP-9) and a serine protease, prolyl endopeptidase (PE) (15, 16). PGP acts on CXCR1 and 2 receptors on neutrophils, similar to IL-8, causing neutrophil influx into areas of injury (14, 15).

Despite these intriguing findings, the impact of this matrix-derived system of inflammation in clinical disease is not currently known. The aim of the present study was to probe for the presence of these peptides and the proteases required for their generation in lung transplantation recipients to determine their potential role in the neutrophilic inflammation observed in BOS. In this report, we demonstrate that the appropriate enzymes required for PGP generation are present at both increased levels and activity in BOS samples compared with other transplant populations. We note a significant correlation between both MMP-9 activity with PGP levels and PE activity with PGP levels in BOS, supporting a direct relationship between these enzymes and PGP generation in this condition. These samples demonstrate increased capacity for neutrophil chemotaxis and have elevated levels of both IL-8 and PGP. Using an Ab against PGP, we are able for the first time to demonstrate the presence of PGP-mediated chemotaxis in BOS. Finally, we highlight the combined effects of IL-8 and PGP in BOS BAL fluid and suggest the possibility of a change in chemokine predominance seen during the
development of BOS. These findings suggest a novel mechanism of neutrophilic inflammation in BOS and may identify disease biomarkers (PE, PGP) to characterize this condition.
Table I. *Pauleau characteristics*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonrejection Transplant Controls</th>
<th>Acute Rejection</th>
<th>BOS</th>
</tr>
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<tbody>
<tr>
<td>No.</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Age</td>
<td>58.4 years (1.9)</td>
<td>48.3 years (3.7)</td>
<td>48.6 years (7.3)</td>
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<tr>
<td>Gender</td>
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<td>38% male</td>
<td>58% male</td>
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<tr>
<td></td>
<td>42% female</td>
<td>62% female</td>
<td>42% female</td>
</tr>
<tr>
<td>Race</td>
<td>86% Caucasian, 14% AA*</td>
<td>75% Caucasian, 25% AA</td>
<td>86% Caucasian, 14% AA</td>
</tr>
<tr>
<td>Type of transplant (single vs bilateral)</td>
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<td>87% single, 15% bilateral</td>
<td>72% single, 28% bilateral</td>
</tr>
<tr>
<td>Indication for lung transplant</td>
<td>28% IPF</td>
<td>37% IPF</td>
<td>59% IPF</td>
</tr>
<tr>
<td></td>
<td>58% COPD</td>
<td>59% COPD</td>
<td>14% COPD</td>
</tr>
<tr>
<td></td>
<td>14% CF</td>
<td>13% Pulm HTN</td>
<td>28% CF</td>
</tr>
<tr>
<td>Average time of lavage from date of transplant</td>
<td>1431 days (464)</td>
<td>1203 days (599)</td>
<td>3 mos before diagnosis: 878 days (205)</td>
</tr>
<tr>
<td>Lung function</td>
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<td>FEV₁ = 1.57L (0.25)</td>
<td>FEV₁ = 2.17L (0.24)</td>
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<tr>
<td></td>
<td>FVC = 2.30L (0.08)</td>
<td>FVC = 2.34L (0.29)</td>
<td>FVC = 2.94L (0.37)</td>
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<tr>
<td></td>
<td>FVC% = 64.1 (5.2)</td>
<td>FVC% = 58.4 (5.9)</td>
<td>FVC% = 72.0 (8.0)</td>
</tr>
<tr>
<td>Immune suppression regimen</td>
<td>72% Prograf, prednisone, and Cellcept</td>
<td>63% Prograf, prednisone, and Cellcept</td>
<td>86% Prograf, prednisone, and Cellcept</td>
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<tr>
<td></td>
<td>28% Prograf, prednisone, and azathioprine</td>
<td>37% Gengraf, prednisone, and Cellcept</td>
<td>14% Gengraf, prednisone, and Cellcept</td>
</tr>
</tbody>
</table>

*Patient populations are presented in these columns with SEMs noted in parentheses. Data are presented as either percentages or means ± SEM.

*AA, African American; Pulm HTN, pulmonary hypertension.*
Materials and Methods

Patient populations

The University of Alabama at Birmingham (UAB) Institutional Review Board approval (IRB X051014005) was obtained before all studies involving human participants and samples. All patients had BAL samples and basic clinical information collected with a unique patient identifier to maintain patient confidentiality. The diagnosis of BOS was made on a decline in forced expiratory volumes 1 s (FEV1) to \(<80\%\) of baseline over a 3-wk period without other identified etiologies. Diagnosis of acute rejection was \(\geq A2\) grade rejection based off of a transbronchial biopsy. A1 grade rejection was not included into any population studied. All study patients had both a negative BAL culture for bacteria and virus. Clinical data are summarized in Table I.

Bronchoscopy and BAL processing

Bronchoscopy and BAL (4 x 25 ml of 0.9% NaCl for a total of 100 ml instilled, \(<30–40\) ml were recovered) were performed in all patients at 3-mo intervals and remnant lavage samples were stored in the Core Facility for Collection, Processing, and Storage of Alveolar Fluid (IRB X041026004). These samples were centrifuged at 200 x g for 10 min and the cell-free BAL was stored in \(<80^\circ C\) until analysis. These samples underwent routine microbiologic testing, examining for bacteria (Gram stain and culture) and the presence of viruses (respiratory and CMV) in samples.
**Materials**

Coomassie Brilliant Blue R-250 was obtained from Bio-Rad. HyClone PBS (1x), 0.067M (PO4) without Ca2+ and without Mg2+ was from Hy-Clone. Goat anti-rabbit Ig-HRP human adsorbed Ab was from Southern Biotechnology Associates. Albumin from bovine serum, Cohn V fraction, was obtained from Sigma-Aldrich. Z-Gly-Pro-pNA was from Chem Impex International. DMEM was from Mediatech.

**Gelatin zymography**

Porcine skin gelatin (Sigma-Aldrich) at 1 mg/ml was added to a 7.5% SDS-polyacrylamide solution before casting. Biologic samples were aliquoted and diluted in nonreducing sample buffer, and 25 μl of sample was added to each lane. All samples were electrophoresed at 12 mA for 1 h. Following electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h at 4°C, then incubated in 50 mM Tris-Cl for 16 h at 37°C. Gels were stained in Coomassie blue for 30 min and subsequently destained for 2 h.

**MMP-8 and MMP-9 activity assay**

Briefly, MMP-8- and -9-specific ELISA-based activity assays were used to quantify specific MMP activity (R&D Systems). Samples were diluted to fit the manufacturer’s sensitivity for individual kits. Both samples and recombinant enzyme standards were prepared and incubated for 2 h at room temperature in 96-well plates coated with mAb for MMP of interest. After incubation, samples and standards were activated with 1 mM 4-aminophenylmercuric acetate, a chemical activator of MMPs, and further incubated for 2 h at 37°C. After incubation, a fluorogenic substrate (Fluor-Pro-Leu- Gly-Leu-Ala-Arg-
NH2) was placed in each well and the plate was incubated at 37°C for 18 h. The plate was then read on a spectrophotometer (excitation and emission wavelength of 320 and 405, respectively, SpectraMax Gemini; Molecular Devices) and data were quantified using standard curves provided with the kits.

Myeloperoxidase (MPO) assay

Briefly, commercially available assays kits were used to quantify MPO activity in clinical samples (Calbiochem). Samples were diluted to fit the manufacturer’s sensitivity for the kit. Both samples and MPO standards were prepared and placed on 96-well plates coated with polyclonal Ab directed to human MPO. After a 2-h incubation, detection reagent was placed in wells for 1 h. The samples’ absorbance was then measured at 450 nm wavelength.

Western blot

All samples were electrophoresed through SDS-polyacrylamide gels (both reducing and nonreducing conditions) and electroblotted onto nitrocellulose membranes. Membranes were blocked in PBS (pH 7.4) containing 5% BSA for 1 h. Once washed, they were incubated with primary Ab (rabbit anti-PE) for 1 h at room temperature. The polyclonal rabbit anti-PE Ab was made for us by EZ Biolab against a synthetic peptide representing residues 190–219 of mouse PE. This Ab detects recombinant human (whose sequence differs from mouse by one residue between aa 190 and 219), but not bacterial PE (whose sequence completely differs from human or mouse PE at residues 190–219). After
incubation, samples were washed and incubated with goat anti-rabbit-HRP secondary Ab
for 1 h. Immunoblots were then developed using ECL chemiluminescent kits (Pierce).

**PE activity assay**

Twenty microliters of BAL was incubated with a specific substrate (2 mM
benzylcarboxy-glycine-proline-p-nitroaniline) at 37°C and 5% CO₂ and cleavage of p-
nitroaniline from the substrate by PE was detected using a spectrophotometer at 410 nm
and compared with a generated standard curve for PE activity.

**IL-8 levels**

Briefly, IL-8 ELISA kits were used to quantify the IL-8 levels in clinical samples (R&D
Systems). Samples were diluted to fit the manufacturer’s sensitivity for the kit. Fifty
microliters of the samples or standards was added to 96-well plates coated with mAb
against IL-8 for 2 h at room temperature. Thereafter, 100 μl of IL-8 polyclonal Ab
conjugated with HRP was added to the wells and incubated for 1 h. Finally, hydrogen
peroxide/chromogen was added to each well and, after 30 min, the absorbance was read
at a wavelength of 450 nm.

**Electrospray ionization liquid chromatography/mass spectrometry/mass spectrometry for
PGP detection**

PGP and N-terminal acetylated PGP (N-α-PGP) were measured in BAL samples using a
MDS Sciex (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu
HPLC. HPLC was done using a 2.1 < 150-mm Develosil C30 column (with buffer A:
0.1% formic acid and buffer B: acetonitrile plus 0.1% formic acid: 0–0.6 min 20% buffer B/80% buffer A, then increased over 0.6–5 min to 100% buffer B/0% buffer A).
Background was removed by flushing with 100% isopropanol plus 0.1% formic acid.
Positive electrospray mass transitions were at 270-70 and 270-116 for PGP and 312-140 and 312-112 of N-α-PGP.

**Neutrophil chemotaxis assay**
Lavage sample was placed in the bottom wells of a 3 μm, 96-well polycarbonate filter plate (Millipore) in 150 μl of DMEM. Two x 10⁵ neutrophils were added in 100 μl of DMEM to the top portion. These were incubated for 1 h at 37°C in 5% CO₂. The upper portion of the plate was removed and micrographs of the migrated cells were made with an Olympus IX70 microscope. Migration was standardized from cell counts such that chemotactic index = cells per high-powered field (experimental)/cells per high-powered field (medium control), as previously described (14). Chemokinesis experiments were preformed using BOS BAL fluid in the upper chamber with BOS BAL fluid in the lower chamber (“checkerboard”). For neutralization assays, anti-IL-8 neutralization Abs were purchased from R&D Systems. A polyclonal anti-PGP-neutralizing Ab was made for us by EZ-Biolabs by coupling N-α-PGP via its C terminus to keyhole limpet hemocyanin. This polyclonal Ab reacts with both N-α-PGP and PGP.

**Statistical analysis**
Descriptive statistics such as means and SEMs for quantitative measures and frequencies and proportions for qualitative measures were derived. Nonparametric testing was used to
compare populations in this study. Comparisons between two groups were performed using the exact Wilcoxon rank sum test for unpaired data and the exact Wilcoxon signed-rank test were used for paired data. In addition, comparisons of proportions between groups were performed using the Fisher exact test. Spearman correlation analysis was used to compare relationships between 1) PE activity and PGP, 2) MMP-9 activity and PGP, 3) change in FEV1 and change in PGP, and 4) change in forced vital capacity (FVC) and change in PGP. Calculations were made using SAS (version 9.1.3; SAS Institute) and SPSS (version 14; SPSS). Values of $p \leq 0.05$ were considered statistically significant.
FIGURE 1. A, Zymogram of representative patient BAL. Left, BOS samples are paired at 3 mo before (a) and at time of diagnosis (b) per individual. Five of seven individuals displayed increased gelatinolytic activity compared with their prior matched samples at the time of BOS. Right, Representative zymogram of four nonrejection individuals and four acute rejection individuals. Overall, none of the nonrejection (zero of seven) or acute rejection (zero of eight) samples displayed any detectable gelatinolytic activity. B, MMP-8 activity in patient populations. BOS samples collected at the time of diagnosis had elevated levels of both basal (*, p < 0.05) and total (†, p < 0.05) MMP-8 activity compared with BOS (3 mo before) and to other transplant populations. C, MMP-9 activity in patient populations. The activity of both the basal (*, p < 0.05) and total (†, p < 0.05) forms of MMP-9 are elevated in BOS BAL at the time of diagnosis compared with BOS (3 mo before) and other transplant populations.
Results

Gelatinolytic activity of proteases was increased in BOS lung transplant patients

To investigate the presence of PGP in clinical lung transplant samples, we began by examining whether or not enzymes necessary for PGP production are present in BAL fluid samples from transplant recipients. Using gelatin zymography, we demonstrated that BAL fluid from BOS patients at the time of diagnosis had a marked increase in gelatinolytic activity as compared with other transplant populations, with a major molecular weight band consistent with the active MMP-9 isoform. This activity was also noticeably increased when compared with matched samples 3 mo before the diagnosis of BOS (Fig. 1A). We have previously demonstrated that MMP-8 or MMP-9 activity seem to be required for PGP generation and that these enzymes can act in concert for more efficient generation of PGP (15, 16). We next examined both the activity and concentrations of these enzymes in our transplant populations. MMP-8 concentrations were elevated in BOS BAL fluid samples compared with other populations ($p < 0.05$). The majority of increased enzyme was detected as a zymogen form that was inducible by 4-aminophenylmercuric acetate (Fig. 1B; $p < 0.05$). In contrast to MMP-8, MMP-9 is constitutively active in BAL fluid of all populations. Interestingly, the average MMP-9 concentrations (146 ng/ml) in all populations were ~10-fold higher than the concentration of MMP-8 (16 ng/ml). However, like MMP-8, MMP-9 activity was several fold lower in all other patient samples including BOS BAL fluid from 3 mo before diagnosis (Fig. 1C; $p < 0.05$). These data suggest that MMP-9 probably plays a prominent role in the generation of PGP after lung transplantation.
FIGURE 2. A, PE detection in pooled BAL fluid from study populations. BAL fluid was probed using a specific polyclonal Ab for PE. Only the BOS samples collected at the time of diagnosis detected PE (~80 kDa). B, PE activity in patient BAL fluid samples relative to transplant controls. BOS samples at the time of diagnosis demonstrate significantly elevated PE activity compared with matched samples collected 3 mo before diagnosis (†, $p < 0.01$) and other transplant populations (*, $p < 0.01$).
PE detection and activity were increased in BOS BAL fluid samples when compared with other patient groups

PE, a serine protease, has been previously described to have a central role in PGP generation (15). We examined whether this enzyme, not previously described in transplantation pathology, was present in the transplant patient samples. Western blot analysis identified an 80-kDa band (consistent with the molecular mass of PE) only present in the BOS samples taken at the time of diagnosis (Fig. 2A). The increased detection of this enzyme was complemented by 4-fold increased activity in BOS BAL fluid at the time of diagnosis compared with normal transplant patients or acute rejection (p < 0.01) and when compared with matched samples from 3 mo before BOS diagnosis (p < 0.01; Fig. 2B). These findings confirm that all of the necessary enzymes for PGP generation were found in BOS BAL fluid.

Classic and novel chemokine levels were elevated in BOS BAL fluid samples

As previously mentioned, BAL from BOS individuals have demonstrated increased polymorphonuclear leukocyte (PMN) counts (8). Indeed, when MPO, a surrogate of neutrophil influx (17), is measured in the transplant patient populations, there was an increased amount seen in BOS vs. other groups (p < 0.05; Fig. 3A). To determine the neutrophil chemoattractants responsible for the increased neutrophil levels observed in these samples, we first examined the presence of IL-8. Levels of IL-8 were ~20-fold higher in BOS samples compared with the matched samples obtained 3 mo before diagnosis (p < 0.05; Fig. 3B) and higher than in samples from control populations. We also examined the presence of the neutrophil chemoattractant PGP in clinical disease
FIGURE 3. A, MPO in BAL fluid samples relative to transplant controls. BOS samples from the time of diagnosis are significantly elevated compared with both nonrejection transplant controls (*, p < 0.05) and matched samples collected 3 mo before diagnosis (†, p < 0.05). B, IL-8 levels in BAL fluid from lung transplant populations. BOS BAL fluid collected at the time of diagnosis demonstrated a 6-fold increase in IL-8 levels compared with samples 3 mo before diagnosis (*, p < 0.05). C, PGP levels in BAL fluid from lung transplant populations. BOS samples demonstrated a >16-fold increase in PGP compared with the individual’s prior matched samples and other transplant populations (†, p < 0.05).
samples using a mass spectrometry technique: electrospray ionization liquid chromatography-tandem mass spectrometry (14–16). PGP levels were 16-fold higher in BOS samples compared with their matched samples 3 mo before diagnosis ($ p < 0.05$) and significantly higher compared with other transplant populations ($ p < 0.05$; Fig. 3C). Only the nonacetylated form of PGP was found in BAL from lung transplant patients (in contrast with our previous reports of N-α-PGP detection in samples from cystic fibrosis (CF) patients).

*Change in PGP correlated with change in lung function in BOS BAL fluid samples*

We next examined whether changes in PGP concentrations correlated with changes in lung function in BOS individuals. We observed a correlation coefficient ($ r $) of $ -0.83 $ ($ p < 0.05 $) for change in FVC and a $ r $ of $ -0.67 $ for change in FEV1 ($ p < 0.14 $). These coefficients were somewhat better than those observed with IL-8 change vs. change in FEV1 ($ r = 0.56; p < 0.25 $) or change in FVC ($ r = 0.73; p < 0.10 $) in these samples.

*PGP levels correlated with both MMP and PE activity in BOS BAL fluid samples*

As we have previously described in a murine model and in CF sputum samples ex vivo (15, 16), PGP generation involves the coordinated activity of MMPs and PE. When we examined the correlation between MMP-9 activity and PGP levels in BOS BAL fluid, the samples demonstrated a strong correlation ($ r = 0.86, p < 0.05 $; Fig. 4A). Similarly, PE activity demonstrated a strong correlation with PGP concentrations in these samples ($ r = 0.96, p < 0.01 $; Fig. 4B).
FIGURE 4. A, Correlation of MMP-9 activity and PGP levels. The PGP levels and MMP-9 activity in BOS samples taken at the time of diagnosis displayed a strong correlation $r$ value of 0.86 ($p < 0.05$). B, Correlation of PE activity and PGP levels. The PGP levels and PE activity in BOS samples taken at the time of diagnosis displayed a strong correlation $r$ value of 0.96 ($p < 0.01$).
This exponential correlation is in keeping with the relationship of an enzyme and its product. Of note, similar $r$ values were seen between MMP-9 activity/PGP and PE activity/PGP in the matched samples 3 mo before BOS, although these correlation coefficients were not as robust as that seen at the time of BOS. These findings suggest that these enzymes operate in vivo to generate PGP over the course of the development of BOS following lung transplantation.

**BOS BAL samples were highly chemotactic for peripheral blood neutrophils ex vivo**

Since we have demonstrated the presence of two neutrophil chemoattractants (IL-8 and PGP) in clinical samples, we examined the neutrophil chemotactic potential of BAL fluid from our study populations. BAL fluid from each of the transplant patient groups was used to perform a chemotaxis assay ex vivo on peripheral blood neutrophils (isolated from normal, nontransplant controls). The BOS samples taken at the time of diagnosis demonstrated a 16-fold higher chemotactic index relative to medium control ($p < 0.01$) and was also higher than transplant controls ($p < 0.05$) and pre-BOS patient population ($p < 0.05$; Fig. 5). These results are consistent with chemotactic activity of both IL-8 and PGP in these populations’ BAL fluid. To verify that these results were due to chemotaxis and not chemokinesis, placement of BOS BAL fluid in the upper well reduced BOS fluid-mediated chemotaxis to the lower well by 82% ($p < 0.05$; data not shown).
Figure 5  Neutrophil chemotaxis of BAL fluid from transplant populations. Samples collected at the time of BOS diagnosis demonstrated 16-fold increase in neutrophil chemotaxis relative to medium control and was significantly elevated compared with BOS 3 mo before diagnosis (†, p< 0.05) and to other transplant populations (*, p< 0.05).
Blockade of IL-8 and PGP ablated the chemoattractive ability of BOS BAL fluid samples.

To determine the degree by which each of these chemokines alone or in concert influenced the chemotactic properties of BOS BAL fluid, we examined pooled samples at the time of BOS with the matched control samples from 3 mo before diagnosis. We investigated the samples’ ability to induce neutrophil chemotaxis in the presence of a specific neutralizing IL-8 Ab and a specific PGP neutralizing Ab developed in our laboratory. Chemotaxis neutralizing curves for each Ab were determined. For the anti-PGP Ab incubated with 100 μg/ml PGP, 1/5000 dilution gave 11% inhibition, 1/1000 dilution gave 92% inhibition, and 1/100 dilution gave 100% inhibition. For the anti-IL-8 Ab incubated with 10 ng/ml IL-8, 1/5000 dilution gave 26% inhibition, 1/1000 dilution gave 90% inhibition, and 1/100 gave 100% inhibition. These values led to the usage of a 1/1000 dilution of each Ab for neutralization experiments. Of note, these Abs demonstrated no cross-reactivity with the alternate chemokine (Fig. 6, A and B). When both Abs were used in combination in either the 3 mo before or at the time of diagnosis BOS BAL, we observed almost complete inhibition of chemotaxis activity (p < 0.05). This suggests that PGP, along with IL-8, are the major PMN chemoattractants in BOS BAL fluid.

By using the specific neutralizing Abs to both PGP and IL-8, we hoped to be able to determine the chemoattractant profile for both time points of the disease. In the samples collected 3 mo before diagnosis, the neutralizing Abs for IL-8 and PGP caused approximately an 81% and 30% reduction in chemotaxis, respectively (Fig. 6C). When matched samples taken at the time of diagnosis were examined, IL-8-neutralizing Ab reduced the chemotactic ability of the samples by <28%, while the PGP-neutralizing Ab
caused a 53% inhibition of neutrophil migration. Only the anti-PGP Ab used in BOS samples at diagnosis demonstrated statistical significance with regard to chemotactic inhibition ($p < 0.05$). In contrast, only the IL-8 Ab used in BOS samples 3 mo before diagnosis demonstrated statistical significance with regard to chemotactic inhibition ($p < 0.05$). These data, therefore, provide some evidence for the importance of PGP as a neutrophil chemoattractant seen in BOS and suggest a shift from an IL-8-biased neutrophil chemotaxis to a PGP-biased neutrophil chemotaxis during the development of BOS.
Discussion

Chronic rejection/BOS in lung transplantation remains the major source of morbidity and mortality in lung allograft recipients (2). Although the role of neutrophils in BOS pathogenesis is not well understood, there is emerging evidence for PMN-derived oxidant injury and protease imbalance in this disease population, potentially leading to airway remodeling (18). The determination of unique pathways involved in this inflammatory and remodeling response may serve to identify unique biomarkers and to elucidate specific therapeutic targets.

Our work confirms that lavage samples from individuals with BOS have increased proteolytic activity. Although different families of proteases have been implicated in the end-organ damage described in post lung transplantation pathology, recent attention has turned to MMPs as mediators of lung damage (19, 20). Although MMP dysregulation appears to contribute to extracellular matrix reorganization in many conditions (21, 22), few studies have investigated MMPs in post lung transplant organ pathology. Studies have noted increased MMP-9 expression in ischemia reperfusion injury, in lung transplant patients with clinical airway obstruction, and in patients with BOS (23–25). There is also evidence that both MMP-8 and MMP-9 are increasingly elevated before the development of BOS in posttransplant individuals (26).

We demonstrate increased MMP-8 and MMP-9 levels and activity in lavage samples from patients at the time of BOS compared with matched samples from 3 mo before diagnosis. Using a novel polyclonal anti-PE Ab and an enzymatic assay to determine PE activity, we describe, for the first time, that PE is both detected and up-regulated in BOS samples. Our current and previous work suggest that increased activity
FIGURE 6. A, PGP- and IL-8- neutralizing Abs do not cross-react with the alternate chemokine. The IL-8- and PGP-specific neutralizing Abs (1/1000 each) completely inhibited IL-8- and PGP-mediated chemotaxis, respectively (*†, p < 0.01). Neither Ab cross-reacts with the alternate chemokine. B, Relative IL-8 and PGP chemotaxis in BOS BAL fluid. IL-8-neutralizing Ab (1/1000) created an 80.6 and 29.6% inhibition of BOS (3 mo before) and BOS (at diagnosis) induced PMN chemotaxis, respectively. In contrast, the inhibition by the PGP-neutralizing Ab (1/1000) was 28.2% (in the 3-mo prior samples) and 53.4% (in the samples at time of diagnosis). Together, the Abs were able to almost completely ablate the chemotactic capacity of the BAL fluid collected 3 mo before (93.2%) and at the time of diagnosis (91.4%) of BOS (†, p < 0.05). Of the individual Abs, anti-IL-8-neutralizing Ab demonstrated a statistically significant reduction in chemotaxis for BOS (3 mo before diagnosis), whereas anti-PGP-neutralizing Ab demonstrated a statistically significant reduction in chemotaxis for BOS at diagnosis (*, p < 0.05).
of these enzymes in clinical samples is important for PGP liberation from collagen in vitro and in vivo (15). The statistically significant correlation coefficients seen with either MMP-9 or PE with PGP levels strongly suggest that these enzymes are operative in PGP production in BOS.

There is also increasing evidence that components of the extracellular matrix may serve as modulators of airway inflammation in lung transplantation rejection. Wilkes and colleagues (27, 28) have described the role of cryptic epitopes of type V collagen as a mediator of immune responses seen during chronic lung transplantation rejection. Attention has also turned to alternate pathways of inflammation involving fragmentation of matrix scaffolding proteins. Nonspecific collagen-derived fragments have been reported to induce neutrophil chemotaxis in murine models (29, 30). In addition, elastin fragments ending with proline-glycine demonstrate the capacity to cause fibroblast and monocyte chemotaxis and, to a lesser degree, neutrophil chemotaxis in models of emphysema (31).

BAL fluid samples from patients at the time of BOS also demonstrate an increased capability to attract neutrophils compared with all other transplant populations. These samples also exhibited high levels of IL-8, a neutrophil chemoattractant well characterized in BOS. Traditional CXCR ligands are felt to play an important role in both neutrophil influx and propagation of injury via activation of other cellular inflammatory mechanisms (10–12). In this study, we show that a extracellular matrix-derived neutrophil chemoattractant, PGP, is elevated in clinical samples from individuals with BOS. To our knowledge, this is the first characterized extracellular matrix (ECM)
fragment described in transplantation rejection with the capability to drive neutrophilic inflammation.

We have also developed a novel detection and inhibitory Ab to PGP which distinguishes this peptide from IL-8. Using this reagent and an IL-8-specific Ab, we demonstrate that during the course of the development of BOS, there is an emerging role of PGP as a prominent neutrophil chemoattractant. Together IL-8 and PGP account for virtually all of the chemotactic activity seen in BOS BAL fluid (Fig. 6C) and may represent logical therapeutic targets. These findings expand our understanding of the role of the ECM as modulators of immune response in lung transplantation rejection, demonstrating that peptide fragments from ECM structural proteins may serve as important mediators of neutrophil recruitment. Our findings also support the concept that collagen-derived chemoattractants might have an increased role in neutrophil influx at the time of airflow reduction in BOS due to the airway remodeling seen in the disease.

One obvious question is whether pretransplant diagnosis may affect posttransplant PGP levels. Although we observe differences in PGP values between populations pretransplant (CF<chronic obstructive pulmonary disease (COPD)<interstitial pulmonary fibrosis (IPF)), all patients with BOS had higher PGP levels compared with other patients with similar pretransplant diagnoses in either the acute rejection or nonrejection populations. These data suggest that the posttransplantation pathology plays a more prominent role in PGP generation than the pretransplant diagnosis.

There are certain limitations to our study. Although most of the presented data is statistically significant between populations studied, the small numbers per group make it difficult to interpret these results for larger cohorts. Also, although correlations were seen
between changes in lung function and changes in chemoattractants in BOS individuals, these findings should be taken with caution as they may represent epiphenomena. Examination of larger populations from multiple centers may allow for determination of the specificity and sensitivity of the levels/activity of PGP, PE, and MMPs in the development of BOS. In addition, future prospective studies with larger cohorts may examine if, over time, PGP levels may predict loss of lung function in posttransplantation individuals. As such, these longitudinal studies of PGP production in chronic rejection/BOS may also be useful to increase our understanding of disease onset and progression and possibly prognosis.

In summary, we demonstrate that PGP is increased in the progressive development of BOS in lung transplant recipients. The elevated levels in BAL fluid in BOS patients, along with the high correlation with generating enzymes and diagnosis of disease, make PGP a potential biomarker and therapeutic target for this condition. We have also shown that, although traditional chemokines regulate neutrophils before BOS, matrix degradation products, such as PGP, have an emerging role in the PMN influx seen in clinical disease. The identification of this novel neutrophil chemoattractant pathway in chronic rejection following lung transplantation sets the stage for future studies to examine the role of this peptide in disease pathogenesis.
Acknowledgments

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Disclosures

The authors have no financial conflict of interest.


A DUAL PROTEASE INHIBITOR/CXCR RECEPTOR ANTAGONIST WITH THERAPEUTIC IMPLICATIONS IN CHRONIC INFLAMMATORY LUNG DISEASE

By

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In Preparation for Submission
Abstract

Dysregulated neutrophilic inflammation is associated with poor outcomes in several chronic pulmonary diseases. Increased protease activity and the presence of neutrophil specific chemokines, interleukin-8 (IL-8) and the tripeptide proline-glycine-proline (PGP), have also been observed as biomarkers of disease. Attempts at blocking the IL-8 pathway of neutrophilic inflammation have largely been met with mixed results at best. For instance, clinical trials utilizing a neutralizing antibody to IL-8 have yielded minimal clinical benefit. Ostensibly this is due to a previous lack of understanding of the PGP mediated pathway for neutrophil recruitment which has been shown to play an equally an important role to that of IL-8 in disease. Consequently, therapeutic modalities are needed that will block both the IL-8 and PGP mediated pathways of neutrophilic inflammation. Recently, the serine protease, prolyl endopeptidase (PE), which is responsible for PGP production, has been described in neutrophils and has been shown to be elevated in cystic fibrosis and bronchiolitis obliterans syndrome, perhaps indicating a feed forward mechanism PGP driven PMN inflammation. This work describes a PE inhibitor, benzyloxycarbonyl-proline-prolinal (ZPP) that is potent in preventing PGP generation from collagen. The particularly novel finding of the present work is that ZPP also appears to be effective in blocking IL-8 and PGP mediated neutrophil recruitment. By using chemotaxis assays, along with radio-receptor assays and in vivo studies, we show that ZPP antagonizes chemokine receptors CXCR1 and 2 and prevents neutrophil migration to IL-8 and PGP. This indicates that there are similarities between the catalytic site of PE and a binding pocket of CXCR1 and/or 2. We initially hypothesized this was possible due to structural relatedness among IL-8, PGP and ZPP. This was confirmed by
experiments showing a PE substrate, Z-G-P-pNA is specifically chemotactic to neutrophils via CXCR1 and 2. To the best of our knowledge, these findings describe for the first time a single compound that is capable of blocking both a pivotal enzyme and receptor in the same pathologic pathway. ZPP affords an exciting opportunity not only for the formulation of novel therapeutics in combating neutrophilic inflammation but also as an example of a new paradigm for the design of single therapeutic compounds that block multiple targets in disease pathways.
Introduction

Chronic neutrophilia, characterized by increased protease activity, superoxide-related damage, and extra-cellular matrix destruction is a hallmark of numerous inflammatory diseases. Chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiolitis obliterans syndrome (BOS), rheumatoid arthritis, and metastatic melanoma are all diseases in which neutrophils play a prominent role (1-4). Under normal conditions, polymorphonuclear leukocytes (PMNs), or neutrophils, are an important component of the innate immune system. They are one of the first responders to a site of infection and are capable of phagocytosis of an invading pathogen, release of anti-microbial enzymes and performing an oxidative burst aimed at destroying a foreign organism (5). Unfortunately, it is the non-specific nature of neutrophils’ cellular armament that makes them potent against both a wide range of invaders, and in certain cases the host’s own tissues. Acute inflammation is the body’s natural response to infection and injury, and the cytokines released by epithelial/endothelial cells in an inflammatory site increase vascular permeability and draw neutrophils into the tissue to deal with infection (6). Mild tissue damage is a side effect of neutrophil recruitment into tissue and is easily healed by the body. It is when there is dysregulated neutrophilic inflammation, and more severe tissue injury occurs, that neutrophils change from effectors of normal homeostasis to mediators of chronic, pathologic disease (7). The classical pathway by which neutrophil recruitment occurs is through the chemokine interleukin-8 (IL-8). IL-8 is a glutamate-leucine-arginine positive (ELR+) CXC chemokine that acts via CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) in humans (4). There have been attempts to blunt the effect of IL-8 in a clinical setting
using a neutralizing monoclonal antibody (mAb), but with limited to no success (8). If IL-8 were truly the only chemokine responsible for neutrophil recruitment, an anti-IL-8 mAb should have been extremely effective in ameliorating conditions associated with increased levels of IL-8. However, in recent work performed by our laboratory it does not appear that IL-8 is the sole chemokine present in chronic neutrophilic inflammation. A collagen breakdown fragment, proline-glycine-proline (PGP), which bears striking structural and sequence similarities to an important functional motif in the ELR+CXC chemokine family, appears to be present and active in multiple clinical diseases. Indeed, in animal models and ex vivo samples PGP appears to play as great a role as ELR+CXC chemokines in causing PMN influx and chemotaxis (1, 3, 9). PGP, and its more potent amino terminal acetylated form (N-α-PGP), are products of a protease cascade of matrix-metalloproteases (MMPs) and a serine protease, prolyl endopeptidase (PE) (9, 10). Additionally, all of the proteases responsible for PGP production are found in neutrophils and have been measured in clinical samples of disease (11, 12). As a result of the discovery of this novel pathway of neutrophilic inflammation, any future therapeutics developed would need to be directed at negating the effects of both chemokine pathways.

It is with this in mind that we undertook the study described in this manuscript. While it would be possible to develop mAbs directed against both IL-8 and PGP, a more useful approach would be to have a single compound capable of ablating the effects of both. Prolyl endopeptidase is a protease that has traditionally been described in the central nervous system (CNS) as a processor of neuropeptides (13-15). It was only recently that our group has described its role in inflammation by liberating PGP from the PPGP repeat seen so frequently in collagen (10). Benzyloxy carbonyl-proline-proline-prolinal
(ZPP) is a specific, noncompetitive inhibitor of PE. Based on the distinct structural similarities between ZPP, PGP and a PE substrate, benzylxycarbonyl-glycine-proline-paranitroaniline (ZGP-pNA) that is chemotactic to neutrophils, we propose that there may be structural similarity between the active site of PE and a binding pocket of CXCR1 and/or 2 which could be exploited in the design of a truly novel type of therapeutic agent. Thus we hypothesize that it may be possible for a single compound, ZPP, to function as both an enzyme inhibitor and a receptor antagonist.
Materials and Methods

Materials

All antibodies were purchased from R & D Systems (Minneapolis, MN). Albumin from Bovine Serum, Cohn V Fraction was obtained from Sigma (St. Louis, MO). Z-Gly-Pro-pNA was from Chem Impex International. DMEM was from Mediatech. Z-Pro-Prolinal was obtained from CalBiochem (Gibbstown, NY) and Enzo Life Sciences (Plymouth Meeting, PA).

PE Activity Assay

100 ng PE was added to 1mM Suc-GP-pNA in phosphate buffer with 10μM BSA with or without ZPP in a total volume of 250μl in a 96-well plate, the plate was placed at 37°C in 5% CO₂ for 1 hour. Absorbance was measured at 410nm using a Tecan Rainbow Spectrophotometer (Mannendorf, Switzerland).

Electrospray ionization liquid chromatography/mass spectrometry/mass spectrometry for PGP detection

PGP and N-terminal acetyl PGP (N-α-PGP) were measured in all samples using an MDS Sciex (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu HPLC. HPLC was performed with a 2.1 x 150 mm Develosi C30 column (with buffer A: 0.1% formic acid and buffer B: acetonitrile plus 0.1% formic acid: 0-0.6 min 20% buffer B/80% buffer A, then increased over 0.6-5 min to 100% buffer B/0% buffer A. Background was then removed by flushing with 100% isopropanol plus 0.1% formic
acid. Positive electrospray mass transitions were at 270-70 and 270-116 for PGP and 312-140 and 312-112 of N-α-PGP.

**Neutrophil Chemotaxis Assays**

The chemokine of interest was placed in the bottom chamber of a 96 well 3 μm filter plate from Millipore in a volume of 150 μl. The filter was placed on top and 2 x 10^5 neutrophils were loaded on top in a volume of 100 μl. The plates were incubated at 37°C in 5% CO₂ for 1 hour. The number of migrated cells were determined using either photomicrographs taken with an Olympus IX70 or an LSRII flow cytometer (BD Biosciences, San Jose, CA).

Antibody neutralization assays were performed by pre-incubation of 100ug/ml (final concentration) of antibody to either CXCR1 and/or 2 for 45 min at 4°C prior to chemotaxis assay. ZPP inhibition of chemotaxis was determined by pre-incubating the cells with ZPP for 45 minutes at 4°C with agitation. Following this a customary chemotaxis assay was performed (see above).

**Mice**

BALb/C mice (The Jackson Laboratory) were kept in the Animal Research Facility (UAB, Birmingham, AL). Food and water were given *ad libitum*. All experimental procedures were in accordance with Institutional Animal Care and Use Committee regulations. All experiments were performed using female mice.
**Isolation of neutrophils from mouse bone marrow**

Balb/C mice were euthanized and both femurs and tibias were perfused using a 24G needle with PBS. The cells were washed and suspended in DMEM on a 62% Percoll gradient and centrifuged at 1000 x g for 30 min at 25°C. The neutrophils were defined as the layer directly above the red cells and PMNs were removed, washed, counted and assessed via differential staining using hemalotaxin.

**In vivo administration of Z-Gly-Pro-pNA**

100 μg of Z-Gly-Pro-pNA was administered intratracheally in 50 μl of PBS with 1% Dioxane (Sigma) and 40% EtOH. Mice were returned to the cage and sacrificed 24 hours later by intraperitoneal (IP) injection of .2ml Nembutol.

**Collection of mouse bronchalveolar lavage fluid**

Mice were euthanized with IP injection of nembutol. A 10 cc syringe connected to a three way stop-cock was attached to a 23 G flexible catheter. The catheter was inserted into the proximal end of the trachea and the lungs were slowly perfused with 3 x 1ml of PBS (room temperature). BAL was collected using an empty 10 cc syringe attached at the third spot on the stop-cock. Cell counts were determined using Trypan Blue staining and a hemocytometer. For further confirmation, cytospins were done of all samples and staining was performed using Trypan Blue (Sigma, St. Louis, MO).
**PGP generation from intact collagen using neutrophil lysates**

4 x 10^6 neutrophils/ml were lysed using 2x freeze-thaw cycles in PBS with 10μg/ml Aprotinin (Sigma). 25μg Type I collagen, 25μg Type II Collagen, with or without 0.25 mM ZPP, 150μl lysates, 10μM BSA, and 5μl 10mg/ml Bestatin was added every 6 hours. After 24 hours at 37°C, the samples were centrifuged, soluble fluid was recovered, washed on 10kDa Millipore filters (Billerica, MA) and analyzed on ESI-LC-MS/MS.

**Radio-Receptor Assay**

1 x 10^6 neutrophils were either pre-incubated with monoclonal antibodies to CXCR1 and/or CXCR2 at 4°C with agitation or directly placed in siliconized microfuge tubes. For controls, either buffer (RPMI 1640 w/ 25mM HEPES and 1% BSA) alone or unlabeled IL-8 was added in a volume of 100 μl for a final concentration of 7.96 ng/ml. For experimental groups, ZPP was added in a volume of 100 μl for final concentrations of 2.4mM or .8mM. Cells were placed at 4°C for 30 minutes before ^{125}I-IL-8 (Perkins Elmer, Waltham MA) was added in 100 μl for a final concentration of 7.8 ng/ml. Cells were returned to 4°C for 90 minutes. Cells were washed three times in 1 ml buffer and CPM was determined using a Packard gamma radiation counter. Non-specific binding was determined using 10 fold excess of unlabeled IL-8.
Results

*N-α-PGP, ZGP-pNA, and ZPP are structurally similar.* Computer modeling of the neutrophil CXCR1 and 2 ligand, N-α-PGP, the PE substrate ZGP-pNA, and the PE inhibitor ZPP demonstrated closely related structures (Fig 1). All three molecules have a ring structure at the amino-terminus with a proline at the carboxy terminus. ZGP-pNA does have a bulky para-nitroaniline group but it extends perpendicular to the plane of the rest of the molecule in this representation. ZPP has a reactive aldehyde group on the C-terminus that forms a covalent bond with the serine of the catalytic triad in the active site of prolyl endopeptidase. The pronounced similarities between all three molecules suggested the idea of a structural relatedness between a N-α-PGP binding pocket of CXCR1 and/or CXCR2 and the PE catalytic site which accommodates ZGP-pNA and ZPP.

*A prolyl endopeptidase substrate is chemotactic for both human and mouse neutrophils.* To begin to investigate possible similarity in the active site of PE and a binding pocket of CXCR1 and CXCR2 we performed neutrophil chemotaxis assays to determine if a PE substrate was chemoattractive for PMNs. Using a previously described assay (3), we demonstrated that ZGP-pNA is dose dependently chemotactic for both human and mouse neutrophils (Fig 2a, p<0.05). The highest dose of ZGP-pNA used approached the chemotactic potential of the classical PMN chemokine IL-8. When ZGP-pNA was instilled in the tracheas of Balb/C mice it caused an 8 fold increase in neutrophils in bronchoalveolar lavage fluid (Fig 2b, p<0.05). To confirm that ZGP-pNA
Figure 1 Three dimensional structures of the neutrophil chemokine N-α-PGP (N-α-PGP), the prolyl endopeptidase inhibitor ZPP, and the PE substrate ZGP-pNA.

Z=benzyloxy carbonyl  pNA=paranitroaniline  Ac=acetyl
2A. ZGP-pNA causes murine and human neutrophil chemotaxis.
Neutrophils were isolated from mouse bone marrow and human peripheral blood and layered at 2 x 10^5 cells/well on the top of a filter and a chemokine of interest was placed in the well beneath. After one hour at 37°C the filter was removed and cells were counted and standardized to a media control. ZGP-pNA dose-dependently caused both human and mouse neutrophil chemotaxis (* p<0.05) in a manner similar to that of IL-8 (20ng/ml)

2B. ZGP-pNA causes in vivo murine neutrophil chemotaxis.
ZGP-pNA (100ug/mouse) was instilled into the tracheas of female BalbC mice. 24 hours later the mice were euthanized and their lungs were lavaged. Cells were pelleted and stained with Trypan Blue and counted using a Leica light microscope. ZGP-pNA caused nearly an 8 fold increase in neutrophil migration into the lungs with no significant change in macrophage presence (* p<0.01).

2C. ZGP-pNA acts via CXC chemokine receptor CXCR2 in mice.
The same chemotaxis assay described above was performed with various CXC chemokines after preincubation of the cells with a neutralizing antibody against the CXC chemokine receptor CXCR2. Like IL-8 and MIP-2, ZGP-pNA mediated chemotaxis was blunted with α-CXCR2 but not IgG control. fMLP (acting via a different receptor) driven PMN migration was unaffected.

2D. ZGP-pNA acts via CXC chemokine receptors CXCR1 and 2 in humans.
Similar to the mouse, a chemotaxis assay utilizing neutralizing Abs to both CXCR1 and 2 was performed with similar results. When both Abs were used in concert they negated almost 70% of ZGP-pNA initiated PMN chemotaxis.
was indeed acting via the same receptors as N-α-PGP and IL-8, neutralizing antibodies against CXCR1 and CXCR2 in humans, and CXCR2 in mice were used to inhibit chemotaxis. Anti-CXCR1 was most effective in blunting chemotaxis to IL-8, N-α-PGP, and ZGP-pNA but when used in conjunction with anti-CXCR2 67% of the chemotactic potential of all three molecules was ablated (Fig 2c, p<0.05). Likewise, the murine neutrophil chemotaxis to known chemokines macrophage inflammatory protein-2 (MIP-2) and N-α-PGP, along with ZGP-pNA was inhibited with anti-CXCR2 neutralizing antibody (Fig 2d, p<0.05). These data suggest that the PE substrate ZGP-pNA is chemoattractive to neutrophils via CXCR1 and CXCR2 (or CXCR2 in mouse) in a manner similar to other PMN specific chemokines.

**ZPP inhibits prolyl endopeptidase activity.** ZPP has previously been described as a potent PE inhibitor by several groups (16-18). In order to confirm the effectiveness of ZPP as an inhibitor of PE we performed both an enzymatic assay utilizing ZGP-pNA as a substrate and an *ex vivo* assay utilizing neutrophil lysates and collagen to generate PGP. We repeated a dose curve of ZPP against PE to confirm that it is indeed capable of inhibiting PE activity at pM concentrations (Fig 3a). Most of the work done with ZPP as a PE inhibitor has focused on the enzymes role in neurodegenerative diseases and has not dealt with its role in inflammation. To evaluate ZPP as a potential therapeutic agent in PGP mediated inflammation we performed an assay in which neutrophil lysates are incubated with collagen to generate PGP (12). Neutrophil lysates were incubated with
**Figure 3**

**3A. ZPP dose dependently inhibits PE activity.**
ZPP was preincubated with PE for five minutes before the addition of a substrate that produces a yellow color upon cleavage by PE. ZPP acts in the sub-nM range with an IC₅₀ of approximately 300pM.

**3B. ZPP blocks PMN production of PGP from intact collagen.**
Neutrophil lysates were incubated for 24 hours at 37°C with collagen with or without ZPP and the samples were passed through 10kDa filters and analyzed via ESI-LC-MS/MS. ZPP ablates the capability of the lysates to produce PGP from collagen. (*p<0.001)
intact Type I and Type II collagen and PGP generation was measured using electro-spray ionization liquid chromatography mass spectrometry/mass spectrometry (ESI-LC-MS/MS). We observed that neutrophils are capable of producing PGP from intact collagen. In the presence of ZPP however, this ability to produce the chemotactic peptide was completely abrogated (Fig 3b, p<0.05). Since ZPP blocks the only enzyme known to be able to produce PGP from collagen fragments it is no surprise the PE inhibitor completely ablates the formation of the tri-peptide. These findings demonstrate that ZPP could be effective in neutralizing the production of PGP by neutrophils seen in chronic inflammatory diseases.

**ZPP inhibits in vitro murine and human PMN chemotaxis to ELR+CXC chemokines and their structural mimetics.** Having discovered that a PE substrate was specifically chemotactic to neutrophils, and having confirmed that a PE inhibitor did indeed block PE activity and the ability of neutrophil lysates to generate PGP from intact collagen we assessed the potential of ZPP as an inhibitor of neutrophil chemotaxis. To investigate this we performed the previously described chemotaxis assays utilizing ZPP to attempt to block PMN migration. ZPP dose dependently, and completely inhibited murine and human neutrophil chemotaxis to ELR+CXC chemokines (MIP-2 and IL-8, respectively) and the PE substrate (ZGP-pNA), but not to fMLP, another neutrophil chemoattractant that acts via a different receptor (Fig 4a, Fig 4b, both p<0.05) (19, 20). Due to the fact that ELR+CXC chemokines specifically act via CXCR1 and CXCR2 this *in vitro* data indicates that there is a significant similarity between a binding pocket of the receptors and the active site of prolyl endopeptidase.
4A. ZPP dose dependently inhibits ELR+CXC chemokines and ZGP-pNA mediated mouse neutrophil chemotaxis.

ZPP was preincubated with murine neutrophils for 45’ at 4°C prior to use in a chemotaxis assay. MIP-2 (10ng/ml), N-α-PGP (100μg/ml), ZGP-pNA (30μg/ml), and fMLP (10μM), were placed in the bottom wells to incite PMN migration. ZPP effectively blocked MIP-2, N-α-PGP, and ZGP-pNA driven chemotaxis but had little to no effect on fMLP stimulation.

4B. ZPP dose dependently inhibits ELR+CXC chemokines and ZGP-pNA mediated human neutrophil chemotaxis.

In the same manner as the mouse, ZPP was preincubated with isolated human peripheral blood neutrophils for 45’ at 4°C. ZPP almost completely ablated IL-8 (20ng/ml), N-α-PGP (30μg/ml), and ZGP-pNA (15μg/ml) driven chemotaxis.

4C. ZPP acts primarily through CXCR1 to block IL-8 binding to its receptor.

Human peripheral blood neutrophils were incubated with indicated amounts of ZPP for 30’ at 4°C prior to addition of 125I-IL-8 (7.8ng/ml) for 90’ at 4°C. CPM were measured using a gamma counter and % inhibition was calculated. Where indicated, cells were pre-incubated with 100μg/ml neutralizing antibody to either CXCR1 or CXCR2 for 45’ at 4°C. This was done to determine where the main site of ZPP action was. ZPP acts primarily at CXCR1, causing no significant change in the 43.89% (* p<0.01) knockdown caused by the antibody alone. When included with α-CXCR2 however, the addition of ZPP increases the inhibition of the Ab alone from 16.9% to 46.2% at the 100μM dose (* p<0.05, † p<0.05).
ZPP competes for $^{125}$I-IL-8 binding on neutrophils in a radio-receptor assay. Utilizing a radio receptor assay, we investigated the effect of ZPP on IL-8 binding to neutrophils. ZPP was pre-incubated with neutrophils before addition of $^{125}$I-IL-8 to attempt to block IL-8 binding to its receptors. Initially we observed a 24% inhibition of total $^{125}$I-IL-8 binding (Fig 4c, p<0.05). To further elucidate if ZPP was binding to both CXCR1 and 2 or a single receptor we employed neutralizing antibodies to CXCR1 and 2 similar to a method previously described (21). Using the monoclonal antibodies alone, or in concert with one another, we determined that ZPP primarily acts on CXCR1 and blocks as much $^{125}$I-IL-8 binding as a monoclonal antibody to CXCR1 (46.27% and 43.89%, respectively) (Fig 5). This suggests that the inhibition of chemotaxis seen by ZPP is due at least in part to a direct interaction with the chemokine receptor CXCR1.
Discussion

The major finding of this work is that a PE inhibitor, ZPP is able to serve a dual purpose as a neutrophil chemokine receptor antagonist. This is a paradigm shifting idea in that there appears to be structural relatedness between the catalytic site of the enzyme and a binding pocket on the receptor. ZPP may serve as a potential therapeutic in PMN inflammatory conditions. Chronic neutrophilic inflammation is a hallmark of a number of debilitating pulmonary conditions. The prolonged increase in proteolytic enzymes, and reactive oxygen species seen with persistent neutrophilia is extremely damaging to the airways and can cause irreversible destruction (27, 28). In diseases like cystic fibrosis, chronic obstructive pulmonary disease, and bronchiolitis obliterans syndrome there is massive remodeling of the airways, increased collagen turnover and an impairment of lung function. In all such diseases a decline in lung function has been shown to correlate with the level of neutrophil influx, neutrophil chemokines, and neutrophil derived proteases present in clinical samples from diseased patients.

Our recent work elucidating a specific collagen fragment (PGP) that is chemotactic for neutrophils has opened the door to a new target for therapeutics in several chronic lung diseases. It is clear that merely targeting IL-8 will not go far enough in ameliorating the pathologies of chronic neutrophilia. Effective therapeutics will have to address both IL-8 and PGP driven neutrophilic inflammation to be able to make any difference in the progression of disease. Our work detailed here describes a novel concept in which a single molecule is capable of blocking not only IL-8, the classic PMN chemokine, but the production of, and receptor for a more recently described PMN chemoattractant, PGP.
These data detail that a commercially available substrate for prolyl endopeptidase is specifically chemotactic for neutrophils both in vitro and in vivo. Using a well established assay for neutrophil migration we show a dose dependant curve for Z-Gly-Pro-pNA induced PMN chemotaxis for both mouse and human neutrophils. When solubilized and administered intratracheally to mice, ZGP-pNA induced neutrophil recruitment into the airways, but did not change macrophage numbers seen in BAL fluid. By utilizing neutralizing antibodies to CXCR1 and 2 in the human, or CXCR2 in the mouse we determined that the PE substrate was acting via the ELR+CXC chemokine receptors. Taken together, these results indicated that there was perhaps some structural similarity between the active site of prolyl endopeptidase and a binding pocket of CXCR1 and/or 2. Obviously, if it were possible to use an inhibitor of PE as a receptor antagonist then it would be an extremely novel therapeutic aimed at the genesis and terminus of a potent pathway of neutrophilic inflammation.

We repeated a dose curve of ZPP’s inhibition of PE activity and confirmed that it has an IC$_{50}$ in the sub-nanomolar range. Following this we showed that ZPP is effective in preventing the generation of PGP from collagen. Our group has previously demonstrated that neutrophils alone are sufficient to generate PGP from intact collagen. Using neutrophil lysates with and without ZPP, here we showed that blocking PE’s action, the final step in pathway of PGP generation, completely neutralizes the ability of neutrophils to produce the chemotactic peptide from the extra-cellular matrix component. These findings suggest that a PE inhibitor has the potential to be a potent therapeutic in blocking a feed forward mechanism of collagen breakdown and neutrophil influx.
Based on the previous data showing a PE substrate is specifically chemotactic for neutrophils we investigated the potential of ZPP as an inhibitor of CXCR1 and 2 driven neutrophil migration. ZPP demonstrated a dose dependant inhibition of both mouse and human neutrophil chemotaxis to a variety of ELR+CXC chemokines, but not to fMLP which does not act via CXCR1 and 2. To confirm that ZPP was indeed acting on the receptors themselves we performed a competition assay with $^{125}$I-IL-8. In our initial experiments we were seeing an inhibition of binding of only approximately 20%. This was obviously disconcerting when taken into account with the near total inhibition of chemotaxis seen in earlier work. We used neutralizing antibodies to each receptor individually to attempt to tease out if ZPP was more effective at blocking one receptor than another. Similar to work previously reported, we observed a 44% knockdown of radiolabeled IL-8 binding when using anti-CXCR1 antibody and 16% decrease when using anti-CXCR2 (21). When ZPP was included in these assays we saw no significant change in binding when using anti-CXCR1 but when anti-CXCR2 was used in concert with ZPP we observed an inhibition of binding like that of anti-CXCR1 antibody alone. ZPP is a small molecule, only 330 MW, and there are most likely several sites that it is capable of interacting with on the exterior of a neutrophil, when some of these sites are taken away, by using a neutralizing antibody to CXCR2, more ZPP is focused at the site it appears more potent at, CXCR1. We interpret these data to indicate that ZPP inhibits IL-8 binding to its receptors at approximately the same level seen using a neutralizing antibody to that of CXCR1. To the best of our knowledge, this is the first time that a specific inhibitor of a protease has been shown to be a receptor antagonist as well.
There are questions raised by this study, in that we see a near 100% inhibition of chemotaxis with ZPP but less blockade of binding of radio-labeled IL-8. This is not the only case where this has been seen however, in previous studies using neutralizing antibodies to CXCR1 and 2 investigators showed a complete knockdown of chemotaxis with much less than 100% inhibition of IL-8 binding (21). It may be that although IL-8 is binding, the receptor is not being activated. Although the receptor blocking affinities are not of optimal magnitude, particularly considering the concentrations required for enzymatic inhibition, ZPP could nonetheless serve as a lead compound to be built upon in the design of future therapeutics.

In conclusion, we have demonstrated a possible relationship between the active site of the serine protease prolyl endopeptidase and a binding pocket of a chemokine receptor. Also, for the first time we introduce the possibility of a single compound capable of blocking the generation of a neutrophil chemokine and its site of action. In fact it may be possible to expand this concept into other systems, for instance; designing a single compound capable of blocking both angiotensin converting enzyme (ACE) and the angiotensin receptor itself as an intervention for hypertension. The ability of a single compound to function as a dual protease inhibitor/receptor antagonist represents a novel element in drug design.
References


GENERAL DISCUSSION

Summary

The manuscripts contained in this dissertation deal with the biology of neutrophilic inflammation and a possible approach to preventing its associated damage. This body of work details the importance of the role of neutrophils, their products, and potential therapeutics in chronic pulmonary inflammation. The initial manuscript delves into the arena of neutrophil proteases; their mechanisms of regulation, release, and action. This book chapter describes current opinions in PMN protease biology and efforts to alter their courses of action. Since the writing of the chapter the experiments detailed in this dissertation have expanded and enhanced several of the concepts put forth in this review. The second paper included in this work describes a specific serine protease, prolyl endopeptidase (PE), for the first time in neutrophils. With the inclusion of PE, this confirms that neutrophils have all of the necessary enzymes to effectively hydrolyze intact collagen and produce the PMN chemokine, PGP. Indeed, when PMNs were stimulated with LPS and incubated with collagen they produced easily quantifiable amounts of the tripeptide. These experiments introduce the novel idea of a possible feed-forward mechanism of dysregulated neutrophilic inflammation where PMNs become responsible for inciting their own recruitment through the production of PGP. Although our group has previously proposed the idea of PGP as a biomarker of disease, the extent to which PGP played a role in the pathophysiological development of a clinical condition was unknown. The third manuscript examines this question in chronic lung transplant
rejection, or bronchiolitis obliterans syndrome (BOS). PGP and the proteases responsible for its production are elevated at the time of diagnosis of BOS compared to both non-rejection patients and patient-matched controls collected three months prior. Not only this, but it appears that in the three month period leading up to diagnosis there is a shift in the chemokine profile from primarily IL-8 driven neutrophil recruitment to a predominantly PGP mediated response in BAL fluids from BOS patients. The availability of these samples allowed our group to examine the biomarker signature of IL-8 and PGP during the natural course of a human disorder. The fourth and final paper included herein investigates blocking both the PGP and IL-8 pathways of neutrophilic inflammation with a single compound, ZPP (Fig 1). We demonstrate that there appears to be a similarity between the catalytic site of PE and a binding pocket of CXCR1 and/or 2. This dissertation markedly expands the knowledge base of PGP mediated PMN inflammation but there are still questions which are looming. Our group has previously described the more potent form of PGP, N-α-PGP in cystic fibrosis and COPD but the mechanism of acetylation is unknown. Additionally, we hypothesize that PMN dyregulation can lead to a feed forward mechanism of inflammation but it is unclear as to whether PGP can lead to the release of protease from neutrophils to cause further collagen breakdown. The following is a brief description of some preliminary data dealing with these questions and indicative of the likely course of this research in the future.
Figure 1. ZPP blocks PE activity and CXC receptor activity.

ZPP functions to prevent the production of PGP by prolyl endopeptidase in addition to negating the effects of IL-8 on neutrophil activation and chemotaxis.
Cigarette Smoke and Its Components Acetylate PGP

Due to the increased amounts of N-α-PGP and chronic neutrophilia observed in COPD, a disease closely associated with cigarette smoking and particulate exposure (73), we investigated the possible role that cigarette smoke was playing in the course of the condition. There are over 4000 compounds that have been described in cigarette smoke, with many reactive oxygen species, toxins, etc. present (74). With this large number of chemically active compounds, it is possible that some of them may be able to perform a chemical acetylation of PGP, creating the more potent N-α-PGP.

To examine this possibility we obtained 3R4F research cigarettes from the University of Kentucky and prepared both cigarette smoke extract (CSE) and cigarette smoke condensate (CSC). CSE is prepared by bubbling cigarette smoke at a rate of 10ml/puff, three times a minute through PBS and sterile filtering it to remove and particulate matter. CSC represents a more lipid soluble form of smoke extract, it is prepared in the same manner except using dimethylsulfoxide (DMSO) as the solvent. PGP, at 100ng/ml in either PBS or DMSO was incubated with CSE or CSC at varying concentrations at 37°C for multiple time points.

We observed that both CSE and CSC caused a dose, and time-dependant chemical acetylation of PGP (Fig. 2a, 2b, 2c). CSC was the more potent form of smoke extract, capable of six-fold higher creation of N-α-PGP than CSE. Additionally, we noted that when CSC was merely placed in the same plate as PGP, not necessarily co-incubated in the same well at 37°C for 24 hours, we found the generation of N-α-PGP. This was prevented when a sealant was placed over the wells to prevent CSC vapor from affecting the control wells (Fig. 2d). The observation of a seemingly volatile compound in
cigarette smoke to cause chemical acetylation of a proline led to investigating whether some possible candidates had already been identified in earlier research.
2A. CSE was prepared by bubbling 1 cigarette/ml of PBS at three puffs/minute and 10ml/puff. A 1x concentration of CSE was defined as having an OD$_{320}$ of 1.0. The indicated concentration of CSE was incubated with 100ng/ml of PGP at 37°C for 24h and N-α-PGP levels were measured using ESI-LC-MS/MS. CSE dose-dependently acetylates PGP (* p<0.01).

2B. CSE at a concentration of 10x was incubate with 100ng/ml PGP for the indicated times at 37°C and N-α-PGP was measured as described above. CSE also time-dependently acetylates PGP.

2C. CSC was prepared in a similar manner as CSE except with DMSO as the solvent. CSC was incubated with PGP at 1, 5, and 10x concentrations for 6 and 24 hours. There was a dose and time dependant acetylation of PGP compared to control (* p<0.05 to control, † p<0.05 to 6h).

2D. CSE at a concentration of 10x was placed in separated wells from PGP (100ng/ml) in a 96-well plate for 24 hours at 37°C and N-α-PGP levels were measured. CSE is capable of indirectly acetylating PGP via CSE vapor. PGP Alone is the sealed plate, CSE Vapor is the unsealed plate.
It was extremely interesting to find two volatile components of cigarette smoke, acrolein and acetaldehyde, that not only were likely to be capable of acetylation of a proline, but also incited IL-8 production and MMP-9 transcription from bronchial epithelial cells (75). These compounds could potentially upregulate both the traditional IL-8 and more recently described N-α-PGP pathway of neutrophilic inflammation. By using the same assay as described above, we were able to determine that both acrolein (data not shown) and acetaldehyde (Fig 3) were components in cigarette smoke extract that could chemically acetylate PGP.

While the confirmation of CSE, CSC and the smoke components acrolein and acetaldehyde as modulators of PGP was significant, it was important to confirm that the formation of N-α-PGP had an effect on neutrophil migration. Using the chemotaxis assay described throughout this manuscript we indeed confirmed that PGP, when incubated with CSC, had a greater chemotactic effect on neutrophils than PGP alone (Fig 4).

These experiments indicate that smoking, especially in relation to COPD where PGP has been shown to be a bio-marker of disease, can have a direct effect on a neutrophil chemokine, modulating its structure, and augmenting its function. Taken in context with the known effect of smoking’s upregulation of IL-8, and MMP production, these data paint a picture of cigarette smoking having multi-faceted pro-inflammatory consequences in lung biology. Future directions in this project will focus on identifying the specific components of cigarette smoke inducing acetylation of PGP and potential therapeutic targets directed at this acetylation process.
Figure 3. Acetaldehyde, a component of cigarette smoke, can acetylate PGP.

Increasing concentrations of acetaldehyde were incubated with 100ng/ml PGP at 37°C for 24 hours. N-α-PGP was measured using mass spectrometry. Acetaldehyde, a known component of cigarette smoke dose-dependently acetylates PGP.
Figure 4. CSC augments PGP mediated neutrophil chemotaxis.

2% CSC was preincubated with PGP for X’ at 37°C prior to inclusion in a chemotaxis assay. PGP or PGP+CSC (both at 100μg/ml) were placed in the bottom well of a multi leveled plate at 37°C for 1 hour with 2 x 10^6 PMN layered in the top well, the top plate was removed and cells were counted using flow cytometry. The addition of CSC significantly increased the chemotactic potential of PGP compared to media control (* p<0.001 to media, † p<.05 to PGP alone). CSC alone is no different than media control.
N-α-PGP Induces MMP Release from Isolated Human Neutrophils

As previously mentioned, it is our hypothesis that PGP/ N-α-PGP actively participate in a self sustaining mechanism of PMN pathology. To this end, we researched the effect of N-α-PGP on PMN degranulation and MMP release. Human peripheral blood neutrophils were obtained from healthy volunteers and isolated using gradient centrifugation. The cells were stimulated for various time points with N-α-PGP and the supernatants were removed and assayed for MMP activity via both zymography and a commercially available activity kit. N-α-PGP caused a time and dose-dependant increase in the MMP activity seen in supernatants collected from neutrophils in both gel zymograms and the activity assay (Fig 5). This was recapitulated in quiescent mouse neutrophils obtained from bone marrow as described in the 4th paper (data not shown). This initially confirms our hypothesis that PGP can cause neutrophils to produce the very enzymes that are necessary for PGP release from intact collagen. Future directions of this project will involve determining the discrete intracellular mechanisms involved in MMP-9 release from PMNs and investigating if PE may also be released in this ligand-dependant manner.

Prolyl Endopeptidase and CXC Receptors as Potential Therapeutic Targets in Chronic Neutrophilic Conditions

The data described above, along with the four papers in this dissertation delineate a central role that a system of neutrophilic inflammation involving prolyl endopeptidase, its product PGP, and CXC receptors play in certain inflammatory conditions. Additionally, in an extremely novel finding, it appears that there is structural similarity
between the catalytic site of prolyl endopeptidase and a binding pocket of CXCR1, and possibly CXCR2. This is particularly important in the development of targeted therapeutics for chronic neutrophilic inflammation in pulmonary disease. As suggested in the fourth paper, it may be possible to identify a single compound with effects at both the genesis and terminus of the PGP pathway, in addition to blunting the effect of IL-8. Shutting off the pathway of PGP production, either by PE or MMP inhibition, or negating the action of PGP and IL-8 on CXC receptors could severely mitigate the chronic neutrophilia associated with conditions such as COPD, CF, BOS; and may have potential impact in heavily neutrophilic conditions outside of the lung such as rheumatoid arthritis, ischemia-reperfusion injury, and aortic aneurysms.

Due to the perceived role of PE in neurological diseases such as Alzheimer’s disease and depression there have been recent advances in designing clinically useful inhibitors of PE (28). A PE inhibitor similar in structure to ZPP, S 17092, is currently in Phase 2 clinical trials. The endpoint measurement being PE activity in plasma and electroencephalograph (EEG) readouts with some preliminary observations indicating an improvement in behavioral function (65). Currently no trials have been performed investigating PGP production or the effect of S 17092 on inflammation. However, considering that S 17092 appears to be well tolerated such trials would not be difficult to undertake. Although ZPP will most likely not be a treatment for chronic PMN inflammation it may prove extremely useful in serving as an archetypical compound for the design of dual therapeutics aimed at both PE and CXC receptors.
Figure 5. Increased MMP-9 activity in culture supernatants from human neutrophils stimulated with N-α-PGP

Human neutrophils isolated from peripheral blood were stimulated with N-α-PGP and IL-8 for different times and then supernatants were collected for MMP-9 assay.

5A. The detection of gelatinolytic activity by gelatin zymography

5B. The detection of gelatinolytic activity by gelatin zymography after human neutrophils were stimulated with Ac-PGP at different dosages for 30 minutes.

5C. The quantification of specific MMP-9 activity by ELISA-based assay. The results showed that N-α-PGP, similar to IL-8, increases gelatinolytic activity in supernatants of neutrophils at three different stimulating times. The MMP-9 activity is elevated in both IL-8 and N-α-PGP mediated neutrophil stimulation. The histograms are the mean values of three experiments. (* p<0.05, compared with PMN only within same time point)

5D. The quantification of specific MMP-9 activity by ELISA. The results showed that N-α-PGP dose-dependently increased gelatinolytic activity in supernatants of neutrophils. The MMP-9 activity is elevated in both IL-8 and N-α-PGP mediated neutrophil stimulation. This is also confirmed by MMP-9-specific ELISA-based activity assays. The pictures and histogram represent a typical zymograph of two experiments.
General References


APPENDIX
NOTICE OF APPROVAL

DATE: June 4, 2009

TO: Blalock, J. Edwin
   MCLM-896 0005
   934-6439

FROM: Judith A. Kapp
   Ph.D., Chair
   Institutional Animal Care and Use Committee

SUBJECT: Title: Therapeutics for Chronic Lung Diseases: New Antagonists of PGP
   Chemokines and CXCR
   Sponsor: NIH
   Animal Project Number: 090608783

On June 4, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>240</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from June 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090608783 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.
Federal regulations require IRB approval before implementing proposed changes.
Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator’s Brochure, questionnaires, surveys, advertisements, etc.).
Complete this form and attach the changed research documents.

Today’s Date: 04.06.09

1. Contact Information
Principal Investigator’s Name: Anit Gaggar, MD PhD BlazerID: agagger@uab.edu
Contact Person’s Name: Matthew Hardison BlazerID: hardison E-mail: hardison@uab.edu
Telephone: 615-429-0491 Fax: 205-994-1444
Campus Address: MCLM 893

2. Protocol Identification
Protocol Title: The Role of Matrix Metalloproteases in Acute and Chronic Rejection Transplantation
IRB Protocol Number: X051014005
Current Status of Project (check only one):
✓ Currently in Progress (Number of participants entered: 90)
☐ Study has not yet begun (No participants entered)
☐ Closed to participant enrollment (remains active)—
  Number of participants on therapy/intervention:
  Number of participants in long-term follow-up only:
  Total number of participants enrolled:

This submission changes the status of this study in the following manner (check all that apply):
☐ Protocol Revision
☐ Protocol Amendment
☐ Study Closed to participant entry
☐ Study Closure
☐ Other, (specify) ______

3. Reason for change
Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted.
✓ Matthew Hardison is currently listed as a co-investigator on the protocol. We would like to make an addendum to add him as a Co-PI for the purposes of his doctoral dissertation in the department of Molecular Physiology and Biophysics. The current working title is “A Dual Protease Inhibitor/CXCR1 and 2 Antagonist with Therapeutics Implications for Neutrophilic Airway Disease.”

4. Does this change revise or add a genetic or storage of samples component?
☐ Yes ☑ No
If yes, please see the Guidebook to assist you in revising or preparing your submission, or call the IRB office at 934-3789.

5. Does the change affect subject participation (e.g., procedures, risks, costs, location of services, etc.)?
☐ Yes ☑ No
If yes, Fiscal Approval Process (FAP)-designated units complete a FAP submission and send to fap@uab.edu. For more on the UAB FAP, see www.uab.edu/ohr.

6. Does the change affect the consent document(s)?
☐ Yes ☑ No
If yes, briefly discuss the changes.
Include the revised consent document with the changes highlighted.
Will any participants need to be reconsented as a result of the changes?
If yes, when will participants be reconsented?

Signature of Principal Investigator

Date 4/4/09

APPROVED

Marilyn Doss, M.A.
Vice Chair - IRB

4/9/09
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Mar 29, 2010
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Philip J. O'Reilly, Matthew T. Hardison, Patricia L. Jackson, Xin Xu, Robert J. Snelgrove, Amit Gaggar, F. Shawn Galin, J. Edwin Blalock
March 3, 2010

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Fax: 205-934-1446  
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