UNIVERSITY OF SOUTHAMPTON

The role of matrix metalloproteinase-9 (MMP-9) in the pathogenesis of chronic obstructive pulmonary disease (COPD)

By

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ABSTRACT

DEPARTMENT OF CELL SCIENCES

THE ROLE OF MATRIX METALLOPROTEINASE-9 IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE.

Paul Francis Mercer.

Chronic obstructive pulmonary disease (COPD) is a progressive and irreversible disease of the airways. Classically the major symptoms result from extracellular matrix destruction resulting in extensive remodelling of bronchiolar walls, loss of alveolar integrity and parenchymal destruction. It has been widely hypothesised that chronic exposure to cigarette smoke initiates inflammation, responsible for an increased proteinase burden and thus pulmonary proteinase/ antiproteinase imbalance. It is this imbalance which is thought to mediate matrix destruction and remodelling.

This thesis has assessed the contribution of matrix metalloproteinase-9 (MMP-9) and its specific inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1) to this process. MMP-9/ TIMP-1 imbalance has been implicated in a variety of diseases involving lung remodelling, including acute respiratory distress syndrome (ARDS), asthma and cystic fibrosis, and there is increasing for involvement in COPD.

This thesis aims to further clarify the MMP-9 and TIMP-1 involvement by analysing a variety of clinical samples from patients with obstructive lung disease including bronchoalveolar lavage (BAL) fluid, sputum and directly resected lung tissue from COPD patients. Where possible MMP-9/ TIMP-1 imbalance has been related to changes in inflammatory cell infiltration and lung function deficit. This thesis illustrates that elevated MMP-9 is indeed associated with disease; finding elevated MMP-9 in both BAL fluid from COPD patients and the lung tissue of smokers with impaired lung function.

Exacerbations or acute worsening of symptoms are important symptomatic events in disease progression, and we have shown a significant MMP-9/ TIMP-1 imbalance during these events. It is likely that during exacerbations neutrophils or lymphocytes, not macrophages or eosinophils are responsible for this proteinase excess.

Our MMP-9 and TIMP-1 analyses concentrated on the balance between MMP-9 and total immunocompetant TIMP-1. TIMP-1 levels were quantified by enzyme linked immunosorbant assay (ELISA), however TIMP-1 functionality could not be determined. Here we have begun to develop an assay to measure levels of both total and active inhibitor, exploring whether the MMP-9/ TIMP-1 balance could be augmented by TIMP-1 inactivation. While this assay requires further development initial results suggest that the amount of active TIMP-1 varies between subjects with COPD, possibly augmenting the proteinase/ antiproteinase imbalance in certain individuals. Additionally the proportion of active TIMP-1 compared to total was found to vary between COPD and asthma subjects suggesting that MMP-9: TIMP-1 imbalance is augmented in different pathologies, to different degrees.

Preface

Parts of the work in this thesis have been published, or are in preparation for publication, in the following:

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Abbreviations.

AP-1	Activator protein-1
BAL	Bronchoalveolar lavage
CD	Cluster designation
COPD	Chronic obstructive pulmonary disease
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbant assay
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HRP	Horse radish peroxidase
IA	Immunoabsorbance assay
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IP	Immunoprecipitation
IQR	Interquartile range
LT	Leukotriene
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MuLV	Murine leukemia virus (reverse transcriptase)
NTP	Nucleotide phosphate
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMSF	Poly methyl sulphoxide
Rnase	Ribonucleic acidase
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus* (DNA polymerase)
TBS	Tris buffered saline
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TMB	Tetra methyl benzedine
TNF	Tumour necrosis factor
TRE	12-O-tetranoyphorbol-13 acetate- response element
WB	Western blot

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Chapter 1.

Introduction

1.1 Overview of chronic obstructive pulmonary disease (COPD).

1.1.1 Definition of COPD.

Chronic obstructive pulmonary disease (COPD) is a major cause of mortality and morbidity world-wide. The term COPD is defined physiologically as chronic airflow obstruction, reflected by a reduction in maximum expiratory flow and slow forced emptying of the lungs. The American Thoracic Society (ATS) definition of COPD highlights two important defining characteristics of the disease [ATS 1995]. Firstly airflow obstruction is progressive; extensive pulmonary damage often occurs over many years before patients are aware of their symptoms. Secondly obstruction is largely irreversible with corticosteroid or bronchodilator treatment; COPD patients exhibit < 15% reversibility, compared to > 15% reversibility found in asthma [Barnes, 1999]. COPD is a heterogeneous condition, caused by 3 important contributory syndromes: chronic bronchitis (chronic simple bronchitis), chronic bronchiolitis (chronic obstructive bronchitis) and emphysema (these will be discussed in greater detail below (1.1.4).

COPD has a wide heterogeneity with each individual presenting with varying degrees of overlap between each of the three syndromes. Complicating diagnoses further, approximately 10% of COPD patients present with contaminating asthma, with individuals showing a comparatively increased bronchodilator and corticosteroid response [Jeffrey, 1998a, O'Byrne and Postma, 1999, Barnes, 1999].

Figure 1.1

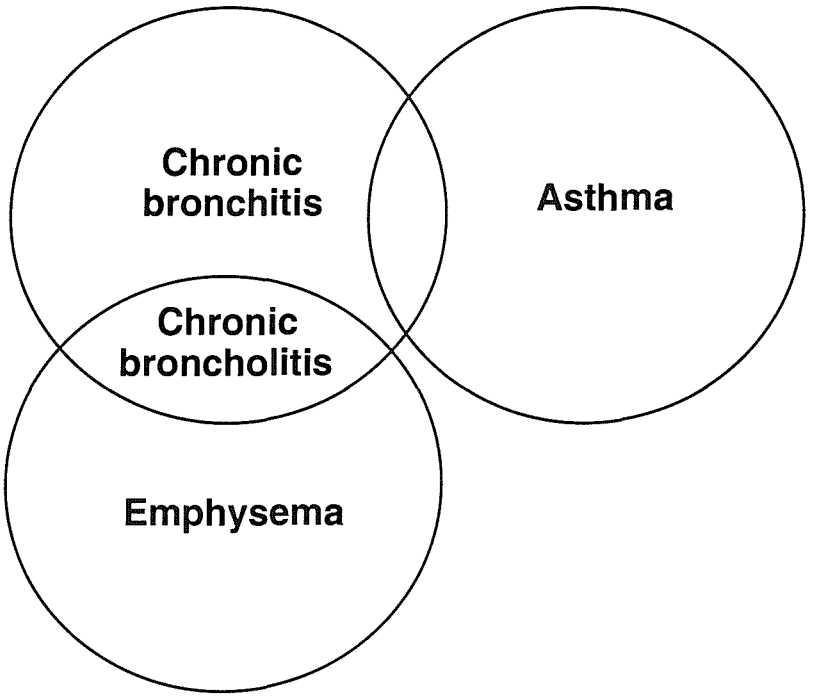


Figure 1.1 Airflow limitation: simplified interrelationships between syndromes contributing to COPD. COPD is caused by the combined contribution of three major clinical syndromes: chronic bronchitis, chronic bronchiolitis, and emphysema. The magnitude of contribution for each syndrome differs from individual to individual. Additionally it is estimated that 10% of COPD patients also have asthma, contributing further to pulmonary pathology. The combination of conditions makes COPD an extremely heterogenous condition.

1.1.2 The epidemiology of COPD.

The disease is a major cause of ill health world-wide and its prevalence is increasing. It is estimated that 14 million people in the United States suffer from the disease; about 12.5 million from chronic bronchitis and about 1.65 million from emphysema, translating to 6% of adult white males and 3% of adult white females [Murray, *et al.*, 1996]. In the UK the disease is responsible for 9% of certified sickness absence from work imposing a financial burden on the National Health Service currently estimated at £818 million per annum [Barnes, 1999]. According to the World Health Organisation COPD is the sixth most common cause of death world-wide, approximately equating to 6% of deaths in men and 4% of deaths in women. In Britain overall mortality is 200-300/100,000 people aged 45-64 years [O'Byrne and Postma, 1999]. Despite the obvious widespread impact of COPD, it has attracted little attention from the medical profession or the pharmaceutical industry. This bias is changing however and relatively recent

research has highlighted a variety of risk factors, causative mechanisms and potential pathologies behind COPD.

1.1.3 Risk factors for COPD.

Risk factors predisposing individuals to develop COPD are under continual review. Cigarette smoking (both passive and active) is accepted as the most important predictor of abnormal decline in lung function and development of the disease after 35 years of age [Snider *et al.,* 1989, Sandford *et al.,* 1997 O'Byrne and Postma, 1999]. Evidence suggests that active tobacco smoking accounts for between 80 and 90% of COPD cases [Snider *et al.,* 1989]. Additionally, active cigarette smoking between the ages of 5 and 20 years is associated with a 5-10% reduction in FEV_1 [O'Byrne and Postma, 1999]. Cigarette smoke is thought to induce 2 types of pathological process in the lung, which will be discussed in more detail later. Firstly it initiates proteolytic destruction of the lung parenchyma leading to enlargement of airspaces and hence emphysema. The second potential effect is to initiate inflammatory narrowing of the peripheral airways, characterised by oedema, mucus hypersecretion, fibrosis, scarring, and obliteration of peripheral airways [Sandford *et al.*, 1997]. However the picture is not as straight forward as *"Smoking causes COPD"* as the condition only affects 10-20% of chronic heavy smokers. Thus while smoking is an important risk factor it is likely that there are other causal mechanisms, which interact with smoking and influence disease susceptibility and severity.

There is evidence that susceptibility to disease has a genetic component. For example case-control studies have shown increased prevalence of COPD in relatives of cases as compared to controls [Tager *et al.,* 1978]. Additionally, there is increased prevalence of reduced lung function in the children of patients who have COPD compared to their spouses [Higgins *et al.,*1975], as well decreasing prevalence of disease with increasing

genetic distance from a diagnosed COPD patient [Tager *et al.,* 1978]. The most important genetic defect linked to the development of COPD was inheritable α_1-antitrypsin deficiency. This was initially described clinically by Laurell and Eriksson in 1963 [Laurell and Eriksson, 1963] and is now is estimated to account for approximately 1% of COPD cases in the United States. Patients who have a genetic deficiency in α_1-antitrypsin (a serum protein, inhibitor of neutrophil elastase normally found in the lung) have a very high risk of developing emphysema at an early age, which is accelerated if they smoke [Laurell and Eriksson, 1963]. Importantly, α_1-antitrypsin deficiency highlights the possible involvement of a proteinase / antiproteinase imbalance in COPD pathology. Weak associations have been found between COPD and mutations in inhibitors such as antichymotrypsin, α_2 macroglobulin, and secretory leukoproteinase inhibitor (SLPI). Detailed research for genetic aberrations is sparse however, and it is likely that many more candidate genes await identification.

It is likely that environmental factors interact with genetic factors and predisposing individuals to COPD. There is evidence for an association between particulate air pollution, occupational exposure to fumes and dusts, and disease development [West, 1995]. Studies have also shown that passive smoking is an important environmental factor for obstructive disease. Children exposed to smoke from their parents have a 1-5% reduction in FEV_1 by the age of 14, and therefore show a higher risk of developing respiratory disease when compared to children of non smoking parents [Tager *et al.,* 1983].

Effectiveness of immune response has also been highlighted as a predictor of disease. For example patients with a history of chest infections are found to be a particular group at risk. Evidence suggests that latent adenoviral infections could either predispose certain individuals to develop COPD [Matsuse *et al.*, 1992] or at least be important factors in disease progression; acting as initiators of exacerbations [Seemungal *et al.,*

2000]. Interestingly genetic studies have highlighted a possible relationship between abnormalities in blood group antigens, HLA antigens and immunoglobulin deficiency and COPD [Sandford *et al.,* 1997]. The effect of such mutations is unclear and often controversial, however they may be indicative of an aberrant immune response. There has also been debate about the role of atopy and non-specific airway hyperresponsiveness in disease development. Atopy is an important factor in persistance of asthma in childhood, and is related to accelerated decline in lung function. Proponents of the argument for atopic involvement argue that it is likely, however unproven, that atopy could enhance the inflammatory response to cigarette smoking in certain individuals [Pride, 1986, Frew *et al.*, 1992, Rijcken, *et al.,* 1995, Weiss, 2000].

1.1.4 General pathology of COPD.

Airflow limitation in COPD is thought to result from a mixture of three contributory conditions: Bronchitis, bronchiolitis, and emphysema.

Chronic bronchitis

This is defined as a productive cough on most days for at least three months over at least two consecutive years, which cannot be attributed to other pulmonary or cardiac causes [West, 1995]. It is caused by chronic hypersecretion of mucus which blocks primarily the central airways [MRC, 1965, Fletcher *et al.*, 1984, West, 1995]. Agents in cigarette smoke are thought to irritate the lung epithelium, increasing mucus secretion from goblet cells via local sensory nerve stimulation (see figure 1.2).

Figure 1.2

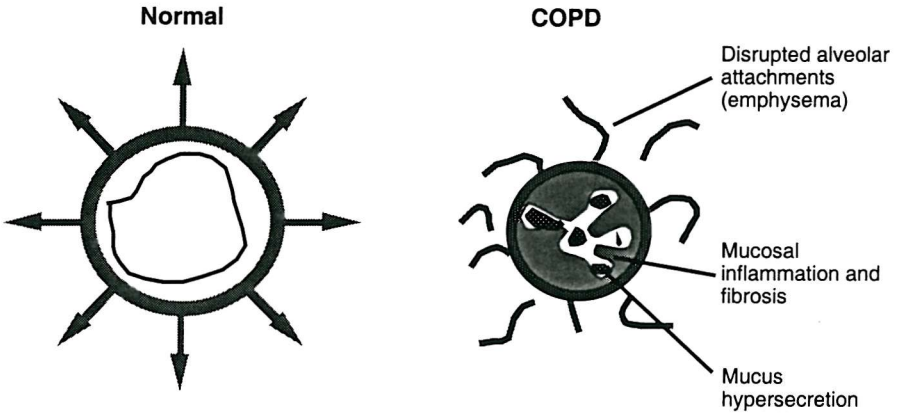


Figure 1.2 The cumulative features of COPD. Airway integrity in normal individuals is maintained by alveolar attachments. In COPD these attachments are disrupted, possibly by proteolytic damage, contributing to airways closure. Peripheral airways are also obstructed by damage resulting from inflammation and mucus obstruction.

Over time the submucosal glands undergo hyperplasia and proliferation, maintaining mucus production at a chronically high level. Additionally, the small airways become narrowed and show evidence of inflammatory cell infiltration [West, 1995]. Activated neutrophils recruited to the epithelial submucosa release neutrophil elastase, a major stimulant of mucus secretion, possibly important in chronically maintaining mucus secretion.

Chronic bronchiolitis and emphysema

Bronchiolitis (or *chronic obstructive bronchitis*) again causes a productive cough and airflow limitation, but is thought to be caused by the obstruction of peripheral airways, in contrast to bronchitis. Obstruction is caused by an aberrant inflammatory response thought to involve macrophages, CD8+ T-lymphocytes and neutrophils, with resultant fibrosis [Buist, 1984]. Chronic bronchiolitis is often found to co-exist with the destruction of alveolar walls (see figure 1.2); emphysema [Fletcher *et al.*, 1984, Snider *et al.*, 1985]. Emphysema is characterised by enlargement of the acinus, the respiratory unit distal to the terminal bronchiole, by the destruction of the acinar walls [West, 1995]. This results in the reduction of driving pressure and obstructive collapse of

peripheral airways causing a reduction in FEV_1 [Fletcher *et al.*, 1984, Snider *et al.*, 1985]. Acinar wall destruction can be characterised as two specific types. In *centriacinar emphysema* destruction is limited to the central part of the acinar lobule with the alveoli remaining unscathed. By contrast *panacinar emphysema* involves widespread destruction of the entire respiratory unit (see figure 1.3).

Figure 1.3

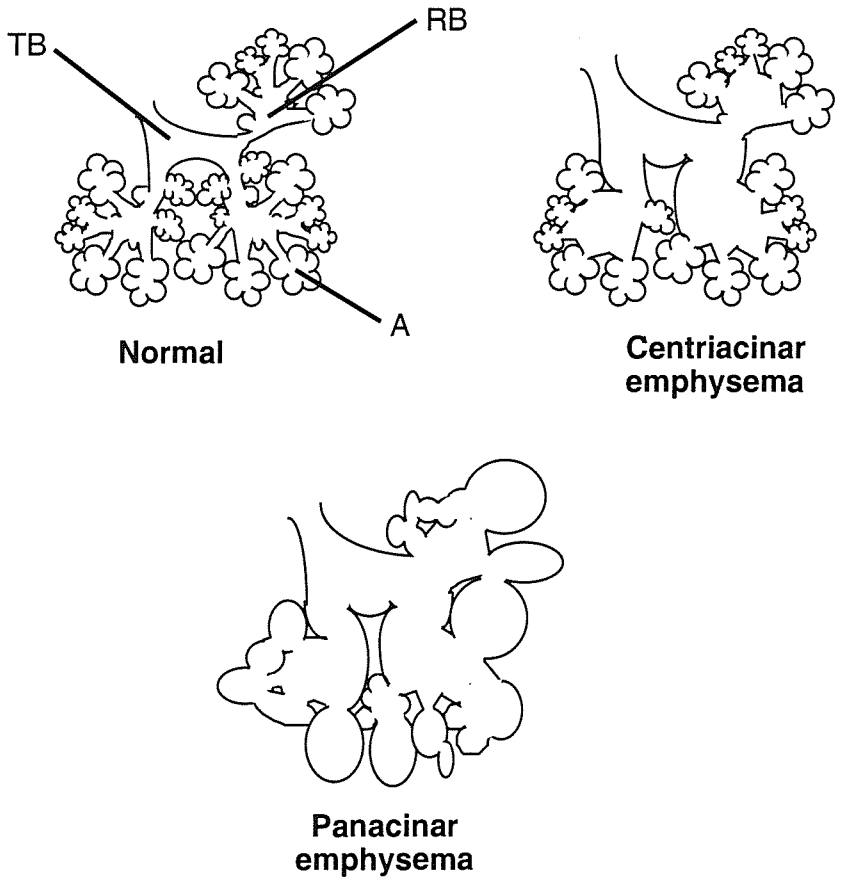


Figure 1.3. Centriacinar and panacinar emphysema. Destruction in centriacinar emphysema is confined to the terminal (TB) and respiratory bronchioles (RB). In panacinar emphysema the peripheral alveoli (A) are also involved. (From West, 1995).

Topographically these two types of emphysematous destruction can be distinguished. Centriacinar emphysema is found to affect the upper regions of the lungs, moving downwards through disease progression, where as panacinar emphysema is found to have no regional preference [West, 1995]. There is debate about the differences between bronchiolitis and emphysema and which of the two is the primary contributor to lung

obstruction [Gelb *et al.,* 1996] however the important underlying feature of each is the aberrant inflammatory response [Jeffrey, 1998a].

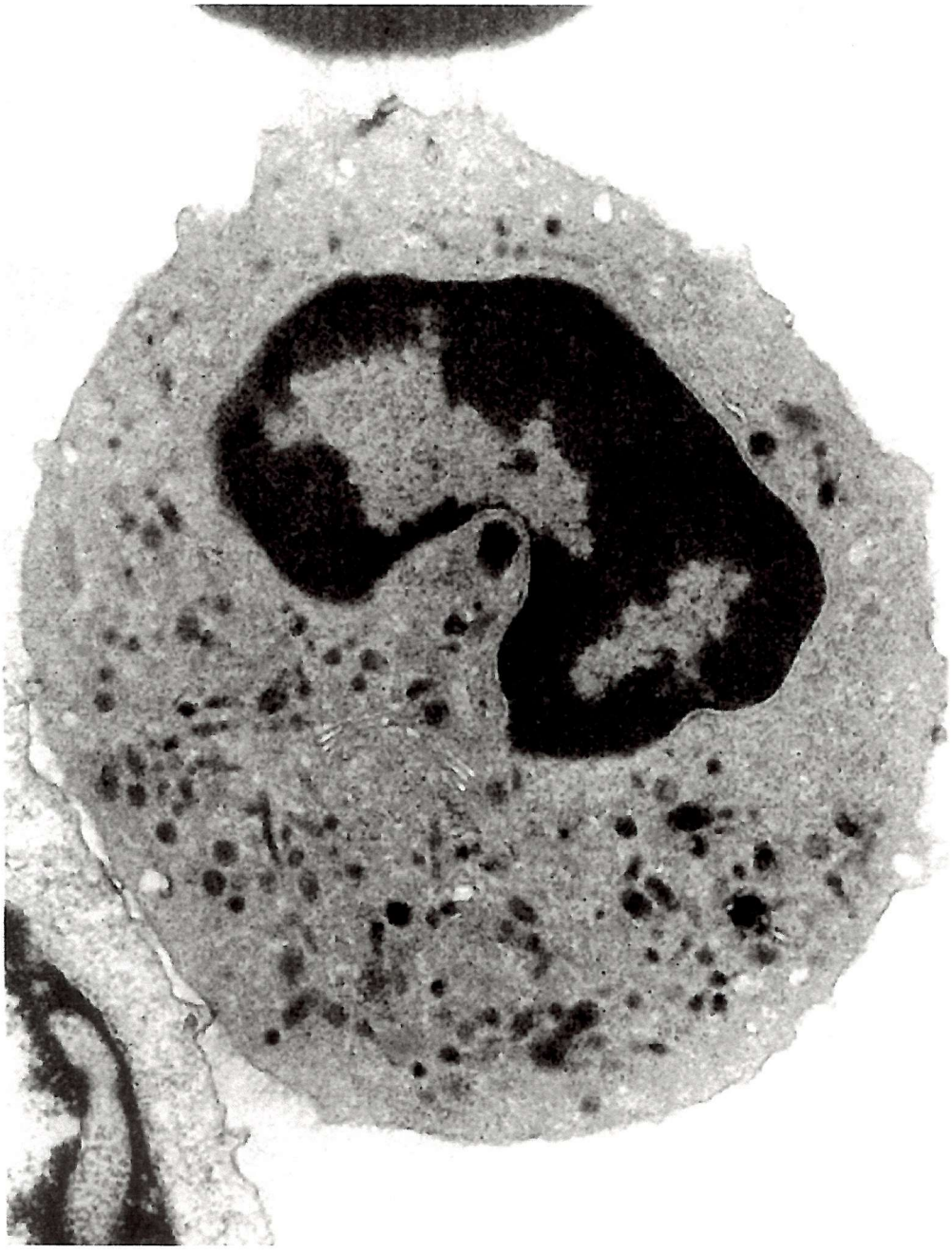
Disease progression: role of exacerbations.

Acute exacerbations are an important cause of morbidity and mortality found in COPD [Seemungal et al.,1998]. Exacerbations are defined by profound symptomatic deterioration often requiring hospitalisation, however little is known about their underlying pathology [Barnes, 1999]. Exacerbations are associated with patients reporting dyspnea and common cold symptoms immediately prior to onset. It is therefore likely that either bacterial or viral infection initiates a chain of events, culminating in increased inflammatory burden in the lung [Seemungal et al., 2000]. Following exacerbation recovery time correlates with severity of exacerbation, possibly reflecting efficiency of clearance of inflammatory cells.

1.2 Cellular Mechanisms in COPD.

As highlighted above the underlying feature of smoking associated COPD is thought to be exposure to cigarette smoke, inducing inflammatory cell recruitment to the lung. Recruited cells in turn release a variety of elastolytic proteinases, such as matrix metalloproteinases (MMPs), creating the potential for widespread matrix destruction. Metalloproteinases such as MMP-1 (interstitial collagenase), MMP-3 (stromelysin), matrilysin (MMP-7), gelatinase-B (MMP-9) and macrophage metallo-elastase (MMP-12) conveyed by inflammatory cells, have the collective ability to degrade all components of the extracellular matrix [Shapiro, 1999]. Therefore the recruitment of particular inflammatory cell types to the lung, the cross talk between them and the aberrant control of the proteinases they release are important factors in tissue damage and COPD progression.

Figure 1.4 facing page. Human neutrophil. Electron micrograph of a human neutrophil, showing the cytoplasmic granules. Magnification x9000. Courtesy of Dr. S. Wilson at Southampton general hospital.



1.2.1 The Neutrophil

1.2.1a Neutrophil development and granule contents.

Neutrophils are non-dividing phagocytic cells with a short life span, estimated to be 24-48 hours after release from the bone marrow. They are distinguishable as cells with a lobed nucleus, and extensively granular cytoplasm. The granules of the neutrophil represent a highly heterogeneous but highly regulated packaging system of storage organelles, containing a variety of secretory proteinases, anti microbial agents, and membrane associated proteins (see figure 1.5, and table 1.1). The organisation of these granules fundamentally affects how neutrophils interact with the external environment.

Neutrophils begin development in the bone marrow under the influence of G-CSF and GM-CSF, IL-6 and IL-3 released from stromal cells. Granules begin to appear as the neutrophil undergoes transition from myeloblast to promyelocyte [Bainton *et al.*, 1971, Borregaard and Cowland, 1997] (see figure 1.5). The first granules to be seen are the primary granules. These are defined by their positive staining for myeloperoxidase. They have a pH of 3.5 - 4, found to contain destructive acid hydrolases and proteinases. In addition they contain the neutral serine proteinases such as neutrophil elastase. The subsequent storage granules to appear in the neutrophil stain negative for myeloperoxidase. These are subdivided into specific (secondary) granules, defined by their contents: lactoferrin and neutrophil collagenase (MMP-8), and gelatinase (tertiary) granules, defined by their high concentration of gelatinase B (MMP-9).

Figure 1.5.

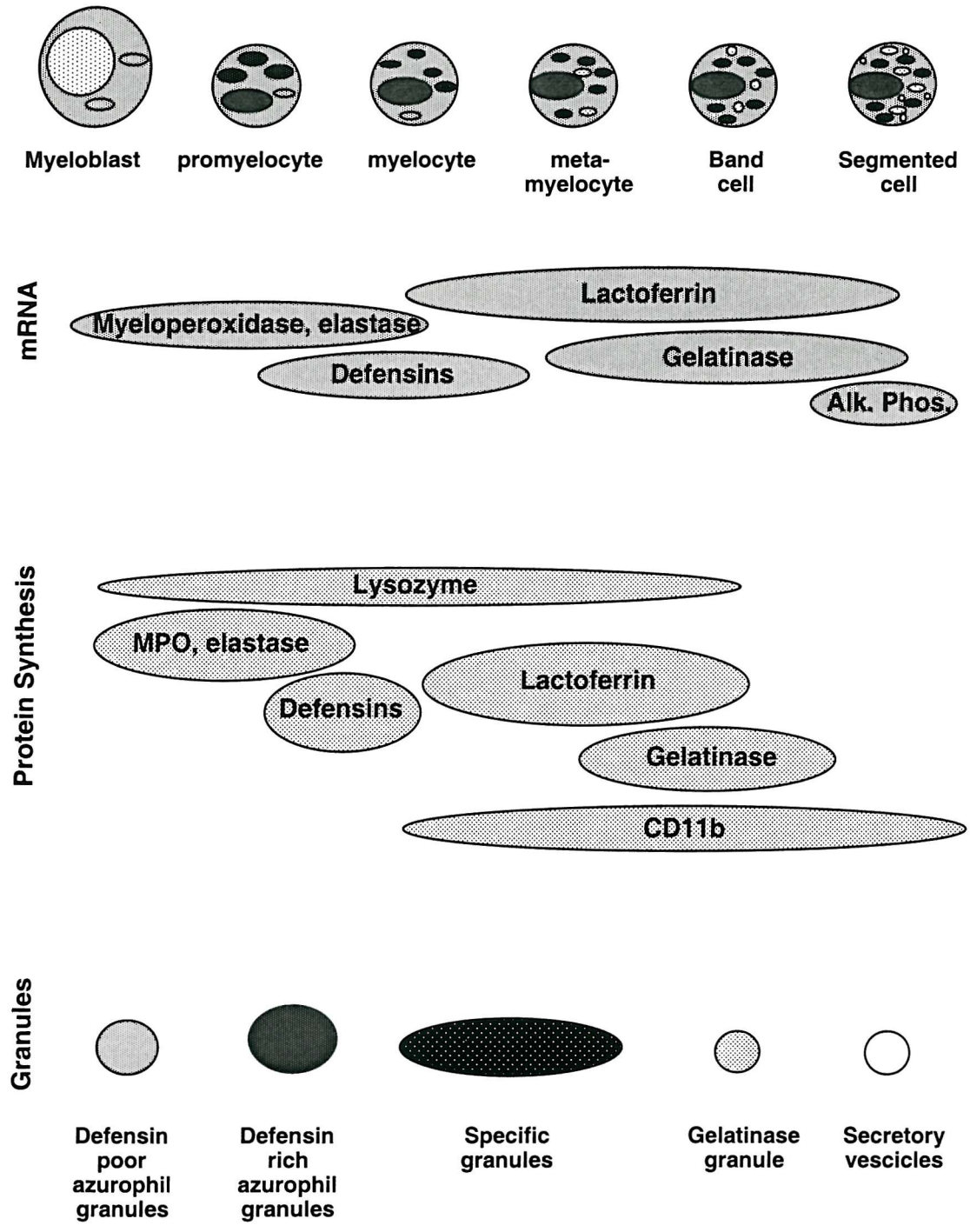


Figure 1.5. Classification of neutrophil granules. Granule proteins are synthesised at specific time points during the maturation of a neutrophil. Specific granules are filled with proteins synthesised at particular time points during neutrophil maturation, and are thus defined by age. Assignment of proteins to granules is not as a result of sorting proteins between individual granule subsets. (Adapted from Borregard and Cowland, 1997)

The specific and gelatinase granules appear second and third respectively during neutrophil development. The appearance of different granules through time suggests that sorting is dependent on distinct biosynthetic windows for each protein; all granule

proteins synthesised at the same time localise to the same granules. Synthesis of the tertiary (gelatinase filled) granules marks the end of cell differentiation; on their completion no further granule synthesis occurs. This static storage of MMP-9 distinguishes neutrophils from macrophages, where MMP-9 synthesis continues throughout the cells active life.

Degranulation of the neutrophil occurs in a reverse order to granule synthesis. The gelatinase granules are exocytosed more readily than specific granules, which in turn are exocytosed preferentially to primary granules. However the most rapidly mobilized intracellular organelles in the neutrophil are the secretory vesicles. These vesicles are essential for neutrophil adhesion and trafficking.

Table 1.1. The constituents and form of the neutrophil granlues.

	PRIMARY	SECONDARY	TERTIARY
SIZE / SHAPE	Spheres / elipsoid, approx 500nm dia.	Spherical (approx 200nm Dia.) or rod shaped (130 – 1000 nm)	
LYSOSOMAL ACID HYDROLASES	β- Glucoronidase, acid phosphatase, cathepsin B, cathepsin D.		
NEUTRAL SERINE PROTEINASES	Elastase, cathepsin G, proteinase 3.	Plasminogen activator	
NEUTRAL METALLO-PROTEINASES		Collagenase (MMP-8)	Gelatinase (MMP-9)
MICROBICIDAL FACTORS	Myeloperoxidase, lysozyme, defensins, cationic proteins.	Lysozyme, cytochrome b	
ADHESION MOLECULES /CHEMOTACTIC FACTOR REC.		Receptors for laminin, fibrinogen, vitronectin, fMet-Leu-Phe receptor. C3 (Mac 1)	
MISC.		Vitamin B12-binding protein, lactoferrin.	

(Adapted from Doherty N.S., Janusz M.J. 1994. Neutrophil proteinase: Their physiological and pathological roles. Immunopharmacology of Neutrophils)

1.2.1b The secretory vesicles and neutrophil migration.

Neutrophils move rapidly into the airways in response to inflammatory stimuli. Emigration of the neutrophil begins with the cell rolling the along the endothelial surface of the blood vessel. This is facilitated by interactions between L-selectin on the

neutrophil surface with E- and P- selectins on endothelial cells [Ley *et al.*, 1995, Simon *et al.*, 1995]. Signalling via these selectins [Simon *et al.*, 1995] or stimulation by inflammatory mediators such as leukotriene B_4 (LTB_4) and interleukin-8 (IL-8) [Sengløv *et al.*, 1993, Borregaard *et al.*, 1994] cause the neutrophil to rapidly change from an L-selectin presenting cell to a CD11b/CD18 (β_2-integrin) expressing cell. CD11b/CD18 presentation causes the neutrophil to firmly adhere to the endothelium [Hughes *et al.*, 1992]. The secretory vesicles are integral to this change of expression from selectin to integrin. The membranes of the highly mobile secretory vesicles are rich in CD11b/CD18 (β_2-integrin) adhesion molecules [Bainton *et al.*, 1987, Sengløv *et al.*, 1992, Borregaard *et al* 1994] and their rapid translocation and incorporation into the plasma membrane results in β_2-integrin expression. It is from here that the neutrophil begins to cross the endothelial barrier and basement membrane beyond into extravascular tissue.

The method of passage through the endothelial cell layer is currently a subject for debate. It was assumed that proteolytic degradation of the basement membrane by MMP-9 would rapidly follow on from adhesion since the gelatinase vesicles are the next most mobile behind the secretory vesicles [Dewald *et al.*, 1982, Sengløv *et al.*, 1992]. This hypothesis was supported by experiments showing that MMP-9 from both neutrophils and lymphocytes was essential for cell passage across synthetic matrigel membranes [Delclaux *et al.*, 1996, Leppert *et al.*, 1995]. Recently, Mackarel and co workers showed that FMLP stimulation indeed caused a large amount of MMP-9 release coupled with migration across endothelial cells. However migration was not actually retarded by MMP-9 inhibitors at concentrations well above those needed for MMP-9 inhibition; implying MMP-9 was not essential for this purpose [Mackarel, *et al.*, 1999]. Therefore despite the highly mobile nature of the neutrophil MMP-9 stores upon activation, the role of the enzyme remains unclear.

1.2.1c The role of neutrophils in COPD

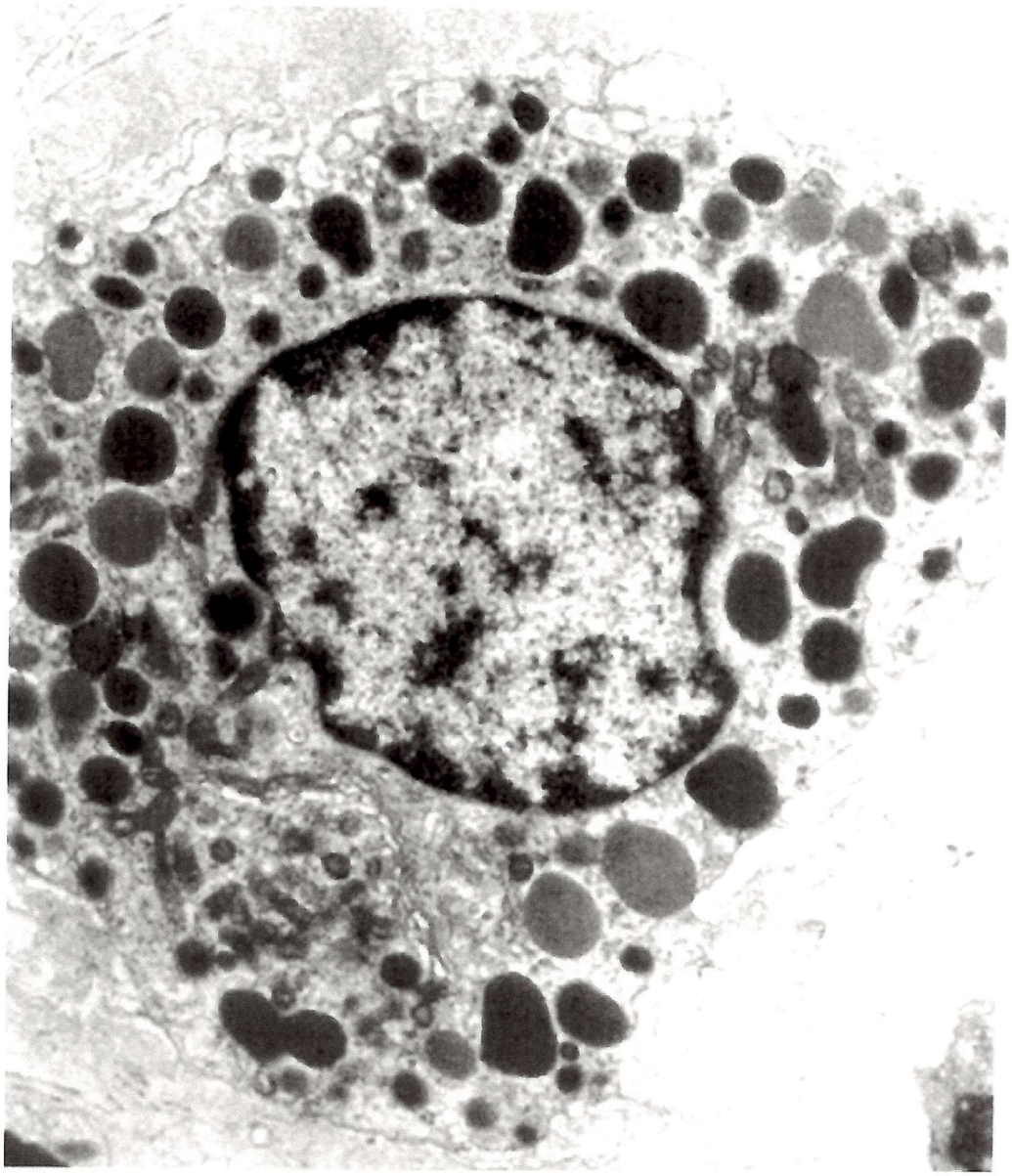
COPD patients show increased numbers of neutrophils in bronchoalveolar lavage fluid [Lacoste, *et al.,* 1993] and induced sputum [Keatings, *et al.,* 1996]. Their role remains to be properly established, however they seem to be able to pass quickly into the airway lumen from circulation [Lacoste, *et al.,* 1993]. This is borne out by high neutrophil numbers in BAL and sputum contrasting with low levels in airway walls, compared to CD4+ and CD8+ T-lymphocyte numbers. This rapid passage could be triggered by release of the neutrophil chemokine interleukin-8 (IL-8) from activated macrophages, [Hallsworth *et al.,* 1994] or epithelial cells exposed to cigarette smoke [Tadeshi *et al.*, 1997], or by the chemotactic effect of nicotine itself [Totti *et al.,* 1984]. Degranulation and release of proteolytic enzymes such as neutrophil elastase [Stockley, 1994] and matrix metalloproteinase-9 (MMP-9) [Tetley, 1993, Finlay *et al.*, 1997b] by waves of migrating neutrophils is likely to cause excessive extracellular matrix damage if unchecked. Conversely, certain authors have found that neutrophil inflammation negatively correlates with progression of emphysema, assessed by both immunohistochemistry [Finkelstein *et al.,* 1995], and CT scan [Selby *et al.,* 1991]. It is postulated therefore that neutrophil inflammation could mark an early phase of tissue damage, and that proteinase release mediated by lymphocytes or macrophages could be the factor behind more chronic lung damage [Finkelstein *et al.,* 1995].

1.2.1d Neutrophil clearance.

In order to limit the amount of tissue destruction caused by inflammation it is important to clear neutrophils from the affected area before they undergo disintegration (necrosis). The macrophage has an important role to play here by clearing neutrophils that have entered apoptosis. During apoptosis neutrophils effectively turn off; loosing the ability to degranulate on external stimulation [Whyte *et al.*, 1993], while still remaining intact.

Phagocytosis of neutrophils by the macrophage at this stage prevents leakage of the destructive neutrophil contents [Kar *et al.*, 1995]. Under normal conditions it also appears that as this phagocytosis occurs the macrophage turns from pro-inflammatory to anti inflammatory; release of pro-inflammatory chemokines (eg IL-8 and TNFα) decreases, and production of chemokines such as TGFβ increases, supressing further neutrophil recruitment [Fadok *et al.*, 1998]. It is possible that tissue damage in COPD could arise from the macrophage population being overwhelmed by apoptosis of large amounts of infiltrating neutrophils giving rise to excess necrosis. Lack of effective clearance of apoptotic cells, could thus lead to leakage of proteinases such as MMPs.

Figure 1.6 facing page. Alveolar macrophage. Electron micrograph of an alveolar macrophage, showing the numerous lysosomes and cytoplasmic granules. Magnification x12,000. Courtesy of Dr. S. Wilson at Southampton general hospital.



1.2.2 The alveolar macrophage.

1.2.2a Macrophage development and lysosomal contents.

The macrophage is the primary phagocytic cell in the immune system; they are found ubiquitously throughout the body dispersed in local tissues and are differentiated according to the particular defensive need of that tissue. Macrophages are the predominant defence cell both in normal human lung and during chronic inflammatory conditions such as COPD, where they are found to comprise 95-98% of the total cell content recovered by bronchoalveolar lavage (BAL) [Shapiro, 1999]. Significantly macrophages are found to concentrate in the respiratory bronchioles where emphysematous lesions first develop, therefore suggesting a major role for them in the pathogenesis of the disease [Cosio and Guerassimov, 1999].

Macrophages begin their development in bone marrow. Chemokines such as colony stimulating factor (CSF), granulocyte macrophage colony stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF) influence initial development and differentiation from committed stem cells. Through development these cells differentiate into monoblast, promonocyte and finally monocytes which pass into the blood stream. Basal release of monocytes can vary from 7 million cells per hour normally to 28 million during inflammation [Holgate and Church, 1993].

The monocytes undergo further development in the blood stream increasing the number of cytoplasmic lysosomes and developing increased phagocytic activity. During development the balance of proteinases in the lysosomes is found to change: In the early stages of development monocytes resemble neutrophils in that they contain serine proteinases such as neutrophil elastase and cathepsin G [Owen, *et al.,* 1994], as well as neutrophil-like azurophilic granules. Monocyte maturation is marked by a decrease in production of these enzymes to nil and increased gene expression of matrix metalloproteinases (MMPs). Final differentiation from monocyte into macrophages is

marked by elevation in MMP-1 and MMP-9 production, and decrease in MMP-7. Additionally synthesis of additional MMPs such as MMP-12 and MMP-3 is initiated [Campbell, *et al.* 1991] (see figure 1.7). Thus monocytes / macrophages show the ability to monitor MMP synthesis depending on the environment and activation state, dysregulation of this expression has the potential to augment matrix degradation which is characteristic of emphysema.

Figure 1.7

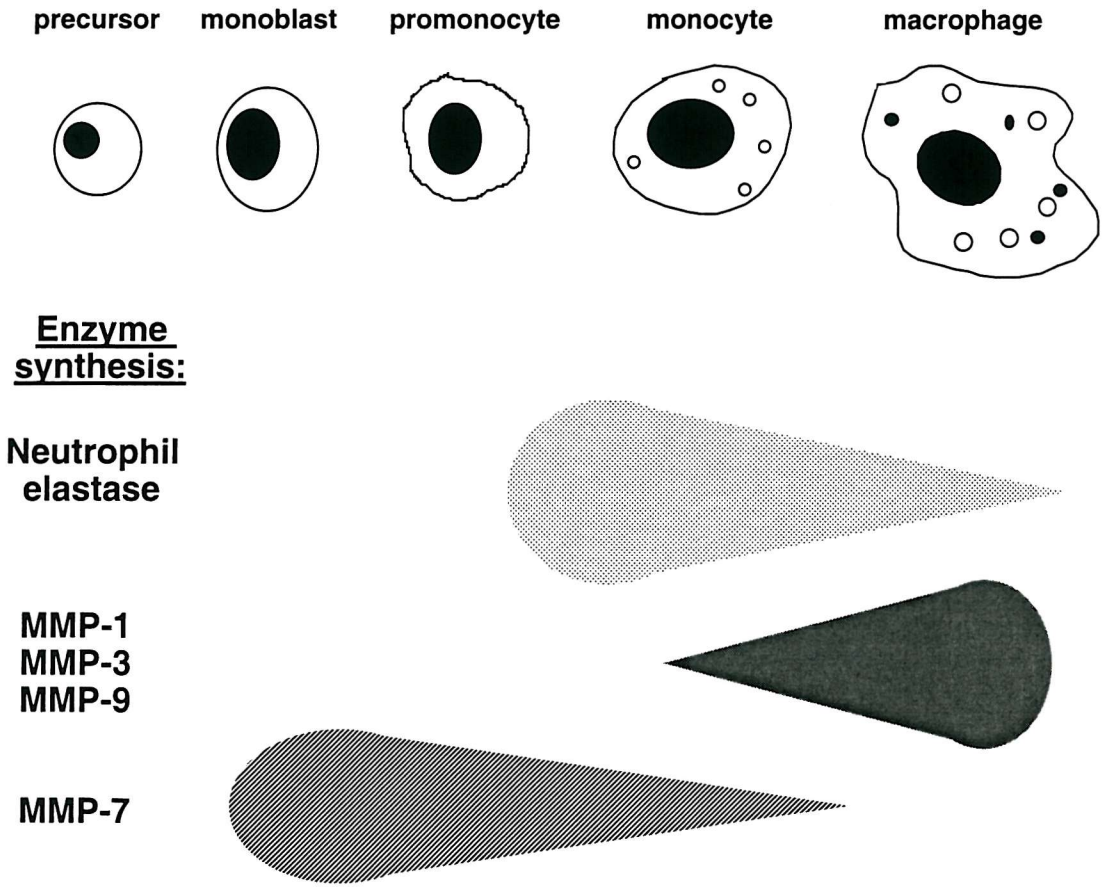


Figure 1.7 Proteinase expression through monocyte/ macrophage development. Distinct time points in the development of monocytes into macrophages are marked by the expression of different lysosomal proteinases. Unlike neutrophils, proteinase synthesis and expression in macrophages continues after the cell has fully differentiated. Immature monocytes express proteinases such as neutrophil elastase and cathepsin G. Upon maturation synthesis of these ceases and production of matrix metalloproteinases is upregulated. As monocytes develop into mature macrophages the relative amounts of MMP-1, MMP-3, MMP-7, MMP-9 and MMP-12 vary further depending on stage of development.

1.2.2b Macrophage activation.

When monocytes first settle down in the tissue as resident macrophages they are in a down regulated state; however elevation of killing ability is thought to be mediated through a powerful positive feedback mechanism. The macrophage has the ability to phagocytose infective agents, processing and presenting antigen to local CD4+ T-cells via MHC class II receptors. Subsequently the stimulated T-cell positively feeds back to the macrophage through the release of lymphokines such as IFNγ or TNFα (see figure 1.8). This fully activates the macrophage, increasing the amount of reactive oxygen species, cationic proteins, and enzymes such as MMPs released per cell.

Figure 1.8

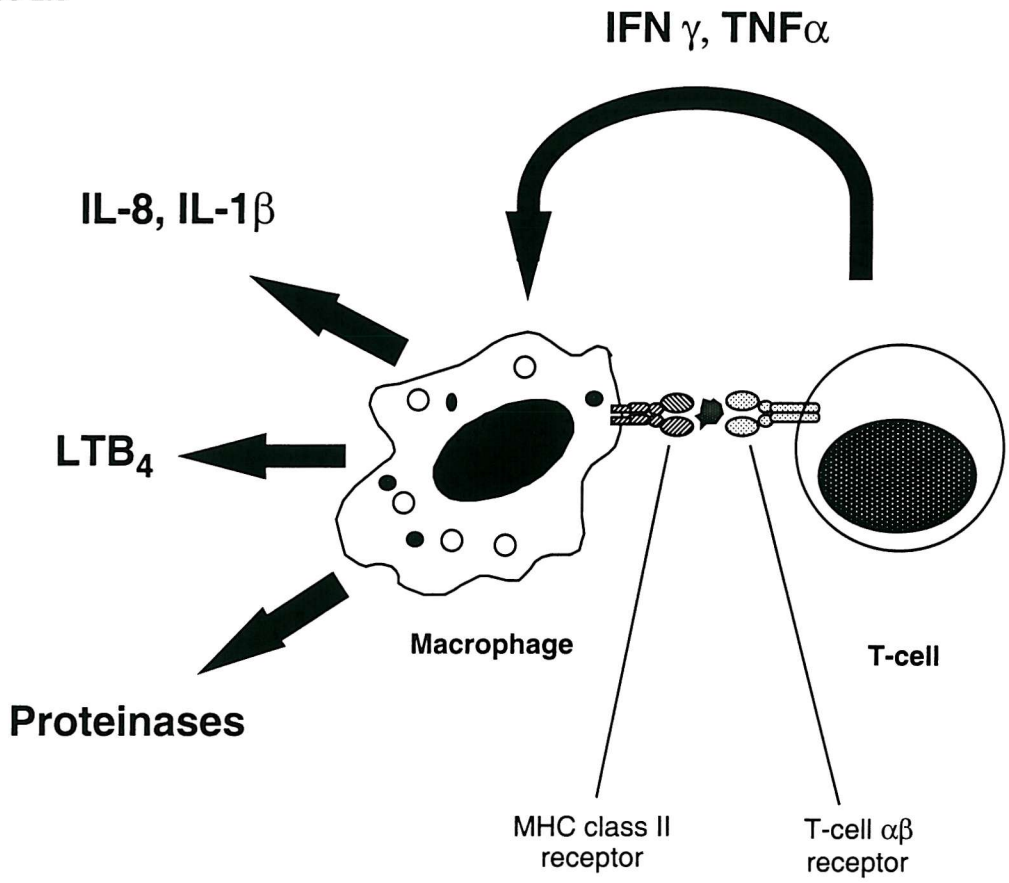


Figure 1.8 Antigen presentation to T-cells. Macrophages present antigenic particles of phagocytosed agents to T-cells via the MHC II receptor. Following recognition of the antigen T-cells release lymphokines such as IFNγ or TNFα, which activates the macrophage. In turn the activated macrophage releases proteinases such as the MMPs, as well as chemokines such as IL-8, LTB_4, TNFα, IL-1β augmenting a wider inflammatory response.

Activated macrophages augment a wider inflammatory response; releasing inflammatory mediators including interleukin-8 (IL-8), and leukotriene B_4 (LTB_4), important in attracting neutrophils or tumour necrosis factorα (TNFα) and interleukin-1β (IL-1β). These cytokines induce MMP production from both macrophages themselves and connective tissue cells, and have been detected at elevated levels in the sputum of COPD patients [Aaron *et al.,* 2001].

1.2.2c The role of macrophages in COPD

There is evidence that alveolar macrophages are affected and activated by cigarette smoke. These activated macrophages could be important in COPD pathology. Populations of alveolar macrophages recovered from the airways of smokers differ in morphology from those recovered from non-smokers. Macrophages from smokers are found to contain a large subpopulation of cells with a far more highly pigmented cytoplasm, and the ability to release more reactive oxygen species than their counterparts obtained from non-smokers [Schaberg *et al.,* 1995]. Interestingly these cells also show increased expression of CD11b/CD18, suggestive of a readily recruitable cell population [Schaberg *et al.,* 1995]. In support of increased, smoking associated recruitment of macrophages, cell numbers are found to be elevated five to tenfold in BAL fluid from smokers with COPD. This infiltration is found to be concentrated in centriacinar zones of the respiratory bronchioles where alveolar wall destruction is found to correlate with increase in macrophage number [Finkelstein, *et al.,* 1995]. It is likely that these macrophages have a dual influence on alveolar remodelling. One influence is by release of their payload of proteinases. The spectrum of the macrophage proteinases changes with developmental stage. Proteinase expression changes from predominantly serine proteinases in blood monocytes, to MMP expression in lung macrophages [Shapiro, 1994]. Enzymes such as MMP-9 [Shapiro, 1994, Finlay

et al., 1997b] macrophage metallo elastase (MMP-12) [Shapiro S.D. 1994, Finlay *et al.,* 1997b] and Interstitial collagenase (MMP-1) [Finlay *et al.,* 1997b] could influence remodelling. Importantly Shapiro and co-workers have demonstrated that macrophoge metalloelastase (MMP-12) knockout mice do not develop emphysema when exposed to cigarette smoke, while normal animals do [Hautamaki *et al.,* 1997]. Additionally Finlay and co-workers identified increased expression of MMP-1 and MMP-9 mRNA in alveolar macrophages from patients with emphysema [Finlay *et al.,* 1997b]. Both lines of evidence are suggestive of involvement of macrophage derived MMPs in destructive lung diseases such as emphysema. The important way macrophages influence remodelling is via the control they exert on other inflammatory cells. Macrophages are important controllers of neutrophil recruitment (described in 1.3.2) and clearance (described in 1.4.3). Lack of effective clearance of apoptotic neutrophils, coupled with excessive infiltration could leave a large number of necrotic cells leaking their proteolytic contents and adding to tissue injury. This could occur if the macrophage response was characterised by monocytes that have had insufficient time to mature into tissue macrophages [Haslett, 1999].

1.2.3 The T-Lymphocyte.

1.2.3a Categories and roles of T-Lymphocytes.

T-Lymphocytes have a pivotal role in controlling inflammatory response. They are distinguished by their expression of CD3 antigens on their surface and are further divided into 2 subsets with distinct functions depending on whether they express the markers CD4+ or CD8+. CD4+ cells have a pro-inflammatory function; they are known to secrete a variety of lymphokines on activation including IFNγ, TNFα, and IL-8 all implicated in COPD. Collectively all of the cytokines released by CD4+ cells have the capacity to augment turnover and activity of all granulocytes. CD4+ cells are

selectively activated by presentation of antigens on the surface of certain cell types. Antigens are presented to these cells by the Major histocompatibility complex class II (MHC class II) molecules, found only on the surface of monocytes, macrophages, dendritic cells and B-lymphocytes (see figure 1.8). CD8+ T-cells have the ability to directly recognise and kill malignant or infected cells, or cells found bearing non self histocompatibility antigens. Unlike the CD4+ lymphocytes, CD8+ cells recognise antigens presented in association with MHC class I antigens on the surface of presenting cells. MHC class I have a far greater distribution than MHC class II antigens, being found on all cell types in the body. Thus CD8+ cells have the ability to respond to malignancy or infection in any cell type.

1.2.3b The role of lymphocytes in COPD.

Interactions between macrophages and T-lymphocytes are thought to be important influencing factors in chronic lung damage (see 1.2.2b). Investigators have found increased numbers of both macrophages and activated T-cells in lung biopsies from chronic bronchitics [Saetta *et al.,* 1993], and have identified a positive correlation between these cell types and extent of lung damage [Finkelstein *et al.,* 1995]. Beyond this however, the knowledge of lymphocyte involvement in COPD is less well defined. Some authors cite CD8+ T-cells as the main infiltrating T-cell sub group in smokers with COPD [O' Shaughnessy *et al.,* 1997, Saetta *et al.,* 1999]. Excessive recruitment of CD8+ T cells may occur in response to repeated viral infection often found to be an underlying feature in the lungs of smokers with COPD [Seemungal *et al.,* 1998]. Increased numbers of CD8+ cells have also been found in the circulation of heavy smokers, with numbers falling following smoking cessation [Hughes *et al.,* 1985]. As a result it has been speculated that CD8+ cells could mediate acute tissue damage in

smokers lungs. Interestingly it has been found that transgenic mice exhibiting induced over expression of Interferon-γ (IFNγ), a product of CD8+ cells, spontaneously develop emphysema. This emphysema mirrors that seen in humans, displaying alveolar enlargement, enhanced lung volumes and pulmonary compliance and a neutrophil/ macrophage rich inflammation [Wang *et al,* 2000]. It was also noted that the emphysema was accompanied by the induction of MMP-9 and MMP-12, cathepsins B,H,D,S and L, and reduction in secretory leucocyte proteinase inhibitor. Thus there was an important shift in the pulmonary proteinase/ antiproteinase balance [Zheng *et al,* 2000].

In COPD patients who have ceased smoking, CD8+ cell reduction [Hughes *et al,* 1985] may be accompanied by an increase in CD4+ cells; as CD4+ cells are found to predominate in the mucosa of ex-smokers[Rutgers *et al.,* 2000]. It has also been speculated that CD4+ lymphocytes, predominant in asthma, may be have a in a particular role during COPD exacerbations [Zhu *et al,* 2001]. CD4+ associated cytokines have been associated with potentially important roles in mediating ongoing chronic inflammation in COPD. These include IL-13 [Zheng *et al,* 2000] and the recently discovered IL-17 [Spriggs *et al.,* 1997].

It has been demonstrated that inducible over expression of IL-13, as with over expression of IFNγ, caused emphysema in adult mice which mirrored that seen in humans. The inflammation was found to be eosinophil and macrophage rich, and an excessive proteinase burden was found to comprise of high levels of MMP-9, MMP-12, MMP-2, MMP-13 and MMP-14. While TIMP-1 production was also induced it was not thought to be of significant magnitude to inhibit the increased proteinase burden.

IL-17 is a recently discovered CD4+ cytokine which induces release of IL-8 from human lung fibroblasts, human bronchial epithelial and venous endothelial cells *in vitro* [Fossiez *et al.,* 1996, Laan, *et al.,* 1999]. Importantly it is found to cause the selective

recruitment of neutrophils into rat airways *in vivo* [Laan *et al.,* 1999] thus potentially increasing MMP-9 burden in the airways .

In addition to marshalling the influx of cells with the capacity to damage tissue, lymphocytes have the ability to cause degradation of the extracellular matrix in their own right. Both CD8+ and CD4+ lymphocytes produce MMP-9 which is selectively modulated by chemokines [Johnatty *et al.,* 1997], thought to mediate basement membrane transmigration [Leppert *et al.,* 1995], and it is conceivable that these enzymes contribute to a proportion of the lung damage. TIMP-1, the innate inhibitor of MMP9 is also produced constitutively by T-lymphocytes. MMP production by CD3+ lymphocytes is known to be induced by IL-2 [Xia, *et al.,* 1996], it is therefore interesting that the IL-2 receptor is found over expressed on lymphocytes in bronchitic airway mucosa, compared with normal mucosa [Saetta, *et al.*, 1993]. In summary, it is possible that lymphocytes are important players in the process of tissue damage seen in COPD. It is probable that their influence is exerted either by augmenting granulocyte infiltration into the COPD airways or by the proteinase-mediated entry of lymphocytes themselves into the tissue.

Figure 1.9

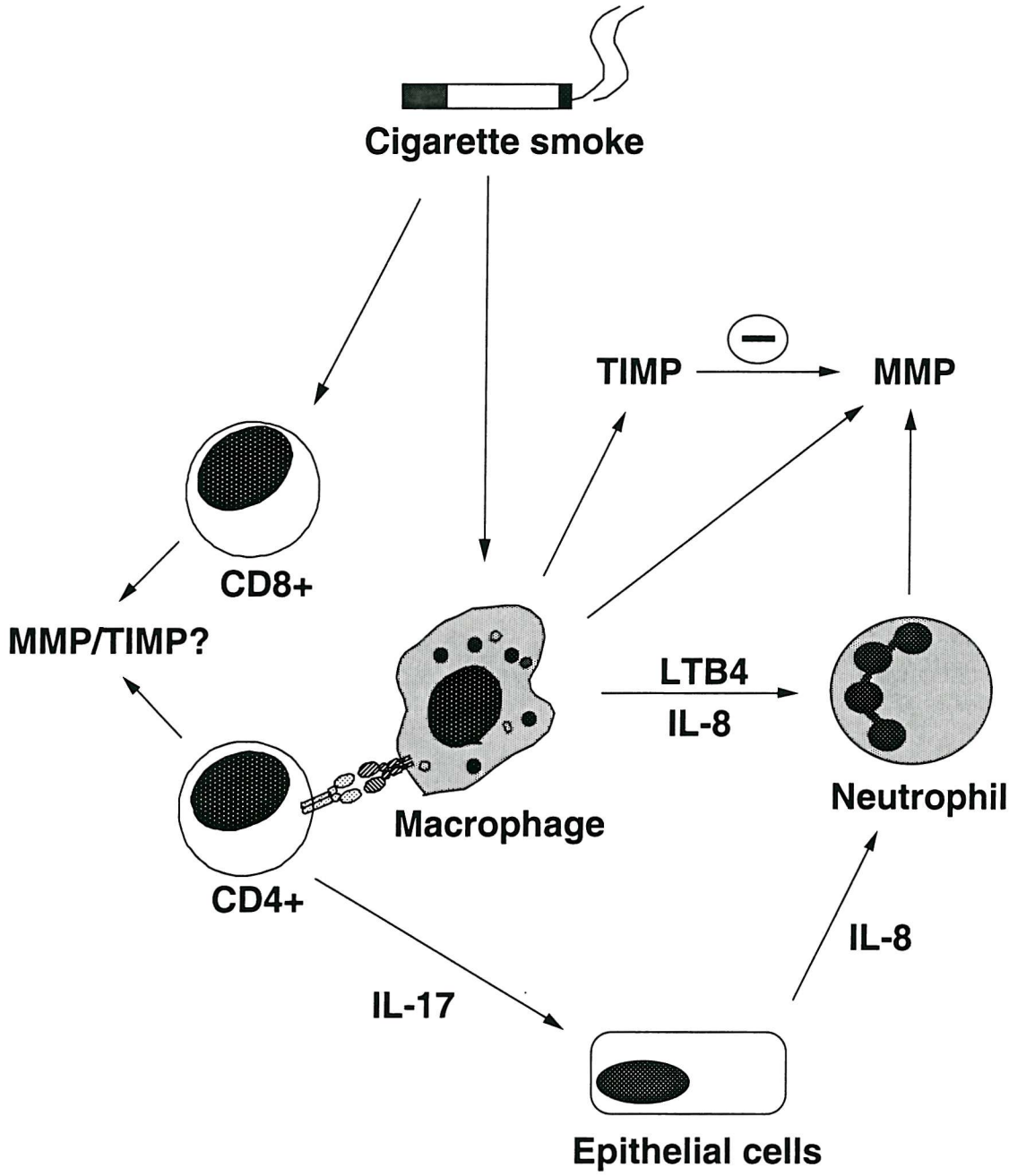


Figure 1.9 Summary of inflammatory mechanisms in COPD. Cigarette smoke causes activation of alveolar macrophages, along with an increase in CD8+ lymphocytes in the lung. CD8+ lymphocytes potentially damage lung tissue by release of lytic agents such as granzyme, perforin enzymes such as the MMPs. Activated macrophages release chemokines such as LTB_4 and IL-8, resulting in neutrophil recruitment to the airways. Additionally CD4+ cells activated by antigen presenting macrophages release IL-17, which in turn induces IL-8 production by epithelial cells. This chain of events again results in neutrophil recruitment to the lung. Degranulation of neutrophils and release of proteinases such as MMPs from activated macrophages results in connective tissue destruction. Additionally release of proteinases including MMPs from activated CD4+ and CD8+ lymphocytes contributes to tissue destruction.

1.2.4 The eosinophil.

Eosinophils are bone marrow derived cells which undergo maturation under the influence of IL-3, IL-5 and GM-CSF [Rothenberg, 1998]. Following maturation they enter the blood for approximately 24 hours and then migrate into tissues. Eosinophils have many potential roles in the tissue, ranging from immunoregulation [Weller *et al.,* 1996], tissue remodelling and repair [Okada *et al.*, 1997] and killing foreign organisms [Rothenberg 1998]. The specific granules in the cytoplasm are endowed with an array of mediators for eosinophil function. They contain up to 20 different cytokines, chemokines and growth factors and large amounts of the tissue toxic proteins including: major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil derived neurotoxin [Erjefalt and Persson, 2000]. Additionally eosinophils contain proteinases such as serine and metalloproteinases, thought to be involved in cell migration through basement membrane [Okada *et al.,* 1997].

Eosinophils are found to predominate in airway and mucosal tissues in asthma, allergic rhinitus, eosinophillic pneumonia and chronic cough [Erjefalt and Persson, 2000], however the evidence for eosinophilia in COPD is conflicting. Although airway eosinophilia has been found during exacerbations in COPD [Saetta *et al.,* 1994 Zhu *et al,* 2001], increase in airway eosinophils during stable COPD has been found in some studies [Lacoste *et al.,* 1993, Balzano *et al.,* 1999] but not in others [Linden *et al.,* 1993, Lusuardi *et al.,* 1994]. It is conceivable that this is a result of overlap between COPD and asthma (see figure 1.1). Interestingly one study identified that sputum eosinophilia in COPD was potentially associated with a slight benefit from corticosteroid treatment [Pizzchini *et al.*, 1998]. The heterogeneity of data suggests that patients exhibiting eosinophilia represent a sub population in general COPD pathology, however firm conclusions regarding eosinophil involvement are hard to draw.

1.3 The matrix metalloproteinases.

1.3.1 Model for pathogenesis: proteinase / antiproteinase imbalance.

Destruction of extracellular matrix and the resultant alveolar remodelling is influenced by the infiltration of the inflammatory cells, highlighted above, and the proteinases they release. The concept that imbalance between proteinases and antiproteinases contributes to lung damage in COPD has been held for 30 years. Firstly in the 1960s Laurell and Eriksson identified that patients with decreased amounts of a protein, subsequently shown to be the neutrophil elastase inhibitor α_1-antitrypsin, were shown to develop early onset emphysema [Laurell and Eriksson, 1963]. Following on from this it was shown that emphysematous lesion could be induced laboratory animals whose lungs had been instilled with either the proteolytic enzyme papain [Gross *et al.,* 1964], or neutrophil elastase [Janoff *et al.,* 1977]. Leading on from these findings many studies have concentrated on neutrophil elastase as the main protagonist in alveolar destruction, while others have extrapolated the findings to look at imbalance between other proteolytic enzymes and their inhibitors. Janoff and co workers demonstrated that elastolytic activity found in BAL fluid from smokers could be inhibited by metal chelators. The conclusion drawn was that matrix metalloproteinases (MMPs) were responsible for a large proportion of elastolytic activity [Janoff *et al.,* 1983].

1.3.2 Normal and pathological function of MMPs.

The matrix metalloproteinases (MMPs) are a family of up to 24 homologous, zinc dependant endopeptidases involved in extracellular matrix turnover and remodelling in both normal and diseased tissue. Collectively the MMPs can degrade all of the protein components of the extracellular matrix. Very low MMP levels are found in normal steady state tissues [Nagase, 1997]. However MMP increased activity is found in tissues undergoing development and repair; such as embryogenesis, ovulation, endometrial

cycling, nerve growth, and wound healing to name a few. Uncontrolled MMP activity is involved in the generation of a range of different diseases including arthritis, cancer, cardiovascular disease, neurological pathologies, ulcerations and liver fibrosis [Nagase, 1999]. There is also evidence for MMP over expression in various inflammatory lung diseases, such as adult respiratory distress syndrome (ARDS), cystic fibrosis, asthma and COPD [Shapiro and Senior, 1999]. In particular attention has focused on the role of MMP-9 in asthma and COPD. Atopic asthma is characterised by allergen induced tissue injury, accompanied with an abberant repair process leading to remodelling and airflow obstruction [Busse, 1993]. Increased concentrations of MMP-9 have been detected in the BAL fluid [Mautino *et al.,* 1997, Lemjabbar, 1999] and sputum [Vignola *et al.,* 1998, Tanaka *et al.,* 2000] of asthmatics compared to normal individuals. Additionally there has been evidence showing that allergen challenge induces a significant increase in detectable levels of MMP-9 in asthmatics [Warner *et al.,* 1997, Becky Kelly *et al.,* 2000]. These data suggest involvement of MMP-9 in remodelling in asthma.

As highlighted above (1.3.1) it is thought that proteinase / antiproteinase balance is a major factor influencing tissue damage in COPD. As with asthma there is considerable evidence for MMP involvement. Increased levels of neutrophil collagenase (MMP-8) and gelatinase-B (MMP-9) have since been identified in the BAL fluid from patients with emphysema [Finlay *et al.,* 1997a]. Increased levels of MMP-9 have also been found in sputum from chronic bronchitics [Vignola *et al.,* 1998], and identified immunohistochemically in lung parenchyma of patients with emphysema [Ohnishi *et al.,* 1998]. The interest in MMPs has been underlined by a variety of studies in transgenic mice. Initial studies found that mice over expressing MMP-1 went on to develop emphysemaous lesions [D'Armiento *et al.,* 1992]. This was followed by studies which macrophage metalloelastase MMP-12 knock out (MMP-12 -/-) mice do not develop emphysema when exposed to cigarette smoke, where MMP-12 +/+ mice do

[Hautimaki *et al.,* 1997]. These studies clearly pointed to the potential role of matrix metalloproteinases in the development of COPD. Recent studies have added to this work, showing that the selective induction of pro inflammatory cytokines IL-13 and IFNγ causes an emphysema like pathology, marked by significant increases in MMPs such as MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 [Zheng *et al,* 2000 and Wang *et al.,* 2000]. The increase in proteinase burden was found to be accompanied by a decrease in antiproteinase screen, however it was also found that treatment of mice with MMP and cysteine proteinase inhibitors prevented the development of emphysema [Zheng *et al,* 2000b].

1.3.3 Cytokine control of MMPs.

The catalytic activity of MMPs is regulated at multiple levels including transcription, secretion, activation and inhibition. An understanding of the transcriptional control of MMPs is of great relevance to understanding the role of the proteinases in inflammatory pathologies. Production of MMPs has been found to be regulated at transcriptional level by a wide variety of cytokines and growth factors [Woessner, 1991; Lacraz *et al.*, 1995; Saren *et al.*, 1996] (see Table 1.2 adapted from O'Connor 1994). For example, both IL-1β and TNFα have been found to increase the release of MMP-9 from human macrophages [Saren *et al.*, 1996]. In addition to this, studies on the human collagenase gene have shown that these cytokines induce an upregulation in collagenase production [Woessner, 1991]. Further investigation has shown that IL-1β and TNFα increase transcription factor binding to an AP-1 binding site, situated 5' to the collagenase gene [Woessner, 1991]. In this model, transcription factors c-Fos and c-Jun bind to AP-1 as a *leucine zipper* heterodimer. The prevention of the synthesis of c-Fos by cycloheximide is found to inhibit the response to either of the cytokines [Woessner, 1991]. Cytokines do not always show a simple augmentation or reduction of one particular MMP; the

effect of TGFβ on fibroblasts gives an example of interconnection of responses. TGFβ causes a decrease in production of collagenase and stromelysin while increasing the production of the gelatinases: MMP-2 and MMP-9, this is found to be exaggerated when given in combination with IL-1 [Chandrasekhar *et al.*, 1988].

Table 1.2. Regulation of MMP and TIMP Expression in stromal cells.

ENZYME / INHIBITOR	UPREGULATION	REPRESSION
Interstitial collagenase (MMP-1)	IL-1β, TNFα, PDGF, EGF, bFGF, TGFβ, NGF	TGFβ, IFNγ, IL-4, glucocorticoids, retinoids.
Gelatinase A (MMP-2)	TGFβ, TNFα, IL-1β	
Gelatinase B (MMP-9)	TGFβ, TNFα, IL-1β	IL-10, IL-4, IFNγ
Stromelysin-1 (MMP-3)	IL-1β, TNFα, PDGF, EGF, bFGF, TGFα, NGF.	TGFβ, IFNγ, glucocorticoids, retinoids.
TIMP*	IL-1β, TNFα, PDGF, bFGF, IL-6, IL-10, TGFβ, (TIMP-1), glucocorticoids, retinoids.	TGFβ, (TIMP-2)

IL = interleukin; TNF = Tumour necrosis factor; PDGF = platelet derived growth factor; EGF = epidermal growth factor; bFGF = basic fibroblast growth factor; TGF = transforming growth factor; NGF = nerve growth factor; IFN = interferon.
* Most studies do not distinguish between TIMP-1 and TIMP-2 .TGFβ is reported to have differing effects on the expression of these inhibitors, causing upregulation of TIMP- 1 and repression of TIMP-2. (From O'Connor and Fitzgerald, 1994).

1.3.4 Molecular biology and structure.

There is a multiplicity of MMPs seen throughout nature, to date a total of 66 have been sequenced of which 24 are found in humans. The human enzymes have counterparts found in other vertebrates, MMPs have been found in invertebrates, and recently in plant sources [Massova *et al.*, 1998].

Most members of the MMP superfamily possess three main highly conserved structural domains (see figure 1.11): an amino terminal propeptide; a catalytic domain; and a hemopexin like domain at the carboxy terminal. MMPs are all secreted as latent zymogens containing a propeptide domain, approximately 80-90 amino acids in length. The propeptide contains the highly conserved sequence PRCG(V/N)PD which interacts with a key zinc ion (known as the catalytic zinc ion) co-ordinated to the catalytic domain of the molecule, the distortion in protein structure prevents access of water molecules to the active site, effectively blocking catalytic activity [Nagase, 1997]. MMP

zymogens must under go stepwise removal of this propeptide before becoming fully active.

Essential features of the catalytic domain are two zinc and one calcium ion which are co-ordinated to histidine residues in the conserved sequence: HEXGHX[L/M]G[L/M]XH. One of the two zinc ions is essential in the catalytic mechanism of MMPs where as the other zinc and the calcium ion are thought to have a role related to the structural integrity of the protein. MMPs have high affinities for these structural zinc and calcium ions [Massova *et al.,* 1998]. The hemopexin like domain of MMPs is the third highly conserved region in the protein and as its name suggests, it shows a high sequence similarity to the plasma protein hemopexin. This domain has been shown to play a functional role in substrate binding and in interactions with the specific inhibitors of the MMPs the Tissue Inhibitors of metalloproteinase (TIMP) [Gomis-Ruth *et al.*,1997, Olson *et al.,* 1997]. The structures of MMP-2 and MMP-9 (also known as gelatinases A and B) are distinct from those of the other MMPs since both contain additional 175 residue inserts in their catalytic regions, which have homology to the collagen binding regions in fibronectin. The inserts are thought to give both MMP-2 and MMP-9 their specificity by enabling the binding of denatured collagen or gelatin [Murphy *et al.*, 1994].

Figure 1.10 facing page. General 3D structure of the MMPs. The 3 major domains of the MMPs are illustrated; the propeptide region is shown as a green ribbon, the catalytic domain is coloured pink and the hemopexin like domain is shown as a yellow ribbon. The catalytic zinc is shown as an orange sphere; the calcium ions in the hemopexin like domains are shown as cyan spheres. (Taken from Massova *et al.,* 1998).

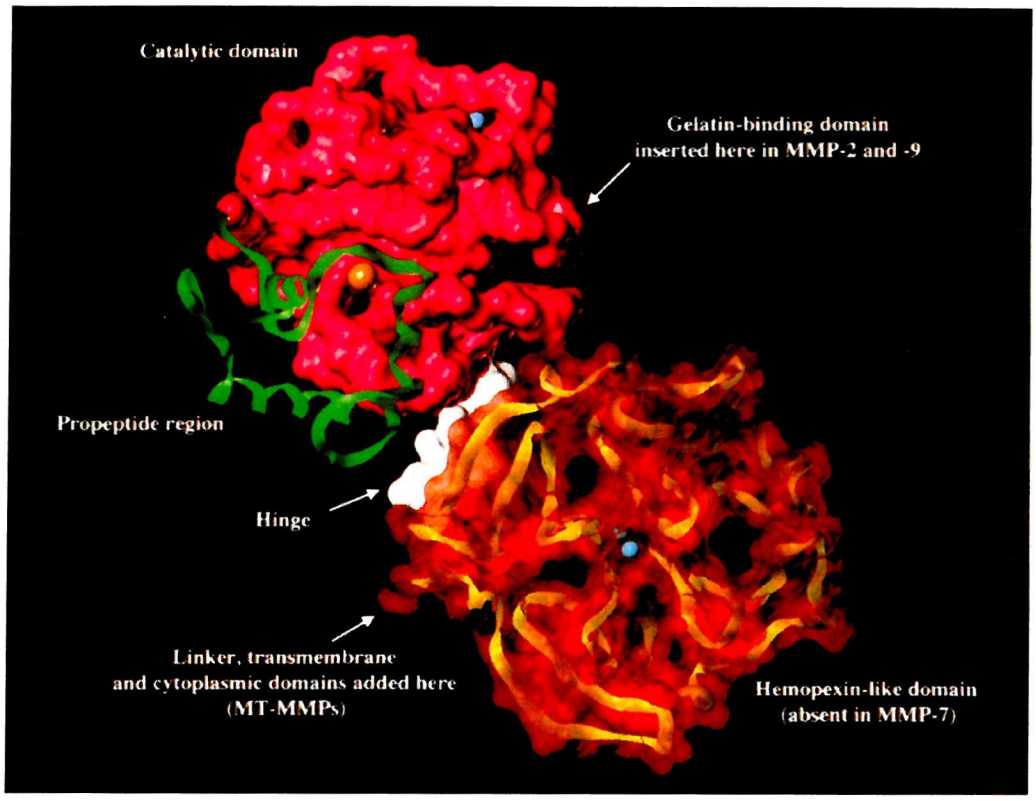

Catalytic domain
Gelatin-binding domain
inserted here in MMP-2 and -9
Propeptide region
Hinge
Linker, transmembrane
and cytoplasmic domains added here
(MT-MMPs)
Hemopexin-like domain
(absent in MMP-7)

Figure 1.11

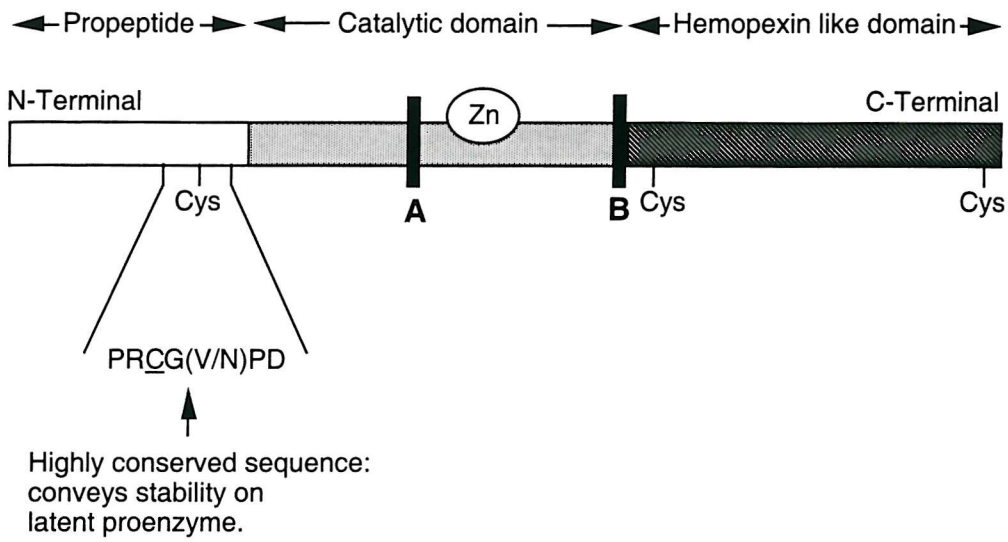


Figure 1.11 Domain structure of MMPs. MMPs have three major domains in their structure: The N terminal propeptide domain (approximately 80 amino acids), the catalytic domain (approximately 170 amino acids), and the C terminal hemopexin like domain (approximately 210 amino acids). The propeptide domain contains a conserved cysteine residue. The tertiary structure of the enzyme is such that this cysteine ligates a zinc ion in the catalytic domain of the enzyme, protecting the active site and necessitating proteolytic removal of the propeptide for MMP activation. The catalytic domains in both MMP-9 and MMP-2 also contain inserts with fibronectin type II homology. These convey substrate specificity for gelatins and collagens. The C-terminal hemopexin like domain interacts with the inhibitors of MMPs (TIMPs), as well as being thought to convey specificity for fibronectin and heparin onto the proteinases (adapted from O'connor and Fitzgerald, 1994 see also figure 1.10 for crystallographic structure of MMPs).

1.3.5 MMP Activation:

All MMPs are released as latent zymogens, and thus an important point of control of MMP activity is at the point of conversion from latent to activated enzyme. Van Wart and Birkedal Hansen working on human fibroblast collagenase proposed that the conserved cysteine residue; Cys^{73} (from the conserved propeptide sequence PRCG[V/N]PD), was co-ordinately bound to the catalytic zinc ion therefore causing the active site to be masked by the propeptide [Van Wart *et al.*, 1990]. This effectively excludes water from the site and inhibits catalytic activity. A key step in MMP activation is the disruption of this co-ordinate bond; this can occur by two different methods (see figure 1.13):

The first of these is a two-stage proteolytic cleavage; the initial phase is fulfilled by an *"activator"* proteinase. This proteinase cleaves a region in the middle of the N-terminal

propeptide, inducing a conformational change in the remaining propeptide, and creating a partially active enzyme. The conformational change renders the peptide susceptible to further proteolytic attack by a second activator proteinase. This proteinase is usually distinct from the first and acts to completely cleave the N-terminal propeptide [Springman *et al.*, 1990, Suzuki *et al.*, 1990]. This second cleavage step can occur in some MMP species by an autocatalytic mechanism, where the remaining propeptide segment is directed towards the active site of the enzyme as a result of the initial conformational change [Woessner, 1991, Nagase, 1997]. The proteinases involved in the activation of a particular MMP, are specific to that MMP, however they vary in type, and cellular source therefore forming a varied activator: proteinase network (see table 1.3).

Table 1.3 Activator proteinases of the MMPs.

Latent MMP	Activator proteinase	
	Initial step	**Final step**
ProMMP-1	Trypsin, plasmin, plasma kallikrein	MMP-3, MMP-2, MMP-7, MMP-10, Chymase.
ProMMP-2	MT1-MMP, MT3-MMP, MMP-1	MMP-2, MMP-7
ProMMP-3	Trypsin, chymotrypsin, plasmin, chymase, neutrophil elastase, pseudolysin, thermolysin (not activated by any MMPs).	MMP-3
ProMMP-7	Trypsin, plasmin, neutrophil elastase.	MMP-3, MMP-7
ProMMP-8	Tissue kallikrein, neutrophil elastase, cathepsin G, trypsin.	MMP-3, MMP-10
ProMMP-9	MMP-1, MMP-2, MMP-3, MMP-7	MMP-1, MMP-2, MMP-3, MMP-7
ProMMP-10	Plasmin, trypsin, chymotrypsin	MMP-10
ProMMP-11		Furin
ProMMP-13	MMP-3, MT1-MMP	MMP-3, MMP-13, MMP-2
ProMT1-MMP		Furin

Different types of latent MMP are activated by interaction with various activator proteinases. Examples of these interactions are listed. (Adapted from Nagase, 1997).

A good example of such an activation network is the activation of MMP-9 (illustrated in figure 1.12). Here the important activator proteinase for proMMP-9 is MMP-3. The cycle starts with MMP-3 being partially activated by neutrophil elastase, trypsin or plasmin. MMP-3 itself completes its activation by autocatalytic cleavage of its N-terminal region. The fully active MMP-3 partially activates MMP-9 by cleaving the N-

terminal region. It then completes the activation process by cleaving the remaining propeptide region. This activation process can be retarded to a degree by the specific inhibitor of MMP-9, TIMP-1. This inhibition can however be overcome by the addition of excess amounts of MMP-3, which destabilises enzyme / inhibitor complex (see section 1.4.5 and figure 1.12). Additionally TIMP-1 can be inactivated by the proteinase neutrophil elastase, leaving MMP-9 susceptible to activation by MMP-3. There are a variety of enzymes implicated in MMP-9 activation (see table 1.3), therefore a variety of activation networks exist for MMP-9 alone. This illustrates the fine balance that must be maintained between a variety of proteinases and inhibitors in order to perturb excess matrix destruction.

An artificial alternative to proteolytic cleavage is activation via the introduction of detergents (such as SDS). This can cause disruption between the zinc and cysteine by unfolding the protein structure and so physically moving the zinc and cysteine apart. In addition reagents such as N-ethylmaleimide (NEM), organomercurials, or hypochlorous acid (HOCl), produced during respiratory burst, reduce the sulphydryl group on the cysteine also causing disruption of the co-ordinate bond [Vallee *et al.*, 1990, Springman *et al.*, 1990].

Figure 1.12, facing page. Model for proMMP-9 activation-involvement of MMP-3 and neutrophil elastase. ProMMP-3 is partially activated by trypsin, plasmin, or neutrophil elastase. Complete activation is brought about by MMP-3 autocatalysis causing complete removal of the propeptide domain, yielding active MMP-3 which has the ability to cause MMP-9 activation. The activation of MMP-9 is perturbed by complexing with its innate inhibitor TIMP-1. Excess amounts of MMP-3 can however destabilise the inhibitorcomplex, causing dissociation and freeing MMP-9, leaving it susceptible to activation.

Alternatively the MMP-9/TIMP-1 complex can be destroyed by neutrophil elastase, preferentially cleaving TIMP-1 complexed with MMP-9. This leaves MMP-9 which is susceptible to activation.

Figure 1.12

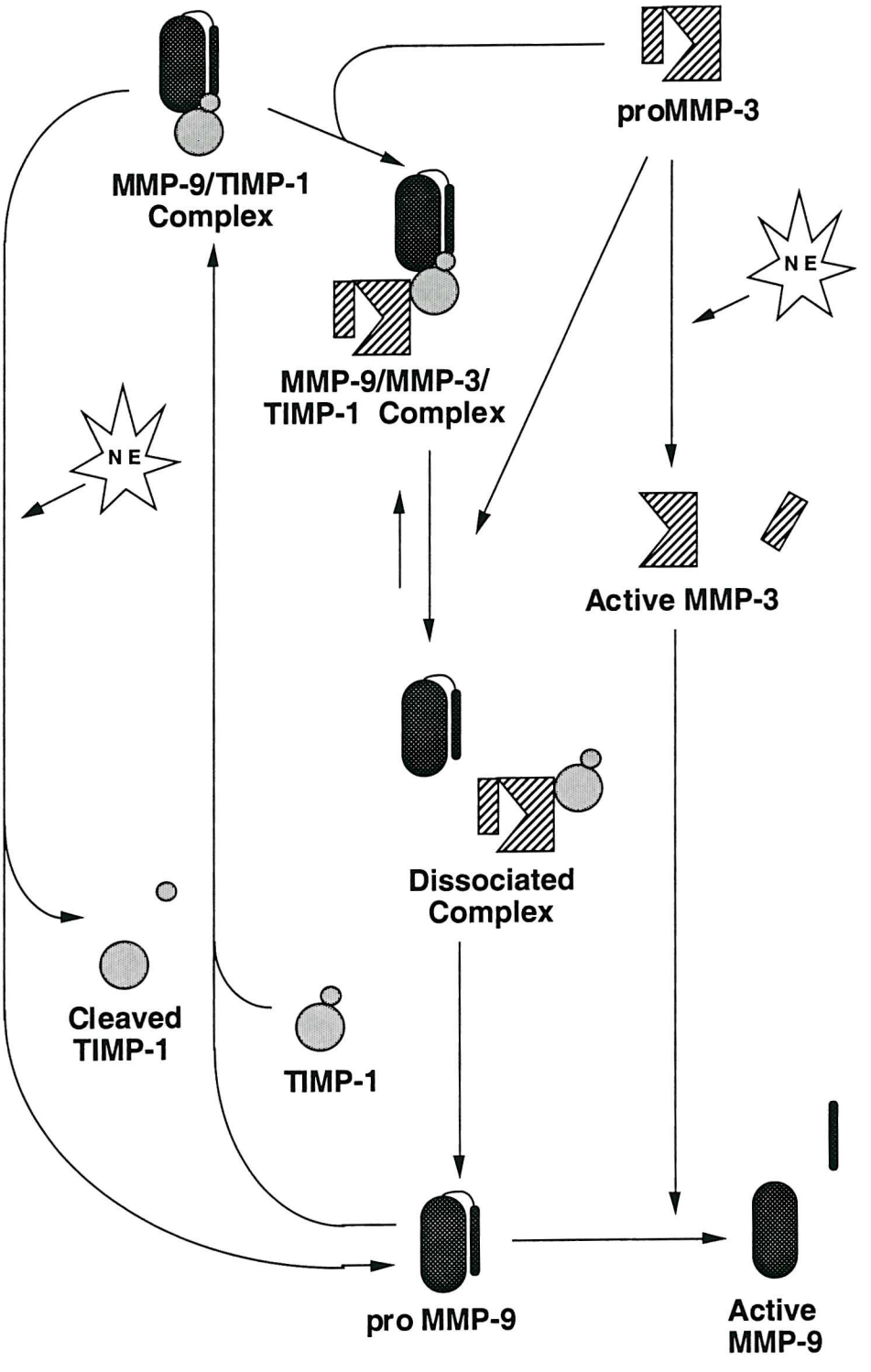

proMMP-3
MMP-9/TIMP-1
Complex
MMP-9/MMP-3/
TIMP-1 Complex
NE
NE
Active MMP-3
Dissociated
Complex
Cleaved
TIMP-1
TIMP-1
pro MMP-9
Active
MMP-9

Figure 1.13

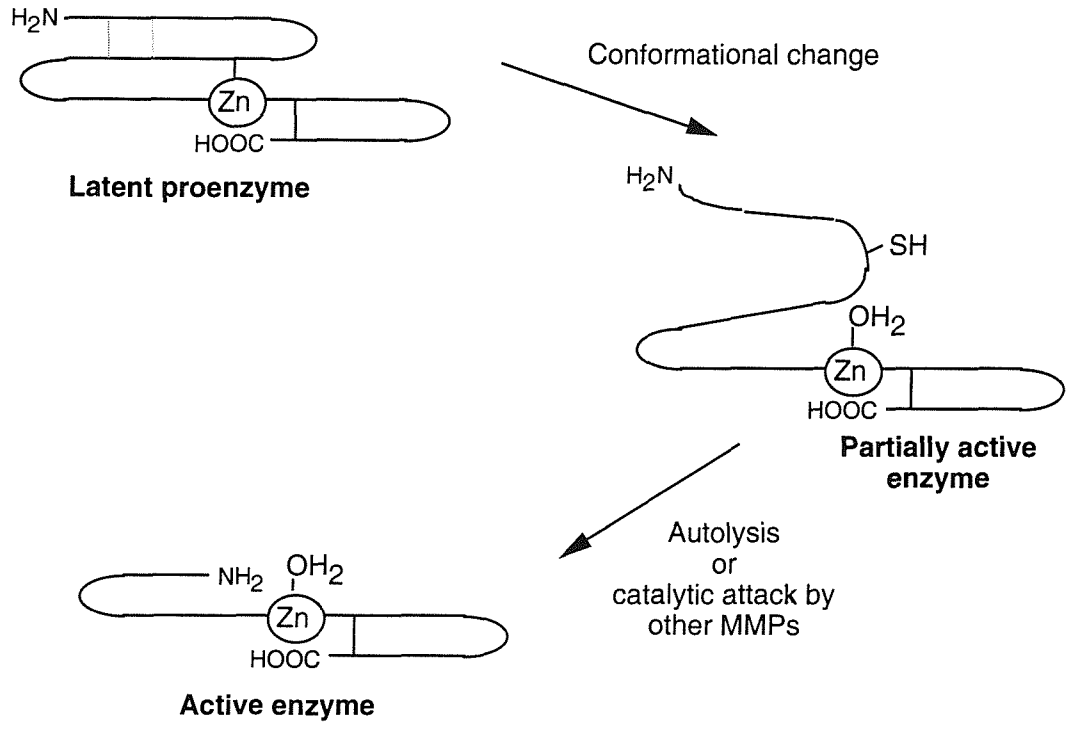


Figure 1. 13. Schematic representation of MMP activation. In latent MMPs the active site of the enzyme is masked by co-ordinate interaction between a highly conserved cysteine residue in the propeptide domain and the catalytic zinc atom in the active site itself. Activation of MMPs involves distortion of this interaction and thus exposure of the zinc atom. This can be achieved chemically by unfolding the structure using physical agents such as SDS, or by inactivating the cysteine residue with sulphydryl reactive chemicals such as N-ethylamide (NEM), oxidised glutathione (GSSG), hypochlorous acid (HOCl) or aminophenyl mercuric acetate (APMA). Two-stage proteolytic cleavage of the propeptide domian by specific activator proteinases also brings about conformational change and ultimate loss of the propeptide segment. This is thought to be the major activation mechanism of MMPs in vivo (adapted from O'connor and Fitzgerald, 1994).

1.4 MMP inhibitors: the tissue inhibitors of metalloproteinase (TIMPs).

1.4.1 Overview of TIMP functions.

Tissue inhibitors of metalloproteinases (TIMPs), as the name implies, are the major inhibitors of matrix metalloproteinase activity found in tissue throughout the body. The other major serum inhibitor of metalloproteinase, α_2 macroglobulin, is prevented from entering the tissues by its large molecular size (720kDa) compared to the smaller TIMP (~20-30kDa) [Ishibashi *et al.*, 1988].

There are 4 different types of TIMP (TIMP-1 through to TIMP-4) expressed in a variety cell types. Stromal cells, including fibroblasts [Welgus *et al.*, 1983], cartilage [Dean *et*

al., 1984], osteoblasts [Uchida *et al.*,2000], synovial [Mcguire *et al.,* 1981] endothelial cells [Hayashi *et al.,* 1996] and epithelial cells [Hayashi *et al.,* 1996, Yao *et al.*, 1997] are important expressers of the inhibitors. In all tissues, normal matrix turnover is maintained in a fine balance between the destructive potential of the MMPs and the restraint of this by TIMPs. It is widely accepted that the over expression of TIMP is a major causative factor in many fibrotic pathologies. For example excessive fibrosis in the synovial cavity in rheumatoid arthritis is thought to be caused by TIMP over expression [Nagase and Woessner 1999], as is the matrix accumulation and fibroblast proliferation found in lung diseases such as idiopathic pulmonary fibrosis (IPF) [Hayashi *et al.*,1996]. Additionally recent studies have suggested that fibrotic airflow obstruction could be caused by an over exuberant TIMP-1 response to an inflammatory / proteolytic insult [Vignola *et al.*, 1998, Becky-Kelly *et al.,* 2000]. As with pathologies caused by proteinase excess (discussed in 1.4.1) these pathologies indicate the importance of proteinase / anti proteinase balance.

As has been discussed, inflammatory cells influence on proteinase / antiproteinase balance in the lung (see 1.2), by influencing their own microenvironment. Importantly TIMP-1 has been identified in lymphocytes [Johnatty *et al.*,1997] alveolar macrophages [Albin *et al.*, 1987, Mautino *et al.*, 1999, Lim *et al.*, 2000] and neutrophils [Treibel *et al.*, 1995, Price *et al.*, 2000]. In terms of COPD, recent studies have found that alveolar macrophages from smokers release higher basal levels of TIMP-1 compared to those obtained from normal subjects [Lim *et al.*, 2000]. While this is matched by an increase in MMP-9 levels from the same cells, the increased TIMP release may represent an attempted compensation for the proteinase burden. Evidence suggests that infiltrating neutrophils also possess such a buffering capacity. Most of the intracellular TIMP-1 found in neutrophils is found to localise to distinct *"TIMP"* vesicles, with smaller amounts found in granules complexed to MMP-9. It is possible that this allows

degranulation of MMP-9 prior to TIMP-1, providing a metalloproteinase control mechanism [Price *et al.*, 2000]. It is conceivable that breakdown of such control mechanisms is an important factor in the proteinase mediated destruction found in COPD.

1.4.2 Regulation of TIMP release.

Regulation of TIMP-1 expression by cytokines and growth factors has been measured in a variety of cell types including various types of fibroblast [Edwards *et al.*, 1987, Bigg *et al.*, 2000], human bronchial epithelial cells [Yao *et al.*, 1997] and macrophages [Lacraz *et al.*, 1995]. Growth factors and anti-inflammatory cytokines favour TIMP-1 expression and thus matrix deposition. For example TGFβ stimulates TIMP-1 production [Wright *et al.*, 1991] while selectively repressing production of MMP-1 and MMP-3 [Edwards *et al.*, 1987]. Retinoic acid has a similar potentiatory effect on TIMP-1 production while also selectively down regulating MMP-9 production [Wright *et al,* 1991b, Frankenberger *et al,* 2001]. It is possible that retinoic acid could form the basis of future drug targets for the selective inhibition of MMP-9 in lung disease [Frankenberger *et al*, 2001]. Additionally, growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) [Edwards *et al.,* 1987] and platelet derived growth factor (PDGF) induce TIMP-1. Interestingly their effects are augmented by TGFβ [Edwards *et al.*, 1987]. The anti-inflammatory cytokine IL-10 is also found to increase TIMP-1 production from human macrophages while inhibiting MMP release [Lacraz *et al.*, 1995, Lim *et al.*, 2000].

Inflammatory factors tip the balance in favour of proteinases. Inflammatory stimulants such as lipopolysaccharide (LPS) or IL-1β, have been found to have little or no effect on levels of TIMP-1 release from both bronchial epithelial cells [Yao *et al.*, 1997], or alveolar macrophages [Lim *et al.*, 2000] while increasing MMP-9. TNFα is found to

exacerbate the proteinase / antiproteinase imbalance by decreasing TIMP-1 production while increasing MMP-9 [Yao *et al.*, 1997].

1.4.3 TIMP-1 structure.

Four TIMPs have been identified, however of these the interactions of TIMP-1 and TIMP-2, with soluble MMPs were the first to be described and remain the best characterised. TIMP-1 is a 28.5kDa protein formed by a backbone of 184 amino acids. There are 12 conserved cysteine residues in this backbone, causing a total of six disulphide bonds to be formed within the structure. This disulphide bond formation organises the TIMP-1 molecule into distinct C and N terminal regions, consisting of 3 domain loops each [Woessner, 1991, Willenbrock and Murphy, 1994]. Additionally this bonding yields an extremely stable molecule which retains full activity even after incubation at pH 2 or boiling at 100°C for 30minutes [Osthues *et al.*, 1992].

1.4.4 MMP-TIMP complex formation.

There have been four specific TIMPs described, and each are found to inhibit a wide variety of MMPs. Whilst each of the TIMPs will inhibit most activated MMPs, distinct functional differences have been ascribed to individual inhibitors. For example, TIMP-1 and –2 were initially studied in relation to their specificity for gelatinases (MMP-9 and –2 respectively). TIMP-1 is considered the innate inhibitor of MMP-9, since it forms a specific complex with pro MMP-9 [Ward *et al.*,1991, Bigg *et al.*, 2000]. Similarly, TIMP-2 will only complex with proMMP-2 [Ward *et al.*, 1991, Bigg *et al.*, 2000]. Conversely, upon activation, active MMP-9 or MMP-2 will readily complex with either TIMP-1 or TIMP-2 [Ward *et al.*, 1991].

TIMP-1 is considered as the innate inhibitor of MMP-9, forming a 1:1 stochiometric complex high affinity (K_i values less than 10^{-9} M) [Woessner, 1991, Wilenbrock and

Murphy, 1994] which proves to be dissociable by SDS-PAGE and treatment with EDTA at acid pH [Murphy *et al.*,1989]. Binding of MMP-9 to TIMP-1 is a biphasic process involving the interaction of both the active site and the hemopexin like C-terminal domain of MMP-9 with the N and C terminal domains respectively of the inhibitor [Gomis-Ruth *et al.*,1997, Olson *et al.*, 1997]. Evidence suggests that the active site of the proMMP-9 molecule forms a low affinity binding site for TIMP-1 (K_d of 7.4 μM for TIMP-1 binding pro (92kDa) MMP-9) and the hemopexin like domain forms a high affinity binding site (K_d of 35nM for TIMP-1 binding pro (92kDa) MMP-9) [Olson *et al.*, 1997]. Interestingly the inhibitor - enzyme affinity of TIMP-1 for activated MMP-9 is greater than for pro MMP-9. In this case the low affinity binding has a K_d value of 3.1 μM and high affinity binding has a K_d value of 23.9nM [Olson *et al.*, 1997]. In agreement with this, competition binding experiments have shown that active metalloproteinase will readily displace pro MMP-9 bound to immobilised TIMP-1 [Ward *et al.*, 1991]. It is conceivable that this differential in affinity between pro and active MMP-9 allows sequestration of TIMP-1 in the tissues wherever pro MMP-9 accumulates [Ward *et al.*, 1991].

1.4.5 TIMP-1 inactivation.

An important factor influencing proteinase / antiproteinase imbalance in inflammation is how much of the measured inhibitor is actually active in the inflammatory milieu. With relevance to this Burnett *et al* reported proportion of TIMP measured by ELISA in lung secretions from bronchitics was inactive [Burnett *et al.*, 1986], thus tipping the balance in favour of proteinase. Despite the relative stability of TIMP-1, the protein being resistant to both boiling and treatment at pH 2 [Osthues *et al.*, 1992], various treatments have been used to inactivate the inhibitor. For example reduction of the twelve conserved cysteine residues in the TIMP-1 backbone using dithiothreitol (DTT)

followed by alkylation with indoacetamide [Dean *et al.*, 1984] breaks the six disulphide bonds crucial for the tertiary structure of TIMP-1, causing loss of activity. Alternatively modification of histidine residues by diethyl pyrocarbonate (DEPC) [Williamson *et al.*, 1983] causes loss of structural integrity, also resulting in inactivation. The implications of these *in vitro* findings in inflammatory disease are limited however.

Oxidative stress, defined as the increased exposure of tissues to oxidants is thought to have an important role to play in COPD [Repine *et al.*, 1997]. Increased amounts of oxidants such as hypochlorus acid, hydroxyl radicals and peroxynitrite are released form both cigarette smoke and the phagocytes of smokers [Repine *et al.*, 1997]. Such oxidants are thought to have a major influence on proteinase / antiproteinase balance by inactivating proteinase inhibitors. Initial work focussed on the inactivation of the neutrophil elastase inhibitor, α_1-antitrypsin. It was shown that phagocyte generated oxidants in smokers, reduced the inhibitory activity of α_1-antitrypsin [Hubbard *et al.*, 1987, Wallaert *et al.*, 1993], by oxidation of methionine residues in the inhibitor structure [Carp *et al.*, 1982]. It has been found that both hypochlorus acid (HOCl) [Shabani *et al.*, 1998] and peroxy nitrite [Frears *et al.*, 1996] also inactivate TIMP-1, suggesting that oxidative inactivation of TIMP-1 occurs during inflammation, in the same way as α_1-antitrypsin inactivation. Proteolytic cleavage is also a potential route of TIMP-1 inactivation. TIMP-1 is a substrate for a variety of proteinases found in the inflammatory environment. TIMP-1 is cleaved by neutrophil elastase [Itoh and Nagase, 1995], trypsin [Murphy *et al.*, 1991, Williamson *et al.*, 1993b] and cathepsin B [Kostoulas *et al.*, 1999], resulting in dramatically reduced inhibitory activity. In the inflammatory environment of the COPD lung, it is likely that both oxidants and proteinases potentiate the activity of MMPs by reducing the inhibitory activity of TIMPs.

1.5 Aims.

As has been highlighted in this chapter, COPD is a progressive inflammatory disease of the lung, characterised by a chronic and irreversible decrease in lung function. Many studies have described the nature of inflammatory involvement in disease, however it remains unclear exactly how inflammation causes lung function decline. It is widely hypothesised that excessive release of proteinases from inflammatory cells may damage the extra cellular matrix, contributing to lung function decline over time. This thesis aims to assess the contribution of MMP-9/ TIMP-1 imbalance to this process by analysing MMP-9 and TIMP-1 levels in a variety of clinical samples from patients with obstructive lung disease. I also aim to relate MMP-9/TIMP-1 imbalance to changes in inflammatory cell infiltration, and where possible deficit in lung function.

Chapter 2.

Materials and Methods.

2.1 Chemicals and reagents

2.1.1 Materials.

Ammonium persulphate, TEMED (N', N, N', N'-Tetraethylmethylne-diamine), acrylamide/bis acrylamide 30% solution, glycine, lauryl sulphate (sodium dodoecyl sulphate-SDS), Triton X-100, Tween-20, gelatin from porcine skin, bovine β casein, bromophenol blue, Coomassie brilliant blue, $CaCl_2$, $MgCl_2$, Broad spectrum, general use proteinase inhibtior cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), transepoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA (1ml of inhibitor cocktail inhibits the equivalent of 1mg of USP pancreatin (P-2714), Phenylmethylsulfonyl fluoride (PMSF), Iodoacetamide, aprotinin, RPMI 1640 medium, penicillin, gentamicin, foetal calf serum and bovine serum albumin, sodium azide, hydrogen peroxide, TRI reagent for RNA extraction, RNAase free chloroform, isopropanol, diethyl pyrocarbonate, RNAase free agarose and ethidium bromide all were purchased from Sigma Chemical Co. (Poole, UK). Methyl benzoate, sodium chloride, Tris, hydrochloric acid, and acetone, was purchased from Merck. RNAase free ethanol was purchased from Fisher. For fixing samples for immunohistochemistry, a JB-4 embedding kit comprising solution A: butoxyethanol monomer, solution B: NN-dimethylanaline monomer in polyethylene glycol and catalyst: benzoyl peroxide was purchased from Park Scientific. Chromogens for immunohistochemistry; aminoethylcarbozole (AEC) liquid system and diaminobenzedine (DAB) liquid system were purchased from BioGenex. EnzCheck™ gelatinase/ collagenase assay kits were purchased from Molecular Probes (Eugene, OR, USA). RNA PCR core kit purchased from Perkin Elmer was used for RT-PCR. Human MMP-9 antigen and human MMP-3 antigen, were purchased from Biogenesis (Poole, UK) Recombinant human TIMP-1 was kindly donated by Prof. Tom McDonald at Southampton General Hospital.

Table 2.1 Table of antibodies used for procedures

Antibody	Clone	Epitope stained	Procedure	Source
CD3	UCHT1	T lymphocytes	IHC	DAKO
CD68	PGM1	Activated macrophages	IHC	DAKO
EG2		Eosinophil Cationic Protein	IHC	DAKO
Neutrophil elastase.	NP57	Neutrophil elastase	IHC	DAKO
MMP-9	4H3	MMP-9	IHC	R&D Systems
MMP-9	Polyclonal	MMP-9	IP	Biogenesis
TIMP-1	63515.111	TIMP-1	IHC/ IA/WB	R&D systems
TIMP-1	102D1	TIMP-1	IHC	Neomarkers
TIMP-1	T2	TIMP-1	IA	Biogenesis
TIMP-1	polyclonal	TIMP-1	IA (capture)	Biogenesis

Table 2.1 procedures denoted by abbreviations; IHC= immunohistochemistry, WB= Western blot, IA=ELISA or substrate binding assay.

2.1.2 Buffers.

The following buffers were used: Electrophoresis / zymography and Western blotting studies; Running buffer for the SDS-PAGE gel contained 0.325M Tris-HCl and 0.1% SDS, and pH was adjusted to 8.8. Stacking buffer contained 0.125 Tris-glycine and 0.2% SDS and the pH was adjusted to 6.8. Electrode buffer contained 25mM Tris base, 192mM glycine and 0.1% SDS; Zymography sample buffer 75mM Tris sample buffer containing 12.5% sucrose and 5% SDS Zymography wash buffer contained 20mM Tris, 2.5% Triton-X 100, the pH was adjusted to 7.8; Zymography wash buffer with calcium contained 20mM Tris, 10mM $CaCl_2$, 5μM $ZnCl_2$, and 1% Triton-X 100, the pH was adjusted to 7.8; Coomassie blue stain contained 0.5% Coomassie brilliant blue in 10% glacial acetic acid and 45% methanol; Gels were destained in 10% glacial acetic acid and 10% methanol. Tyrode's buffer for electrophoretic transfer contained 25mM Tris, 190mM glycine and 20% methanol. Buffers for immuno histochemistry: Tris buffered saline contained 0.13M NaCl, 5mM Tris and 4mM HCl, pH was adjusted to 7.6; Tris-HCl buffer contained 50mM Tris with pH adjusted to 7.6; endogenous peroxidase inhibitor contained 0.1% sodium azide and 0.3% hydrogen peroxide; blocking medium was made up of Dulbecco's modified Eagles medium, containing 20% foetal calf serum and 1% bovine serum albumin.

2.2 Preparation of clinical samples.

2.2.1 Preparation of sputum samples.

Sputum samples from patients with COPD were provided by Dr. Angshu Bhowmik and colleagues at the London Chest Hospital. All patients experienced exacerbation symptoms during the course of an ongoing study at the hospital and sputum samples were induced before, during or after exacerbation and coded accordingly. Sputum samples were processed as per Bhowmik *et al* 2000. Briefly, The sputum was incubated with four times its weight of 0.01M DTT in HBSS, at 4°C for 15 minutes. The volume of HBSS was then doubled (ten fold dilution of original sputum sample) and incubated for a further 5 minutes. The suspension was then filtered through 50μm nylon gauze to remove mucus and debris without removing any of the cells and centrifuged at 790g for 10 minutes. The cell free supernatant was removed and stored at -70 °C. The cell pellet was resuspended, total and viable cell count was determined. Cytospins were made from the cell suspension and stained with Diff-Quik to obtain a differential cell count.

Sputum samples obtained from cystic fibrosis patients and normal volunteers at Southampton General hospital were used to compare inflammatory markers with COPD sputum.

2.2.2 Preparation of bronchoalveolar lavage (BAL) samples.

BAL samples were obtained from patients with a range of different lung diseases during routine bronchoscopy. During the procedure 120mls of sterile saline was flushed into the alveoli via a bronchoscope and as much as possible was retrieved. The BAL fluid was analysed for cell content and then centrifuged at 800g for 20 minutes. The cell free supernatants were aliquoted, stored at -70°C and removed when required for analysis.

2.2.3 Characterisation of human lung tissue.

Lung tissue was removed from patients undergoing resection for carcinoma; tissue used was from the non-tumourous normal margin tissue surrounding the tumour site. Data relating to asthmatic state, smoking history, lung function, age and sex was obtained for patients where possible. Patients were characterised on the basis of their FEV_1/ FVC ratio. FEV_1/ FVC <70% indicated obstructive lung function, where as FEV_1/ FVC > 70% indicated non obstructive lung function.

2.2.4 Preparation of lung tissue homogenates for protease analysis.

Small fragments of characterised human lung tissue (approximately 50mg) were finely chopped using dissecting scissors and weighed. An ice-cold solution of PBS containing 0.1% triton and a commercially available proteinase inhibitor cocktail (Sigma P-2714) was added; 100µl PBS-triton was added per 50mg of tissue. The samples were homogenised on ice using a sonicator set at amplitude of 3 microns; 12 cycles of 10 seconds sonication followed by 20 seconds rest ensured minimal heating of the tissue sample. Following sonication the samples were centrifuged at 15,000g for 15 minutes at 4°C, and supernatants removed for storage. Efficient protein extraction from the tissue pellet was ensured by 4 subsequent washing / centrifuging steps in equal volumes of PBS- triton. Samples were stored at -70°C prior to analysis.

2.3 Analysis of MMP levels.

2.3.1 Method for gelatin zymography.

Detection of MMP-9 was by gelatin zymography using methods described by Kleiner and Stetler-Stevenson. [Kleiner and Stetler-Stevenson, 1994]. Briefly, samples were separated under denaturing conditions on an SDS poly acrylamide gel, in which 0.1% gelatin was allowed to co-polymerize with 7.5% acrylamide. A 4 % acrylamide

stacking gel was poured on top. Samples were prepared in 5x tris sample buffer containing 12.5% sucrose and 5% SDS, and a bromophenol blue stain was added to render samples visible on loading. An MMP-9 control (100ng/ml) and molecular weight standards were loaded alongside the samples under investigation. Samples or standards were loaded in volumes of 10µl. Following electrophoresis at 30mA for approximately 1 hour, the gels were washed twice in triton containing buffer to remove any SDS and allow proteins to refold. The gels were subjected to a further wash in a calcium / zinc containing buffer, and incubated over night in the same buffer at 37°C to allow activation of the gelatinases. Following incubation the gels were stained in Coomassie blue stain (see 2.1.1) this had the purpose of fixing the gelatinases, preventing further degradation of the gelatin, as well as staining undigested gelatin.

2.3.2 Method for casein zymography.

MMP-3 was examined using casein zymography with 0.01% casein co-polymerized with 12% acrylamide. A 4 % acrylamide stacking gel was poured on top. Casein gels were pre run before the samples were loaded due to the high electrophoretic clearance of casein, the samples were then prepared as in section 2.3.1 and a 10µg/ml MMP-3 standard included. Following electrophoresis of the samples, the gels were washed and incubated for 16 hours as outlined in the protocol for gelatin zymography (see 2.3.1). Following incubation the gels were stained in Coomassie blue stain (see 2.3.1).

2.3.3. Quantitation of zymography.

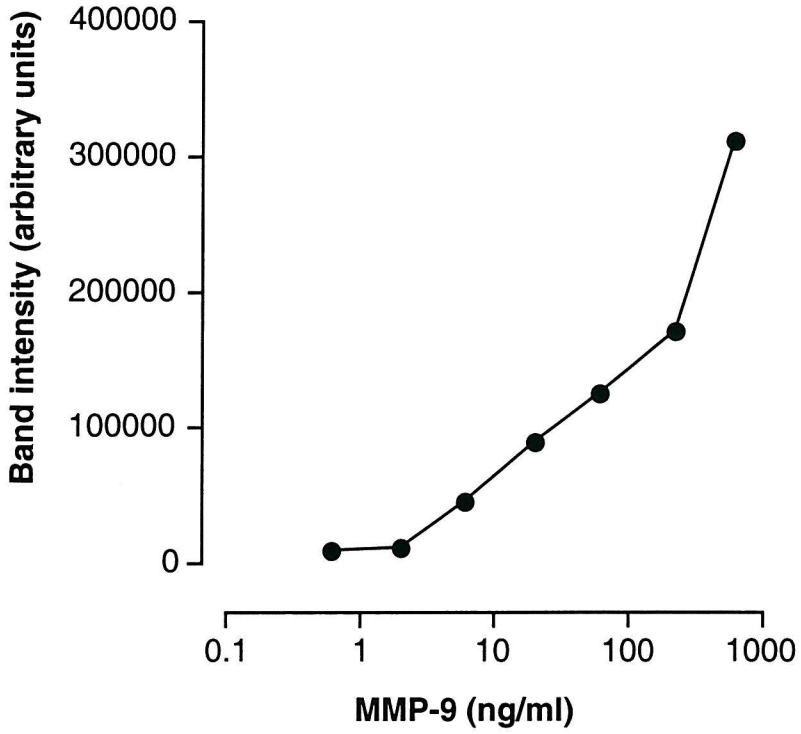
Following staining, zymograms were analysed using a scanning densitometer. Typically, a lane with no sample was used as a background and the individual lanes identified using computer software (scan analysis). Each lane was integrated vertically,

and the background subtracted. Total area under the curve was obtained for each lane and normalised against a 100ng/ml MMP-9 (or 10μg/ml MMP-3 for casein zymography) standard run on each gel.

Experiments were conducted to assess the linearity of the zymography assay. Increasing concentrations of MMP-9 were run on zymograms and analysed using densitometry. Density of bands was proportional to amount of MMP-9 loaded up to 200ng/ml (see fig 2.1). Casein zymography to detect MMP-3 activity is a far less sensitive technique than gelatin zymography. Reflecting this the limit of detection for casein zymography was found to be 650 ng/ml (data not shown).

The coefficient of variation for zymography was 5 percent for samples run on the same gel and 11 percent when run on different gels.

Figure 2.1



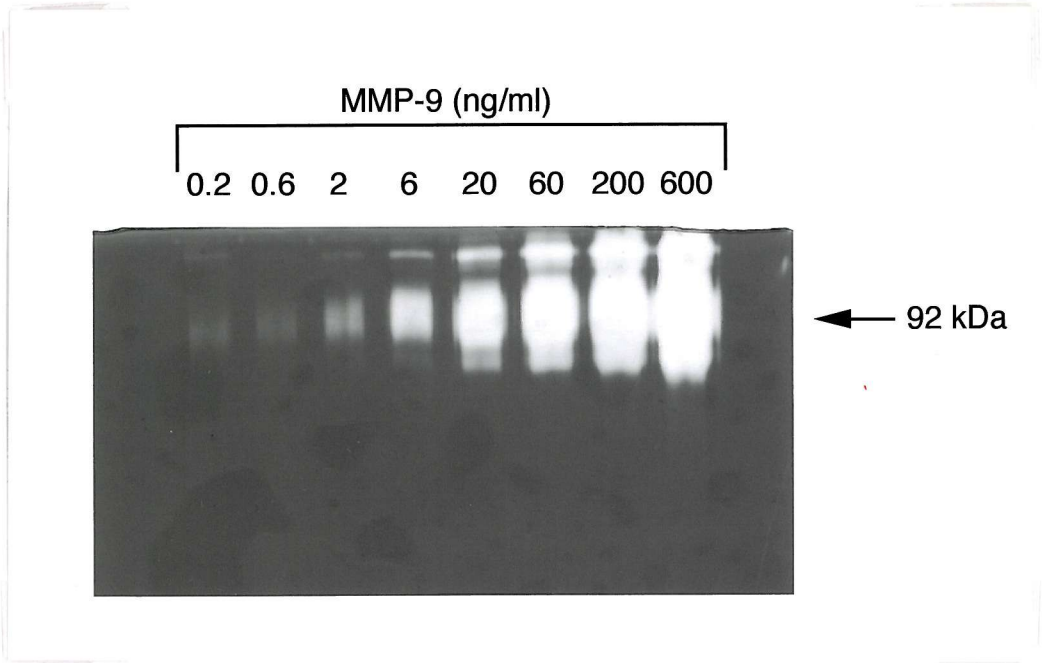


Figure 2.1 Sensitivity of zymography. Samples containing a range of concentrations of MMP-9 were separated on a 7.5% SDS-polyacrylamide gel containing 0.1% gelatin. Following staining for non degraded gelatin using Coomassie blue the band intensity was measured using densitometry. The gel and the standard curve obtained are both exhibited above. Concentration of MMP-9 (ng/ml) shows a linear relationship between 200 and 2 ng/ml.

2.3.4 Validation of zymography by immunoprecipitation.

The candidate MMPs could be identified by their molecular weight. For gelatin zymography bands of MMP-9 activity were present at 280kDa (band 1), 128kDa (band 2), and 92kDa (band 3). MMP-2 activity was found to migrate to 72kDa (band 4). Using casein zymography MMP-9 activity was found at 95 kDa and MMP-3 activity at 45 and 30kDa. Their identity was confirmed by immunoprecipitation. Briefly, in the case of MMP-9; 5μl of antiMMP-9 (4mg/ml) was incubated with 20μl of sample or distilled water (negative control) for 2 hours, on ice. Following incubation, MMP-9 sample or water control were spun at 12,000g for 5 minutes at 4°C, the supernatants were recovered, and run on a zymography gel (see 2.1.4) alongside an untreated sample (positive control). Immunoprecipitation of MMP-2 and MMP-3 required the addition of 5μl of protein-A agarose to the incubation. Casein is a substrate for a range of proteases, thus this method does not exhibit the specificity of gelatin zymography. Reflecting this, figure 2.3 illustrates a range of enzymes detectable by casein zymography, including MMP-9, MMP-3, trypsin and neutrophil elastase.

Figure 2.2

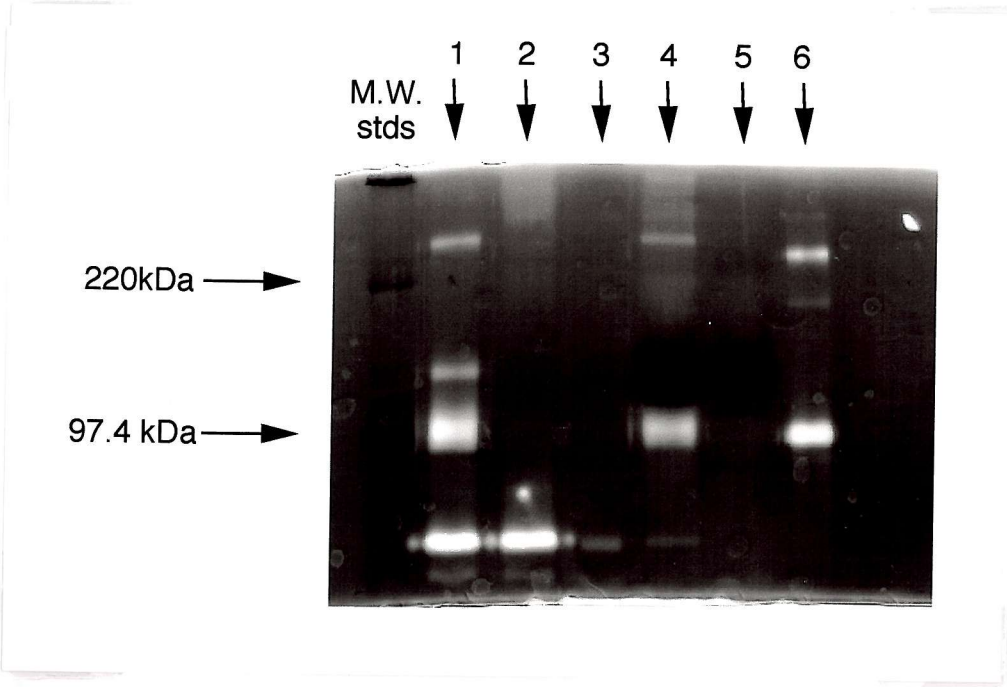


Figure 2.2 Immunoprecipitation of MMP-9 and MMP-2 from homogenised human lung fragments. Following immunoprecipitation supernatants were recovered and separated on a 7.5% SDS-polyacrylamide gel containing 0.1% gelatin.. From left to right: Sample containing MMP-2 and MMP-9 (lane 1), same sample incubated with anti-MMP-9 (lane 2), anti MMP-9 alone (lane 3), sample incubated with anti MMP-2 (lane 4), anti MMP-2 alone (lane5) MMP-9 standard (100ng/ml).

Figure 2.3

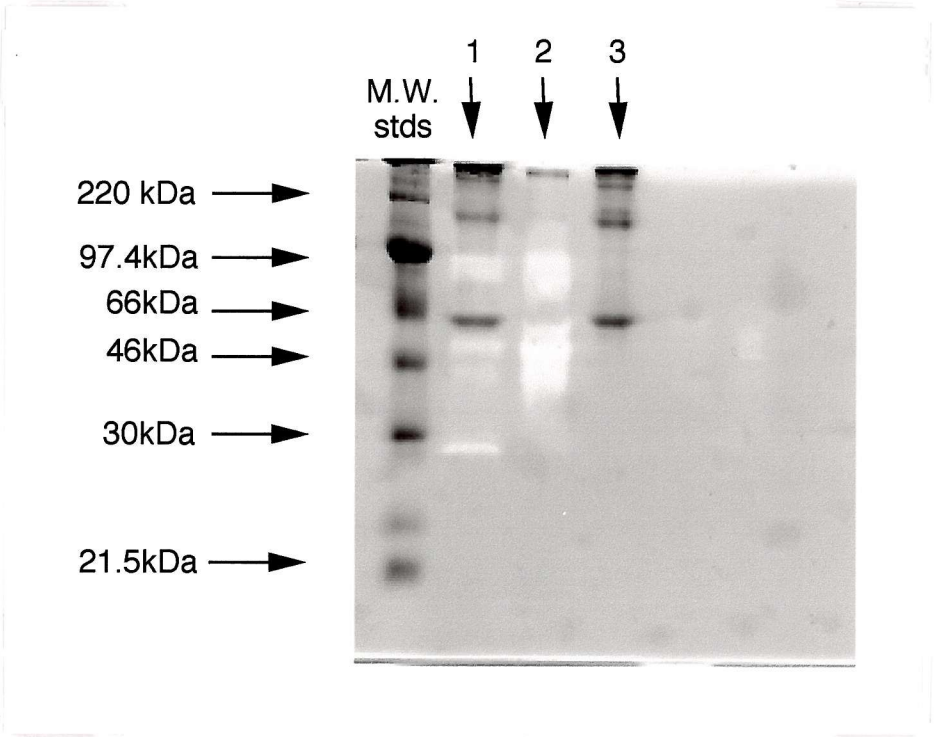


Figure 2.3. Immunoprecipitation of MMP-3 from sputum samples. Following immunoprecipitation, supernatants were recovered and separated on a 12% SDS-polyacrylamide gel containing 0.01% casein. From left to right: sample and anti MMP-3 (lane1), sample alone (lane 2), anti MMP-3 (lane 3).

Figure 2.4

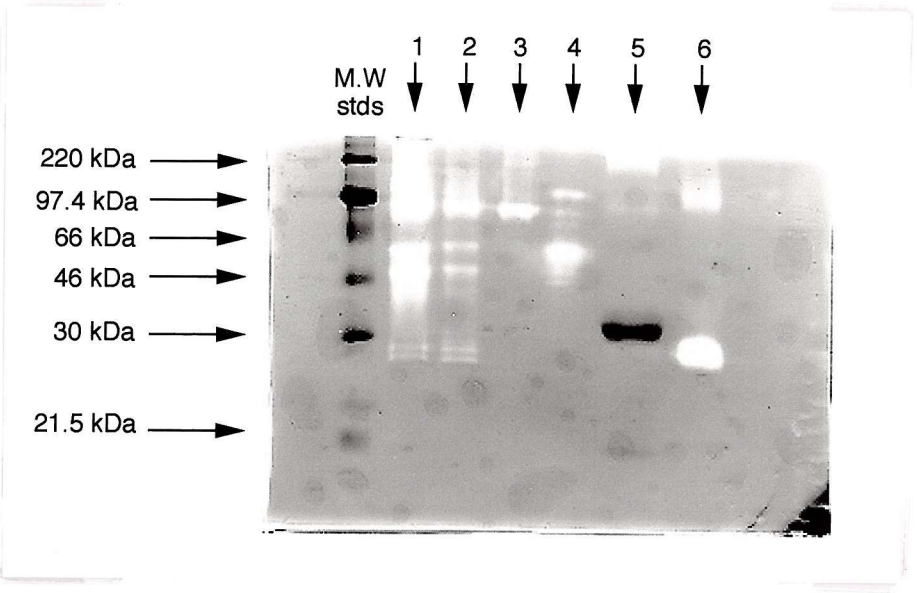


Figure 2.4 Enzyme activity visible on a casein zymogram. A range of samples were separated on a 12% SDS-polyacrylamide gel containing 0.01% casein illustrate which particular enzymes could be visualised. From left to right: concentrated sputum (lane1), dilute sputum (lane 2), MMP-9 1µg/ml (lane 3), MMP-3 10µg/ml (lane4), tryptase 0.16 units/lane (lane 5), neutrophil elastase 0.15 units/lane (lane 6).

2.3.5 Analysis of net gelatinase activity using soluble substrate assay.

Analysis of net gelatinase / collagenase activity was achieved by use of the EnzChek™ assay system. The system was based around the degradation of DQ gelatin - fluorescein conjugate; gelatin so heavily labeled with fluorescein that fluorescence was quenched. This substrate was then digested by active gelatinase (or collagenase) to yield fluorescent peptides. Thus, increase in fluorescence was proportional to gelatinase / collagenase activity. The protocol was identical to that in the accompanying literature. Briefly, 40µl of diluent and 10µl of DQ gelatin (1mg/ml) was pipetted into wells, this was followed by 50µl of sample or gelatinase standard. The plate was incubated at 37°C for 3 hours. Digestion products from the DQ gelatin had absorption maxima at 495nm,

and emission maxima at 515nm. EnzChek™ system can detect enzyme activity down to $2x10^{-3}$ U/ml, where 1 unit of enzyme is defined as the amount of enzyme required to liberate 1μmole L-leucine from collagen, in 5 hours at 37°C, pH 7.5.

2.4 Analysis of TIMP-1 levels.

2.4.1 Measuring total immunocompetent TIMP-1 by ELISA.

Samples were analysed using a TIMP-1 ELISA system, obtained from Amersham Life Sciences (Amersham, UK). The assay was reported to recognise total immuno competent TIMP-1. The lowest reference on the standard curve 3.13ng/ml and the highest was 100ng/ml. The protocol followed was identical to that in the accompanying literature. Briefly, 50μls of sample or standard was pipetted into the appropriate wells. Following incubation for 2 hours at 20-25°C the plate was washed thoroughly and blotted before 50μl of anti TIMP-1/peroxidase conjugate was added to each well. Following further incubation, 2 hours at 20-25°C, the plate was again washed, blotted and 50μl of the horseradish peroxidase substrate 3, 3', 5, 5' tetra-methyl benzedine (TMB) was added to each well. The reaction between HRP and TMB was terminated by the addition of 1M H_2SO_4 and the OD of each well was read at 450nm.

2.4.2 Analysis of immunocompetent TIMP-1 on Western blot.

Immunocompetent TIMP-1 was analysed using Western blotting. Briefly, samples were separated under denaturing conditions on an SDS poly acrylamide gel containing 12% acrylamide. A 4 % acrylamide stacking gel was poured on top. Samples were prepared in 75mM sample buffer containing 12.5% sucrose and 5% SDS, and a bromophenol blue stain was added to render samples visible on loading. Samples were loaded in volumes of 10μl. Following electrophoresis at 30mA for approximately 1 hour, gels

were removed and emmersed in Tyrode's buffer for 20 minutes. Nitrocelluloes membrane was also immersed in preparation for transfer. Following immersion a transfer sandwich of gel and nitrocellulose membrane was tightly assembled between filter paper and support pads. The complete sandwich was positioned in a transfer tank with the membrane closest to the positive electrode. Following transfer at 400mA for 2 hours, the nitrocellulose membrane was removed, washed twice in 0.1% PBS-Tween and incubated overnight in 0.1% PBS-Tweeen containing 5% milk.

The following day the nitrocellulose membrane was washed three times with 0.1% PBS-Tween and incubated with a mouse monoclonal antibody to human TIMP-1 (R&D systems) (1 μg/ml). Following a 2 hour incubation at room temperature and further washing in 0.1% PBS-Tween the membrane was incubated with a biotinylated antibody to the mouse monoclonal (APBiotech; RPN 1177), for a further 2 hours. Following washing the membrane was subsequently incubated with streptavidin-bitinylated HRP complex (APBiotech; RPN1051), for 1 hour to increase signal resolution. The nitrocellulose membrane was treated with chemiluminesence reagent; ECL plus (APBiotech; RPN 2132). Bands were visualised on X-ray film.

2.4.3 Quantitation of TIMP-1 activity by novel inhibitor binding assay.

Wells were coated with MMP-9 (400ng/ml) in 50mM Tris-HCL buffer (pH 7.5) containing 5% (w/v) polyethylene glycol (PEG) and incubated at 4°C for 5 hours. The plate was washed thoroughly and blocked overnight with 30% soya milk in 0.1% TBS-Tween at 4°C. The plates were washed and blotted and 100µl of sample or recombinant TIMP-1 standard was pipetted into the appropriate wells; the lowest reference on the standard curve was 0.78ng/ml and the highest 100ng/ml. After incubation at 4°C for 2 hours the wells were washed out, 100µl detecting monoclonal antibody for TIMP-1 applied and the plate allowed to incubate for a further 2 hours at

4°C. Following primary antibody binding, the wells were sluiced again, 100µl of the second stage HRP conjugated antibody added and the plate incubated as above. Following a final wash, the horseradish peroxidase substrate TMB was added to each well; a blue colour developed which was proportional to the amount of TIMP-1 bound to MMP-9. This reaction was stopped by the addition of 1M sulphuric acid, yielding a yellow colour, which could be read at 450nm. Assay development and validation is described in chapter 6 (Figure 6.2 gives a schematic representation of the assay)

This protocol was run in parallel with an ELISA developed in house, measuring total immunocompetent TIMP-1. The protocol was identical to the one outlined above, except that polyclonal antibody to human TIMP-1 was coated onto wells (2µg/ml in bicarbonate coating buffer, pH 9.5) and used to capture total immunocompetent TIMP-1.

2.5 Immunohistochemistry

2.5.1a Acetone fixation and GMA embedding of lung tissue.

Biopsies of human lung tissue were placed in ice cold acetone containing protease inhibitors - phenylmethyl-sulphonyl fluoride (PMSF) (2mM) and iodoacetamide 20mM)- and stored overnight at -20°C. Following storage the sample was put in acetone at room temperature for 15 minutes and then in methyl benzoate for a further 15 minutes. The sample was then immersed in a mixture of JB4 solution A (see 2.1.1) and 5% methyl benzoate for 6 hours during which time the solution was changed 3 times. The sample was finally embedded in resin; prepared by mixing JB4 solutions A and B (see 2.1.1) in the presence of the catalyst benzoyl peroxide, polymerization occured at 4°C overnight. Embedded samples were desiccated and stored at -20°C. 2µM thin sections were obtained using a glass bladed microtome, floated onto ammonia water (1:500), and mounted on poly-L-Lysine coated slides. Toluidine blue staining was used to assess tissue morphology.

2.5.1b Alcohol fixation and paraffin embedding of lung tissue.

Sections of bronchial epithelium were fixed and embedded at the place of origin; the Alfred hospital, Victoria, Australia. Briefly specimens were fixed in a solution containing 60% absolute ethanol, 30% chloroform and 10% glacial acetic acid for 1 hour. From here they were subject to six separate immersions in absolute alchohol; for 15 minutes per immersion and at a temperature of 37°C. The specimens were then immersed in 2 separated changes of xylene, for 15 minutes and 30 minutes respectively. The sections were then submerged in molten wax, at 60°C for 2 periods of 45 minutes. 10μM thin sections were obtained using a wax cutting microtome, these were then floated onto warm water (37°C), and mounted onto poly L lysine coated slides.

2.5.2a Staining of GMA embedded lung tissue

Following cutting and mounting the sections were treated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide; inhibiting any endogenous peroxidase activity. Slides were then washed and treated with blocking medium to inhibit non-specific binding. Following blocking primary antibodies were applied at the appropriate dilutions and allowed to incubate at room temperature over night.

The next morning slides were washed and second stage antibodies applied, these were allowed to incubate for 2 hours at room temperature before being washed again. Streptavidin biotin-peroxidase complexes were added for a further 2 hour incubation period.

2.5.2b Staining of paraffin embedded lung tissue.

Following cutting and mounting slides are allowed to dry for a minimum of 24 hours. Slides were then treated with xylene to remove paraffin and rehydrated through a series of graded alcohol washes. Endogenous peroxidase activity was blocked by the

application of a solution of 0.5% hydrogen peroxide in methanol. Following washing avidin solutuion and then biotin were added, followed by blocking medium to inhibit non-specific binding. Following washing primary antibodies were applied at appropriate dilutions and incubated on the slides overnight at 4°C. The following day slides were drained and washed, secondary biotinylated antibodies were applied at appropriate dilutions and allowed to incubate at room temperature for 30 minutes. Following subsequent washing streptavidin biotin-peroxidase complexes were applied at appropriate dilutions and allowed to incubate at room temperature for a further 30 minutes. Slides were washed in preparation for application of chromogen (see 2.5.2c)

2.5.2c Application of chromogen to processed slides.

Following washing the chromogen aminoethyl carbozole (AEC) was added (instructions for mixing as per manufacturer), and allowed to incubate for 20 minutes (30 minutes in the case of paraffin embedded samples) at room temperature. The slides were then immersed and washed in runing tap water, counter stained in Mayer's haematoxylin stain and rinsed again in tap water. Upon drying the slides were treated with crystal mount and baked until the crystal mount was dry and hard. Coverslips were glued on top of the sections and allowed to set in place overnight.

2.5.3 Assessment of stained slides.

Immunoreactivity was assessed in the lung parenchyma from lung resection samples and the lamina propria. of bronchial biopsy specimens. Positively staining cells were counted in the bronchial biopsies and the area measured by the Leica Q-win system and related software, cell counts were expressed as median numbers of cells per mm^2. The heterogeneity and small size of patient groups made exact quantitative analysis difficult, thus analysis was semi-quantitative.

2.6 Molecular biology.

2.6.1 Preparation of mRNA from human lung tissue.

Prior to use human lung fragments were stored in RNA stabilising solution at -20°C. Human lung tissue was homogenised using an Ultra-Turrax homogeniser in 1ml of TRI reagent and mRNA was isolated as per manufacturers instructions. Briefly, the homogenate / TRI reagent suspension was left to stand for 10 minutes. 200µl of chloroform was then added to each sample and the tubes centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase was aspirated into new tubes and 500µl of isopropanol added to each sample. The tubes were left at room temperature for 10 minutes and then centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed and the pellet washed in 75% ethanol, then the tubes were respun at 7,500g and 4°C. The supernatant was again aspirated and the pellets air dried. 20µl of water was added to redissolve the mRNA and the pellets were stored in this form at -70°C.

2.6.2 Identification MMP-9 and TIMP-1 by RT-PCR

MMP-9 expression in human lung was investigated using primers designed in house. TIMP-1 primers were previously described by Yao *et al.,* 1998. Primers were optimized against commercially available human lung derived mRNA.

To ensure that there was no contamination of mRNA in samples by cellular DNA, PCR was performed on RNA which had not been subjected to reverse transcription.

Primers for the MMP-9 were designed to translate a 690bp product, primers for TIMP-1 were designed to translate a region of 399bp.

Table 2.2 Primers used for identification of MMP-9 and TIMP-1 expression.

	Forward primer	Reverse primer
TIMP-1	GGGGACACCAGAAGTCAACCAGA	CTTTTCAGAGCCTTGGAGGAGCT
MMP-9	TGACGCCGCTCACCTTCACTC	CTGTCAAAGTTCGAGGTGGTA

The primers used for RT-PCR are shown above from 5' to 3'.
The optimised reaction conditions for reverse transcription have been summarised in table 2.2

Table 2.3 Reaction conditions for reverse transcription.

reaction component	concentration of reaction component
MuLV reverse transcriptase	**1.0U/□l**
10XPCR BufferII	**1x**
25mM MgCl2	**5 mM**
DNTP	**1 mM**
Random hexamers	**2.5 μM**
Nuclease free water	
RNAase inhibitor	**1U/□l**
RNA	**1 μl (1□g)**

Thermal cycling parameters for reverse transcription were as follows:

1 Incubation 25°C - 10 minutes
2 Reverse transcription 42°C - 45 minutes
3 Inactivation 99°C - 5 minutes

The optimised reaction conditions for PCR have been summarised in table 2.4

Table 2.4 Reaction conditions for PCR.

reaction component	concentration of reaction component
AmpliTaq DNA polymerase	0.025U/μl
10XPCR BufferII	1x
25mM $MgCl_2$	2mM
Forward primer	0.2μM
Reverse primer	0.2μM
Nuclease free water	

Thermal cycling parameters for PCR were as follows:

1. Melt 96°C - 30 seconds.
2. Anneal 60°C - 30 seconds.
3. Elongation 72°C - 45 seconds.

30 cycles performed.

4. Final elongation 72°C - 10 minutes.

Following completion of the cycles, the PCR products were separated on a 1% agarose gel impregnated with ethidium bromide (0.5μg/ml) for DNA visualisation.

2.7 Statistical analyses.

Statistical analysis of results was carried out using Statview™ software. Non parametric Mann -Whitney analysis was carried out on unpaired data, and Wilcoxon Signed Rank analysis was carried out on paired data. The gross outcomes of bivariate associations were analysed by Spearman Rank correlations and the net effects of multiple variables were analysed using multiple regression where appropriate. $P<0.05$ was generally considered as significant.

Chapter 3.

MMP-9 and TIMP-1 in bronchoalveolar lavage (BAL) fluid.

3.1 Introduction.

Chronic obstructive pulmonary disease (COPD) is a disease characterised by progressive extracellular matrix (ECM) remodelling and loss of functional alveoli. The precise pathology behind this remodelling is poorly understood to date, however it is thought likely that proteinases released from infiltrating inflammatory cells have a key role to play [Stockley, 1994]. Initially damage was attributed to neutrophil elastase [Tetley, 1993], however additional studies have highlighted the involvement of matrix metalloproteinases (MMPs) in the disease process [Jannoff *et al.*, 1983, D'Armiento *et al.*, 1992; Shapiro, 1994]. The clinical significance of these studies became apparent with studies by Finlay *et al.*, who demonstrated elevated levels of MMP-9 and collagenase in bronchoalveolar lavage (BAL) fluid from patients suffering from emphysema [Finlay *et al.*, 1997a]. This present study confirms and adds to these results by assessing MMP-9 levels in BAL from patients with COPD, and correlating this with levels of tissue inhibitor of metalloproteinase-1 (TIMP-1), the cognate inhibitor of MMP-9, in the same individuals. In addition the relationship between MMP-9, TIMP-1 and numbers of infiltrating inflammatory cells has been analysed.

3.2 Methods.

Samples of BAL fluid were obtained from a group of 22 COPD patients in Jena Germany. The group consisted of current and ex smokers with a median age of 59 (IQR= 11.5). BAL was also carried out on a group of 10 normal non smokers with a median age of 25 (IQR= 5).

Both normal subjects and COPD patients were lavaged with 100 ml of pre-warmed saline. Following retrieval the BAL fluid was filtered through a two layer sterile gauze into sterile plastic vials, centrifuged for 10 minutes at 500g, in a microcentrifuge at 4°C. The cell free supernatants were removed, aliquoted and frozen at -70°C until required for analysis. Cytological examination of BAL fluid was performed after cytocentrifugation and staining with May-Gruenswald-Giemsa. Relative subpopulations of leukocytes were determined by a differential cell count of 1000 cells. Levels of total MMP-9 were measured by gelatin zymography and total immuno-competent TIMP-1 was measured by ELISA, as described previously (see section 2.4).

3.3 Results.

3.3.1 MMP-9 in BAL fluid.

MMP levels were investigated using gelatin zymography. Figure 3.1 indicates that MMP-9 levels were significantly higher in BAL from COPD patients (median: 22.27ng/ml; IQR: 82.51ng/ml) compared to normal controls (median: 3.2ng/ml; IQR: 11.07ng/ml) ($P< 0.005$). A wide inter-individual variability in MMP-9 levels in the COPD group is observed. COPD shows an intrinsic heterogeneity in disease severity, and it is conceivable the wide range of data is a reflection of this.

Figure 3.1

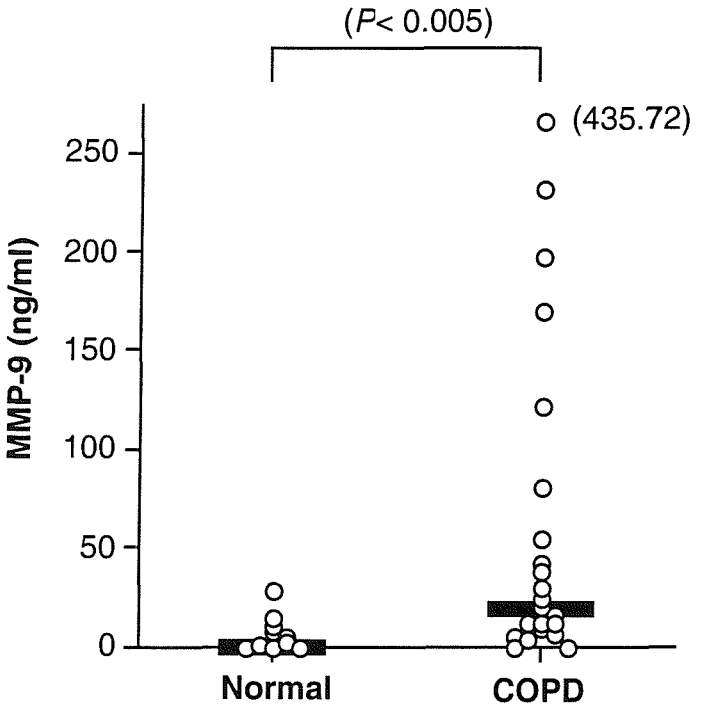


Figure 3.1 MMP-9 levels in BAL fluid. Levels of MMP in BAL fluid from a group of patients suffering from COPD (n=22) and a group of control subjects (n=10) was analysed by gelatin zymography. MMP-9 levels were expressed as ng/ml and values of MMP-9 beyond the range of the graph axes are indicated in brackets. Groups were compared by the non-parametric Mann Whitney U Test, $P< 0.05$ was considered statistically significant. Median values are marked.

The major gelatinolytic activity in BAL fluid was found to be MMP-9, which was confirmed by immunoprecipitation (data not shown). MMP-9 activity was found in three bands: at 92kDa, 130kDa and 220kDa. This pattern corresponds to that found by previous authors, and is thought to represent polymeric MMP-9 (220kDa), neutrophil derived MMP-9 complexed to lipocalin (130kDa) and free proMMP-9 (92kDa). Active MMP-9 (82kDa) was not readily distinguishable on the zymograms and thus levels of active gelatinase were measured using a commercially available assay (see 2.3.5). Levels of active gelatinase measured using this method were found to be below the lowest level of detection in all of the samples tested (data not shown).

Additionally 10 out of the 22 COPD patients showed MMP activity at 72kDa, this band was confirmed as MMP-2 by immunoprecipitation.

3.3.2 TIMP-1 in BAL fluid.

The activity of MMP-9 in tissue is controlled by its cognate inhibitor TIMP-1. Levels of total immunocompetent TIMP-1 were measured in BAL using a commercially available ELISA. Figure 3.2 shows that there was no significant difference in total levels of TIMP-1 in BAL from COPD patients (median: 17.60ng/ml; IQR: 23.44ng/ml) or BAL from normal controls (median: 8.71ng/ml; IQR: 8.53ng/ml).

Figure 3.2

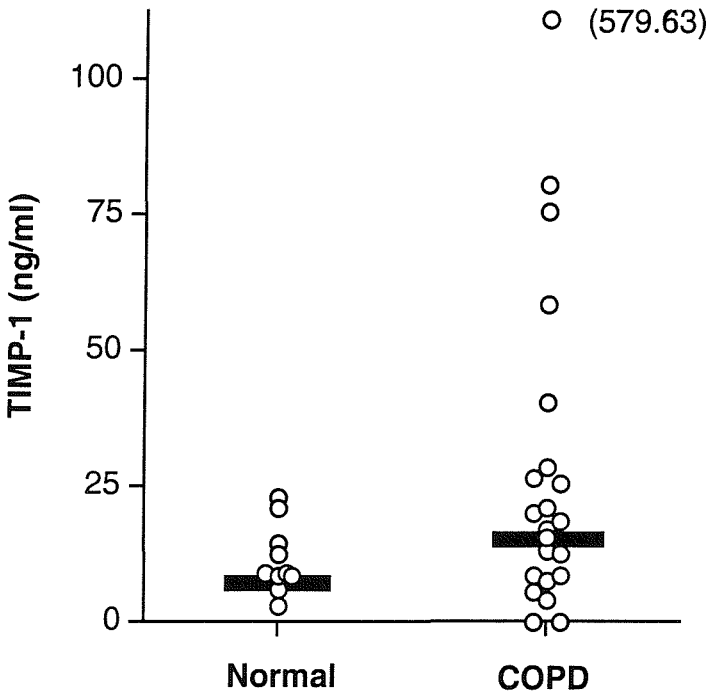


Figure 3.2 TIMP-1 levels in BAL fluid. Levels of immunocompetent TIMP-1 in BAL fluid from a group of patients suffering from COPD (n=22) and a group of control subjects (n=10) was analysed by ELISA. TIMP-1 levels were expressed as ng/ml, values beyond the range of the graph axes are indicated in brackets. Median values are marked.

The data illustrated in figures 3.1 and 3.2 was used to calculate the molar ratio of MMP-9 to TIMP-1 in BAL from each individual patient. Two individuals with COPD had TIMP-1 levels below the level of detection for the commercially available ELISA and so could not have the molar ratios accurately calculated. Following calculation, the molar ratio of MMP-9/TIMP-1 was significantly higher in COPD patients (median: 0.23; IQR: 0.5) than normal individuals (median: 0.12; IQR: 0.24).

Figure 3.3

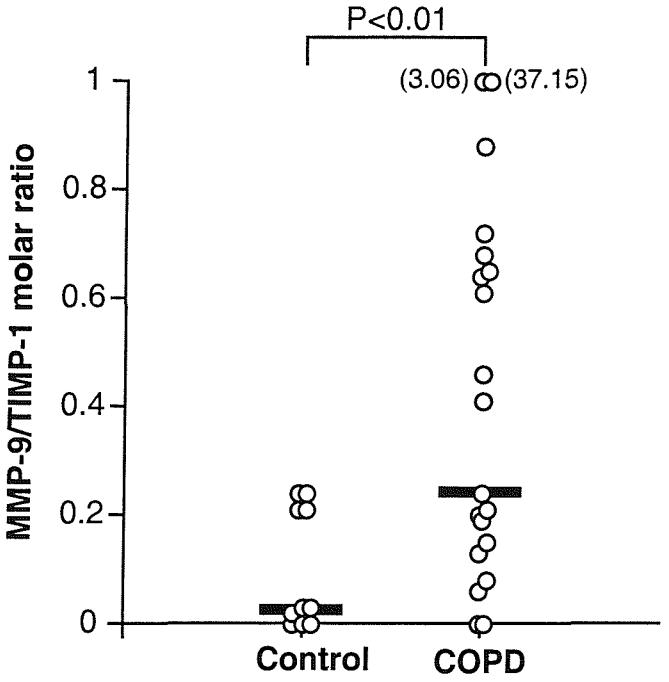


Figure 3.3 MMP-9/TIMP-1 molar ratios in COPD BAL. Data on MMP-9 and TIMP-1 levels in BAL from each individual were used to calculate the molar ratio of MMP-9/ TIMP-1. Values beyond the range of graph axes are indicated in brackets. Median values are marked as solid bars. Data were statistically analysed using the non-parametric Mann Whitney U Test, $P<0.05$ was considered statistically significant.

Molar ratios in both control and COPD populations remain below 1. This indicates that despite the trend towards MMP-9/ TIMP-1 imbalance seen in COPD patients, levels of immunocompetent TIMP-1 exceed MMP-9 at the alveolar level.

3.3.4 Cytology of BAL fluid.

Cell counts were obtained from 15 of the COPD patients studied. Macrophages were found to infiltrate BAL in greatest numbers, followed by neutrophils. No significant populations of eosinophils were detected in BAL fluid (data not shown).

Table 3.1 Median cell counts in COPD BAL.

	Neutrophil	Macrophage	Lymphocyte
Median	$2.1x10^4$	$1.1x10^5$	$2.0x10^3$
IQR	$3.0x10^4$	$2.8 x10^5$	$1.2x10^3$
N	15	15	15

Relative subpopulations of leukocytes were determined by differential cell count of 1000 cells performed after cytocentrifugation and staining with May-Gruenswald-Giemsa. Median values are expressed as cells/ml and correlation with MMP-9 or TIMP-1 was analysed using the non-parametric Spearman Correlation.

No correlation was found between MMP-9, TIMP-1 and the major inflammatory cells: macrophages, neutrophils and lymphocytes.

3.4 Discussion.

Proteinase: antiproteinase balance in the lung is believed to be an important mediator in the pathogenesis of COPD. More recently attention has focused on the role of MMPs, especially the gelatinases (MMP-9 and MMP-2). Both asthma and COPD are diseases which involve airway remodelling. Importantly elevated levels of MMP-9 have been found in BAL fluid from asthmatics and released in increased amounts from important inflammatory cells such as neutrophils and eosinophils [Mautino *et al.,* 1997, Warner *et al.*, in press]. This suggests MMP involvement in the remodelling process. While remodelling in asthma can lead to epithelial shedding and fibrosis, remodelling in COPD involves break down of the extracellular matrix leading to parenchymal destruction, loss of alveolar attachments, basement membrane integrity and ultimately airway collapse. MMP-9 has the capacity to degrade type IV collagen, a major component of basement membranes and thus could potentially have a profound influence on extracellular matrix remodelling. Importantly elevated levels of MMP-9 have been found in BAL from patients with emphysema [Finlay *et al.*1997a, Betsuyaku *et al.,* 1999], or COPD as defined by the American Thoracic Society (ATS) [Segura-Valdez *et al.,* 2000], in comparison with BAL from healthy individuals. This study extends and confirms these findings. We found significantly higher levels of MMP-9 in BAL fluid from COPD patients than normal individuals (Figure 3.1) ($P<0.005$). All samples were taken from patients with stable COPD, despite this however a wide heterogeneity was observed in the amount of MMP-9 measured in BAL from different individuals. This mirrors the findings of other studies and reflects the variation in the pathology of the disease [Finlay *et al.*,1997a]. The wide-ranging MMP-9 burden could

result from different levels of inflammatory response, influenced by the interplay of a variety of factors. These include smoking status and history or differing levels of colonisation by COPD typical pathogens such as rhinovirus, or *Haemophillus influenzae* [Bhowmik *et al.*, 2000]. Alternatively certain individuals may have components of asthma, contaminating and adding to their COPD pathology [Jeffrey, 1998a]. The effects of such artefacts are unknown and largely impossible to dissect out of such a study.

Previous authors studying MMP-9 levels in COPD BAL have not analysed levels of the cognate inhibitor TIMP-1. TIMP-1 is an important inhibitor of MMP-9 in the lung forming a 1 to 1 complex with either the pro form of MMP-9, preventing its activation, or with the active enzyme, inhibiting MMP-9 activity. This study indicated that there was no significant difference in levels of immunocompetent TIMP-1 between either the COPD patients or normal individuals (Figure 3.2). The net result of this being more molecules of MMP-9 per molecule of TIMP-1 in COPD patients compared to normals. Interestingly, despite this shift in favour of proteinase, TIMP-1 remains in control since the calculated molar ratios remain less than 1 in the majority of COPD patients studied (1 individual had a molar ratio greater than 1). On initial observation, high amounts of TIMP coupled with low amounts of MMP means that there is little chance for enzyme activity, and thus matrix degradation. However conclusions must be drawn with caution since subtle changes could occur in the inflammatory environment, affecting proteinase: inhibitor balance. Here we have measured levels of immunocompetent TIMP-1 in BAL. TIMP-1 however could be inactivated by a variety of factors including proteinases; neutrophil elastase chymotrypsin and trypsin are known to cleave TIMP-1 [Okada *et al.*, 1988, Williamson *et al.*, 1993], and the free radical by products of oxidative stress such as hypochlorus acid and peroxynitrite

[Shabani *et al.,* 1998]. Inhibitor inactivation could alter the MMP-9/ TIMP-1 balance in favour of excess proteinase and remodelling, without affecting the amount of TIMP-1 detectable by conventional ELISA (see also Chapter 6). Such issues must be taken into consideration when interpreting this data. Despite BAL giving a good general picture of alveolar inflammation, subtle pericellular changes may be distorted due to a dilution effect. For example, enzymes are stored in extremely high concentrations in the granules of inflammatory cells; it is estimated that neutrophil elastase is stored at a concentration of approximately 5mM, far outweighing physiologic extracellular concentrations of innate neutrophil elastase inhibitors (32μM) [Liou *et al.*, 1996]. Thus proteinase concentrations are likely to be extremely high in the immediate environment of a degranulating cell, far outweighing inhibitor concentrations. As the enzyme diffuses away from the cell, MMP: TIMP ratio reaches equilibrium. Therefore the opportunity for MMP activation will be greatest in the area close to the cell surface; a phenomenon described as quantum proteolysis [Liou *et al.*, 1996]. Additionally, recent work has found that MMP-9 and TIMP-1 are compartmentalised into distinct neutrophil vesicles [Price *et al,* 2000], speculating that proteinase and inhibitor release could occur at subtly different time intervals. If MMP-9 release occurred slightly before TIMP-1 there would clearly be an MMP-9 TIMP-1 imbalance in the pericellular environment, not reflected by the large sample volume in BAL.

Finally the cellular source of MMP-9 and TIMP-1 in BAL was investigated (Table 3.1). Previous authors have found that MMP-9 in BAL from COPD patients shows a significant association with levels of the neutrophil specific protein lipocalin. This suggests that neutrophils are a major source of MMP-9 [Finlay *et al.*1997a, Betsuyaku *et al.,* 1999], however a relationship between MMP-9 and cell number proved impossible to establish [Betsuyaku *et al.,* 1999].

Similarly in this current study, no correlation was found between MMP-9, TIMP-1 and any of the major inflammatory cells including neutrophils, macrophages and eosinophils. It is of note that BAL measures mediator and cell levels in the airways, however it is unlikely that degranulating cells are to be found exclusively in the airway lumen. For example cells could be releasing their contents as they are sequestered in the microvasculature, or alternatively as they migrate from the blood stream into the lung. It is likely that MMP-9 and TIMP-1 from all of these sub populations of cells accumulate in the airspaces and are subsequently measured in BAL. In contrast however only those cells which have successfully migrated into the airspaces are accounted for in cell counts from BAL. This ultimately leads to dissociation between cell counts and mediator levels in BAL.

In summary the results in this chapter have illustrated that MMP-9 levels are significantly elevated in individuals with COPD when compared to a group of normal individuals. Molar TIMP-1 levels do not change between the two groups, and seem consistently high enough to abolish molar levels of free MMP-9. While this is the case however, limited conclusions can be drawn as to whether the proteolytic effects of MMP-9 are inhibited in the pericellular environment.

Chapter 4.

MMP-9, TIMP-1 and inflammatory changes during exacerbation.

4.1 Introduction.

Exacerbations or periodic and acute worsening of symptoms represent an important feature in the progression of chroinc obstructive pulmonary disease (COPD). They are commonly characterised as acute increases in dyspnea, cough, and sputum production with increased purulence of sputum. They are reported to be experienced by patients between one and four times per year, on average [Anthonisen *et al.,* 1987] and alarmingly the current mortality rate of COPD patients admitted to hospital with exacerbations is estimated at 14% [Fabbri *et al.*, 1998]. The aetiology of exacerbations is unsure. There is evidence to suggest exacerbations are acute inflammatory events triggered by viral infection [Seemungal, *et al.*, 1998] or bacterial colonisation [Soler *et al.*, 1998, Stockley, 1998], however, recent evidence conflicts with this theory, suggesting the inflammatory response actually occurs independently of demonstrable respiratory infection [Aaron *et al.* 2000]. Whatever the trigger, increased inflammation during exacerbation conceivably results in increased proteolytic burden, contributing to the remodelling seen in COPD. Information on protease changes close to the onset of exacerbation is sparse, in part because bronchoscopy is virtually impossible to perform on these patients. Analysis of induced sputum forms a safe and reproducible alternative to study airway lining fluid [Bhowmik *et al.*, 1998, Bhowmik *et al.*, 2000] during such episodes, and this study utilises sputum to analyse changes in matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels. Patients were split into two groups: sampling sputum before and during an exacerbation (n=13) or during exacerbation and 4-6 weeks following (n=13). Levels of MMP-9 were assayed and correlated with inflammatory cell infiltrate. The amounts of the specific inhibitor of MMP-9, TIMP-1 were also measured, thus allowing analysis of MMP-9: TIMP-1 balance through the course of an exacerbation. Additional assays measured levels of MMP-3 and active gelatinase through the course of an exacerbation.

4.2 Methods.

Induced sputum samples from a group of twenty COPD patients, part of a cohort of patients followed long term since October 1995 in the East London COPD study [Bhowmik *et al,* 1998], were kindly donated by Dr Angshu Bhowmik, at the St Bartholomew's Hospital. Inclusion criteria for the study were: FEV_1 <70% predicted for age and height, β_2 agonist reversibility < 15% or 200ml with airflow obstruction evidenced by an FEV_1/FVC ratio <70%. Patients with a history of asthma, bronchiectasis, carcinoma of the bronchus or other significant respiratory disease were excluded. All patients studied suffered acute exacerbations in COPD. Exacerbations were defined as any two of three major symptoms - increase in dyspnea, sputum purulence and sputum volume; or at least one major symptom in conjunction with either a cold (increase in nasal discharge or congestion), wheeze, sore throat, cough or fever [Anthonisen et al 1987].

The cohort of 20 patients experienced a total of 26 exacerbations, during which sputum samples were taken. For 13 of these exacerbations, paired samples had also been obtained from patients during periods of stable COPD prior to the exacerbation (see table 4.1a for clinical details). For the 13 remaining exacerbations paired sputum samples were obtained from patients 6-8 weeks following the exacerbation (see table 4.1b for clinical details). The characteristics of all subjects during both stable COPD and during exacerbation are reported in table 4.1.

Additionally sputum samples taken from a group of 6 healthy subjects, with no smoking history (median age 23) were used as negative controls. Cystic fibrosis (CF) patients are known to have excessive MMP-9 burden in their lungs [Delacourt *et al.,* 1995], and sputum from a group of 12 CF patients was used as positive controls for descriptive analyses.

Table 4.1a. Clinical details of subjects sampled prior to and during exacerbation

Subject No.	Age (Yr).	Sex (M/F)	Smoking (Pack year)	FEV_1 (% pred) Stable	FEV_1 (% pred) Exacerb.	FEV_1/FVC (%) Stable	FEV_1/FVC (%) Exacerb.
pre1	56	F	35	45.14	36.57	46.77	42.34
pre2	53	F	30	39.67	34.00	37.19	37.09
pre3	64	M	20	33.00	23.30	45.00	41.67
pre4	73	M	78	58.39	56.57	41.78	46.41
pre5	60	M	23	25.61	22.43	17.63	16.8
pre6	75	M	26	33.45	37.63	25.95	32.64
pre7	74	F	51	47.14	58.57	60.74	52.34
pre8	74	M	83	20.89	22.94	20.27	23.02
pre9	84	M	18	73.21	66.03	56.46	56.33
pre10	63	M	10	41.48	44.32	73.37	73.93
pre11	77	M	16	42.11	45.15	59.41	52.74
pre12	64	M	66	27.27	34.94	49.23	59.13
pre13	72	M	58	41.61	48.06	61.43	61.32
Median	72	3F/ 10M	30	41.48	37.63	46.77	46.41
IQR	13		43	16.01	23.67	28.51	22.87

Table 4.1b. Clinical details of subjects sampled during and 4-6 weeks after exacerbation.

Subject No.	Age (Yr).	Sex (M/F)	Smoking (Pack year)	FEV_1 (% pred) Exacerb.	FEV_1 (% pred) Stable	FEV_1/FVC (%) Exacerb.	FEV_1/FVC (%) Stable.
post1	61	M	150	19.11	19.74	23.35	33.69
post2	69	M	53	39.13	35.12	46.62	53.57
post3	60	M	23	23.45	26.95	19.87	17.06
post4	68	M	51	44.66	41.11	47.88	44.83
post5	77	M	128	28.35	35.82	23.52	25.95
post6	77	M	128	30.97	41.79	34.87	28.71
post7	74	M	3	43.75	43.75	65.94	54.82
post8	75	M	26	19.86	22.34	25.11	25.3
post9	63	M	105	33.03	36.34	45.64	45.66
post10	74	M	18	19.52	18.15	19.72	19.92
post11	68	M	16	31.92	28.34	30.63	25.89
post12	63	M	10	44.32	45.45	73.93	76.92
post13	77	M	16	54.95	54.05	52.36	59.41
Median	69	13M	26	31.92	35.82	34.87	33.69
IQR	13		100.50	22.38	18.13	26.69	28.60

Sputum supernatants were taken from a group of COPD patients during periods of stable COPD and exacerbations. Clinical details such as smoking history, changes in lung function (as indicated by change in FEV_1 and ratio of FEV_1/ FVC) were noted when samples were taken. These data are listed above. Patients fell into 2 distinct groups: those sampled both prior to and during exacerbation (Table 4.1a, pre1-pre13), or alternatively patients sampled during exacerbation and 4-6 weeks later (Table 4.1b, post1-post13). Neither FEV_1 nor FEV_1/ FVC were found to differ significantly from stable periods of COPD to periods of exacerbation.

4.3 Results

4.3.1 MMP levels in COPD sputum.

Initial analyses in figure 4.1 looked at variation in MMP-9 levels between groups of COPD patients and normal subjects. Striking differences were seen between the levels of MMP-9 in the sputum of normal individuals (median= 0μg/g sputum) and COPD patients sampled during exacerbation (median= 17.10μg/g) and 4-6 weeks later (median= 25μg/g) (P<0.005). There was a striking 20 fold difference in the highest median MMP-9 level in COPD, and median MMP-9 recorded in cystic fibrosis patients (median= 420μg/g). Differences in MMP-9 levels between normal individuals and CF patients reached statistical significance (P< 0.005).

Figure 4.1

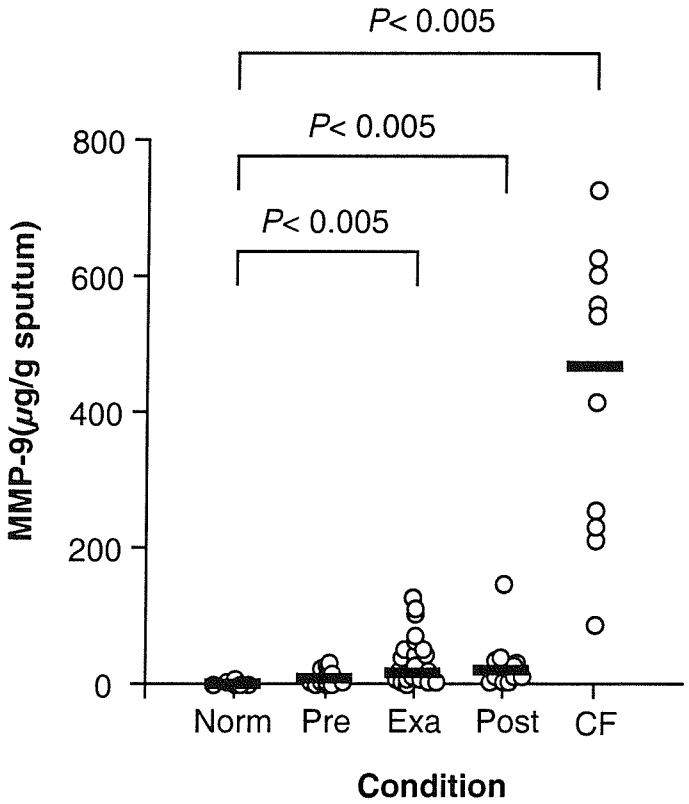


Figure 4.1. MMP-9 levels in sputum from COPD patients. MMP-9 levels were measured in sputum supernatants using gelatin zymography. Samples grouped as either before an exacerbation (n=13), during exacerbation (n=26), or 4-6 weeks after exacerbation (n=13). MMP-9 levels were also measured in normal controls (n=6) and cystic fibrosis patients (n=10) Median values are marked as solid bars. Data were analysed by the non-parametric Mann Whitney Test, P< 0.05 was considered to be statistically significant.

Figure 4.2 illustrates the analysis of MMP-9 levels in paired samples of sputum taken from patients either before and during exacerbation or during and after exacerbation. This allowed the analysis of MMP-9 levels through the progression of disease. There was a statistically significant increase in MMP-9 during an exacerbation ($P<0.01$); 11 out of 13 patients studied showed an increase in MMP-9 levels from baseline to during an exacerbation causing median values to increase from 10.49μg/g to 17.10μg/g (Figure 4.2).

Figure 4.2

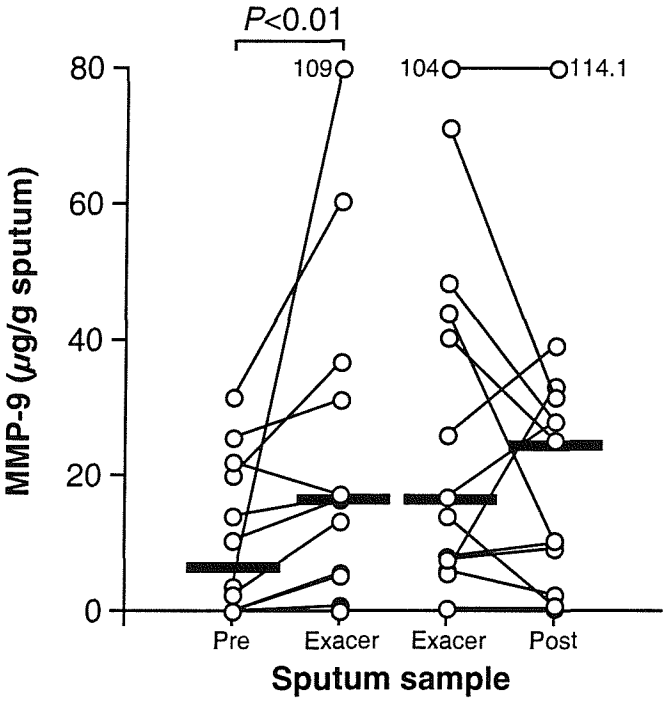


Figure 4.2 MMP-9 levels in COPD sputum during exacerbation. MMP-9 levels were measured in sputum from COPD patients using gelatin zymography. Samples were taken from patients prior to and during exacerbation (n=13). In a separate group MMP-9 levels were measured in samples taken during exacerbation and 4-6 weeks later (n=13). Data were expressed as μg/g and median values marked as solid bars. Data were analysed by the non-parametric Wilcoxon Signed Rank Test, $P<0.05$ was considered statistically significant.

We also found similar MMP-9 levels during exacerbation in the second set of samples, with a median of 17.00μg/g. Interestingly, 4-6 weeks later, amounts had not significantly changed, in fact the median level increased slightly to 25μg/g (Figure 4.2). This value reflects a rise seen in 6 patients, and a fall in 7 out of the 13 studied, and

suggests that efficiency of MMP-9 clearance from the lungs after an exacerbation varies from individual to individual.

MMP-3 is an important enzyme since it is known to activate MMP-9. MMP-3 levels were measured through the course of an exacerbation, using casein zymography. This has limitations since it is far less sensitive than gelatin zymography, in terms of both limits of detection and specificity of enzyme identification (see 2.3.2). Limitations in specificity meant that only weak signals for enzyme activity were obtained and no significant variation in levels of MMP-3 were seen during the course of an exacerbation.

4.3.2 TIMP-1 levels in COPD sputum.

Control of MMP function has a very important part to play in tissue remodelling diseases such as COPD. An important point of control of the activity of MMP-9 is inhibition by its cognate inhibitor TIMP-1.

Figure 4.3

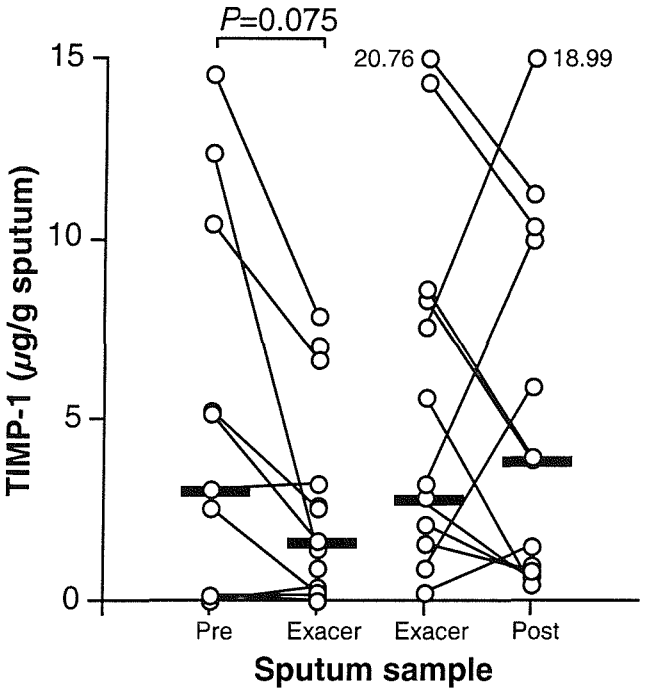


Figure 4.3. TIMP-1 levels in COPD sputum during exacerbation. Immunocompetent TIMP-1 levels (μg/g) were measured in paired sputum samples, taken from COPD patients prior to and during an exacerbation in COPD (n=13). In a separate group TIMP-1 levels were measured in samples taken during an exacerbation and 4-6 weeks later (n=13). Median values are marked as solid bars. Data were analysed by a non parametric Wilcoxon signed rank test.

As with MMP-9, levels of immunocompetent TIMP-1 were measured through the progression of the disease. A commercially available ELISA was used to analyse this and the data illustrated in figure 4.3. There was a decrease in levels from 3.51 μg/g before to 1.54 μg/g during exacerbation, this fall approached statistical significance with *P*= 0.075 (Figure 4.3). Interestingly there was no significant change in TIMP-1 levels in sputum sampled both during and after exacerbation; median levels showed a very slight increase from 3.20μg/g to 3.90μg/g (Figure 4.3). The MMP-9 increase seen in figure 4.2, coupled with the lack of significant TIMP-1 variation seen in figure 4.3

suggests that an MMP-9: TIMP-1 imbalance could exist during an exacerbation in COPD.

4.3.3 Molar ratio of TIMP-1:MMP-9 in COPD sputum

Figure 4.4

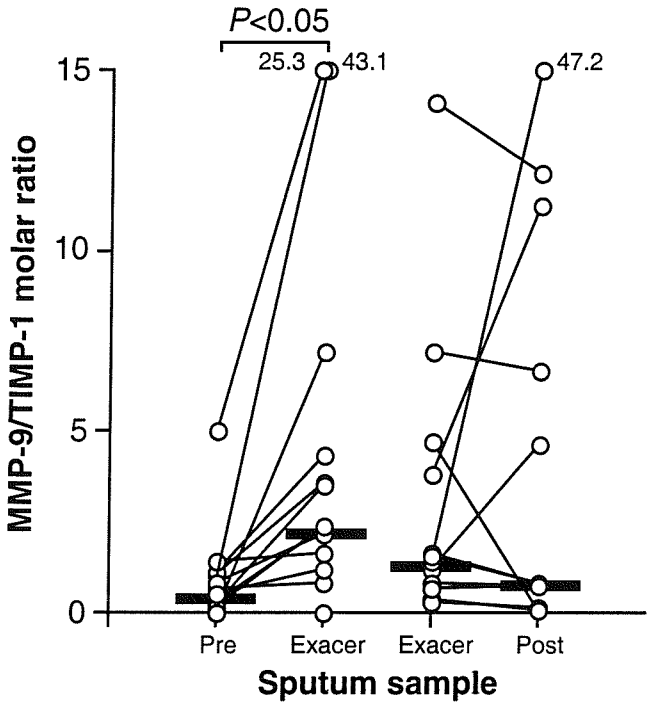


Figure 4.4. Molar ratio between MMP-9 and TIMP-1 in COPD sputum. Data on MMP-9 and TIMP-1 levels in BAL from each individual were used to calculate the molar ratio of MMP-9:TIMP-1. Values beyond the range of graph axes are indicated on the figure. Median values are marked as solid bars. Data were statistically analysed using the Wilcoxon Signed Rank Test, P<0.05 was considered as statistically significant.

The data from figures 4.2 and 4.3 was used to calculate the molar ratio of MMP-9/TIMP-1 (Figure 4.4). This data indicates COPD that exacerbations are marked by a molar imbalance of MMP-9 with respect to TIMP-1. Significantly the median MMP-9: TIMP-1 ratio is 4 fold higher in supernatants sampled during exacerbation (median = 2.35), than in supernatants taken before exacerbation (median = 0.59) ($P< 0.05$), where the balance is in favour of TIMP-1. In the second group of paired samples taken during exacerbation and 4-6 weeks later there was no significant change in median molar ratio. Median ratio was calculated as 1.4 during exacerbation and 0.83 afterwards. It is

possible therefore that protease imbalance exists for some time after an exacerbation, and may even increase further in certain individuals.

4.3.4 Gelatinase activity in COPD Sputum.

In order to assess the activity of MMP-9 in sputum a soluble substrate Enzchek assay (Molecular Probes) was used which looks at levels of net gelatinase and collagenase activity in clinical samples. The major limitation of the assay was that it had a low signal to noise ratio, therefore reproducible positive results were only obtained from patients with the highest levels of MMP-9 in sputum. This meant that activity levels could only be measured in 9 patients sampled prior to and during exacerbation, and 6 patients in the separate group sampled during exacerbation and 4-6 weeks later (Figure 4.5).

Figure 4.5

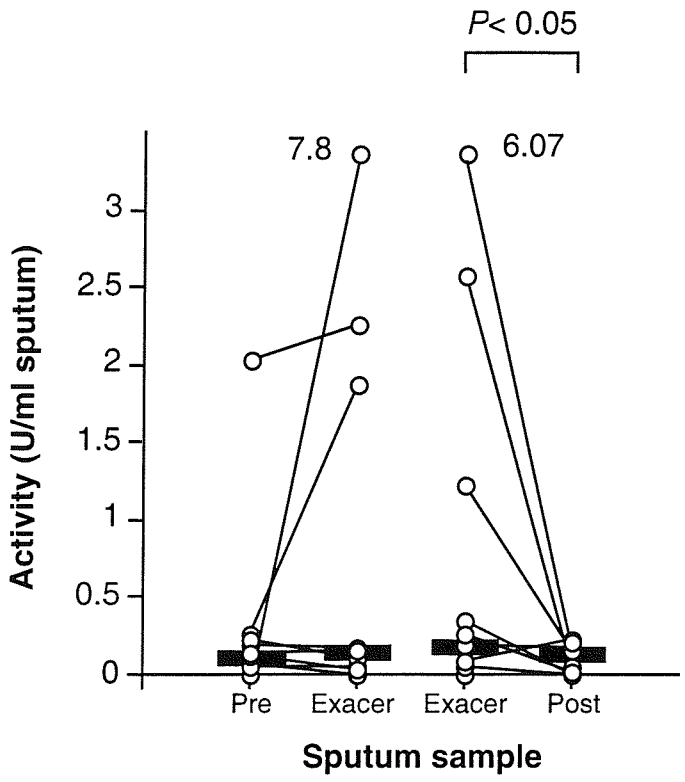


Figure 4.5. Gelatinase activity levels in COPD sputum. Levels of active gelatinase/ collagenase (U/g)* were measured using the Enzchek soluble substrate assay, in sputum from COPD patients at baseline, and during exacerbation (n=9), or during exacerbation and 4-6 weeks later (n=6). Data were analysed by a non parametric Wilcoxon signed rank test. $P< 0.05$ was considered as significant.
*1 unit of enzyme is defined as the amount of enzyme required to liberate 1μmole L-leucine from collagen, in 5 hours at 37°C, pH 7.5.

Interestingly in contrast to the MMP-9 increase from before to during exacerbation (Figure 4.2 & 4.4). Figure 4.5 indicates there was no significant change in activity over the same period. Only 3 out of the 9 patients actually showed an increase in activity, with median levels changing from 0.14U/g at baseline to 0.18U/g during an exacerbation. Following exacerbation there is a decrease in gelatinase activity, showing a fall from a median level of 0.26U/g to 0.17U/g ($P<0.05$); reflecting a reduction in 5 out of 6 patients. These data are difficult to analyse since they only reflect a sub sample of the original patient groups.

4.3.4 Inflammatory cell infiltration in COPD sputum.

Figure 4.6

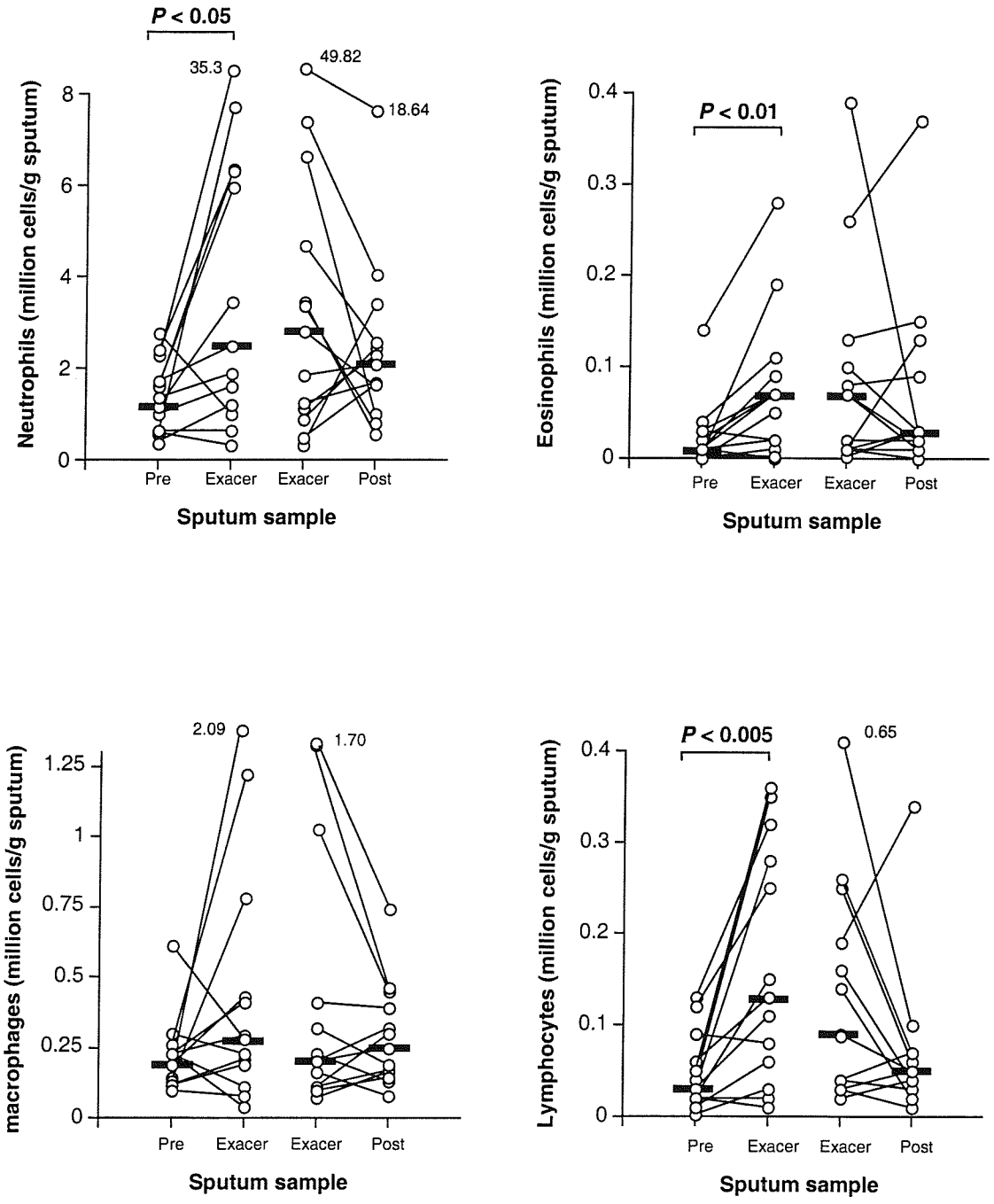


Figure 4.6 Inflammatory cell changes during an exacerbation. Differential cell counts were carried out on paired sputum samples taken from COPD patients prior to and during an exacerbation in COPD (n=13). In a separate group, paired samples were taken during an exacerbation and 4-6 weeks later (n=13) and again differential cell counts carried out. Median values are marked as solid bars. Data were expressed as million cells/ g of sputum and analysed by the non-parametric Wilcoxon Signed Rank Test and $P< 0.05$ was considered statistically significant.

The data presented in figure 4.6 and table 4.2 show infiltration of neutrophils, eosinophils and lymphocytes into supernatants significantly increases during an exacerbation (Table 4.2a and b, Figure 4.6). Interestingly median numbers of each of these cell types have fallen 4-6 weeks after exacerbation, however this fall in number does not reach statistical significance. Macrophages appear to represent a distinct cell population with median values remaining unchanged through the course of an exacerbation. It is evident that there are exceptions to this general rule with 3 individuals in particular showing a greater than 3 fold increase, however these data do not alter the level of statistical significance.

Table 4.2a Cell counts prior to and during exacerbation

Cell type	Pre-exacerbation	Exacerbation	*P*-value
Neutrophil	1.37	2.50	0.05
Macrophage	0.23	0.28	NS
Eosinophil	0.01	0.07	0.01
Lymphocyte	0.03	0.13	0.005

Table 4.2b Cell counts during exacerbation and 4-6 weeks later

Cell type	Pre-exacerbation	Exacerbation	*P*-value
Neutrophil	2.80	2.08	NS
Macrophage	0.20	0.25	NS
Eosinophil	0.07	0.05	NS
Lymphocyte	0.09	0.03	NS

Differential cell counts were obtained from induced sputum samples obtained both prior to and during exacerbation (Table 4.2a, n=13). This was repeated on a separate set of supernatants taken from individuals sampled both during exacerbation and 4-6 weeks later (Table 4.2b n=13). Median values (expressed as million cells/g sputum) and statistical significance of changes in cell number for each group are listed (Table 4.2a and table4.2b).

All of the inflammatory cells analysed are known to be sources of MMP-9 [Dahlen *et al.*, 1998 (neutrophils), Finlay *et al.*, 1997b (macrophages), Ohno *et al.* 1997 (eosinophils), Leppert *et al.* 1995 (lymphocytes)]; the potential contribution of each cell type to MMP-9 burden during an exacerbation in COPD was analysed. A variety of statistical analyses were conducted to study relationships between cells and amounts of MMP-9. Firstly, the gross effect of each cell type on MMP-9 levels was measured using non-parametric Spearman Rank Correlation.

Figure 4.7

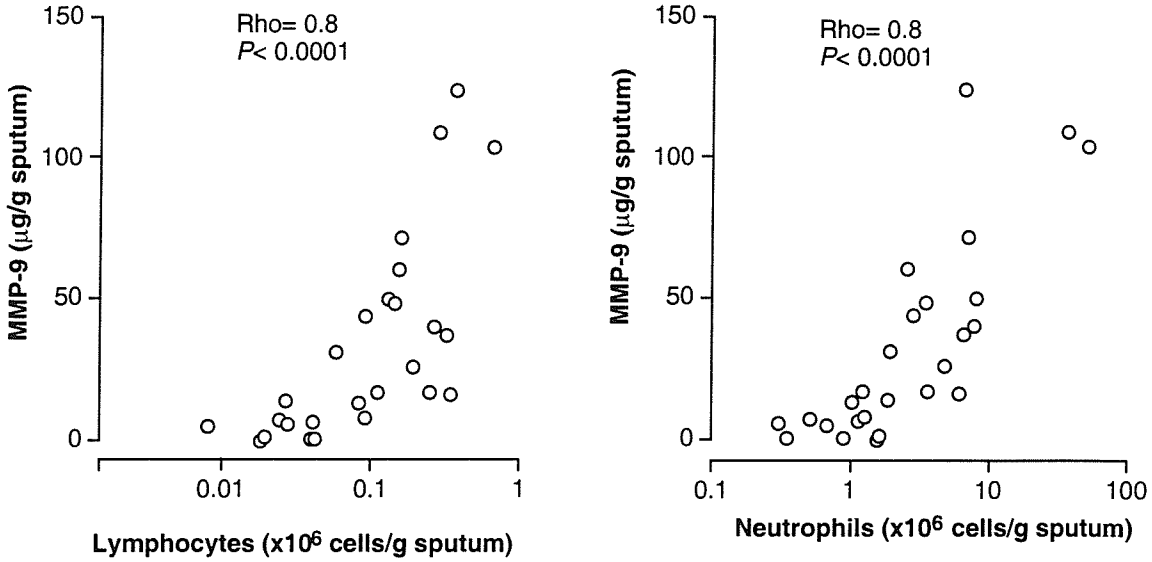


Figure 4.7 Relationship between cell infiltration and MMP-9 levels in COPD sputum during an exacerbation. Differential neutrophil (4.9a) and lymphocyte (4.9b) counts were carried out on sputum samples from COPD patients during exacerbation (n=26), and corrected for weight of sputum. Measurement of MMP-9 levels was carried out on cell free sputum supernatants. Cell count data were expressed as 10^6 cells/g and MMP-9 data as µg/g. Data were analysed by the non-parametric Spearman Rank Correlation $P<0.05$ was considered as statistically significant.

Table 4.3 Relationship of MMP-9 to inflammatory cells

Correlation	Rho	*P* value
MMP-9 Vs Neutrophils	0.83	<0.0001
MMP-9 Vs Macrophages	0.46	<0.05
MMP-9 Vs Eosinophils	0.45	<0.05
MMP-9 Vs Lymphocytes	0.79	<0.0001

The relationship between MMP-9 and different inflammatory cell types was analysed during exacerbation in COPD. Analysis was by non parametric Spearman Correlation. Both Rho and *P* values are listed for analysis of relationship significance. $P< 0.005$ was considered highly significant.

There was no significant relationship between MMP-9 and cell number either before or after exacerbation, for any of the cell types. In marked contrast during an exacerbation, MMP-9 levels showed a highly significant relationship with both neutrophils (Rho= 0.8, $P< 0.0001$), and lymphocytes (Rho= 0.8, $P<0.0001$) (Figure 4.7). The relationship between MMP-9 and either macrophages or eosinophils was statistically significant, however far less striking than with neutrophils and lymphocytes with Rho values only approaching 0.5 and $P< 0.05$ (Table 4.2).

It is probable that interactions between different inflammatory cell types, influence the total MMP-9 pool. With this in mind the relative relationships of the different cell

populations were analysed. Analysis indicated that neutrophil and lymphocyte populations have a highly significant relationship with one another (Rho=0.8, $P<0.0001$) during exacerbation.

4.4 Discussion

Patients with COPD are prone to periodic exacerbations of their illness with worsening of their respiratory symptoms [Anthonisen *et al.*, 1987, Fabbri *et al.*,1998, Bhowmik *et al.*, 2000]. Increased airway inflammation seen in exacerbations is presumed to play an important role in the pathogenesis of airflow obstruction, however relatively little is known about the exact mechanisms underlying these episodes. The characterisation of the inflammatory elements involved in an exacerbation is important in defining what drives COPD. By virtue of the non-invasive nature of sputum collection, this study has been the first to analyse change in MMP-9 and TIMP-1 close to the onset and during exacerbation. Additionally, changes in protease/ antiprotease balance have been related to change in inflammatory cell infiltration in the same sputum samples.

From the data presented it is clear that over the course of an exacerbation there is a marked increase in MMP-9. The data show a significant increase ($P<0.01$) in median levels of MMP-9 (Figure 4.2). Importantly over this same period of time there is no corresponding increase in levels of immunocompetant TIMP-1 (Figure 4.3). This results in a protease/ antiprotease imbalance during an exacerbation, reflected by a significant change in molar ratio of MMP-9: TIMP-1, ($P<0.05$) (Figure 4.4). Despite this change in molar ratio there seems to be no significant change in levels of active gelatinase over this time period (Figure 4.5), however questions about the signal to noise ratio of this assay limit the conclusions that can be drawn. Additionally, it is possible that the active gelatinase remains tightly bound to their substrates in the airway

walls, acting as a solid phase store which is not liberated into the sputum [Allan *et al* 1995, Nagase 2000 (unpublished discussion)].

Previous studies on bronchoalveolar lavage (BAL) fluid [Lacoste, *et al.,* 1993 Betsuyaku *et al.,* 1999] and induced sputum [Keatings, *et al.,* 1996 and 1997] from COPD patients have identified increased numbers of neutrophils and increased amounts of degranulation markers when compared to normal subjects. Data presented here supports and adds to previous studies on sputum showing that neutrophil influx increases further during an exacerbation (Figure 4.6) [Saetta *et al.,* 1994]. It is thought that neutrophils represent a highly mobile cell population, passing quickly from the circulation into the airway lumen [Lacoste, *et al.,* 1993] in reaction to stimuli such as TNFα and IL-8 which are found to increase during exacerbations [Aaron *et al.,* 2000]. Neutrophils are important since they store and release MMP-9 [Dahlen *et al.*, 1998, Shapiro and Senior 1999] and have previously been associated with airway obstruction in COPD. Importantly the pattern of neutrophil accumulation during an exacerbation is similar to the increase of MMP-9 (Figure 4.2 and 4.6). Intriguingly MMP-9 levels and neutrophil number actually exhibit a very strong relationship during exacerbation (Figure 4.7). This supports the hypothesis that neutrophils accumulating in the airways during an exacerbation are potentially an important source of MMP-9. The relationship between MMP-9 and neutrophils is interesting in the light of the observation that in BAL there was no association was seen between cells and MMP-9 (see 3.3.4 and Betsuyaku *et al.,* 1999). In sputum a statistically significant association is only observed during an exacerbation, reflecting the acute nature of the inflammatory episode where all of the neutrophils contributing to the MMP-9 pool have passed into the airways. In the sputa taken before or after exacerbation there is no association between MMP-9 and neutrophils. It has been argued that a lack of correlation between MMP-9 and specific cells in BAL may result from cells, randomly distributed between the microvasculature,

the lamina propria and epithelium or in the airways, contributing to a common MMP-9 pool [Betsuyaku, *et al.,* 1999]. It may be that lack of a significant inflammatory signal at time points outside of an exacerbation results in a similar random distribution of cells, thus there is no correlation between MMP-9 and neutrophils. For instance, prior to exacerbation sputum levels of the neutrophil chemoattractants TNF-α and IL-8 are low compared to levels during exacerbation. Levels fall back to baseline values when measured up to a month following an episode [Aaron *et al.,* 2000]. Such fluctuations in cytokines could be reflected in this study by similar increase and decrease in neutrophil numbers. Following exacerbation, it is likely that neutrophil clearance is more efficient in some individuals than others. It is also possible that neutrophils are cleared from the airways more rapidly than secreted MMP-9. This kinetic would leave a basal level of remnant MMP-9 in the airways following the clearance of degranulated neutrophils, destabilising any apparent relationship between MMP-9 and neutrophils after exacerbation.

Curiously, both accumulation of neutrophils during exacerbation, and the increase in MMP-9 correlates tightly with an accumulation of lymphocytes in the sputum. While it is not known whether the lymphocytes observed were predominantly CD4+ or CD8+ an argument could be constructed that these lymphocytes are initiating neutrophilia. Recent reports have suggested that CD4+ lymphocytes could play an important role in neutrophil influx into airways via the release of IL-17 [Linden *et al.,* 2000, Laan *et al.,* 1999], this cytokine is thought to stimulate IL-8 release from bronchial epithelial cells, drawing neutrophils to the airways. It is conceivable that during an exacerbation the CD4+ load in the bronchi increases, thus inducing an increase in neutrophils. Contrary to this theory, Finkelstein and co authors have identified that parenchymal destruction correlates with an increased CD8+ and macrophage burden [Finkelstein *et al.,* 1995]. It is possible that these 2 different inflammatory patterns reflect different stages in the

pathogenesis of disease: neutrophils and CD4+ representing an acute exacerbation dominated phase, macrophages and CD8+ a more chronic emphysematous phase. Alternatively they may differ as a result of the different areas of the lung from which they are sampled.

Evidence that change in MMP-9 levels during exacerbation are neutrophil driven comes from analysis of the macrophage population. Despite being known to be important sources of MMPs; there is evidence for macrophage derived MMP-9 in asthma [Mautino *et al.*, 1997], MMP-9 mRNA [Finlay *et al.*, 1997b] and MMP-12 in emphysema [Shapiro *et al.*, 1994], this study found only a weak association between MMP-9 and macrophage number. While macrophages may provide a source of MMP-9 their role is less clear cut than with the other cells studied (Table 4.3). Out of the four cell types studied, macrophages were the only population which did not change, a finding made by previous authors [Saetta *et al.*, 1994]. (Figure 4.6 and Table 4.2a.).

Although COPD is associated with neutrophillia, exacerbations in COPD are also associated with increased eosinophilia [Saetta *et al.*, 1994]. Correspondingly, a significant increase in sputum eosinophils was measured in this study also (Figure 4.6). Eosinophils have previously been highlighted as a source of MMP-9 in asthmatic inflammation [Ohno, *et al.*, 1997, Shute, *et al.*, 1997]. In contrast however this study observed only a weak association between MMP-9 and eosinophils during an exacerbation, suggesting that these cells are not a major source of MMP-9 in COPD and possibly distinguishing this condition from asthma (Table 4.3).

In summary this study has found that during an exacerbation in COPD there is an increased MMP-9 burden in the airways, which combined with unchanging TIMP-1 leads to MMP-9/TIMP-1 imbalance in favour of MMP-9. In addition this study has analysed cell changes through exacerbation, and postulated that an interplay between

infiltrating neutrophils and lymphocytes is important in driving the proteolytic imbalance.

Chapter 5.

MMP-9, TIMP-1 and inflammation in COPD lung.

5.1 Introduction.

Analysis of protease and inhibitor levels in BAL and sputum provides a good general picture of the amount released into the extracellular environment in chronic obstructive pulmonary disease. However this does not take into account matrix metalloproteinase-9 (MMP-9) or its cognate inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1) which may remains bound to extracellular matrix (ECM) or unreleased from cells. This chapter presents data from investigations focussing on MMP-9 and TIMP-1 levels in resected parenchymal lung tissue, and how this potentially relates to lung function deficit. Initial analyses focussed on homogenates of normal margin tissue samples taken from patients undergoing resection for lung cancer. The data obtained was correlated with lung function and smoking history data for each subject. Following on from this, MMP-9 and TIMP-1 expression was analysed in some of the samples using immunohistochemistry, providing a picture of proteinase and antiproteinase localisation in the lung parenchyma as well as the architectural relationship of inflammatory cells. Additionally, samples of bronchial epithelium were obtained from the primary bronchi of emphysematous lungs removed during transplant procedures. Expression of MMP-9, TIMP-1, and inflammatory cell distribution was compared in emphysema with bronchial samples taken from normal individuals, obtained post mortem following sudden death. This adds to the analysis of parenchymal samples, providing additional information on MMP-9 distribution in the upper respiratory tract.

5.2 Methods.

Table 5.1a. Patient details for non smokers

Subject No.	Age (Yr).	Gender. (M/F)	Smoking. (Pack year).	FEV_1/FVC (%)
NON1	27	F	n/a	75
NON2	62	F	n/a	89
NON3	67	M	n/a	78
NON4	84	F	n/a	72
NON5	64	M	n/a	76
Median	64	2M/3F		76
Range	57			18

Tissue samples were taken from a group of 5 non smokers (denoted NON1- NON5). Patient details including age, sex and lung function based on the ratio of FEV_1/FVC are displayed.

Table 5.1b. Patient details for current smokers.

Subject No.	Age (Yr).	Gender. (M/F)	Smoking. (Pack year).	FEV_1/FVC (%)
SM1	70	M	-	76
SM2	64	M	49	74
SM3	58	M	44	73
SM4	57	M	-	96
SM5	57	F	84	72
SM6	41	F	-	77
SM7	70	M	-	51
SM8	67	F	39.75	63
SM9		M	98	61
SM10	47	M	-	50
SM11	45	F	-	54
Median	61	7M/4F	49	72
Range	15.5		49	223

Tissue samples were taken from a group of 11 current smokers (denoted SM1- SM11). Patient details including age, sex, smoking history (No. of pack years) and lung function based on the ratio of FEV_1/FVC are listed. Number of pack years are omitted where data is unavailable.

Table 5.1c. Patient details for ex smokers.

Subject No.	Age (Yr).	Gender. (M/F)	Smoking. (Pack year).	FEV_1/FVC (%)
EX1	78	M	-	67
EX2	52	F	-	59
EX3	62	F	-	57
EX4	74	F	15	61
EX5	74	M	-	69
EX6	72	F	-	71
EX7	57	F	-	84
EX8	78	M	-	74
EX9	72	M	52	83
EX10	77	M	34	84
EX11	62	F	10	77
Median	72	5M/6F	24.5	71
Range	15		36.2	23

Tissue samples were taken from a group of 11 ex smokers (denoted EX1-EX11). Patient details including age, sex and lung function based on the ratio of FEV_1/FVC are listed. Number of pack years are omitted where data was unavailable.

Lung tissue was removed from 27 patients (14M/13F) undergoing resection for carcinoma; tissue used was from non-tumourous normal margin tissue surrounding the tumour site. Data relating to smoking history, lung function, age and sex was obtained for patients where possible (see Table 5.1).

Tissue samples were processed as previously described (see 2.2.4). Briefly tissue samples were homogenized at a temperature of 4°C in PBS containing 0.1% w/v triton X-100. MMP-9 levels were analysed using gelatin zymography and TIMP-1 levels measured using a commercially available ELISA. Levels of both MMP-9 and TIMP-1 were expressed as ng/mg tissue. In addition some lung tissue samples were processed in glycol methacrylate for resin embedding and analysis by immunohistochemistry.

As well as measuring levels of native protein in tissue samples, extraction of RNA and RT-PCR was used to analyse levels of gene expression (as described in 2.6.2). Actin expression (1kbp product) was used as an internal control. Primers for the MMP-9 were designed to transcribe a 690bp product, pimers for TIMP-1 were designed to transcribe a product of 399bp.

Table 5.2 Primers used for identification of MMP-9 and TIMP-1 expression.

	Forward primer	Reverse primer
TIMP-1	GGGGACACCAGAAGTCAACCAGA	CTTTTCAGAGCCTTGGAGGAGCT
MMP-9	TGACGCCGCTCACCTTCACTC	CTGTCAAAGTTCGAGGTGGTA

The primers used for RT-PCR are shown above from 5' to 3'.

Additional immunohistochemistry was carried out on bronchial sections, in order to give an idea of MMP-9 and TIMP-1 in the respiratory tract. Bronchial samples were taken from the resected lungs of COPD patients undergoing lung transplant at the Alfred hospital, Victoria, Australia, and donated for analysis by Prof. John Wilson. These individuals were characterised as having either emphysema (n=4), or α_1 anti

trypsin deficiency induced emphysema (n=3) (see Table 5.3 for age and gender details). Post mortem tissue samples were obtained from normal individuals and used as controls (n=4) age and gender details of control subjects were not available.

Table 5.3 Patient details for Australian emphysema resection group.

Subject No.	Age. (Yr).	Gender. (M/F)	Diagnosis
EM1	41	M	emphysema
EM2	-	-	emphysema
EM3	45	M	emphysema
EM4	63	M	emphysema
α_1 AT 1	48	M	α_1-AT deficiency
α_1 AT 2	49	M	α_1-AT deficiency
α_1 AT 3	50	F	α_1-AT deficiency
Median	48.5		
Range	22		

Bronchial sections were taken from 7 emphysematous lungs resected during transplant surgery. 4 individuals were diagnosed as having smoking induced emphysema (denoted as EM-1-EM4) and 3 diagnosed as having α_1-antitrypsin deficiency (denoted as α_1 AT 1- α_1 AT 3). Patient details including age, sex and type of emphysema are listed above. Details of the normal individuals were unavailable.

Bronchial sections were fixed in ethanol and embedded in paraffin wax. Immunohistochemical staining of the samples, using the streptavidin biotin peroxidase detection system, was used to analyse the expression of MMP-9 and TIMP-1, and related this to expression of inflammatory cell markers such as neutrophil elastase (neutrophils), and CD68 (activated macrophages).

5.3 Results.

5.3.1 MMP-9 levels in current and ex-smokers vs non smokers.

Patients were grouped according to their smoking history, and differences in MMP-9 levels between each group was analysed. As can be seen in figure 5.1 levels of MMP-9 did not significantly vary between groups of smokers or non-smokers.

Figure 5.1

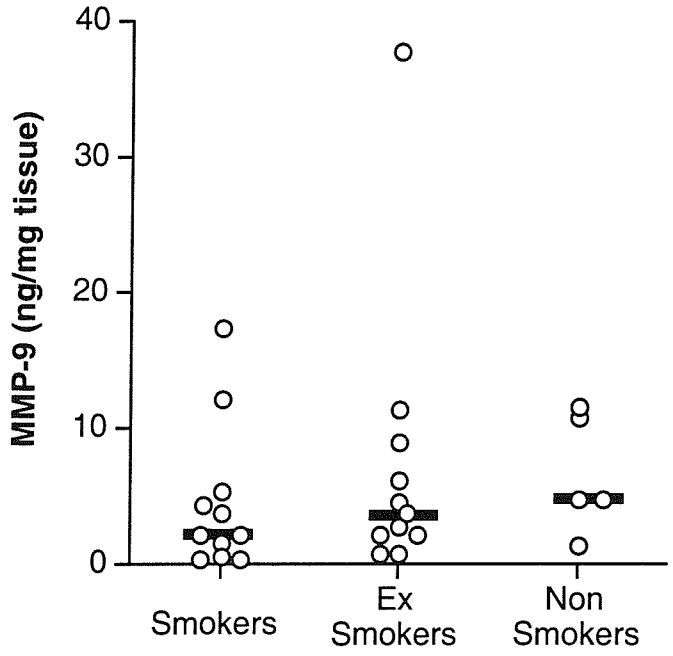


Figure 5.1 MMP-9 levels in current, ex and non smokers. Fragments of lung tissue from smokers (n=11), ex smokers (n=11) and non smokers (n=5) were homogenised at 4°C in PBS containing 0.1w/v Triton X-100. MMP-9 levels in the homogenate were measured by gelatin zymography. Values were expressed as ngMMP-9/mg tissue and the median values are marked by solid black lines. The groups were compared statistically using the non-parametric Mann Whitney Test for unpaired data.

Patients undergoing resection for lung carcinoma are primarily smokers and therefore a limiting factor in this study was the small size of the non-smoking group. While data from this study indicates that smoking history does not influence MMP-9 levels, greater numbers of non-smokers would be needed for a more statistically robust analysis.

5.3.2 MMP-9 levels in current and ex smokers.

Figure 5.2

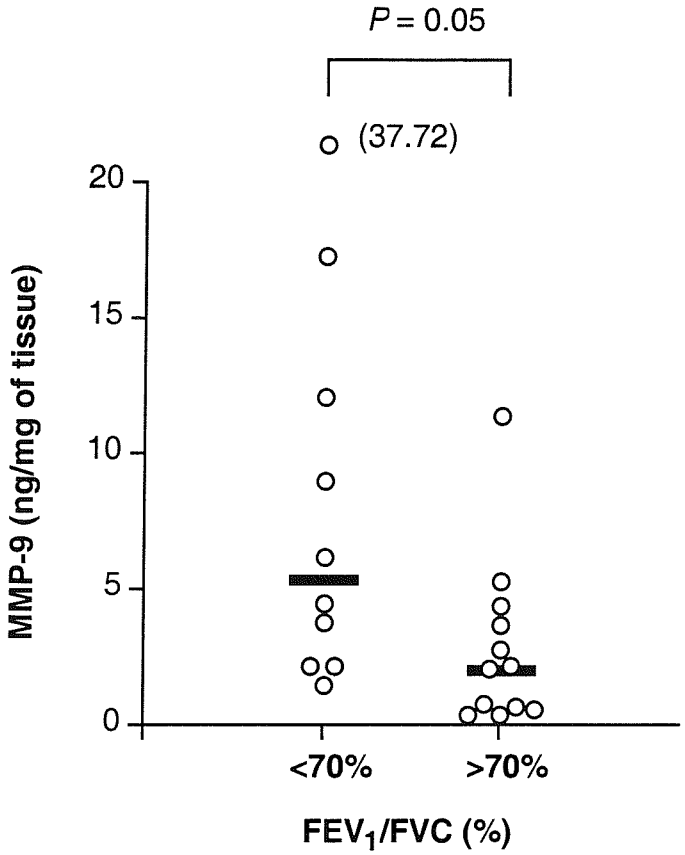


Figure 5.2 MMP-9 in lung tissue homogenates from current and ex smokers. Fragments of lung from 22 current and ex smokers were homogenised at 4°C in PBS containing 0.1w/v Triton X-100. Levels of MMP-9 were measured by gelatin zymography and expressed as ngMMP-9/mg tissue values beyond the range of axes are indicated in brackets. The data were grouped according to the lung function of the subjects FEV_1/ FVC <70% (obstructive) (n=10), or FEV_1/ FVC >70% (non obstructive) (n=12). The median values for each group are marked by black lines. The data was statistically analysed using the non-parametric Mann Whitney U Test for unpaired data.

The set of twenty-two current and ex smokers were grouped according to their lung function. Individuals with FEV_1/FVC <70% were said to have obstructive lung function, whereas those with FEV_1/FVC >70% were said to have non-obstructive lung function. It can be seen in figure 5.2 that there were higher levels of MMP-9 in the group with obstructive lung function (FEV_1/FVC <70%) (median= 5.28ng/mg tissue, IQR= 11.33ng/mg tissue) than the group with normal lung function (FEV_1/FVC >70%) (median =2.06ng/mg tissue, IQR =3.64ng/mg tissue). Differences in MMP-9 levels were found to be statistically significant (P=0.05 by Mann Whitney).

These results suggest that variation in levels of MMP-9 in lung tissue is associated with a deficit in lung function.

5.3.3 TIMP-1 levels in current and ex smokers.

In previous chapters it was reported that median levels of TIMP-1 in the BAL fluid from COPD patients did not differ significantly from those found in normal controls (see 3.3.2). It was also found that TIMP-1 levels in individuals did not change significantly during exacerbations (see 4.3.2). These previous findings are added to here with data showing that TIMP-1 levels did not significantly differ between groups of patients with obstructive or non obstructive lung function (Figure 5.3).

Figure 5.3

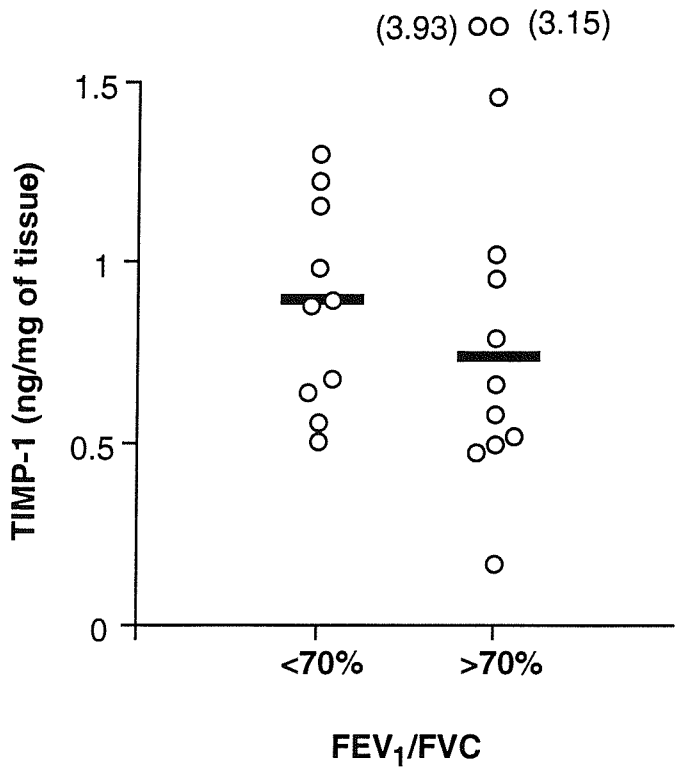


Figure 5.3 TIMP-1 levels in lung tissue homogenates. Fragments of lung from 22 current and ex smokers were homogenised at 4°C in PBS containing 0.1w/v Triton X-100. TIMP-1 levels were measured in the homogenates using a commercially available ELISA and expressed as ngMMP-9/mg tissue. Values beyond the range of axes are indicated in brackets. The data were grouped according to the lung function of the subjects FEV_1/ FVC <70% (obstructive) (n=10), or FEV_1/ FVC >70% (non obstructive) (n=12). The median values for each group are marked by black lines. The data was statistically analysed using the non-parametric Mann Whitney U Test for unpaired data.

Median TIMP-1 levels of 0.88ng/mg of tissue (IQR= 0.6) in the group with obstructive lung function compared to 0.73ng/mg of tissue (IQR = 0.85) in the non-obstructive group.

5.3.4 MMP-9/ TIMP-1 ratios in current and ex smokers.

The data illustrated in figures 5.2 and 5.3 was used to calculate the molar ratio of MMP-9 to TIMP-1 in the tissue homogenates.

Figure 5.4

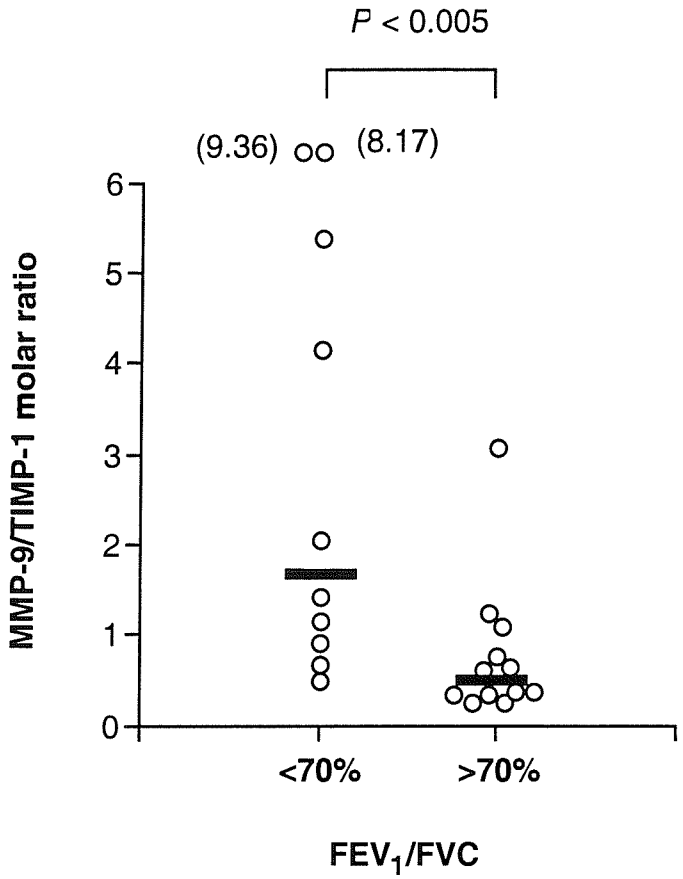


Figure 5.4. MMP-9/ TIMP-1 molar ratios in lung tissue homogenates. Data on levels of MMP-9 and TIMP-1 in the homogenized lung from 22 current and ex smokers was used to calculate the molar ratio of MMP-9 to TIMP-1. Values beyond the range of graph axes are indicated in brackets. Median values are marked as solid bars. Data were statistically analysed using the non-parametric Mann Whitney U Test for unpaired data, $P<0.05$ was considered statistically significant.

The molar ratio was significantly higher in the lungs of patients with obstructive lung function (median: 1.74; IQR: 5.22) than patients with normal lung function (median:

0.5; IQR: 0.67). The median MMP-9/ TIMP-1 molar ratio is far higher than in either sputum or BAL (see Figure 5.4). These data indicate that in individuals with impaired lung function there is a reservoir of MMP-9 either as a solid phase store, adhered to the ECM, or contained within cells. This reservoir exceeds any antiprotease screen provided by TIMP-1 and suggests that MMP-9/ TIMP-1 imbalance in the tissue could be associated with obstructive lung function (FEV_1/ FVC <70%).

5.3.5 MMP-9 and TIMP-1 gene expression.

Analysis of MMP-9 and TIMP-1 gene expression in lung tissue of current and ex smokers could potentially provide important information on the genetic basis of proteinase: antiproteinase imbalance in disease. We hoped to gain an idea of levels of gene expression in the tissue samples from current and ex smokers. Figure 5.5 illustrates the RT-PCR products from 5 randomly selected lung tissue samples.

Figure 5.5

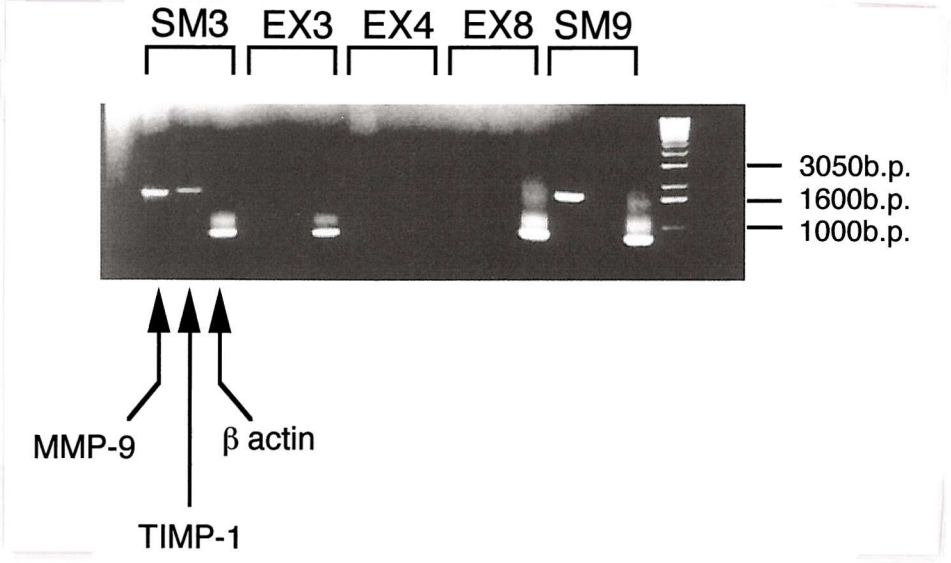


Figure 5.5 RT-PCR of human lung. Samples human lung tissue samples were removed from 5 individuals (from left to right SM3, EX3, EX4, EX8, SM9; see table 5.1 for clinical details) during lung resection procedures, and mRNA was extracted by TRIZOL extraction. RT-PCR was performed on each of the mRNA samples, with primers specific for transcription of either β actin (internal control), TIMP-1, or MMP-9 products. The results were visualised on a 1% agarose gel, impregmatesd with ethidium bromide (0.5μg/ml)

Studies repeatedly yielded inconsistent levels of gene expression probably resulting from poor RNA preservation in samples. This could be caused by a variety of factors. For example adequate and immediate freezing of tissue samples was impossible to arrange between Southampton and either the operating theatre or laboratory staff in London. This was coupled to the fact that the lung tissue- particularly that of heavy smokers- was often in a poor condition, often with visible blackening and tissue damage, presenting a continual risk of RNA degradation. Additionally the collagenous nature of lung tissue also made effective RNA extraction difficult. Consequently gene products were found to be poor in quality as illustrated in Figure 5.5, either not expressing one or other of the mRNA products of interest while the internal control is positive, (samples.B, D and E, Figure 5.5) or not expressing any product at all (sample C, Figure 5.5).

Primers for MMP-9 and TIMP-1 should transcribe fragments of 690bp and 399bp respectively, however both primer sets yielded products of approximately 1.6kbp. Removal and sequencing of the 1.6kbp bands showed the presence of introns, suggesting DNA contamination. The difficulties experienced in obtaining pure mRNA from human lung prevented these studies from progressing further.

5.3.6 Immunohistochemistry on lung tissue.

Immunohistochemistry is an extremely powerful technique allowing the visualisation of proteins in their native tissue. In this study we have analysed tissue samples obtained from two distinct areas of the lung namely the parenchyma; indicating the alveolar expression of proteinases, and the primary bronchus; indicating the epithelial localisation of proteinases in the upper respiratory tract.

Parenchymal tissue samples were obtained from the same group of patients as the homogenate samples. These samples were fixed in acetone and embedded in glycol

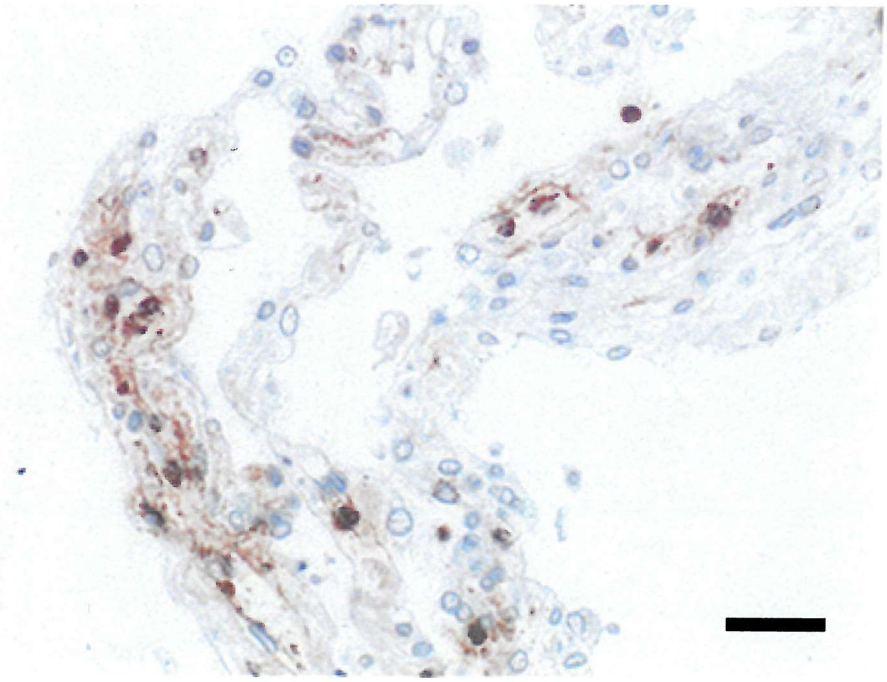
methacrylate (GMA). Bronchial tissue was obtained from a group of patients diagnosed as having COPD, or post mortem tissue from a group of normal healthy individuals. These specimens were fixed in cold ethanol and embedded in paraffin wax.

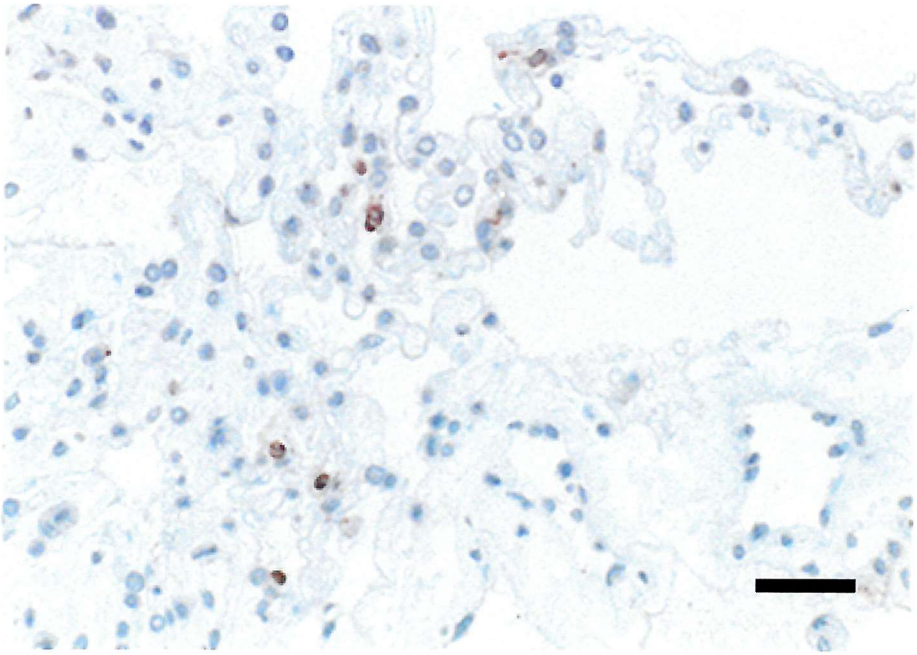
5.3.6a MMP-9 and TIMP-1 in lung parenchyma.

The staining presented here is from two individuals, representing the two poles of the study groups in the lung homogenate experiments described above. Both were stained with monoclonal antibodies for either MMP-9 or TIMP-1 (see Table 2.1, section 2.1.1). Figure 5.6 (upper panel) shows MMP-9 staining in a sample obtained from a male, 70 years of age, with a smoking history (SM7; see table 5.1b). This individual presented with an FEV_1/FVC of 51% indicating obstructive disease. There is intense MMP-9 staining in infiltrating inflammatory cells however, the cuts were not sequential and so identification of cellular MMP-9 sources is difficult. Interestingly, there was evidence of extensive extracellular immunoreactivity, indicating MMP-9 released from inflammatory cells and binds to the ECM in large amounts.

The control sample (Figure 5.6, lower panel) was from a female, 27 years of age, who was extremely active, and had never smoked (NON1; see table 5.1a). The FEV_1/FVC for this individual was 75%, indicating normal lung function. As can be seen there was little MMP-9 immunoreactivity in this sample. This pattern of MMP-9 staining is strikingly different to that found in the obstructive lung (Figure 5.6 lower panel).

Figure 5.6 facing page. MMP-9 staining in lung parenchyma. Staining for MMP-9 in the lung parenchyma from a 70 year old individual (SM7; see table 5.1b) with obstructive lung function and 50 year smoking history (upper panel). Compared to MMP-9 staining in lung parenchyma from a 27 year old individual (NON1; see table 5.1a) with good lung function and no smoking history. *Bar* represents 50μm.

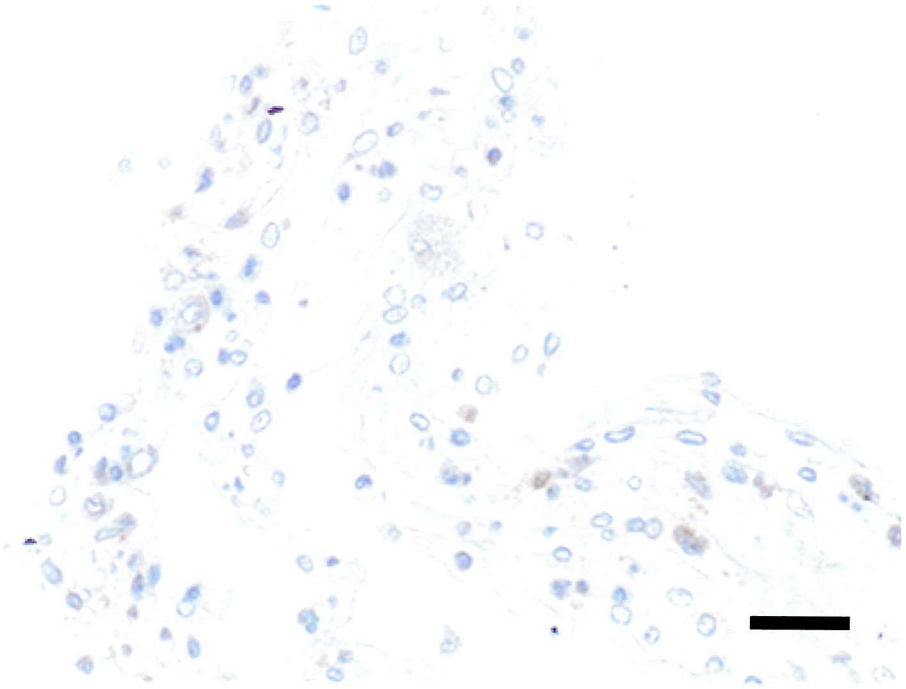


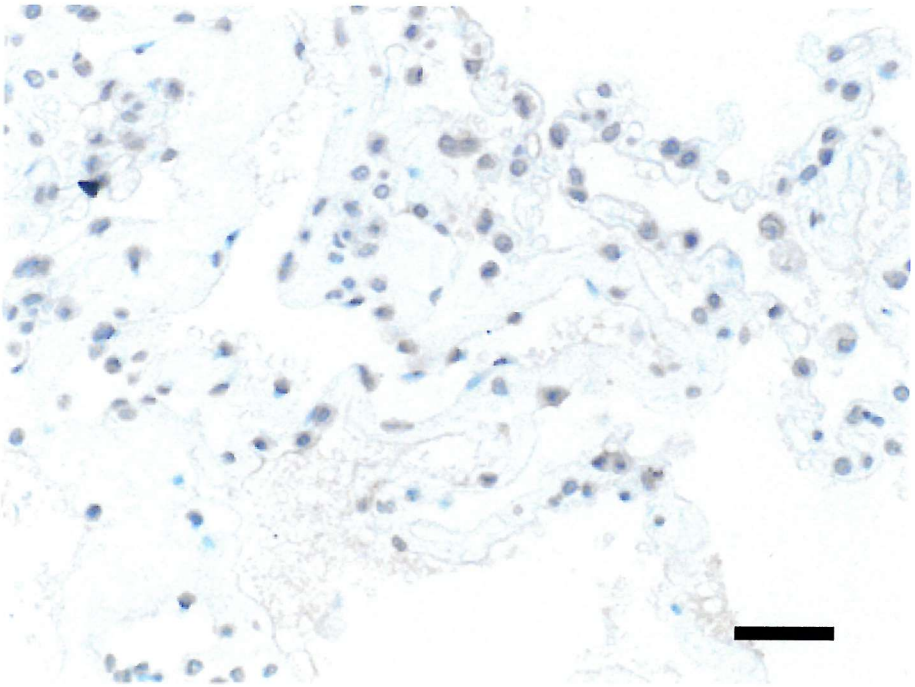


Both the samples from the impaired and normal lung were stained with a monoclonal antibody to TIMP-1. Figure 5.6 (lower panel) shows staining in the control subject where as Figure 5.6 (upper panel) shows staining in the lung of the smoker. Staining for TIMP-1 was mainly found in the extracellular environment, with little immunoreactivity found within cells.

In contrast to what was seen with staining for MMP-9 there seems to be little difference in TIMP-1 immunoreactivity between control and obstructive lung (Figure 5.7). The striking difference in MMP-9 staining between the two samples and the similarity in levels of TIMP-1, reflects the trends found in the homogenised lung samples.

Figure 5.7 facing page. TIMP-1 staining in lung parenchyma. Staining for TIMP-1 in the lung parenchyma from a 70 year old individual (SM7; see table 5.1b) with obstructive lung function and 50 year smoking history (upper panel). Compared to TIMP-1 staining in lung parenchyma from a 27 year old individual (NON1; see table 5.1a) with good lung function and no smoking history. *Bar* represents 50μm.

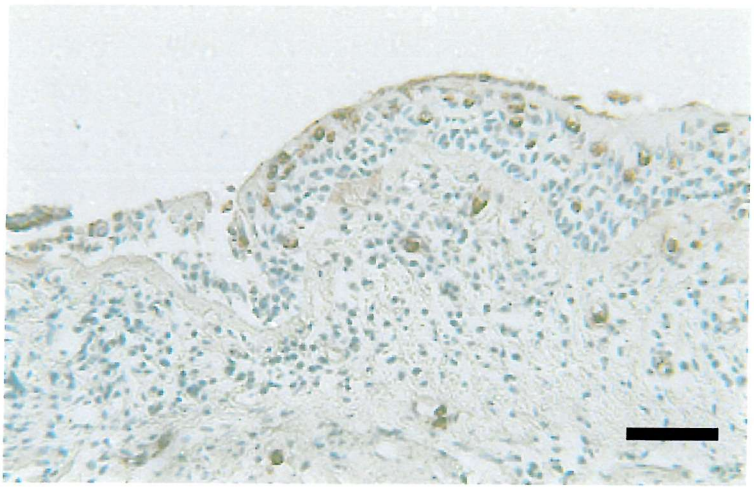


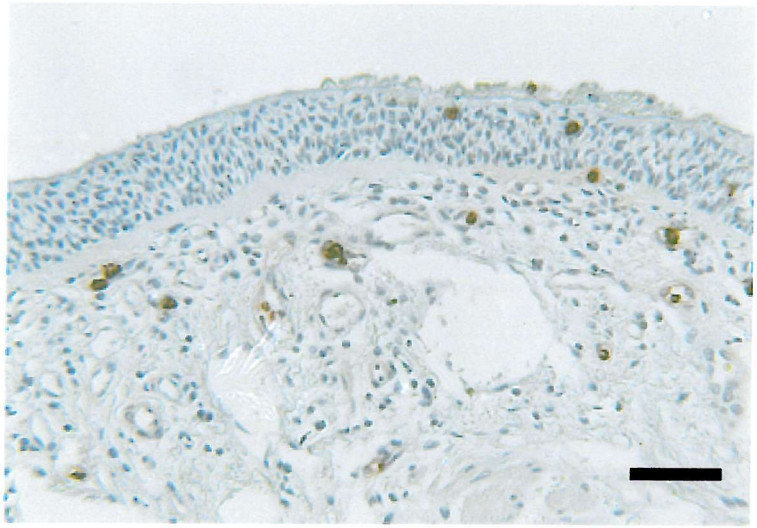


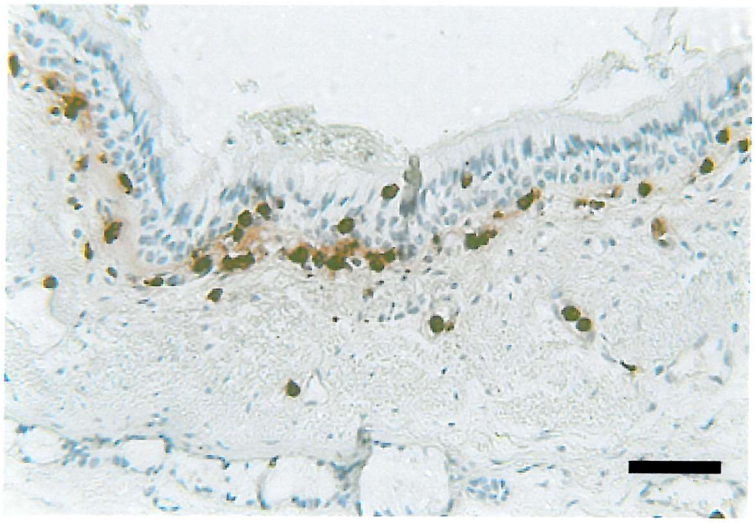
5.3.6b MMP-9 and TIMP-1 in bronchial epithelium.

Bronchial samples were taken from the primary bronchi of four normal controls and seven COPD patients undergoing transplant. The COPD patients were characterised as having either emphysema (n=4) or α_1 antitrypsin deficiency induced emphysema (n=3). Sections were stained with monoclonal antibodies to MMP-9 and TIMP-1 as well as inflammatory cell markers: neutrophil elastase and CD68 (for activated macrophages). On analysis of section morphology, the lamina propria below the basement membrane was well preserved in all of the specimens analysed. It was common for the epithelial layer to be absent from specimens, possibly having been sloughed off during biopsy retrieval. Distinction between areas of alveolar and glandular tissue was often difficult. As a result immunoreactivity could only be analysed confidently in the lamina propria. MMP-9 staining in sections from a normal control subject (Figure 5.8, upper panel) and an individual with α_1 antitrypsin deficiency (Figure 5.8 middle panel) are compared with an emphysematous section (Figure 5.8 lower panel) are illustrated below.

Figure 5.8 facing page. MMP-9 staining in human bronchial epithelium. Expression of MMP-9 in human bronchial epithelium. Sections of human bronchial epithelium were taken from a normal individual (upper panel), denoted NORM3 in table 4.5a; a patient with α_1 antitrypsin deficiency induced emphysema (middle panel), denoted α_1AT-2 in table 5.4b; and a patient with smoking induced emphysema (lower panel), denoted EM4 in table 5.4c. Sections were stained with monoclonal antibodies to MMP-9. *Bar* represents 50μm.

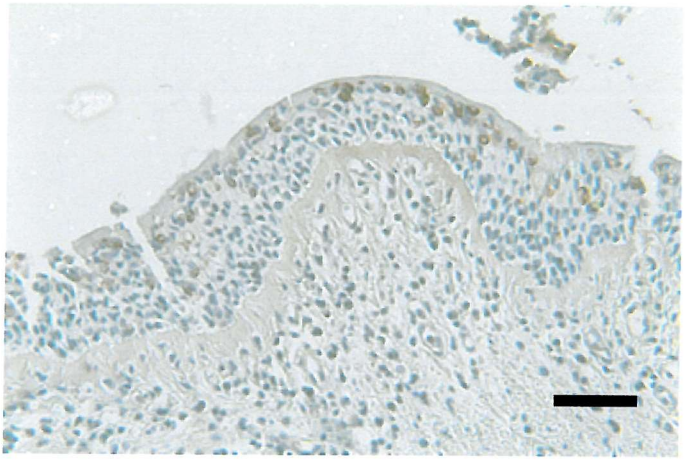


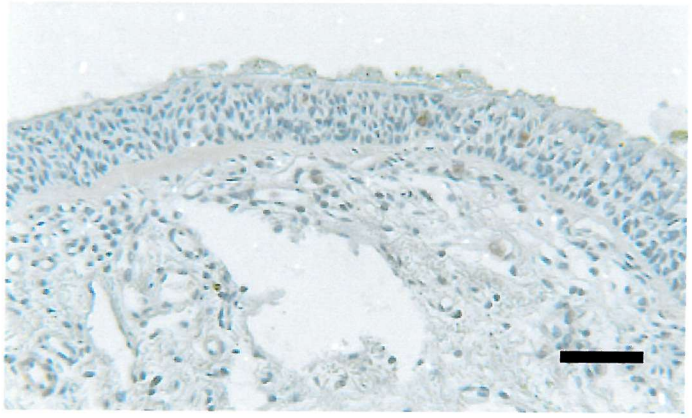


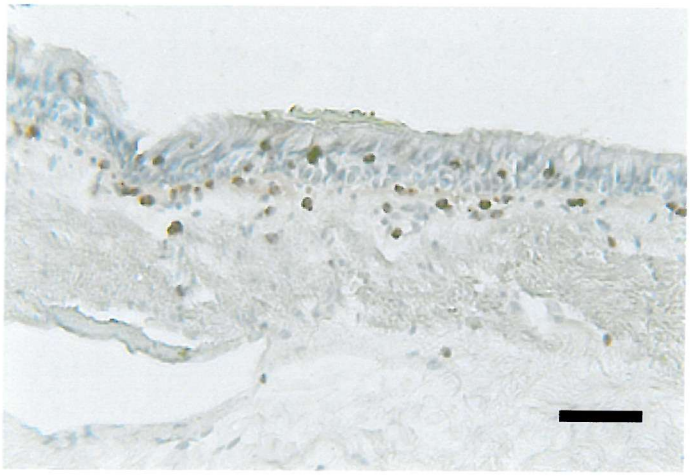


Staining for TIMP-1 was conducted in parallel with staining for MMP-9. Initial staining experiments with a monoclonal antibody to TIMP-1 showed little intracellular expression of the inhibitor, however yielded intense staining in mucus glands. This staining pattern was consistent in all samples studied. While it is conceivable that TIMP-1 levels should be high in secreted mucus, the lack of intracellular expression was suspicious. To address this experiments with a second monoclonal antibody to TIMP-1 were conducted to identify areas of TIMP-1 staining. This antibody showed less localisation of TIMP-1 in mucus glands, but identified a positive signal for intracellular TIMP-1, inside infiltrating inflammatory cells (Figure 5.9). While staining for TIMP-1 was not as intense as MMP-9, the localisation of staining was found to be in similar regions. Figure 5.9 shows staining for TIMP-1 in a normal individual (Figure 5.9 upper panel), an individual with α_1 antitrypsin deficiency (Figure 5.9, middle panel), and an individual with smoking induced emphysema.

Figure 5.9 facing page. TIMP-1 staining in human bronchial epithelium. Expression of TIMP-1 in human bronchial epithelium. Sections of human bronchial epithelium were taken from a normal individual (upper panel), denoted NORM3 in table 4.5a; a patient with α_1 antitrypsin deficiency induced emphysema (middle panel), denoted α_1AT-2 in table 5.4b; and a patient with smoking induced emphysema (lower panel), denoted EM4 in table 5.4c. Sections were stained with monoclonal antibodies to TIMP-1. *Bar* represents 50μm.







For each biopsy the number of cells staining postitive for MMP-9, TIMP-1, neutrophil elastase and CD 68 were counted. The counted area was measured by the Leica Q-win system and related software and cell counts converted to cells/mm^2.

Table 5.4a. Positive staining in normal control bronchial sections.

subject	MMP-9	TIMP-1	NE	CD68
NORM1	69	-	100	185
NORM2	173	-	134	565
NORM3	64	57	70	175
NORM4	361	-	582	XS
median	121	-	117	375

Table 5.4b. Positive staining in α_1 antitrypsin deficient sections.

Subject	MMP-9	TIMP-1	NE	CD68
α_1 AT-1	59	9	109	240
α_1 AT-2	105	38	126	63
α_1 AT-3	185	111	-	XS
median	105	38	109	150

Table 5.4c. Positive staining in emphysematous sections.

subject	MMP-9	TIMP-1	NE	CD68
EM1	96	153	93	140
EM2	237	181	268	144
EM3	438	126	342	657
EM4	531	307	468	510
median	337	153	305	327

The number of cells expressing MMP-9, TIMP-1, neutrophil elastase and CD 68 were counted in bronchial samples obtained from normal controls (Table 5.4a; NORM1-NORM4), patients with α_1-antitrypsin deficient emphysema (Table 5.4b; α_1 AT-1- α_1 AT-3), and smoking induced emphysema (Table 5.4c; EM1-EM4). Cell counts were corrected for the area of lamina propria analysed and expressed in cells/mm^2. XS denotes too many cells to accurately count.

The highest levels of MMP-9 expression are seen in bronchial epithelium of individuals with smoking induced emphysema (median=337 cells/mm^2, table5.3c). This compares with lower density staining in samples from both normal individuals (median =121 cells/mm^2, Table 5.4a) and patients with α_1-antitrypsin deficiency (median =105 cells/mm^2, Table 5.4b).

The groups studied were small in size due to availability of samples; therefore differences in MMP-9 staining did not reach statistical significance. A simple power calculation was performed on the data, which suggested that a study incorporating nineteen COPD and normal subjects would be needed to make statistical comparisons

between groups.

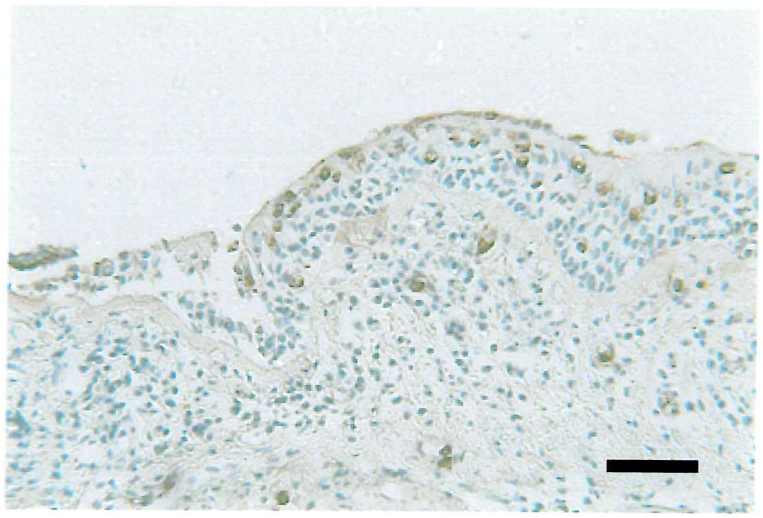
Staining for TIMP-1 was found to be greatest in the smoking induced emphysema sections (median= 153 cells/mm^2). There are fewer cells positively staining for TIMP-1 compared to MMP-9 across all 11 samples studied.

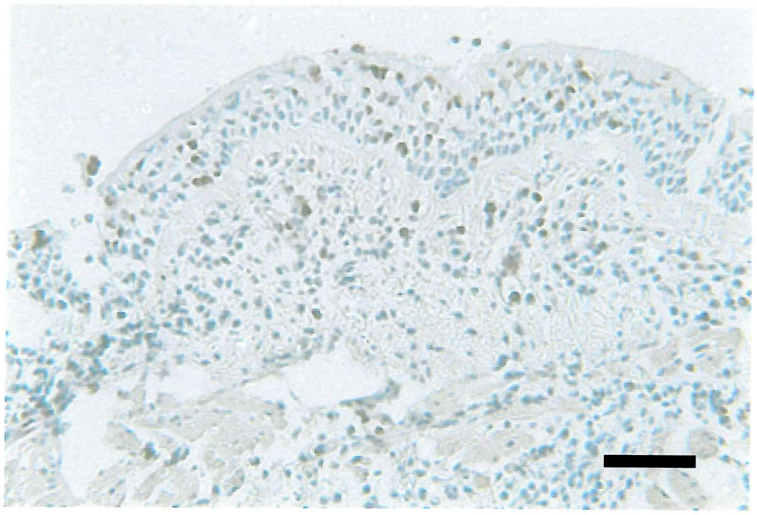
Co-localisation analysis of sequential sections was conducted to identify inflammatory cells contributing to MMP-9 burden. The markers used were neutrophil elastase (neutrophils), CD68 (activated macrophages), eosinophil cationic protein (ECP) (eosinophils), and CD3 (lymphocytes). It was consistently found that there was little or no staining for either ECP or CD3, however it is likely that this reflects an antigen retrieval problem rather than a true result. All sections stained positive for varying amounts of both neutrophil elastase and CD68. Samples from normals showed high amounts of staining for CD68; identifying activated macrophages (median = 375 cells/mm^2, Table 5.4a and Figure 5.10 lower panel) compared to lower levels of neutrophil elastase (median =117cells/mm^2, Table 5.4a, Figure 5.10, middle panel). Neutrophil elastase showed a similar low pattern of expression to MMP-9 in these individuals (median= 121 cells/mm^2, Table 5.4a, Figure 5.10, upper panel).

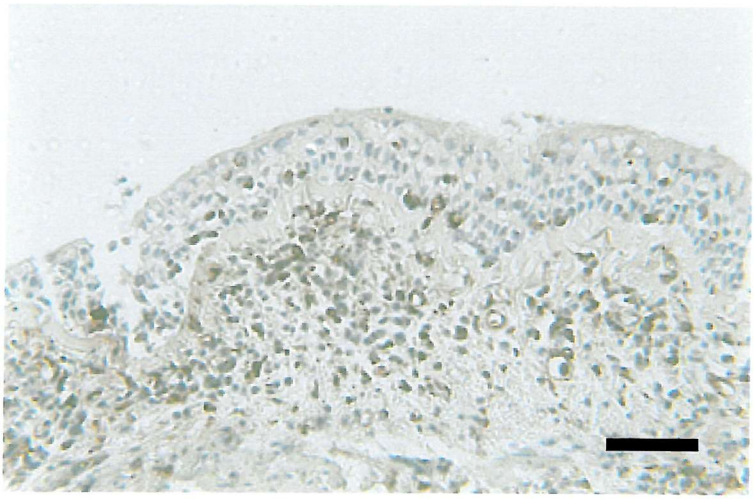
In the α_1 antitrypsin deficient sections, the cellular infiltration was similar to the controls. These individuals showed similar patterns of low density staining for both neutrophil elastase (median = 109 cells/mm^2, Table 5.4b Figure 5.11, middle panel) and MMP-9 (median = 104 cells/mm^2, Table 5.4b, Figure 5.11, upper panel). This was accompanied variable levels of expression for CD68 (median= 150 cells/mm^2, Table 5.4b Figure 5.11, lower panel).

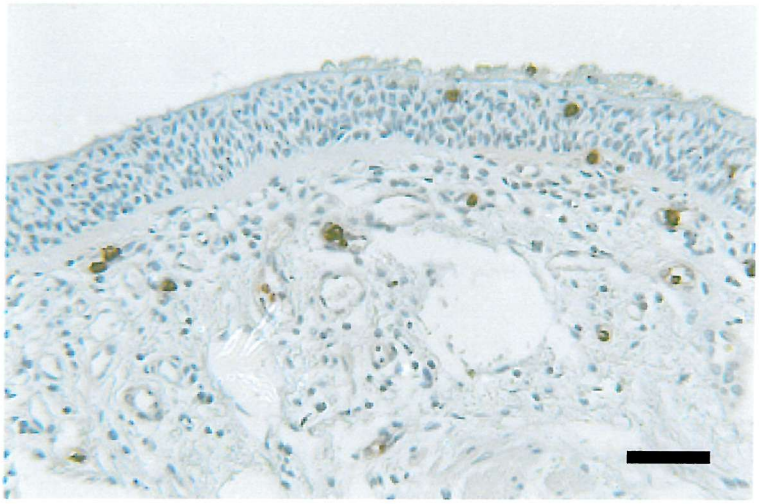
Figure 5.10 facing page. Co-localisation staining in normal bronchial epithelium. Sequential sections of bronchial epithelium were obtained from a normal individual (denoted NORM3 in table 5.4a) and stained with monoclonal antibodies for MMP-9 (upper panel), neutrophil elastase (middle panel) and CD68 (lower panel). This illustrates the co-localisation of MMP-9 with inflammatory cell markers in normal bronchial epithelium.

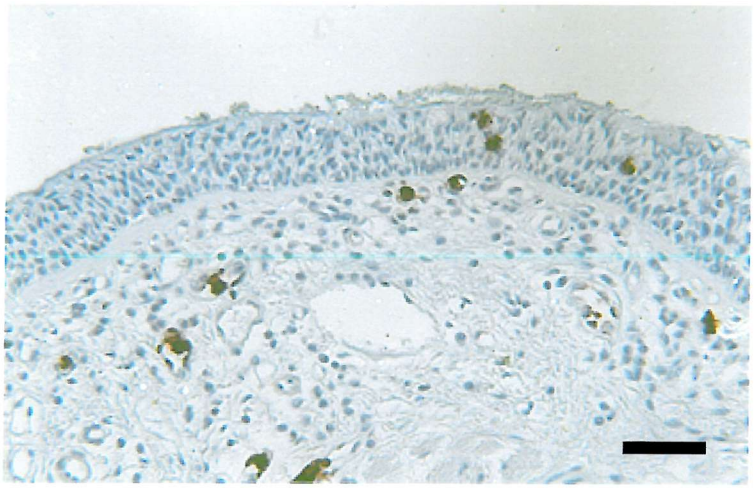
Figure 5.11 (following page). Co-localisation staining in bronchial epithelium from an α_1 antitrypsin deficient individual. Sequential sections of bronchial epithelium, from an individual with α_1 antitrypsin deficiency induced emphysema (denoted α_1 AT-2 in table 5.4b) were stained with monoclonal antibodies for MMP-9 (upper panel), neutrophil elastase (middle panel), and CD 68 (lower panel). This illustrates the co-localisation of MMP-9 with inflammatory cell markers in α_1 antitrypsin deficiency. *Bar* represents 50μm.

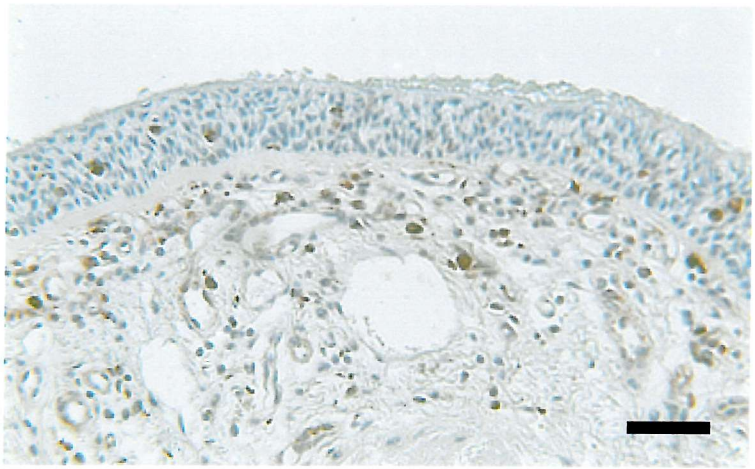








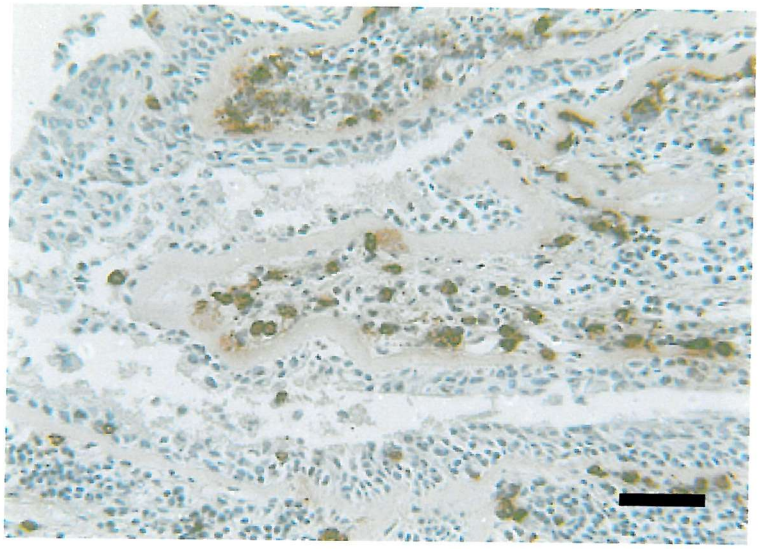


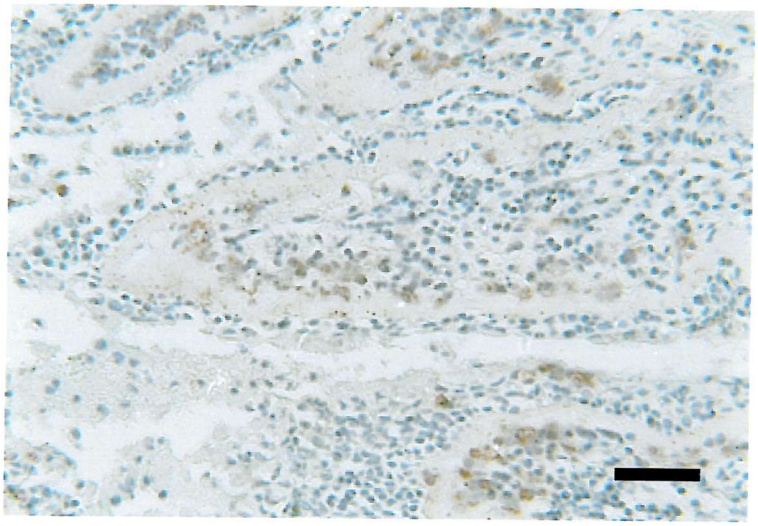


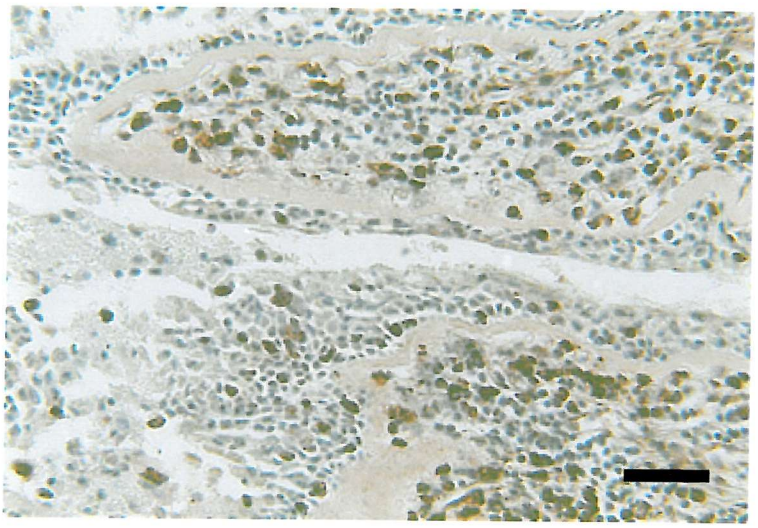
Samples from emphysematous bronchi showed high median levels of both neutrophil elastase (median =304 cells/mm^2, Table 5.4c, Figure 5.12 and 5.13, middle panels) and CD68 expression (median =327 cells/mm^2, Table 5.4c, Figure 5.12 and 5.13, lower panels). Inter -individual analysis of the emphysema samples indicated that in some individuals MMP-9 could clearly associated with neutrophilic inflammation (illustrated in Figure 5.12), whereas in others it is less easy to distinguish whether neutrophils or macrophages represent the source of MMP-9 (illustrated in Figure 5.13). It is possible that the different neutrophil/ macrophage ratios illustrated represent 2 sub pathologies involved in smoking induced emphysema.

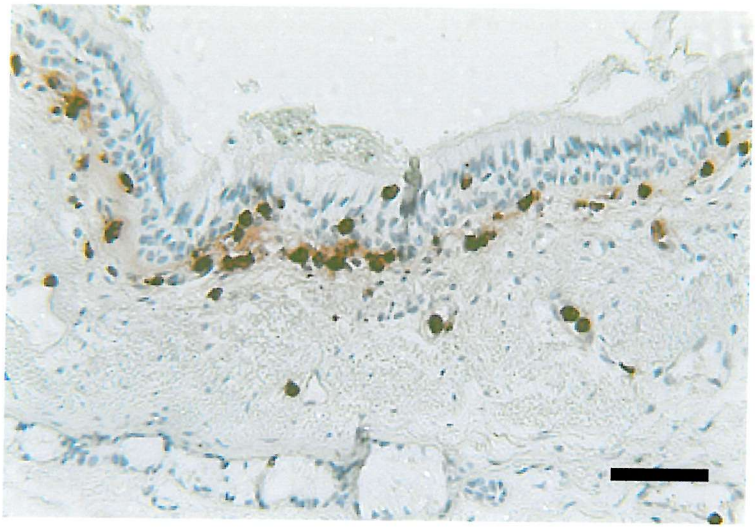
Figure 5.12 facing page. Co-localisation staining in bronchial epithelium from individuals with emphysema. Sequential sections of bronchial epithelium were obtained from an individual with smoking induced emphysema (denoted EM3 in table 5.4c)and stained with monoclonal antibodies for MMP-9 (upper panel), neutrophil elastase (middle panel) and CD68 (lower panel). This illustrates the co-localisation of MMP-9 with inflammatory cell markers in an emphysematous individual who has large amounts of macrophage infiltration compared to neutrophil infiltration.

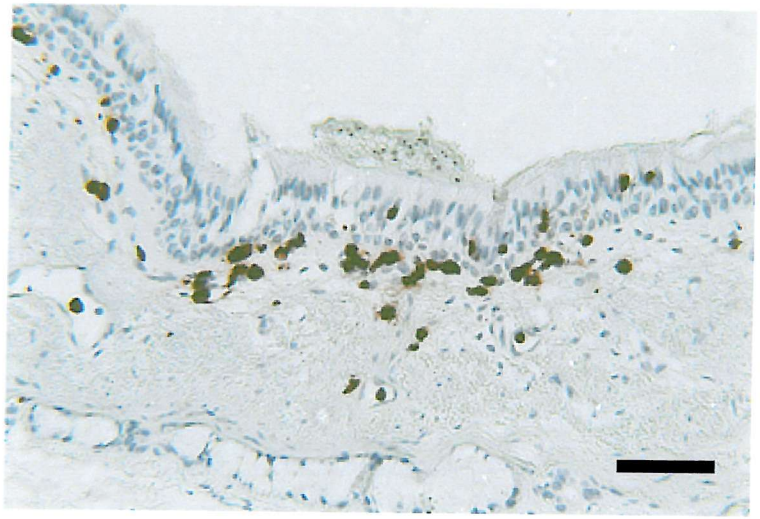
Figure 5.13 (following page). Sequential sections of bronchial epithelium, were obtained from a second individual with smoking induced emphysema (denoted EM4 in table 5.4c) and stained with monoclonal antibodies for MMP-9 (upper panel), neutrophil elastase (middle panel), and CD 68 (lower panel). This illustrates the co-localisation of MMP-9 with inflammatory cell markers an emphysematous individual with similar levels of macrophage and neutrophil infiltration. Together the staining patterns in figures 5.12 and 5.13 illustrate the heterogeneity of inflammation in emphysema. *Bar* represents 50μm.

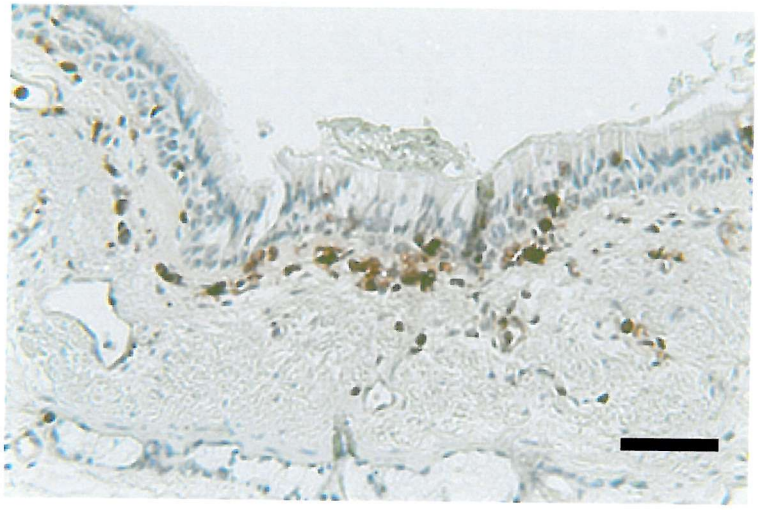












5.4 Discussion.

Previous studies have shown that the degree of airflow obstruction is correlated with severity of inflammation in a group of smokers [Di Stefano *et al.*, 1998], and that inflammation persists even after smoking cessation [Turato *et al.*, 1995]. Here we show that protease: antiprotease imbalance in lung parencyma is associated with lung function deficit. It is important to note however that high proteinase levels do not necessarily correlate with a history of smoking. When groups were compared on the basis of whether they were current, ex or non-smokers there were no significant differences in MMP-9 found. Non smoking normal controls are extremely hard to obtain, particularly in a population specifically requiring resection for carcinoma. Hence our group of normal individuals is very small which makes comparison to smokers very difficult. This is compounded by the fact that the normals, despite being non smokers, are likely to have come into contact with a variety of irritants which could have triggered a subclinical inflammation, possibly leading to increased levels of MMP-9. Patient histories taken from the tissue donors prior to resection are brief and may well miss the development of minor acute inflammation. There is also evidence for the development of inflammation and chronic bronchitis in people who have never smoked, which matches that seen in smokers [Lusuardi *et al.*, 1994]. It may be that this type of inflammation could be an additional artefact, causing increased levels of MMP-9 in our non-smoking subjects. On analysis of the samples from patients with a known smoking history (current or ex smokers) it was found that proteinase imbalance associates with patients who have an obstructive lung function (defined by FEV_1/FVC <70%). This is important evidence suggesting that MMP-9 is associated with some form of parenchymal damage and loss of function.

Nineteen patients were studied in total; some people with smoking histories being more adversely effected than others. This group was too small to allow the detailed analysis

of smoking history (i.e. pack years, type of cigarette smoked) on protease: antiprotease balance. However, it could clearly form the basis of an extremely powerful analysis into why some smokers have developed a proteinase burden, and loss of lung function, while others have not.

Previous chapters have highlighted potential MMP-9/ TIMP-1 imbalance in disease, sampling from different areas of the lung; namely the lower airways and alveoli (using BAL), and upper airways (using sputum). In this chapter immunohistochemistry has been used to further investigate protease: antiprotease distribution. Previous investigations have found tissue expression of MMP-9 in asthma [Dahlen *et al.*, 1999], however to date the only study which we found to have looked at distribution in COPD patients surprisingly reported that MMP-9 immunoreactivity was absent [Ohnishi *et al.*, 1998]. The aim of our initial investigations was to supplement data from our lung homogenate studies with visual information on proteinase and inhibitor distribution in parenchyma. For this we processed parenchymal tissue from a normal non-smoking individual, and a chronic smoker with impaired lung function. These individuals represented the two poles of the normal and impaired study groups. It must be noted that in this small study we did not employ standardisation techniques such as: analysis of multiple sections from around the tumour area, or the fixation of tissue under a constant perfusion pressure as have been employed on larger scale studies [Finkelstein *et al.*, 1995]. In both individuals intense staining for MMP-9 was found to localise to infiltrating inflammatory cells. It is striking that the amount of MMP-9 in the smoker with impaired lung function is far greater than in the normal individual. Interestingly the MMP-9 immunoreactivity was spread diffusely through the ECM in the impaired individual, a pattern that contrasted with staining in the normal. Previous studies on biopsies from asthma patients [Dahlen *et al*, 1999] found a similar diffuse staining pattern. The authors suggested that this arose from binding of MMP-9 to collagen I

fibrils. Collagen I is not a substrate for MMP-9, however binding in this way is proposed to confer retention, stability, and thus prolonged bioactivity; augmenting its proteolytic role [Murphy *et al,* 1991]. Staining for TIMP-1 in both samples is significantly less intense than MMP-9. It is possible to see faint intracellular localisation with additional staining around epithelial cell membranes, and concentrated in extracellular secretions. While this faint staining provides some evidence that TIMP-1 is present it is difficult to make judgements about extent of the existence of an MMP-9/ TIMP-1 imbalance.

To gain a picture of proteinase distribution in the central airways, in addition to the parenchyma, we analysed expression of MMP-9, TIMP-1 and markers for inflammatory cells in bronchial epithelium. Samples of the primary bronchus were taken from the resected patients undergoing lung transplant for emphysema. These were analysed in parallel with bronchial samples from normal individuals, taken post mortem following sudden trauma. MMP-9 expressing cells were found infiltrating the lamina propria of patients with emphysema in greater density than in normal sections or sections from α_1-antitrypsin deficient lungs. This is important since it emphasises the increased MMP-9 burden in COPD. Additionally expression of MMP-9 was consistently higher than TIMP-1 in all of the samples analysed. In an attempt to identify the contributors to this proteinase burden, MMP-9 positive cells were assessed in sequential sections with staining for neutrophil elastase, CD68 (activated macrophages), eosinophil cationic protein and CD3. Previous authors have correlated decline in lung function with infiltration of neutrophils and macrophages in the airways of smokers [Stanescu *et al.*, 1996, Di Stefano *et al.*, 1998] and additional investigations have identified neutrophils as major MMP-9 producing cells [Dahlen *et al.*, 1999]. Interestingly the preliminary findings in our study suggest that neutrophils are an important source of MMP-9 both in normal and diseased lung, and it is conceivable that

an increase in MMP-9 expression in the COPD lung results primarily from increased neutrophil infiltration. Interestingly the relative ratio of macrophages to neutrophils varied between individuals with smoking induced emphysema, making it difficult to define to deffine exactly whether neutrophils or macrophages contributed to MMP-9 burden. It is possible that differences in neutrophil: macrophage ratio reflects different stages of emphysema. COPD develops progressively and there is evidence to suggest that peripheral airways damage in young smokers is marked by macrophage accumulation [Niewoehner *et al.,* 1974] and that disease progression is marked by macrophage and lymphocyte accumulation in the airway wall [DiStefano *et al.,* 1996]. Worsening airflow limitation has also been correlated with increasing numbers of neutrophils [DiStefano *et al.*, 1998]. It is possible that even when a patient is diagnosed as having emphysema different types of cell infiltration contribute at different stages to MMP burden.

In summary this chapter has begun to illustrate that an MMP-9/ TIMP-1 imbalance exists at the tissue level in the lungs of current and ex smokers with an impaired lung function, as well as in the central airway walls of individuals with emphysema.

Chapter 6.

TIMP-1 activity: An inhibitor-binding assay.

6.1 Introduction.

Under normal conditions TIMP-1 forms a high affinity complex with MMP-9 dissociable only with SDS or EDTA treatment [Woessner, 1991, Wilenbrock and Murphy, 1994]. Complex formation inhibits proteolytic activity as well as preventing activation of the MMP zymogen. It is extremely important for the balance between matrix degradation and proliferation that TIMP-1 retains the ability to efficiently bind to its cognate proteinase: MMP-9.

The structure of TIMP-1 is based around 6 disulphide bonds, which hold the molecule in a tight double looped conformation [Osthues *et al.,* 1992]. While on the one hand conferring stability (see Figure 6.1), these bonds are also an Achilles heal, since they are open to modification by both reducing agents [Dean *et al.*, 1983], and oxidants [Shabani *et al.,* 1998, Frears *et al.*, 1996] thus providing important points for TIMP-1 inactivation. Importantly, many of the agents capable of destabilising TIMP are pathological factors in COPD. For example, a characteristic of COPD is oxidative stress caused by oxidants either present in cigarette smoke or released by phagocytes such as hypochlorus acid, hydroxyl radicals, peroxy nitrate and hydrogen peroxide [Repine *et al.*, 1997]. Clearly there is great potential for oxidative inactivation of TIMP-1 by these toxic agents.

Additionally TIMP-1 is a substrate for a variety of proteinases found in the inflammatory environment of the COPD lung. For example TIMP-1 is cleaved by neutrophil elastase [Itoh and Nagase, 1995], cathepsin B [Kostoulas *et al.*, 1999], trypsin [Murphy *et al.*, 1991]. TIMP-1 is readily cleaved by trypsin; containing 18 possible sites for tryptic cleavage [Williamson *et al.,* 1993]. Truncated N-terminal TIMP-1 containing residues 1-127 is known to retain binding activity for MMP-9 [Murphy *et al.,* 1991], however it is unknown whether particular tryptic cleavage products retain binding activity.

Figure 6.1

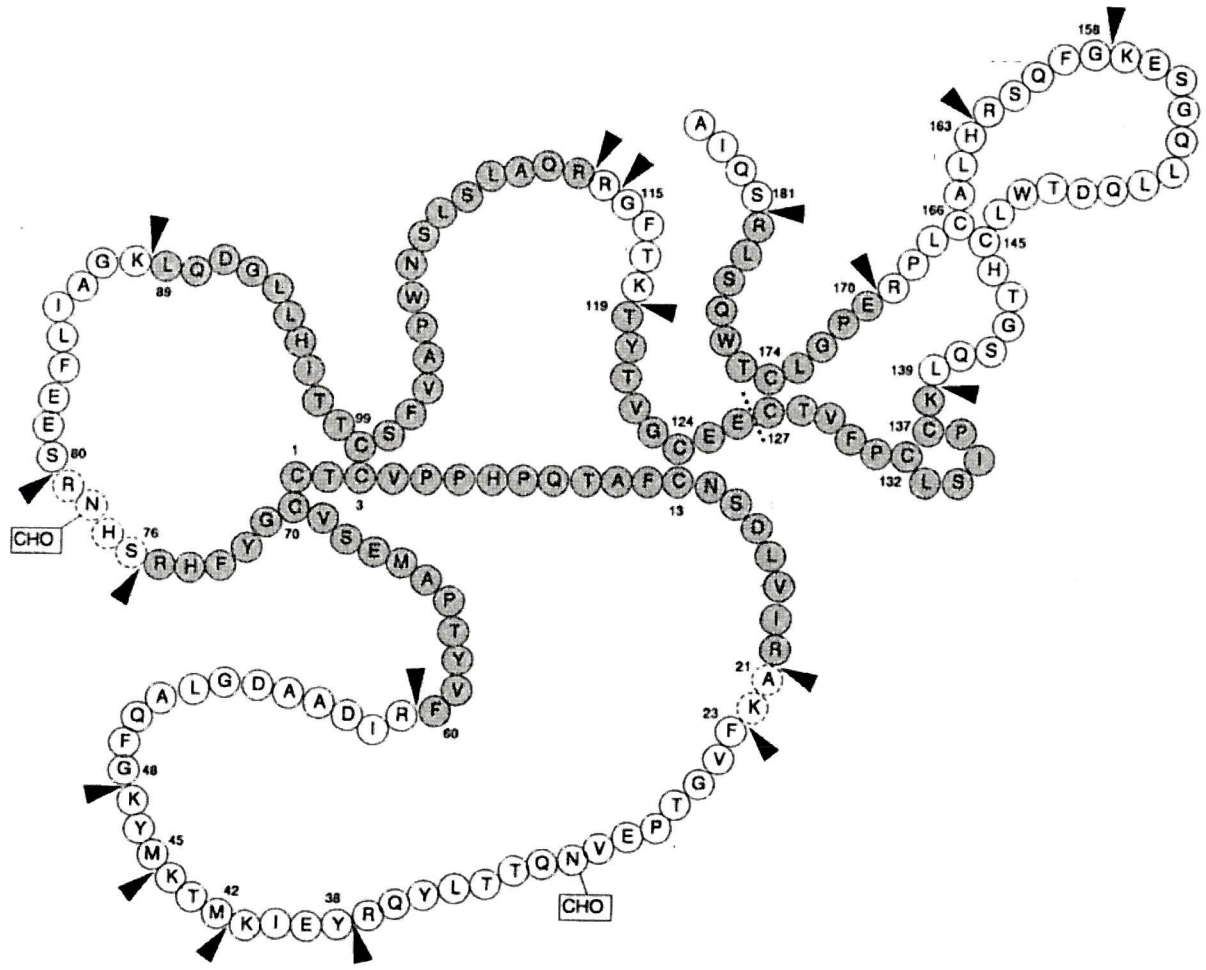


Figure 6.1 Amino acid structure of TIMP-1. The structure of TIMP-1 is extremely stable, with the structure having 6 cysteine-cysteine disulphide bonds. TIMP-1 is however readily susceptible to trypsin cleavage, and the trypsin cleavage sites are illustrated by triangles.

It is conceivable that both oxidants and proteinases together play a role in reducing the inhibitory capacity of TIMP in the COPD lung. Clearly when investigating the destructive potential of MMP-9: TIMP-1 imbalance it is important to assess how much inhibitor is capable of binding to and inhibiting proteinase activity. This was highlighted in one study that compared the collagenase inhibitory activity, in COPD bronchoalveolar lavage (BAL) fluid and sputum, to levels of total TIMP-1. The authors found that in both sputum and BAL fluid TIMP-1 levels exceeded collagenase inhibitory activity, suggesting that a proportion of inhibitor was inactivated in the disease [Burnett *et al.*, 1986]. The study measured inhibitory activity by measuring the ability of samples to inhibit the digestion of C^{14} labelled collagen, by rabbit collagenase. The purpose of the present study was to develop an inhibitor-binding assay to analyse TIMP-1 activity in lung secretions. The assay is based on the ability of active TIMP-1 to bind latent MMP-9 coated onto a conventional 96 well plate. This binding could be visualised and quantified using specific antibodies to TIMP-1 and a commercially available chromogen.

6.2 Methods.

6.2.1 MMP-9 binding assay for TIMP-1.

A full method for this assay is provided in chapter 2 (see 2.4.2a), however a brief overview of the assay method is as follows:

Wells were coated with MMP-9 in 50mM Tris-HCL buffer (pH 7.5) containing 5% (w/v) polyethylene glycol (PEG). Following blocking for non-specific binding, samples containing both functional and non-functional (TIMP-1 unable to bind MMP-9) were added to the plate and MMP-9:TIMP-1 complexes allowed to form. Following this, the plates were washed removing any unbound TIMP-1. Bound TIMP-1 could then be detected using a monoclonal antibody, which itself could be detected by a HRP conjugated secondary antibody. Addition of the chromogen TMB caused a colour change, quantifiable at an optical density of 450nm, which was proportional to the amount of bound TIMP-1. This value was compared to a known concentration of TIMP-1 on a standard curve.

This method was run in parallel with an ELISA developed in house, measuring total immuno competent TIMP-1. The protocol for the measurement of total TIMP-1 was identical to the inhibitor-binding assay, except that MMP-9 coated onto the plastic wells was replaced with polyclonal antibody to human TIMP-1 (2μg/ml in bicarbonate coating buffer, pH 9.5) used to capture the inhibitor.

Figure 6.2

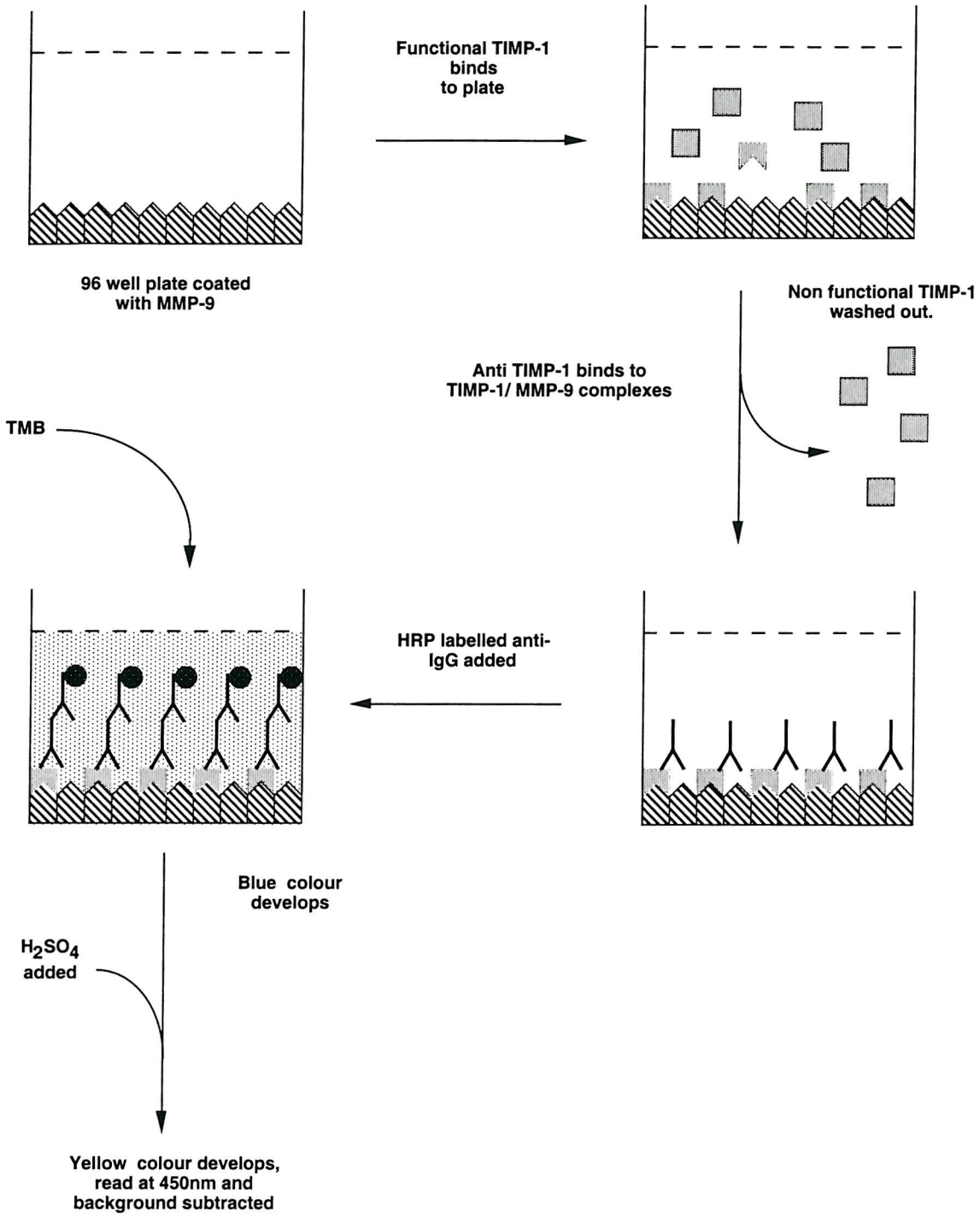


Figure 6.2 Schematic diagram of the TIMP-1 inhibitor-binding assay. The wells in a 96 well plate were coated with excess MMP-9 and following blocking for non specific binding, samples containing active TIMP-1 were added to the coated wells. Functional TIMP-1 bound to the MMP-9 coating, and TIMP-1 impaired from binding was removed by washing. A monoclonal antibody to TIMP-1 was used to detect bound TIMP, which itself was detected by a HRP conjugated secondary antibody. The horseradish peroxidase substrate 3, 3', 5, 5' tetra-methyl benzedine (TMB) was used to visualise bound TIMP-1 by colour change, which was proportional to the amount of bound inhibitor.

6.3 Results.

6.3.1 Assay development.

Initial experiments were conducted to identify suitable monoclonal antibody for the detection of TIMP-1: MMP-9 complexes. Antibody 1 (AB1) was described by the manufacturers (Biogenesis, clone T2) as a neutralising antibody for TIMP-1 activity. Though the exact epitope map for this antibody was not released by the manufacturers, its neutralising capacity would suggest an N-terminal domain position, as the N-terminus is thought to form the major active site of TIMP-1 [Murphy *et al.,* 1991]. The epitope for AB2 (R&D Systems clone: 63515.111) was also unknown. MMP-9 at a concentration of 200ng/ml was bound to the plate, and following blocking for non-specific binding, concentration curves of TIMP-1 ranging from 1.56ng/ml to 50ng/ml applied to the plate. Following complex formation the detecting antibodies were applied to separate standard curves at concentrations recommended by the manufacturers for ELISA (see Figure 6.2).

Figure 6.3

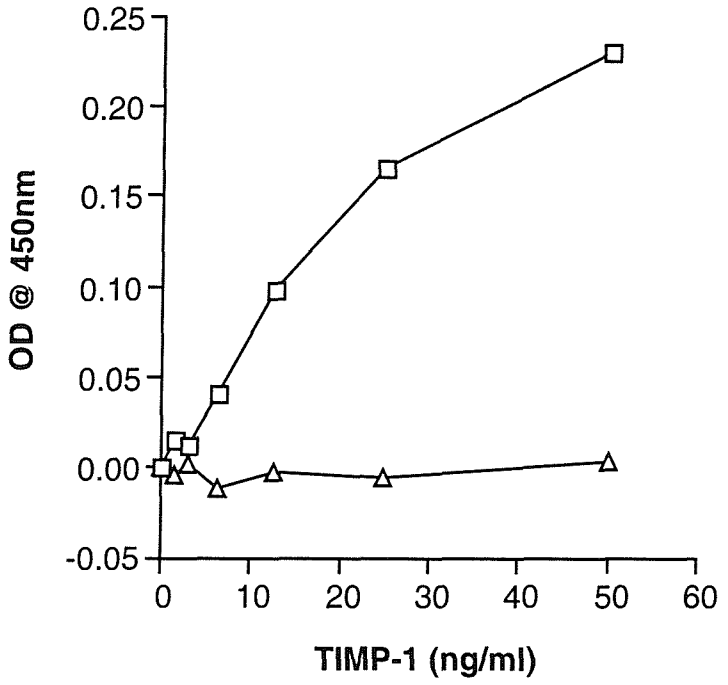


Figure 6.3 Comparison of detection antibodies for TIMP: MMP-9 complexes. Concentrations of TIMP-1 ranging between 1.56ng/ml and 50ng/ml were applied to MMP-9, coated onto a microplate (200ng/ml). Antibodies, designated as AB1 (0.2μg/ml, triangles) and AB2 (0.5μg/ml, squares) were used to detect TIMP-1 which had formed complexes with the coated MMP-9. Values are given in units of optical density (OD) at 450nm and represent the duplicate results of one experiment.

AB2 (R&D systems clone: 63515.111) proved most efficient at detecting complexes formed between TIMP-1 and MMP-9 coated onto a microplate. It is conceivable that access to the active site epitope for the T2 antibody would be inaccessible once complex formation has occurred, explaining the lack of signal.

The low signal from the assay meant that signal to noise ratio was poor. Further experiments were conducted to increase the signal to noise ratio, by increasing the amount of MMP-9 coated onto the microplate. MMP-9 at 3 different concentrations (400ng/ml, 200ng/ml and 100ng/ml) was applied to the surface of wells. Following blocking for non-specific binding, TIMP-1 was applied to the microplate in concentrations ranging from 0.6-500ng/ml. AB2 was used to detect TIMP: MMP-9 complexes.

Figure 6.4

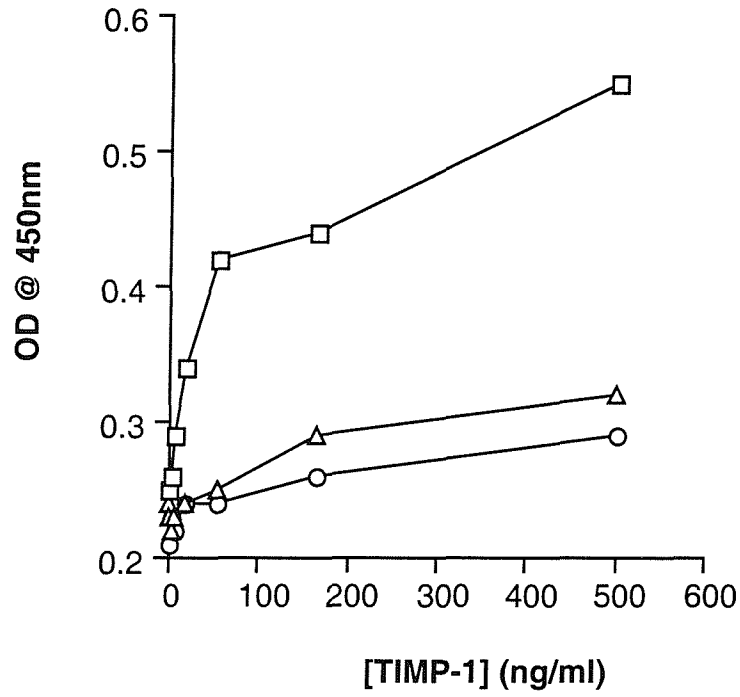


Figure 6.4 Selection of the optimal coating concentration of MMP-9. MMP-9 was diluted to concentrations of 400ng/ml (squares), 200ng/ml (triangles) and 100ng/ml (circles) and applied to separate lanes of a 96 well microplate. Following blocking to prevent non-specific binding, standard curves of TIMP-1 were prepared, ranging from 500- 0.6ng/ml, and applied to the coated MMP-9. Monoclonal antibody specific for TIMP-1 was used to detect bound inhibitor. The data represent the duplicate results of one experiment.

Clearly 400ng/ml of bound MMP-9 gave the best separation of optical density values of the three MMP concentrations used, becoming saturated at approximately 100ng/ml of TIMP-1. Higher concentrations of MMP-9 would not be viable due to the high cost of MMP-9.

In a parallel ELISA assay, immunocompetent TIMP-1 was captured by polyclonal antibody (antibody concentration 2μg/ml), as opposed to the MMP-9 (400ng/ml) used in the inhibitor-binding assay. The detection antibody was the same as that used in the inhibitor-binding assay (R+D systems clone: 63515.11) and the concentration of detection antibody was identical (0.5μg/ml).

6.3.2 Effect of inactivating agents on TIMP-1 binding.

Experiments were conducted to analyse the effects inactivating agents on TIMP-1: MMP-9 complex formation (Figure 6.5). Samples of TIMP-1 at a concentration of 5μg/ml were treated with trypsin (1μg/ml in Tris-HCL buffer, pH 7.5) and incubated at 37°C for a period of 30 minutes. The trypsin cleavage of TIMP-1 was stopped in separate samples at 30 seconds, 1 minute, 5 minutes, 10 minutes and 30 minutes by the addition of aprotinin (1μg/ml). In a separate experiment TIMP-1 at a known concentration (10μg/ml) was treated with 10mM hydrogen peroxide and 0.1% w/v DTT. Mixtures were incubated for 30 minutes at 37°C. From each reaction mixture 10μl of sample was recovered and loaded onto a Western blot. The results from these experiments are illustrated in Figure 6.5.

Western blotting of untreated TIMP-1 consistently yielded the 2 bands, one band at 25kDa and one at approximately 50kDa. The reason for the existence of a higher molecular weight band is unclear, however the fact that it completely disappears with DTT treatment could suggest it is some form of TIMP-1 homodimer.

Treatment of TIMP-1 with trypsin over 30 minute time course results in the degradation of immunocompetent TIMP-1 (Figure 6.5, top left panel). It is of note that the monoclonal antibody used is unable to detect cleaved fragments of TIMP-1. It is possible that the epitope for this monoclonal antibody is located in a region readily exposed to tryptic cleavage.

Figure 6.5

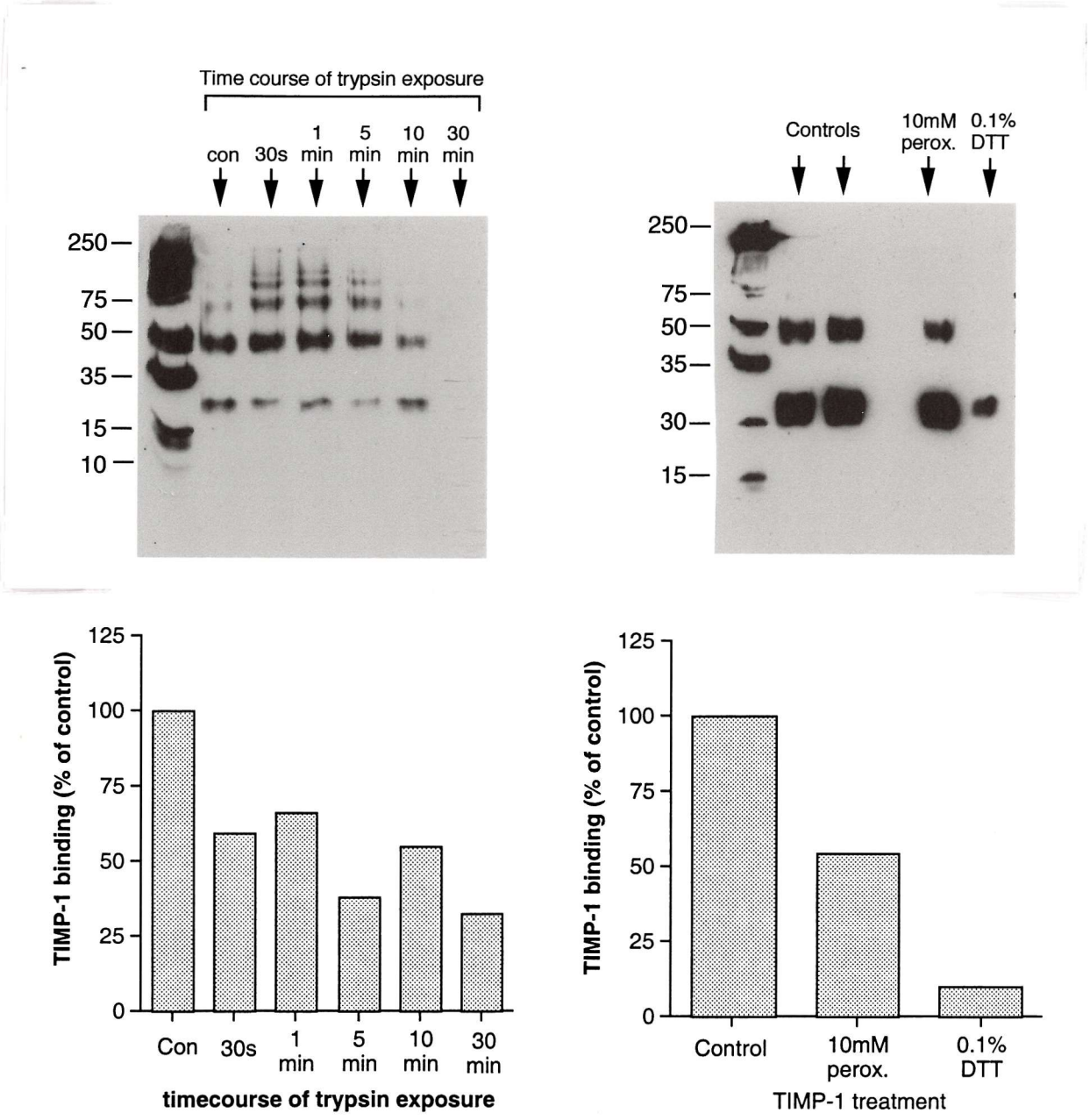


Figure 6.5 The effects of Inactivating agents on the immunocompetence and activity of TIMP-1. Samples of TIMP-1 (5μg/ml) were treated with trypsin (1μg/ml) and incubated at 37°C for a period of 30 minutes. The trypsin cleavage of TIMP-1 was stopped in separate samples at 30 seconds, 1 minute, 5 minutes, 10 minutes and 30 minutes by the addition of aprotinin (1μg/ml). The resultant reaction mixtures were separated by SDS-PAGE and immunocompetent TIMP-1 identified by Western blot (upper left panel). The TIMP-1 activity in each treated sample was assessed by the ability of TIMP-1 to complex with MMP-9 (400ng/ml) coated onto a 96 well plate (lower left panel). Data are expressed as % of control (untreated TIMP-1) activity. In a separate experiment TIMP- (10μg/ml) was treated with 10mM hydrogen peroxide and 0.1% w/v DTT. TIMP-1 immunocompetence was assessed by Western blot (upper right panel) and activity by ability to complex with MMP-9 coated onto a 96 well plate (lower right panel), as previously described.

The remaining sample was diluted and 100µl samples were pipetted into wells coated with MMP-9 for analysis of inhibitor-binding (as described in section 6.2). The protocol for detecting TIMP-1 on both the Western blot and binding assay was identical.

Trypsin pre-treatment of TIMP-1 reduced the ability of the inhibitor to bind MMP-9 (Figure 6.5, bottom left). This mirrored the data from the Western blots which indicated a reduction in TIMP-1 immunocompetency following trypsin treatment. Reduction of TIMP-1 with 0.1% DTT for 30 minutes also resulted in greatly reduced immunocompetency and binding ability of the inhibitor, probably as a result of disulphide bond cleavage.

In contrast to treatment with trypsin and DTT, incubation of TIMP-1 with 10mM hydrogen peroxide had little effect on the antigenicity of TIMP-1 as shown on Western blots (Figure 6.5, top right panel). Despite this, hydrogen peroxide caused a 50% reduction in the ability of TIMP-1 to bind MMP-9 (Figure 6.5 bottom right panel). This observation is notable since oxidation is the only inactivation method tested which resulted in a reduction of activity, without a significant reduction in antigenicity.

6.3.3 Total and active TIMP-1 in clinical samples.

The inhibitor-binding assay was run in parallel with an ELISA measuring total immunocompetent TIMP-1. Values for TIMP-1 activity could be compared to total levels of immunocompetent TIMP-1, and percentage activity calculated. Standard curves were constructed, and were typically similar in shape, both being linear between 1.56ng/ml and 25ng/ml (Figure 6.6).

Figure 6.6

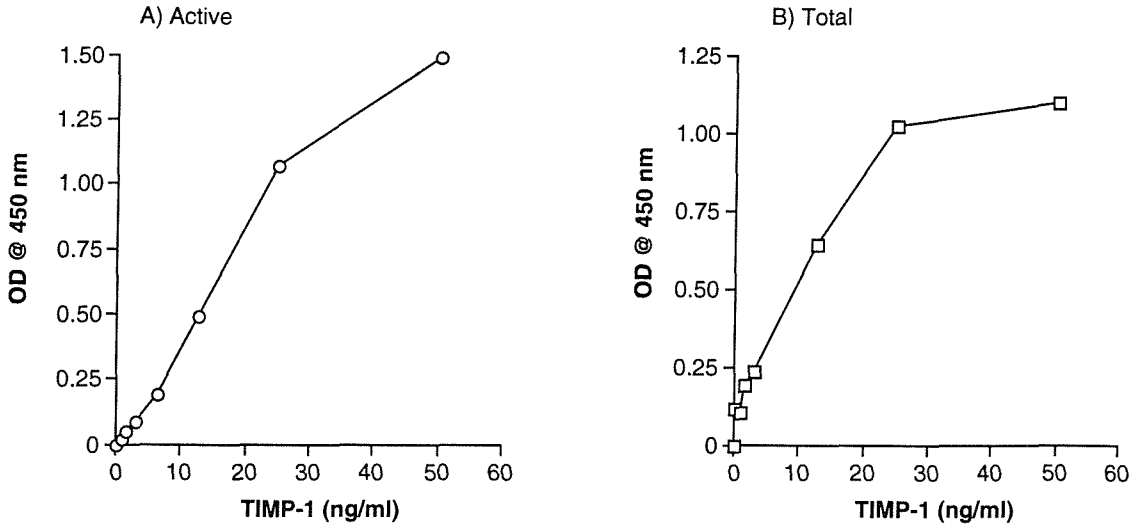


Figure 6.6 Typical standard curves from the TIMP-1 inhibitor-binding assay. 96 well microplates were coated with either proMMP-9 (400ng/ml (Biogenesis)) or polyclonal antiTIMP-1 (2μg/ml (Biogenesis)), to assay for active (Figure 6.6 right hand panel) and total TIMP-1 (Figure 6.6 left hand panel) respectively. Following blocking to prevent non-specific binding recombinant TIMP-1 was applied to each plate at concentrations ranging from 1.6 to 50ng/ml, bound TIMP-1 was detected with a commercially available monoclonal antibody (R+D systems). Typical standard curves are displayed above; the right hand panel illustrates active TIMP-1 binding to MMP-9, left hand panel illustrates TIMP-1 captured by polyclonal antiTIMP-1. The curves from both assays are linear between 1.6ng/ml and 25 ng/ml.

Following initial development, the TIMP-1: MMP-9 binding assay was used to investigate if there were measurable differences between levels of total immuno competent and active TIMP-1 in lung disease. Initial experiments concentrated on COPD BAL from a group of 20 COPD patients.

Analysing the group of COPD patients as a whole, there was no significant difference between median levels of total and active TIMP-1. Median levels of total TIMP-1 measured 5.3ng/ml (IQR: 6.4ng/ml) compared to a value for active TIMP-1 of 5.1ng/ml see table 6.1 (IQR: 4.5ng/ml).

Table 6.1 Levels of total and active TIMP-1 in COPD BAL.

Grouping	(n)	Total TIMP-1 (ng/ml)	Active TIMP-1 (ng/ml)
Grouped activity values	20	5.3 (6.4)	5.1 (4.5)
> 100% activity	8	4.2 (5.9)	6.6 (7.5)
< 100% activity	12	5.7 (5.7)	3.4 (3.4)

Table 6.1. Levels of total and active TIMP-1 were measured in BAL fluid taken from a group of 20 COPD patients. Further analysis showed that this group could be subdivided into subjects with significantly lower levels of active TIMP-1 compared to total (<100% activity, n=12) or subjects who had TIMP-1 activity levels greater than total (>100% activity, n=8). Median values were calculated and interquartile range (IQR) for each group is quoted in brackets.

When the data from each individual was analysed, it was found that 12 individuals had TIMP-1 activity levels lower than values for total TIMP (see table 6.1). The remaining eight subjects had activity levels which exceeded values for total TIMP-1 (see table 6.1). The reasons for greater than 100% recovery of activity in these samples is unknown. Further assay validation compared TIMP-1 activity in BAL from mild asthmatics to activity in COPD.

Figure 6.7

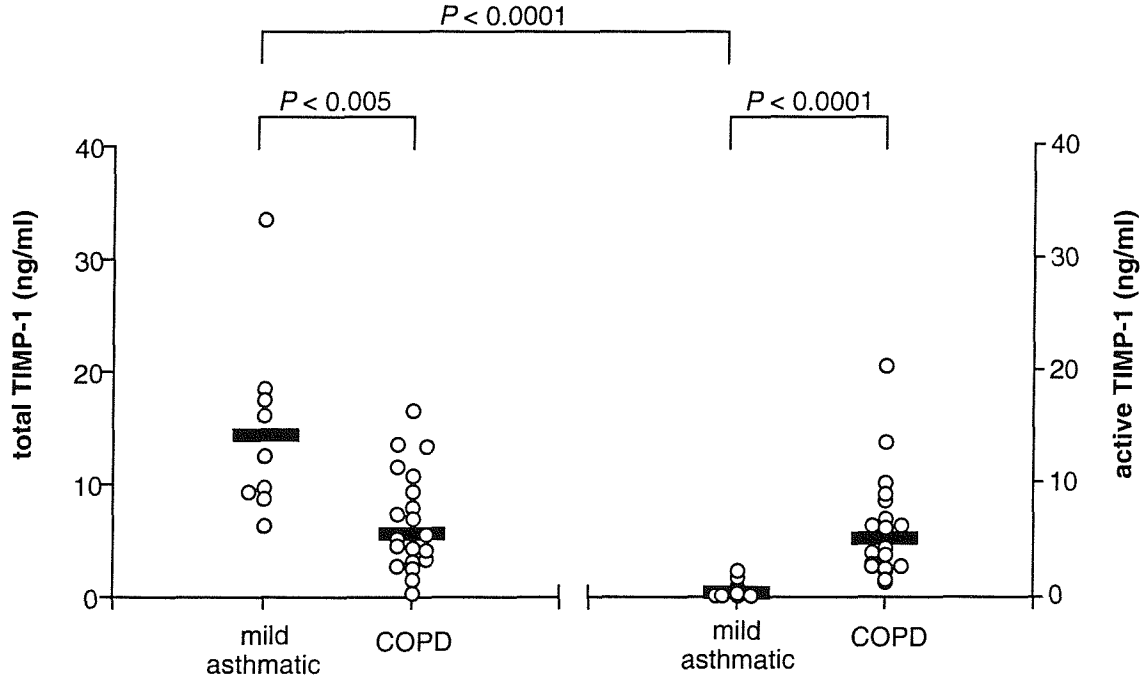


Figure 6.7 Total and active TIMP-1 in mild asthmatics and COPD patients. BAL fluid was obtained from a group of 10 mild asthmatics and 20 COPD patients and assayed for levels total TIMP-1 (left hand panel) and active TIMP-1 (right hand panel) in parallel assays. Values for total TIMP-1 were expressed in ng/ml, median values are marked. Inter-group differences were statistically analysed by the non-parametric Mann Whitney U Test for unpaired data and intra-group differences were analysed by the non parametric Wilcoxon Signed Rank Test. $P< 0.05$ was considered significant for all analyses.

BAL fluid from healthy normals was not used due to a shortage of normal samples. BAL from COPD patients, where median levels of total and active TIMP-1 remain at similar, contrasted starkly with what was seen in asthma (Figure 6.7). In asthmatic BAL total TIMP-1 a median of 14.2ng/ml, compared to a median activity of only 0.41ng/ml ($P< 0.0001$). This indicated that only 3% of the immunocompetent TIMP-1 measured was actually active.

Clearly this assay is able to identify a deficit of activity for TIMP-1 in certain inflammatory conditions, however why this deficit is not as readily apparent in COPD as it is in asthma is unknown.

6.4 Discussion

In assessing proteinase/ antiproteinase imbalance, it is important to ascertain how much of the total immunocompetent antiproteinase (as measured by conventional ELISA) is capable of forming a complex with its cognate proteinase. Reduction in the amount of active inhibitor, not apparent using conventional ELISA, could enhance the destructive potential of proteinases in pathologies such as COPD. We have begun to develop an assay to quantify the amount of TIMP-1 capable of complexing MMP-9 (active TIMP-1). We have also attempted to compare these values to total levels of TIMP-1, measured by conventional ELISA.

Initial experiments looked at the effects of inactivating agents on TIMP-1 immunocompetency and ability to form complexes with MMP-9. Pre-treatment with trypsin caused a significant reduction in the amount of TIMP-1 visible on a Western blot, with no TIMP-1 bands visible after 30 minutes of treatment at 37°C. Trypsin pre-treatment also significantly reduced the ability of TIMP-1 to complex with MMP-9. However despite the fact that the Western blot showed no evidence of TIMP-1, the inhibitor retained approximately 30% of control binding activity, even after treatment

with trypsin for 30 minutes. It is likely that this is a reflection of the sensitivity of the plate assay compared to Western blotting. There was no evidence of low molecular weight *"cleavage"* fragments following treatment, suggesting that trypsin mediated inactivation involves destruction of the detecting antibody epitope. There are 3 large regions of the TIMP-1 molecule which are not part of the trypsin resistant core of the (see Figure 6.1). The regions span residues 21-59, 76-88, and 139-170, it may be that the epitope for the TIMP-1 antibody lies in one of these areas of predicted tryptic cleavage. It may be important to identify an antibody that detects both intact and cleaved TIMP-1, during further assay development. There are however difficulties in this approach. The antibody would ideally be raised to the tryptic core of TIMP, making up large portion of the N-terminal domain. However, the N-terminal domain is thought to form much of the active site of TIMP-1 [Murphy *et al.*,1991], and this study has shown that access for an antibody to the active site could be hindered by TIMP-1 forming a complex with MMP-9.

In contrast to trypsin pre-treatment, inactivation of TIMP-1 with hydrogen peroxide significantly reduced its ability to bind MMP-9, while not affecting the molecule's immunocompetency. This finding is important, since it indicates that oxidant mediated damage causes a loss of activity which can be readily quantified by this assay.

A quantitative ELISA was developed to measure total immunocompetent TIMP-1, in parallel with the quantitative analysis of TIMP: MMP binding. Standard curves for both the inhibitor-binding assay and the total ELISA were found to be similar and linear for concentrations of TIMP-1 from 1.56ng/ml to 25ng/ml. Initially bronchoalveolar lavage (BAL) samples from COPD patients were analysed in the assays. Some individuals had lower levels of active TIMP-1 compared to total. Unexpectedly however, a proportion of the samples analysed showed TIMP-1 activity values greater

than the total. The reason for this discrepancy is unknown. However, there is a suggestion in the literature that efficiency of TIMP-1 binding to polyclonal coating IgG is reduced when TIMP-1 is in an existing complex with MMP-9 [Murphy *et al.*, 1991]. Consequently, TIMP: MMP complexes, naturally formed in BAL, may be causing signal reduction in the total TIMP-1 ELISA when compared with a standard curve of free TIMP-1. It is important in further development to find monoclonal antibodies that could overcome the differences in binding efficiency. Despite this need, initial testing on our stock of commercially available monoclonal antibodies has failed to identify an efficient TIMP-1 capture antibody, to match the polyclonal used.

During the further validation of this assay intriguing differences have been identified between levels of total and active TIMP in the BAL fluid from mild asthmatics compared to COPD patients. Total levels of TIMP were significantly higher in the BAL fluid of the mild asthmatics compared to the COPD patients. Interestingly however, when levels of active TIMP-1 were measured it was found that only approximately 3% of total TIMP-1 in asthmatic BAL retained MMP-9 binding activity. This large deficit in TIMP-1 activity compares with only a 50% reduction in the activity of another important antiproteinase, α1- antitrypsin, also seen in asthma [Furr, 2001]. The activity deficit in asthmatics is interesting when compared with COPD patients. The unilateral activity deficit seen in the asthmatics is contrasted in COPD by extremely heterogeneous data. Looking at the COPD data as a whole group, there was no significant difference between median levels of total and active TIMP-1. However when this group was looked at more closely, some individuals showed greater than 100% retention of activity, while the others showed activity deficit. Clearly there are differences in the pathologies both asthma and COPD which may contribute to the different TIMP profiles in either condition. For instance asthma is considered as primarily eosinophillic, where as COPD is neutrophillic and it may be that different

types of inflammation affect the TIMP antiproteinase screen to different degrees. However, it may also be true that different inflammatory processes grouped together as COPD affect the TIMP antiproteinase screen in differing ways.

In summary, we have begun the development of an assay to analyse the amount of total immunocompetent TIMP-1 measured by conventional ELISA that is actually capable of complexing with MMP-9. While clearly improvements must be made before we can assess the implications on proteinase / antiproteinase balance it has given an intriguing insight into TIMP-1 activity in different inflammatory processes.

Chapter 7.

General Discussion.

7.1 Discussion.

Chronic obstructive pulmonary disease (COPD) is defined by the American Thoracic Society (ATS) as a progressive airflow obstruction, due to chronic bronchitis or emphysema, which displays a degree of irreversibility [ATS Statement]. The progressive and irreversible airway resistance, characteristic of COPD, results from destruction of extracellular matrix resulting in extensive remodelling of bronchiolar walls and parenchymal destruction [Bosken *et al.*, 1992, Shapiro, 1994]. These processes are linked to cigarette smoking, however the precise mechanisms by which smoking causes destruction and remodelling are the subject of much research [for review see Barnes 1998, Jeffrey, 1998a, O' Byrne and Postma, 1999]. The major theory, in its simplest terms is that in certain individuals chronic exposure to smoke initiates the influx and activation of a variety of inflammatory cells. Cigarette smoking is associated with a fivefold increase in total inflammatory cells recovered from BAL, predominantly comprising resident macrophages [Shapiro and Senior, 1999]. In addition to macrophages, neutrophils are also thought to be important mediators of COPD pathology. They form a highly motile group of cells, rapidly transiting in large numbers into the lung from the blood stream during acute inflammatory episodes [Stockley, 1994]. Activation of these neutrophil and macrophage populations involves the release of a variety tissue of destructive agents, including proteinases such as serine proteinases, cathepsins and the matrix metalloproteinases (MMPs) [Tetley, 1993]. Continued proteolytic insult from infiltrating inflammatory cells is thus thought to contribute to the tissue destruction seen in COPD.

The involvement of matrix degrading proteinases in COPD is well established in the literature. Reports from as early as the 1960s describe individuals with a genetic

defect causing an excess of the elastin degrading serine proteinase; neutrophil elastase, compared to its specific inhibitor α_1-antitrypsin. The imbalance subsequently caused the development of early onset emphysema [Laurell and Eriksson 1963]. A large body of research into COPD pathology in later years has concentrated on the imbalance between neutrophil elastase and its inhibitors (α_1 antitrypsin and secretory leukoproteinase inhibitor) and the role of elastin degradation in lung destruction [for reviews see Tetley, 1993, Stockley, 1994, Barnes 1998]. Recently research has focused on the role of other proteinases with the ability to degrade alternative matrix constituents, with members of the MMP family of enzymes highlighted as important in the disease process. The family consists of 24 homologous zinc dependent enzymes, which are collectively able to degrade all components of the ECM. MMPs were first implicated as potentially important active components in COPD pathology when it was observed that up to 50% of elastolytic activity in BAL fluid from smokers could be removed by treatment with metal chelators [Jannoff *et al.*, 1983]. Later research using transgenic mouse technology identified MMPs likely to be involved in the pathology of emphysema. Importantly transgenic mice over expressing MMP-1 were observed to develop emphysematous lesions in the lung [D'Armentio *et al* 1992]. MMP-12 was also implicated as a proteinase involved in smoke associated emphysema when MMP-12 -/- mice exposed to cigarette smoke were unharmed while wild type (MMP-12 +/+) developed emphysema [Hautamaki *et al.*, 1997].

Furthermore, MMP-9 is also an important candidate for involvement in COPD pathology. MMP-9 is a 92kDa gelatinase with major matrix substrates being gelatin, collagenase IV/V entactin and proteoglycan. Most MMPs are transcriptionally regulated by growth factors, cytokines and ECM components. MMP-9 is an important exception to this, being synthesised constitutively during neutrophil development and stored in the secondary granules where they are poised for rapid release [Shapiro and Senior, 1999].

It has been suggested that sustained periods of neutrophil infiltration, and subsequent granule release could be early factors in COPD contributing to tissue damage. Elevated levels of MMP-9 have been found in BAL from patients with emphysema, in comparison with normals [Finlay *et al.*1997a, Betsuyaku *et al.,* 1999]. Importantly these increased MMP-9 levels show a significant association with levels of the neutrophil specific marker lipocalin, suggesting that both neutrophils and the MMP-9 they store, could be important factors in COPD progression [Finlay *et al.*1997a, Betsuyaku *et al.,* 1999].

This study supports published literature and extends the data through measurement of MMP-9 and its cognate inhibitor, the tissue inhibitor of metalloproteinase-1 (TIMP-1) at a variety of sites in the COPD lung. This utilises bronchoalveolar lavage (BAL), sputum from patients through the course of an exacerbation in symptoms, and lung tissue homogenates from smokers and ex smokers with obstructive lung function. BAL provides an indirect method of sampling and analysing MMP-9 released into the lumen of small airways and alveoli, by contrast sputum analysis reflects this in the upper respiratory tract. The added advantage of sputum analysis through periods of exacerbation was that it allowed the tracking of proteinase levels through distinct stages in disease progression. This complements and contrasts with BAL sampled from patients during stable COPD. Additionally analysis of MMP-9 and TIMP-1 levels in lung tissue homogenates and histological tissue sections from patients with COPD, adds to bronchoalveolar lavage and sputum studies. This allows insight into the effect on proteinase / antiproteinase balance of MMP and TIMP which may be unaccounted for in lung secretions or lavage. This may include MMP or TIMP tightly bound to extracellular matrix or cell surfaces, or alternatively remains unreleased from cells.

An important finding in this study is the significantly elevated levels of MMP-9 in individuals with COPD as compared to normal controls. MMP-9 burden has destructive implications for the ECM, with the major matrix substrates being collagenases IV and V, entactin and proteoglycan. Additionally MMP-9 has the potential to influence the activity of other proteinases, as it has been shown to inactivate the specific inhibitor of neutrophil elastase, α_1 antitrypsin, *in vitro* [Desrochers *et al* 1992]. This mechanism of serpin degradation has recently been highlighted as an important process in basement membrane destruction leading to blistering in inflammatory skin disease [Liu *et al,* 2000]. Though this is a different physiological system, it may also be involved in COPD pathology. Whether released into the alveolar and airway lumen, bound to the ECM or found within infiltrating inflammatory cells awaiting release. It must be noted that while levels of MMP-9 are significantly higher in disease, there is a wide heterogeneity in BAL, sputum and tissue homogenates. Such a range of values has also been reported by all authors investigating MMP-9 levels in COPD BAL [Finlay *et al.,* 1997a, Betsuyaku *et al.,* 1999] and sputum [Vignola *et al.,* 1998]. It should be considered that COPD is a heterogeneous condition with complicating components such as asthma [Jeffrey 1999], basal viral and bacterial infections [Bhowmik *et al.*, 2000] and therefore probable that our data mirror this diversity of disease status.

A major point of control of MMP-9 activity in the tissue environment is the interaction with its cognate inhibitor TIMP-1. In BAL fluid sampled from patients with stable COPD there were more molecules of MMP-9 per molecule of TIMP-1 in disease than in normal patients. Despite this, the calculated molar ratios of proteinase to inhibitor remained below 1 in the majority of COPD patients, indicating that TIMP-1 remains in excess. This could indicate that the destructive potential of the proteinase is controlled by a corresponding increase in inhibitor during stable COPD. Additionally it

may also be that analysis of BAL gives slight distortion of kinetic events in the pericellular environment since it samples a large pool of luminal constituents in a relatively large volume. For example recent work on neutrophils has suggested that TIMP-1 and MMP-9 are compartmentalised into distinct vesicles formed late in cell differentiation [Price *et al.,* 2000]. They are both vesicles which degranulate early upon neutrophil stimulation, however the fact that both are spatially separated may raise the possibility of selective degranulation, with MMP-9 release which may occur before TIMP-1 [Price *et al,* 2000]. Additionally it is hypothesised that proteinase concentration close to the cell far outweighs inhibitor concentration. As the enzyme diffuses away from the cell, protease: inhibitor ratio reaches equilibrium. Thus enzyme activity will be greatest in the area close to the cell surface; a phenomenon described as quantum proteolysis [Liou and Campbell, 1996]. These scenarios are impossible to predict if measuring proteinase / antiproteinase levels in whole BAL, however the slight shift in the balance in favour of MMP-9 in COPD may reflect a far more significant shift at the pericellular level.

In order to redress any MMP excess in COPD, possible future therapies may entail management with synthetic MMP inhibitors. Non selective MMP inhibitors such as marimastat (BB-2516) and batimastat (BB-94) have proved efficacious in MMP related conditions such as some metastatic cancers [Cawston, 1996], however are complicated by side effects. Tolerated inhibitors are required for use in the predominantly aged and immobile population, suffering from COPD [Barnes, 1999]. In addition to inhibiting MMP activity *per se,* levels of MMP and inhibitor have been shown to be influenced by compunds such as retinoic acid (RA). RA was found to upregulate TIMP-1 while downregulating MMP-9 in cells from bronchoalveolar lavage (BAL) [Frankenburger *et al.,* 2001] Administration of RA may also prove to have an effect alleviating oxidative stress, an additional symptom of COPD.

In attempting to ascertain a cellular source for MMP-9 in BAL our study found no association between MMP-9 levels and inflammatory cells numbers. This was in agreement with a previous investigation also attempting to correlate MMP-9 with inflammatory cells where no correlation was observed [Betsuyaku *et al.* 1999]. It must be considered when interpreting this data that while BAL samples a pool of inflammatory components in the airways, it is unlikely that all the cells contributing to the mediator pool are actually located in those airways. For example during stable COPD, during which time BAL samples can be safely taken, MMP-9 releasing cells are likely to be in a variety of tissue locations. A proportion of cells will be sequestered in the pulmonary microvasculature, whereas others may be migrating through the mucosa, with only a minority in the lumen *per se*. BAL samples only those cells washed from the airways, however it also samples mediators such as MMP-9 released from cells in and around the airway and which may diffuse into the lumen. In such a situation dissociation between MMP-9 and cell numbers is likely to occur. The dissociation between MMP-9 and cell number seen in BAL contrasts starkly with what was observed in sputum during an inflammatory event; an exacerbation. These episodes are important events in disease progression, with frequency of occurrence negatively correlating with quality of life [Seemungal *et al* 1998]. During these episodes inflammatory burden significantly increases, in particular neutrophil numbers have been shown to rise [Balzano *et al*, 1999]. Importantly this study has shown that exacerbations are associated with a significant increase in neutrophils which correlates with a significant increase in MMP-9. The subsequent increase in proteinase burden due to MMP-9 is not accompanied by an increase in levels of TIMP-1, thus the antiproteinase screen is clearly diminished in airways during this period. These data would suggest that neutrophils are an important source of MMP-9 but not TIMP-1 under these physiological conditions. Additionally it was observed that MMP-9 levels remain

elevated for a period of up to six weeks following an exacerbation in some of the individuals tested. This could suggest that either inefficient clearance of the cells involved in the initial inflammatory episode or a maintained inflammatory burden is keeps MMP-9 at potentially damaging levels in some individuals. Such a maintained insult has clear implications for chronic matrix destruction and remodelling.

The number of lymphocytes in sputum during an exacerbation also rose, compared to baseline values, and intriguingly showed a significant association with levels of both neutrophils and MMP-9. The implications of these correlations are unclear, however an attractive hypothesis for disease progression is that an inflammatory stimulus could activate lymphocytes, which in turn attracts neutrophils. These infiltrating neutrophils release excess proteinases such as MMP-9 contributing to tissue destruction disease progression. CD4+ lymphocytes have been highlighted in recent reports as influencing neutrophil chemotaxis and activation by production of a newly described cytokine,interleukin-17 (IL-17) [Laan *et al.,* 1999]. IL-17 induces expression of interleukin-8 (IL-8) in bronchial epithelial cells. IL-8 is thought to be an important chemoattractant for neutrophils in inflammatory airways disease [Richman-Eisenstat *et al.,* 1993], and thus upregulation of IL-8 production by IL-17 could be important in influencing neutrophil migration. Additionally the CD4+ associated cytokine IL-13 has been implicated in causing MMP and cathepsin dependant emphysema when over erxpressed in transgenic mice [Zheng *et al.*, 2000]. While both the lymphocyte subtype and cytokine profile are uncharacterised in this study, the cited literature along with the reporting of a possible CD4+ type response in COPD exacerbation [Zhu *et al.,* 2001] could point to potential pathway for neutrophil activation, during exacerbation. This requires further investigation.

Exacerbations form very important treatment targets in COPD. Airway damage in the disease is generally in advanced stages and likely irreversible once symptoms present,

hence treatments aimed at slowing further disease progression are the most obvious treatment target. Exacerbations represent important stages in disease progression, and therefore possible measurable treatment targets. It is widely accepted that the increased inflammation observed during exacerbation is triggered by a bacterial or viral infection [Anthonisen, 1987], hence the treatment of choice has been antibiotics [Anthonisen, 1987]. Recently however it has been suggested that inflammation occurs independently of infection, in which case anti inflammatory therapy could form an important option [Aaron *et al.*, 2001]. Anti-inflammatory intervention in the form of short term, high dose, corticosteroid treatment has been shown to have little clinical benefit in stable COPD, having no effect on levels of neutrophil chemoattractant cytokines such as IL-8, as well as levels of MMP-9 and TIMP-1 [Culpitt *et al* 1999]. However there is possibly a distinction between stable COPD and exacerbation, with steroids being found to reduce symptoms and improve lung function during exacerbation [Thompson *et al.*, 1996]. This suggests that patients during exacerbation could be steroid responders, where they would not necessarily be in stable disease. Additionally Llwellyn-Jones and co-workers found that short term, high doses of the steroid fluticasone proprionate administered to patients with stable COPD, reduced the neutrophil chemoattractive potency of sputum, possibly by an effect on locally produced cytokines. This effect was coupled with an increase in antiproteinase activity [Llwellyn-Jones *et al.*, 1996]. It is conceivable that during an exacerbation in COPD this chemoattractive inhibition could be amplified, possibly resulting in a positive treatment effect which could cause reduction in neutrophil number and hence MMP-9 burden. Further studies on the cellular effects of steroids during exacerbations are clearly necessary.

While the neutrophil is renowned as the key inflammatory cell in COPD [Hunninghake, 1983, Stockley, 1994, 1998] the eosinophil predominates in asthma [Ohno *et al.*, 1997]. This study found that eosinophil number increased during

exacerbation mirroring findings from Saetta and co workers who reported a 30 fold increase in eosinophils in bronchial biopsies taken during exacerbation compared to baseline [Saetta *et al.*, 1994]. The authors postulated that this implicated eosinophils in COPD inflammation, however we found that eosinophils did not show a close association with MMP-9. This could mean that in COPD infiltrating neutrophils have a role that is distinct from that seen in asthma [Ohno *et al.*, 1997]. Immunohistochemical analysis shows that both eosinophils and neutrophils express immunoreactivity for MMP-9; however these data suggest that only neutrophils release MMP-9 during an exacerbation.

Of the four cell subtypes (macrophages, neutrophils, eosinophils and lymphocytes) studied macrophages were the only population which did not change through the course of an exacerbation. Additionally macrophages formed a weak association with MMP-9 levels. Macrophages appeared to form a resident unchanging population in contrast to the highly mobile, quickly sequestered and rapidly cleared neutrophils. As neutrophils seem to be such a dominant source of MMP-9 in COPD exacerbations it is difficult to ascertain how much macrophages contribute to total proteinase burden.

Homogenizing lung tissue fragments directly samples the total pool of MMP-9 and TIMP-1. This entails liberating pools of MMP-9 and TIMP-1 bound to matrix or remaining within cells. These pools of proteinase and inhibitor may not be accounted for in the analysis of lavage or secretions found in sputum. Importantly, in these homogenates there was found to be a molar excess of MMP-9 over TIMP-1, associated with a group of current and ex smokers who displayed an obstructive lung function ($FEV_1/FVC < 70\%$). Importantly the imbalance identified did not seem to be influenced by the fact that some of the group no longer smoked. In agreement with this, it has been suggested that inflammation is ongoing in groups of ex smokers; with numbers of

neutrophils, macrophages, and eosinophils found to be higher in sputum, BAL and bronchial biopsies from ex smokers when compared to healthy controls [Rutgers *et al.,* 2000]. Immunohistochemical analysis of tissue samples from some of the same individuals showed MMP-9 present within infiltrating inflammatory cells, and also bound in large amounts to the ECM, in individuals with impaired lung function. These results suggest that the ECM could act as a solid phase MMP-9 store, ready for release during disease. Immunohistochemical analysis on bronchial biopsy specimens from COPD patients also point towards increased levels of MMP-9 compared to normal controls. Staining for MMP-9 in these specimens mainly localised with staining for neutrophil elastase, identifying neutrophils as important contributors to MMP-9 burden in the mucosa. In a proportion on the individuals it was difficult to differentiate whether MMP-9 was solely associated with macrophages, or whether there was also an additional macrophage source.

Collectively all of these data show that there is an increased MMP-9 burden in the lungs of COPD patients which has the ability to overwhelm the TIMP-1 antiproteinase screen. Infiltrating inflammatory cells such as neutrophils which have previously been highlighted as being involved in COPD pathology are clearly important sources of this enzyme and an important target for novel therapy. Cytokines such as IL-8 and LTB_4 are important in neutrophil recruitment to the airways, and specific inhibitors to both of these are currently under development, and may assist in reduction of a neutrophil based proteinase burden [Barnes, 1999, White *et al.,* 1998]. Alternatively drugs such as PDE4 inhibitors display inhibitory effects on lymphocyte and macrophage as well as neutrophil function, and thus could prove important broad spectrum drugs in decreasing inflammation in COPD, and thus MMP burden [Torphy 1998].

The vast majority of studies so far into MMP: TIMP imbalance in COPD, analyse total levels of immunocompetant protein in lung washes and secretions. Clearly however this balance may also be influenced by interactions in the inflammatory milieu, which bring about further MMP activation, or alternatively TIMP inactivation. It is possible that mediators released by inflammatory cells inactivate TIMP-1 altering the MMP-9:TIMP-1 balance in favour of excess protease and remodelling. For example, cleavage by neutrophil elastase [Okada *et al.*, 1988] or cathepsin B [Kostoulas, *et al.*, 1999] or oxidation [Shabani *et al.*, 1998] are all factors which are known to inhibit TIMP-1 functionality. Interestingly, one previous study found that the concentration of total TIMP-1 in sputum from COPD patients exceeded collagenase inhibitory activity from the same samples. This suggested that a proportion of inhibitor was inactivated in the disease [Burnett *et al.*, 1986], favouring matrix degradation. In contrast to the methods used by Burnett and co workers, this study used a substrate binding assay to analyse TIMP-1 activity. The assay was based on the fact that only functional TIMP-1 was able to bind latent MMP-9 coated onto a conventional 96 well plate, and binding was visualised and quantified using specific antibodies to TIMP-1 and a commercially available chromogen. Control experiments showed that TIMP-1 fragments could not be identified on a Western blot by the monoclonal antibodies used and therefore the assay could not easily distinguish between total functional TIMP and TIMP subject to proteolytic inactivation. Inactivation by oxidation however involved little cleavage of the TIMP-1 and little epitope destruction. This treatment did however result in 50% inhibition of the ability of TIMP-1 to bind to MMP-9. The assay therefore demonstrated its ability to identify TIMP-1 inactivated by oxidation. Analysis of BAL samples from COPD patients showed that inactivation of BAL derived TIMP-1 occurs in COPD. Interestingly, we found that amounts of inactivated TIMP varied widely between subjects possible that this is a reflecting of heterogeneity of inflammation in the group

of patients. This assay requires additional validation and testing on BAL and sputum from normals and individuals with other inflammatory lung diseases, for example sampling from asthmatics, however initial results are encouraging.

7.2 Conclusion.

It is clear that MMP-9 has an important role to play in COPD pathology. We have independently sampled from three different sites in diseased lung, using sputum, bronchoalveolar lavage and tissue samples. Data from each of these has consistently shown that individuals with COPD have significantly higher levels of MMP-9 in their lungs than normals. It is likely that the main cellular source of MMP-9 through disease progression is the neutrophil. This places immense burden on the inhibitor of MMP-9; TIMP-1. Not only is it the case that MMP-9: TIMP-1 imbalance underlies COPD, but also that it can be further augmented by inactivation of TIMP-1. Here we have developed a novel inhibitor binding assay to analyse this phenomenon. Early results indicate that inhibitor inactivation occurs in the diseased lung of certain individuals, however we do not know as yet whether the amount or rate of inactivation is different from that seen in normal individuals and further work is needed to elucidate this.

This study is important as it has highlighted the important role played by MMP-9 in COPD progression. However this enzyme represents one part of a network of proteinases each contributing to tissue destruction. Understanding of the different stimuli, roles and interactions of each of these is needed for full understanding of COPD pathology.

7.3 Further work.

A) This study has shown that MMP-9 levels increase during exacerbation, in parallel with neutrophil infiltration. The aim of further studies should be to build on this data with more detailed studies on MMP/ TIMP-1 involvement in these events. A more rigorous programme of sputum sampling, following patients at monthly intervals with extra samplings during exacerbation, would provide a more robust profile of inflammatory events. These well characterised samples could be used to analyse what controls neutrophil infiltration and MMP-9 increase. For wxample it would be interesting to study whether particular lymphocyte sub groups or novel cytokines correlate with change in neutrophil number. It would also be important to compare and contrast the inflammatory/ MMP profile of sputum taken from stable COPD patients with sputum from regular exacerbators.

B) During this study we have begun to develop a well characterised archive of lung tissue samples, taken from a group of patients undergoing resection for lung carcinoma. Importantly this samples from a cross section of subjects with a variety of smoking histories and standards of lung function. Much of this archive has been embedded for immunohistochemistry and could form the basis of a wider immunohistochemical study into proteinase/ antiproteinase imbalance and inflammation in lung parenchyma. If tissue preservation conditions could be improved it would be interesting to analyse relative expression of MMP-9 and TIMP-1, using semiquantitative RT-PCR. This would give valuable information as to whether MMP-9/ TIMP-1 imbalance was at the level of protein synthesis, or whether it was a post translational phenomenon.

The group of paraffin embedded samples of bronchial epithelium is being continually added to. This is forming a well characterised set of samples which,

with further study, could provide insight into MMP-9/ TIMP-1 balance in central airways.

C) We have begun to develop an inhibitor binding assay to assess the activity of TIMP-1 in clinical samples. Further validation and development of this assay is required. Primarily work should focus on why, in some subjects, the recovery of TIMP-1 is greater in the activity assay than on total TIMP-1 ELISA. This work would entail experimenting with a variety of capture antibodies and binding conditions, obtaining optimum binding of immunocompetent TIMP-1 and improving signal to noise ratio.

Chapter 8.

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