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Serine protease inhibitors in the upper and lower airways

With specific focus on
Secretory leukocyte protease inhibitor and α1-antitrypsin

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DOCTORAL THESIS

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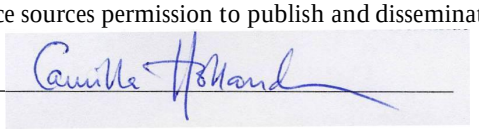
Signature  Date_____

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Malmö 2006

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Everybody can learn from science,

but it all depends on how one uses the knowledge

Contents

CONTENTS 7

ABBREVIATIONS 8

SUMMARY 9

LIST OF STUDIES 10

ACKNOWLEDGEMENTS 11

1. BACKGROUND 13

1.1 THE AIRWAYS 13
1.2 DISEASES OF THE AIRWAYS 13
1.3 PATHOPHYSIOLOGICAL ASPECTS OF DISEASES OF THE AIRWAYS 14
1.4 CYTOKINES AND CHEMOKINES IN AIRWAY INFLAMMATION 18
1.5 PROTEASES AND ANTI-PROTEASES IN AIRWAY INFLAMMATION 20

2. SPECIFIC BACKGROUND 24

2.1 SECRETORY LEUKOCYTE PROTEASE INHIBITOR (SLPI) 24
2.2 α1-ANTITRYPSIN (AAT) 26

3. AIMS 29

4. MATERIALS 30

4.1 CELLS AND CELL CULTURES 30
4.2 CLINICAL MATERIAL 31
4.3 SPECIFIC REAGENTS 32

5. EXPERIMENTAL PROCEDURES 36

6. STATISTICAL ANALYSIS 39

7. RESULTS 40

7.1 REGULATION OF SLPI PRODUCTION (STUDIES I AND III) 40
7.2 BIOLOGICAL ACTIVITIES OF SLPI AND AAT (PROLASTIN) (STUDY II AND III) 41
7.3 CONCENTRATIONS OF SLPI AND AAT IN PLASMA AND BRONCHIAL LAVAGE FLUID (STUDY IV AND V) 46

8. CONCLUDING REMARKS 50

9. SAMMANFATTNING PÅ SVENSKA 51

10. REFERENCES 53

ORIGINAL ARTICLES

Abbreviations

AAT	Alpha-1-antitrypsin
ACT	Alpha-1-antichymotrypsin
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary disease
EPC	Epithelial cell
FCS	Fetal calf serum
FEV_1	Forced expiratory volume in 1 second
fMLP	formyl-Met-Leu-Phe
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
ICAM-1	Inter-cellular adhesion molecule-1
IL	Interleukin
LPS	Lipopolysaccharide
LTB4	Leukotriene 4
M AAT	M variant of alpha-1-antitrypsin
MC	Mast cell
MMP	Matrix metalloprotease
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PiMM	Wild type AAT variant
PiZZ	Homozygous AAT-deficiency variant
RANTES	Regulated-upon-activation, normal-T-cell expressed and secreted protein
SLPI	Secretory leukocyte protease inhibitor
TIMP	Tissue inhibitor of metalloproteases
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
Z AAT	Z variant of alpha-1-antitrypsin
α2M	α2-Macroglobulin

Summary

Clinical and experimental studies implicate proteases and their inhibitors in the physiology and pathology of the airways**.** Inflammatory cells, such as neutrophils, monocytes/macrophages, epithelial and mast cells, release various proteases. It is well known that increased production or activity of proteases, or inactivation and decreased production of protease inhibitors may lead to protease/anti-protease imbalance and tissue damage. In human airways protease inhibitors include those produced locally by mucosal epithelial cells, serous cells, and bronchiolar goblet cells, such as secretory leukocyte protease inhibitor (SLPI), and those produced mainly by the liver that can reach the airways via passive diffusion, such as α1-antitrypsin (AAT). SLPI is a significant component of the anti-neutrophil elastase shield in the respiratory tract and is therefore complementary to the AAT, a major inhibitor of human neutrophil elastase and protease 3. Inherited, severe AAT deficiency is a recognised genetic risk factor for the development of chronic obstructive lung disease (COPD), whereas no gene polymorphism has been found for SLPI. There is, therefore, no indication of whether a deficit in SLPI could be responsible for the development of COPD in patients that otherwise have sufficient levels of AAT. To test the hypothesis that a compensatory increase in levels of SLPI might favourably improve the protease/protease-inhibitor balance in AAT deficient individuals, we analysed plasma levels of SLPI in healthy subjects with and without severe AAT deficiency. The finding that plasma SLPI did not differ between the groups studied led to the conclusion that SLPI does not compensate for the deficiency of plasma AAT. SLPI, however, is produced locally in the airways and its levels are regulated by various pro-inflammatory stimuli. SLPI levels in bronchial lavage fluid from asthma and COPD patients are remarkably higher than in plasma. Therefore plasma collected when the individual is healthy (asymptomatic) may not be the appropriate biological fluid in which to measure SLPI. In addition to anti-protease activities, both SLPI and AAT, exhibit other anti-inflammatory properties *in vitro* and *in vivo*. Therefore, an understanding of the mechanisms involved in AAT and SLPI protein expression, release and consumption may provide important knowledge on the dynamics of the regulation of the protease/anti-protease and other systems involved in the inflammatory response. Findings in our studies revealed that SLPI expression and release by epithelial cells are dependent on the interaction of epithelial and mast cells and that endotoxin (LPS) significantly increases SLPI levels in nasal lavage. Interestingly, while AAT was found to inhibit LPS-induced, pro-inflammatory monocyte and neutrophil responses *in vitro*, and to suppress nasal chemokine (IL-8) release in LPS-challenged individuals, *in vivo*, SLPI effects shown to be much more complex. *In vitro*, SLPI significantly inhibited LPS-stimulated IL-8 release from monocytes but had no effect in neutrophil models. Indeed, SLPI itself stimulated neutrophil, but not monocyte, chemotaxis and adhesion. These findings further expand our knowledge on the regulatory effects of SLPI and AAT during inflammatory reactions, effects which may be potentially more biologically profound than their ability to inhibit proteases.

List of studies

This thesis is based on the following original articles, referred to in the text by their roman numbers:

I. *Hollander C*, Nyström M, Janciauskiene S, Westin U:
Human mast cells decrease SLPI levels in type II - like alveolar cell model, in vitro. Cancer Cell International 2003, 3(1):14.

II. Nita I, *Hollander C,* Westin U, Janciauskiene SM:
Prolastin, a pharmaceutical preparation of purified human α1-antitrypsin, blocks endotoxin-mediated cytokine release. Respiratory Research 2005, 6(1):12.

III. *Hollander C*, Westin U, Janciauskiene SM:
Secretory leukocyte protease inhibitor's effects on human neutrophils and monocytes. Submitted.

IV. *Hollander C*, Sitkauskiene B, Sakalauskas R, Westin U, Janciauskiene SM:
Plasma and bronchial lavage fluid concentrations of inflammatory markers in patients with COPD and Asthma. Submitted.

V. *Hollander C*, Westin U, Wallmark A, Piitulainen E, Sveger T, Janciauskiene S:
Plasma levels of serine protease inhibitors in healthy and COPD subjects with and without α1-antitrypsin deficiency. Submitted.

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1. Background

1.1 The airways

The respiratory system controls breathing and respiration, and also cleans, warms, and moistens air on its way to the lungs. The respiratory system can be subdivided into an upper and lower respiratory tract. The upper part consists of the nose, nasal cavity, pharynx (throat), larynx, and upper part of the trachea (windpipe). The lower part consists of the lower part of the trachea, bronchi, and lungs (which contain bronchioles and alveoli). The airways are lined with a layer of epithelial cells which comprise an important primary line of defense to the entire respiratory tract. This superficial cellular layer consists primarily of mucus-producing (goblet) cells and ciliated cells (Figure 1) which function in a coordinated fashion to entrap inhaled particles and remove them from the airways. The proteins that are present in epithelial lining fluid derive from mucous and serous cells in submucosal glands, goblet cells, Clara cells, epithelial cells, and other cells within the mucosa (plasma cells, mast cells, phagocytes, and fibroblasts) [1, 2].

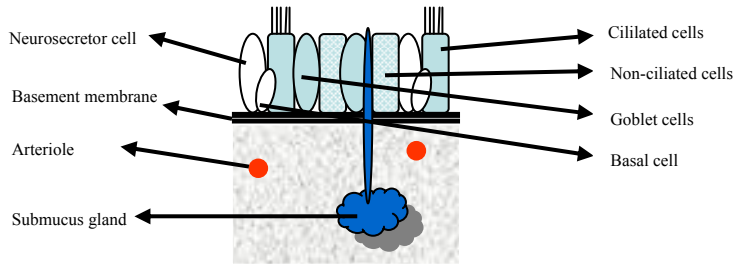

Figure 1. Ciliated and mucus cells covering the airways.

1.2 Diseases of the airways

The respiratory system is sensitive to air pollutants, bacteria and viruses. A wide range of acute, infectious and chronic respiratory diseases afflict many individuals. Respiratory diseases in adults, such as chronic obstructive respiratory disease (COPD), constitute a major burden in terms of morbidity and mortality in the developing world [3, 4].

1.3 Pathophysiological aspects of diseases of the airways

1.3.1 Inflammation of the airways

Inflammation is a characteristic feature of respiratory tract diseases such as bronchial asthma, chronic obstructive lung disease, acute bronchitis and bronchiolitis, idiopathic pulmonary fibrosis and emphysema. Recent studies suggest a relationship between upper and lower airway inflammation [5, 6].

The inflammatory process in the airways is complex and involves the release of many mediators, including cytokines, chemokines, adhesion molecules, enzymes and others, as well as the processes of cell migration, activation and degranulation. The main histological features of airway inflammatory diseases are epithelial alterations, goblet-cell and submucosal gland hyperplasia, smooth-muscle cell hyperplasia and hypertrophy, subepithelial fibrosis, microvascular proliferation, cartilage changes, airway wall oedema, and inflammatory cell infiltration [7-9]**.** The inflammatory infiltrates characteristically contain a substantial population of eosinophils as well as T-cells, monocytes, and neutrophils [10]. There is no doubt that all together these cells play an important role in the development, course, and treatment of inflammatory diseases of the airways.

1.3.2 Inflammatory cells

Inflammatory diseases are dependent upon multiple cell types, including initiator, effector and regulatory cells. All of these cells can be recruited individually, as part of responses to tissue insults.

Mast cells

The biological functions of mast cells (MC) include a role in innate immunity, tissue repair and angiogenesis [11]. Mast cells arise in the bone marrow, enter the circulation as CD34+ mononuclear cells that are positive for stem-cell factor, travel to mucosal and submucosal sites, and undergo tissue-specific maturation [12]. Mast cells differentiate and mature locally in most tissues, especially those that are exposed to the external environment, such as the airways [11, 13]. For example, some studies have reported that mast cells are present in increased numbers in the airways of patients with asthma [14].

There are at least two subpopulations of mast cells; mast cells containing tryptase located in the mucosa (MC_T) and mast cells with both tryptase and chymase found in the connective tissue (MC_{TC}) [15]. The MC_{TC} cells are predominant in the nasal mucosa while MC_T are more prevalent in the peripheral lung tissue [16-19].

Although the role of these enzymes is not fully defined, inhibitors of tryptase have been shown to modulate the response of the airway to allergen [20]. Mast cells also produce cytokines, histamine, prostaglandins, leukotrienes, platelet activating factor, proteoglycans and acid hydrolases which all contribute to different immunological and pathological processes [21, 22]. Several mast cell-products have the potential to adversely affect the growth and function of smooth muscle. For example, the mast cell–derived autacoid mediators histamine, prostaglandin D_2, and the cysteinyl leukotrienes are potent spasmogens of airway smooth muscle, and the mast cell–specific serine protease tryptase has been shown to induce broncho-constriction, airway remodelling, and airway hyper-responsiveness [23, 24].

Epithelial cells

The airway epithelium is not just a mechanical barrier, but also a regulator of tissue response to external stimuli. The activated respiratory epithelium has been shown to produce a number of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-8 [25], and other inflammatory mediators, including arachidonic acid products, nitric oxide, endothelin-1, and transforming growth factor (TGF-β) [26]. Clara cells (epithelial secretory cells in the large and small airways) secrete leukocyte protease inhibitor (SLPI) [27].

Interferon-γ and TNF-α produced by the epithelium have been shown to provoke epithelial cells to produce lipid mediators, reactive oxygen species, reactive nitrogen species, and cytokines as secondary mediators. These latter can further promote hypersecretion of mucus and inflammation [28, 29].

Neutrophils

Neutrophils are short-lived cells (up to 48 hours) produced in the bone marrow where they develop from stem cells via three stages (myeloblasts, promyelocytes and metamyelocytes) to mature neutrophils and are then released into the blood stream [30, 31].

In the airways neutrophils are widely accepted to be a central effector cell [32, 33]. Under normal conditions, the role of neutrophils is to kill and eliminate endogenous and exogenous stimuli such as airborne pollutants, allergens, and microbes by employing phagocytosis, a

respiratory burst, and the release of cytotoxic mediators and proteases. These defense mechanisms are dependent solely on the ability of the neutrophils to adhere to and migrate through a normally tight microvascular endothelial barrier. Neutrophils are the primary cell type that is recruited to the airways during the acute stages of respiratory infections [34, 35] and this is likely in response to chemotactic factors, such as IL-8 and LTB_4 [36, 37]. Numerous studies have shown that neutrophil-derived serine proteases, such as elastase, cathepsin G and protease-3, contribute to airway damage by direct activation of the epithelium [38], up-regulation of mucus secretion [39] and induction of glandular hyperplasia [31]. In particular, neutrophil elastase has been shown to provoke secretion of mucins by isolated airway epithelial cells and glands from several species [40].

Eosinophils

Eosinophilopoiesis begins in the bone marrow and is regulated by IL-3, IL-5, and granulocyte–macrophage colony-stimulating factor [41]. To participate in the inflammatory response, the eosinophils migrate from the circulation to the airways [42]. The mature eosinophils have intracellular granules that are sources of inflammatory proteins, including major basic protein, eosinophil-derived neurotoxin, peroxidase, and cationic protein. Major basic protein, in particular, can damage airway epithelium, intensify bronchial responsiveness, and cause degranulation of basophils and mast cells. The eosinophil is a rich source of leukotrienes which contract airway smooth muscle, increase vascular permeability, and may recruit more eosinophils to the airway [43].

Monocytes/macrophages

Monocytes develop from pluripotent stem cells in the bone marrow under the influence of growth factors, and less than a day after formation monocytes enter the blood stream. The monocytes in peripheral blood constitute 4-8 % of the total leukocyte population.

Depending on the local signals in their micro-environment, monocytes secrete a variety of macromolecules and products of low molecular weight that mediate inflammation and repair [44]. It has been reported that in patients with chronic obstructive pulmonary disease (COPD) the migration of monocytes to growth-related protein is significantly increased compared to monocytes from healthy subjects [45]. It has also been found that there are significantly higher levels of monocyte chemo-attractant protein-1 (MCP-1) and growth-related proteins in the sputum from COPD patients compared to healthy subjects and smokers [46].

Monocytes circulate for a short time (8 to 71 hours) [47] and migrate through the blood vessel walls into various organs and then differentiate into macrophages. Thus, macrophages are not a constant population of cells, but are regularly renewed by the influx of monocytes from the blood stream.

Macrophages are the predominant defence cell in the normal lung and are increased during conditions associated with chronic inflammation. Macrophage numbers have been shown to be elevated in the lungs of smokers and patients with COPD, where they accumulate in the alveoli, bronchioli and small airways [48]. Furthermore, there is a positive correlation between macrophage number in the alveolar walls and the mild-to-moderate emphysema status in patients with COPD [49]. For example, in bronchoalveolar-lavage of patients with COPD, macrophage numbers are found to be increased by 5 to 10 times as compared to controls [50]. Macrophages are suggested to play a role in driving the inflammatory process by the release of chemotactic factors and by recruiting neutrophils [51]. Like many metalloproteases (MMPs), macrophage elastase (MMP-12) is able to degrade extracellular matrix components, such as elastin, and is involved in tissue remodelling processes. Studies using animal models of acute and chronic pulmonary inflammatory diseases, pulmonary fibrosis and COPD, have provided results that MMP-12 is an important mediator of the pathogenesis of these diseases [52].

A more recent theory suggests that alveolar macrophage-derived MMPs mediate inflammation by releasing TNF-α from macrophages with subsequent neutrophil influx, endothelial activation and tissue breakdown caused by the neutrophil-derived proteases.

T-lymphocytes

Dependent on the phenotype, T-lymphocytes can produce a spectrum of cytokines, including IL-1, IL-4, IL-5, IL-10, and interferon-γ (IFN- γ), and affect other pro-inflammatory cells [53].

T-lymphocytes also stimulate endothelial cells to secrete IL-8 and monocyte chemo-attractant protein-1 (MCP-1), which may further promote the migration of monocytes and neutrophils [54].

T-cells may directly adhere to endothelium, increase its permeability and favour the extravasations of inflammatory cells [55]. It has been shown that both $CD4^+$ (T-helper) and $CD8^+$ (suppressor/cytotoxic) T-cells are increased in the airways and lung parenchyma of patients with COPD, with a predominance of $CD8^+$ cells [56, 57]. In asthma patients there is a

predominance of $CD4^+$ cells which express IL-4, IL-5, and IL-13, and are associated with an increased number of eosinophils [58].

Dendritic cells/antigen presenting cells

The dendritic cells are bone marrow-derived, migratory, non-phagocytotic cells that are present in most tissues. The density of dendritic cells in the airways is related to the extent of antigen exposure, being greatest in the proximal airways, and diminishing towards the periphery. A variety of pro-inflammatory stimuli including exposure to bacterial and viral infection [59, 60], cigarette smoking [61] and allergen inhalation in previously sensitised animals [59, 60], induce a marked increase in the density of dendritic cells in the lung. Increased numbers of respiratory mucosal dendritic cells are found in allergic rhinitis and asthma, and these cells express the high affinity IgE receptor [62].

Upon encounter with inhaled antigens, dendritic cells of the airway migrate to the regional lymph nodes of the lung and present the antigens to B- and T-lymphocytes to induce either Th1 or Th2 immune responses [63, 64]. The dendritic cells can polarize naïve T-cells into either T-helper type (Th1) or Th2-effector cells [65, 66], and are increasingly recognized as having a central role in the establishment of T-cell memory and peripheral immune tolerance [67, 68].

1.4 Cytokines and chemokines in airway inflammation

Cytokines and chemokines are produced and secreted by a variety of cell types, including neutrophils [69], monocytes, macrophages, endothelial cells, and epithelial cells [70]. It has been demonstrated that the supernatants of cultured airway epithelial cells contain measurable amounts of cytokines such as IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) [71]. Upon epithelial cell stimulation, the expression of these molecules is increased in order to direct inflammatory cells to the site of an injury. Mast cells produce several cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, interferon-γ and tumour necrosis factor-α (TNF-α) [72]. The potential for the extracellular release of these cytokines points to the possibility that mast cells contribute to both acute and chronic allergic inflammation. A number of laboratories have

established the importance of the Th2-derived cytokines IL-4, IL-5, and IL-13 in mediating the airway inflammatory response for allergic lung inflammation [73-75].

Tumour necrosis factor-α (TNF-α)

TNF-α, which was originally identified as a product of activated macrophages, is also produced by endothelial cells, mast cells, leukocytes, and the airways epithelium [76]. Among multiple biological effects, TNF-α can cause the enhanced expression of adhesion molecules, ICAM-1 (inter-cellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), on the pulmonary endothelium and airway epithelium and the enhanced expression of E-selectin on eosinophils and neutrophils [77]. Furthermore, in airway epithelial cells, TNF-α has been shown to stimulate the secretion of GM-CSF, IL-6, and IL-8 [78].

Chemokines

Chemokine, IL-8, is produced by alveolar macrophages, lymphocytes, and epithelial cells, and it was the first chemokine shown to be produced by neutrophils [79]. It is well-documented that cytokines and growth factors (e.g., TNF-α, IL-1, and GM-CSF), chemo-attractants (e.g. Platelet activating factor, PAF, and Leukotriene$_4$, LTB$_4$), proteolytic enzymes, various bacteria, fungi, protozoa, and viruses possess the capacity to trigger the secretion of IL-8 by neutrophils [80]. Although IL-8 is chemotactic for eosinophils, basophils, and T-lymphocyte subsets [81], its main action is suggested to be in neutrophil recruitment and activation, causing a release of granule enzymes, an increase in respiratory burst activity, the up-regulation of adhesion molecules, and the increased adherence of neutrophils to endothelial cells [82].

The chemokines RANTES (regulated-upon-activation, normal-T-cell expressed and secreted protein), macrophage inflammatory protein 1α, and the eotaxins are central to the delivery of eosinophils to the airway [83]. These chemo-attractants are produced by epithelium, macrophages, lymphocytes, and eosinophils. Chemokines have been detected on cells and in airway tissue from patients with asthma. It was shown that that the constitutive expression of RANTES was greater in the airways of patients with asthma than in normal subjects [84]. Immunohistochemical studies of airway-biopsy specimens from normal subjects, allergic patients with asthma and patients with non-allergic asthma, have shown that epithelial cells,

endothelial cells, and macrophages were the primary sources of eotaxin, eotaxin-2, RANTES, and monocyte chemotactic proteins 3 and 4 [85].

1.5 Proteases and anti-proteases in airway inflammation

Proteases

Proteases play an essential role in modulating the turnover of extracellular matrix. They are involved in regulating fundamental cellular functions such as apoptosis, cell growth and activation and also in protein secretion and phagocytosis [86]. Proteases are classified on the basis of their catalytic mechanisms as serine, aspartic, metallo, threonine and cysteine proteases and are localized either extra-cellularly, at the cellular surface, in the cytoplasm of cells or within specific sub-cellular structures such as lysosomes (Table 1). The inflammatory process in the respiratory airways includes the release of proteases that regulate the adhesion of molecules, and the process of cell migration, activation and degranulation. The destruction of tissue in inflammatory diseases of the airways is to a large extent mediated by an excess of serine proteases and matrix metallo-proteases [49, 87-89].

The major sources of proteases within the lung are inflammatory cells, such as neutrophils, mast cells, macrophages and lymphocytes [50, 90, 91]. Other cells, including epithelial, endothelial and fibroblasts, also synthesise proteases [92, 93]. Serine proteases, including neutrophil elastase, cathepsin G and protease 3, are located in primary granules within neutrophils [94]. Some of the metallo-proteases, MMP-8 and MMP-9, are also packaged into specific and gelatinase granules in the neutrophil [95]. Neutrophil proteases are released either intra-cellularly into phagolysosomes, or extra-cellularly following cellular activation [96]. Differentiation of monocytes into macrophages results in the loss of the serine protease complement of these cells. However, this is replaced by an ability to synthesise other proteases including a number of MMPs and elastolytic cathepsins [97, 98].

Serine proteases with trypsin-like (tryptase) and chymotrypsin-like (chymase) properties are major constituents of mast cell granules. Tryptases and chymases have been shown to promote vascular permeability [99], to contribute to tissue remodelling through selective proteolysis of matrix proteins and through activation of protease-activated receptors and of

matrix metallo-proteases [99]. Tryptase and other proteases such as chymase have recently been reported to be involved in the proliferation of airway smooth muscle cells [100].

Matrix metallo-proteases (MMPs) are zinc-dependent endopeptidases, a major group of proteases known to regulate extra-cellular matrix (ECM) turnover and therefore they have been suggested to be important in the process of lung disease associated with tissue remodelling. The MMP family can be classified into distinct sub-classes: collagenases (MMP-l, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), membrane-type MMP (MMP-14 to MMP-25), matrilysin (MMP-7), and macrophage metallo-elastase (MMP-12).

Most studies on airway proteolytic enzymes have focused on serine proteases (neutrophil elastase, protease 3 and cathepsin G) and on MMPs (matrix metallo-proteases; MMP-2 is referred to as gelatinase A, MMP-9 as gelatinase B and MMP-12 as macrophage metallo-elastase) [101]. However, much less is known about the role and activity of papain-like, cysteine proteases, such as cathepsins B, H, K, L and S [102, 103]. Lung cathepsins B, K, L and S are expressed intra-cellularly by resident macrophages, fibroblasts and epithelial cells [104, 105], while cathepsin H is mostly in type II pneumocytes [106, 107]. It has been shown that cigarette smoking causes macrophages to release cathepsins B and S [108], and cathepsin L activity has been found in the BAL fluid of smokers [109]. It has been proposed that cathepsins K, L and S, like neutrophil elastase and MMPs, may be involved in the extra-cellular degradation and/or remodelling of the ECM [102, 110-112]. Recently Bühling and collaborators have proposed that cathepsin K is critical for lung matrix homoeostasis [86].

Proteases	Name	Protease inhibitor
Serine proteases	Neutrophil elastase Cathepsin G Protease 3 Trysin Chymotrypsin Tissue kallikrein Urokinase-type plasminogen activator	α1-antitrypsin (AAT) α1-antichymotrypsin (ACT) Secretory Leukocyte Protease Inhibitor (SLPI) Elafin Plasminogen activator inhibitors α_2- macroglobulin
Cysteine proteases	Cathepsin B, C, H, K, L, S Caspases	Cystatin A – F Kininogens α_2-macroglobulin
Metallo-proteases	MMP-1 MMP-3 MMP-7, -8, -9, -10 MMP-12 MMP-13 Collagenase-1 macrophage metalloelastase Stromelysin-1	α_2-macroglobulin Tissue inhibitors of metalloproteases (TIMP)
Aspartic proteases	Cathepsin D, E Napsin A Pepsinogen C (gastricin)	Pepstatin

Table 1. Proteases and protease-inhibitor in the airways.

Anti-proteases

To contain the potential injurious effects of proteases, the anti-proteases developed in a parallel network consisting of "alarm" and "systemic" inhibitors. The latter is synthesised mainly in the liver, among them α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin, plasminogen activator inhibitor-1 and others [25]. The former group includes members of the anti-leukoprotease family, known as secretory leucocyte protease inhibitor (SLPI), and elastase-specific inhibitor, also known as elafin or skin-derived anti-leukoprotease (SKALP). These inhibitors are synthesized and secreted locally at the sites of injury in response to primary cytokines such a TNF-α and IL-1, and therefore they are considered to be a first wave of local, inducible defence in the anti-protease network [113]. The inhibitors of the MMPs are α-2 macroglobulin and the family of specific tissue inhibitors of MMPs (TIMPs)

[114]. Similarly, cysteine protease activity is regulated by their natural inhibitors of the cystatin superfamily including both extra-cellular cystatin C and kininogens [115, 116].

Under normal conditions, anti-proteases prevent the deleterious effects of proteases, as they are present in higher concentrations than the proteases, providing an “anti-protease screen”. An increased protease activity implies either increased production or activity of proteases, or decreased production of anti-proteases. Thus, when the protease concentration in the local milieu overwhelms the local anti-protease protective screen this leads to an excessive extra-cellular activity and tissue damage [117].

2. Specific background

2.1 Secretory leukocyte protease inhibitor (SLPI)

Protein, structure and biological activity

SLPI is one of the two members of the anti-leukoprotease superfamily of protease inhibitors (the other being ESI/elafin) and is a highly basic (pH > 9.5), acid-stable but alkaline-labile protein [118]. SLPI is an 11.7-kDa, non-glycosylated protein consisting of 107 amino acid residues and comprising two domains. It contains 16 cysteine residues that form eight disulphide bridges [119] (Figure 2).

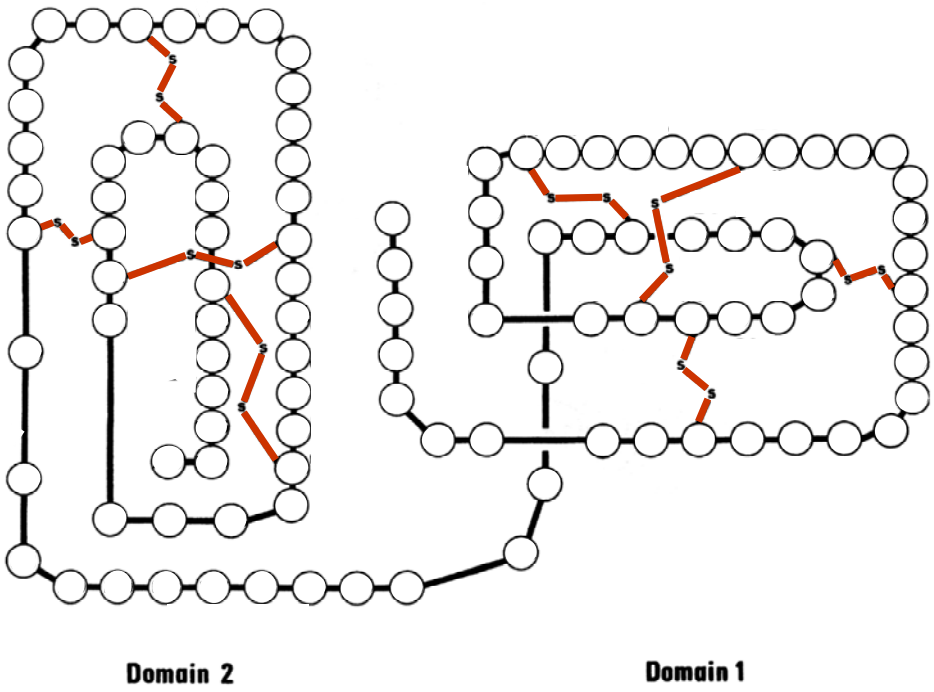

Figure 2. Schematic presentation of the SLPI protein, modified from Grutter et al, 1988 [119]. *(Disulphide-bridges are marked in red).*

SLPI, through its C terminal domain, is a serine protease inhibitor and provides significant protection against neutrophil elastase. Studies targeted at the physiological role of SLPI in the normal lung suggest that SLPI provides the major anti-elastase protective screen at the epithelial surface of the upper respiratory tract [120, 121]. SLPI also inhibits the serine

protease cathepsin G, trypsin, chymotrypsin and chymase as part of its anti-protease effects [122-124]. Furthermore, SLPI inhibits the activity of the elastin-bound elastase [125].

In addition to its potent anti-protease activity, SLPI has important anti-bacterial, anti-viral and anti-inflammatory properties. The N-terminal domain of SLPI has modest anti-microbial activity *in vitro* against gram-negative and gram-positive bacteria [126]. SLPI has been shown to inhibit human immunodeficiency virus (HIV) infectivity of monocytes by blocking viral DNA synthesis [127-129]. Saliva is rich in SLPI and it is suggested that this accounts for the low viral transmission rates via saliva [127, 130]. In addition to its anti-protease activity, SLPI has been shown to exhibit anti-inflammatory properties, including down-regulation of TNF-α expression by lipopolysaccharide (LPS) in macrophages and inhibition of nuclear factor (NF)-κB activation in a rat model of acute lung injury [131]. SLPI has recently been shown to enter cells, becoming rapidly localized at the cytoplasm and nucleus where it affects NF-κB activation by binding directly to NF-κB binding sites in a site-specific manner [132]. It has also been found that pre-treatment with SLPI greatly reduces inflammation in both liver and lungs in a mouse model of hepatic ischaemia/reperfusion injury [133].

However the anti-inflammatory and anti-elastase effects of SLPI can be diminished by oxidation or cleavage [134]. SLPI is susceptible to protease cleavage. Cathepsins B, L, and S have been shown to cleave and inactivate SLPI. For example, analysis of epithelial lining fluid samples from individuals with emphysema indicated the presence of active cathepsin L and cleaved SLPI [135].

Distribution in cells and tissue

Expression of the SLPI gene has been examined in superficial epithelial cells from human airways [136-138]. Several studies have demonstrated that SLPI is expressed in the serous cells of tracheal and bronchial submucosal glands [27, 139, 140], the Clara and goblet cells of the bronchiolar and bronchial lining epithelium [141, 142], and in the serous cells of human nasal mucosa [143]. So far, no gene polymorphisms have been reported for SLPI.

SLPI has been purified from different sources, including parotid, cervical, seminal and lung secretion [144]. SLPI is the third most abundant, innate, immunity protein of respiratory secretions after lysozyme and lactoferrin. It is estimated that SLPI is present at concentrations of 0.1 to 2 μg/ml in airway lavage fluid [145, 146] and 2.5 μg/ml in nasal secretions [147, 148]. It is also found in salivary glands [149] and middle ear secretions [150, 151], cervical

mucosa [152] and vesicular seminalis [153]. Complexes of SLPI with elastase have been found in purulent bronchial secretions [154].

2.2 α1-antitrypsin (AAT)

Protein, structure and biological activity

α1-antitrypsin (AAT), also referred to as α_1-protease inhibitor, is the most abundant serine protease inhibitor in human plasma. AAT consists of a single polypeptide chain of 394 amino acid residues, molecular weight about 52 000 Da, containing one free cysteine residue, no disulphide bridges, and three asparagine-linked carbohydrate side-chains [155, 156]. AAT belongs to the serpin (serine protease inhibitor) - super-family of proteins, whose membership is based on the presence of a single common core domain consisting of three β-sheets and 8-9 α-helices, and a set of highly unusual structural and functional properties that result from the presence of this core domain. Mechanistically, AAT, like other serpins, have an exposed polypeptide segment, the reactive site loop, which is susceptible to protease attack. Cleavage of the scissile bond in the loop of most serpins results in a large conformational change in which the reactive site loop migrates and is inserted into a pre-existing β-sheet. These major structural changes are necessary for the formation of the stable protease–serpin inhibition complex (Figures 3 and 4).

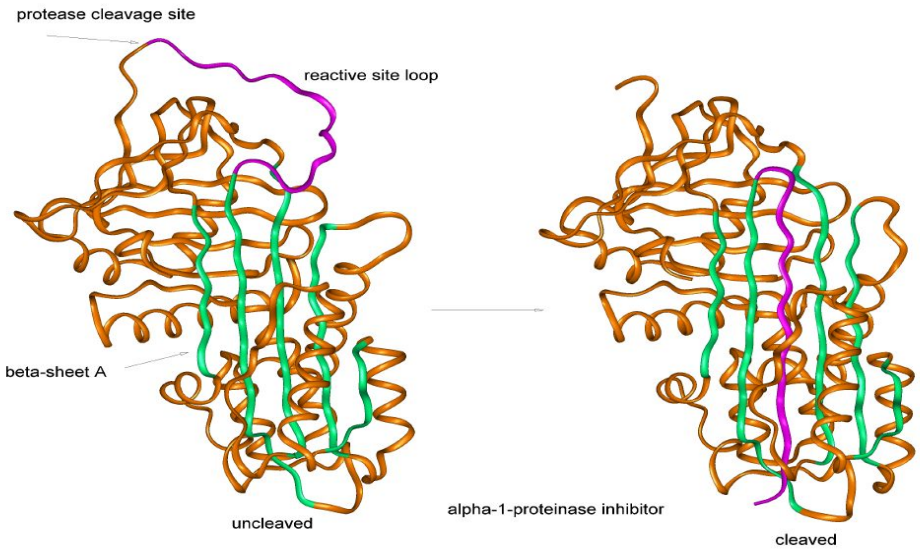

Figure 3. Proteolytical cleavage of AAT results in a large conformational change in which the reactive site loop migrates and is inserted into a pre-existing β-sheet.

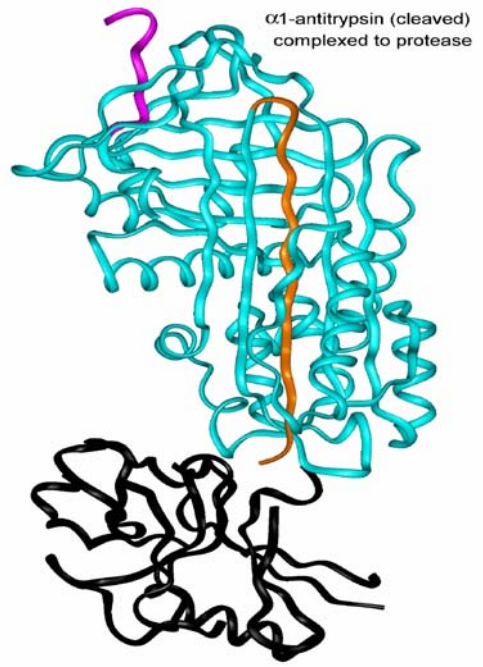

Figure 4. Formation of a stable protease-serpin inhibition complex.

Until recently, neutralization of serine protease activity, particularly neutrophil elastase and protease 3, which are up-regulated during the inflammatory response, was assumed to be the primary function of AAT [157]. For example, AAT controls approximately 90 % of neutrophil elastase activity [158]. It also inhibits pancreatic serine proteases such as pancreatic elastase, trypsin, chymotrypsin, kallikrein, thrombin and plasmin.

AAT increases rapidly to concentrations exceeding three- to four-fold above the normal (average 1.34 mg/ml) during inflammation, infection and malignant diseases [159]. Alterations of the AAT molecule that compromise its structure and/or secretion and thereby reduce its concentrations, are known to predispose the individual to inflammatory disease, including emphysema, liver disease, panniculitis and vasculitis [160]. Inherited, severe AAT deficiency (PiZ deficiency) is the only proven genetic risk factor for the development of COPD [161]. Asthma is also common in patients with AAT deficiency and is usually associated with COPD [162].

Distribution in cells and tissue

AAT is mainly produced in liver parenchyma cells. To a lesser extent, AAT is synthesized by blood monocytes, macrophages, pulmonary alveolar cells, intestinal epithelial cells and the cornea [163-165]. The normal daily rate of synthesis is approximately 34 mg/kg body weight, leading to a plasma concentration ranging from 0.9 - 1.75 mg/ml, with a half-life of 3 to 5 days. AAT is present in various biological fluids, including saliva, tears, milk, semen, and bile [166]. The distribution of the protein in the tissues is not uniform, for example, AAT diffuses through endothelial and epithelial cell walls and is shown to be present in the epithelial lining fluid at concentrations of 10 to 15 % of total serum AAT [167].

As an acute-phase reactant, tissue and circulating AAT levels increase within hours (3- to 4-fold above normal) in response to inflammation or infection [159] while, for example, local levels of AAT have been shown to increase up to 11-fold [168]. Human neutrophils, monocytes and alveolar macrophages can increase the expression of AAT in response to inflammatory mediators, such as cytokines (IL-6, IL-1 and TNF-α) and endotoxins [169].

There is growing support that AAT, in addition to its anti-protease activity, may have other functional activities. For example, AAT has been demonstrated to stimulate fibroblast proliferation and procollagen synthesis, to up-regulate human B cell differentiation into IgE- and IgG4-secreting cells [170], to interact with the proteolytic cascade of enzymes involved in apoptosis [171] and to express contrasting effects on the post-transcriptional regulation of iron between erythroid and monocytic cells [172]. AAT is also known to inhibit neutrophil superoxide production, induce macrophage-derived interleukin-1 receptor antagonist release [173] and reduce bacterial endotoxin and TNF-α-induced lethality *in vivo* [174].

3. Aims

The overall aim of these studies was to extend our knowledge on the biological role of the serine protease inhibitors, Secretory Leukocyte Protease Inhibitor (SLPI) and α1-antitrypsin (AAT). The investigations were focused on the anti-inflammatory activities of SLPI and AAT and their quantitative measure in health and disease. The specific aims of the present investigation were:

In cell culture models:

1. To elucidate the connection between mast-epithelial cell interaction and SLPI expression and release.
2. To determine the effects of α1-antitrypsin on endotoxin (LPS)-stimulated human primary monocyte and neutrophil cytokine and chemokine release.
3. To study SLPI effects on human monocytes and neutrophil IL-8 release, adhesion, chemotaxis and apoptosis.

In human biological fluids:

1. To measure SLPI and IL-8 concentrations in nasal lavage of healthy subjects exposed to endotoxin (LPS).
2. To quantify plasma levels of α1-antitrypsin, α1-antichymotrypsin and SLPI in asymptomatic and COPD cases with wild type MM and deficiency ZZ and SZ α1-antitrypsin.
3. To analyse plasma and BAL fluid levels of α1-antitrypsin, SLPI and inflammatory biomarkers in patients with asthma and COPD.

4. Materials

4.1 Cells and cell cultures

Cell lines (study I)

Human mast cells HMC-1, established from a patient with mast cell leukaemia, were obtained from Dr. J.H. Butterfield, Mayo Clinic, Rochester, MN, USA. The cells were cultured in Dulbecco's medium supplemented with 10 % iron-supplemented fetal calf serum, 1.2 mM α-thioglycerol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B and fungizone, at 37 °C with 5 % CO_2.

A type-II alveolar cell line (A549, CCL 185^{TM}, ATCC), established in 1972 from human lung carcinoma, was cultured in a RPMI-1640 medium supplemented with 10 % fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B and fungizone, at 37 °C with 5 % CO_2.

Human primary monocytes (studies II and III)

Human blood monocytes were isolated from buffy coats using Ficoll-Paque PLUS (Pharmacia, Sweden). Briefly, buffy coats were diluted 1:2 in PBS with addition of 10 mM EDTA and layered on Ficoll. After centrifugation at 400 *g* for 35 min at room temperature, the cells in the interface were collected and washed with PBS-EDTA. Cell purity and amounts were determined in an Autocounter AC900EO cell counter (Swelabs Instruments AB, Sweden). The granulocyte fractions were less than 5 %. Cells were seeded in RPMI 1640 medium supplemented with penicillin 100 IU/ml; streptomycin 100 μg/ml; non-essential amino acids 1x; sodium pyruvate 2 mM and HEPES 20 mM (Gibco, UK). After 1h 15 min, non-adherent cells were removed by washing with PBS supplemented with calcium and magnesium. Fresh medium was added and the cells were used for the experiments.

Human neutrophils (studies II and III)

Neutrophils were isolated from the peripheral blood of healthy donors using Polymorphprep^{TM} (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturers'

recommendations. Neutrophils were harvested as the lower cellular band above the red cell pellet and washed by centrifugation with PBS. Residual erythrocytes were removed by a hypotonic lysis using ice-cold 0.2 % NaCl for 30 s, followed by the addition of an equal volume of 1.6 % NaCl to restore isotonicity. Purified neutrophils were washed in PBS and then re-suspended in RPMI-1640 containing glutamax. The neutrophil purity was typically 90 % as determined by an AC900EO AutoCounter (Swelab Instruments, Sweden) and cell viability exceeded 95 % according to trypan blue staining.

4.2 Clinical material

Nasal lavage (studies II and III)

The procedure for nasal lavage was performed according to a method described by Wihl and co-workers [175]. With a 60 ml syringe to which an olive was adapted, 8 ml of saline was used for the lavage of each nostril. To prevent lavage spilling into the throat, the subject was bent forward at an angle of 60° during the procedure. Equilibrium was maintained between the mucosal lining and the lavage fluid by injecting the saline gently into the nasal cavity and drawing it five times back into the syringe. The lavage was performed in both nostrils and samples were collected in a test tube, centrifuged at 1750 rpm, at 6 °C for 10 min and immediately frozen at - 80 °C.

The Regional Bioethics Committee approved of the study, and written informed consent was received from all participants.

Bronchial lavage fluid (study IV)

To perform BAL, the local upper airways were anaesthetised with 5 ml of 2 % lidocaine (Grindex, Latvia). The bronchoscope (Olympus, USA) was wedged into the segmental bronchus of the middle lobe and 100 ml of sterile saline solution was infused. Fluid was gently aspirated immediately after the infusion had been completed and was collected in a sterile container, immediately filtered using 48 μm nylon gauze (Milipore, North Ryde, NSW, Australia) and centrifuged at - 4 °C for 10 minutes. The supernatants were removed and frozen at -70 °C.

The Regional Bioethics Committee approved of the study, and written informed consent was received from all participants.

Plasma (studies IV and V)

Blood was collected in tubes containing EDTA (B-D Vaccutainer System, Franklin Lakes, N.J., USA), and centrifuged at 2000 *g* at 4 °C for 10 min. The aliquots of plasma were immediately frozen at -80 °C and stored until assayed.

4.3 Specific reagents

Proteins

Purified recombinant Secretory Leukocyte Protease Inhibitor (SLPI) was obtained from the Otorhinolaryngology Department, Malmö University Hospital, Malmö, Sweden. The quality of SLPI was confirmed by 12 % SDS-PAGE electrophoresis under reducing conditions (Figure 5).

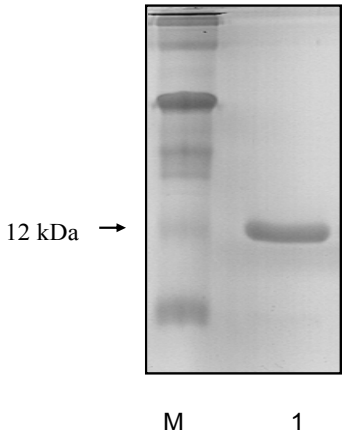

Figure 5. 12 % SDS-PAGE; M: Molecular size markers (M - 204.5, 130.7, 89.9, 37.8, 31.5, 17.7 and 6.7 kDa); 1: SLPI (2 µg/well).

Anti-elastase activity was assessed spectrophotometrically (Spectrophotometer DU 600, Beckman). SLPI was incubated with pancreatic elastase at a 25:1 molar ratio for 5 min at room temperature, in 0.1 M Tris buffer at pH 8. After addition of chromogenic elastase

substrate (SAPNA, succinyl-(Ala)3-*p*-nitroanilide), the absorbance was measured at 405 nm, for 300 seconds. SLPI (23.4 μg/ml) inhibited 75 % of the elastase (1 μg/ml) activity.

Purified human plasma alpha1-antitrypsin (AAT) was obtained from the Department of Clinical Chemistry, Malmö University Hospital, Sweden and from Calbiochem (USA). The purity of the AAT preparations was > 97 % and their inhibitory activity > 75 %. Native AAT was diluted in PBS at pH 7.4. To ensure the removal of endotoxins, AAT was subjected to Detoxi-Gel AffinityPak columns according to instructions from the manufacturer (Pierce, IL, USA). Purified batches of AAT were then tested for endotoxin contamination with the Limulus amebocyte lysate endochrome kit (Charles River Endosafe, SC, USA). Endotoxin levels were less than 0.2 enzyme units/mg protein in all preparations used.
Polymeric AAT was produced by incubating native AAT at 60 °C for 10 hours. Polymers were confirmed on non-denaturing 7.5 % PAGE gels (Figure 6A), and native AAT was analysed on 7.5 % SDS-PAGE (Figure 6B).

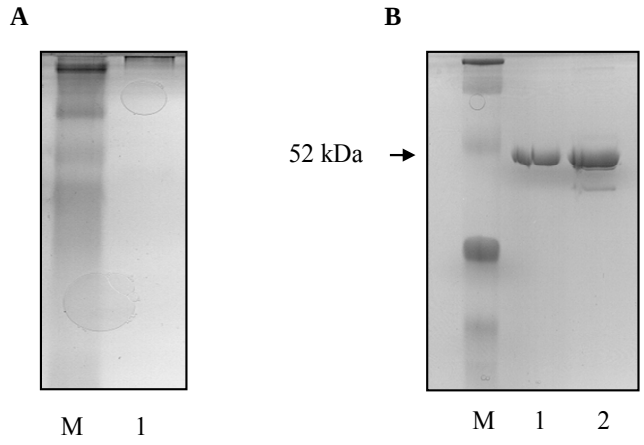

Figure 6. Polymeric (A) and native (B).
***A.** Non-denaturing 7.5 % PAGE gel, M: Molecular size markers (M - 204.5, 130.7, 89.9, 37.8, 31.5, 17.7 and 6.7 kDa); lane 1: Polymerized AAT (2 μg/well).*
***B:** SDS-PAGE 7.5 % gel, M: molecular marker as described in A; lane 1: Native AAT (2 μg/well), lane 2: Prolastin (2 μg/well).*

Prolastin®, purified and pooled human AAT, was a gift from Bayer Corporation (Bayer Corporation, Elkhart, IN, USA, Lot nr. 26 N3 PT2). This vial of Prolastin contained 1059 mg of functionally active AAT, as determined by its capacity to inhibit porcine pancreatic elastase.

Antibodies

Polyclonal goat anti-SLPI antibody was obtained from the Department of Histopathology, Malmö University Hospital;

Monoclonal mouse anti-tryptase (MAB 1254), polyclonal rabbit anti-human AAT (A 0022), polyclonal rabbit anti-human ACT (A 0012), peroxidase-Conjugated rabbit anti-goat immunoglobulins (Lot 038), non-immunized mouse IgG, Biotinylated rabbit anti-goat IgG antibody, avidin DH biotinylated horseradish peroxidase (P 0364) were obtained from (DAKO, Glostrup, Denmark).

Monoclonal mouse anti-human mast cell 229 kindey juxtaglomerular CE were procured from Swant, Switzerland and anti-human SLPI antibody (AF 1274) were purchased from R&D Systems, England.

Pro-inflammatory agents

Lipopolysaccharide (product number L 2654, 95 % purity, Sigma-Aldrich), a major constituent of the wall of most gram-negative bacteria. It is a highly immunogenic antigen with the ability to enhance immune responses to soluble antigens.

Zymosan (product number 97340 Sigma-Aldric), an insoluble polysaccharide fraction of yeast cell walls, used in assay of phagocytosis.

fMLP (N-formyl-Met-Leu-Phe, product number F3506, purity ≥ 97 %, Sigma-Aldrich), inducer of leucocyte chemotaxis and macrophage activator. Receptors that bind formyl-peptides are found on phagocytic neutrophils and have recently been identified on cells of the intestinal mucosa.

Leukotriene B_4 (product number L0517, purity ≥ 97 %, Sigma-Aldrich), stimulates chemotaxis, aggregation and degranulation of polymorphonuclear leukocytes.

Other reagents

Porcine pancreatic elastase (E0127, Sigma-Aldrich), enzyme.

Fibronectin (product number F 2518 Sigma-Aldrich), is an adhesion glycoprotein of the extracellular matrix.

Cytochalacin B (product number C6762, purity > 98 %, Sigma-Aldrich), cell permeable fungal toxin that disrupts contractile microfilaments by inhibiting actin polymerization.

Calcein, AM (product number C3100 MP Invitrogen), a cell-permeable dye that can be used to determine cell viability in most eukaryotic cells. In live cells the non-fluorescent calcein AM is converted to green-fluorescent calcein, after acetoxymethyl ester hydrolysis by intra-cellular esterases.

5. Experimental Procedures

Cell co-culture (study I)

A transmigration model was used in which mast cells HMC-1 migrated across the top of the transwell filter (pore size 3 µm) towards the type II alveolar cell line, A549 (Figure 7), for predetermined durations (24, 48, 72 and 96 hours).

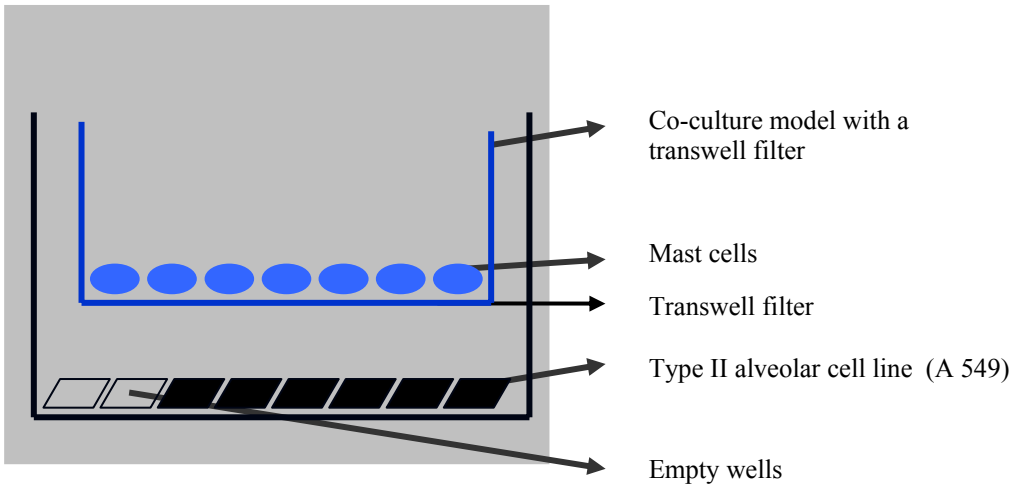

Figure 7. Cell co-culture.

Chemotaxis (study III)

Neutrophil Chemotaxis was performed in NeuroProbe, 96-well, disposable, chemotaxis chambers fitted with 3 µm diameter pore, polycarbonate filters. A suspension of neutrophils was placed in the upper wells, and test substances or medium alone were placed in the lower wells. The chamber was then incubated in a 5 % CO_2 atmosphere at 37 ºC for 75 minutes. Neutrophil Chemotaxis was analyzed by microscopy and confirmed by determining cell-associated myeloperoxidase activity in migrated cells.

Neutrophil adherence (study III)

The adherence of calcein-labelled neutrophils to fibronectin was measured according to Howard et al. [176]. Purified neutrophils were labelled with calcein-AM for 25 minutes at 37 ºC and re-suspended in RPMI-1640-Glutamax-1 medium and 0.1 % (W/v) BSA. Aliquots of cells were added to the fibronectin-coated 96-well plates containing medium alone or test

proteins. The plates were then incubated at 37 ºC for 25 minutes under static conditions. Adherent cells were analyzed with a fluorescence spectrophotometer, excitation of 485 nm and an emission of 520 nm.

Neutrophil apoptosis (study III)

Purified neutrophils were re-suspended in RPMI medium containing 10 % FCS for 2, 6 and 24 hours. Three or four smears (each made of 3 µl cell-suspension) were made from each sample using a "Hemaprep ™" (Blood smearing instrument for *in vitro* diagnostic J.P. Gilbert Co, USA). The slides were dried in air, stained with May-Grünwald/Giemsa and the number of apoptotic cells counted under light microscopy (x 400). Neutrophils undergo apoptosis spontaneously during *in vitro* incubation, therefore, to evaluate apoptosis induced by SLPI or positive control, we calculated the specific percentage of apoptotic cells as follows:

[% apoptosis of the treated neutrophils] -

[% spontaneous apoptosis] / (100 % - % spontaneous apoptosis) x 100

Immuno-histochemistry (study I)

To detect SLPI in mast cells, we used primary goat antibody against SLPI, and the secondary biotinylated rabbit anti-goat IgG antibody. Finally a DH biotinylated horseradish peroxidase (ABC) complex was added. Visualisation of the immuno reaction was accomplished by addition of the diaminobenzidine tetrahydrochloride (DAB). To monitor the differentiation, the mast cells were stained for tryptase using a monoclonal mouse anti-human tryptase and secondary labelled anti-mouse antibodies. Nitrotetrazolium/bromochloridolylphosphate (NBT/BCIP) was used as a substrate. In control experiments the specific antibodies were used after pre-incubation with SLPI or tryptase.

Quantitative Enzyme Linked Immunosorbant Assay (ELISA) (studies II, III, IV and V)

Commercially available ELISA kits were used to determine the concentrations of SLPI and the pro-inflammatory cytokines TNF-α, IL-8, IL-1β in biological fluids and cell culture supernatants. The minimum detectable concentration for SLPI was 62.5 pg/ml, for TNF-α - 15.6 pg/ml, for IL-8 - 31.2 pg/ml and for IL-1β -15.6 pg/ml.

Immunoelectrophoresis (studies IV and V)

Plasma levels of ACT and AAT were determined using rocket-immuno electrophoresis as described by Laurell CB [177, 178]. Briefly, aliquots of plasma were run for 1.5 h at 200 V on 1 % agarose gels containing anti-human AAT or anti-human ACT antibodies. Gels were pressed between filter paper and dried before staining with Coomassie Blue. To quantify AAT and ACT, the distance between the tip of the rocket-shaped immuno-precipitates and the application well, were measured. Standard curves were generated by serial dilutions of a standard (Seronorm, Sero AS, Norway) run in parallel with the samples on every gel. The coefficient of variation percentage (% CV) for the inter- and intra-batch variability was 7.9 % and 5.8 %, respectively.

Electrophoresis and Western blot (studies I, II and III)

Proteins were separated on 7.5 % or 12 % homogenous native or SDS-PAGE gels. Gels were stained with Coomassie Blue or transferred to a polyvinylidene difluoride membrane (Millipore, Millipore Corporation, Bedford, MA 01730) using a semi-dry blot electrophoretic transfer system. Western blot was performed using specific antibodies. The immuno-complexes were visualised with secondary horseradish peroxidase-conjugated antibodies and developed with the ECL Western Blot analysis system (Amersham, UK) or substrate 3,3'-diaminobenzidine.

mRNA extraction (study I)

Total RNA was isolated and quantified from A549 cells cultured alone and in co-culture. The cells were lysed, total RNA was extracted using a single-step method based on the acid-guanidinium thiocyanate-phenol-chloroform extraction described by Chomoczynski and Sacchi [179]. The total RNA yield was quantified at 260 and 280 nm. The transcript levels of SLPI and β-actin were evaluated by slot blot, using a Magnagraph nylon membrane according to the manufacturer's instructions [180].

Protein determination

Protein concentrations were determined using the Coomassie® Plus assay reagent kit (PIERCE Biotechnology, USA) according to manufacturer's recommendations.

6. Statistical analysis

The statistics were performed using either parametric or non-parametric methods, depending on whether the data had a normal distribution. Results are presented as mean ± standard error of mean (SEM) values, and N equals the number of subjects or experiments.
The Statistical Package for Social Sciences (SPSS for Windows, release 11) was used for the statistical calculations.

7. Results

7.1 Regulation of SLPI production (studies I and III)

Background

Secretory Leukocyte Protease Inhibitor (SLPI) is produced locally in serous cells of the sub-mucosal glands, by non-ciliated cells of the bronchial epithelium, by neutrophils [181] and peritoneal macrophages [182]. Little is known concerning the regulation of SLPI expression and release in the airways. It has been shown that SLPI is up-regulated by pro-inflammatory stimuli including lipopolysaccharide (LPS), TNF-α, IL-6 and IL-1β *in vitro* [113, 183]. Neutrophil elastase has also been found to increase SLPI transcript levels in primary and transformed human airway epithelial cells in a time- and dose-dependent manner [113, 184]. These observations suggest that the sensitive regulation of SLPI, in relation to local levels of proteases, may play an important role in minimising tissue destruction.

Aims

To investigate the effects of the interaction between mast cells and epithelial cells on SLPI release and expression (study I) and to measure the levels of SLPI in nasal lavage fluid obtained from LPS-challenged, healthy individuals (study III).

Results and Discussion

The finding that mast cells are SLPI-positive strengthens earlier observations showing that SLPI and other chymase inhibitors, i.e. α1-antichymotrypsin and α1-antitrypsin, are present in stimulated mast cells [185]. With this as a background we have further investigated the effects of mast cells on the property of the epithelial-presenting cell, A549, to express and release SLPI. To our surprise we found that epithelial cell co-culture with mast cells results in a gradual, time-dependent decrease of the amount of SLPI in the co-culture medium. However although, SLPI was decreased at the protein level, the SLPI mRNA in epithelial cells was up-regulated. It is important to note that the mast cell-conditioned medium had no influence either on SLPI protein levels or on SLPI mRNA expression in epithelial cells. Thus, mast cells have an effect on SLPI levels only when they are in close contact with SLPI-producing epithelial cells.

It has been shown that neutrophil elastase increases SLPI mRNA expression, while it decreases SLPI protein release, *in vitro* [186]. In addition, studies by Hill and co-workers, have found that SLPI concentration does not decrease until the elastase activity of the samples is in excess of 50 nM [187]. The relationship between SLPI and elastase therefore seems not to be linear, indicating that a certain amount of elastase and/or other enzymes is needed in order to induce epithelial damage or interfere with epithelial cell metabolism which would result in a decreased SLPI secretion and/or increased degradation.
SLPI levels have been found to be decreased in nasal secretion after antigen challenge *in vivo* [188] and also in bronchial alveolar lavage obtained from asthmatics compared to healthy subjects [189]. In study III we have quantified SLPI in nasal lavage fluids obtained from healthy volunteers before and after a single dose of endotoxin (LPS) challenge. We found that LPS-induced nasal provocation results in a significant elevation of SLPI levels.

Conclusion
A better understanding of the mechanisms involved in SLPI protein expression, release and consumption may provide important knowledge regarding the dynamics of the regulation of the protease/anti-protease systems *in vivo.*

7.2 Biological activities of SLPI and AAT (Prolastin®) (studies II and III)

Background
The production of pro-inflammatory molecular species, such as free radicals, cytokines, chemokines and proteases in the inflammatory environment usually occurs due to the presence of a combination of sources, including bacterial by-products, complement factors and inflammatory and non-inflammatory cells (Figure 8).

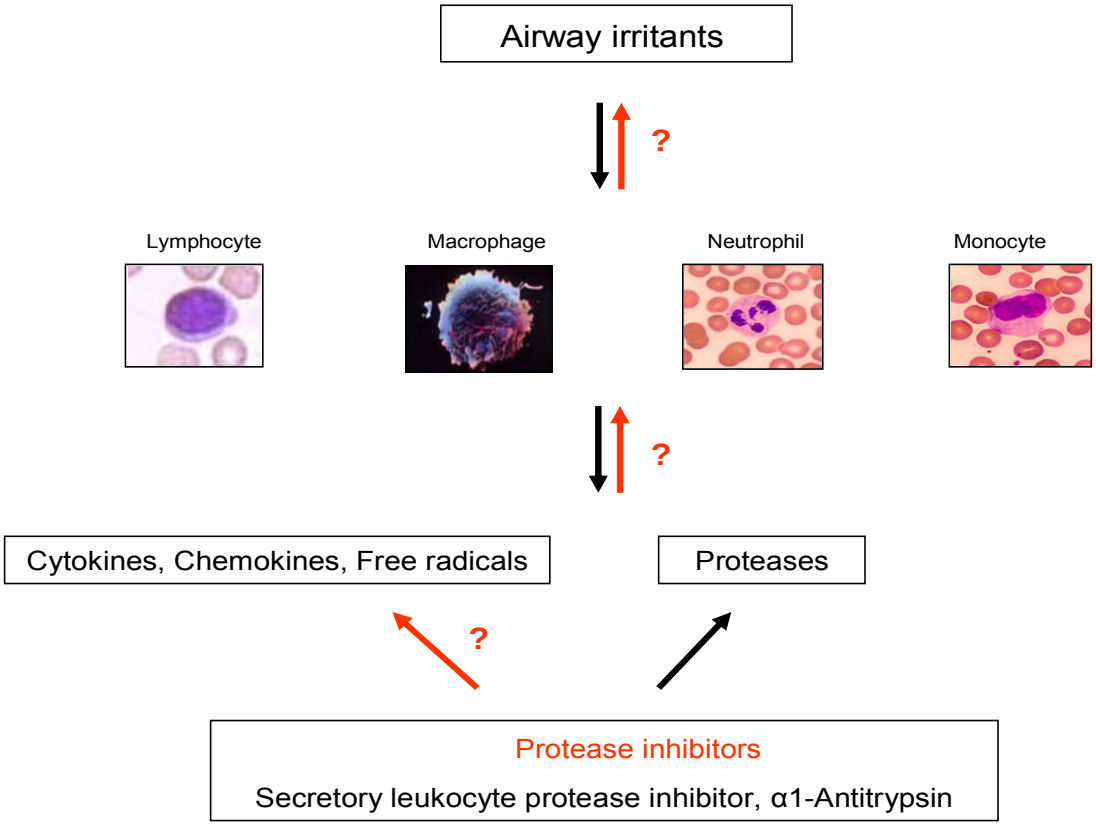

Figure 8. Roles of Secretory leukocyte protease inhibitor and α1-antitrypsin in airway inflammation.

α1-antitrypsin (AAT) is one of the major inhibitors of serine proteases, both in the circulation and in tissue. As such, the protective effects of AAT are generally attributed to the inhibition of destructive proteolytic processes during inflammation. Recent observations, however, suggest that the function of AAT may extend beyond its inhibitory activity, with down-regulation of inflammatory cell activation via receptor-mediated events. Secretory leukocyte protease inhibitor (SLPI) also appears to act more than just as an anti-protease, but rather as a molecule with broader anti-inflammatory properties (Figure 8). Recent scientific evidence suggests that SLPI may have a broad spectrum of antibiotic activities that includes anti-retroviral, bactericidal, and anti-fungal activity. SLPI plays a role in limiting elastase-induced pulmonary inflammation and may therefore act as a complement to AAT. Various functional activities of AAT and SLPI are presented in tables 2 and 3.

Functional activities of Secretory Leukocyte Protease Inhibitor	Reference
Binds to NF-κB binding sites in monocytes and inhibits p65 binding	[132]
Down-regulates prostaglandin, and matrix metalloprotease synthesis by monocytes	[190]
Inhibits HIV-1 replication in cultured human monocytes	[127-129, 191]
Expresses anti-microbial activity *in vitro* against Gram-negative and Gram-positive bacteria	[126]
Suppresses macrophage responses to bacterial lipopolysaccharide (LPS)	[131, 192]
Reduces inflammation in both liver and lungs in a mouse model of hepatic ischaemia/reperfusion injury	[133]
Inhibits HIV-1 activity in various mucosal secretions	[193]
Decreases the liver-metastasising potential of carcinoma cells	[194]
Promotes wound healing	[195]
Inhibits joint damage and inflammation	[196]
Reduces viral transmission rates via saliva	[130]
Pro-malignant activity	[197]

Table 2. Activities of Secretory Leukocyte Protease Inhibitor (SLPI).

Functional activities of α1-antitrypsin	Reference
Modulates neutrophil Elastase-mediated phosphatidylserine receptor cleavage which is necessary for the recognition and clearance of apoptotic neutrophils	[198]
Blocks the cytotoxicity of neutrophil defensins and their ability to up-regulate IL-8 production	[199]
Acts as an antioxidant	[200]
Acts as an immunoregulator	[201]
Stimulates fibroblast proliferation and extracellular matrix production	[202]
Binds to secreted enteropathogenic *Escherichia coli* proteins EspB and EspD and reduces their mediated hemolysis of red blood cells	[203]
S-nitrosylated AAT exhibits growth of Gram-positive and -negative bacteria	[204]
Inhibits neutrophil activation	[205]
Inhibits LPS-induced Cytokine and Chemokine Release from human monocytes	[206]
Inhibits Moraxella catarrhalis MID protein-induced Tonsillar B-cell Proliferation and IL-6 release	[207]
Inhibits angiogenesis and tumour growth	[208]
Protects mice from TNF-α or LPS-induced lethality	[209]
Inhibits both matrix degradation and connective tissue breakdown, in C57-BL/6 mice models	[210]
Prevents the PMN inflammatory influx and connective tissue breakdown in a mouse model of acute silica-induced inflammation	[173]
Ameliorates cigarette smoke-induced emphysema in mice	[211]
Prolongs islet allograft survival in mice	[212]
Modulates cellular immunity and prevents type 1 diabetes in non-obese diabetic mice	[213]
Expresses anti-apoptotic activity	[214]
Suppresses bacterial proliferation in a rat model of chronic *Pseudomonas aeruginosa* lung infection	[215]
Reduces the incidence of lung infections in patients with emphysema related to AAT deficiency	[216]
Interacts with Cryptosporidium parvum and inhibits C. parvum infection	[217, 218]

Table 3. Activities of α1-Antitrypsin (AAT).

Aims

To study the biological activities of SLPI and AAT in LPS-activated monocyte and neutrophil models *in vitro*, and to investigate AAT (Prolastin®) effects on nasal IL-8 release in LPS-challenged individuals, *in vivo*.

Results and Discussion

Findings from study II provide *in vitro* evidence that native AAT (inhibitor) and a modified (non-inhibitory i.e. temperature inactivated) form of AAT can block the release of an array of chemokine and cytokines from LPS-stimulated monocytes. Similarly, Prolastin®, a preparation of human AAT which is used for augmentation therapy, significantly inhibits endotoxin-induced IL-1β and TNF-α release by monocytes and IL-8 release by neutrophils *in vitro*. To explore these findings we examined whether Prolastin® inhibits responses to LPS in the nasal airway, *in vivo*. Our results show that instilled, defined amounts of endotoxin (25 μg/per nostril) induce time-dependent nasal IL-8 release in normal subjects. However, 30 min after challenge with LPS, Prolastin® (2.5 mg/per nostril) is instilled and no induction of nasal IL-8 release is found over the duration of the study. We were not able to determine from these experiments whether Prolastin® is directly suppressing IL-8 release or suppressing another inflammatory response that leads to IL-8 release. Our finding suggests that effects of Prolastin directed against endotoxin-stimulated inflammatory responses may be beneficial.

In study III we evaluated the effects of SLPI on chemokine, IL-8 release, chemotaxis, adhesion and apoptosis in neutrophil and monocyte models, *in vitro*. We found that SLPI has no significant effect on IL-8 release from LPS-stimulated neutrophils either at a short (2 and 6 h) or at the long (24 h) cell incubation. However, within 2 and 6 h, monocyte exposure to LPS, led to the remarkable induction of IL-8 release while LPS-stimulated IL-8 release was strongly diminished in the presence of SLPI. SLPI was without effect after 24 h, implying that SLPI delays chemotactic cytokine, IL-8, release from stimulated monocytes rather than inhibits its production.

Most interestingly we found that SLPI itself elicits the chemotaxis and adhesion of neutrophils to fibronectin, *in vitro*. Although chemotactic effects of SLPI were lower in magnitude compared to LTB4, they may still be of great physiological importance, *in vivo*. In contrast to SLPI, AAT was found to inhibit neutrophil chemotaxis and adhesion in similar experimental models *in vitro* [205].

Conclusion

The results of these studies further support the assumption that SLPI and AAT play a central role in inflammation, not only as the regulators of protease activity, but also as the modulators of endotoxin-induced, pro-inflammatory responses. The finding that SLPI, in contrast to AAT, has a differential effect on monocytes and neutrophil pro-inflammatory responses emphasizes the potentially new role of SLPI *in vivo*.

7.3 Concentrations of SLPI and AAT in plasma and bronchial lavage fluid (studies IV and V)

Background

Secretory leukocyte protease inhibitor (SLPI) is found in various biological fluids, including bronchial and nasal secretions [219], tears [220], cervical mucus [221], seminal plasma [222] and ascites [223]. For example, in the epithelial lining fluid of the upper airway of normal individuals SLPI concentrations are between 72 - 144 μg/ml [224], in nasal secretions 10 - 80 μg/ml [147], in human tear fluids 5 - 10 μg/ml [220], in saliva 4 - 24 μg/ml [129], and serum 27 - 47 ng/ml [183, 195]. α1-antitrypsin (AAT) is mainly produced in liver parenchyma cells. To a lesser extent, AAT is synthesized by blood monocytes, macrophages, pulmonary alveolar cells, intestinal epithelial cells and the cornea [165, 168, 225]. Identification of AAT expression in a variety of human tissues e.g. kidney, stomach, small intestine, pancreas, spleen, thymus, adrenal glands, ovaries and testes, and demonstration of *de novo* synthesis of AAT by human cancer cell lines suggest that the transcription of its gene is not limited to a single tissue. The normal daily rate of synthesis is approximately 34 mg/kg body weight, leading to a plasma concentration ranging from 0.9 - 1.75 mg/ml, with a half-life of 3 to 5 days.

Individuals with plasma AAT values below 0.7 mg/ml are considered to be AAT deficient. Over 100 alleles of AAT have been identified to date, of which at least 20 affect either the amount or the function of the AAT molecule *in vivo*. To classify allele expression, a protein inhibitor (Pi) system has been developed to describe diverse genotypes, based on the migration-rate of the protein in an electric field. According to the location of the migrated

protein, a letter designation is assigned (*eg*, *M* for middle, *Z* for the slowest or most cathodal types).

The genes are inherited as co-dominant alleles (products of both genes can be found in the circulation). Therefore, individuals heterozygous for the Z allele (MZ) have 30–40 % while individuals homozygous for the Z allele (ZZ) have only 10 –15 % of normal plasma MM AAT levels (5). The intermediate and severe AAT deficiency phenotypes result mainly from combinations of S-, Z- and null alleles.

Prevalence in Sweden of most the common AAT variants [226]

MM (93/100)

MZ (4.6/100)

SS (1/1600)

SZ (1/750)

ZZ (1/1600)

The lack of AAT in the circulation is suggested to result in uncontrolled serine protease activity and development of lung emphysema. The results available are moderate or weak for the relationship between AAT deficiency and bronchial asthma, bronchiectasis and systemic vasculitis and several other diseases, such as rheumatoid arthritis, intracranial and abdominal aneurysms, arterial dissections, psoriasis, chronic urticaria, mesangiocapillary glomerulonephritis, pancreatitis and pancreatic tumours, multiple sclerosis, and other conditions occasionally reported.

Individuals with severe AAT deficiency have at least a 20-fold increased risk of developing lung disease, especially if they smoke [227]. It has been hypothesized that compensatory increases in other protease inhibitors and/or decreased leukocyte activity may reduce the severity of AAT deficiency by favourably affecting the overall protease/protease-inhibitor balance in AAT-deficient individuals. Serial reports from the prospective follow-up of individuals with AAT deficiency up to age 26 years have been published. These showed that at the age of 8 and 18, individuals with severe ZZ and moderate SZ AAT deficiency, have a higher plasma concentration of α2MG, antithrombin III and ACT [228, 229], while at the age of 26 years these individuals have higher plasma SLPI and lower neutrophil lipocalin levels compared to age-matched, healthy MM subjects [230].

The major anti-neutrophil elastase shield is provided by AAT in peripheral lung and SLPI in the airways [231]. Thus, it has further been speculated that compensatory, higher levels of SLPI in AAT deficient individuals may play an important role in protecting these individuals from developing of lung diseases.

SLPI was first purified from secretions of patients with COPD and cystic fibrosis suggesting that SLPI is important in the pathophysiology of lung disease. However, the emerging role of mast cell and leukocyte serine proteases in asthma, as well as the functional and physical properties of SLPI suggest that this molecule is also important in the pathophysiology of asthma. For example, the acid stability of SLPI allows the inhibitor to remain functionally active under acidic inflammatory conditions. With a pI > 9, SLPI may also bind tissue sites attacked by proteases, thus facilitating prolonged inhibition of protease activity in the airways. Although studies measuring SLPI in asthmatic patients are scarce, animal models of allergic diseases have shown SLPI to be of benefit in inhibiting both early- and late-phase inflammatory events [232].

Aims

To further evaluate the hypothesis that, in subjects with inherited AAT deficiency, increased plasma levels of other protease inhibitors, such as SLPI, are compensating for the genetically induced AAT deficiency, and to analyse and compare the concentrations of SLPI in plasma and BAL fluid in patients with asthma and COPD.

Results and Discussion

As expected, we found that plasma levels of AAT differ significantly depending on AAT genetic variant: MM > SZ > ZZ ($p<0.001$). It is also important to point out that plasma AAT levels were found to be similar in all asymptomatic, AAT deficient subjects independent of age (n=48, mean age 32, 0.27±0.05 mg/ml, and n=10, mean age 53, 0.2±0.03 mg/ml). However, we found no significant difference in plasma SLPI levels between subjects with severe AAT deficiency (32 year old subjects from the prospective follow-up study, T Sveger) and subjects with wild type AAT (age and gender matched controls). One possible explanation might be that SLPI is produced locally in the airways and therefore plasma may not be the relevant biological fluid in which to measure SLPI levels. On the other hand, plasma SLPI was measured in samples obtained from young healthy subjects, thus one can not exclude that plasma collected from the same individuals under inflammatory conditions

(i.e. with a common cold or infection) may show different concentrations and correlations between AAT and SLPI. For example, increased levels of ACT, SLPI and α2-MG were found in COPD patients. We also found that plasma levels of SLPI are higher in COPD patients than in controls ($p<0.001$). Moreover, our findings show that SLPI levels in COPD patients are higher independent of the genetic variant of AAT. Studies using larger groups of patients are necessary in order to clarify whether plasma SLPI is inversely related to AAT concentrations.
We also compared plasma and BAL fluid levels of SLPI in patients with asthma and COPD. Our results show that concentrations of SLPI in plasma and BAL fluid did not differ between COPD and asthma groups. However, in both patient groups SLPI concentrations in BAL fluid were significantly higher than in plasma. In both patient groups, the concentration of SLPI in BAL fluid correlated with the concentration of IL-8, while in patients with asthma the serum concentrations of SLPI correlated with serum sCD14.

Conclusions

So far, our findings do not support the hypothesis that in healthy (asymptomatic) subjects with severe AAT deficiency, plasma levels of SLPI are higher than in age-matched subjects with wild type AAT. Alternatively, because SLPI is produced locally in the airways, plasma SLPI levels when analysed in young healthy subjects may not reflect the real situation. Therefore, this theory remains to be strengthened through further study.
The finding that in asthma and COPD patients the concentration of SLPI is significantly higher in BAL fluid than in plasma further points to the importance of selecting a correct biological fluid when analysing specific biomarkers.
Analysis of SLPI levels in combination with other biomarkers using larger groups of patients, and also including patients, who manifest clinical features of both COPD and asthma, may help to identify the new pathophysiological distinctions between these diseases.

8. Concluding remarks

A characteristic destruction of tissue in inflammatory diseases of the airways is to a large extent mediated by an excess of serine and matrix metalloproteases. Therefore, the regulation of proteolytic enzyme activity by endogeneous inhibitors is a prerequisite for the maintenance of tissue integrity, and for the repair of tissue damage. Protease inhibitors that provide protection against the extracellular activity of serine proteases include α1-antitrypsin (AAT), and Secretory Leukocyte Protease Inhibitor (SLPI). Whereas AAT is produced mainly by the liver and reaches the tissues via passive diffusion, SLPI is produced locally. SLPI is found in considerable amounts in nasal, bronchial and cervical mucous, and in saliva. The carboxyl-terminal domain of SLPI manifests inhibitory activities against chymotrypsin, trypsin, granulocyte and pancreatic elastase, cathepsin G and mast cell chymase, whereas anti-inflammatory, anti-bacterial and anti-fungal activities appear to reside in its amino-terminal domain. Local and circulating levels of SLPI have been shown to be increased in patients with chronic obstructive inflammatory disease, particularly those with lung cancer. However the biological significance of these observations is not known.

The known primary function of AAT is to neutralize over-expressed neutrophil serine protease activity, specifically neutrophil elastase and protease 3. Therefore, the alterations of the AAT molecule which affect its structure and/or secretion and thereby reduce its functional levels result in a pathological change. There is ample clinical support that inherited, severe AAT deficiency predisposes to chronic obstructive pulmonary disease. Today there is increasing indication that AAT, in a manner similar to SLPI, is able to initiate effects other than protease inhibition. However, to strengthen this new concept and make it useful for clinical application, we still need to answer important questions: how broad are anti-inflammatory properties of these inhibitors, are the anti-inflammatory effects of AAT and SLPI cell-specific, which cellular receptor(s) and mechanism are involved?

We believe that the answers to these questions will open the door to new basic knowledge on SLPI and AAT as endogenous anti-inflammatory proteins, and will provide scientific bases to explore new therapies for individuals with inherited AAT deficiency, but also for other clinical conditions. In addition, a better understanding of the biological role and clinical importance of SLPI and AAT may improve they usefulness as clinical biomarkers for the evaluation of inflammatory processes of the airways.

9. Sammanfattning på svenska

Kliniska och experimentella studier har visat att proteaser och deras inhibitorer deltar i mycket hög grad i luftvägarnas fysiologi och sjukdomsutveckling. Inflammatoriska celler, såsom neutrofiler, monocyter/makrofager, epitel- och mastceller, friger olika proteaser. Det är allmänt känt att ökad produktion eller aktivitet av proteaser eller inaktivering och minskad produktion av proteas-hämmare kan leda till proteas/proteas-hämmar obalans och nedbrytning av vävnad. Proteas-hämmare utgörs av dem som produceras lokalt av slemhinnan, som till exempel sekretorisk leukocyt proteas inhibitor (SLPI), och de som huvudsakligen produceras i levern och når luftvägarna via passiv diffusion, som till exempel α1-antitrypsin (AAT). SLPI utgör en viktig komponent i försvaret mot neutrofilt elastas och är därför ett komplemet till AAT. Ärftig, allvarlig AAT-brist är en känd genetisk faktor för utvecklingen av kronisk obstruktiv lungsjukdom (KOL). Det finns ingen känd genetisk brist av SLPI.

För att pröva en hypotes om kompensatorisk ökning av nivåer i SLPI positivt skulle kunna förbättra proteas/proteas-hämmar balansen hos AAT-bristande individer, analyserade vi plasmanivåer av SLPI hos friska försökspersoner med och utan allvarlig AAT-brist. Resultaten visade att SLPI i plasma ej skillde mellan de studerade grupperna, vilket ledde oss till slutsatsen att SLPI ej kompenserar för brist av plasma AAT. Som stöd för detta tankesätt fann vi att SLPI koncentrationer i bronksköljvätska från astma och KOL patienter var anmärkningsvärt högre än i plasma. Vi fann också att SLPI i plasma var högre hos KOL-patienter oberoende av genetisk variant av AAT-brist. Det är av betydelse att analysera SLPI i rätt biologiskt matrial.

Utöver sina funktioner som proteas-hämmare har både SLPI och AAT visat sig inneha anti-inflammatoriska egenskaper. Att förstå mekanismerna involverade i protein-uttrycket av AAT och SLPI, deras frisättning och konsumtion kan ge viktiga kunskaper i regleringen av proteas/proteas-hämmare och andra händelser i den inflammatoriska reaktionen. Resultaten från våra studier avslöjade att SLPI-uttryck och -utsöndring av epitelceller är beroende av samverkan mellan epitel och mastceller. I nästa försöksmodell stimulerades friska försökspersoner med endotoxiner (LPS) i näsan. Vi fann ökningar av SLPI nivåer i nässköljningsvätska.

Vi fortsatte våra försök med att studera hur monocyter och neutrofiler svarar på endotoxin-stimulering med och utan SLPI och AAT. Resultaten visade att AAT hämmade LPS-

inducerad pro-inflammatorisk monocyt och neutrofil respons. Observationer visade också att AAT inhiberade chemokiner (IL-8) frisättning hos LPS-stimulerade individer. SLPIs effekter var mycket mer komplexa. SLPI hämmade LPS-stimulerad IL-8 frisättning från monocyter, men hade ingen effekt på neutrofiler. Faktum var att SLPI själv stimulerade neutrofilers, men inte monocyters, migration och adhesion. Dessa fynd styrker hypotesen att SLPIs biologiska effekter är beroende av celltyp. Sammanfattningsvis styrker våra studier kunnskapen om att SLPI och AAT utövar andra effekter än enbart deras förmåga att vara proteas-hämmare.

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Human mast cells decrease SLPI levels in type II – like alveolar cell model, *in vitro*

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Abstract

Background: Mast cells are known to accumulate at sites of inflammation and upon activation to release their granule content, e.g. histamine, cytokines and proteases. The secretory leukocyte protease inhibitor (SLPI) is produced in the respiratory mucous and plays a role in regulating the activity of the proteases.

Result: We have used the HMC-1 cell line as a model for human mast cells to investigate their effect on SLPI expression and its levels in cell co-culture experiments, *in vitro*. In comparison with controls, we found a significant reduction in SLPI levels (by 2.35-fold, $p < 0.01$) in a SLPI-producing, type II-like alveolar cell line, (A549) when co-cultured with HMC-1 cells, but not in an HMC-1-conditioned medium, for 96 hours. By contrast, increased SLPI mRNA expression (by 1.58-fold, $p < 0.05$) was found under the same experimental conditions. Immunohistochemical analysis revealed mast cell transmigration in co-culture with SLPI-producing A549 cells for 72 and 96 hours.

Conclusion: These results indicate that SLPI-producing cells may assist mast cell migration and that the regulation of SLPI release and/or consumption by mast cells requires interaction between these cell types. Therefore, a "local relationship" between mast cells and airway epithelial cells might be an important step in the inflammatory response.

Background

The inflammatory process in the respiratory airways includes the release of several mediators such as chemoattractants, cytokines and proteinases that regulate the adhesion of molecules, and the processes of cell migration, activation and degranulation. The characteristic destruction of tissue in inflammatory diseases is to a large extent mediated by an excess of neutral serine proteinases and matrix metalloproteinases (MMP) [1–4]. The serine proteinases also contribute to the activation of MMPs, which are typically released in a latent form [5]. Therefore, the regulation of proteolytic enzyme activity in the respiratory airways by endogeneous inhibitors is a prerequisite for the maintenance of tissue integrity, and for the repair

of tissue damage. Proteinase inhibitors that provide protection against the extracellular activity of serine proteinases include alpha1-antitrypsin (AAT), secretory leukocyte proteinase inhibitor (SLPI) and elafin/skin–derived anti-leukoproteinase (SKALP). Whereas AAT is produced mainly by the liver and reaches the tissues via passive diffusion [6], SLPI and elafin/SKALP are produced locally [7–12]

SLPI is found in considerable amounts in nasal, bronchial and cervical mucous, in saliva, and in seminal fluid [7,9,13–16]. There is increasing evidence that SLPI has numerous functions that are not related to its protease-inhibitory activity. SLPI is a non-glycosylated, hydrophobic, cationic 12 kDa protein, consisting of two homologous cystein-rich domains of 53 and 54 amino acids [17]. The carboxyl-terminal domain of SLPI manifests inhibitory activities against chymotrypsin, trypsin, granulocyte and pancreatic elastase, cathepsin G and mass cell chymase [18–22], whereas anti-inflammatory, anti-bacterial and anti-fungal activities appear to reside in its amino-terminal domain [23,24]. SLPI is shown to reduce LPS-induced TNFα production in the macrophage cell line [25,26], to suppress the production of prostaglandin E2 and metalloproteinase in monocytes [27], and also to antagonize up-regulation of nuclear transcription factor (NF-κB) activation [28]. Lentsch and co-workers have demonstrated that SLPI attenuates the acute inflammatory response caused by the deposition of IgG immune complexes in the lungs [29]. In addition, Ashcroft and associates found that SLPI might play a crucial role in wound healing [30]. Recently SLPI has also been shown to inhibit HIV-1 replication in cultured human monocytes [23]. The up-regulation of SLPI by bacterial lipopolysaccharides, and cytokines such as TNFα and IL-1β, combined with a broad spectrum antibiotic activity against gram-positive and gram-negative bacteria, suggest it to be a potent anti-microbial "defensin-like" peptide produced by the lungs. States of impaired healing are characterized by excessive proteolysis and often bacterial infection, leading to the hypothesis that SLPI may also have a role in this process.

Historically, SLPI was first purified from secretions of patients with chronic, obstructive pulmonary disease and cystic fibrosis [18], and it was suggested that SLPI being a major anti-elastase inhibitor of the bronchi, is an important molecule for protecting the respiratory epithelium [13,15]. In contrast to α1-antitrypsin, SLPI blocks elastin-bound elastase in the alveolar walls, which might also protect against the development of emphysema [31]. The interaction between SLPI and elastase is reversible, probably facilitating the transfer of neutrophil elastase to α1-antitrypsin [32]. It is important to point out that neutrophil elastase has been found to increase SLPI mRNA

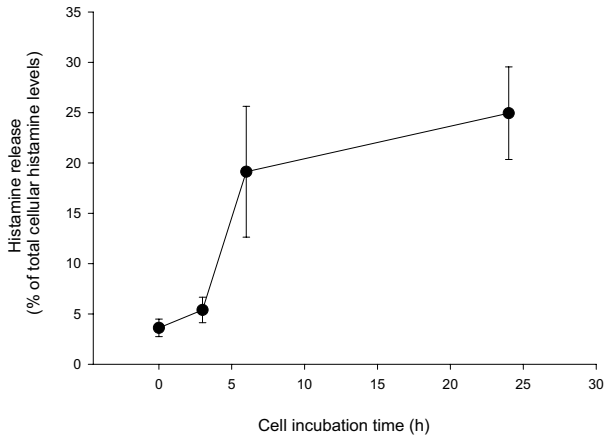

Figure 1
Histamine release from HMC-1 cells. Histamine release was measured at different time points: 0, 3, 6 and 24 h and calculated as a percent of the total cellular histamine content. Each point represents mean ± S.D. of five or six separate experiments. Histamine release increases from 4 % to 25 % over time ($p < 0.05$).

expression in lung epithelial cells *in vitro*, but this increase in SLPI expression was accompanied by a decrease in SLPI protein release [33]. The local induction of SLPI might be important to break the cycle of inflammation. However the mechanisms involved in the regulation of SLPI expression and release still remain to be elucidated. It has been shown that SLPI is up-regulated by pro-inflammatory stimuli including LPS, TNFα, IL-6 and IL-1β, *in vitro* [25,26,34,35]. Corticosteroids have also been found to enhance SLPI mRNA levels in airway epithelial cells leading to the suggestion that antiinflammatory effects of corticosteroids may be related to the stimulated SLPI levels [36]. The demonstration that neutrophil defensins increase SLPI release from the bronchial epithelial cells supports the idea that leukocytes play a prominent role in the regulation of SLPI production [37].

Contradictory results have been presented concerning the levels of SLPI during allergic rhinitis in antigen-challenged atopic subjects. For example, lower SLPI levels were found in the bronchial secretions of asthma patients [38]. Other studies indicated that levels of SLPI are also lower in nasal secretion of allergic rhinitis patients compared to healthy controls. After allergen challenge SLPI seems to decrease in the nasal secretions in atopic subjects, which probably indicates mucosal damage [39]. However, the question why SLPI levels is decreased during certain allergic reactions, still remains to be answered.

Table 1: Time dependent SLPI release from the mast cells, HMC-1, and type II epithelium cells, A549, cultured alone

Incubation time	HMC-1 cells			A549 cells		
(h)	SLPI (pg/ml)			SLPI (pg/ml)		
	Mean		SD	Mean		SD
24	-	±	-	6.7	±	0.26
48	-	±	-	14.5	±	0.9
72	0.17	±	0.07	14.4	±	1.5
96	0.2	±	0.06	38.75	±	0.98

*) mean and standard deviation of 2 experiments

Allergic inflammation, including rhinitis, asthma, anaphylaxis and urticaria are all disorders associated with mast cell activation [40]. Mast cells are multifunctional cells capable of secreting a wide variety of cytokines, chemokines and growth factors [41,42]. The mediators released by mast cells can independently, and in synergy with macrophage- and T-cell-derived cytokines, induce much of the inflammatory pathology and serve to stimulate a complex immune response [43,44]. Mast cells are the primary initiating cell of IgE-mediated hypersensitivity. Allergen binding to, or the cross-linking of IgE on the surface of mast cells, which is bound to the high affinity IgE-receptor, leads to the rapid release of inflammatory mediators that further provoke a profound immunological and inflammatory process. There are indications that SLPI can inhibit IgE-mediated histamine release from rodent and human nasal mucosa mast cells [45,46]. SLPI may also counterbalance the proteolytic activities caused by protease leakage from the cells [47]. Mast cell and leukocyte serine proteinases are shown to be elevated in the airways of asthmatic patients [40,48]. Individuals with reduced anti-proteinase activity as a result of AAT deficiency, have an increased propensity to develop asthma [49,50]. Together, these findings indicate that proteinase-antiproteinase imbalance in the airways contributes to the pathophysiological responses in the airways. Because SLPI provides a potent, broad-spectrum inhibitory activity against mast cell and leukocyte serine proteinases, this protein is suggested to be an effective protector against antigen-induced inflammatory responses in the airways. The purpose of this study is to further elucidate how local SLPI levels may be influenced during mast cell interaction with epithelial cells. A co-culture model was used, in which we studied mast cells HMC-1 affect on SLPI levels released from the type II alveolar cell line (A549) derived from human lung carcinoma.

Results

Mast cell characterisation

Most studies performed on the mechanisms of the mast cell degranulation are based on the release of histamine. Histamine is electrostatically linked to the protein heparin complex in a manner, which allows it to be released very easily. As shown in figure 1, a spontaneous histamine release from HMC-1 cells consistently increases with incubation time (up to 24 h) from 4% to 25% relative to the total cellular histamine content. Continues cell incubation (up to 96 h) showed no further changes in histamine release (data not shown). In addition, the supernatants from the HMC-1 cells alone did not contain SLPI after cell culture for 24 and 48 h, and only trace amounts of SLPI are detected after 72 and 96 h of incubation (Table 1). The ability of HMC-1 cells to express SLPI was also confirmed by immunohistochemical analysis (Fig. 2A and 2B).

Cell co-culture experiments

Epithelium cells, A549, cultured alone for 24, 48, 72 and 96 h increased the SLPI release from 6.7 ± 0.26 to 38.8 ± 0.98 pg/ml (Table 1). Next, we studied the effect of mast cells on SLPI levels by using a transmigration model in which mast cells HMC-1 migrated across the topside of the transwell filter towards the A549 cells. The results obtained with this model show that, during the first 48 h of cell co-culture, mast cells did not migrate into the lung epithelium cell culture (Fig. 3A). However, as shown in figure 3B, cell co-culture for 72 h resulted in a slight mast cell transmigration as indicated by the presence of immunoreactive HMC-1 cells among the A549 cells. This was, furthermore, confirmed by the co-culture of these cells for 96 h after which a large number of immunoreactive mast cells was detected among the A549 cells (Fig. 3C). It should be pointed out that HMC-1 cells showed no ability to transmigrate through a "blank" transwells. Moreover, cells which had not migrated into the polyester membrane inserts displayed immunoreactivity to tryptase for all incubation times, from 24 to 96 h, showing that under the chosen experimental conditions mast cells preserved

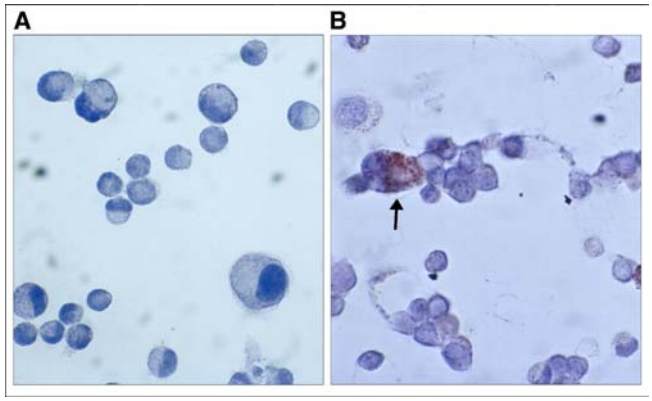

Figure 2
Localisation of SLPI in HMC-1-mast cells. A, the control slides were incubated with specific antiserum previously adsorbed with SLPI (1/500) and no positive staining for HMC-1 cells was found (original magnification × 500). B, HMC-1 cells stained with polyclonal goat-anti-SLPI antibody (1/500) show immunoreactivity for SLPI (original magnification × 500).

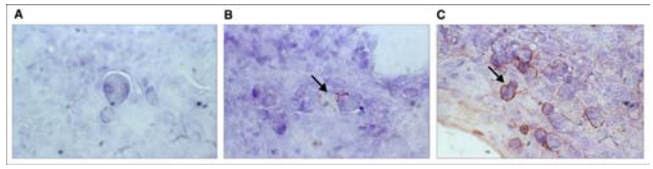

Figure 3
Mast cell HMC-1 migration towards the lung type II epithelium cells, A549. Specimens stained with a mouse anti-human mast cell 229; kidney juxtaglomerular CE diluted 1/5000 show immunoreactivity for HMC-1 mast cells. A, HMC-1 are not detected among the lung carcinoma cells after 24 h of cell co-culture. B and C, Solitary cells and a large number of cells can be detected after 72 and 96 hours of cell co-culture, respectively. Original magnification × 200. Mast cell immunoreactivity indicated by arrow.

their capacity to differentiate (not shown). We further examined the effect of mast cell trans-migration on the amount of SLPI released by the A549 cells into cell culture media. Only a slight decrease in SLPI levels was observed during 72 h of cell co-culture compared to the SLPI-producing cells alone (Fig. 4). However, when cells were co-cultured for 96 h a significant decrease in SLPI levels was found. As shown in figure 3, SLPI levels decline by 2.35-fold ($p < 0.01$) in the presence of mast cells relative to the A549 cells alone.

Effects of conditioned media on SLPI levels

In the next series of experiments we aimed to investigate if concentration of SLPI can be influenced by the incubation of A549 cells with HMC-1-conditioned medium for various time periods (from 24 to 96 h). In this case, a medium collected from mast cells at different time points was added to the A549 cells and allowed to act for 24, 48, 72 and 96 h. As shown in Figure 5, the culture of epithelium cells in the mast cell-media caused no changes in SLPI levels.

SLPI mRNA expression by A549 cells

To determine whether the decrease of SLPI levels in co-culture with mast cells involves changes in SLPI mRNA expression in A549 cells, we monitored the SLPI mRNA/β-Actin ratio in A549 cells alone and in co-culture over the period of 24 to 96 h. As shown in figure 6, the levels of SLPI mRNA expression were relatively unchanged when A549 cells were cultured alone. By contrast, increased SLPI mRNA levels were observed in co-culture experiments after 96 h (1.58-fold, $p < 0.05$) compared to 24 or 48 h. Under the same experimental conditions, we also examined whether addition of conditioned medium from HMC-1 cells effects SLPI mRNA expression in same way. Consistent with our earlier observations (Fig. 5) showing that A549 cells incubated with conditioned medium from mast cells did not change SLPI protein levels, we found that under these experimental conditions SLPI mRNA levels are not changed either (data not shown).

Discussion

Mast cells are widely distributed within the connective tissue, with a preferential localization adjacent to the small blood vessels. They play a central role in inflammatory and allergic reactions, and are involved in tissue remodelling during wound healing [40]. The mast cell responses involve the ingestion and killing of adherent substances, unlike that of traditional phagocytic cells. Concomitant with this endocytic activity, inflammatory mediators are released by the mast cells.

Mast cells constitute a heterogeneous group of cells containing several proteases, i.e. tryptase, carboxypeptidase, cathepsin G and chymase [51]. Mainly there are two kinds of mast cells, those in the connective tissue (MC_{TC}) and those in the mucosa (MC_T). They differ in protease content, MC_{TC} cells contain tryptase and chymase, whereas MC_T cells contain only tryptase. The MC_{TC} cells are predominant in the nasal mucosa while MC_T cells are more prevalent in the peripheral lung tissue. Mast cells have also been found to interact with different types of cells, including fibroblasts, endothelial cells, lymphocytes, macrophages, neutrophils, eosinophils, nerve cells and cancer cells [40,43,52,53]. However, the adhesive

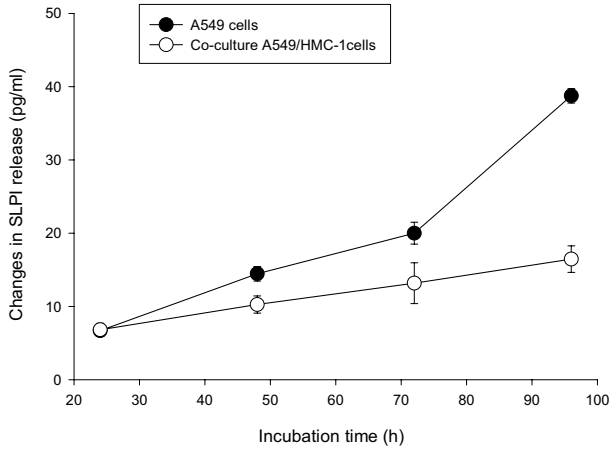

Figure 4
Effects of mast cells HMC-1 on the ability of the A549 cells to release SLPI. The SLPI release from the A549 cells was decreased in co-cultured with HMC-1 cells, compared to the A549 cells alone ($p < 0.006$). The most pronounced fall in SLPI production was observed after 96 hours of cell co-culture (**$p < 0.01$). Each point represents mean of six or five separate experiments.

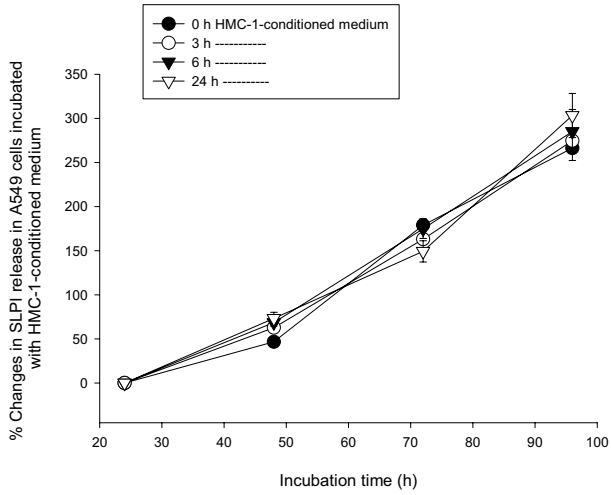

Figure 5
Effects of HMC-1-conditioned medium on SLPI levels in A549 cells. Medium from HMC-1 cells cultured for 0, 3, 6, 8 and 24 h was used to incubate A549 cells for various time periods (from 24 to 96 h). The mast cell-media caused no changes in SLPI levels. Each point represents mean of three independent experiments

mechanisms initiating cell-cell interaction and the consequences of this are not well understood.

The present study was designed to investigate the adherence of mast cells, HMC-1, to the cell line A549, which represents epithelial cells within the respiratory tract, and to investigate the effects of this cell-cell interaction on SLPI levels.

The mast cell line HMC-1 is known to express a number of β1- and α-integrins as well as other receptors which permit the binding of these cells to the extracellular matrix compounds [53–55]. The factors that stimulate mast cell migration still remain largely undefined, although recent reports have implicated the transforming growth factor-β family (TGFβ) as the potential candidate for acting as mast cell chemotaxin, recruiting mast cells into inflammatory reactions [56]. In accordance with other studies, we found that HMC-1 mast cells growing in suspension adhere efficiently and spread on top of cell monolayers, in our case on top of A549 cells. After 72 h of HMC-1/A549 co-culture, solitary HMC-1 cells were detected among the A549 cells, while after 96 h a large number of mast cells was found to be adhering to the A549 cells. The maintenance of HMC-1 cell maturity was verified by the tryptase immunoreactivity each of the periods of cell culture.

The role of mast cells as primary effector cells in IgE-dependent, immediate hypersensitivity is well established [57]. The discovery that mast cells can release a wide variety of immune mediators, including proteases, cytokines, chemokines and growth factors, suggests an additional role of mast cells in modulating late-phase reactions and other chronic inflammatory processes [40,42]. Here we also demonstrate by immunocytochemistry that HMC-1 mast cells are SLPI-positive, and trace amounts of SLPI were found in the cell culture supernatants collected after 72 and 96 h of culture. Although the amount of SLPI released by few activated mast cells had no importance for the present study, this observation still extends earlier findings showing that SLPI and other chymase inhibitors, i.e. α1-antichymotrypsin and α1-antitrypsin are present in stimulated mast cells and protect the microenvironment against chymase activity [47]. Previously it has been shown that chymase degranulates mast cells, induces histamine release and an increase in SLPI concentration [58]. Neutrophil elastase was also found to increase SLPI transcript levels in primary and transformed human airway epithelial cells in a time- and dose-dependent manner [34,36]. These observations suggest that the sensitive regulation of anti-proteases, such as SLPI, in relation to local levels of proteases, may play an important role in minimising tissue destruction.

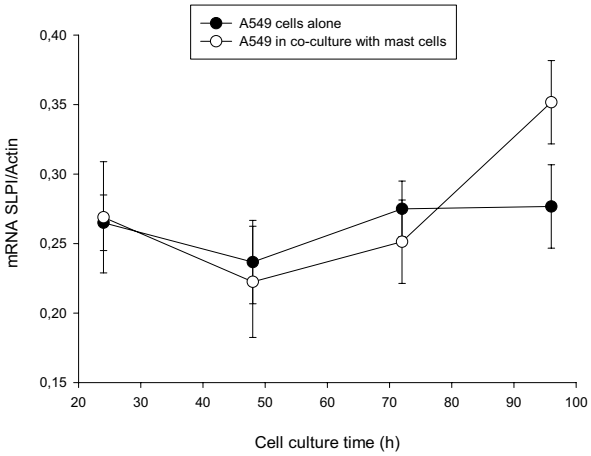

Figure 6
SLPI mRNA expression by lung epithelium, A549, cells. Increased SLPI mRNA levels in co-culture experiments were observed after 96 h (* $p < 0.05$), compared to 24 and 48 hours. No change in SLPI mRNA levels was found when lung carcinoma cells were cultured alone.

With these results as a background we have further investigated the effects of mast cells on the capacity of epithelial-presenting cells, A549, to express and release SLPI. In our experimental model, SLPI-producing epithelial cells were cultured for determined time points in conditioned media obtained from mast cells or in co-culture with mast cells. By using this approach, we were able to show that under cell co-culture conditions SLPI levels in cell culture media are gradually decreased, although the expression of mRNA SLPI increases. A significant diminition in SLPI levels (by 2.35-fold) was observed after 96 h of cell co-culture, although the SLPI mRNA was up-regulated (by 1.58-fold). In contrast, the A549 cell culture in the mast cell conditioned medium for the time periods chosen had no influence either on SLPI protein levels or on SLPI mRNA expression. By measuring histamine release and tryptase activity in media from mast cells cultured alone for up to 96 h, we were able to show that these cells are not activated. Histamine levels plateaued after 24 h of cell culture which explains why the conditioned media collected from mast cells and added to the SLPI producing cells (A549) had no effect on SLPI levels.

Based on these findings one can conclude that mast cells exert an inhibitory effect on SLPI levels only when they are in close contact with SLPI-producing, A549, cells. Since our primary goal was to find out if mast cell interaction with epithelial cells by itself can induce expression of SLPI and reduce its levels in media, we specifically did not investigate mast cell exogenous activation under these conditions. This indirectly shows that SLPI is either consumed (for example degraded or in complex with enzymes) or its release is inhibited. Studies on this point are in progress in our laboratory.

Recently, van Wetering and co-workers have shown that neutrophil elastase increases SLPI mRNA expression, while it decreases SLPI protein release, *in vitro* [37]. On the other hand, studies by Hill and co-workers, have indicated that SLPI concentration does not decrease until the elastase activity of the samples is in excess of 50 nM [48]. The relationship between SLPI and elastase is therefore found not to be a simple linear, indicating that a certain amount of elastase and/or other enzymes is needed in order to induce epithelial damage or interfere with epithelial cell metabolism resulting in a decreased SLPI secretion [12,32,34].

SLPI levels were found to be decreased in nasal secretion after antigen challenge *in vivo* [39] as well as in bronchial alveolar lavage obtained from asthmatics compared to healthy subjects [38]. In the airways of allergic patients, mast cells are found in the close proximity of airway epithelial cells, which may indicate that mast cells and epithelial cells influence each other's properties. Together, previous findings, that SLPI levels are lower in inflammatory loci in airways and data from our experimental model, that co-culture of mast cells with SLPI-producing epithelial-like, A549, cells, results in decreased SLPI levels in cell co-culture media suggest that decrease in local antiprotease activity might sustain mucosal damage in reactions in which mast cells are participating.

Conclusion

Airway inflammation, present in asthma, bronchitis, and bronchiectasis, is characterised by the presence of activated inflammatory cells. The proteinases, presumably leaking from the cells during their migration from the blood into the extracellular space, can be detrimental to the connective tissue. Proteinase inhibitors, such as SLPI, produced locally in the airway epithelium, are thought to be important in minimising proteolytic damage during inflammation. On the other hand, proteinases may also play a role in the regulation of the antiproteinase profile at the epithelial site. Thus, the auto-regulatory loop observed, namely that the up-regulation of SLPI mRNA parallels apparent down-regulation of the levels of the SLPI in the mast/epithelial like cell, A549, co-culture model, will need further investigations. An understanding of the mechanisms involved in SLPI protein expression, release and consumption may provide important knowledge regarding the dynamics of the regulation of the protease/antiprotease systems.

Materials and Methods

Cell cultures

The human mast cells HMC-1, established from a patient with mast cell leukaemia, were obtained from Dr J.H. Butterfield, Mayo Clinic, Rochester, MN, USA [59] The cells were cultured in 75 cm^2 flasks in Iscove's modified Dulbecco's medium (Gibco BRL, Paisly, UK) supplemented with 10% (v/v) iron-supplemented fetal calf serum (FCS) (Gibco BRL), 1.2 mM α-thioglycerol (Sigma, St Louis, MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B and fungizone (Gibco BRL), in humidified air with 5% CO_2, at 37°C. At confluence, the cells were centrifuged at 2000 g for 5 min, washed in PBS, re-suspended in cell culture medium and counted in a Bürker chamber. The viability of the HMC-1 cells was ≥ 95%.

SLPI producing a type-II alveolar cell line (A549) derived from human lung carcinoma, was cultured in RMPI-1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B and fungizone at 37°C in an atmosphere of humidified air saturated with 5% CO_2. The cells were subcultured every 4–5 days by tripsinization and were used for experiments after reaching confluence.

Human type II lung epithelium cells (A549) and HMC-1 co-culture models

Epithelium cells (A549) were cultured in the lower compartment of the cell co-culture plates (Nunc, Wiesbaden, Germany) to obtain confluent monolayers. A constant amount of HMC-1 cells (10^6 cells/ml) was seeded on fibronectin-coated polycarbonate transwell filters (pore size 3 μm and 12 mm in diametre). The cells were co-cultured in an Iscove's modified Dulbecco's medium for 24, 48, 72 and 96 h. The cell viability was analysed after 96 h of cell co-culture by a trypan blue staining, and was found to be 90 % and 95% for the mast cells and for the lung epithelium cells, respectively. For the controls, A549 cells and mast cells were cultured separately in co-culture plates under the same experimental conditions as described above. The experiments were repeated six times.

SLPI expression by A549 cells alone and in co-culture models

Total RNA was isolated and quantified from A549 cells cultured alone and in co-culture. The cells were lysed in 1.5 ml lyses buffer (4 M guanidinum thiocyanate, 25 mM sodium citrate (pH 7), containing 0.5% antifoam A and 100 mM 2-mercaptoethanol). Total RNA was extracted using a single-step method based on acid-guanidinum thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi [60]. The total RNA yield was quantified at 260 and 280 nm. The transcript levels of SLPI and β-actin were evaluated by slot blot, using a Magnagraph (MSI) nylon membrane according to the manufacturer's instructions. Briefly, 5 μg of total RNA was mixed with 100 μl of dilution buffer containing 7.4% formaldehyde-7 and SSPE (150 mM sodium chloride, 10 nM sodium phosphate and 1 mM EDTA), denatured for 5 min in boiling water, cooled very fast (0°C) for 2 min and loaded onto the nylon membrane. SLPI mRNA was detected with a cocktail of an equimolar mixture of three single stranded oligonucleotide probes (British Biotechnology Products LTD, Oxon, UK). The probes were based on the antisense sequence and modified at the 5' end with digoxigenin. The digoxigenin-labelled β-actin probe was purchased from Roche, catalogue number 1498045. The blots were exposed to Kodak XAR-5 X-ray film (Sigma Chemical, St. Louise, MO). Autoradiographs were analysed using the Fuij film LAS-100, Luminescent Image analyser and Image reader macintosh version 1.0 was used to determine the densitometric units for both SLPI and β-actin. The data represent the mean SLPI/β-actin ratio.

SLPI and histamine quantification assays

SLPI was quantified in the cell supernatants obtained from each experimental condition. Analyses of SLPI were performed by using the quantitative sandwich ELISA kit according to the manufacture recommendations (R&D systems, Inc, USA). The minimum detectable dose of SLPI was less than 25 pg/ml. The mast cell degranulation was verified by the amount of histamine released. Histamine was quantified in the supernatants obtained from the mast cells cultured alone after the various incubation time points. Histamine was measured by a sensitive radioenzyme assay based on the conversion of histamine to [3H] methylhistamine in the presence of the enzyme histamine – N-methyltransferase using S-adenosyl-L-[methyl-3H] methionine as the methyl donor, using a commercial radioimmunoassay (RIA)-kit (Immunotech, KEMILA, Sollentuna, Sweden). Histamine secretion is expressed as a percentage of total cellular content (cell lysate plus spontaneous release) and is corrected for spontaneous release.

Immunohistochemistry

To monitor the differentiation of the mast cells we stained cells for tryptase after the various incubation times of 24, 48, 72 and 96 h. The mast cells were also stained for SLPI in order to eliminate the possibility that these cells themselves produce significant amounts of this protein. To confirm mast cell migration, the lung carcinoma cells, co-cultured with mast cells for various time periods, were immunohistochemically stained with a monoclonal anti-mast cell antibody.

To block unspecific staining, the slides were incubated with 5% or 10% of normal horse serum for 20 minutes at room temperature. Polyclonal goat-anti-SLPI antibody

(produced at our laboratory) was used as primary antibody at dilutions of 1/500 and 1/1000, a monoclonal mouse anit-tryptase antibody, Mab 1254 (DAKO, Glostrup, Denmark) diluted 1/1000 was added and allowed to react for 90 min at room temperature. To identify migrated mast cells, a mouse anti-human mast cell 229; kidney juxtaglomerular CE (Swant, Switzerland) diluted 1/5000 was applied under the conditions described above. Control slides were incubated with the buffer or non-immunised mouse IgG (negative control), instead of primary antibody. The slides were washed and the second labelled antibody was applied and left for 30 min at room temperature. The slides were incubated with biotinylated rabbit anti-goat IgG antibody (5 mg/ml buffer) and then incubated with avidin DH biotinylated horseradish peroxidase (ABC) complexes. After this, the slides were washed again and stained with 0.06% 3,3'-diamino-benzidine-tetrahydrochloride (DAB) for 20 min and mounted. In addition, the control slides were incubated with specific antiserum previously adsorbed with SLPI.

Human type II lung epithelium cells, A549, cultured in HMC-1-conditioned medium

Lung epithelium, A549, cells and mast cells were cultured and prepared as described above. A549 cells were re-seeded into the six-well plates and grown till confluence. The supernatants collected from the mast cells after different periods of time (0, 3, 9 and 24 h) were added to the A549 cells for a further incubation of 24, 48, 72 and 96 h. A549 cell controls were cultured in a cell growth medium for the same length of time. SLPI levels were measured in the supernatants collected from the HMC-1 cells before and after addition to the lung carcinoma cells. These experiments were repeated four times.

Statistics

Regression coefficients were calculated for each SLPI release curve from each of the experiments. The hypothesis i.e. the differences in regression coefficient were tested with a non-parametric Wilcoxon's paired rank sum test. The Mann-Whitney U-test was calculated on the results of 24, 48, 72 and 96 h of cell culture. Results are expressed as the mean ± SD of at least four to six independent experiments. P values exceeding 0.05 were considered not significant.

Abbreviations

SLPI, secretory leukocyte protease inhibitor; MMP, matrix metalloproteinases; AAT, alpha1-antitrypsin; IL, interleukin; TNFα, tumor necrosis factor; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assays.

Authors' contributions

HC and NM, carried out the cell culture experiments and immunohistochemistry and drafted the manuscript, SJ, performed the statistical analysis and presentation of the data, described and interpreted data, UW, participated in study design, data evaluation and coordination.

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II

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Prolastin, a pharmaceutical preparation of purified human α1-antitrypsin, blocks endotoxin-mediated cytokine release

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Abstract

Background: α1-antitrypsin (AAT) serves primarily as an inhibitor of the elastin degrading proteases, neutrophil elastase and proteinase 3. There is ample clinical evidence that inherited severe AAT deficiency predisposes to chronic obstructive pulmonary disease. Augmentation therapy for AAT deficiency has been available for many years, but to date no sufficient data exist to demonstrate its efficacy. There is increasing evidence that AAT is able to exert effects other than protease inhibition. We investigated whether Prolastin, a preparation of purified pooled human AAT used for augmentation therapy, exhibits anti-bacterial effects.

Methods: Human monocytes and neutrophils were isolated from buffy coats or whole peripheral blood by the Ficoll-Hypaque procedure. Cells were stimulated with lipopolysaccharide (LPS) or zymosan, either alone or in combination with Prolastin, native AAT or polymerised AAT for 18 h, and analysed to determine the release of TNFα, IL-1β and IL-8. At 2-week intervals, seven subjects were submitted to a nasal challenge with sterile saline, LPS (25 μg) and LPS-Prolastin combination. The concentration of IL-8 was analysed in nasal lavages performed before, and 2, 6 and 24 h after the challenge.

Results: *In vitro*, Prolastin showed a concentration-dependent (0.5 to 16 mg/ml) inhibition of endotoxin-stimulated TNFα and IL-1β release from monocytes and IL-8 release from neutrophils. At 8 and 16 mg/ml the inhibitory effects of Prolastin appeared to be maximal for neutrophil IL-8 release (5.3-fold, $p < 0.001$ compared to zymosan treated cells) and monocyte TNFα and IL-1β release (10.7- and 7.3-fold, $p < 0.001$, respectively, compared to LPS treated cells). Furthermore, Prolastin (2.5 mg per nostril) significantly inhibited nasal IL-8 release in response to pure LPS challenge.

Conclusion: Our data demonstrate for the first time that Prolastin inhibits bacterial endotoxin-induced pro-inflammatory responses *in vitro* and *in vivo*, and provide scientific bases to explore new Prolastin-based therapies for individuals with inherited AAT deficiency, but also for other clinical conditions.

Background

α1-antitrypsin (AAT) is a glycoprotein, which is the major inhibitor of neutrophil elastase and proteinase 3 [1,2]. AAT is mainly produced in liver cells, but also in extrahepatic cells, such as monocytes, macrophages and pulmonary alveolar cells [3,4]. The average concentration of AAT in plasma in healthy individuals is 1.3 mg/ml, with a half-life of 3 to 5 days. AAT is an acute phase protein, and its circulating levels increase rapidly to concentrations exceeding 2 mg/ml in response to inflammation or infection [5]. Individuals with plasma AAT values below 0.7 mg/ml are considered to be AAT deficient [6,7]. Over 75 alleles of AAT have been identified to date, of which at least 20 affect either the amount or the function of the AAT molecule in vivo [6-8]. A very common deficiency allele is termed Z, which differs from the normal M in the substitution of Glu 342 to Lys [7,9,10]. This single amino acid exchange causes spontaneous polymerization of the AAT, markedly impeding its release into the circulation [11]. The retained material is associated with hepatic diseases [12], while diminished circulating levels lead to antiproteinase deficiency and higher susceptibility to elastase mediated tissue injury [13,14]. The alleles of AAT are inherited in an autosomal codominant manner [2]. Therefore, individuals heterozygous for the Z allele (MZ) have 30–40% whereas individuals homozygous for the Z allele (ZZ) have only 10–15% of normal plasma AAT levels [15-17]. Tobacco smoke and air pollution have long been recognised as risk factors for the development of chronic obstructive pulmonary disease (COPD); the only proven genetic risk factor, however, is the severe Z deficiency of AAT [18,19]. Cigarette smokers with AAT-deficiency develop COPD much earlier in life than smokers with the normal AAT genotype [8,10,11].

The pulmonary emphysema that is associated with inherited AAT deficiency is intimately linked with the lack of proteinase inhibitor within the lungs that is available to bind to, and inactivate, neutrophil elastase. On the basis of clinical observations involving patients with inherited AAT deficiency and various experimental studies, the elastase-AAT imbalance hypothesis became widely accepted as the explanation for lung tissue destruction in emphysema [20,21]. There is now increasing evidence that an excessive activity of various proteolytic enzymes in the lung milieu, including members of the serine, cysteine and metalloprotease families, may damage the elastin network of lungs [14]. Since the severe ZZ and intermediate MZ AAT deficiency accounts for less than 1–2% and 8–18% of emphysema cases, it is believed that the protease-antiprotease hypothesis provides a rational basis for the explanation of the development and progression of emphysema in general [22,23].

Based on the protease-antiprotease hypothesis, augmentation therapy of emphysema with severe AAT deficiency was introduced during the 1980s [24]. Intravenous administration of a pasteurized pooled human plasma AAT product (Prolastin; Bayer Corporation; Clayton, North Carolina) is used to increase AAT levels in deficient individuals [25]. The major concept behind augmentation therapy is that a rise in the levels of blood and tissue AAT will protect lungs from continuous destruction by proteases, particularly neutrophil elastase [26]. For example, anti-elastase capacity in the lung epithelial lining fluid has been found to increase to 60–70% of normal in homozygous Z AAT-deficient individuals subjected to augmentation therapy [26,27]. Whether this biochemical normalization of AAT levels influences the pathogenic processes of lung disease is still under debate. The most recent results, however, suggest that Prolastin therapy may have beneficial effects in reducing the frequency of lung infections and reducing the rate of decline of lung function [28,29].

There is growing evidence that AAT, in addition to its antiproteinase activity, may have other functional activities. For example, AAT has been demonstrated to stimulate fibroblast proliferation and procollagen synthesis [30], to up-regulate human B cell differentiation into IgE-and IgG4-secreting cells [31], to interact with the proteolytic cascade of enzymes involved in apoptosis [32,33] and to express contrasting effects on the post-transcriptional regulation of iron between erythroid and monocytic cells [34]. AAT is also known to inhibit neutrophil superoxide production [35], induce macrophage-derived interleukin-1 receptor antagonist release [36] and reduce bacterial endotoxin and TNFα-induced lethality *in vivo* [37,38]. We recently demonstrated, *in vitro*, that both native (inhibitory) and non-inhibitory (polymerised and oxidised) forms of AAT strongly inhibit lipopolysaccharide-induced human monocyte activation [39]. AAT appears to act not just as an anti-proteinase, but as a molecule with broader anti-inflammatory properties. Data presented in this study provide clear evidence that Prolastin, a preparation used for AAT deficiency augmentation therapy, significantly inhibits bacterial endotoxin-induced pro-inflammatory cell responses *in vitro*, and suppresses nasal IL-8 release in lipopolysaccharide-challenged individuals, *in vivo*.

Materials and Methods

α1-antitrypsin (AAT) preparations

α1-antitrypsin (Human) Prolastin® (Lot 26N3PT2) was a gift from Bayer (Bayer Corporation, Clayton, North Carolina, USA). This vial of Prolastin contained 1059 mg of functionally active AAT, as determined by capacity to inhibit porcine pancreatic elastase. Prolastin was dissolved in sterile water for injections provided by

manufacture and stored at +4°C. Purified human AAT was obtained from the Department of Clinical Chemistry, Malmö University Hospital, Sweden. Native AAT was diluted in phosphate buffered saline (PBS), pH 7.4. To ensure the removal of endotoxins, AAT was subjected to Detoxi-Gel AffinityPak columns according to instructions from the manufacturer (Pierce, IL, USA). Purified batches of AAT were then tested for endotoxin contamination with the Limulus amebocyte lysate endochrome kit (Charles River Endosafe, SC, USA). Endotoxin levels were less than 0.2 enzyme units/mg protein in all preparations used. The concentrations of AAT in the endotoxin-purified batches were determined according to the Lowry method [40]. Polymeric AAT was produced by incubation at 60°C for 10 h. Polymers were confirmed on non-denaturing 7.5% PAGE gels.

Monocyte isolation and culture

Monocytes were isolated from buffy coats using Ficoll-Paque PLUS (Pharmacia, Sweden). Briefly, buffy coats were diluted 1:2 in PBS with addition of 10 mM EDTA and layered on Ficoll. After centrifugation at 400 *g* for 35 min, at room temperature, the cells in the interface were collected and washed 3 times in PBS-EDTA. The cell purity and amount were determined in a cell counter Autocounter AC900EO (Swelabs Instruments AB, Sweden). The granulocyte fractions were less than 10%. Cells were seeded into 12-well cell culture plates (Nunc, Denmark) at a concentration of 4×10^6 cells/ml in RPMI 1640 medium supplemented with penicillin 100 U/ml; streptomycin 100 μg/ml; non-essential amino acids 1×; sodium pyruvate 2 mM and HEPES 20 mM (Gibco, UK). After 1 h 15 min, non-adherent cells were removed by washing 3 times with PBS supplemented with calcium and magnesium. Fresh medium was added and cells were stimulated with lipopolysaccharide (LPS, 10 ng/ml, J5 Rc mutant; Sigma, Sweden) in the presence or absence of various concentrations of Prolastin (0–16 mg/ml), constant concentration of native or polymerised AAT (0.5 mg/ml) for 18 h at 37°C, 5% CO_2.

Neutrophil isolation and culture

Human neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorphprep TM (Axis-Shield PoC AS, Oslo, Norway) as recommended by the manufacture. In brief, 25 ml of anti-coagulated blood was gently layered over the 12.5 ml of Polymorphprep TM and centrifuged at 1600 rpm for 35 min. Neutrophils were harvested as a low band of the sample/medium interface, washed with PBS, and residual erythrocytes were subjected to hypotonic lysis. Purified neutrophils were washed in RPMI-1640- Glutamax-1 medium (Gibco-BRL Life Technologies, Grand Island, NY) supplemented with 0.1% bovine serum albumin (BSA) and resuspended in the same medium. The neutrophil purity was more than 75% as determined on an AutoCounter AC900EO. Cell viability was > 95% according to trypan blue staining.

Neutrophils (5×10^6 cells/ml) were plated into sterile ependorf tubes. Zymosan was boiled, washed and sonicated. Opsonized zymosan was prepared by incubating zymosan with serum (1:3) in 37°C water bath for 20 min. After, zymosan was centrifuged, washed with PBS and resuspended at 30 mg/ml. Cells alone or activated with zymosan (0.3 mg/ml) were exposed to various concentrations of Prolastin (0–8 mg/ml), and native or polymerised AAT preparations (0.5 mg/ml) for 18 h at 37°C 5% CO_2. Cell free supernatants were obtained by centrifugation at 300 *g* for 10 min, and stored at -80°C until analysis

Cytokine/chemokine analysis

Cell culture supernatants from monocytes and neutrophils stimulated with LPS or zymosan alone or in combination with Prolastin, native or polymerised AAT were analysed to determine TNFα, IL-1β and IL-8 levels by using DuoSet ELISA sets (R&D Systems, MN, USA; detection levels 15.6, 3.9, and 31.2 pg/ml, respectively).

Subjects

Seven subjects (four females and three males) of 26–50 (median 38) years of age, non-smokers, non-allergic volunteers participated in the study. All subjects gave written informed consent before participation in the study. None of the subjects has a history of respiratory disease and none took any medication at the study time.

Study Design

At 2-week intervals each subject was submitted to a nasal challenge with sterile saline, LPS and LPS-Prolastin combination. All experimental sessions were done in the same room. On each provocation day, the nose was inspected and cleaned with 8 ml of isotonic NaCl. Between nasal lavages the subjects stayed in the same building and asked to keep away from known sources of nasal irritants. The night was spent in their own homes. All participants completed a symptom questionnaire. In the first session, the baseline lavage was taken after instillation to each nostril of 8 ml of sterile isotonic NaCl. In the next session, the subjects were challenged with LPS from Escherichia coli serotype 026:B6, Lot 17H4042 (Sigma-Aldrich, USA). The provocation solution was prepared prior to use. LPS was added to 8 ml of sterile 0.9% NaCl to obtain a final concentration of 250 μg/ml, and 100 μl of the provocation solution was sprayed into each nostril, using a needle-less syringe. In the third session, the subjects were first challenged with LPS, as described above, and after 30 min with 2.5 mg of Prolastin into each nostril. Lavage samples were taken with instillation to each nostril of 8 ml of sterile isotonic NaCl after 2, 6 and 24 h followed by assessment of symptoms by a questionnaire. All subject

completed a symptom questionnaire with questions about nasal and eye irritation, and throat and airway symptoms. None of the participants reported symptoms of nasal, eye or throat irritations, and no general symptoms such as muscle pain, shivering, were mentioned.

Nasal Lavage

The procedure for nasal lavage was performed according to a method described by Wihl and co-workers [41]. Each nasal cavity was lavaged separately with a syringe (60 ml) to which a plastic nasal olive was connected for close nostril fitting. To prevent lavage spilling into the throat, the subject was bent forward at an angle of 60° during the procedure. Equilibrium was maintained between the mucosal lining and the lavage fluid by injecting the saline gently into the nasal cavity and drawing it back five times into the syringe. The lavage was performed in both nostrils and samples were collected into a test tube. The samples were then centrifuged at 1750 rpm, 6°C for 10 min and immediately frozen at -80°C. The protein concentration in the lavage fluids was measured by Lowry method and IL-8 levels were determined by DuoSet ELISA sets (R&D Systems, MN, USA; detection levels 31.2 pg/ml).

Statistical Analysis

Statistical Package (SPSS for Windows, release 11.5, SPSS Inc., Chicago) was used for the statistical calculations. The differences in the means of cell culture experimental results were analysed for their statistical significance with the one-way ANOVA combined with a multiple-comparisons procedure (Scheffe multiple range test). The equality of means of experimental results in healthy volunteers were analysed for statistical significance with independent two sample t-test and repeated measures of ANOVA using the SPSS MANOVA procedure http://www.utexas.edu/cc/docs/stat38.html. Tests showing $p < 0.05$ were considered to be significant.

Results

Concentration-dependent effects of Prolastin on LPS-induced cytokine release from human monocytes

Various concentrations of Prolastin (0–16 mg/ml) were added to adherent-isolated human monocytes with or without LPS (10 ng/ml). Cells stimulated with LPS alone served as a positive control, while PBS stimulated monocytes served as negative controls. As illustrated in figures 1A and 1B, simultaneous incubation of monocytes with LPS and Prolastin resulted in a reduction in TNFα and IL-1β release compared to the cells stimulated with LPS alone. Inhibition of LPS-induced cytokine release by Prolastin was concentration-dependent and was typically observed over a concentration range of 0.5–16 mg/ml. At 16 mg/ml the inhibitory effects of Prolastin appeared to be maximal for both TNFα (10.7-fold, $p < 0.001$) and IL-1β (7.3-fold, $p < 0.001$), compared to LPS alone.

Inhibitory effects at 0.5 mg/ml of AATs on LPS-mediated IL-1β and TNFα release

We recently found that simultaneous incubation of monocytes with LPS and either the inhibitory (native) or non inhibitory (polymeric) form of AAT resulted in a reduction in TNFα and IL-1β release compared to the cells stimulated with LPS alone. At 0.5 mg/ml the effects of native and polymerised AAT appeared to be maximal (41). Therefore, we selected a 0.5 mg/ml concentration of Prolastin, native and polymerised AAT, and compared their effects on LPS-stimulated cytokine release at 18 h. As shown in figures 2A and 2B, LPS triggered a significant release of TNFα and IL-1β ($p < 0.001$ v medium alone) by monocytes. At 0.5 mg/ml, native and polymerised AAT remarkably inhibited LPS-induced TNFα and IL-1β release ($p < 0.001$) (Fig. 2). The inhibitory effect of Prolastin (0.5 mg/ml) on LPS-stimulated TNFα release was comparable in magnitude to that of native or polymeric AAT, whereas its inhibitory effect on LPS-induced IL-1β release did not reach significance.

Concentration-dependent effects of Prolastin on neutrophil IL-8 release

The effects of Prolastin (0–8 mg/ml) on human neutrophil IL-8 production are shown in Figure 3A. Neutrophils stimulated with opsonized zymosan (0.3 mg/ml) released a large amount of IL-8 ($p < 0.001$), compared to controls. Prolastin inhibited IL-8 release by neutrophils stimulated with opsonized zymosan (Fig 3A). This inhibition was concentration-dependant, with maximal suppression of IL-8 release (5.3-fold, $p < 0.001$ compared to zymosan treated cells) at 8 mg/ml.

Inhibitory effects at 0.5 mg/ml of native, polymeric AAT and Prolastin on zymosan-mediated IL-8 release

Neutrophils were stimulated with zymosan (0.3 mg/ml) or AATs (0.5 mg/ml) either alone or in combination for 18 h and IL-8 protein determined. As illustrated in figure 3B, polymeric and native AAT and Prolastin significantly inhibited the release of IL-8 protein by activated neutrophils. In terms of maximal effect, native AAT >polymerised AAT>Prolastin. It must be noted that native, polymeric AAT and Prolastin alone showed no effect on neutrophils, relative to non-treated buffer controls (data not shown).

Inhibition of the LPS-induced increase in nasal IL-8 release by Prolastin

To assess the effect of Prolastin on LPS-induced nasal provocation, IL-8 levels in nasal lavages were measured. Nasal instillation 25 μg per nostril of LPS alone or in combination with 2.5 mg/ml of Prolastin was performed in non-smoking and non-allergic volunteers (n = 7, 4 females and 3 males). The IL-8 release in response to LPS challenge increased over time compared to baseline levels

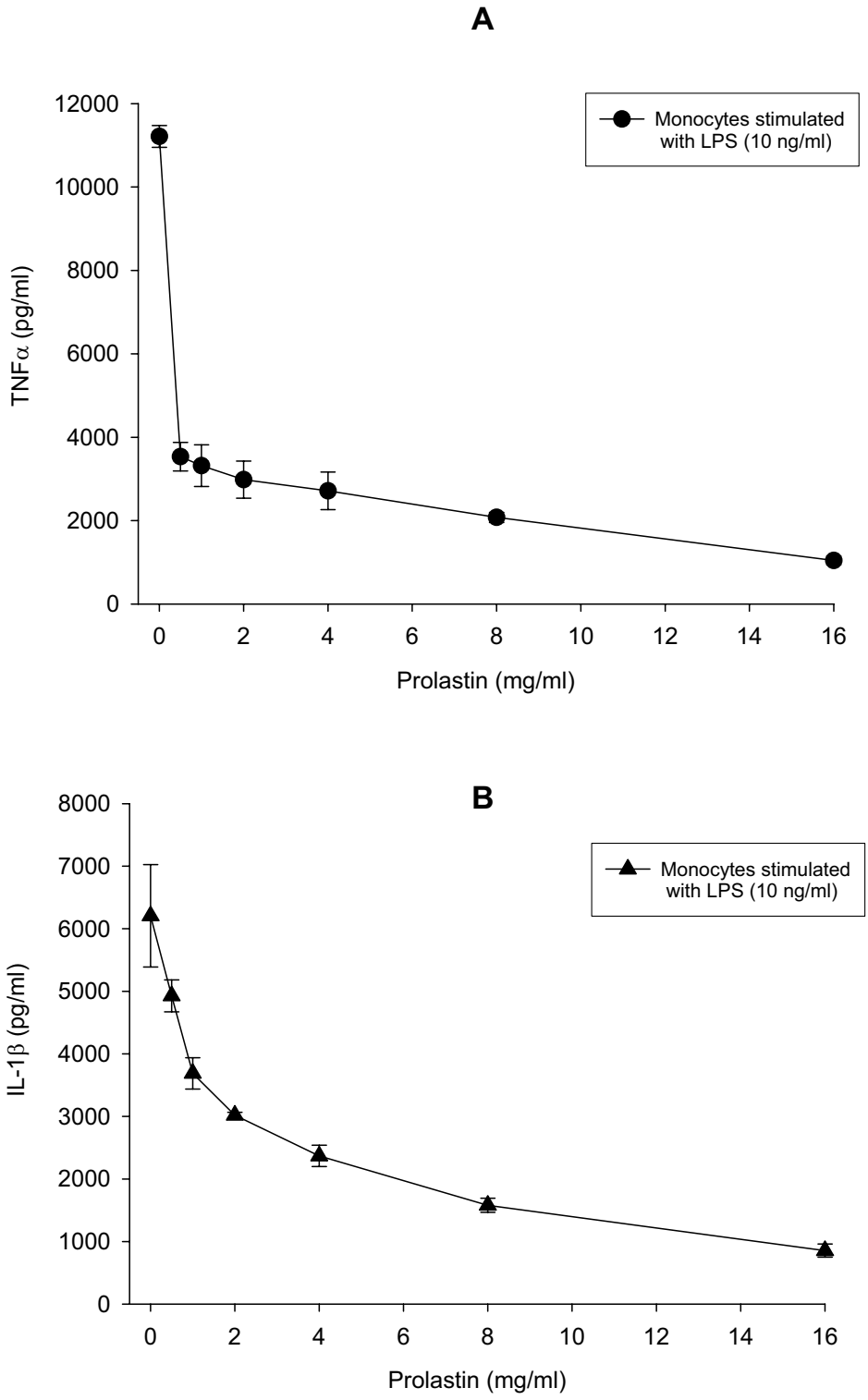

Figure 1
A concentration-response inhibition of lipopolysaccharide-stimulated TNFα (A) and IL-1β (B) release by Prolastin in human blood monocytes. Isolated blood monocytes were treated with LPS (10 ng/ml) alone or together with various concentrations of Prolastin (0–16 mg/ml) for 18 h. TNFα and IL-1β levels were measured by ELISA. Data are the means of quadruplicate culture supernatants ± S.E. and are representative of three separate experiments.

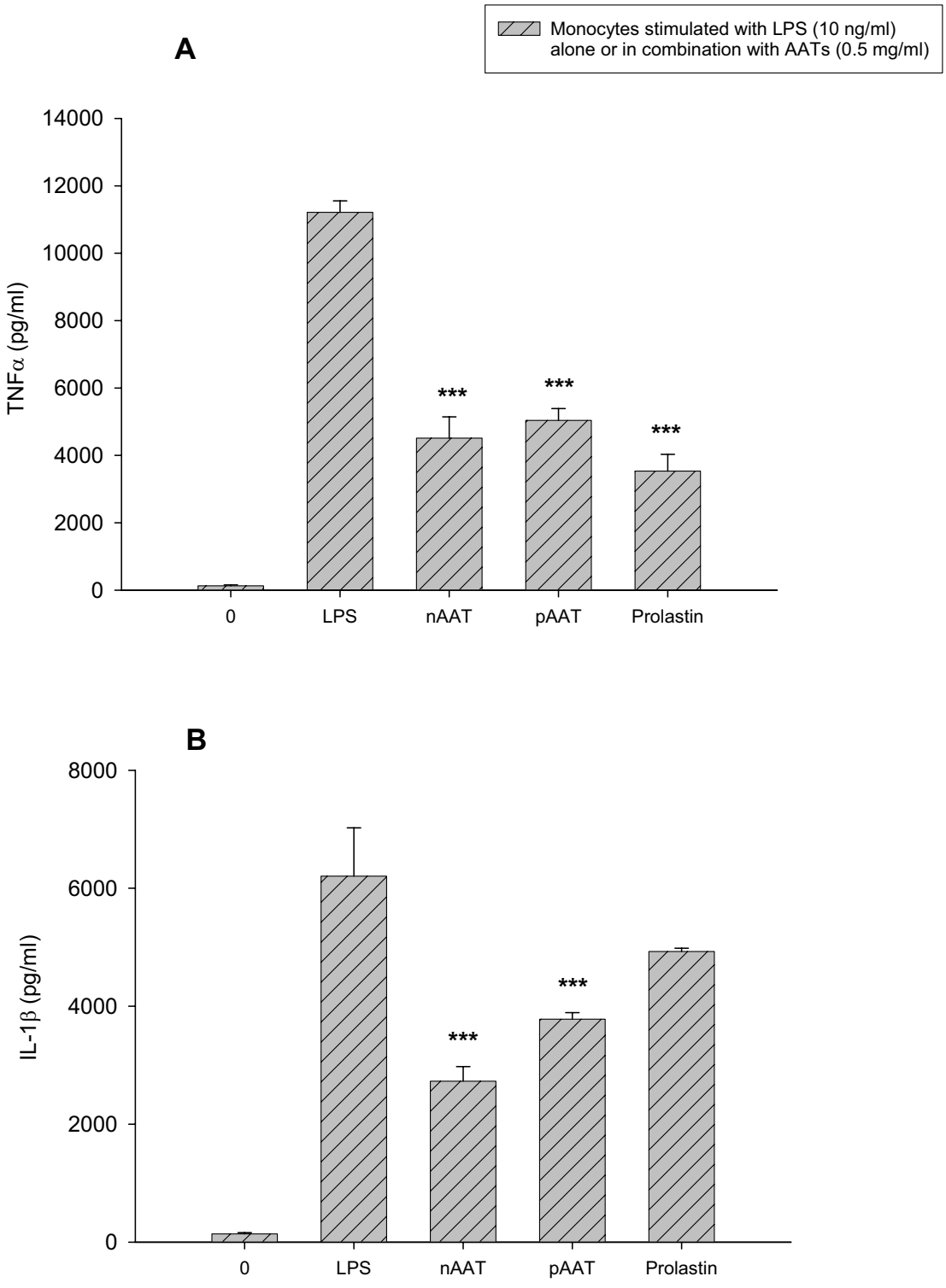

Figure 2
Comparisons of the effects of native (nAAT), polymeric (pAAT) and Prolastin on lipopolysaccharide – stimulated TNFα (A) and IL-β (B) production by human blood monocytes isolated from four healthy donors. Isolated blood monocytes were treated with LPS (10 ng/ml) alone or together with 0.5 mg/ml nAAT, pAAT or Prolastin for 18 h. TNFα and IL-1β levels were measured by ELISA. Each bar represent the mean ± S.E. *** $p < 0.001$.

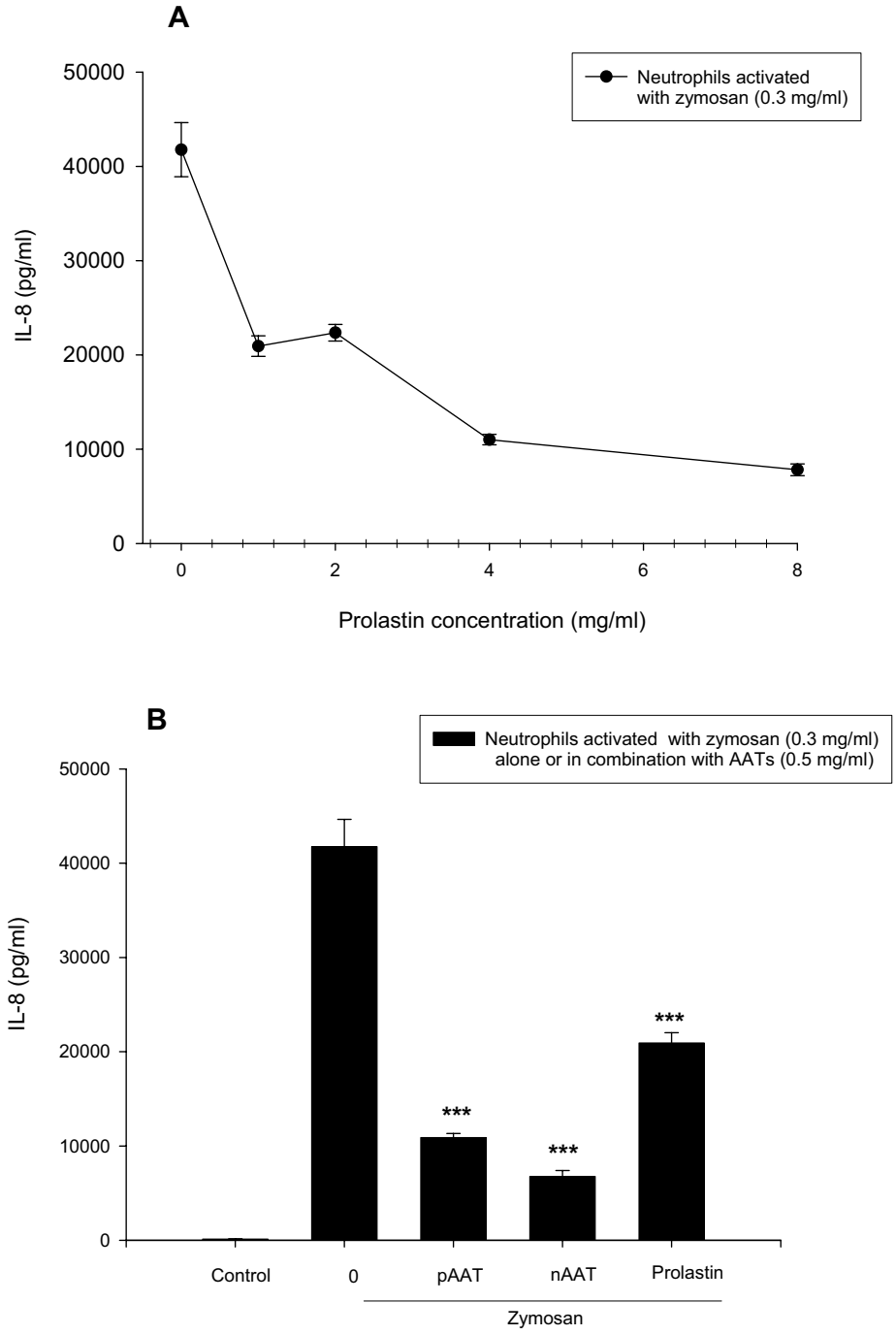

Figure 3
Effects of AATs on neutrophils activated with zymosan. (A) Concentration-dependent effects of Prolastin on IL-8 release from neutrophils activated with opsonised zymosan. Freshly isolated blood neutrophils were treated with zymosan (0.3 mg/ml) alone or together with various concentrations of Prolastin (0–8 mg/ml) for 18 h. IL-8 levels were measured by ELISA. Data are the means of quadruplicate culture supernatants ± S.E. and are representative of three separate experiments. (B) Effects of opsonised zymosan alone or together with native (nAAT), polymeric (pAAT) AAT or Prolastin on IL-8 release from neutrophils. The release of neutrophil IL-8 was measured in cell free supernatants as described in Materials and methods. Neutrophils were treated for 18 h with a constant amount of zymosan (0.3 mg/ml) alone or together with nAAT, pAAT or Prolastin (0.5 mg/ml) for 18 h. IL-8 levels were measured by ELISA. Each bar represents the means ± S.E. of three separate experiments carried out in duplicate repeats. *** $p < 0.001$

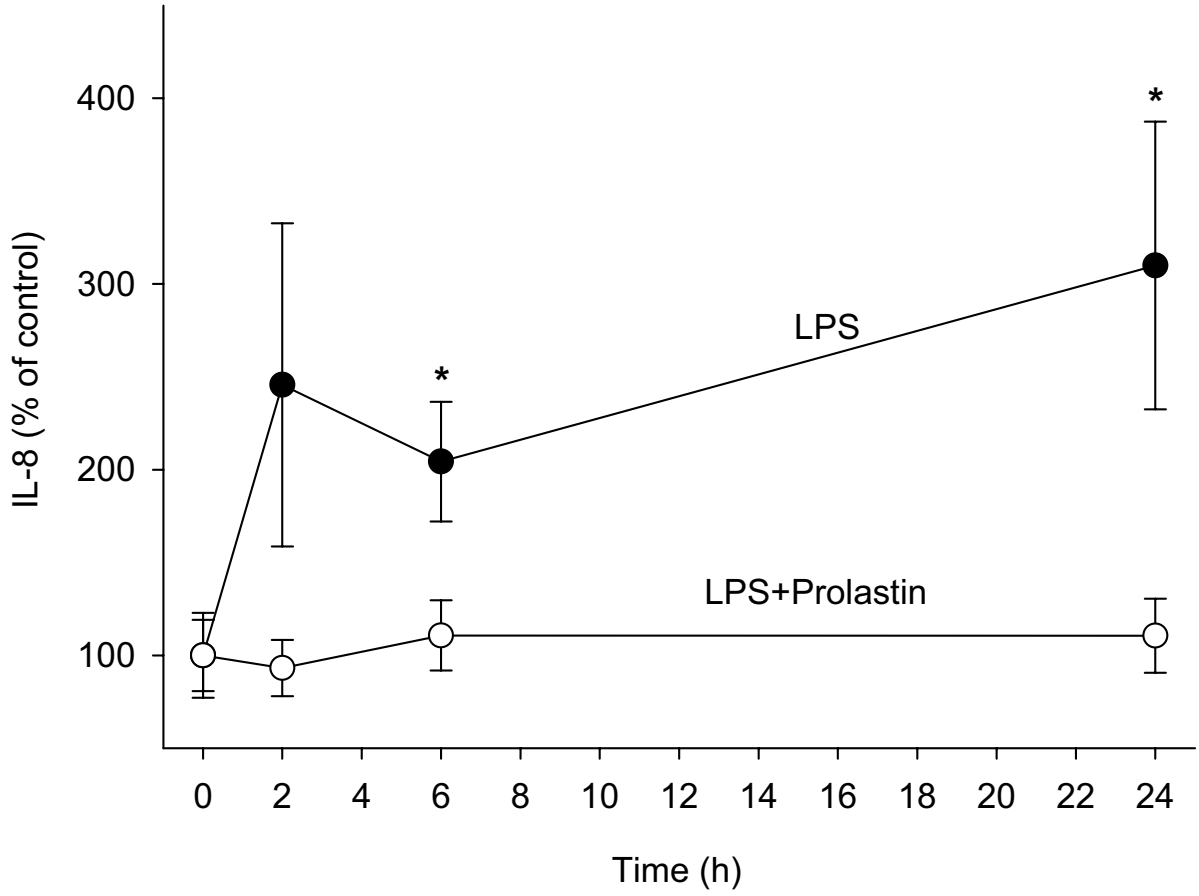

Figure 4
IL-8 analysis in nasal lavage of subjects challenged with LPS alone or LPS+Prolastin combination. Seven healthy volunteers were treated with LPS (25 μg/nostril) or with LPS followed 30 min later with Prolastin (2.5 mg/nostril), nasal lavage was collected at different time points (0, 2, 6 and 24 h) as described in Material and Methods. The concentration of IL-8 (pg/ml) was measured by ELISA. IL-8 values are expressed as a ratio of IL-8 concentration at selected time point and the basal level. Independent two sample t-test shows after 6 and 24 h significantly higher levels of IL-8 in subjects treated with LPS compared to LPS+Prolastin. * $p < 0.05$

(Fig. 4). The levels of IL-8 increased already after 2 h of LPS challenge (245.7% ± 87) and remained higher after 24 h (310 ± 77.5) compared to baseline (100% ± 19.2). By contrast, when IL-8 levels were examined in LPS-Prolastin-treated lavage samples, no significant changes in IL-8 release were observed compared to baseline. In the presence of Prolastin, the LPS effect on IL-8 release was inhibited ($p < 0.05$) (Fig. 4).

Disscussion

There is now, however, ample evidence that serine proteinase inhibitors (serpins), in addition to their well established anti-inflammatory capacity to regulate serine proteinases activity, may possess broader anti-inflammatory properties. Several studies have shown that the biological responses of bacterial lipopolysaccharide (endotoxin) *in vivo* may be sensitive to serpins. For example, the serpin antithrombin, has been shown to protect animals from LPS-induced septic shock and also to inhibit IL-6 induction by LPS [42,43]. Our recent study provided first *in vitro* evidence that native (inhibitor) and at least two modified (non-inhibitory i.e. polymeric and oxidised) forms of AAT can block the release of an array of chemokine and cytokines from LPS-stimulated

monocytes [39]. These studies therefore further support a central role of serpins in inflammation, not only as the regulators of proteinase activity, but also as the suppressers of endotoxin induced pro-inflammatory responses. In line with these findings, we demonstrate here that Prolastin, a preparation of human AAT which is used for augmentation therapy, significantly inhibits endotoxin-induced pro-inflammatory effects *in vitro* and *in vivo*.

Stimulation of human monocytes and neutrophils with bacterial endotoxin results in the release of a range of inflammatory mediators including the pro-inflammatory cytokines (*e.g.* IL-6, IL-1β and TNFα) and the chemokines (*e.g.* MCP-1 and IL-8) [44-46]. Together, these play a crucial role in the recruitment and activation of leukocytes and the subsequent release of harmful proteases that may further perpetuate the inflammatory process. We found that Prolastin significantly inhibits endotoxin-induced IL-1β and TNFα release by monocytes and IL-8 release by neutrophils *in vitro*. The Prolastin exhibited these anti-inflammatory properties in a concentration-dependent manner. Its maximal effects were observed with 16 mg/ml in the monocyte model and with 8 mg/ml in the neutrophil model, since doubling these concentrations did not significantly modify the intensity of the effects. Indeed, Prolastin markedly prevented endotoxin-induced cell activation at 0.5–4 mg/ml concentrations, implying that these lower concentrations of Prolastin might also be sufficient to inhibit endotoxin effects. It is worth noting that in order to reduce a potential risk of transmission of infectious agents the Prolastin preparation is heat-treated in solution at 60° ± 0.5 for not less than 10 h. Data from *in vitro* studies show that heat-treatment results in AAT polymerization and loss of its inhibitory activity [47,48]. Therefore, in our experimental model we compared anti-inflammatory effects of Prolastin with those of native and heat treated (60°C 10 h) AATs. At concentrations used (0.5 mg/ml), no significant difference was found between the effects of Prolastin and native or heat-treated (polymeric) AAT on endotoxin-induced monocyte TNFα and neutrophil IL-8 elevation. The median concentrations of endotoxin-stimulated IL-1β levels also decreased in the presence of Prolastin but failed to reach statistical significance. In general, inhibitory effects on endotoxin-stimulated monocyte IL-1β and neutrophil IL-8 release were better pronounced by native AAT compared to polymeric AAT or Prolastin. Similarly, in our previous study we found that in terms of maximal effect, native AAT >polymerised AAT>oxidized AAT were efficient in inhibiting LPS-stimulated TNFα and IL-1β, and IL-8 release from monocytes [39]. Further studies will be necessary to better evaluate how temperature, pH or other physicochemical challenges may influence anti-inflammatory effectiveness of AAT preparations.

To explore our hypothesis that AAT functions as a potent inhibitor of endotoxin-induced effects, we examined whether Prolastin also inhibits responses to LPS in the nasal airway, *in vivo*. In particular, we were interested in concentrations of the neutrophil chemoattractant, IL-8. Endotoxin (or LPS) from gram-negative bacteria is a common air contaminant in a number of occupational conditions, especially those in which exposure to animal waste or plant matter occurs [44,49-51]. Levels of LPS in such environments may exceed 20 μg/m^3 air and may be associated with respiratory symptoms and nasal inflammation in exposed persons [52]. For example, nasal inflammation as evaluated by an increased influx of inflammatory cells into the nasal airway and increased IL-8 levels, has been described in persons occupationally exposed to LPS [51]. Moreover, it has been suggested that constitutive levels of IL-8 might further enhance responses to an inflammatory stimulus, such as LPS [53]. A number of experimental studies have shown that a nasal instillation of LPS causes the cytokine and chemokine reaction [54,55]. In our pilot study we also showed that instilled defined amounts of endotoxin (25 μg/per nostril) induce time-dependent nasal IL-8 release in normal subjects. Two hours after LPS instillation the IL-8 levels in nasal lavage reached more than twice the basal level and remained higher during all the times studied. However, during the next session, when 30 min after challenge with LPS, Prolastin (2.5 mg/ per nostril) was instilled, no induction of nasal IL-8 release was found compared to the basal levels. Furthermore, the protective ability of Prolastin did not disappeared over study time. We cannot determine from these experiments whether Prolastin is directly suppressing IL-8 release or suppressing another inflammatory response that leads to IL-8 release; nonetheless, our finding suggests that effects of Prolastin directed against endotoxin-stimulated inflammatory responses may be beneficial.

Thus, data from both *in vitro* and *in vivo* experiments provide novel evidence that the Prolastin preparation is a potent inhibitor of endotoxin effects. The major concept behind augmentation therapy with pooled plasma-derived AAT has been that a rise in the level of AAT in subjects with severe inherited AAT deficiency would protect the lung tissue from continued destruction by proteinases (i.e. primarily leukocyte elastase) [7,56,57]. Recent findings provide evidence that augmentation therapy with AAT reduces the incidence of lung infections in patients with AAT-related emphysema [28,58]. Furthermore, Cantin and Woods have reported that aerosolized AAT suppresses bacterial proliferation in a rat model of chronic *Pseudomonas aeruginosa* lung infection [59]. Stockley and co-workers demonstrated that a short-term therapy of AAT augmentation not only restores airway concentrations of AAT to normal, but also reduces levels of leukotriene B4, a major mediator of neutrophil recruitment and

activation. Interestingly, authors have suggested that the efficacy of AAT augmentation may be most beneficial in individuals with the most inflammation [29,60]. Data presented in this study clearly show that Prolastin inhibits endotoxin-stimulated pro-inflammatory responses, and thus provides new biochemical evidence supporting the efficacy of augmentation therapy. The current findings also suggest that Prolastin may, in fact, be used for broader clinical applications than merely augmentation therapy.

Abbreviations

AAT, α1-antitrypsin; COPD, chronic obstructive pulmonary disease; LPS, lipopolysaccharide; ZZ, homozygous AAT-deficiency variant; MM, wild type AAT variant; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Authors' contribution

Izabela Nita, performed cell culture experiments, made contribution to acquisition of data;

Camilla Hollander, made substantial contribution to patient study design, material collection and analysis; Ulla Westin, contributed to study design and data interpretation; Sabina Janciauskiene, contributed to conception and study design, data interpretation and wrote the article

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IV

Plasma and bronchial lavage fluid concentrations of inflammatory markers in patients with COPD and Asthma

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Abstract

Background: Airway inflammation is associated with an increased expression and release of inflammatory reactants that regulate processes of cell migration, activation and degranulation. The purpose of this study was to quantify bronchial lavage (BAL) fluid and plasma levels of chemokine (IL-8), Secretory Leukocyte Protease Inhibitor (SLPI), soluble intracellular adhesion molecules-1 (sICAM-1) and sCD14, as surrogate markers of inflammatory and immune response in asthma and chronic obstructive pulmonary disease (COPD) patients with similar disease duration time.

Methods: Biomarkers in plasma and BAL fluid from asthma (n=13) and COPD (n=25) patients were measured using commercially available ELISA kits.

Results: We found that in asthma and COPD groups the concentrations of IL-8 and SLPI are significantly higher in BAL fluid than in plasma, while levels of sICAM-1 and sCD14 in BAL fluid are significantly lower than in plasma. Of these 4 measured biomarkers, only the BAL IL-8 was higher in COPD patients when compared to asthma ($p<0.05$). In both groups, BAL IL-8 correlated with SLPI ($r=0.577$, $p<0.01$ and $r=0.589$, $p<0.05$, respectively). In patients with COPD the BAL sICAM-1 correlated with sCD14 ($r=0.576$, $p<0.01$), while in asthma patients the plasma SLPI correlated with sCD14 ($r=0.68$, $p<0.01$), and BAL sICAM-1 correlated with FEV1/FVC ($r=0.418$, $p<0.01$).

Conclusion: Our findings point to the importance of selecting a correct biological fluid when analysing specific biomarkers, and also show the correlations between measured markers that may be useful to distinguish asthma and COPD.

Key words: asthma, COPD, inflammatory biomarkers

Introduction

Airway inflammation is a cardinal pathophysiological feature in patients with asthma and chronic obstructive pulmonary disease (COPD). Both diseases are associated with an increased expression and release of inflammatory reactants, including cytokines, chemokines, proteases and their inhibitors, and adhesion molecules that regulate processes of cell migration, activation and degranulation [1,2]. Recent studies show similar cellular responses in asthma and COPD, with raised levels of neutrophils in more severe forms of asthma [3,4] and raised counts and activation of eosinophils in COPD [5-7]. Monocytes/macrophages are a significant component of the inflammatory infiltrate in COPD [8] and several studies show that monocytes/macrophages are in an activated state in asthma and orchestrate immune reactions [9,10]. For instance, levels of CD14, a marker of monocyte/macrophage activation, [11] have been shown to be increased in asthma patients during acute asthma attacks [12-14]. IL-8 occurs at high levels in COPD and is a selective attractant of neutrophils and IL-8 levels in induced sputum are correlated with the extent of neutrophilic inflammation and with disease severity (% predicted FEV_1) [15,16]. IL-8 has also been suggested to play a role in asthma, where it is reported to be involved in lymphocyte, eosinophil and basophile activation and migration to the inflammatory site.

Adhesion molecules are important in the recruitment and migration of leukocytes from circulation to the inflammatory sites. Elevated cell surface expression of intercellular adhesion molecule-1 (ICAM-1) has been demonstrated in asthmatic airways [17,18] and increased levels of soluble ICAM-1 (sICAM-1) has been found in asthmatic sputum [19]. Whether ICAM-1 is up-regulated to the same extent in COPD remains uncertain, as the studies published so far have yielded conflicting results [18-20].

Proteases and their inhibitors are also implicated in the pathophysiology and airway pathology of COPD as well as asthma [21]. Secretory leukocyte protease inhibitor (SLPI), also known as antileukoprotease, is a naturally occurring serine proteinase inhibitor produced by mucosal epithelial cells, serous cells, and bronchiolar goblet cells in human airways [22,23]. Historically, this inhibitor was first purified from lung secretions of patients with COPD and cystic fibrosis. However, emerging evidence suggests that SLPI is also important in the pathophysiology of asthma because it exhibits broad spectrum inhibitory activity against mast cell and leukocyte serine proteases implicated in asthma pathology [24-26].

Thus, inflammation in the respiratory tract involves epithelial cells, resident macrophages, eosinophils, and neutrophils, and provides many cell-released markers that can be used to follow pathophysiological changes related to the disease. However, an understanding of the relationship between the complex array of cells and mediators involved in asthma and COPD is not yet fully dissected which makes difficult to find a specific and sensitive biomarker or a panel of biomarkers that can reflect intensity of these pathological processes and can help to predict the individual outcome. It is well known that the validation of a specific biomarker(s) requires the choice of appropriate biological medium for analysis, therefore we aimed to measure and to compare plasma and BAL fluid concentrations of SLPI, sICAM-1, IL-8 and sCD14 -as potential inflammatory markers in asthma and COPD patients.

Materials and methods

Subjects

The studied group consisted of COPD (n=25) and asthma (n=13) hospital outpatients, age >35 years from the Department of Pulmonology and Immunology, Kaunas Medical University Hospital, Lithuania. The COPD diagnosis was based on the classification of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [27]. Patients included in the study had a forced expiratory volume in one second (FEV_1) of < 80% of predicted and a

FEV_1/FVC (forced vital capacity) ratio of < 70% of predicted, classified as moderate to severe COPD (GOLD stages II-IV). Adult asthmatics with a history of asthma of six months or more were diagnosed according to the standards of the American Thoracic Society [28]. All patients were without inhaled and systemic steroids for at least one month before the study. None of the subjects showed signs of acute respiratory infection at least one month before the investigation.

The exclusion criteria were α1-antitrypsin deficiency, dyspnoea of other origin (including cardiovascular disorders, pneumonia, interstitial lung disease, pleural disease, upper airways obstruction, neuromuscular disease and anaemia) and bronchial carcinoma. Number of smoking pack-years was calculated as the product of tobacco use (in years) and the average number of cigarettes smoked per day/20 (years x cig. per day/20). The study was approved at the Regional Bioethics Committee and written informed consent was received from all participants.

Lung function testing

Pulmonary function was tested using a pneumotachometric spirometer "Custo vitM" (Custo Med, Germany) with subjects in the sitting position, and the highest value of FEV_1 and FVC from at least two technically satisfactory maneuvers differing by less than 5 % was recorded. Normal values were characterized according to Quanjer [29]. Subjects had to avoid the use of long-acting β_2-agonists for at least 48 hours and short-acting β_2-agonists for at least 8 hours prior to the test.

Bronchoscopy and bronchoalveolar lavage processing

Subjects were not allowed to drink or eat at least 4 hours prior the bronchoscopy. Smoking was not allowed at least 10 hours before the procedure. To perform BAL, the local upper

airways were anaesthetized with 5 ml of 2% Lidocaine (Grindex, Latvia). All bronchoscopic examinations were performed in the morning. The bronchoscope (Olympus, USA) was wedged into the segmental bronchus of the middle lobe and 5 boluses of 20 ml sterile saline solution was infused. Fluid was gently aspirated immediately after the infusion had been completed and was collected into a sterile container. The fluid was immediately filtered using 48 μm nylon gauze (Millipore, North Ryde, NSW, Australia) and centrifuged at -4°C for 10 minutes. Supernatants were removed and frozen in Eppendorf tubes at –70°C for further investigation. All patients underwent bronchoscopic procedures without any significant complication.

Analysis of plasma and bronchial lavage (BAL) fluid

The plasma and BAL fluid concentrations of IL-8, sICAM-1, sCD14 and SLPI were determined using commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, USA) according to the manufacturer's instructions. Assays were performed in duplicate and the optical density at 450 nm, with a background correction at 570 nm, was determined using a microplate reader (Labsystems iEMS Reader MF). The readings for each standard and sample were averaged and the average of the zero standard was subtracted. The detection limits of the IL-8, sICAM-1, sCD14-1 and SLPI kits were 31.2, 15.6, 125 and 62.5 pg/ml respectively.

Statistical Analysis

Statistical analysis was performed using the SPSS software (version 12.0.1 for Windows, SPSS Inc., Chicago, I11, USA). Normal distribution of the variables was tested using the Kolmogorov-Smirnov test and since log-transformation of non-normally distributed variables did not improve the normal distribution, non-parametric tests were used. The Kruskal-Wallis

test was used for comparisons between more than two groups and if significance was reached, the Mann-Whitney U test, with correction for multiple comparisons (Bonferroni), was used for group-wise comparisons. Correlations were investigated using the Spearman rank order correlation test. Data is presented as mean and range.

Results

Patient characteristics

Table 1 gives the demographic data, disease duration, lung function and smoking habits of the investigated asthma and COPD patients. The asthma patients were younger than the COPD group ($p=0.026$), had more females than males (69.2% vs 8.7%, respectively) and included significantly fewer smokers. As expected, COPD patients had significantly lower FEV1 ($p<0.001$) compared to asthma patients. No significant difference was found between asthma and COPD patients in disease duration time (8.6 ±2.5 vs 7.8 ±1.3 years), plasma AAT levels (1.47±5.6 vs 1.38±5.4 mg/mL) and the concentration of total BAL fluid proteins (62.9 ± 11.9 vs 52.4 ± 6.7 μg/mL)

Concentrations of IL-8 in BAL and plasma

The concentration of BAL IL-8 was significantly higher in the group with COPD than in the group with asthma (mean±SE: 0.104 ±0.03 vs 0.03± 0.01 ng/mL, $p=0.04$). No significant difference was observed between the two groups in plasma IL-8 concentrations, although the mean levels were higher in COPD than in the asthma patients (mean±SE: 0.09±0.07 vs 0.016±0.01 ng/mL). In both groups IL-8 concentrations were significantly higher in BAL fluid than in plasma (COPD $p=0.011$ and asthma $p=0.034$).

Concentrations of SLPI in BAL and plasma

As shown in table 2, we found no significant difference in BAL and plasma SLPI concentrations between the studied groups. However, SLPI concentration was higher in BAL fluid compared to plasma in both COPD (72.6 vs 40.3, statistically non significant) and asthma (64.7 vs 41.95, $p=0.04$) groups.

Concentrations of sICAM-1 in BAL and plasma

We found no significant differences between COPD and asthma groups in plasma and BAL concentrations of sICAM-1 (Table 2). In both groups plasma sICAM-1 levels were higher compared to BAL (COPD: 152.8 vs 4 pg/mL, $p<0.001$ and asthma: 161 vs 6.8, $p<0.01$).

Concentrations of sCD14 in BAL and plasma

We found no significant difference in BAL and plasma concentrations of sCD14 between COPD and asthma groups. In both groups BAL and plasma levels of sCD14 were higher in plasma compared to BAL fluid (COPD: 1531 vs 363.2 pg/mL, $p<0.001$ and asthma: 1433 vs 292, $p<0.01$, respectively).

Relationship between measured BAL and plasma biomarkers

When the patients were considered as a single group, independent of diagnosis, the concentration of BAL fluid IL-8 was found to be significantly correlated with BAL SLPI ($r= 0.572$, $p<0.01$) (Fig 1A) and BAL sICAM-1 correlated with BAL sCD14 ($r=0.442$, $p<0.01$) (Fig 1B). Moreover, plasma SLPI directly correlated with plasma sCD14 ($r=0.332$, $p<0.05$), but inversely correlated with BAL sCD14 ($r=-0.365$, $p<0.05$).

Relationship between studied biomarkers in the group with asthma

The serum concentrations of SLPI significantly correlated with serum sCD14 ($r=0.68$, $p<0.01$). Moreover, the BAL concentration of IL-8 correlated with BAL SLPI ($r=0.589$, $p<0.05$) and BAL sICAM-1 correlated with FEV1 (percent predicted) ($r=0.38$, $p<0.05$) and FEV1/FVC ($r=0.418$, $p<0.01$).

Relationship between studied biomarkers in the group with COPD

In the group with COPD, the BAL concentrations of IL-8 significantly correlated with BAL SLPI ($r=0.577$, $p<0.01$) and BAL concentrations of sICAM-1 correlated with BAL sCD14 ($r=0.576$, $p<0.01$). In addition, negative correlation was found between BAL concentration of sCD14 and serum SLPI ($r= -0.429$, $p<0.05$) and positive correlation between plasma SLPI concentration and FEV1/FVC ($r=0.408$, $p<0.05$). No association was observed between measured biomarkers and pulmonary function or smoking habit.

Discussion

Previously it has been demonstrated that IL-8 levels are increased in COPD patients [15,30], and that increased levels of IL-8 in sputum samples correlate with the airway bacterial load and myeloperoxidase released from activated neutrophils [31]. Recently, Schulz and collaborators [32] have shown that in subjects with COPD the constitutive and stimulated IL-8 release is significantly higher compared to 'healthy' smokers and control subjects. Consistent with this, we also found higher concentrations of BAL fluid IL-8 in the patients with COPD than in those with asthma. It is important to note that our patient groups differ significantly in age, gender, and smoking habits; *i.e*. the asthma group includes predominantly

non-smoking females while the COPD group is primarily smoking males. Thus, it is possible that the higher IL-8 levels in COPD patients are related to the above differences between the patient groups. It has been demonstrated that cigarette smoke induces the release of IL-8 from cultured human bronchial epithelial cells [33], and BAL specimens from non-asthmatic smokers have greater concentrations of neutrophils, macrophages, and a number of cytokines, including IL-1ß, IL-6, IL-8, and monocyte chemoattractant protein-1 than non-smokers [34]. However, we found no association between BAL fluid or plasma IL-8 levels and smoking history in the COPD group, suggesting that the mechanism for IL-8 release may depend on several factors, and not just simply on smoking alone. Furthermore, the BAL IL-8 level did not correlate with age and gender. The molecular mechanisms that trigger, maintain, and regulate the expression and release of IL-8 have not yet been fully elucidated. For instance, bacterial endotoxin and tumour necrosis factor-alpha (TNF-α) are known to increase IL-8 production by epithelial cells [35]. In addition, it has been proposed that neutrophil elastase may stimulate epithelial cells to produce more IL-8 [36]. Neutrophil elastase has also been shown to increase SLPI transcription in primary human airway epithelial cells [24,26], and we found a significant direct correlation between BAL fluid IL-8 and SLPI concentrations in both COPD and asthma groups. However, the positive correlation of SLPI and IL-8 may be independent of elastase activity, because SLPI, like IL-8, may also be up-regulated by bacterial endotoxins and cytokines such as IL-1 and TNFα [37,38]. Therefore, in order to determine whether BAL IL-8 alone or in combination with other biomarkers, such as SLPI, is a suitable biomarker to be used in COPD, better understanding of the pathological process is needed.

Inflammation in COPD and asthma extends beyond the airways [39] and higher baseline levels of several circulating inflammatory markers have been shown in COPD and asthma patients [40]. It is possible that biomarkers reflecting systemic rather than local inflammation

in the lungs may be of greater value in diagnosis. In support of this, the results of Little and collaborators [41] show that high levels of plasma intercellular adhesion molecule-1 (sICAM-1) and IL-8 in BAL fluid correlate with the development of chronic lung disease and with the severity of disease.

Adhesion of inflammatory cells to the endothelium is regulated not only by chemokines, such as IL-8, but also by at least three adhesion molecule families, including selectins, integrins and the immunoglobulin super family (ICAM-1 and vascular cell adhesion molecule-VCAM-1). It has been proposed that adhesion molecules such as sICAM-1 (CD54), are up regulated in COPD and asthma as part of ongoing inflammation, and thereby cause increased tissue entry of inflammatory cells [42,43]**.** Increased levels of sICAM-1 in plasma and BAL from patients with COPD have been reported [44]. Serum concentrations of sICAM-1 and sVCAM-1 were also found to be increased in acute asthma [45]. We found no significant difference in plasma and BAL fluid sICAM-1 concentrations between the COPD and asthma groups. However, it is important to note that the BAL fluid concentrations of sICAM-1 correlated significantly with lung function measured as FEV1 (percent predicted) and FEV1/FVC ratio in patients with asthma. This finding is difficult to interpret, but it has been shown that in untreated asthmatics, ICAM-1 is over-expressed in bronchial epithelial cells [18]. The asthma patients included in our study were untreated for at least one month prior to sample collection and were in stable condition. This may explain why sICAM-1 was found to be directly correlated to lung function in asthma patients.

In the COPD group, BAL fluid sICAM-1 correlated with soluble (sCD14), a surface protein found on activated monocytes/macrophages and neutrophils, which serves as the cellular receptor for lipopolysaccharide (endotoxin) [46]. CD14-dependant mechanisms of inflammation have been discussed in acute respiratory distress syndrome [47], sarcoidosis [48] and asthma [49]. The soluble form of CD14 (sCD14) is generated by proteolytic

shedding of the membrane-associated form (mCD14) during cellular activation and can be detected proportionately in the BAL fluid [50]. It is not clear, however, whether BAL sCD14 originates locally or represents leakage from the peripheral blood. We found significantly higher levels of sCD14 as well as sICAM-1 in plasma compared to BAL, and therefore we cannot exclude that these proteins are less valuable as biomarkers when measured in BAL fluid.

The observed differences in BAL fluid and plasma levels of measured biomarkers and correlated ratios of these markers are tempting to speculate on their potential importance in monitoring and/or distinguishing patients with COPD and asthma. In general, the methods to measure markers of airway inflammation include invasive methods, such as bronchoalveolar lavage (BAL) which will sample the proximal airways and the distal small airways as well as the alveolar spaces, and the non-invasive methods, such as the examination of blood. Many markers of inflammation in BAL fluid may have a stronger signal than the same markers in blood and *vice versa,* therefore the information gained from such assays might be difficult to interpret. For instance, in the present study we have found that in asthma and COPD groups the concentrations of IL-8 and SLPI are significantly higher in BAL fluid than in plasma while levels of sICAM-1 and sCD14 in BAL fluid are significantly lower than in plasma. It is well known that the major sites of SLPI synthesis are Clara and goblet cells of the surface epithelium [51,52], and the serous cells of the submucosal glands [53-55]. Therefore, it is reasonable to believe that analysis of plasma SLPI may only partially reflect the ongoing pathological process in the airways, and therefore too insensitive to monitor the actual state of disease. The relevance of circulating IL-8, sICAM-1 and sCD14 and their relation to locally produced in COPD and asthma remain much less clear. Thus, the applicability of these

biomarkers in respiratory diseases will much depend on the selection of a correct biological fluid when analysing them.

Authors' contributions

CH carried out the analysis, participated in the interpretation of data and helped to draft the manuscript. BS and RS collected patient material. UW helped in the interpretation of data and in the design of the study. SJ designed the study, carried out the final statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1 A and B. The relationship between the BAL fluid IL-8 and SLPI (A) and BAL fluid sCD14 and sICAM-1 (B) concentrations in study subjects (n=41).

Table 1. Patient demographics

Diagnose	Asthma	COPD
Number of patients	13	25
Females/Males	9/4	2/23
Age (years)	54.3 ± 11	64 ± 8.8
Duration of the disease (years)	8.6 ± 2.5	7.8 ± 1.3
Smoking history		
Never smokers	12	6
Smokers	1	19
FEV1% of predicted[b]	84.1 ± 25	58.3 ± 10
FVC% of predicted	91 ± 20	75 ± 17
FEV1/FVC% ratio	77.4 ± 7.7	60 ± 9

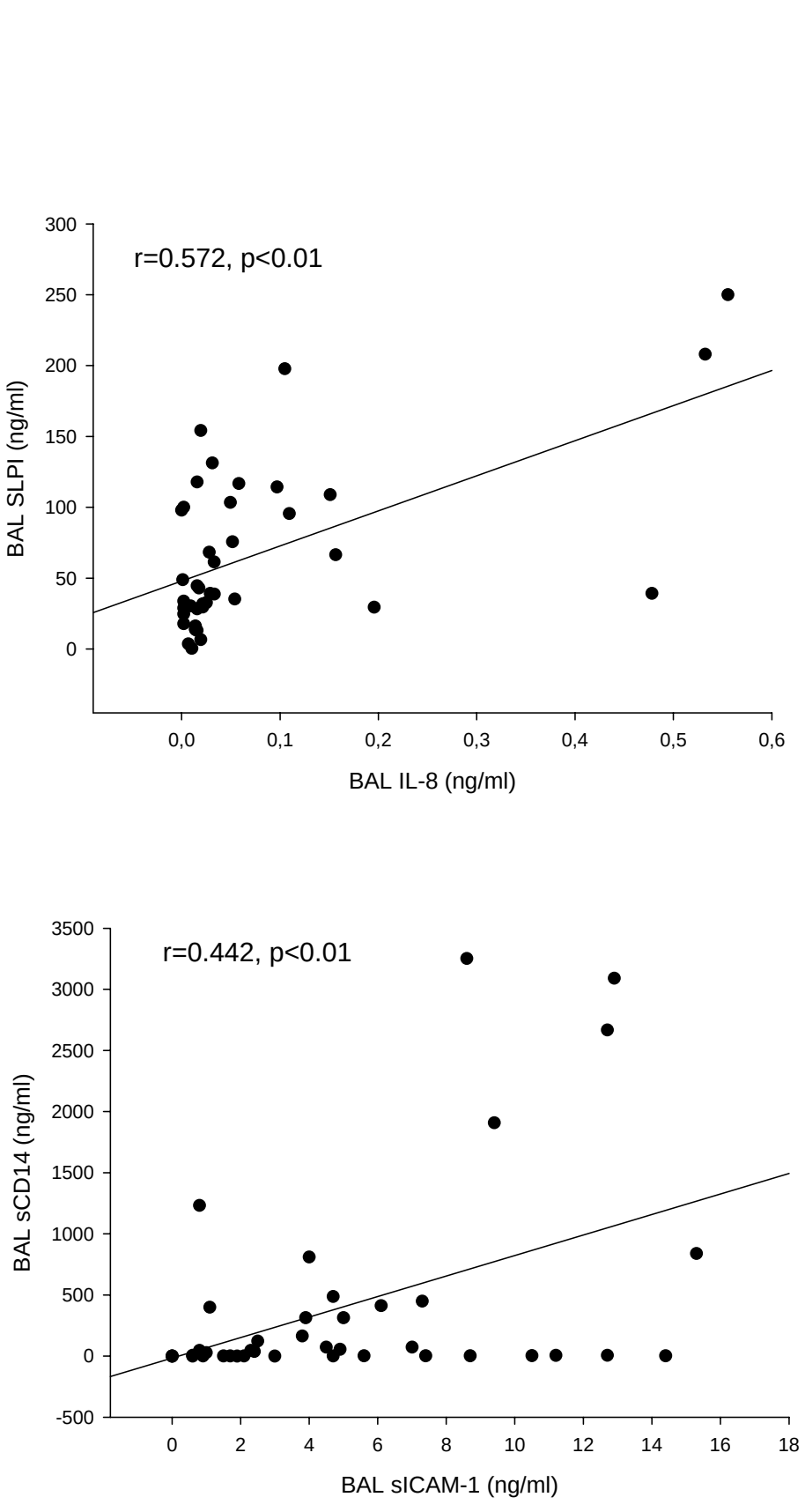
a) Data is presented as means ± SD.
b) Measured after bronchodilatation.

Table 2. Concentrations of measured variables in plasma and BAL fluid

Measured Protein (ng/ml)	COPD (n=25)		Asthma (n=13)	
	Serum	BAL	Serum	BAL
SLPI	40.34 (26.1-65)*	72.58 (0.4-250)	41.95 (28.6-60.1)	64.7 (17.9-116.8)
IL-8	0.09 (0.001-1.78)	0.104 (0.002-0.56)	0.016 (0.001-0.13)	0.03 (0-0.11)
sICAM-1	153 (92-224)	4 (0-15.3)	161 (102.4-314)	6.8 (0.9-14.4)
sCD14	1531 (750-2180)	363 (0-2667)	1433 (1065-1859)	292 (0.5-3091)

*Values are expressed as mean and (range)

Figure 1


A
r=0.572, p<0.01
BAL SLPI (ng/ml)
BAL IL-8 (ng/ml)
300
250
200
150
100
50
0
0,0
0,1
0,2
0,3
0,4
0,5
0,6
B
r=0.442, p<0.01
BAL sCD14 (ng/ml)
BAL sICAM-1 (ng/ml)
3500
3000
2500
2000
1500
1000
500
0
-500
0
2
4
6
8
10
12
14
16
18

V

Plasma levels of alpha1-antichymotrypsin and Secretory leukocyte proteinase inhibitor in healthy and COPD subjects with and without severe α1-antitrypsin deficiency

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Key words: alpha1-antitrypsin deficiency, COPD, protease inhibitors

Abstract

Background: Individuals with severe Z α1–antitrypsin (AAT) deficiency have a considerably increased risk of developing chronic obstructive lung disease (COPD). It has been hypothesized that compensatory increases in levels of other protease inhibitors mitigate the effects of this AAT deficiency. We analysed plasma levels of AAT, α1-antichymotrypsin (ACT) and secretory leukocyte protease inhibitor (SLPI) in healthy and COPD subjects with and without AAT deficiency.

Methods: Studied groups included: 71 asymptomatic AAT-deficient subjects (ZZ, n = 48 and SZ, n = 23, age 31±0.5) identified during Swedish neonatal screening for AAT deficiency between 1972 and 1974; age-matched controls (MM, n=57, age 30.7±0.6); older asymptomatic ZZ (n=10); healthy MM (n=10, age 53± 9.6); and COPD patients (ZZ, n=10, age 47.4 ± 11 and MM, n=10, age 59.4 ± 6.7). Plasma levels of SLPI, AAT and ACT were analysed using ELISA and immunoelectrophoresis.

Results: No significant difference was found in plasma ACT and SLPI levels between the healthy MM and the ZZ or SZ subjects in the studied groups. Independent of the genetic variant, subjects with COPD had elevated plasma levels of SLPI and ACT relative to healthy controls (49.7±7 vs 40.6±9 ng/ml, $p<0.001$ and 0.52 ± 0.19 vs 0.39 ± 0.11 mg/ml, $p<0.05$, respectively).

Conclusion: Our findings show that plasma levels of SLPI are not elevated in healthy young subjects with genetic AAT deficiency compared MM controls and do not appear to compensate for the deficiency of plasma AAT.

Introduction

Serine proteases play key roles in coagulation, fibrinolysis, and in kinin and complement activation. The activities of these enzymes are controlled at least in part by specific serine protease inhibitors, most of which are serpins. The relationship between serine protease and serpin inhibitor levels has been examined extensively, since an imbalance between them is linked to local tissue injury and to many pathologies, including cancer, autoimmune diseases, chronic obstructive lung disease (COPD), inflammation and infectious diseases [1-5].

The serine protease inhibitor found in highest concentration in plasma is α1-antitrypsin (AAT). Severe AAT deficiency in the homozygous Z variant, which differs from the wild type M variant in the substitution of Glu-342 by Lys, was first recognized as a hereditary condition predisposing to COPD on the basis of low plasma levels (10% of normal) of AAT [6]. This single amino acid mutation perturbs the folding and tertiary structure of AAT, leading to spontaneous polymerization and cellular retention. Thus, it is a failure in the secretion rather than biosynthesis of AAT that leads to the deficiency [6, 7]. AAT is mainly synthesized by liver cells and the Z variant of AAT is retained as inclusion bodies in the endoplasmic reticulum of hepatocytes[8]. These Z-AAT polymers are hypothesized to be cytotoxic and cause liver damage with variable clinical presentation ranging from neonatal cholestasis to liver cirrhosis and hepatocellular carcinoma in adults [9-11].

Individuals with severe AAT deficiency have at least a 20-fold increased risk of developing lung disease, especially if they smoke [12]. AAT is the most important inhibitor of neutrophil elastase and proteinase 3, and it was suggested that proteases from activated neutrophils, inadequately regulated by AAT, cause destruction of lung tissue [13]. Other serine protease inhibitors, such as α1-antichymotrypsin (ACT), α2-macroglobulin (α2MG), antithrombin and antiplasmin, also play important roles in controlling serine protease activity.

While these latter inhibitors are produced mainly by the liver and reach tissues by passive diffusion [14], other serine protease inhibitors, such as Secretory Leukocyte Proteinase Inhibitor (SLPI) and elafin/SKALP, are produced locally by airway epithelial cells [15, 16]. It was proposed that SLPI, as a major anti-elastase inhibitor of the bronchi, is important for protecting the respiratory epithelium [17, 18]. In contrast to AAT, SLPI blocks elastin-bound elastase in the alveolar walls, which might also protect against the development of COPD [19].

It has been hypothesized that compensatory increases in other protease inhibitors and/or decreased leukocyte activity may reduce the severity of AAT deficiency by favourably affecting the overall protease/protease-inhibitor balance in AAT-deficient individuals[20-22]. To further test this hypothesis that compensating increases of other protease inhibitor concentrations occur in cases of genetically caused AAT deficiency, we determined plasma AAT, ACT and SLPI levels in different age groups of healthy and COPD adult patients with and without AAT deficiency.

Materials and Methods

Study Subjects

The first study group included 71 asymptomatic, 31 year old, AAT-deficient subjects : ZZ, n = 48, 24 females and 24 males, age 31.1± 0.52 years and SZ, n = 23, 12 females and 11 males, age 31± 0.58 years, identified in the Swedish neonatal screening study during 1972-1974 [20-22]. Clinical examination, spirometry, and routine blood tests were performed by a chest physician at the local hospitals. An age-matched control group MM (n=57, 33 females and 24 males, age 31.7± 0.6 years) was recruited from the general population living in the south of Sweden. No significant differences in lung function (FEV_1 and FEV_1/FVC%) was found among the studied subject groups with ZZ, SZ and MM variants of AAT.

The second study group consisted of 20 healthy MM AAT adults (11 males and 9 females, aged 53± 9.6 years), 10 asymptomatic ZZ AAT adults (5 males and 5 females, aged 53± 9.6 years) and 20 COPD patients, among them 10 patients with ZZ AAT (5 males and 5 females, aged 47.4 ± 11 years) and 10 patients with MM AAT (5 males and 5 females, aged 59.4 ± 6.7 years). COPD was diagnosed according to the NHLBI/WHO Workshop guidelines[23]. The ZZ and MM COPD patients had a forced expiratory volume in one second (FEV1) ≤80% of that predicted and a FEV1/Forced vital capacity ratio (FVC) ≤70%, while asymptomatic ZZ individuals had normal lung function. All COPD patients included in the study were in a stable, non-exacerbated phase of the disease. The exclusion criteria were liver diseases, vasculitic or other extra-pulmonary diseases. The control subjects showed no evidence of any disease and had no respiratory symptoms; none of them was on medication and all had MM variant and normal plasma concentration of AAT. The ZZ individuals were recruited from the Swedish AAT Deficiency Register. The MM COPD individuals were outpatients at the Department of Respiratory Medicine, University Hospital, Malmo. The healthy volunteers were recruited from the hospital staff and their relatives. All individuals gave a signed, informed consent to take part in this study, which has been approved by the research ethical committee of Lund University, Sweden.

Blood sample collection

Blood was taken by venipuncture, plasma was directly separated by centrifugation and stored at -20ºC or –80°C until assayed. AAT-phenotyping was performed by isoelectric focusing at the Department of Clinical Chemistry, University Hospital.

Assay of SLPI

Secretory leukocyte proteinase inhibitor (SLPI) was analysed using ELISA kits (R&D Systems Europe Ltd, Abingdon, UK) according to the manufacturer's instructions. Absorbance was measured spectrophotometrically at 450 nm using a microplate reader (Labsystems). The minimum detectable level of SLPI was 0, 0625 ng/ml.

Quantitative analysis of ACT and AAT

The plasma concentration of ACT and AAT was determined by the rocket immunoelectrophoresis method based on a quantitative estimation of proteins by electrophoresis in 1% agarose gel containing monospecific antibodies against ACT or AAT at a concentration of 5.5 μg per square gel area [23]. The ACT concentration was quantified by counting the height of the rocket-shaped precipitation zone. For the calibration curve a standard plasma Seronorm™ with a known concentration of ACT and AAT was used.

Statistical analysis

The statistical package SPSS for Windows (release 11.5, SPSS Inc., Chicago) was used for statistical calculations. Differences in the means were analysed for their statistical significance with the one-way ANOVA combined with a multiple-comparisons procedure (Scheffe multiple range test). The equality of means was analysed for statistical significance with an independent two sample t-test and Pearson correlation analysis. Tests showing $p < 0.05$ were considered to be significant. Data are expressed as mean ± SD.

Results

To test the hypothesis that circulating levels of other serine protease inhibitors are elevated in the AAT-deficiency state and may compensate for the deficiency of AAT, we measured plasma levels of AAT, ACT and SLPI in both healthy and COPD adult patients with and

without AAT deficiency. We analysed these inhibitors in two groups of individuals, the first group consisting of 31 year old asymptomatic AAT deficiency individuals identified by the Swedish neonatal AAT screening study, and the second consisting of older asymptomatic and COPD AAT deficiency subjects from the Malmö AAT Deficiency Register. Age and gender matched individuals with wild type MM AAT were used for comparison.

As expected, plasma levels of AAT in 31 year old subjects were ranked: MM>SZ>ZZ and are significantly different among the groups [$F(2/125)=216$, $p<0.001$] (Table 1).

It is noteworthy that AAT levels were similar in all asymptomatic ZZ AAT cases independent of age (0.27±0.05 mg/ml, n=48, mean age 32 years and 0.2±0.03 mg/ml, n=10, mean age 53 years), while ZZ COPD patients had 25% higher plasma AAT levels compared to asymptomatic ZZ cases, although this was not statistically significant. We found no significant difference in plasma levels of SLPI and ACT between deficiency and normal AAT subjects in the absence of COPD (Table 2A). For the COPD cases (Table 2B), the only difference between normal and AAT deficiency populations is a marginally (within standard deviation) higher plasma ACT level in the cases with normal AAT compared to those with AAT deficiency [$t(17)=2.5$, $p<0.05$)].

We also compared levels of ACT and SLPI in healthy and COPD individuals independent of the genetic variant of AAT. As shown in Table 3, levels of both protease inhibitors were found to be elevated in COPD patients relative to healthy subjects (ACT $t(19.4)=2.8$, $p<0.05$ and SLPI $t(167)=4.2$, $p<0.001$). Previous studies have also demonstrated increased levels of ACT, SLPI and α2-MG in COPD [24-27].

Discusion

The potentially damaging effects of high levels of serine proteases at a site of injury are controlled by protease inhibitors. The α2-MG, ACT and AAT are systemic inhibitors

synthesized in the liver [28], while SLPI and elafin/ESI/SKALP are potent elastase inhibitors produced at the site of injury as a first wave of local, inducible defence in the protease inhibitor network [15, 29]. AAT plays a critical role in inactivating elastase released by activated airway neutrophils, thereby suppressing inflammation and protecting airway tissue from enzyme-induced damage [30, 31]. ACT, another inflammatory serine proteinase inhibitor, also protects the lung against proteolytic attack. Point mutations in the ACT gene that result in plasma deficiency are associated with COPD [32]. In contrast to AAT or ACT deficiency, no polymorphisms have been reported for SLPI or elafin. Therefore, the question remains open as to whether a deficit in either SLPI or elafin contributes to the development of COPD in patients who otherwise have sufficient levels of AAT and ACT.

Reports published from the prospective follow-up study of ZZ and SZ individuals up to age 26 years, focusing on clinical health, lung and liver function tests and plasma markers of the protease/protease inhibitor balance, have shown that ZZ and SZ subjects had significantly higher plasma concentrations of α2-MG, ACT and antithrombin III [20] at age 8 and 18 compared with MM control subjects. Recent findings at age 26 for ZZ and SZ subjects with normal lung function and only marginal deviations in liver test results showed significantly higher plasma SLPI levels, but not α2-MG, compared to age matched healthy MM subjects [20]. In contrast, at age 31, we find in this study that ZZ and SZ individuals have no significant difference in plasma SLPI and ACT levels compared to MM controls. The higher plasma α2-MG, SLPI and ACT levels reported in AAT deficiency subjects at younger ages were previously attributed to an unidentified compensatory mechanism that protects lung tissue against proteolytic injury under conditions of AAT depletion. Our results show that this differential is not observed at age 31 and older, and raise the question of whether the

marginal differences in protease inhibitor levels observed at younger ages between AAT deficient subjects and normal are of clinical importance.

The increased protease inhibitor plasma levels in young AAT deficiency subjects relative to normal subjects could be linked to age rather than to a ZZ or SZ phenotype. For instance, α2-MG levels in cord blood and in young children have been found to be very high and to fall fairly rapidly from age 15 to 20 years, and to continue to decline gradually until the age of 30 to 40 [33-35]. Similar measurements in another study found concentrations of α2MG in normals to be high in youth, reach their minimum in middle age, and gradually increase with old age [36]. It has been shown that plasma α2MG levels in ZZ AAT subjects dropped from 310% at age 8 years to 215% at 18 years and to normal 100% levels at adult age. This pattern tracked that observed in MM AAT control 8-year olds in whom the plasma concentrations of α2-MG and α2-antiplasmin were found to be increased up to 252% and 125% of adult levels (100% corresponding to 2.5 mg/ml and 70 μg/ml, respectively)[20] and to decline to normal at adult age. These data indicate an inherent elevation of α2-MG in the young that is not exclusive to the AAT deficient population.

It is also important to point out that, for example, when subjects were 26 years old, but not 18 and 31 years old, SLPI was found to be significantly higher in AAT-deficient subjects than in wild type controls. These inconsistent findings during the follow-up studies suggest that plasma may not be the relevant biological fluid to measure SLPI levels.

Our data show small increases in ACT and SLPI, as well as AAT, concentrations in normal MM subjects with COPD relative to well subjects. These differences exceed those for the same parameters for ZZ subjects with and without COPD and suggest that the COPD disease state could confound conclusions drawn from comparative studies seeking to attribute phenotypes (elevated protease inhibitor levels) to genotypes (ZZ, ZS AAT deficiency).

Conclusion

We conclude that there is no clear evidence for compensatory up-regulation of protease inhibitors in subjects with inherited AAT deficiency. Thus, the age related changes in circulating levels of protease inhibitors and their likely impact on individual susceptibility to lung disease remains to be confirmed through further studies.

Authors' contributions

CH carried out the analysis, participated in the interpretation of data and helped to draft the manuscript. EP and TS collected patient material. UW and AW helped in the interpretation of data and in the design of the study. SJ designed the study, carried out the final statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations

AAT, alpha-1-antitrypsin; PiZZ, homozygous AAT-deficiency variant; PiMM, wild type AAT variant; ACT, alpha-1-antichymotrypsin; COPD, chronic obstructive pulmonary disease; α2-MG, alpha2-macroglobulin; SLPI, Secretory leukocyte proteinase inhibitor; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity;

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Table 1. Plasma levels of serine protease inhibitors in 31 year old asymptomatic AAT-deficiency and age and gender matched wild-type AAT subjects.

AAT-variant	N	F/M	AAT (mg/ml) Mean ± SD	ACT (mg/ml) Mean ± SD	SLPI (ng/ml) Mean ± SD
ZZ	48	24/24	0.27 ± 0.05	0.41 ± 0.11	40.2 ± 9.6
SZ	23	12/11	0.64 ± 0.14	0.39 ± 0.08	40.7 ± 8.7
MM	57	33/24	1.44 ± 0.43	0.38 ± 0.08	41.0 ± 7.6

Table 2. Plasma levels of serine protease inhibitors in older healthy (asymptomatic) and COPD subjects with and without severe Z AAT deficiency.

Healthy (asymptomatic) subjects

AAT-variant	N	AAT (mg/ml) Mean* ±SD	N	ACT (mg/ml) Mean ± SD	N	SLPI (ng/ml) Mean ± SD
ZZ	10	0.20 ± 0.03	10	0.40 ± 0.20	9	44.0 ± 15
MM	20	1.37 ± 0.20	15	0.37 ± 0.15	13	38.2 ± 9.9

COPD subjects

AAT-variant	N	AAT (mg/ml) Mean* ±SD	N	ACT (mg/ml) Mean ± SD	N	SLPI (ng/ml) Mean ± SD
ZZ	10	0.32 ± 0.17	10	0.42 ± 0.12	10	48.5 ± 8.4
MM	10	1.73 ± 0.22	9	0.62 ± 0.21	9	51.1 ± 6.1

N-subject number

Table 3. Plasma levels of serine protease inhibitors in healthy and COPD subjects independent of genetic variant of AAT

Subjects	Number	ACT (mg/ml) Mean* ± SD	Number	SLPI (ng/ml) Mean ± SD
Healthy	153	0.39 ± 0.11	150	40.6 ± 9.1
COPD	19	0.52 ± 0.19	19	49.7 ± 7.3