Serine and Cysteine Proteases and Their Inhibitors as Antimicrobial Agents and Immune Modulators

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Abstract Proteases are not merely restricted to digestive purposes and remodeling of extracellular matrix and tissues, but are also key factors for the induction of physiological immune responses. This induction can be direct, through the degradation of pathogens within phagolysosomes, or indirect, through the activation of key pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). Unfortunately, excess production of proteases leads to maladaptive host responses and excess tissue inflammation and damage. Although the mechanisms described here will apply to a variety of different organs, we will deal chiefly with processes occurring in the lung, in pathological conditions such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). To combat these deleterious effects of proteases, the host fortunately produces antiproteases, which directly counteract the proteolytic activities of proteases. In addition to this "straightforward" effect, novel "defensin-like" activities for these molecules are clearly now emerging, as it has recently been demonstrated that protease inhibitors can themselves help in restoring tissue homeostasis by inducing innate and adaptive responses, such as through their interaction with dendritic cells (DCs).

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N. Vergnolle and M. Chignard (eds.), *Proteases and Their Receptors in Inflammation*, Progress in Inflammation Research, DOI 10.1007/978-3-0348-0157-7_2,

Keywords Adjuvant • Antiproteases • Asparagine endopeptidase (AEP) • Dendritic cell (DC) • Elafin • Immune responses • Inflammation • Macrophage • Neutrophil elastase (NE) • Proteases • Secretory leukocyte protease inhibitor (SLPI) • Toll-like receptor (TLR)

1 Introduction

Proteases are classified on the basis of catalytic mechanism, and five known distinct classes are described: metallo, aspartic, cysteine, serine, and threonine. In humans, metallopeptidases are extremely diverse as they encompass 24 families, whereas cysteine proteases are represented by 19 families, serine proteases 17, and aspartic and threonine peptidases are represented by three families. For further generic information about this "degradome," we refer the reader to recent reviews including [1] and [2].

Until recent times, the action of proteases was believed to be restricted to digestive purposes, extracellular modeling and/or remodeling of tissues, mainly through proteolytic activity on interstitial molecules, occurring throughout homeostasis and development or, in aberrant maladaptive circumstances, during disease pathogenesis. This view has clearly become untenable as proteases are clearly involved in a myriad of homeostatic as well as pathological processes. Similarly, several novel physiological functions have been attributed to endogenous antiproteases including antimicrobial and immunomodulatory activities.

We will discuss in this chapter the actions of proteases and antiproteases on physiological immune induction and inflammatory processes, as well as proteases-driven maladaptive responses. Although the mechanisms described here will apply to a variety of different organs, we will deal chiefly with processes occurring in the lung, as the protease/antiprotease balance in other tissues will be addressed by other contributors in this issue.

2 Toll-Like Receptors and Dendritic Cells in the Induction of Immune Responses

Mucosal surfaces are the first barriers against infections and their role is paramount in the prevention of systemic dissemination of pathogens. To perform this role in an unchallenged naive host, the latter uses both innate and adaptive immunity. The innate immune system is genetically programmed to detect invariant features of invading microbes. In contrast, the adaptive immune system, which is composed of T and B lymphocytes, employs antigen receptors that are not encoded in the germline but are generated de novo in each organism. Thus, adaptive immune responses are highly specific. The best-characterized microbial sensors are the so-called PRRs of the innate immune system, which detect relatively invariant

molecular patterns found in most micro-organisms [3]. These structures are referred to as pathogen-associated molecular patterns (PAMPs). Microbial pathogens are recognized through multiple, distinct PRRs that can be broadly categorized into secreted, transmembrane, and cytosolic classes. The transmembrane PRRs include the TLR family and the C-type lectins. TLRs in mammals are either expressed on the plasma membrane or in endosomal/lysosomal organelles [4]. Cell-surface TLRs recognize conserved microbial patterns that are accessible on the cell surface, such as lipopolysaccharide (LPS) of gram-negative bacteria (TLR4), lipoteichoic acids of gram-positive bacteria and bacterial lipoproteins (TLR1/TLR2 and TLR2/TLR6), and flagellin (TLR5), whereas endosomal TLRs mainly detect microbial nucleic acids, such as double-stranded RNA (dsRNA) (TLR3), single-stranded RNA (ssRNA) (TLR7), and dsDNA (TLR9) [5–8].

Innate immune cells bearing TLRs include DCs, macrophages, and neutrophils, among others. DCs are crucial immune cells detecting micro-organisms and linking innate to adaptive immunity. TLR signaling is linked to MyD88- and TRIF-dependent signaling pathways that regulate the activation of different transcription factors, such as nuclear factor (NF)-κB. Specific interaction between TLRs and their ligands activates NF-κB resulting in enhanced inflammatory cytokine responses, induction of DC maturation (e.g., upregulation of CD40, CD80, CD83, and CD86) and chemokine receptors (e.g., CCR7) [9]. These features have for a long time indicated that, in particular, TLR triggering switches the immature DC phenotype to an inflammatory phenotype that is capable of inducing adaptive immune responses, instructing both antigen-specific $CD4^+$ and $CD8^+$ T-cell responses and humoral responses.

2.1 Role of TLR9 in Inflammation and Immunity

Some studies suggest a role for TLR9 in the triggering of innate immune response to protozoan parasites as well as for some bacteria and viruses. For example, TLR9 is required for the development of the Th1-type inflammatory responses that follow oral infection with *Toxoplasma gondii* in mice from some inbred strains and is also implicated in the control of parasitemia during infection with *Trypanosoma cruzi*. The hemozoin pigment of *Plasmodium* or some parasite DNA associated with the pigment results in signaling through TLR9. More recently it has been shown that the early natural killer (NK) cell response to infection with *Leishmania donovani* was dependent on the secretion of IL-12 by myeloid DCs triggered in response to TLR9 stimulation [10]. TLR9-deficient ($TLR9^{-/-}$) mice have been recently described to be more susceptible to infection with *Leishmania major*. DCs lacking TLR9 failed to be activated by *L. major* probably suggesting that the DNA of *L. major* is a TLR9 ligand. Furthermore, *L. major*-infected $TLR9^{-/-}$ DCs were unable to stimulate $CD4^+$ T cells [11]. TLR9 ligands are known to be ssDNA carrying unmethylated CpG motifs [12]. A vast array of data indicates that TLR9 plays a key role in DNA-induced immunity and links it with a role in acquired

immunity through the activation of various cell types such as plasmacytoid DCs (pDCs), conventional DCs (cDCs), and B cells. Analysis of TLR9$^{-/-}$ mice revealed that TLR9 is essential not only for proinflammatory cytokines production and other inflammatory responses but it also plays a role in the induction of Th1 acquired immune response and in the proliferation of B cells. In addition, TLR9 also recognizes bacterial and viral DNA. In particular, TLR9 cooperates with TLR2 to induce innate immune response against *Mycobacterium tuberculosis*. TLR9 also plays an important role in the fight against infections with *Brucella*, *Streptococcus pneumoniae*, and could be involved in recognition and clearance of *Helicobacter*. TLR9-mediated antiviral responses are largely documented. Indeed, mouse cytomegalovirus, herpes simplex virus type 1 and 2, and adenovirus are recognized by TLR9 on pDCs which produce high amount of interferon (IFN)-α in response to this stimulation. Recently, natural DNA repetitive extragenic sequences from *Pseudomonas aeruginosa* have been shown to strongly stimulate TLR9 [13]. In addition, signaling through TLR9 appears to be important in *P. aeruginosa* keratitis, and silencing TLR9 signaling reduces inflammation but contributes to decreased bacterial killing in the cornea [14].

3 Role of Proteases in the Induction of Immunity

3.1 Cysteine Proteases

Cysteine proteases were historically shown to have an important role in antigen presentation and the induction of immunity [15]. They are constitutively expressed in most cell types, especially in macrophages and DCs. They contain a cysteine thiol as part of their catalytic site and are related to papain and belong to the C1 family. Among them, cathepsins B, C, F, L, H, K, L, S, V and W have been isolated. Some of these enzymes are endopeptidases, whereas others are either amino or carboxy exopeptidases (see Table 1). Another endopeptidase named asparagine

Table 1 Lysosomal proteases

Cathepsin	Location	Family	Cleavage pattern	Phenotype/function
B	Lysosomes	Cysteine	Carboxypeptidase	Lysosomal apoptosis pathway and tumor spreading
C	Endo/lysosomes	Cysteine	Aminopeptidase	Serine protease activation
F	Lysosomes	Cysteine	Endopeptidase	Ii processing
K	Lysosomes	Cysteine	Endopeptidase	TLR9 signaling
L(V)	Lysosomes	Cysteine	Endopeptidase	CD4 and NK T cells tymic selection
S	Endo/lysosomes	Cysteine	Endopeptidase	MHC class II pathway, Ii chain processing
X(Z)	Endo/lysosomes	Cysteine	Carboxypeptidase	T-cell migration
D,E	Lysosomes	Aspartic	Endopeptidase	Lysosomal storage, early cell death
AEP	Endo/lysosomes	Cysteine	Asparagine sites	MHC class II pathway, cathepsins maturation and TLR processing

endopeptidase (AEP) or legumain is unrelated to the papain-like cysteine protease family such as cathepsin B and L and is grouped together with the caspases, separases, and some bacterial proteases in clan CD [16–18]. Most of these enzymes are synthesized as precursors and targeted to the endocytic pathway. For example, the N- and C-terminal propeptides of AEP are auto-cleaved in the lysosomal compartments to generate a 46 kDa mature form, which can be further processed into a 36 kDa fragment [19].

Acidic pH is a prerequisite for maturation of most of these enzymes and so their greatest activity is found in lysosomal compartments. Their main function is to provide ligands for the MHC class II-restricted antigenic pathway. MHC class II molecules access the endolysosomal compartments to bind peptides and display them on the surface of DCs to trigger $CD4^+$ T-cell response. Indeed, the uptake of exogenous antigen into DCs is followed by protease-mediated degradation in endolysosomal compartments. These proteases also process the invariant chain (Ii), a chaperone molecule which associates with MHC class II molecules in the endoplasmic reticulum (RE). Cathepsin L and cathepsin S are the best characterized proteases to proteolyse Ii [20]. The endolysosomal proteases have probably a redundant role in the selection of the peptides which will be presented at the DCs surface. However, there are examples where some antigens require a particular protease. Indeed, AEP is unique among lysosomal cysteine proteases, in that it is insensitive to leupeptin and cleaves on the carboxyl terminal sides of asparagine residues. AEP initiates the processing of tetanus toxin in human B cells, destroys an immuno-dominant peptide of myelin basic protein (MBP – an autoantigen implicated in the autoimmune disease multiple sclerosis) and performs the early steps of degradation of the Ii chain in human B-EBV cells [21–23].

3.2 Asparagine Endopeptidase, TLR7/9 Pathway and Antigen Presentation in DCs

DCs are heterogeneous and consist of various DC subsets among which TLR expression and function differ. pDC is a DC subset which differs from cDC and can produce vast amounts of type I interferon upon bacterial and viral infection. pDCs only express TLR7 and TLR9. Thus, pDCs can be regarded as a DC subset specialized for detecting nucleic acids mainly through TLR7/9. In mice, cross-presentation has been considered a unique property of cDCs. This crucial mechanism in microbial immunity allows exogenous antigen to be delivered into the MHC class I pathway to initiate cytotoxic T-cell response. However, recently, it has been shown that stimulation by TLR 7/9 also licences pDCs to cross-present [24].

Little is known about how endosomal TLRs and their ligands are targeted to the endocytic pathway. TLRs are sensitive to chloroquine, a lysomotropic agent that neutralizes acidic compartments indicating a role for endo/lysosomal proteases for their signaling. Indeed, recent findings have described the importance of proteolysis

for TLR9 function [25, 26]. It has recently been shown that mouse TLR9 is non-functional until it is subjected to proteolytic cleavage in the endosomes. Upon stimulation, full-length TLR9 is cleaved into a C-terminal fragment which is highly dependent on AEP in DCs. A recruitment of TLR9 and a boost in AEP activity, which was induced shortly after TLR9 stimulation, was shown to promote TLR9 cleavage and correlated with an increased acidification in endosomes and lysosomes. Moreover, mutating a putative AEP cleavage site in TLR9 strongly decreases its signaling in DCs suggesting perhaps that a direct cleavage of TLR9 by AEP is required for this process. These results demonstrated that TLR9 requires a proteolytic cleavage for its signaling and identified a key endocytic protease playing a critical role in this process in DCs [27]. Interestingly, in contrast, TLR9 processing does not rely on AEP in macrophages probably because of the already highly acidic milieu found in the endocytic pathway of macrophages in comparison to DCs, thus allowing many proteases (and not only AEP) such as cathepsins B, L, K and S to perform TLR9 degradation [25, 27] and thus, TLR9 proteolysis has been proposed to restrict receptor activation to endosomal/lysosomal compartments and to prevent TLRs from responding to selfnucleic acids. Other endosomal TLRs, and in particular TLR7, are also probably subjected to a similar proteolytic maturation but this remains to be fully investigated (unpublished data).

Several studies have suggested that intracellular TLRs can be targeted directly from the ER, where they reside, to endosomes in which they signal. Relatedly, mouse and human genomic studies have identified UNC93B1, which encodes for a 12-membrane spanning molecule highly conserved in the ER, as a key regulator in the transport of endosomal TLRs. The third mutation (UNC93B mutation) results in a phenotype where no signaling occurs via the intracellular TLRs 3, 7 and 9 and also diminishes presentation of exogenous antigen [28, 29]. However, the exact role played by UNC93B1 in these processes remains to be fully elucidated.

4 Proteases and Maladaptive Inflammation

Proteases produced by inflammatory cells such as neutrophils and macrophages play a crucial role in the first line of defense against invading bacteria, fungi and protozoa, either by directly killing pathogens or by inducing immune recognition, e.g., via TLRs. Individuals with cyclic neutropenia, a disease characterized by mutations in the gene encoding neutrophil elastase (NE), commonly experience recurrent bacterial infections, highlighting their critical importance in this respect. Neutrophils contain at least four types of granules: azurophil granules, specific granules, gelatinase granules, and secretory granules [30, 31]. In addition to proteases, these granules are an important reservoir of other antimicrobial proteins, such as defensins, and components of the respiratory burst oxidase [32]. It has also been suggested that these granules contain a wide range of membrane-bound receptors (e.g., CD11b/CD18 [33] and *N*-formyl-methionyl-leucyl-phenylalanine

[fMLP] receptor) for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation [30, 32]. In addition to these molecules, a novel antimicrobial mechanism for neutrophils has recently been described, with the demonstration that neutrophils form neutrophil extracellular traps (NET) that could potentially bind, disarm and kill pathogens extracellularly [34–37]. DNA is the major structural component of NETs and it provides the backbone on which the proteinaceous effectors such as proteases are anchored to [34].

Although all of the effects described above are beneficial to the host, chronic and persistent presence of neutrophils is a hallmark of lung pathologies such as COPD and CF. There is certainly an excess of neutrophil chemoattractants such as IL-8 and leukotriene B_4 (LTB_4) recovered in bronchoalveolar lavage (BAL) fluid of these patients [38, 39]. Bacteria present in high concentrations in these pathologies also provide additional chemoattractants for neutrophils. Furthermore, neutrophils may survive longer in the airways of CF/COPD patients because of the production of excess concentrations of granulocyte macrophage-colony stimulating factor (GM-CSF) and the relative lack of IL-10, which, when present, promotes neutrophil apoptosis [38–41]. Moreover, cleavage of the phosphatidylserine receptor (PSR) and CD14 by NE could specifically disrupt phagocytosis of apoptotic neutrophils by macrophages [42, 43]. On the other hand, the decreased mucociliary clearance in CF/COPD leads to longer retention of apoptotic neutrophils causing them to necrose, hence releasing their toxic agents, e.g., NE, into the affected airways. In turn, NE contributes to the vicious circle of chronic inflammatory airway disease by inducing mucin production in airway epithelial cells [44–46]. Mucins, normally beneficial in microbial infections, by binding and removing bacteria via the mucociliary ladder, can be detrimental in chronic pathologies, by clogging the airways and providing an appropriate milieu for bacterial growth and colonization [47]. NE also reduces ciliary beat frequency resulting in marked disruption of epithelial cells [48], and induces goblet cell metaplasia which is dependent on its proteolytic activity [49–52].

In addition to the direct deleterious effect of proteases (such as NE) on innate immune effectors, these mediators also have a negative effect on immune cells such as DCs. For years, the nature of the elusive lung DCs was poorly understood, but with increasing interest in the role of adaptive immunity in the pathophysiology of human CF, COPD and emphysema, interest in further characterization of specific DC subsets in normal and diseased lungs arose [53–55]. In that context, we and others have shown that NE could be instrumental in the elicitation of this breach in host defense, through its action on DCs. Indeed, we demonstrated that NE is able to disable mature DC function by reducing the level of DC surface costimulatory molecules (CSMs), interfering both with the ability of immature DCs to mature in response to bacterial LPS and by reducing the allostimulatory activity of these cells, resulting in reduced Th1 cytokine production [56]. Similarly, neutrophils and culture supernatants of unprimed/primed neutrophils are able to downregulate human monocyte-derived DCs allostimulatory function in vitro [57]. This effect was associated with the amount of NE released by neutrophils, which in turn

converted immature myeloid DCs into transforming growth factor (TGF)-β1-secreting cells [57]. These in vitro observations are further supported by an earlier report showing that APCs isolated from BAL fluid of CF patients were unable to present antigen and stimulate T-cell responses [58], despite appropriate responses from systemic APCs (monocytic cells). However, although the characteristics and functional properties of lung DCs can be easily studied in animal models, very few and in most cases contradictory data from their human counterparts are currently available [55].

4.1 Neutrophil Elastase

Human NE is a serine protease found in the azurophil granules of the neutrophil. The highly cationic glycoprotein product contains 218 amino acids and four disulfide bridges, and is a member of the serine protease family [59]. The catalytic site of the NE molecule is composed of the triad His41-Asp99-Ser173, in which the γ-oxygen of serine becomes a powerful nucleophile, able to attack a suitably located carbonyl group on the target substrate [60]. Neutrophils release NE upon exposure to various cytokines and chemoattractants, including tumor necrosis factor (TNF)-α, interleukin (IL)-8, C5a, LPS, and a tripeptide derived from bacterial wall fMLP [61]. The concentration of NE in neutrophils exceeds 5 mM [62], and each neutrophil contains approximately 400 NE-positive granules. Although NE is most abundant in neutrophils, small amounts are expressed by monocytes and T cells [63, 64]. NE has broad substrate specificity and is capable of degrading a wide range of extracellular matrix proteins, including elastin, collagen (types I–IV), fibronectin, laminin, and proteoglycans. Additionally, many biological molecules like cytokines and their receptors contain putative cleavage sites for neutrophil serine proteases. Indeed, as expected, many receptors, cytokines and other molecules have been found to be natural substrates for NE (Table 2).

Like the cysteine protease family described above, NE possesses potent microbicidal activity and is speculated to assist with phagocytosis of pathogens by activated neutrophils [65]. To determine the contribution of NE in combating bacterial infections, NE-deficient ($NE^{-/-}$) mice were generated [62] and shown to be more susceptible to sepsis and death following intraperitoneal infection with gram-negative (*Klebsiella pneumoniae*, *P. aeroginosa*, and *Escherichia coli*) but not gram-positive (*Staphylococcus aureus*) bacteria. NE is required for maximal intracellular killing of *P. aeruginosa* by neutrophils, as it degrades the major outer membrane protein F, a protein with important functions, including porin activity, maintenance of structural integrity, and sensing of host immune system activation [66]. In addition, in vitro incubation of NE with *E. coli* leads to a loss of bacterial integrity and lysis of bacteria [62]. Indeed, the primary sequence of outer membrane protein A (OmpA) amino acid has multiple NE-preferred cleavage sites and NE was shown to directly degrade purified OmpA of *E. coli* in vitro [62]. Furthermore, NE degrades virulence factors of enterobacteria such as *Salmonella enterica* serovar

Table 2 Summary of the expanding list of natural neutrophil elastase (NE) substrates

Target	Hypothetical biological function	References
Receptors		
Proteinase-activated receptor-1 (PAR-1)	Inactivation, modulation of response	[76, 150]
PAR-2	Inactivation, modulation of response	[150–152]
PAR-3	Inactivation, modulation of response	[153]
IL-2Rα	Inhibiting cellular response and prolongation of cytokine half-life time	[154]
TNF-RII	Inhibiting cellular response and prolongation of cytokine half-life time	[155]
C5aR (CD88)	Inhibition of chemotaxis, feedback mechanism	[156]
CR1 (CD35)	Inhibition of complement signaling	[157]
Urokinase R (CD87)	Regulation of cell migration	[158]
Granulocyte-colony stimulating factor receptor (G-CSF-R)	Growth inhibition	[159, 160]
CD43 (sialophorin)	Regulation of adhesion	[161, 162]
CD14	Inhibition of LPS-mediated cell activation/apoptotic cell recognition	[163]
CD2, CD4, and CD8	Impairment of T lymphocytes	[164]
CD40, CD80, and CD86	Impairment of DCs	[56]
Soluble IL-6 receptor	Regulation of inflammation	[165]
CXC chemokine receptor 1 (CXCR1)	Regulation of cell migration	[166]
Cytokines/chemokines		
TNF-α	Regulation of inflammation	[167]
IL-2	Regulation of inflammation	[63]
IL-6	Regulation of inflammation	[168]
IL-8	Regulation of inflammation	[169]
IL-12p40	Regulation of inflammation	(unpublished)
G-CSF	Growth inhibition	[159]
Integrins/others		
Intercellular adhesion molecule-1 (ICAM-1)	Regulation of adhesion	[170, 171]
Vascular endothelium cadherin	Regulation of adhesion	[172]
Proepithelin	Regulation of wound healing	[173]
Tissue factor pathway inhibitor (TFPI)	Regulation of coagulation and intravascular thrombus growth	[174]
Matrix metalloprotease-9 (MMP-9)	Regulation of proteolysis	[175]
Tissue inhibitor of metalloprotease-1 (TIMP-1)	Regulation of proteolysis	[175]
Basic fibroblast growth factor (bFGF)	Regulation of angiogenesis	[176]
Vascular endothelial growth factor (VEGF)	Regulation of angiogenesis	[176, 177]
Laminin-332 (laminin-5)	Regulation of cell migration	[178]
Surfactant protein D (SP-D)		[179]

(continued)

Table 2 (continued)

Target	Hypothetical biological function	References
	Regulation of inflammation/innate immunity	
Insulin receptor substrate-1 (IRS-1)	Regulation of cell growth	[180]
von Willebrand factor (VWF)	Regulation of cell hemostasis	[181]
Cut homeobox 1 (CUX1)	Regulation of gene expression	[182]
Plasma factor XIII (FXIII)	Regulation of coagulation	[183]
AlphaIIb β3	Regulation of adhesion	[184]

Typhimurium, *Shigella flexneri*, *Yersinia enterocolitica* and *Streptococcus pneumoniae* [67]. Thus, in the absence of NE these bacteria escape from the phagolysosome leading to their increased survival in the cytoplasm of infected neutrophils [68]. Finally, NE is able to suppress flagellin transcription in *P. aeruginosa*. Flagellin suppression by NE could elucidate how and why CF patients undergo cyclical exacerbations of the inflammatory lung disease caused by *P. aeruginosa*. When neutrophil numbers and thus NE concentrations are low, *P. aeruginosa* may proliferate, assemble a flagellum, and release flagellin, stimulating a robust inflammatory response in the patient's airways [69].

4.1.1 NE Signaling Activity

It has been suggested that NE signals via the cell surface membrane-bound TLR4 [70], by activating the NF-κB signaling pathway [71–73]. A more recent study, however, proposed that IL-1R1/MyD88 signaling and inflammasome activation, but not TLRs, are critical for NE-induced lung inflammation and emphysema in murine models [74]. Additionally, NE has been reported to induce apoptosis, thus contributing to the pathogenesis of inflammatory injury in the respiratory tract. NE-induced apoptosis of lung epithelial cells is mediated by a proteinase-activated receptor-1 (PAR1)-triggered pathway involving activation of NF-κB and p53, and a PUMA- and Bax-dependent increase in mitochondrial permeability leading to activation of distal caspases [75, 76].

4.2 Endogenous Protease Inhibitors

To modulate the multiple activities of proteases (including NE), either beneficial, but also potentially deleterious (see above), the body synthesizes antiproteases. We will concentrate our discussion on NE inhibitors, as other inhibitors will be described in this issue by other contributors. These NE inhibitors can be broadly classified into two groups, the "alarm" and the "systemic" antiproteases. Systemic antiproteases, such as α1-protease inhibitor (α1-PI), are produced mainly by hepatocytes. However, during infection, the activity of locally produced mucosal

alarm antiproteases such as SLPI and elafin may add an extra edge to the host defense armamentarium, as will be discussed below (reviewed in [77]and [78]).

4.2.1 Alarm Antiproteases

SLPI and elafin alarm *antiproteases* have been isolated and characterized under a variety of names in adult and fetal tissues [78]. They belong to the family of whey-acidic protein (WAP) proteins and are produced by epithelial cells and cells of the immune system. Importantly, alarm antiproteases are generated locally in areas of infection or neutrophil infiltration and are upregulated by pathogen- and inflammation-associated factors, including cytokines and NE itself [79]. In addition to their antiprotease properties, and because of their biochemical characteristics (heavily disulphide-bonded, low molecular mass cationic peptides, present at mucosal sites), elafin and SLPI have recently been proposed to possess "defensin/cathelicidin-like" properties [77, 78, 80].

Elafin

Elafin was simultaneously isolated from the skin of psoriatic patients [81, 82] and from the sputum of COPD subjects [83, 84]. Elafin gene was cloned and sequenced by Saheki and colleagues in 1992 [85] and by Sallenave and Silva in 1993 [86], and shown to code for a 117-amino acids protein, of which the first 22 amino acids represent a hydrophobic signal peptide. Elafin is produced as a 9.9-kDa full-length non-glycosylated cationic protein composed of an N-terminal "cementoin" domain which facilitates transglutaminase-mediated cross-linkage on to polymers or extracellular matrix components and a globular C-terminus, containing the protease inhibitor moiety [87]. The elafin molecule shares ~40% homology with SLPI and has been shown to be a more specific inhibitor of proteases than SLPI, since it inhibits NE, porcine pancreatic enzyme, and proteinase 3 [83, 88, 89], but does not inhibit cathepsin G, trypsin, or chymotrypsin [83, 88].

The regulation of elafin expression during inflammation has been well studied. In vitro, bronchial and alveolar epithelial cells produce little elafin protein, but the quantity of elafin recovered from the supernatant can be greatly enhanced by addition of the inflammatory cytokines IL-1 and TNF-α [79]. These cytokines induce similar increases in expression of elafin from keratinocytes in vitro [90]. The c-jun, p38 mitogen-activated protein (MAP) kinase, and NF-κB pathways are thought to be implicated in the elafin response to inflammatory cytokines [91–93]. Of note, the cytokine-mediated increase in elafin production by epithelial cells is greater than the increase in SLPI production [79]. Hence, whereas SLPI has been described as providing a baseline antiprotease shield and can be isolated from bronchial lavage samples from healthy individuals [94–96], elafin might be of greater significance during an inflammatory challenge to the lungs. In keeping

with this notion, elafin mRNA expression in bronchial epithelial cells is increased by free NE, which is found in abundance at times of inflammation [97, 98].

Although inhibition of NE activity has historically been considered to be the primary role of elafin, recent work has highlighted further properties of this cationic molecule. Simpson and colleagues [99] demonstrated that elafin has antibacterial activity against gram-negative *P. aeruginosa* and gram-positive *S. aureus*, and further established that, while antiprotease activity resides exclusively in the C-terminus, the majority of antimicrobial activity of elafin resides in its N-terminal domain [99]. In support of these findings, supernatants of *P. aeruginosa* could induce elafin production in human keratinocytes, and elafin inhibits growth of *P. aeruginosa* in vitro, but not *E. coli* [100, 101]. Further, adenovirus (Ad)-mediated augmentation of human elafin in murine lungs was shown to protect the lungs against *P. aeruginosa*-mediated injury, and also reduced bacterial numbers. Similarly, overexpression of elafin using the Ad-strategy dramatically improved the clearance of *S. aureus* in vitro and in vivo [102]. In these studies, concomitant anti-inflammatory activities have been demonstrated, which can probably be explained by an inhibition of the AP-1 and NF-κB pathways [103, 104]. More recently, using wild-type and CD14 knockout mice, Wilkinson and co-workers demonstrated the opsonic activity for elafin against *P. aeruginosa*, both in vitro and in vivo [105]. In an extension of these data, there is evidence that elafin binds both smooth and rough forms of LPS in vitro and could potentially modulate immune responses depending on the microenvironment [106].

We have also shown that elafin exhibits chemotactic activity for leukocytes locally in the lung [107, 108], while, conversely, downregulating inflammation systemically [108]. In keeping with this immunomodulatory activity, we demonstrated that overexpression of elafin in murine lungs results in a higher number of $CD11c^{+}/MHCII^{+}$ DCs with an activated phenotype, as evidenced by expression of higher levels of co-stimulatory molecules CSMs (CD80 and CD86), and higher levels of Th1-biased cytokines IL-12p40, TNF-α, and IFN-γ in their broncholaveolar (BAL) fluids [109].

Secretory Leukocyte Protease Inhibitor

Secretory leukocyte protease inhibitor (SLPI) is an 11.7-kDa protein that was first isolated from human parotid gland secretions [110]. SLPI orthologs have also been demonstrated in mice, rats, pigs, and sheep [111–113]. It is a non-glycosylated, highly basic, acid-stable, cysteine-rich, 107-amino acid, single-chain polypeptide [110]. The tertiary structure of the SLPI molecule resembles a boomerang, with each arm carrying one domain [114]. The four-in-each-domain disulfide bridges formed between the cysteine residues, as well as the two-domain interaction, contribute to the conformation and efficacy of the molecule [115]. SLPI provides a significant component of the human antiprotease shield within the lung. Through its C-terminal domain, SLPI gives significant protection against proteases, such as NE and the serine protease cathepsin G [116]. SLPI is produced by various

inflammatory cells, such as neutrophils [117], mast cells [118], and macrophages [119]. It is estimated that SLPI is present at concentrations of 0.1–2 μg/ml in BAL fluid [120, 121] and 2.5 μg/ml in nasal secretions [122].

It is believed that SLPI also shields the tissues against inflammatory products by downregulating the macrophage responses against bacterial LPS. Patients with sepsis have elevated circulating SLPI levels and LPS is the key mediator in bacterial endotoxic shock [96, 123, 124]. LPS seems to induce SLPI production by macrophages directly or by way of IL-1β, TNF-α, IL-6, and IL-10 [125, 126]. SLPI, like elafin, in turn inhibits the downstream components of the NF-κB pathway by protecting the inhibitor of NF-κ (I-κB) from degradation by the ubiquitin-proteosome pathway [103]. SLPI is believed to enter cells, becoming rapidly localized to the cytoplasm and nucleus where it affects NF-κB activation by binding directly to NF-κB binding sites in a site-specific manner [127]. Thus, SLPI renders macrophages unable to release pro-inflammatory cytokines and nitric oxide [125]. These data have been confirmed by in vivo studies demonstrating that SLPI knockout mice show increased susceptibility to endotoxic shock, and macrophages and B lymphocytes from the same mice show increased activation after administration of LPS [128].

In addition to its NE inhibitory and immunomodulatory activities, SLPI, like elafin, possesses broad-spectrum antibactericidal, antiviral, and antifungal properties [115, 129–134].

The Systemic Antiprotease α1-Protease Inhibitor

The systemic antiprotease α1-PI (also called α1-antitrypsin) is a 52-kDa secreted glycoprotein and is the prototypic member of the serine protease inhibitor (serpin) superfamily of proteins, which has a major role in inactivating NE and other proteases, such as cathepsin G and proteinase 3. Although some epithelial surfaces and cells of the immune system may produce small quantities of systemic antiproteases, such as α1-PI [135, 136], these inhibitors are produced primarily by hepatocytes [137, 138]. The production of α1-PI by alveolar macrophages is upregulated by pro-inflammatory cytokines and bacterial LPS [139]. Also, the cytokine oncostatin M is a major inducer of α1-PI in bronchial epithelial cells [135, 140].

The importance of α1-PI in the lung has historically been inferred from genetic studies: α1-PI deficiency is a genetic disorder that affects about 1 in 2,000–5,000 individuals. α1-PI deficiency is characterized by a decrease in levels of secreted α1-PI, which results in early-onset of emphysema in affected individuals. Although it was originally believed that genetic emphysema was caused by this decreased secretion of α1-PI in the respiratory tract, leading to unopposed and prolonged NE activity [141], recent evidence suggests that the mutated Z variant of α1-PI, when polymerized, may be pro-inflammatory when secreted, acting as an important chemoattractant for neutrophils in the α1-PI-deficient lung and adding to the excessive neutrophil and NE burden [137, 142].

In addition to its role as an antiprotease, like elafin and SLPI, α1-PI possesses important pleiotropic anti- or pro-inflammatory properties, depending upon the conditions. These effects include blocking of the pro-inflammatory effects of human NE [143, 144], and regulating expression of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-1β, and monocyte chemotactic protein (MCP)-1 by monocytes [145, 146]. Both the native and polymerized forms of α1-PI have been shown to possess similar effects as monocyte stimulators, with pro-inflammatory effects at low doses, and anti-inflammatory activities at physiologically normal doses [145]. This strengthens the concept that some of the apparently contradictory effects of these inhibitors reported in the literature may be due to differences in dosage between experimental protocols.

Lastly, α1-PI could also inhibit alveolar cell apoptosis in vivo [147]. Thus direct inhibition of active NE [75] and caspase-3 [148] by α1-PI may represent a novel anti-apoptotic mechanism relevant to disease processes characterized by excessive structural cell apoptosis, oxidative stress, and inflammation in the airways [149].

5 Conclusions

Here, we have described the important role of proteases in immune functions, not only in the direct degradation of micro-organisms and antigen presentation, but also in the induction of inflammatory responses. We have also discussed the importance of protease inhibitors in the modulation of maladaptive responses caused by extracellularly released proteases. Finally, we described novel bioactivities of elastase inhibitors, such as antimicrobial and adjuvant-like functions. These latter functions are likely to be exploited further for the treatment of individuals prone to developing CF and COPD, especially to combat frequent episodes of lung infections, either in a therapeutic (antimicrobial activity) or prophylactic (vaccination) fashion.

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http://www.springer.com/978-3-0348-0156-0

Proteases and Their Receptors in Inflammation
Vergnolle, N.; Chignard, M. (Eds.)
2011, XIV, 310 p., Hardcover
ISBN: 978-3-0348-0156-0
A product of Springer Basel