CHARACTERIZATION OF PROTEINASE ACTIVATION PEPTIDES AND THEIR POTENTIAL AS DIAGNOSTIC MARKERS OF DISEASE

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ABSTRACT

Prostate cancer is the second leading cause of cancer death in men. While prostate specific antigen (PSA) is currently the best biomarker available, its use has many limitations. This study investigates the biosynthesis, secretion and activation of PSA. PSA is secreted as a pro-enzyme containing a seven amino acid activation peptide (APLILSR). **Because APLILSR is removed extracellularly *in vivo*, the hypothesis was tested that it may be detected in the blood or urine.** Our data indicates that APLILSR is filtered from the bloodstream by the kidney, and is detectable in the urine of patients with prostate cancer, but not controls.

Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease of unknown etiology. Matrix metalloproteinases (MMPs) are a family of proteinases that regulate extracellular matrix turnover and are believed to contribute to IPF. **For this reason, the hypothesis that levels of MMP activation peptides will increase in patients with IPF was tested.** To test these hypotheses, urine from mice were collected and an ELISA was used to quantify MMP activation peptides. These experiments show that the activation peptides of MMP-2, MMP-7, MMP-8 and MMP-9 are increased in mice with pulmonary fibrosis compared to control mice. The data also showed that that the activation peptides of MMP-2, MMP-7, and MMP-9 are increased in the urine of human patients with IPF compared to healthy controls.

These data suggest that urine detection of MMP activation peptides is feasible and correlates with disease.

Because urinary detection of the activation peptides of proteinases are indicative of proteinase activation *in vivo*, the new hypothesis that the accurate measurement of proteinase activation peptides will be relevant clinically arises, and that such measurements may aid in the diagnosis of disease and serve as a marker for following disease progression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α1-ACT	alpha-1-antichymotrypsin
α2M	alpha-2-macroglobulin
Asb	asbestos
BALF	bronhoalveolar lavage fluid
Bleo	bleomycin
BMP	bone morphogenetic protein
BPH	benign prostatic hypertrophy
CaP	prostate cancer
CART	classification and regression tree
COPD	chronic obstructive pulmonary disease
CTGF	connective tissue growth factor
DL_{CO}	carbon monoxide diffusing capacity
ECM	extracellular matric
ELISA	enzyme-linked immunoassay
FVC	forced vital capacity
GSH	intracellular glutathione
HP	hypersensitivity pneumonia
HRCT	high-resolution computed tomography
IFN-γ	interferon-γ
IPF	idiopathic Pulmonary Fibrosis
LMMP	lysosome associated matrix metalloproteinase
MMP	matrix metalloproteinase
NAC	N-aceytlcysteine
NC	normal control
NE	Neutrophil elastase
NSIP	nonspecific interstitial pneumonia
PSA	prostate specific antigen
ROC	receiver operating characteristic
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamind gel electrophoresis
Sal	saline
TIMP	tissue inhibitor of metalloproteinases
TGF-ß	transforming growth factor-ß
TNF-α	tumor necrosis factor-α
WT	wild type

1. INTRODUCTION

1.1 PROTEASES

Proteases are enzymes that catalyze the hydrolytic breakdown of proteins into smaller peptide fractions and amino acids. This process is known as proteolysis. Proteases are essential for the synthesis of many proteins. They control the composition, size, shape and fate of proteins in an extremely selective manner by cleaving peptides at very specific sequences of amino acids under a preferred set of environmental conditions. This highly specific and limited substrate cleavage is termed proteolytic processing. Proteinases are a subclass of proteases that cleave proteins whereas peptidases are able to cleave smaller peptides.

1.1.1. Proteinase Biology

There are over 500 known proteinases which account for 2% of the human genome(1). A majority of human proteinases are secreted as inactive zymogens that require a biochemical change for activation. Activation of secreted proteinases usually occurs extracellularly where part of the precursor enzyme is cleaved in order to activate it. The amino-acid chain that is released upon activation is called the activation peptide.

On the basis of the mechanism of catalysis, proteinases are classified into six distinct classes: aspartic, metallo, glutamic, cysteine, serine and threonine proteinases. Proteinases in the first three classes use an activated water molecule as a nucleophile to attack the peptide bond of the substrate. In the remaining classes the nucleophile is a catalytic amino-acid residue (Cys, Ser or Thr, respectively) that is located in the active site (2).

1.1.2. Proteinase Activation

Most proteinases are synthesized as larger zymogens. Upon activation, the zymogen is cleaved and an inhibitory peptide is removed. In some cases, activation involves dissociation of an inhibitory protein. Activation may occur after a proteinase is delivered to a particular compartment within a cell or in the extracellular space. Proteinases can be activated by other proteinases sometimes of the same type. This is an important method of regulating proteinase activity.

1.1.2.1.Serine Proteinase Catalysis

Serine proteinases exist as two families, the 'trypsin-like' and the 'subtilisin-like' that have independently evolved a similar catalytic device (3). The catalytic mechanism of serine proteinases is based on the function of the catalytic triad. The catalytic triad is located in the active site of the enzyme, where catalysis occurs, and is preserved in all serine proteinase enzymes (4). The triad consists of three essential amino acids: a histidine (His 57), a serine (Ser 195) and an aspartic acid (Asp 102) (FIGURE 1). These amino acids are critical for the functional activation of these proteinases.

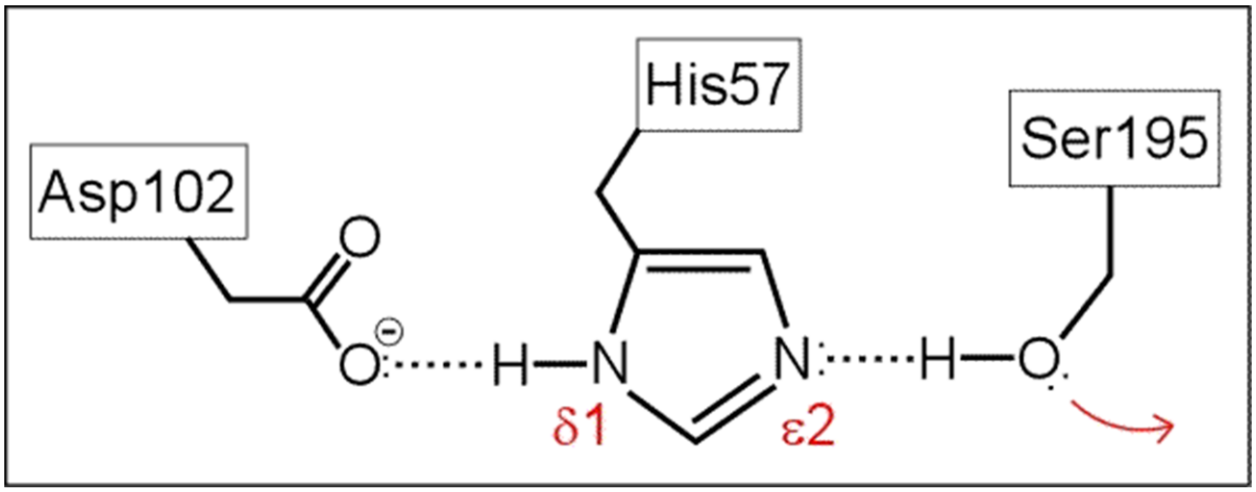

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Figure 1. The catalytic triad of a serine proteinase is made up of three amino acids that enable enzyme activity. Note: the arrow shows the electrons that attack the carbonyl carbon of the peptide bond.

During serine proteinase catalysis several intermediates are generated. The catalysis of the peptide cleavage is a non-linear reaction where the polypeptide being cleaved binds as a substrate, the N-terminus activation peptide is released, water binds, and then the C-terminus active protein is released (FIGURE 2). The active site of serine proteinases is shaped as a cleft where the polypeptide substrate binds (5).

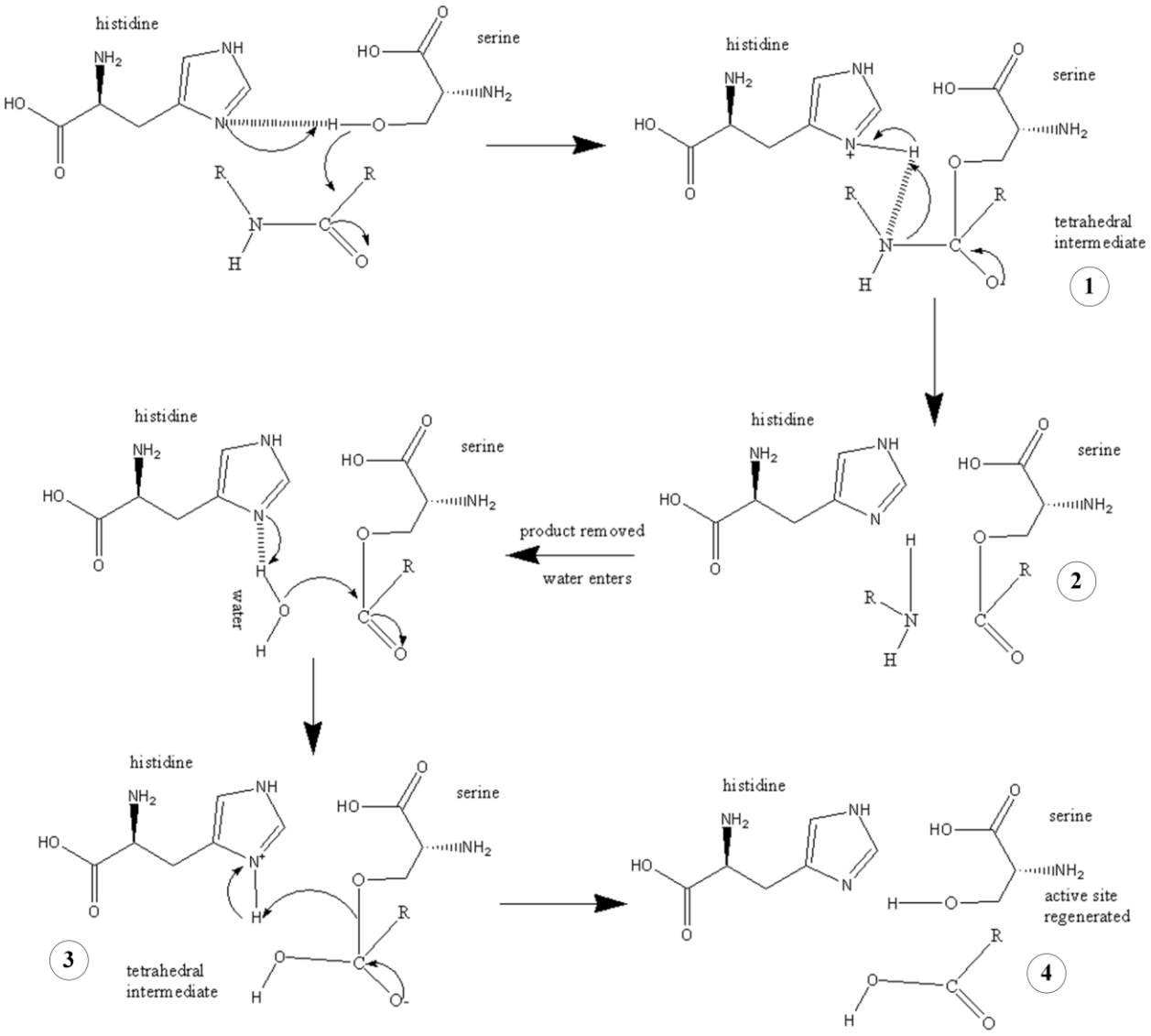

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Figure 2. Catalysis of serine proteinases involves several intermediate steps mediated by each member of the catalytic triad. First, an –OH group on the serine acts as a nucleophile to attack the carbonyl carbon of a substrate's peptide bond where the hydrogen is accepted by the nitrogen on the histidine. From this a tetrahedral intermediate is formed (1). The covalent electrons that were involved in the nitrogen – carbon bond of the peptide are now free to attack and break the hydrogen bond to the histidine forming an acyl-enzyme intermediate (2). Once the product is removed, water replaces the N-terminus of the cleaved peptide facilitating an interaction between the water, carbon, and nitrogen from the histidine producing another tetrahedral intermediate (3). Lastly, the serine – carbonyl carbon bond attacks the histidine hydrogen from the new intermediate leaving the peptide carbon free to re-form a double bond with its oxygen resulting in the freeing of the C-terminus of the peptide (4).

1.1.2.2.Metalloproteinase Catalysis

Metalloproteinases, or zinc proteinases, are proteolytic enzymes whose catalytic mechanism involves a metal. Most metalloproteinases are zinc-dependent. However, some use cobalt. The metal ion is directed to the protein via three histadine imidazole ligands. A labile water molecule takes up a fourth position. During catalysis, the Zn^{2+} promotes a nucleophilic attack on the carbonyl carbon by the oxygen atom of a water molecule at the active site. An active site base facilitates this reaction by extracting a proton from the attacking water molecule (FIGURE 3). Significantly, oxidants are able to activate MMPs. They are also able to inactivate them by oxidizing the active cysteine residue. Metalloproteinases include the digestive enzymes carboxypeptidases, matrix metalloproteinases (MMPs), and a proteinase that is associated with the membrane of lysosomes (LMMP).

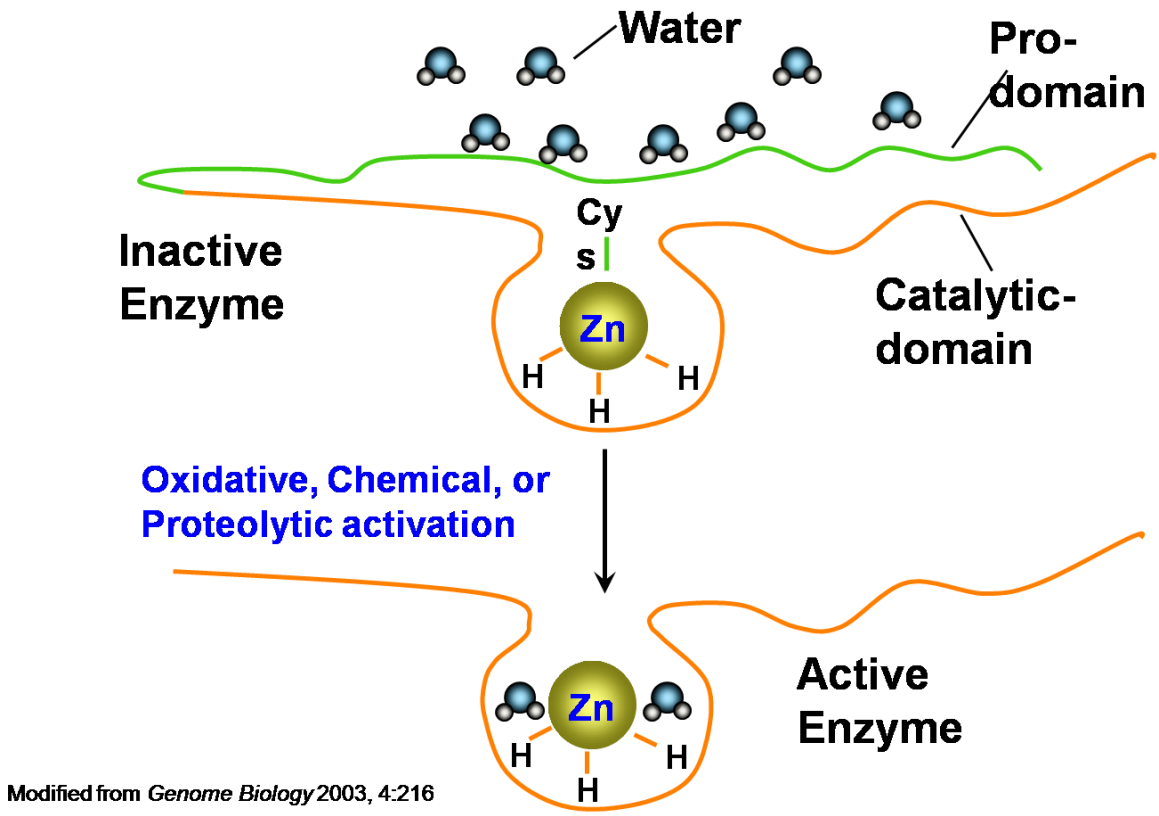

Figure 3. The conserved cysteine at the carboxyl terminus of the prodomain holds a thiol group that functions as a fourth ligand for the catalytic zinc in the active site. This binding keeps water out and the enzyme inactive. When the prodomain is removed via proteolysis the cysteine-zinc interaction is broken and water replaces the thiol group at the zinc atom allowing the protein to perform its enzymatic activity.

1.1.3. Proteinase Inhibition

Most proteinase inhibitors are proteins with domains that enter or block a proteinase active site to prevent substrate access. Tissue inhibitor of metalloproteinases (TIMPs) are inhibitors of metalloproteinases that are secreted by cells. A domain of the inhibitor protein interacts with the catalytic Zn^{2+}. Serpins are widely distributed proteins that utilize a unique suicide mechanism to inhibit serine or cysteine proteinases. To inhibit proteolysis, a large

conformational change in the serpin accompanies cleavage of its substrate loop. This leads to disruption of the proteinase active site, preventing completion of the reaction. The serpin remains covalently linked to the proteinase as an acyl-enzyme intermediate (FIGURE 2.2). Serpins are widely distributed within and outside of cells, and can have different roles, including regulation of thrombosis, fibrinolysis, and inhibition of apoptosis (6-8).

Alpha-2-macroglobulin (α_2M) is able to bind to and inactivate a variety of proteinases including serine-, cysteine-, aspartic-, and metalloproteinases. α_2M has a 35 amino-acid bait region that enables its binding to proteinases. When the bait region is cleaved, α_2M undergoes a conformational change trapping the proteinase inside. The entrapped proteinase remains active but steric hindrance prevents it from cleaving large proteins. It is possible, however, for very small substrates to gain access and be cleaved by an α_2M-bound proteinase (9). Following the conformational change, the proteinase- α_2M complex is recognized by macrophage receptors and rapidly cleared from the system (10).

1.1.4. Proteinases in Disease

While proteinases are known to play pivotal regulatory roles in human processes such as conception, digestion, maturation and even death they are also thought to contribute to the formation and regulation of a number of human diseases. In over 50 human proteinases, it has been found that a single amino acid mutation is sufficient to cause a genetic disease. A mutation in the trypsin-like proteinase matriptase has been shown to be sufficient to cause iron-refractory iron deficiency anemia in humans (11). Interestingly, matriptase is also the first membrane bound proteinase known to act as an oncogene (12). The metalloproteinase ADAMTS-13 (also known as von Willebrand factor-cleaving proteinase) is responsible for cleaving the large blood

clotting protein von Willebrand factor. Alterations in this proteinase can lead to thrombotic thrombocytopenic purpura (TTP), another form of familial anemia (13, 14). Mutations in other members of the ADAMTS family can lead to a variety of other disorders including arthritis and other joint diseases (15, 16).

Additionally, other genetic or environmental conditions that alter the levels of proteinases such as mutations in their inhibitors have also been shown to contribute to disease initiation or progression. Diseases caused by serpin disregulation are so well documented they are termed "serpinopathies." Mutations of a serpin can cause misfolded or aggregated proteins leading to many different diseases. Familial emphysema is caused by a deficiency in α1-antitrypsin (17). This type of disease is caused by uncontrolled proteinase activity and tissue destruction brought about by the lack of active serpin function. Thrombosis can also occur with a deficiency of the serpin anti-thrombin (18). Moreover, liver cirrhosis and other diseases marked by cell death and tissue damage can occur when protein aggregates form due to the absence of efficient protein clearance clogging up the endoplasmic reticulum of cell (17, 19).

Proteinases are critical in cancer development. Proteolysis is a central cofactor for neoplastic progression resulting from a cascading effect of proteinase activation (8). These include proteinases such as cysteine and serine proteinases, which converge leading to amplification of metalloproteinase proteolytic activities. Total tumor proteolysis then contributes to tumor progression by mediating tissue remodeling, inflammation, angiogenesis, and acquisition of invasive capabilities, cell survival, and proliferation (8). Proteinases have also been shown to be potential diagnostic and/or prognostic indicators of disease. The kellikrin

family of proteinases, of which prostate specific antigen (PSA) is a member, has long been used as a diagnostic marker for prostate cancer.

Proteinases have been implicated in numerous other diseases including bacterial and viral infections, stroke, coronary artery disease, cardiovascular and neurodegenerative disorders including Alzheimer's disease, and many inflammatory and respiratory conditions. Additionally, studies show that MMP activation contributes to the pathogenesis of pulmonary diseases such as idiopathic pulmonary fibrosis and asbestosis (20-23)(see below).

1.2. PROSTATE CANCER

Prostate cancer (CaP) is the most common cancer in men and has the second highest cancer-related death rate in the United States, surpassed only by lung cancer (24). CaP is classified as an adenocarcinoma and most often develops in the peripheral zone of the prostate. A specific cause of CaP has not been found but the most significant risk factor associated with the disease is age (25). Other risk factors are genetics, race, diet, lifestyle and medications. Some genetic factors found to be related to the onset of CaP include alterations in the AR (26), BRCA1, and BRCA2 genes (27). Currently, the most useful screening tool for CaP is serum PSA measurement (28, 29). Despite the extensive use of this method, 30% of men with CaP have locally advanced or metastatic disease at the time of diagnosis. These men are substantially less likely to be cured than men diagnosed with localized disease. The use of serum PSA testing has many limitations. There is a very high false positive rate associated with this test. Approximately 70% of men with “abnormal” PSA levels (above 4 ng/ml) do not have CaP. In addition, PSA testing has a significant false negative rate. More than 20% of men with normal PSA values

(between 2.5 and 4 ng/ml) have CaP (30-32). The complex biology of PSA makes assessments of stage and prognosis difficult for individual CaP patients. Inaccuracies in predicting pathologic stage and the biology of prostate cancer often result in over treatment of some men and under treatment of others. A better understanding of PSA biosynthesis, regulation, and clearance will enhance efforts to develop a more sensitive and specific test for prostate cancer.

1.3. PROSTATE SPECIFIC ANTIGEN (PSA)

PSA is a 33 kDa serine proteinase which is similar in structure to the trypsin-like tissue killikreins but has substrate specificity similar to that of chymotrypsin (33). PSA is produced by the epithelial cells of the prostate (34) and is one of the most abundant proteins in the seminal fluid. The active enzyme is likely involved in dissolution of the gel forming proteins semenogelin I and II in the semen.

The cDNA sequence encoding PSA predicts an N-terminal 7 amino acid activation peptide (APLILSR) (35), although it has not been detected in purified PSA (36-39). Analogous to other serine proteinases, the activation involves a conformational change initiated by proteolysis of the Arg_7Ile_8 peptide bond (FIGURE 4). Activation of serine proteinases is usually tightly regulated. Most serine proteinases are secreted as inactive precursors and activated extracellularly. However, some serine proteinases are processed intracellularly and secreted in their active form (40). It has recently been determined that PSA is in fact activated extracellularly (41, 42). This finding allows for the possible detection of the PSA activation peptide in biological samples.

PSA is detected in the plasma in three distinct forms (i) free-PSA; (ii) PSA-α_1-antichymotrypsin complexes (PSA-α_1ACT) and (iii) PSA-α_2-macroglobulin complexes (PSA-α2M) (43-45). Of these three major serum forms, only free-PSA and PSA-α_1ACT are immunodetectable by current commercial assays. Native PSA-α2M complexes are not recognized by PSA antiserum by ELISA because of the unique nature of the complex (46, 47). However, PSA-α2M can be detected following denaturation of the complex. The plasma half-life of α_1ACT- and α2M- proteinase complexes are short because they are rapidly removed by hepatocyte receptors (48-51). The plasma clearance of these complexes is independent of the proteinases involved. The α2M complexes are cleared from the circulation by the low density lipoprotein receptor (52, 53). Serpin complexes are recognized by two serpin receptors: SR2, which recognizes and eliminates proteinases-α_2-antiplasmin complexes, and SR1 which recognizes complexes between proteinases and α1-proteinase inhibitor, anti-thrombin III, heparin cofactor II, or α_1-ACT (50, 51). These receptors usually maintain undetectable levels of proteinase-inhibitor complexes in the blood. The level of PSA-α_1-ACT during malignant disease may rise to several hundred ng/ml. It is therefore likely that pathological PSA levels result from saturation of the clearance mechanisms. It follows that the PSA concentration depends both on how much PSA gains access to the blood stream and how efficiently it is removed. However, to date, the impact of clearance mechanisms has not been well studied. This clearance rate is likely to depend upon the overall health of the patient, including physical condition, body weight, and alcohol and tobacco consumption. These factors may account for some of the serum PSA variability between individuals.

Current use of PSA testing is directed toward detecting the three major PSA forms in the blood (free-PSA, PSA-α_1-ACT, and PSA-α2M) as well as complexes of PSA with other serine proteinase inhibitors including Inter-α-inhibitor and α_1-proteinase inhibitor (51). The use of PSA as a screening or diagnostic test for the presence of CaP, however, has several limitations. PSA is known to interact with other proteins in the blood. These interactions affect the half-life and interfere or prevent detection (54, 55). In 2003, Mistry et al. performed a meta-analysis to determine the sensitivity and specificity of PSA and digital rectal examination as screening tests for CaP (56). When pooling the results of 13 articles, they found the sensitivity of PSA to be 72.1% and the specificity to be 93.2%. These are the highest rates determined thus far for PSA however these results did include physical examination. When determining serum PSA alone as a test, they found the positive predictive value to be only 25.1%.

1.4. IDIOPATHIC PULMONARY FIBROSIS (IPF)

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown etiology. IPF is a progressive life threatening disease that is characterized as excessive deposition of fibrotic tissue in the interstitium with some, typically chronic, associated inflammation (57). The incidence of IPF is at least 29/100,000 in the general population and the median survival rate for individuals diagnosed with IPF is around 3 years (58). There is currently no cure or significant treatment for this disease (59) and no effective method to monitor progression in IPF patients. Currently, due to the lack of available biomarkers, the diagnosis of IPF is made by lung biopsy or via radiographic evidence of disease. Historically, IPF has been seen as a gradually progressive disease. Some patients with IPF, however, experience a rapid

deterioration of lung function and accelerated death. These episodes have been termed acute exacerbations of IPF.

IPF is characterized by diffuse interstitial fibrosis with variable levels of inflammation, honeycomb changes, fibroblastic foci and a pattern of usual interstitial pneumonia with clinical features including diffuse interstitial infiltrates on chest radiographs, honeycombing seen on high-resolution CT (HRCT) scans and a restrictive impairment with reduced gas exchange on pulmonary function test results (60). Thickening of the alveolar septa disrupts efficient gas exchange in the lung and is thought to be initiated by endogenous or environmental stimuli. Risk factors for the disease are age, gender, smoking (61), genetic factors, and infection (62). The two main symptoms of IPF are shortness of breath and cough. Additional symptoms may include weight loss, chest pain or tightening, and/or fatigue. IPF typically occurs after the age of 50 and slightly more often in men than in women.

Treatment options for IPF are minimal. The most commonly used therapy is corticosteroids. Corticosteroids have many side effects, however, and have not proven to be very beneficial. Lung transplantation has the most data suggesting a survival benefit (57, 63-65). Pharmacological agents that are designed to treat lung scarring may be promising in the treatment of IPF but these are still in the experimental phase (66). Likewise, treatments intended to suppress inflammation have had only limited success in reducing the fibrotic progress of IPF (67-71). Because of the limited treatment options available, there are more than 25 national clinical trials underway to either determine genetic variations of the disease in hopes of finding

therapeutic targets, or to test the efficacy of new therapies for the disease or a process of the disease (66). There are currently no FDA approved drugs for the treatment of IPF.

1.4.1. Animal Models of Pulmonary Fibrosis

Human IPF maintains a few pertinent features of disease, including temporal heterogeneity of the fibrotic lesions, progressive nature of the disease, and the development of myofibroblastic foci (57, 60, 72). Experimentally, there are a number of *in vivo* models used to study pulmonary fibrosis. Two models that have been used extensively are the bleomycin and asbestos models. Unfortunately, there is no single animal model that recapitulates all of the hallmark features of the human disease.

1.4.1.1. Bleomycin Model

The use of bleomycin as an inducer of murine pulmonary fibrosis is the most well characterized of the animal models (73). A single intratracheal dose, typically 1.25–4 U/kg, is sufficient to cause pulmonary fibrosis in 14 days (20). While bleomycin induced pulmonary fibrosis has clinical similarities to human IPF it creates a temporally homogeneous fibrotic lung which is discordant with human manifestations (74). Additionally, it has been shown that injury can begin to resolve after 28 days which varies from the progressive nature of human disease (73, 75, 76).

1.4.1.2. Asbestos Model

Intratracheal instillation or inhalation of asbestos is also an established model of pulmonary fibrosis in mice. Crocidolite, amosite, and chrysotile asbestos have all been shown to initiate fibrosis (77-79). The type of asbestos used may, however, be a factor in the development of fibrosis as it has been shown that the longer the fiber, the more prominent the fibrosis (77, 78). Animals subjected to asbestos show clinical characteristics of pulmonary fibrosis in just 7 days. An advantage of the asbestos model over the bleomycin model is that asbestos induces temporal heterogeneity of fibrosis as seen in human IPF and the fibrosis does not resolve with time (80).

1.5. MATRIX METALLOPROTEINASES IN IPF

Matrix metalloproteinases (MMPs) are a family of at least 25 proteinases that regulate extracellular matrix turnover. Several studies have shown that MMP activation contributes to the pathogenesis of pulmonary fibrosis in animal models (22, 81, 82). Also, there are many studies that have found increased MMP expression and activation in human IPF lungs, blood and bronchoalveolar lavage fluids (21, 82-85).

Most MMPs including MMP-2, MMP-7, MMP-8, and MMP-9 are secreted as pro-enzymes with a signal sequence and pro peptide of about 80 amino acids that gets cleaved extracellularly upon activation (Table 1). All members of the MMP family share a common catalytic core with a Zn^{2+} in its active site. The pro peptide domain contains a sequence (PRCGxPD) termed the cysteine switch which contains a conserved cysteine that is involved in chelating the active Zn^{2+} site. Full MMP activation is brought about by disruption of the cysteine-zinc interaction and removal of the pro peptide (86). Proteolysis of the prodomain can be brought about by furin (87),

plasmin (88), and other metalloproteinases (89, 90). Modification of the cysteine switch by other means such as reactive oxygen and nitrogen species can also activate MMPs (91-93).

Activated MMPs react rapidly with proteinase inhibitors including tissue inhibitor of metalloproteinases (TIMPS) and α_2-macroglobulin in the blood. These protein complexes are removed from the circulatory system by receptor-mediated endocytosis (94). While MMPs are easily detected in the blood, this rapid clearance likely interferes with the measurement of the active proteins in the urine or bloodstream resulting in the inability to accurately detect the total amount of MMP activation occurring simply by measuring the steady state amount in biological fluids.

Table 1. Partial list of matrix metalloproteinases involved in pulmonary fibrosis and their pro peptide weights

Enzyme	Family Name	Molecular Weight (Pro peptide)
MMP-2	Gelatinase	8934.42
MMP-7	Matrilysin	8822.25
MMP-8	Collagenase	9210.61
MMP-9	Gelatinase	8339.62

1.6. CONCLUSIONS

Prostate cancer and idiopathic pulmonary fibrosis are just two of the many diseases that have been shown to be associated with the activation of proteinases. This wide field of research is currently limited by the inability to noninvasively and quantitatively detect the steady state amounts of associated proteinases in the body. It is likely that methods to measure proteinase

concentrations are confounded by the rapid clearance of proteinase-inhibitor complexes in the serum. For this reason, this study explores an alternative method for the detection of proteinase activity *in vivo*. Results presented in this dissertation show that the activation peptides of proteinases, once cleaved, are cleared through simple renal filtration and detectable in the urine in a disease specific manor. This method of detection may prove to be not only clinically relevant for the diagnosis of disease but may also significantly contribute to the scientific research of proteinase activation.

2. RATIONAL AND HYPOTHESIS

There are a multitude of diseases that are influenced by the activation of proteinases, some of which do not have adequate biomarkers available for diagnostic or prognostic use. For those that do, such as PSA for the detection of prostate cancer, serum protein levels as a measurement of disease is likely limited by complex clearance mechanisms as discussed above. For this reason investigating the utility of measuring just the activation peptide of proteins in the urine may prove to be a valuable addition to the currently limited options available to clinicians faced with diagnosing or treating these diseases.

The activation peptide concentration should be representative of total proteinase activation, therefore, we hypothesize that detection of the activation peptides of proteinases will give a more accurate representation of protein activation due to their simple renal clearance as opposed to the complex clearance mechanisms of the full length active protein.

Although PSA activation is a well-studied indicator of prostate cancer, the hypothesis tested here is that pathological PSA levels are a result of saturated clearance mechanisms and that PSA concentration depends both on how much PSA gains access to the blood stream and how efficiently it is removed. Therefore, detection of the activation peptide of PSA should prove to be a more sensitive marker of disease than serum detection of the active PSA protein. In a similar situation, it is known that MMP activation contributes to idiopathic pulmonary fibrosis initiation and progression. For this reason, we hypothesize that the accurate detection of activation of these proteinases will also be relevant clinically and may allow for earlier detection and/or following progression of disease.

3. MATERIALS AND METHODS

3.1. REAGENTS

ECL Western blotting detection reagents were from Amersham (Arlington Heights, IL). RPMI Medium 1640, RPMI 1640 select amine kit, Dulbecco's phosphate buffered saline, Earls' balanced salt solution, penicillin-streptomycin from Gibco (Grand Island, NY). Epidermal growth factor, L-glutamine was from Sigma (St. Louis, MO). PSA antiserum was produced by DAKO Corporation (Carpinteria, CA). Human metastatic prostate adenocarcinoma (LNCaP) cells were obtained from American Type Culture Collection (Rockville, MD). Radiochemicals were from DuPont/NEN (Boston, MA). α_1-ACT was purified as previously described (95). Urine samples for the detection of PSA activation peptide were collected from Duke University Urology Clinic. Human prostatic tissues were obtained from Duke University Medical Center for purification of PSA. Pathologists confirmed histology of each tissue independently.

All MMP antibodies were commercially available. A rabbit polyclonal to the MMP-8 propeptide domain was from Abcam (Cambridge, MA). A mouse monoclonal antibody to the N-terminus of MMP-2 (APSPIIKFPGD-VAPKTDK) was obtained from Thermo Scientific (Fremont, CA). A rabbit monoclonal antibody to the propeptide domain of MMP-9 was from Novus Biologicals (Littleton, CO). A mouse monoclonal antibody to the propeptide domain of MMP-7 (EYSLFPNSPKWTSKV) was purchased from R&D Sysytems (Minneapolis, MN). Propeptides corresponding to the N-terminus of each MMP were synthetically made by Genscript (MMP-2, 7, 9)(Piscataway, NJ) or Abcam (MMP-8)(Cambridge, MA). All antibodies cross react with human and mouse. For ELISA assays, Costar 96-well RIA/EIA plates (Costar, Cambridge, MA) were used.

3.2. ANIMAL STUDIES

3.2.1. Plasma Clearance Experiments

^{125}I-APLILSR (0.25 μg), ^{125}I-α_1ACT (2.0 μg), or a 1:1 molar basis of PSA-^{125}I-α_1ACT (2 μg and 0.97 μg respectively) was injected intravenously into the lateral tail vein of 50-60 day old CD-1 mice. Identical volumes of blood were collected by heparanized capillary tubes from the retro-orbital venous plexus. Radioactivity was measured in a γ-radiation counter and expressed as percentage radioactivity present compared to the first sample, drawn approximately 5 seconds after injection.

3.2.2. Pulmonary Fibrosis Inducing Treatment

Asbestos and bleomycin treatments were approved individually by the University of Pittsburgh Institutional Animal Care and Use Committee. Male C57BL/6 mice 8-10 wks. old (Taconic, Germantown, NY) were anesthetized with brief exposure to isoflurane (Abbot Laboratories, Chicago, IL) and then treated with a single 0.1-mg dose of crocidolite asbestos, 0.05 units of bleomycin, or 0.9% saline by intratracheal instillation as previously described (96). Mice were euthanized with an injection of 5 mg of pentobarbital (Sigma) at day 35. Lungs were inflation fixed with 10% buffered formalin, incubated overnight in 70% ethanol, and embedded in paraffin by the University of Pittsburgh's Research Histology Services.

3.2.3. Urine Collection

Mice were individually placed on a 12 inch x 12 inch square of PARAFILM®(Pechiney Plastic Packaging, Menasha, WI) an allowed to urinate. Immediately after urination, the mouse was removed and the voided urine was aspirated using a Nichipet EX (Nichiryo, Maryland Heights, MO) and transferred into a sterile micro-centrifuge tube and stored on ice until all urines were collected. Urine volumes ranged from 10 µl-300 µl per mouse. Samples were taken in the morning on day 0 prior to treatment and at approximately the same time on days 1-7, 13, 14, 17, 20, 23, 26, 29, 32, and 35 following treatments. Samples were frozen at –20°C for storage. Prior to analysis, the samples were thawed, and creatinine concentrations were determined using a commercial kit (R&D Systems Minneapolis, MN) according to manufacturer's instructions. Urine was tested for presence of blood and leukocytes using Multistix 9 Urinalysis Strips (Bayer) and samples containing blood or leukocytes were excluded. Urine samples were centrifuged using Microcon Centrifugal Filter Devices Ultracel YM-10

(Millipore Corporation, Billerica, MA). Retentate was discarded and the flow through (<10 KDa) was used for analysis.

3.3. HUMAN STUDIES

3.3.1. Sample Collection

Urine samples for the detection of PSA activation peptide were collected from Duke University Urology Clinic. Human prostatic tissues were obtained from Duke University Medical Center for purification of PSA. Pathologists confirmed histology of each tissue independently.

Sixty nine urine samples were analyzed in the IPF study, including samples from patients diagnosed with IPF (n = 39) and controls (n = 30). IPF groups and controls were comparable with respect to age both in terms of the mean (64, 59) and the range (40-81, 50-82). IPF urine was collected according to the institutional bioethical guidelines pertaining to clinical material from the University of Pittsburgh Simmons Center for Interstitial Lung Disease under an approved IRB (IRB0610029, University of Pittsburgh). Control urine was purchased from Bioreclamation Inc. (Hicksville, NY). Samples were collected in sterile containers and immediately frozen at –80°C. The samples were thawed, and creatinine concentrations were determined according to manufacturer's instructions (R&D Systems Minneapolis, MN). Urine containing blood or leukocytes as determined by Multistix 9 Urinalysis Strips (Bayer) was excluded. Protein concentration of urine was determined by the Bradford method using bovine serum albumin as the standard. Urine samples were centrifuged using Microcon Centrifugal

Filter Devices Ultracel YM-10 (Millipore Corporation, Billerica, MA). Retentate was discarded and flow through (<10 KDa) was used for analysis.

3.3.2. Pulmonary Function Tests and IPF Diagnosis

Assessments of pulmonary function were performed according to the American Thoracic Society (ATS) guidelines, using standard equipment. The FVC and DL_{CO} were expressed as the percent predicted values. Radiographic IPF diagnosis was made by a chest radiologist and pulmonologist using conventional HRCT scans of the chest without intravenous contrast. Discrepant readings were subjected to a second review by both experts to determine consensus classification. Histopathologic diagnosis was determined by evaluation of lung biopsy that was paraffin embedded, cut, and stained with hematoxylin and eosin according to the standard protocols used in the University of Pittsburgh Department of Surgical Pathology. Each diagnosis was designated by a lung pathologist.

3.4. BIOCHEMICAL AND CELLULAR STUDIES

3.4.1. Purification of PSA

Steps were performed at 4°C. Prostate tissue (100 g) was homogenized (Vitris, Tempest) in 300 ml of 0.05 M Tris-HCl (pH 7.4) containing 0.1 M NaCl and 0.01 M EDTA. The homogenate was filtered through cheesecloth and cleared by centrifugation. The supernatant was digested with 0.1 mg/ml RNAse, 0.2 mg/ml DNAse and 0.005 M MgCl for 4h. Following 4h incubation the supernatant was dialyzed overnight against 0.01 M HEPES, pH 8. The sample was clarified by centrifugation and applied to a Q-Sepharose FF (Pharmacia, Piscataway, NJ)

column (2.5 x 20 cm) equilibrated in 0.01 M HEPES, pH 8. The charged column was washed in equilibration buffer and then developed with a linear gradient (total volume of 2 l) from 0 M NaCl to 0.4M NaCl. Fractions of 4 ml were collected and tested for PSA by western blotting. The active fractions were pooled and concentrated by ultrafiltration (Amicon, Bedford, MA) and applied to a S-200 HR gelfiltration (Pharmacia, Piscataway, NJ) column (2.5 x 150 cm) equilibrated in 50 mM HEPES, 150 mM NaCl. Fractions of 4 ml were collected and assayed for PSA by western blotting. The PSA containing fractions were pooled and dialyzed into 10 mM HEPES, pH 8 and separated on a MONO-Q 5/5 HR (Pharmacia, Piscataway, NJ) connected to a Pharmacia FPLC system. The column was equilibrated in 10 mM HEPES and developed using a linear gradient from 0 M NaCl to 400 mM NaCl.

3.4.2. Preparation of Pro-PSA Antiserum

A peptide, APLILSRC, corresponding to the N-terminal activation peptide of PSA was synthesized (Bio Synthesis, Lewisville, TX), purified by RP-HPLC (RP-300, 4,6 mm column), and analyzed by mass spectrometry. The peptide was coupled to ovalbumin at a molar ratio of 10:1 using *m*-maleimidobenzoyl-*N*-hydoxysuccinimide ester (Pierce, Rockford, IL). Coupling was confirmed by SDS-PAGE. The purified peptide-ovalbumin complex was used for injection into rabbits (1 mg/injection). Antibodies from the final bleed were purified on a Protein G column.

3.4.3. Polyacrylamide Gel Electrophoresis

The supernatants from immunoprecipitates were recovered by centrifugation and separated by SDS-PAGE through 5-15% gradient gels (97). The gels were stained, destained,

dried, and subjected to imaging on a PhosphorImager (Molecular Dynamics 410A). Immunoprecipitates for radiosequence analysis were transferred to Immobilon membranes. Following electrophoresis, the Immobilon membranes were dried and exposed directly to X-ray film overnight at -70 °C.

3.4.4. Metabolic Labeling and Pulse-Chase Analysis

Human metastatic prostate adenocarcinoma (LNCaP) cells were maintained in RPMI Medium 1640 (ATCC, Rockville, MD) supplemented with 10 % fetal bovine serum, epidermal growth factor (5 mg / 500 ml), L-glutamine (150 mg / 500 ml) and 1 % penicillin-streptomycin in 5 % CO_2. For standard biosynthetic radiolabeling, cells were grown in 50 mm tissue culture plates until 80% confluent. The cells were washed twice with Earls' balanced salt solution, and incubated for 30 min in RPMI without fetal bovine serum or the amino acids that would be used for metabolic labeling. Cells were incubated for 5 min with either [^{35}S]Met or [^{35}S]Cys. For radiosequence analysis [^{35}S]Cys was added to the proteins. At the end of the labeling period, cells were rinsed twice with serum free RPMI and chased with "cold" complete medium according to the study.

3.4.5. Lysis and Immunoprecipitation

Conditioned medium was collected and frozen. Cell lysates were prepared by three rapid freeze-thaw cycles in high salt buffer containing 0.5% Triton X-100 and a proteinase inhibitor cocktail (40, 98). Prior to immunoprecipitation, the samples of lysates and conditioned medium were cleared by the addition of a pre-immune serum followed by the addition of protein-G Sepharose 4 FF (Pharmacia, Piscataway, NJ). The supernatants were incubated overnight with

the relevant specific antiserum. The next day protein-G Sepharose 4 FF was added and immunoprecipitates were collected by gentle centrifugation. The immunoprecipitates were then washed several times and bound proteins were released from the protein G Sepharose 4 FF by boiling in SDS sample buffer or by 100 mM glycine-HCl (pH 2.7) before SDS-PAGE.

3.4.6. Radiosequence Analysis

These analyses were performed as previously described (40, 98). Briefly, following immunoprecipitation and SDS-PAGE the [^{35}S] labeled proteins were electrotransferred to Immobilon membranes (99). The proteins were identified by autoradiography and bands of interest were excised and analyzed by automated Edman degradation in an Applied Biosystems 477A sequencer (Foster City, CA). The anilinothiazolinone (ATZ) amino acids released after each cycle were collected and counted for [^{35}S]. In the experiments destined for radiosequence analysis the metabolic labeling were performed using [^{35}S]Cys which is positioned as residue 14 in the PSA zymogen. Subsequent radiosequence analysis of the bands and release of radioactive ATZ-amino acid in the anticipated cycle of Edman degradation provided identification of the protein band.

3.4.7. Radiolabelling

Approximately 100 µg of α_1ACT and of the N-terminal activation peptide (synthesized by NEN^{TM} Life Science Products, Boston, MA) were labeled with ^{125}I. α_1ACT was labeled using Iodo-beads (Pierce, Rockford, IL) and the peptide (APLILSR) was labeled using the Bolton-Hunter method according to manufacturers recommendations. (Pierce, Rockford, IL).

3.4.8. Dot blotting

Membranes were developed using the ECL Western blotting kit from Amersham™ (Piscataway, NJ). Briefly, following transfer to PVDF membranes the membranes were blocked for 1 h in 20 mM Tris-Cl, 137 mM NaCl, pH 7.6 containing 0.1 % Tween (TBS-T buffer) and 5% of the supplied blocking reagents. The membrane was washed in TBS-T buffer before the primary antibody was added (1/2000 dilution). Following 1 h of incubation, the membrane was washed in TBS-T buffer and horseradish peroxidase labeled second antibody was added (1/20,000 dilution). The membranes were incubated for 1 h and washed with TBS-T buffer and developed using the supplied reagent.

3.4.9. Enzyme-linked Immunosorbent Assays (ELISAs)

Costar 96-well RIA/EIA plates (Costar, Cambridge, MA) were incubated overnight at 4 °C with sample to be tested, in a total volume of 50 µl. Wells containing known concentrations of activation peptide were simultaneously analyzed. Coated plates were washed and blocked with PBS 1% BSA, 5% sucrose, 0.05% NaN_3 (blocking buffer) for 2 h at 37 °C. Plates were washed with blocking buffer then incubated with 100 µl of MMP antisera specific to the activation peptide of interest diluted in blocking buffer for 1h at 37 °C. The plates were washed and incubated for 1 h using 100 µl (1/2000 dilution) of horseradish peroxidase coupled antibody (either anti-mouse IgG or anti-rabbit IgG). After washing with blocking buffer and PBS, the substrate *o*-phenylenediamine dihydrochloride (Sigma) was added. Horseradish peroxidase activity was read at 450 nm using a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA).

3.5. STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation. The Wilcoxon rank-sum test was used to identify which of the four markers univariately distinguish IPF samples from controls. Data were analyzed using the R language for statistical computing (http://www.r-project.org/) (100). Classification and regression trees (CART) methodology was used to identify potential combinations of markers that could be used to distinguish IPF from controls. CART was performed using the rpart package for recursive partitioning. Classification performance was assessed using the ROCR package (http://rocr.bioinf.mpi-sb.mpg.de/).

4. PSA AND ITS ACTIVATION PEPTIDE HAVE DIFFERENT CLEARANCE CHARACTERISTICS

4.1. PROSTATE SPECIFIC ANTIGEN IS ACTIVATED EXTRACELLULARLY

The cDNA sequence encoding PSA predicts an N-terminal 7 amino acid activation peptide (35) (FIGURE 4), although this putative activation peptide has not been detected in purified PSA (36-39). PSA is secreted as an inactive pro-enzyme and contains the Ala-Pro-Leu-Ile-Leu-Ser-Arg- N-terminal activation peptide. To confirm that this is in fact the case and that the activation peptide is removed extracellularly after secretion, both intracellular and secreted PSA were characterized.

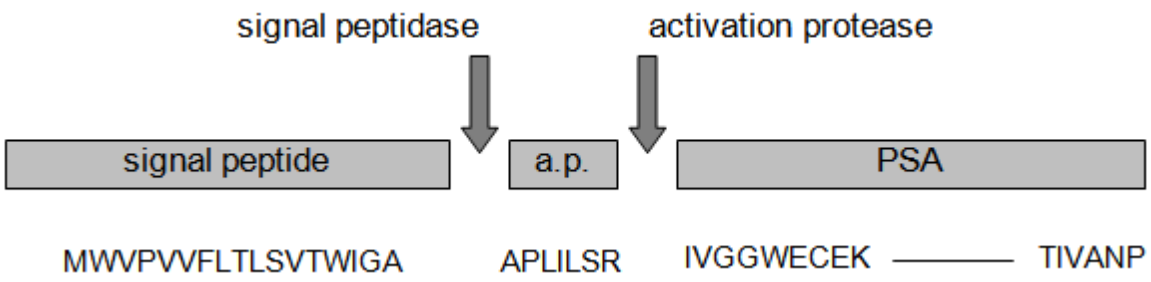

Figure 4. Proteolytic processing of PSA. Schematic diagram of pro-PSA. Pro-PSA is composed of 244 amino acid residues. The signal peptide and the putative activation peptide (a.p.) are indicated. The arrows indicate the positions of the expected proteolytic cleavage sites. The solid bar labeled PSA represents mature, active PSA, with the majority of the sequence abbreviated.

This analysis was performed using a polyclonal PSA antibody (FIGURE 5A) and antisera specific to the activation peptide (FIGURE 5B). PSA was able to be immunoprecipitated from both the cell lysates and medium, demonstrating that PSA was produced and that it was not degraded following secretion (FIGURE 5A). A pulse chase experiment was used to investigate the kinetics of the activation, using antibodies directed specifically against the activation peptide (FIGURE 5B). The analysis of the gels suggested that the pro-PSA was detected intracellularly and, significantly, also in the cell culture medium. The results indicate that PSA does not undergo any N-terminal processing events in this culture system. This was confirmed by radiosequence analysis of both intracellular and secreted PSA (FIGURE 6). The activation of pro-PSA as an extracellular event allows for the detection of the dissociated activation peptide in the extracellular space.

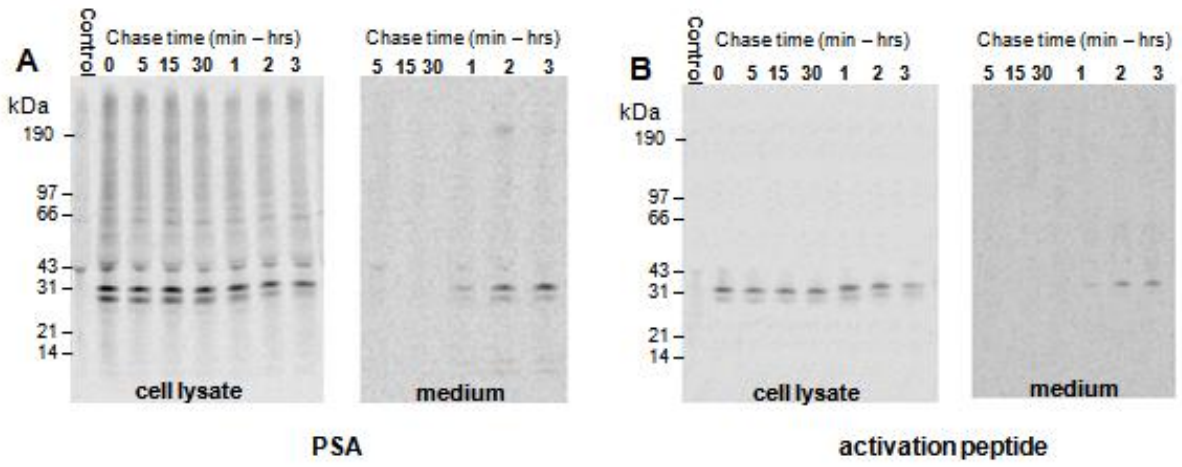

Figure 5. LNCaP cells biosynthetically radiolabeled using a pulse-chase protocol. The cells were chased for the indicated times and the lysates and medium were probed with specific antisera to the whole PSA (top panels) and to the activation peptide (bottom panels). The samples were analyzed by reduced SDS-PAGE. Following electrophoresis the gel was dried and subjected to imaging on a PhosphorImager. It is apparent that the cells produce and secrete pro-PSA as both antisera recognize a band of ~36 kDa. Note the direct correlation in the temporal pattern of production and secretion using antibodies against PSA vs. the activation peptide. There is no evidence of free activation peptide, which only appears on a ~36 kDa band. The doublet sometimes observed reflects carbohydrate heterogeneity.

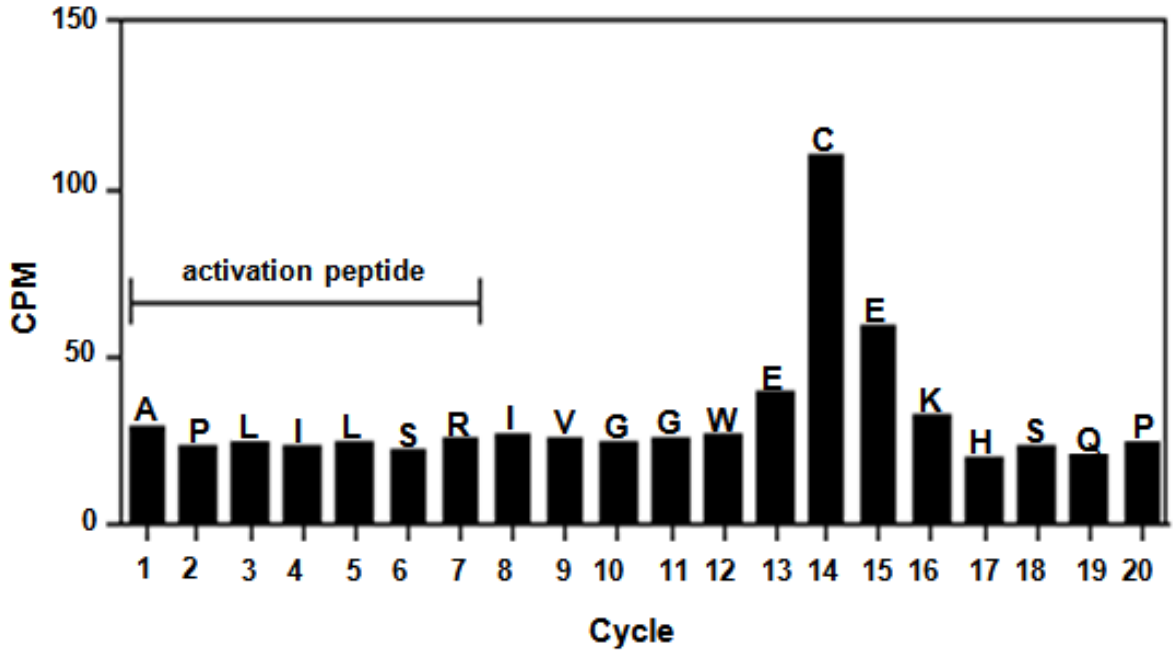

Figure 6. N-terminal radiosequence analysis. Radiosequence analysis of biosynthetically radiolabeled pro-PSA immunoprecipitated from the medium of LNCaP cells. The cells were pulse labeled with [^{35}S]Cys and subjected to immunoprecipitation, electrophoresis, autoradiography and radiosequencing. The vertical bars represent the level of [^{35}S] radioactivity released during each cycle of Edman degradation. The N-terminal protein sequence of pro-PSA is indicated above each vertical bar to facilitate interpretation. The results confirm that the immunoprecipitated band is pro-PSA. Significantly, newly secreted PSA contains the N-terminal activation peptide; whereas, purified PSA from prostate tissue does not. This suggests the activation peptide is removed extracellularly.

4.2. ELIMINATION OF PSA-α_1ACT COMPLEXES

When comparing the clearance rate of ^{125}I-α_1ACT to the clearance rate of PSA-^{125}I-α_1ACT complexes in mice (FIGURE 7), it was seen that the half-life of α_1ACT alone is much longer than that of the protein-inhibitor complex. The half life of α_1ACT alone is several days whereas α_1ACT in complex with PSA has a half-life of around 20 minutes. It is logical to deduce that when PSA secretion increases above a certain point its clearance mechanism becomes saturated. Only following this saturation is when PSA-α_1ACT complexes start to accumulate. This is

evident by our experiment where PSA-^{125}I-α_1ACT complex was injected with a 500-, 1000- and 2000-percent excess of unlabeled "cold" PSA-α_1ACT complexes (FIGURE 7).

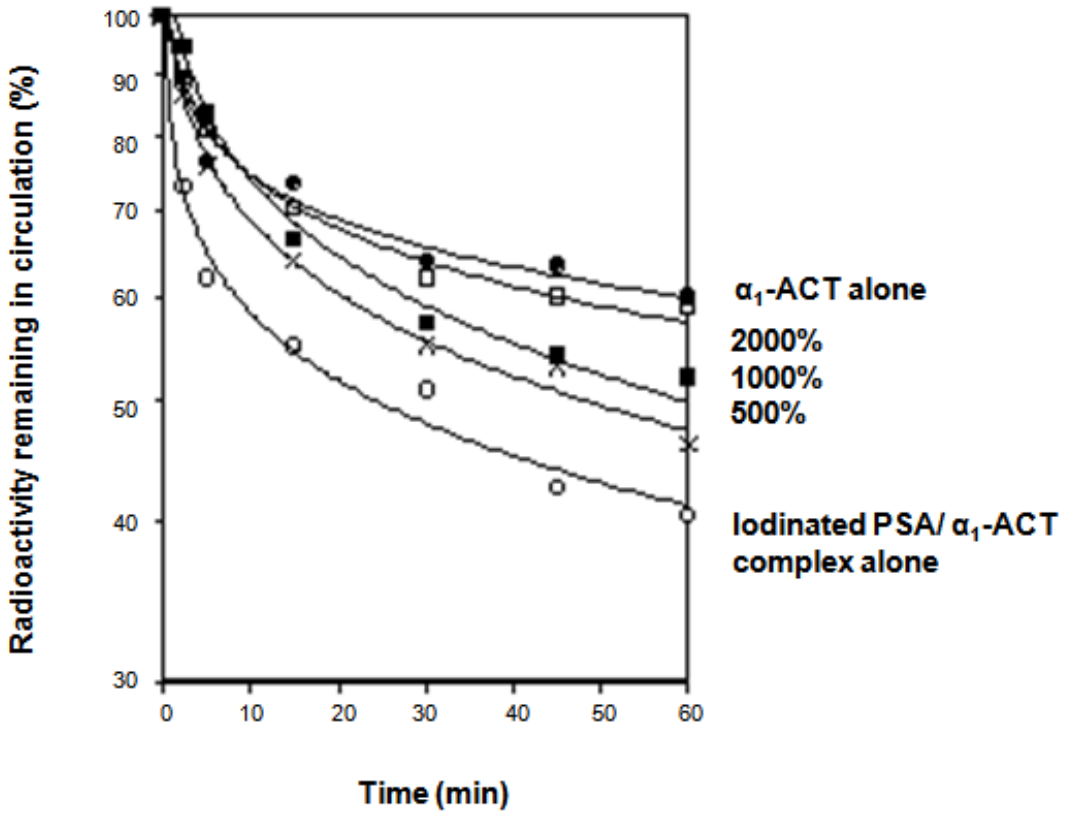

Figure 7. Plasma elimination of PSA- α_1ACT complexes. α_1ACT was injected alone (●) and in complex with active PSA (○). The half-life of the PSA-α_1ACT complex was significantly reduced compared to native α_1ACT. To mimic a situation in which more and more PSA is secreted, PSA-^{125}I-α_1ACT complexes were injected with a 500 (x), 1000 (■), and 2000 (□) percent molar excess of "cold" PSA-α_1ACT complexes. These experiments demonstrate that PSA-α_1ACT complexes are rapidly removed from the blood and do not begin to accumulate until the clearance mechanism is saturated. Thus, a threshold exists below which increased levels of PSA-α_1ACT complexes can not be detected due to rapid clearance.

4.3. PLASMA ELIMINATION OF THE ACTIVATION PEPTIDE OF PSA

In order to determine if the clearance of the activation peptide was also affected by the level of peptide in the blood, plasma elimination of the activation peptide (APLILSR) was studied by injecting ^{125}I labeled activation peptide (APLILSR) into the lateral tail vein of a mouse (FIGURE 8). Plasma elimination of ^{125}I-labeled activation peptide was followed for 1 hour, revealing that the half-life of the peptide in the plasma was less than 2 minutes. The ^{125}I-labeled activation peptide was also injected with a 1000- and 2000-percent excess of unlabelled ("cold") activation peptide. The clearance rate was not significantly affected by the level of activation peptide in the blood stream, suggesting a non-receptor mediated clearance mechanism. Following plasma elimination experiments, the organs of the test mice were examined for radioactivity using a γ-counter. The predominance of radioactivity in bladder and kidney indicates that the peptide is removed from the blood stream by renal filtration (FIGURE 9).

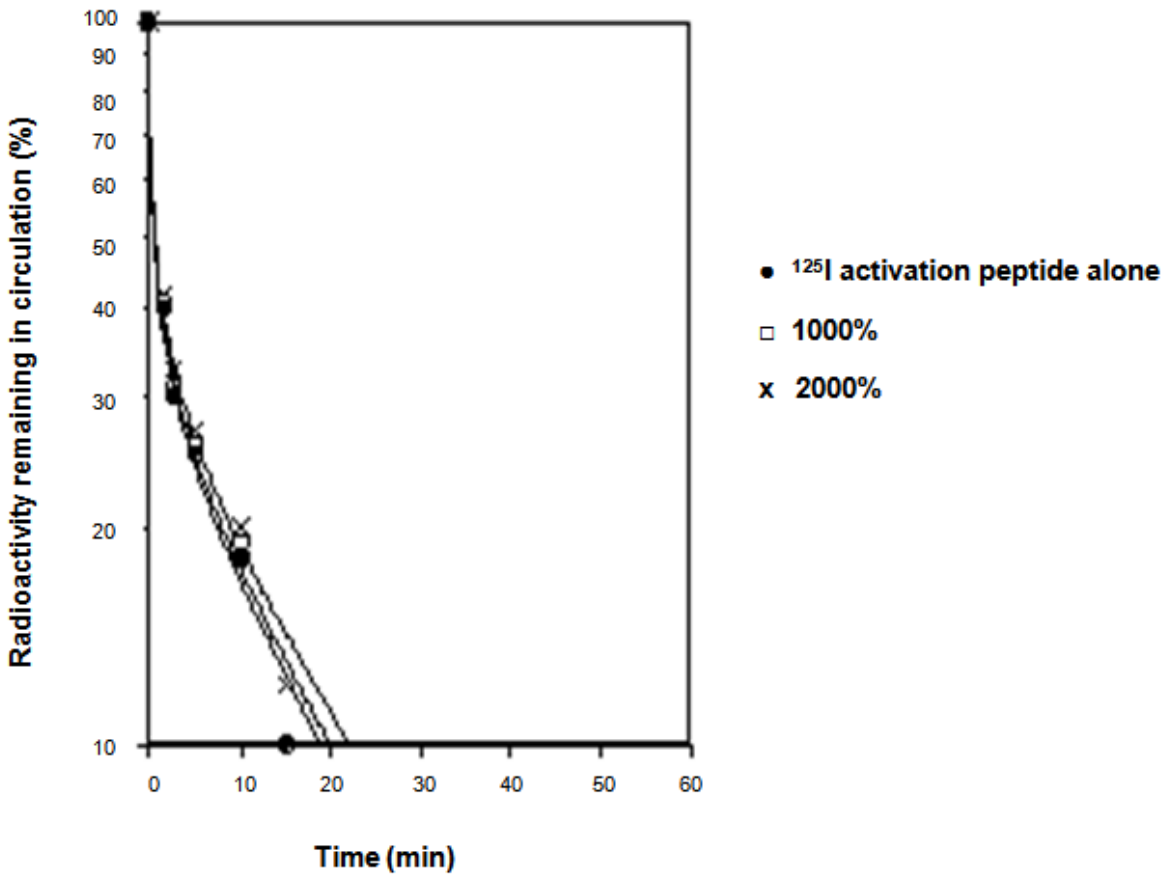

Figure 8. Plasma elimination of activation peptide. ^{125}I labeled activation peptide was injected into the lateral tail vein and the plasma elimination was monitored. The half life of the activation peptide injected alone was less than 2 min (●). ^{125}I labeled peptide was co-injected with a 1000 (□) and 2000 (x) percent excess of cold activation peptide. The half-life of the peptide was not affected significantly by the amount of peptide injected demonstrating that unlike plasma PSA, the clearance of plasma activation peptide appears not to be receptor mediated. Therefore, levels of activation peptide may correlate directly with of amount of pro-PSA produced.

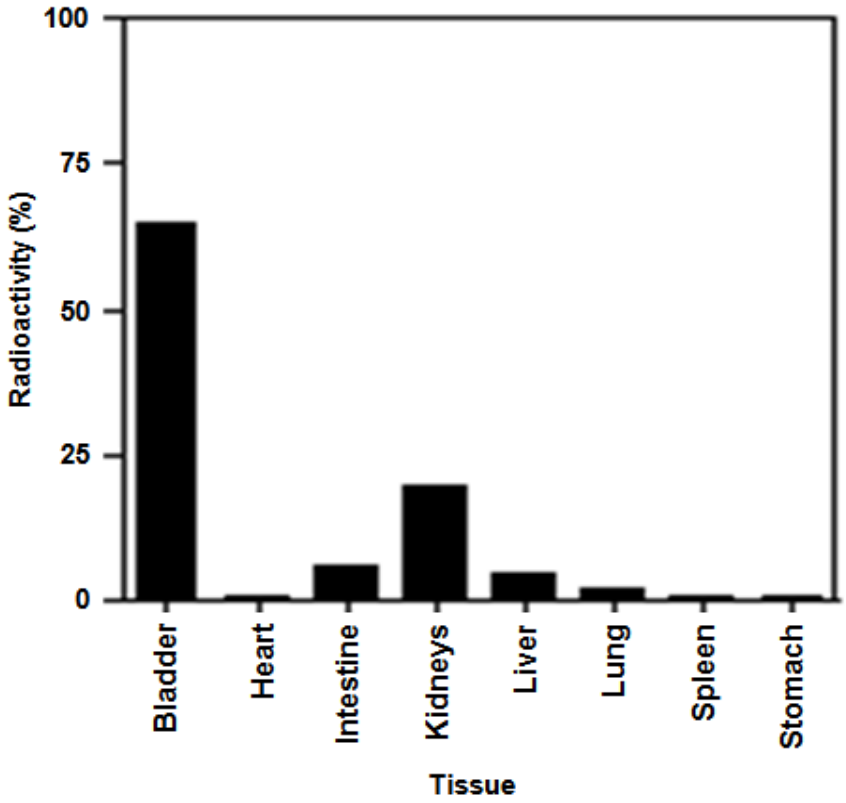

Figure 9. Tissue distribution of the ^{125}I-activation peptide. Following the plasma elimination experiments the mice were perfused and the organs were removed and counted in a γ-counter. The accumulation of the peptide in the bladder and kidneys indicate that the activation peptide is removed from the plasma by renal filtration.

4.4. CONCLUSIONS

The above study indicates that PSA is activated extracellularly and shows that the activation peptide of PSA is available for detection in extracellular fluids. When PSA is in complex with its main inhibitor (PSA-α_1ACT) it is rapidly removed from the circulatory system. Additionally, it is clear that serum PSA concentration is dependent on how much PSA gains access to the bloodstream and how efficiently it is removed by receptor-mediated mechanisms. Demonstrating

that the PSA-α_1ACT complex in a mouse model is rapidly cleared from the plasma suggests that the complex is only detectable in the serum after the clearance mechanisms become saturated. In contrast to PSA itself, the mechanism responsible for the removal of the activation peptide is mainly a passive filtration event in contrast to the active receptor mediated endocytosis required for the removal of the PSA-α_1ACT complex. Moreover, in mice, the plasma clearance of the activation peptide is not influenced by saturation kinetics like serum PSA complex levels.

5. THE ACTIVATION PEPTIDE OF PSA IS DETECTABLE IN BIOLOGICAL SAMPLES

5.1. THE ACTIVATION PEPTIDE OF PSA IS IN THE URINE OF PROSTATE CANCER PATIENTS BUT NOT CONTROLS

Because the activation peptide of PSA is removed extracellularly and is cleared through renal filtration, it follows that it should be detectable in the urine. It is known that PSA is up regulated in prostate cancer but, as we have shown, may only be elevated in the serum after its clearance mechanisms are saturated. Because the activation peptide of PSA is not affected by such clearance issues its presence in the urine should correlate with the presence of prostate cancer. In order to test whether PSA activation peptide is measurable in the urine of patients with prostate cancer, the activation peptide was synthesized and a polyclonal peptide antiserum was prepared in rabbits. The specificity of the antisera was first verified by dot blot against the synthetic activation peptide, which was tested in concentrations ranging from 0.01 - 5.0 ng of peptide (FIGURE 10A; increasing concentration of activation peptide from dot 1 to dot 9). The antisera produced a dose-dependent reaction. Urine samples from seven control subjects with no

evidence of prostate disease and eight patients with prostate cancer were tested for the presence of the activation peptide as discussed above. The activation peptide was detected in the urine (FIGURE 10B) of the cancer patients, but not in the urine (FIGURE 10C) of controls.

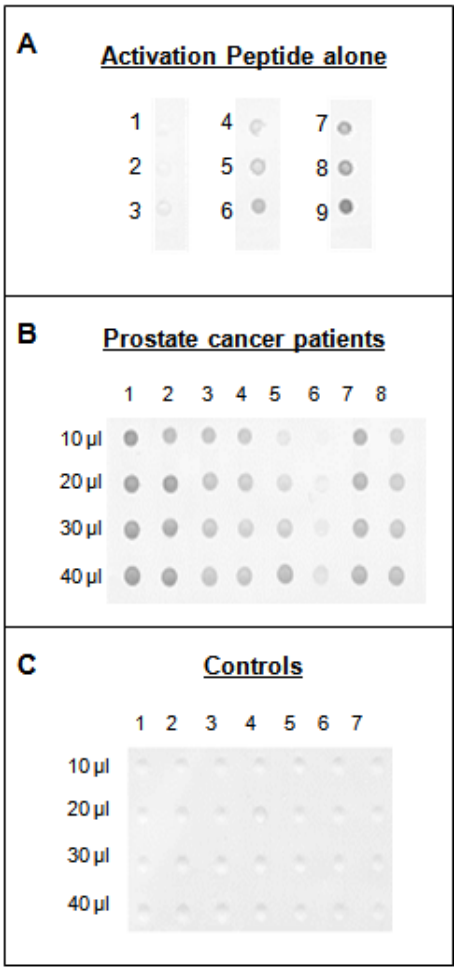

Figure 10. Detection of PSA activation peptide by dot blotting. The reactivity of the activation peptide antisera against synthetic activation peptide added to control urine samples was tested at concentrations ranging from 0.01 - 5 ng of peptide and the antiserum produced a dose dependent sensitive reaction. (A). Following these tests, urine from prostate cancer (B) and control patients (without prostate disease) (C) were analyzed. This shows that urine detection of the PSA activation peptide is feasible.

5.2. CONCLUSIONS

The activation peptide is easy to detect within the urine and represents a noninvasive sampling procedure, amenable to large-scale clinical use. Most significantly, the detection of the activation peptide in urine of subjects correlates with the presence of prostate cancer.

6. URINE MMP ACTIVATION PEPTIDE CONCENTRATIONS CAN BE MEASURED BY AN ENZYME LINKED IMMUNOASSAY

Like PSA, MMPs are proteinases that are implicated in disease. Specifically, MMP-2, MMP-7, MMP-8, and MMP-9 have been shown to be associated with IPF. For this reason, we hypothesized that increased urinary levels of the activation peptides of MMPs may be associated with IPF in a manner similar to the PSA activation peptide in prostate cancer. MMP-2, 7, 8, and 9 map to different chromosome regions of the human genome but are individually conserved across species with 79% (MMP-2), 75% (MMP-7), 80% (MMP-8) and 96% (MMP-9) homology to the respective mouse MMPs. The protein structure of human MMPs includes a pro domain that consists of 80 to 90 amino acids (TABLE 1). Also like PSA, the pro domain or activation peptide is cleaved from the latent protein upon activation and therefore should be detectable in the biological fluids. To quantitate the release of MMP activation peptides into the urine, we used antibodies against the pro domain of each MMP.

6.1. ELISA IS A SENSITIVE WAY TO MEASURE MMP ACTIVATION PEPTIDE CONCENTRATIONS

Standard curves, using serial dilutions of synthetic peptide corresponding to the pro peptide domain of MMP-2, MMP-7, MMP-8, and MMP-9 in phosphate buffered saline (PBS) starting with 10 ng/ml for MMP-2, 7, and 8 activation peptides, and 1ng/ml for MMP-9 activation peptide, determined the sensitivity of the assays to be <1 ng/ml. This means that we are able to effectively detect a concentration less than 1 ng of activation peptide per mL of urine. Sensitivity of these assays was defined as the lowest MMP activation peptide concentration that could be differentiated from zero (assay blank/PBS) by Student's *t*-test. A representative figure for the standard curve of MMP-2 is shown (FIGURE 11).

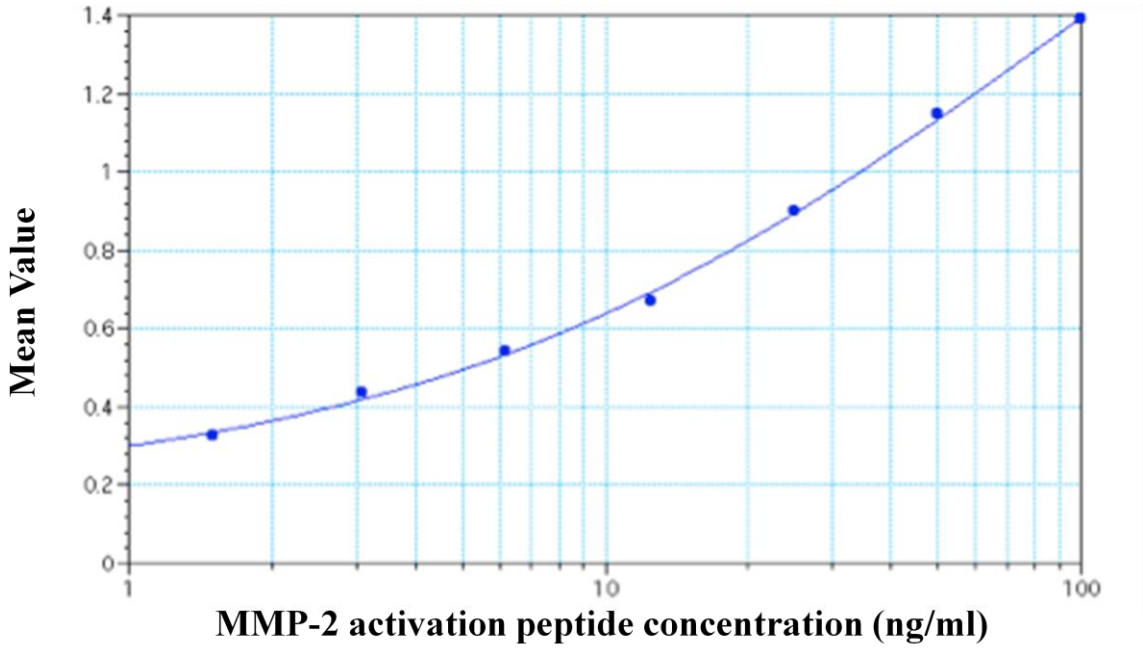

Figure 11: Standard curve for MMP2 activation peptide ELISA showing detection of MMP2 activation peptide to about 1ng/ml (R^2 = 0.999).

4-P Fit: $y = (A - D)/(1 + (x/C)^B) + D$

6.2. ELISA IS A VALID METHOD FOR THE DETECTION OF MMP ACTIVATION PEPTIDE CONCENTRATIONS

The reproducibility of the assay was shown by assessing the precision profile of each MMP activation peptide. Urine samples that were known to have a concentration above the midpoint of the detection range for each MMP activation peptide were used. The inter- and intra-assay coefficient of variances ranged from 5.7% to 7.9% (n = 10) and from 6.3% to 9.9% (n = 2 in 10 different plates), respectively (FIGURE 12A, B).

To determine the dilution linearity, a known positive urine sample containing between 2 and 3.5 ng/ml corresponding to medium concentrations of MMP activation peptide was used at dilutions of 1:5, 1:10, and 1:20. The sample gave results close to linearity (r = 0.95–0.99) (FIGURE 12C) strengthening the validity of each assay.

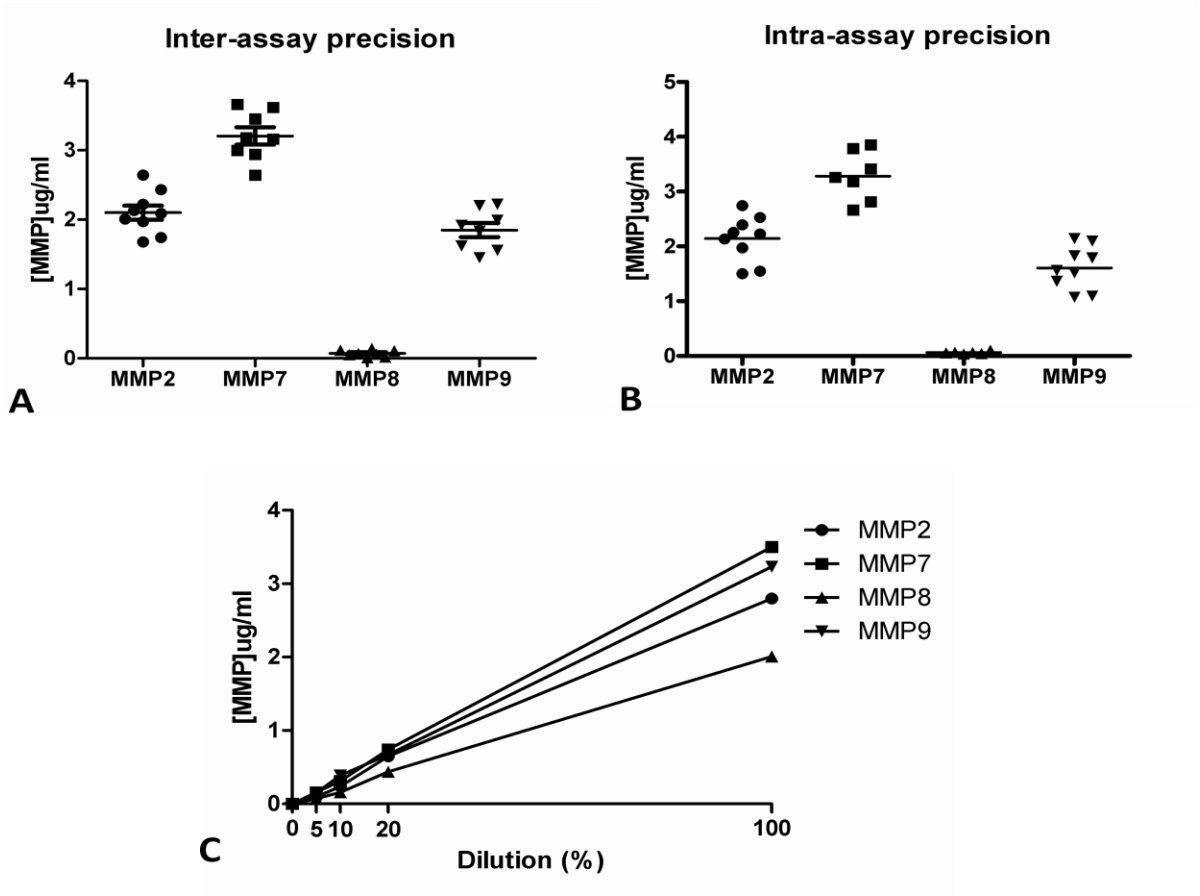

Figure 12. Independent validation of each of the MMP activation peptide ELISAs
Validation profiles of MMP activation peptide ELISAs. When determining precision, each of the assays had a coefficient of variance less than 10% in both the (A) inter-assay test and the (B) intra-assay test. (C) All four MMP activation peptide assays had sufficient dilution linearity.

6.3. CONCLUSIONS

Before any results can be obtained using a new assay, it must first be proven to be a valid way to measure the data. In this case, an ELISA is used to measure the activation peptides of MMPs in hopes of correlating their concentrations to disease state. The validity is determined by assuring that you can measure low enough levels of your peptide to correspond to biological levels in the urine. This is what determines the sensitivity. Additionally you must be sure that each result is reproducible by repeating a measure multiple times and on multiple days. Lastly, the ability to dilute a known positive sample serially and see linear results when assayed adds to the validity of the test. Based on all of these experiments, one can be sure that one is, in fact, accurately measuring the MMP activation peptide concentrations in the urine of patients.

7. INCREASED URINE MMP ACTIVATION PEPTIDE CONCENTRATIONS ARE ASSOCIATED WITH FIBROTIC DISEASE

It has been well established that MMP activity is associated with fibrotic diseases, specifically IPF. There are many ways that MMPs have been implicated in the disease process. For example, the gelatinases (MMP-2 and MMP-9) have known involvement in basement membrane degradation and turnover. The breakdown of the basement membrane is believed to be an early event in pulmonary fibrosis (101) as it may allow for the migration of inflammatory cells and the mobilization of fibroblasts into the lung (102). To confirm that MMPs are relevant in IPF and to determine if the activation peptide levels of MMPs in the urine also correlate with disease state, we measured MMP activation peptide levels in the urine of mice using two models of pulmonary fibrosis.

7.1. ACTIVATION PEPTIDES FROM MMP-2, MMP-7, MMP-8, AND MMP-9 ARE INCREASED IN THE URINE OF MICE FOLLOWING PULMONARY INJURY

To determine if the activation peptides of MMP-2, MMP-7, MMP-8 and MMP-9 are detectable in the urine of mice following pulmonary injury the same ELISA was used for the human samples. For this study, urines were collected from C57BL/6 mice prior to and following asbestos and bleomycin induced pulmonary injury. The mice were treated intratracheally with either 0.1-mg of asbestos, 0.05 units of bleomycin, or 0.9% saline only. Urines were collected at day 0 prior to treatment and then everyday post treatment. The results show that all of the markers assessed are significantly increased in the urine of mice with pulmonary injury compared to the urine of mice treated only with saline. At day 14, when these mice are exhibiting detectable fibrosis (FIGURE 13A), it is clear that the activation peptide levels are significantly increased in the urines of the injured mice compared to controls (FIGURE 13B).

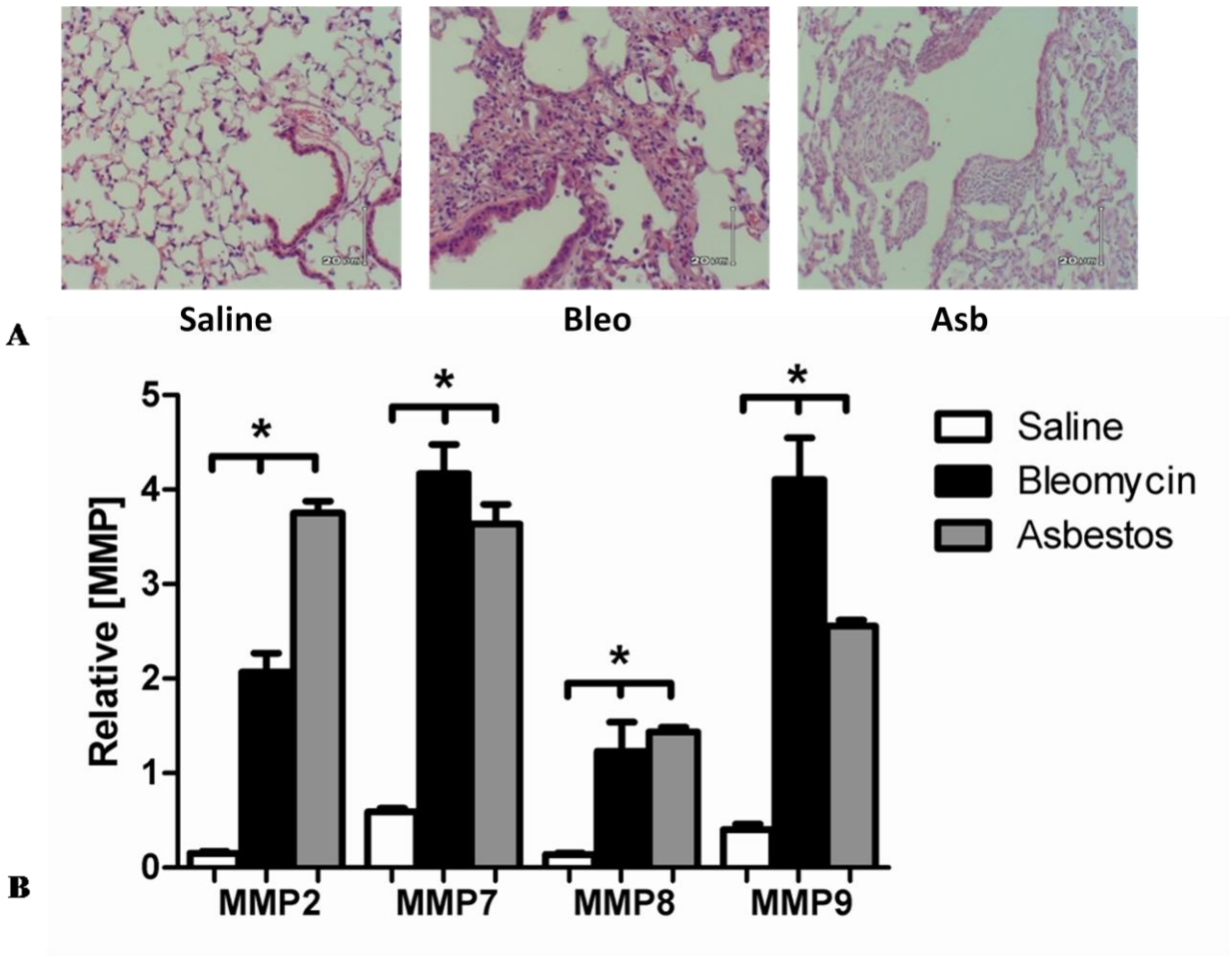

Figure 13. MMP activation peptides are detectable in the urine of mice
14 days following intratracheal instillation, mice treated with bleomycin and asbestos exhibit fibrosis (A). Activation peptides of MMP2, MMP7, MMP8 and MMP9 are detectable in the urine of mice (n=5) via ELISA. All four are significantly increased in the urine of mice treated with bleomycin (black bars) or asbestos (gray bars) compared to the urine of mice treated only with saline (white bars) (*p<.05) (B).

7.2. MMP ACTIVATION PEPTIDE CONCENTRATIONS ARE DIFFERENT BETWEEN IPF PATIENTS AND CONTROLS

7.2.1. Patient Characteristics

Demographic data, urine MMP activation peptide concentrations, urine creatinine levels and pulmonary function test results are summarized in Table 2. IPF patients were diagnosed via lung biopsy or radiographic evidence and normal controls (NC) were healthy age range matched with a similar gender distribution. Pulmonary function tests reveal that there is no significant correlation between urine MMP activation peptide concentrations and forced vital capacity (FVC%) or carbon monoxide diffusing capacity (DL_{CO}%).

Table 2. Patient Characteristics and MMP Activation Peptide Levels

Variable	IPF	NC
Age	65.7 ± 11.4	59.5 ± 8.8
Sex, male/female	29/13	24/6
Creatinine	183.3 ± 74.2	132 ± 75.5
PFT Fvc%	66.74 ± 13.94	-
PFT DlCo%	50.49 ± 17.23	-
[MMP-2 activation peptide]*	2.6 ± 2.0	0.76 ± 0.61
[MMP-7 activation peptide]*	3.6 ± 2.6	0.97 ± 1.0
[MMP-8 activation peptide]*	0.88 ± 1.0	0.6 ±0 .74
[MMP-9 activation peptide]*	3.3 ± 2.4	0.87 ± 1.1

*** Activation peptide concentrations are relative to individual creatinine concentrations (ng/mL)**

7.2.2. MMP-2, MMP-7, and MMP-9 Activation Peptide Relative Concentrations Are Higher in IPF Patients Compared to Controls

To determine whether urine MMP-2, MMP-7, MMP-8, and MMP-9 activation peptide concentrations were higher in IPF patients compared to controls, their levels were measured in 42 samples from patients with IPF and 30 healthy age range matched controls via ELISA. The resulting concentrations are relative to each patient's urine creatinine level. Univariately, the relative urine concentrations of the activation peptides of MMP-2 ($p < .001$), MMP-7 ($p < .001$), and MMP-9 ($p < .001$) are significantly higher in IPF patients compared to controls (FIGURE

14). MMP-8 activation peptide levels were slightly increased in IPF urines but this increase was not significant.

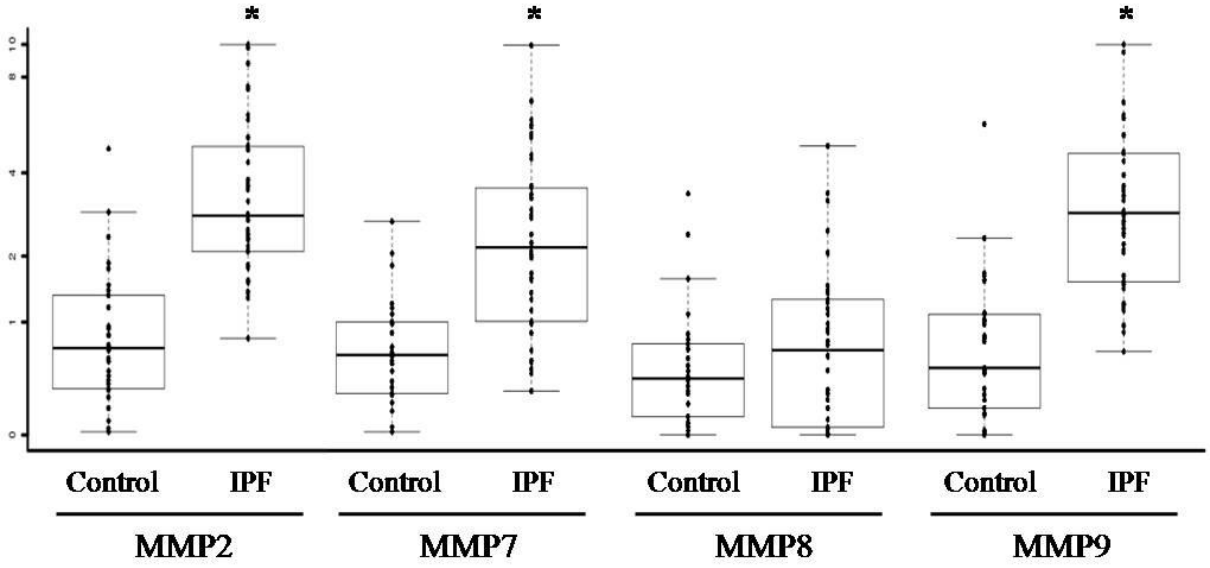

Figure 14. Urine MMP Activation Peptides Distinguish IPF Patients From Controls
The relative concentration of MMP2, MMP7, and MMP9 activation peptides are significantly higher in the urine of IPF patients (n=42) compared to the urine of healthy age matched controls (n=30) as detected by ELISA.

Recursive partitioning (CART) was used to determine whether these four markers in the urine comprise a combinatorial classifier to correctly classify IPF patients from controls. The results suggest that these markers in the urine can be used to distinguish IPF from control with high sensitivity (97.6% CI (0.874, 0.999)) and specificity (96.7% CI (0.828, 0.999)). Low relative concentrations of MMP-7 activation peptide alone (≤1.255) correctly exclude 41 of 42 IPF patients but incorrectly classify one normal sample as IPF and one IPF sample as control,

whereas the combination of high relative urine concentrations of MMP-7 (≥1.255), MMP-2 (≥0.985), and MMP-9 (≥1.135) exclude all controls but one. Therefore, if MMP-7 is low, then a randomly selected case is almost guaranteed not to be an IPF patient (FIGURE 15). Further, if MMPs -7, -9, and -2 are all simultaneously high, a randomly selected case is almost guaranteed to be an IPF patient. Relative urine concentrations of MMP-8 activation peptide are not independently important. Receiver operating characteristic curves (ROCs) agree that a combination of the four markers, most significantly the combination of the activation peptides of MMP-2, MMP-7, and MMP-9 correctly classify IPF patients from controls (FIGURE 16).

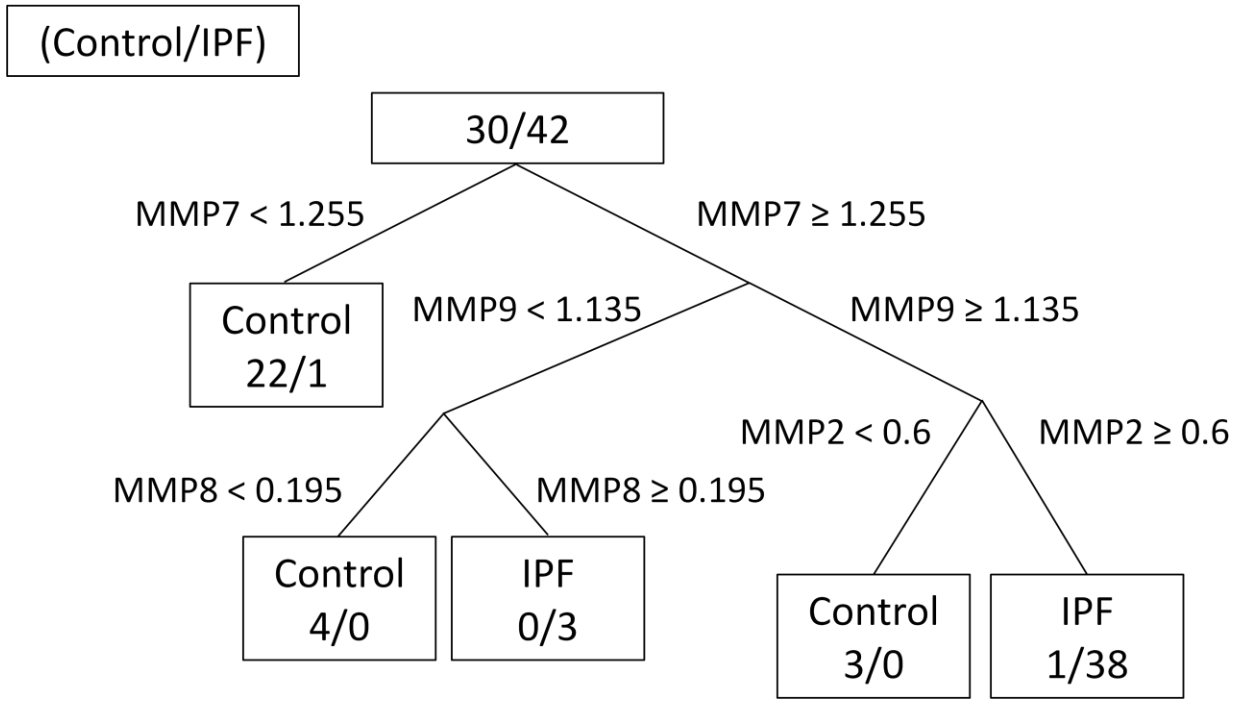

Figure 15. Urine MMP Activation Peptides Distinguish IPF Patients From Controls
A classification tree obtained by CART when applied to relative urine MMP activation peptide concentration from IPF patients and controls shows that these markers can be used as classifiers to correctly identify IPF patients from controls. All data are presented as control/IPF and are based on urine MMP activation peptide levels divided by urine creatinine levels (µg/ml).

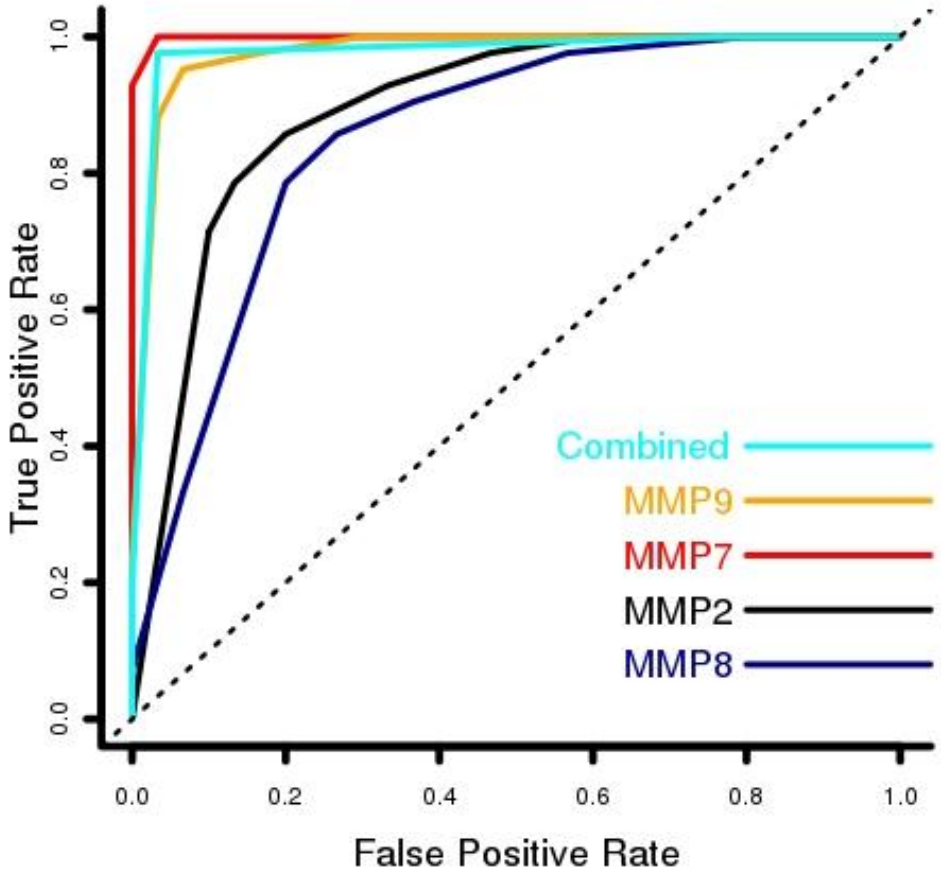

Figure 16. Urine MMP Activation Peptides Distinguish IPF Patients From Controls
ROC curves for using each of the four markers, or their combination, to classify samples as IPF or control. Sensitivity, or true positive rate, is potted on the *y*-axis, and false positive rate, or 1 – specificity, is plotted on the *x*-axis. The area under each ROC curve is equal to the numerator of the Mann-Whitney *U*-statistic comparing the marker distributions between IPF and control samples.

7.3. CONCLUSIONS

IPF is a fatal lung disease without viable treatment options. For this reason, researchers have been trying to identify biomarkers of disease that will shed light on its pathogenesis, aid in the development of therapies, or offer better diagnostic or prognostic tools. MMP disregulation has been shown to be associated with IPF and may provide an avenue to explore the processes of

disease. It was shown that the activation peptides of MMPs are detectable in the urine and correlates with the presence of disease. Because the PSA model determined that serum proteinase detection is confounded by complex clearance mechanisms, it is likely that the detection of MMP activation peptides will give a more clinically relevant measure of MMP activation *in vivo* that is not confounded by these clearance mechanisms.

8. DISCUSSION AND FUTURE DIRECTIONS

Proteinases are involved in a diverse range of key biological processes, including blood coagulation, hormone processing, fertilization, cell growth, and apoptosis among others. In addition, the levels of proteinases and/or their inhibitors in the body critically regulate initiation or progression of several disease states including cancer. Proteolysis facilitates malignant invasion into the vasculature by tumor cells by breaking down of extracellular matrix barriers in the interstitial stroma and basement membrane. The degradation of the basement membrane is a complex process that requires the production, release, and activation of a number of extracellular matrix degrading enzymes. An inappropriate overexpression of these enzymes almost invariably occurs in all malignant tumor cells (103). Also, highly invasive tumor cells have an elevated capacity to degrade the surrounding extracellular matrix (ECM), which they do so by the synthesis of a variety of proteinases that digests the ECM proteins (103). Because of their key role, proteinases have been shown to be potential diagnostic and/or prognostic indicators of specific diseases. Prostate-specific antigen (PSA) and matrix metalloproteinases (MMPs) are proteinases that have found wide application as biomarkers of diagnosis and prognosis of prostate cancer and idiopathic pulmonary fibrosis (IPF), respectively.

Unfortunately, the biochemical nature of proteinases complicates the ability to detect them in the blood (104) (FIGURE 17). The data presented here show that, due to their simple renal clearance, activation peptides of proteinases can be found in the urine and may be used for biomarkers of disease.

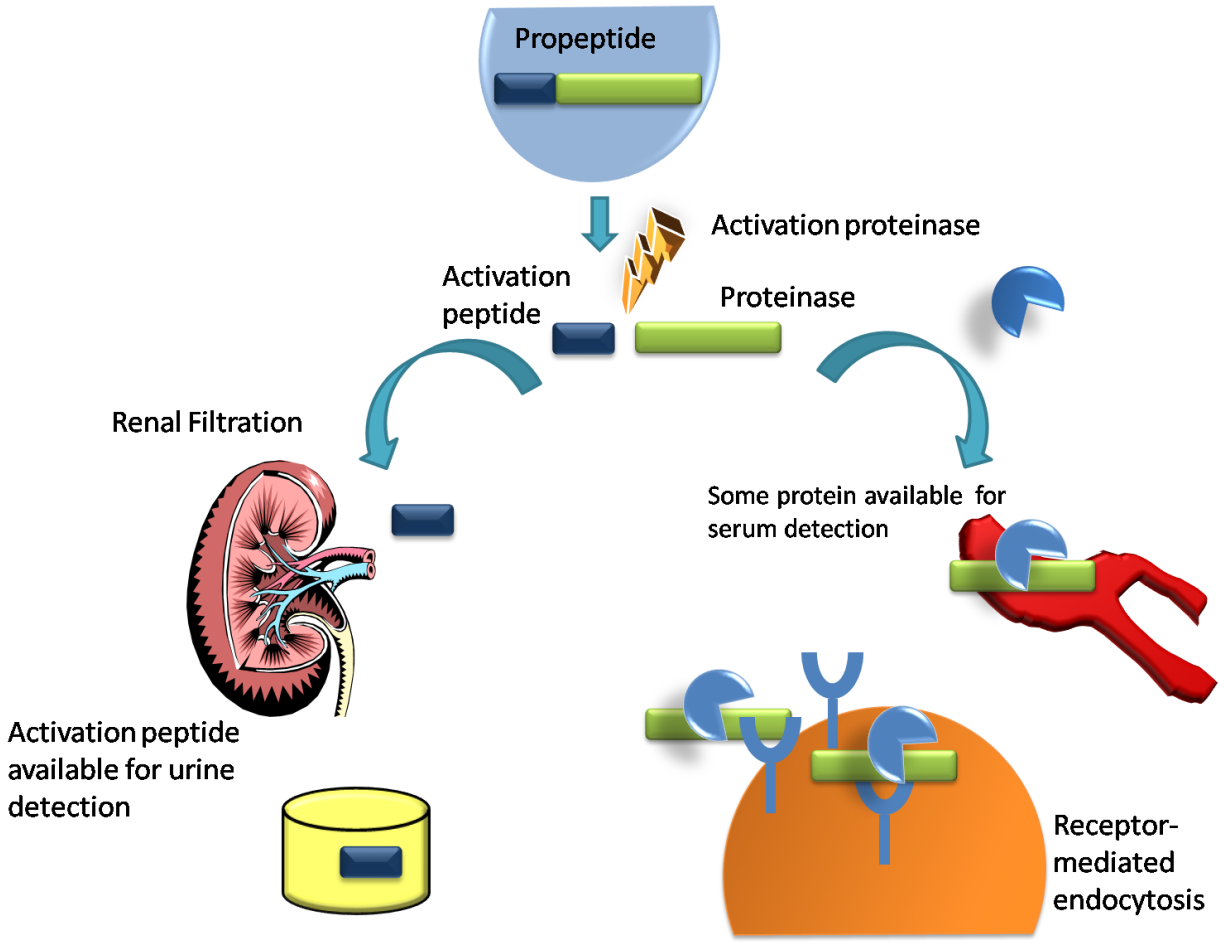

Figure 17. Once an extracellularly activated zymogen is secreted from a cell it is activated by an activation protease. Upon activation, the active protease is quickly bound by its inhibitors in the blood stream where they then bind their respective receptors and are cleared through endocytosis. The activation peptide of the zymogen, however, is cleared through simple renal filtration and can be found intact in the urine.

8.1. PSA ACTIVATION PEPTIDE BIOSYNTHESIS AND CHARACTERIZATION

Prostate cancer is the most frequently diagnosed malignancy in men in North America and Northwestern Europe and the second leading cause of cancer death in males (105). The identification of patients who are at high risk as well as the prediction of tumor progress is critical for treatment and disease management. This is currently being done using PSA in serum as a surrogate marker in the early diagnosis and management of prostate cancer. However, its limited specificity necessitates additional biomarkers to be identified to supplement or potentially replace serum PSA testing for accurate diagnosis of prostate cancer. Laxman et al. have put forth the idea of "multiplexing" or combining several biomarker tests for more accurate detection of prostate cancer (106).

Activated PSA rapidly complexes with proteinase inhibitors e.g., α_1ACT in the blood and is cleared from the circulatory system by receptor-mediated endocytosis. Purified proteins, *in vitro*, do not form complexes as readily (107). The free, noncomplexed form of PSA constitutes only a minor fraction of the serum PSA, as serum ACT occurs in excess. The clearance rate of the PSA- α_1ACT complex is significantly faster than for native α_1ACT. Experiments show that the half-life of α_1ACT alone was up to several days whereas for the PSA- α_1ACT complex it was as low as 20 min (FIGURE 7).

Our experiments suggest that, in general, PSA-α_1ACT complexes are cleared very rapidly from the blood. However, we found that the rapid clearance is dependent on the levels of PSA-α_1ACT complex. The capacity of the clearance mechanism is unknown, but it is evident that it eventually becomes overwhelmed. Indeed PSA complexes only begin to accumulate after very high levels are introduced. These results imply that rapid removal of PSA from the circulation in the early stages of prostate cancer, when there is a low tumor cell burden and lower levels of PSA production, may form a heretofore-unrecognized problem with conventional PSA testing. It is possible that, in cases of prostate cancer associated with normal serum PSA concentrations, the plasma elimination mechanism has not yet been saturated, so that PSA is quickly removed from the bloodstream.

Hypothesizing that one of the reasons for the errant serum PSA values could be the rapid clearance mechanisms, this study looked at the measurement of the 7-amino acid activation peptide of PSA (APLILSR) in the urine as a potential additional biomarker for CaP. Recent progress in the technological tools for peptide detection, quantification, and identification has enabled easy and accurate evaluation of urine biomarker proteins as compared to the insufficient resolution of many earlier techniques such as two-dimensional electrophoresis (108). The dot blotting technique that was used in this study revealed the presence of PSA activation peptide in the urine of prostate cancer patients but not in the control samples. This demonstrated the practicability of using urinary PSA- activation peptide as a screening tool for prostate cancer particularly for early stage diseases, and to serve as a potential sensitive measure for monitoring recurrence of disease.

At first glance the rapid clearance of PSA-α_1ACT complex observed in the present study, could be contrary to previous studies performed in male patients undergoing radical prostatectomy, where a relatively slow clearance of the PSA-α_1ACT complex was observed (109, 110). However, in these studies it is likely that they were monitoring clearance of PSA in patients who had saturated their clearance mechanisms. This is consistent with the relative high concentrations of initial serum levels of complexed PSA in the patients. Furthermore, our hypothesis is consistent with the fact that on a longer time scale, the decrease in complexed PSA concentration is much more pronounced in the patients (109, 110) i.e. once the system was no longer saturated clearance accelerated. The clearance rate is also likely to depend upon the overall health of the patient, including physical condition, body weight, and alcohol and tobacco consumption. These factors may account for some of the variability of serum PSA levels between individuals. This rapid clearance of PSA complexes limits the utility of serum PSA as an early diagnostic tool. Although serum PSA testing plays a useful role in monitoring treatment response and relapses in a given individual, the complex clearance mechanisms of serum PSA may also interfere with the detection of early recurrence monitoring as well. On the other hand, these factors are not expected to significantly affect the half-life of the activation peptide. The mechanism responsible for the removal of the activation peptide is mainly a passive filtration event in contrast to the active receptor mediated endocytosis required for the removal of the PSA-α_1ACT complex. Moreover, the data presented here show in mice that plasma clearance of the activation peptide is not influenced by saturation kinetics like serum PSA complex levels.

Our results suggest that the presence of the PSA activation peptide in urine may be a reliable indicator of secreted and activated PSA with fewer confounding factors than serum PSA analysis. This activation peptide is cleaved from PSA during activation of pro-PSA in the extracellular space. The rapid and simple kinetics of clearance suggest that it does not interact with other proteins, but is cleared from the blood by simple renal filtration. Therefore screening for the presence of the activation peptide in urine may give a more accurate representation of PSA production. Additionally, it represents a noninvasive sampling procedure, amenable to large-scale use.

It is important to note that the ratio of free PSA to total PSA in the serum has been shown to be helpful in discriminating CaP from benign prostatic hypertrophy (BPH) (111, 112) where an increase in free PSA is suggestive of benign disease. Because a majority of free PSA is either enzymatically inactive proPSA ((-7)proPSA) or a significantly nicked variant of proPSA ((-4,-5)proPSA) (113), detection of the full 7 amino acid activation peptide in the urine would correspond to amounts of PSA that have been activated. This would include active PSA that has subsequently bound to α_1ACT and has been cleared or is still present in the blood as well as PSA that is undetectably bound to α_2M. This is of great interest as there is currently no simple assay that can detect PSA bound to both α_1ACT and α_2M.

Because proPSA has been shown to have different forms, one must consider that an antibody against APLILSR may also detect slightly truncated forms of the activation peptide in the urine. Because it would be difficult to discern between 5 and 7 amino acid peptides in an immunoassay, it is possible that the APLIL peptide that is cleaved from the (-2)proPSA form

will also be detected in the urine with an antibody against APLILSR. While this would seemingly implicate a lower specificity for detection, it has been shown that the presence of (-2)proPSA in the serum is a sensitive indicator of cancer (114). This detection might therefore increase the sensitivity of the assay to detect prostate cancer.

It is therefore proposed that an assay to detect APLILSR will constitute a clinically sensitive method of screening for cancers associated with increased levels of secreted PSA. As proteinase activation is a common occurrence in many other diseases, this activation peptide detection technique may also prove useful for the diagnosis or monitoring of other proteinase specific conditions.

In summary, while serum PSA screening is useful, particularly in following treatment response in individuals with known prostate cancer, it is clear that a better understanding of mechanisms involved in PSA biology and regulation will lead to improved prostate cancer screening. Our biosynthesis and clearance studies indicate that prior to detectable elevations of serum PSA in patients, there is likely significant pathology developing in the prostate, and elevated serum PSA levels develop only after saturation of hepatic clearance mechanisms. A saturation-independent method of detecting increased PSA secretion would enhance early detection of prostate cancer and would improve treatment outcomes. We propose that urine detection of the PSA activation peptide may represent a sensitive and reliable early detection method that would also be useful in following treatment response in patients with known prostate cancer.

8.2. FUTURE DIRECTIONS: ALTERNATE PSA ACTIVATION PEPTIDE DETECTION METHOD

While the ability to detect the activation peptide of PSA in the urine and have it predict disease state is certainly useful, for true clinical utility the urinary PSA activation peptide concentrations will need to be individually determined. In order to do this a sensitive quantitative assay will need to be developed. The data presented here show that an ELISA is an appropriate method to quantitatively detect the PSA activation peptide (FIGURE 18), but a more robust antibody is needed in order to determine meaningful results. Ideally, two monoclonal antibodies against the activation peptide will need to be produced in order to develop a sandwich ELISA. Following the development of such an assay, measuring the concentrations of the PSA activation peptide may have use in predicting disease severity and disease recurrence in addition to its diagnostic value.

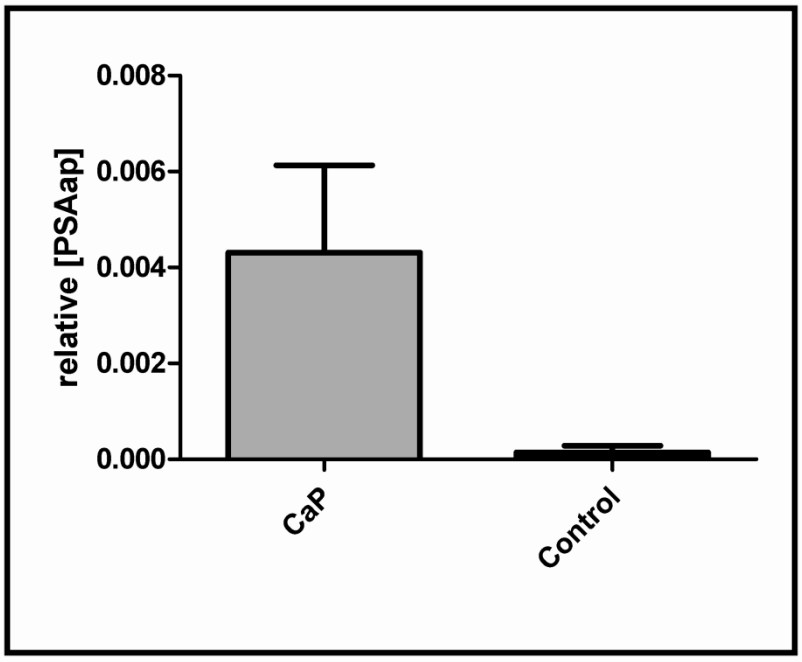

Figure 18. The relative concentration of PSA activation peptide (activation peptide/creatinine ng/ml) is higher in the urine of prostate cancer patients compared to healthy controls as determined by ELISA. The detection limit of the assay is not low enough to determine significance of these results.

Another important point to explore is the stability of the PSA activation peptide in the urine. Because we are able to detect the activation peptide in the urine via dot blotting, it is evident that there is some peptide stability. However, based on our results, we cannot be sure that there is not some subset of the peptide being degraded in the urine. As mentioned above, if the peptide is reduced to its five amino acid form its detection will still hold clinical value by suggesting the amount of (-2)proPSA in the serum. More significant truncation of the peptide will likely, however, confound results. For this reason, it will be necessary to perform experiments to determine the stability of the peptide. These experiments can be done in the same fashion done to determine the stability of the trypsinogen activation peptide in the urine (115).

The trypsinogen activation peptide has been used as a urinary marker for severe acute pancreatitis and has been shown to correlate with the severity of the attack (116-118). But it has been shown that the activation peptide is relatively unstable and hence, does not make a good marker (115).

8.3. PATHOPHYSIOLOGY OF IPF

Pulmonary fibrosis, consequent to lung injury, is initiated by migration, adhesion, and proliferation of fibroblasts in the alveolar interstitium. Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic fibrosing interstitial pneumonia. It is a chronic and usually fatal disorder characterized by impaired oxygen transfer, alveolar collapse, and interstitial fibrosis (American Thoracic Society/European Respiratory Society Consensus Classification, 2002). The clinical features of IPF are quite variable (119) and as the name suggests, the etiology of the disease is unknown. Although the precipitating causes of IPF are indefinite, one prevailing hypothesis is that the initial injury is linked to abnormal repair of damaged alveolar epithelium and irreversible pulmonary structural remodeling. Thus, the course of IPF is mainly associated with the production, deposition, and proteolysis of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of MMPs [TIMPs]), transforming growth factor (TGF)-β1, and cytokines are postulated to play key roles in the pathophysiology of IPF. MMPs and TIMPs have been shown to participate in the parenchymal destruction and repair processes resulting in ECM remodeling (120). MMPs are a family of around 25 structurally related, ECM-degrading, zinc-dependent metalloenzymes with different, yet overlapping, substrate specificities. In addition to IPF, many of these proteinases have been

implicated in other pathological conditions, including cancer (121, 122), and several different vascular diseases (123).

8.4. IPF AND MATRIX METALLOPROTEINASES

MMPs are involved in the initiation of proteinase cascades that impact several substrates thereby playing a central role in several interrelated processes observed in fibrosis such as ECM remodeling, basement-membrane breakdown, epithelial-cell apoptosis, cell migration, and angiogenesis (124-126). Proteinase activity, in general, is regulated *in vivo* by an alteration of the rate of enzyme synthesis and degradation, activation of (pre-) proforms, and binding with endogenous inhibitors. The progressive fibrosis seen in IPF has been shown to occur due to an imbalance between the synthesis and degradation of extracellular matrix caused by an erratic regulation of MMPs and their inhibitors, TIMPs (127). Several MMPs are strongly up-regulated in IPF, e.g., MMP-3, -7, -8 and -9 (126, 128), which has increased attention as to what these MMPs may be doing to promote or inhibit fibrosis. It was originally thought that MMPs would be protective against IPF as they could potentially degrade the fibrotic tissue (129, 130). More recent studies have shown, however, that MMPs actually directly contribute to development of fibrosis. This process, while seemingly counterintuitive, is now the prevailing thought (131-133).

A majority of the MMPs with the exception of the membrane-bound MMPs and stromelysin 3, which are secreted in active form, are secreted as latent zymogens and attain catalytic properties within the ECM. Non-proteolytic compounds such as organomercurial chemicals, several proinflammatory agents such as oxidants, detergents and protein denaturants

(SDS), or other already active MMPs or other proteinases may break open the cysteine to zinc bond to trigger proMMP activation. Experimental models of lung injury have suggested an important role for MMPs with increased gelatinolytic and collagenolytic activities being observed in the lung (134). Endogenous tissue inhibitors of MMPs (TIMPs) specifically form complexes with and inhibit active forms of MMPs. Such complexes are rapidly cleared preventing accurate biological detection of MMP activation. In contrast, the mechanism of clearance of the activation peptides released from the MMPs is unknown. A major drawback for clinical research on IPF is the late presentation of clinical symptoms by which stage the disease is essentially untreatable and has a poor prognosis (135). The generally rapid progression of the disease associated with high mortality raises the importance of initiating optimal therapy early. Furthermore, diagnosis of IPF is rendered difficult because of many other lung diseases with similar symptoms, including several other interstitial lung diseases. Hence, it is imperative to understand better the underlying cellular, molecular, and genetic mechanisms so as to be able to effectively monitor the progress of the disease and, more importantly, to develop methods for earlier detection, which may allow for the development of more effective therapies.

8.5. PROTEINASE MEDIATORS

The actual mechanism of IPF development is not yet understood. The earlier held view that IPF is another inflammation-driven form of lung fibrosis does not seem wholly valid especially because steroids and immunosuppressants are of little help in controlling IPF. The accepted hypothesis now relates chronic epithelial damage to the process of lung fibrosis. The lack of knowledge regarding the etiology of this fibrotic disease has been the major stumbling

block for the development of appropriate antifibrotic therapies. Simply stated, fibrotic disease is thought to be normal wound healing gone awry. Normal wound healing is tightly controlled by a complex set of interactions involving a network of profibrotic and antifibrotic cytokines and secreted proteins. According to a comprehensive review of the pro- and antifibrotic protein mediators of wound healing and fibrosis, the major profibrotic proteins consist of transforming growth factor-ß (TGF-ß) and connective tissue growth factor (CTGF) while the major antifibrotic proteins include tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (136). TGF-ß, a potent fibrogenic cytokine which provokes fibroblasts to produce as well as contract ECM, is a key mediator of the fibrotic response. The protein CTGF secreted by human endothelial cells which is induced by TGF-ß is considered a downstream mediator of the effects of TGF-ß on fibroblasts (136).

The protein α-smooth muscle actin (α-SMA) is produced by wound fibroblasts (or myoblasts) which helps wound closure by inducing the contraction of extracellular matrix. α-SMA as well as collagen type I syntheses are triggered by TGF-ß1 under the influence of the matrix protein fibronectin (137). In addition to the above mentioned profibrotic mediators, several antifibrotic mediators are also involved in the process of wound healing. For example, as a part of the wound healing response, the proinflammatory cytokine TNF-α is increased by macrophages (138). Furthermore, immediately after injury, the proinflammatory cytokine IFN-γ released by T cells suppresses collagen synthesis (139). Thus, the involvement of TGF-ß signaling is central to wound healing and fibrotic responses. On the other hand, the MMPs, in addition to enhancing ECM turnover and promoting tissue remodeling, are also involved in the release and or activation of cytokines and profibrotic growth factors including TGF-ß1 (136).

All the proteins involved work as a highly complex network and new drug intervention strategies to combat chronic fibrosis will likely have to consider the cytokine components of this network. Providing evidence that overexpression of the active, but not latent, TGF-ß in rat lung results in prolonged and severe interstitial and pleural fibrosis, (140) have shown that targeting active TGF-ß and steps involved in TGF-ß activation may be valuable therapeutic strategies. Because MMP activity can regulate this growth factor, it follows that monitoring the activation of MMPs via activation peptide detection in the urine may prove to be another useful method to monitor disease processes.

8.6. BIOMARKERS FOR IPF

There are currently no biomarkers available clinically for the monitoring of IPF disease progression. The typical means to monitor disease are based on outward symptoms including increasing dyspnea and declining pulmonary function tests. These symptoms could however be the result of a different respiratory illness or infection. Identifying clinically useful biomarkers in bronchoalveolar lavage or blood has been challenging because of the complexity of IPF (135). The plasma concentrations of two metalloproteinases, MMP1 (collagenase) and MMP-7 (matrilysin), were found to be elevated in patients with IPF. These levels were higher than in controls or patients with other chronic fibrotic lung diseases, including chronic obstructive pulmonary disease, sarcoidosis, and HP (85). Plasma MMP-7 levels were increased in patients with IPF with asymptomatic disease as well, but the concentrations were lower than in symptomatic disease. Also, the levels correlated with reduced gas diffusion and lung volumes suggesting that MMP-7 in plasma may be used as an early marker of disease and disease

progression (85). Due to the potential problems associated with measuring proteinases in the blood, as previously described, it would certainly be beneficial to have an additional way to monitor MMP-7 levels. Assaying urinary concentrations of the activation peptide of MMP-7 would be an appropriate adjunct to this method.

A specific biomarker for IPF would be extremely useful for both early detection screening and in determining if patients were actually suffering from accelerated progression of their disease. It has been shown that patients with different progression patterns of their disease (slow progressors vs. rapid progressors) have different gene expression patterns suggesting that the variability of IPF progression must be considered when treating these patients (141). In addition it has been shown that rapid disease progression is a likely cause of death in IPF patients (142). Therefore, the ability to monitor patients biochemically for actual disease progression would have extreme advantages. It is plausible that this assay could be used to monitor patients longitudinally for acute exacerbations to determine if a particular level of MMP activation peptide present in the urine suggests an impending acute exacerbation. Similar work in the BAL fluid has already shown that active MMP-9 may be a predictor of fast progression (141).

Several MMPs are known to be upregulated in acute lung injury models but the exact role of these MMPs in the development of the lung injury is unclear (143). The present study showed for the first time the presence of increased amounts of activation peptides of MMP-2, MMP-7, and MMP-9 in the urine of mice with pulmonary fibrosis compared to WT mice and also in the urine of human patients with IPF compared to healthy controls. Although MMPs are believed to contribute to IPF, monitoring their activation is complicated by proteinase inhibition and

clearance. Thus, measuring levels of the activation peptides of MMPs represents a better overall measure of MMP activation. We have previously shown in the PSA study that small activation peptides are filtered into the urine. Because MMP activation peptides are also relatively small, we chose to measure urinary levels of the activation peptides for MMPs. It is likely however, that these activation peptides will also be present in the bronchoalveolar lavage fluid (BALF).

Gelatinases are believed to participate in remodeling and/or degradation of the ECM (144). The gelatinolytic activity of MMP-2 has been presumed to be related to the invasive as well as the metastatic properties of the cancers while MMP-9, another gelatinase, has been implicated in the pathogenesis of the lung injury (143)**.** An upregulation of collagenase 1 and 2 and gelatinases A and B, has been suggested to contribute to the pathogenesis of COPD through the remodeling of airways and alveolar structures. Henry et al. have reported MMP-8 to be the major contributor to the bronchoalveolar lavage fluid collagenase activity in the airways of patients with idiopathic pulmonary fibrosis and initiator of collagen destruction and remodeling leading to the development of pulmonary fibrosis (23). The present study did not, however, find any significant increase in MMP-8 activation peptide in urine. Of the members of the MMP family, active MMP-2 and MMP-9 can provoke the disruption of basement membranes thereby facilitating fibroblast invasion into the alveolar spaces. The present study has shown significant increases in the activation peptides of both MMP-2 and MMP-9 in urine supporting the convention that these MMPs are being activated to a greater level in patients with IPF.

An overexpression of MMP-9 by alveolar macrophages has been reported to play an active role in lung remodeling in IPF (145). The plasma concentrations of MMP-7 has been suggested

as a plausible biomarker for monitoring disease progression and facilitating early diagnosis (135). MMP1 and MMP-7 have been suggested to be the main components of the peripheral blood protein signature in IPF, whose overexpression can distinguish IPF from other chronic lung diseases (85). To the contrary, it has been demonstrated that the increased expression of MMP-7 is not a unique phenomenon of IPF, but a common feature of interstitial fibrotic disorders (146). Because MMPs in the blood should be cleared rapidly, analogous to our PSA studies, detection of elevated levels of these MMPs in the blood may only occur after saturation of clearance mechanisms. It is possible that these confounding factors are what are responsible for such conflicting results whereas activation peptide detection may be a reliable additional way to measure MMP-7 activity. Our study showed the presence of significantly elevated levels of activated peptide of MMP-7 in urine of IPF patients.

Because there is currently no appreciable therapy for IPF, additional markers of disease could aid in the development of new therapeutic targets. Proteinase inhibitors are commonly used in the treatment and prevention of viral infections and also have the potential to treat cancers (147, 148). It is clear the MMPs play a role not only in the progression of IPF but also in the initiation of disease. MMP inhibition has already shown to be protective against asbestos- and bleomycin-induced fibrosis (22, 149). For this reason, it is possible that the inhibition of certain MMPs may be a viable option in the treatment of IPF.

Biomarkers and surrogate markers can provide important complementary information in combination with traditional clinical and laboratory data. Relatively noninvasive and easy-to-perform tests make the best biomarkers. Such tests usually involve blood or urine specimens, can

be measured serially, and have a fast turnaround. Modern high-throughput proteomic technologies allow the complete profiling of low molecular weight peptides in biological fluids. These techniques work better with urine, however, because it is a less complex fluid compared to serum which has a wide component of proteins, the predominant ones being albumin and Ig that usually overwhelm the less abundant signals (150). Combined with the proper statistical analyses these techniques may potentially be used to identify biomarkers indicative of disease states.

8.7. FUTURE DIRECTIONS: IPF

8.7.1. IPF Biomarkers

The fact that there are no available biomarkers for this disease makes it very difficult to monitor patients' response to therapy. Radiography and symptom resolution are currently what is used to monitor patients through their course of treatment, however these methods are unable to show biochemical response and therefore cannot determine if the disease itself is being treated or just the symptoms. Biomarkers to predict therapeutic responses in patients with IPF would have a tremendous impact on future clinical trials by making them more cost effective (reduce need for expensive radiographic studies, pulmonary function studies, and other physiologic studies that are not that sensitive to detect changes) and also aid in determining if a trial should be continued if no effect is seen.

The biomarkers studied here were selected because they have previously been shown to be related to the development of pulmonary injury (21, 23, 82, 85, 125, 128, 143, 144). As such, it would not be surprising to find these markers present in other pulmonary diseases such as HP, NSIP, COPD, and sarcoidosis. In order to determine clinical diagnostic utility of this assay, we

will have to expand our population to include patients with other pulmonary diseases. While it is possible that the levels of MMP activation peptides will be up regulated regardless of type of lung injury, we expect that urine MMP-7 activation peptide concentrations will be elevated in IPF patients compared to patients with COPD and sarcoidosis as it has been shown that elevated MMP-7 in the peripheral blood is sufficient to distinguish IPF from the later two diseases (85). It would be interesting to compare urinary MMP-7 activation peptide levels to MMP-7 levels in the plasma longitudinally to determine if one increases before the other. Inferring from our previous studies with PSA, it is likely that the MMP-7 levels that have been measured in the plasma do not represent all of the MMP-7 production as MMP-7 is inhibited and cleared from the system once activated. One would expect then that urinary levels of MMP-7 activation peptide would increase prior to plasma MMP-7 levels. It would certainly be of value to assay for other proteinases that may be involved in disease initiation and progression. At least two other MMPs are known to be up regulated in pulmonary fibrosis, MMP-1 and -13 (23). Additionally, the serine proteinase neutrophil elastase (NE) has been shown to contribute to pulmonary fibrosis processes (151, 152). These proteinases are all activated extracellularly therefore their activation peptides should also be detectable in the urine.

Future studies should also attempt to correlate gelatinolytic and collagenolytic activities in lung tissue with urinary activation peptides. This could be done by combining techniques such as gelatin zymography to analyze proenzymes and activated enzymes and urine activation peptide estimation. Zymography results could also be compared to urinary MMP activation peptide concentrations and MMP protein concentrations in the plasma to further evaluate the biosynthesis of each MMP. It would also be important to devise suitable studies with, for

instance, specific MMP-deficient mice to examine the source of active metalloproteinases including MMP-2, -7 and -9 so as to understand their role in the pathophysiology of IPF.

Urinary proteomics is a powerful non-invasive tool for diagnosis and monitoring of several human diseases. Signatures of urinary MMP activation peptides could hold an abundance of information that would be helpful in the development of new biomarkers useful in clinical investigations of IPF and other interstitial lung diseases. Urinary activation peptide signatures would possibly be helpful in identifying the point in time when predisposition to IPF is developing into disease. There is also a possibility that such biomarkers would be able to help identify potential trigger events in IPF.

8.7.2. IPF Therapeutics

The transforming growth factor (TGF)-β and bone morphogenetic proteins (BMPs), members of a superfamily of cytokines and present in different isoforms, are essential components of the tissue regeneration machinery with specific functions such as wound healing, extracellular matrix remodeling, and the control of epithelial– mesenchymal interactions during embryogenesis. In particular, isoform BMP-4 plays an essential role in lung development. In the adult lung, TGF-β is involved in the regulation of extracellular matrix synthesis and degradation while BMPs also have a role to play. A sustained activation of TGF-βs together with an abnormal expression of BMPs are associated with chronic diseases such as fibrosis and cancer. Changes in the TGF-β/BMP balance have been shown to lead to the development of fibrosis. Suitable modification of the concentration of either of these two parameters holds promise as a viable therapeutic option. It is known that in IPF, BMP-2 is down-regulated whereas BMP-4 is

enhanced, and so is the concentration of gremlin, a BMP antagonist (153). Gremlin can bind BMP-2, -4, and -7 and inhibit their activity. BMP-7 signaling is down-regulated in response to up-regulation of gremlin; but, upon restitution of activity, was found to inhibit the progression of fibrosis in mice. This restoration of BMP-7 signaling in fibrotic lung could, therefore, be deemed a potential means of treatment of IPF patients (154). Myllärniemi et al. observed that BMP-7 treatment significantly reduces fibrosis *in vivo* in asbestos-exposed mice (155) while, according to Murray et al., BMP-7 inhibits renal fibrosis but not bleomycin-induced fibrosis in the lung (156).

Another novel mode of therapy for pulmonary fibrosis that is undergoing extensive research currently is the use of proteinase inhibitors. Neutrophil elastase (NE) is one such important proteinase which is under investigation. A specific NE inhibitor has been reported to effectively prevent development of pulmonary fibrosis in bleomycin- treated mice (157). The most prevalent proteinase inhibitor in serum is α-1 antitrypsin. This natural proteinase inhibitor regulates the activity of neutrophil elastase that hydrolyzes elastin in the lungs. Individuals with congenital α-1 antitrypsin deficiency have a proteinase-antiproteinase imbalance in their lungs, which leads to early onset progressive lung disease. They develop emphysema as a result of increased elastase activity in the lungs. CT lung density images have demonstrated significant benefit from α-1 antitrypsin augmentation (or replacement) treatment (158). However, the augmentation therapy with the natural proteinase inhibitor does not cure α-1 antitrypsin related emphysema, but it slows the progression of this disease.

Another important hypothesis implicates an oxidant–antioxidant imbalance to contribute to the disease process in idiopathic pulmonary fibrosis (159-161). TGF-ß decreases the intracellular glutathione (GSH) content in murine embryo fibroblasts leading to an increase in collagen I mRNA content and collagen protein production. Inhibiting GSH depletion with *N*-acetylcysteine (NAC), or by addition of GSH or GSH ester could abrogate TGF-β-stimulated collagen production. GSH plays a major role in antioxidant defense. Therefore, the above observations suggest that reactive oxygen species (ROS) are involved in TGF-ß - stimulated collagen production and that GSH depletion mediates TGF-β-stimulated collagen production probably by facilitating ROS signaling (162). Also, inhalation treatment with GSH or NAC, a precursor of GSH, has been found to successfully attenuate experimental lung fibrosis and significantly improve pulmonary function in patients with lung fibrosis. Furthermore, a multicenter European study has also suggested, based on the effect of the antioxidant *N*-acetylcysteine on the progression of IPF, that the cellular redox state may significantly affect the progression of the disease (161). The study further indicated that cellular redox balance could have critical effects on gene expression and thereby on the synthesis of various compounds related to pulmonary fibrosis. Continued investigation into therapeutic approaches to inhibit ROS-mediated reactions in the initiation and progression of lung fibrosis, and, in particular, evaluation of several new synthetic antioxidants (e.g., SOD mimetics) as potential drugs for treatment of IPF has been recommended (161).

To further evaluate the therapeutic benefits of BMP, proteinase inhibitors or antioxidants for IPF, it would be necessary to identify proper biomarkers for monitoring the progression/regression of the disease. At present, no suitable marker has been identified. MMP

activation peptides in urine described in the present study hold remarkable promise in that direction, being easy to sample and analyze. Significantly, the ability to detect the activation peptides of MMPs in the urine enables researchers to follow treatment overtime to determine therapeutic response over the course of a study.

Additionally, to enhance the efficacy of MMP activation peptides as biomarkers, comparative studies need to be carried out on MMP activation peptides levels in idiopathic pulmonary fibrosis patients suffering from acute exacerbations of their disease and those with stable IPF. Depending on whether significantly elevated levels of MMP activation peptides are found in patients with acute exacerbations of IPF, further studies requiring serial monitoring of the biomarker MMP activation peptides need to be conducted in IPF patients to determine if the biomarkers start increasing prior to acute exacerbations. A positive outcome of such studies would suggest that monitoring MMP activation peptides would be a useful biomarker in predicting acute exacerbations in IPF patients and potentially help to develop therapies to prevent the onset of acute exacerbations all together.

8.8. CLINICAL IMPLICATIONS

The pathology of lung fibrosis crucially involves the remodeling of interstitial collagen. Therefore, determining which of the MMP collagenases contribute to collagenolysis in fibrosis, singly or collectively, is important to further investigate their potential roles. Evaluation of the activation peptide moieties in urine give an accurate idea of the MMPs being activated during the process. Activation peptides of other collagenases such as MMP1 or MMP13 were not evaluated in this study but they could be potential biomarkers (23). An abnormal bronchiolar proliferation

is indicated by the upregulation of the activation peptide of MMP-9 in urine since an excessive activation of the gelatinase, MMP-9 could result in the remodeling of airways and alveolar structures (163). The presence in urine of the activation peptide of MMP-2, another gelatinase enzyme which is known to be responsible for the degradation of extracellular matrix (ECM) including basement membrane (126), could be an indication of the possible involvement of MMP-2 in parenchymal remodeling.

Investigations of IPF conducted using the bronchoalveolar lavage fluids are technically somewhat straightforward but the compulsory invasiveness of the technique is a definite drawback. Activation peptides of MMPs in the urine as biomarkers would score better in this regard.

Because there are numerous diseases associated with disregulation of proteinases, the ability to accurately quantify proteinase activation could serve to be extremely beneficial across the boards. The MMP family alone has been implicated in disease progression in many other diseases including, cancers and cardiac diseases. The spread of breast cancer, and the initiation of acute leukemias, for example, have been attributed in part to MMPs (121, 122). While an assay to measure the activation peptides of MMPs may hold great benefit for these diseases as well, it also potentially makes a diagnostic assay for one particular disease less specific. Studies would have to be performed to evaluate the urinary activation peptide signatures of the diseases known to be influenced by MMP activation. Ideally, each disease would have an individual urinary activation peptide signature that could discern between diseases. For this to occur, it is probable that the panels of markers would have to be larger. Individuals, with arthritis,

infections, neurodegenerative diseases, and diabetes all have proteinases known to be differently modulated compared to non diseased controls (164-168). Activation peptide concentrations in the urine could potentially be measured for all of these diseases giving new options for diagnosis, prognosis, or therapeutic targets.

Clinical trials aim to find new therapies to lessen symptoms, stop disease progression, or even cure disease. Currently, for diseases without adequate treatment options and without viable biomarkers such as IPF, trials are only able to determine therapeutic utility by monitoring symptoms. In the case of IPF, the most specific symptom that is measured in trials is mortality. Interferon gamma has been used in many trials as a treatment for IPF. While there has been a trend seen towards increased survival (169) the mortality rate has not been significantly reduced with this treatment and is not recommended for therapy (170). Knowing whether a drug was working prior to the patient's death would of course be a valuable resource. Not just for the patient's health but also for the cost of conducting a failing clinical trial. We have shown that PSA is not detectable in the serum until its clearance mechanisms are saturated which greatly affects the accuracy of standard serum testing. While this is likely the case for other serum proteinases, this is not an issue for urinary detection of activation peptides for these proteins. For this reason, we feel that introducing activation peptide detection as an adjunct to other methods for monitoring therapeutic response could provide a valuable tool for clinical trials and beyond.

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