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Protean proteases: at the cutting edge of lung diseases

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ABSTRACT   Proteases were traditionally viewed as mere protein-degrading enzymes with a very restricted spectrum of substrates. A major expansion in protease research has uncovered a variety of novel substrates, and it is now evident that proteases are critical pleiotropic actors orchestrating pathophysiological processes. Recent findings evidenced that the net proteolytic activity also relies upon interconnections between different protease and protease inhibitor families in the protease web.

In this review, we provide an overview of these novel concepts with a particular focus on pulmonary pathophysiology. We describe the emerging roles of several protease families including cysteine and serine proteases.

The complexity of the protease web is exemplified in the light of multidimensional regulation of serine protease activity by matrix metalloproteases through cognate serine protease inhibitor processing. Finally, we will highlight how deregulated protease activity during pulmonary pathogenesis may be exploited for diagnosis/prognosis purposes, and utilised as a therapeutic tool using nanotechnologies.

Considering proteases as part of an integrative biology perspective may pave the way for the development of new therapeutic targets to treat pulmonary diseases related to intrinsic protease deregulation.

Introduction

Proteases represent almost 2% of the human genome, comprising 565 members [1, 2]. Based on their different catalytic mechanisms, five major classes of proteases are known in mammals, including serine, cysteine, metallo, aspartic, and threonine proteases, the three first families being the most widespread in humans [3]. Protease activity requires tight regulation (figure 1): elevated protease levels, actively contributing to disease progression, constitute a common feature shared by a broad range of pulmonary pathologies, including idiopathic pulmonary fibrosis (IPF), cystic fibrosis, emphysema or infection [4]. The underlying mechanisms of such observations have long remained elusive: indeed, proteases were traditionally viewed as mere protein-degrading enzymes with a restricted spectrum of substrates. During the past decade, a major expansion in protease research from different fields dramatically shifted this paradigm. The study of degradomics (the protease substrate repertoire) revealed that the nature of the dedicated substrates of proteases, and the biological effects triggered by their processing, are exceptionally diverse [5]. Proteases are now considered key components of regulatory mechanisms, through irreversible cleavage of substrates resulting in their activation, inactivation, or modulation of function. The serine proteases of the coagulation cascade constitute an archetypal example: beyond blood clot formation, these proteases contribute to a variety of pathophysiologies, including IPF, through the irreversible activation of their cellular receptors, the protease-activated receptors [6, 7]. Knowledge of the cellular effects of other protease families, such as cysteine proteases or matrix-metalloproteases (MMPs), began to unfold only recently. Additionally, recent evidences show a regulation of protease activity through interconnection of protease cascades (the protease web).

In this review, these new concepts of integrative protease biology are discussed from the perspective of pulmonary pathophysiology. First, we highlight the striking role in lung pathogenesis of cysteine proteases, which were traditionally circumscribed to lysosomal degradation. We describe the recently discovered involvement of macrophage and neutrophil elastases, which (despite their names) actually belong to different clans, in cystic fibrosis. Defined localisation of protease activity is exemplified by the involvement of A disintegrin and metalloproteinase (ADAM) proteases in lung inflammation. Next, we focus on the main protease inhibitors, including serpins, to summarise the mechanisms underlying serpinopathies, and review recent evidence on serpin regulation by MMPs, thereby bolstering the concept of the protease web. These data are recapitulated in table 1. Finally, we highlight how deregulated protease activity during pulmonary pathogenesis may be exploited for diagnosis/prognosis purposes and utilised as a therapeutic tool using nanotechnologies.

Unusual suspects: the emerging role of cathepsins, neutrophil and macrophage elastases, and ADAMs in lung diseases

Cysteinyl cathepsins: endolysosomal degradation and beyond

It is only very recently that an unexpected role of cathepsins in lung diseases has emerged. Human cysteine cathepsins are a group of 11 papain-like lysosomal proteases including cathepsins B, H, L, C, X, F and O, and are ubiquitously expressed in most tissues, whereas cathepsins V, K, S and W have a more limited expression profile. Such diverse, tissue-specific expression suggests that their action is not limited solely to bulk protein degradation, but that they are also involved in the regulation of specific cellular functions. All of this allows cathepsins to regulate a broad variety of important physiological processes, including intracellular protein turnover, immune response, bone remodelling and prohormone processing [8, 9].

With respect to lung pathophysiology, cathepsins have a critical role in major histocompatibility class II-mediated antigen processing and presentation [3, 10, 11]. In addition, cathepsins, especially cathepsin K, have been found to have also major roles in the processing of TLR receptors [10, 12, 13]. In cystic fibrosis, levels of cathepsins B, L and S are increased in patient bronchoalveolar lavage fluid (BALF) [14], with bronchial epithelium acting a source of cathepsin S [15]. However, their relative contribution to extracellular proteolysis (as secreted proteases) *versus* intracellular proteolysis (as lysosomal proteases) remains to be determined, as does the balance between degradation and/or processing of specific substrates. Over the past decade, the role for cathepsins K and B in IPF has also emerged. In IPF patients, cathepsin K is increased in fibroblasts from fibrotic tissues. Unexpectedly, in the murine model of bleomycin-induced pulmonary fibrosis, cathepsin K deficiency associates with more severe lesions. Indeed,

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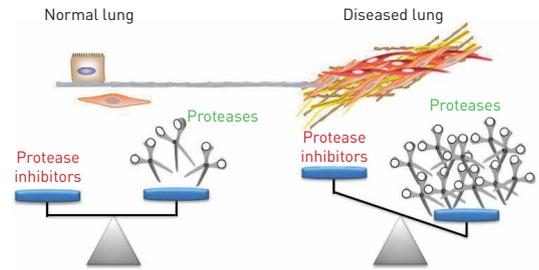
FIGURE 1 More-than-normal protease activity in lung pathogenesis. In the normal lung (left), proteases (represented by scissors) are involved in lung homeostasis and their activity is tightly regulated by (among others) cognate protease inhibitors. In a diseased lung (right), elevated protease activity, which can occur through increased protease expression and/or loss in cognate protease inhibitor, results into imbalanced protease activity.

cathepsin K has in fact a pivotal protective role in pulmonary homeostasis through collagen cleavage thereby contributing to the airway structural integrity [16–18]. Increased expression of cathepsin B was also observed in IPF patient-derived fibroblasts. Cathepsin B contributes to myofibroblast differentiation by triggering activation of the transforming growth factor (TGF)-β1-driven canonical Smad 2–3 pathway [19]. Accordingly, cathepsin B inhibition and/or genetic silencing diminishes α-smooth muscle actin expression and fibroblast differentiation.

From the above, the use of cathepsin pharmacological inhibitors seems an obvious therapeutical intervention. However, the off-targets and/or side-effects of cathepsin inhibitors remain to be fully elucidated. For instance, cathepsin S inhibitors efficiently reduce lung inflammation but can result in cartilage and bone degradation [20]. Cathepsin K inhibitors, developed for the treatment of osteoporosis, target cathepsin K collagenolytic activity and may impair collagen I turnover, thus favouring lung fibrosis [21]. A reversible cathepsin B inhibitor, VBY-376, demonstrated potent activity in a mouse model of CCl_4-induced liver fibrosis [22]. Promising phase I trials for treatment of hepatic fibrosis have also been obtained with VBY-376 [23]. Since cathepsin B-driven responses are common in pulmonary and hepatic fibrosis, the use of VBY-376 may be appropriate for pulmonary fibrosis treatment.

Overall, it has emerged from the past decade that, beyond lysosomal degradation, cathepsins are crucial actors in pulmonary homeostasis and further research is warranted to better delineate their role in pulmonary pathophysiology.

Neutrophil and macrophage elastases in cystic fibrosis: old dogs, new tricks

An imbalance between neutrophil-derived proteases, including neutrophil elastase (NE), cathepsin G (CatG) and proteinase 3 (PR3), and extracellular inhibitors is considered an important pathogenic mechanism for a number of lung diseases [24–26]. It is assumed that unopposed CatG, NE and PR3 collectively cause severe lung damage and emphysema [27], further underscoring their importance in pathology (which has been the subject of a previous review [28]). A fourth elastase-related protease, NSP4, expressed in the lung, has been recently identified. Its proteolytic effects, controlled by natural serine protease inhibitors of the serpin type, are most likely limited to the intracellular and pericellular microenvironment of activated neutrophils. The natural substrates of NSP4, however, remain to be determined [29]. Recent studies on mouse NE revealed the existence of a new variant, created by the spontaneous conversion process of the single-chain form of NE into a specific two-chain form by autoprocessing. The existence of this two-chain NE form could adversely affect the protease–antiprotease balance in the lung during NE release, and may lead to the development of more efficient anti-elastase therapies [30].

Strikingly, recent studies in infants and preschool children with cystic fibrosis also identified the increased activity of NE as a key risk factor of the onset and progression of early bronchiectasis [31, 32]. In the context of chronic lung disease, NE is involved in a number of processes, including induction of interleukin (IL)-8 expression, increased mucin secretion, and inactivation of innate airway proteins and cell surface receptors, all of which, combined, identify NE as a pro-inflammatory molecule [33–40]. Similarly, in other chronic lung diseases, including chronic obstructive pulmonary disease (COPD), alpha-1 antitrypsin (A1AT) deficiency and bronchiectasis, NE is thought to contribute to disease pathogenesis [41–43].

However, it is in cystic fibrosis airways where the most definitive role regarding the deleterious effects of NE has been most clearly demonstrated, as shown by the AREST CF group [32]. The development of the βENaC-overexpressing (βENaC-Tg) mouse, featuring cystic fibrosis-like airway surface dehydration, phenocopies key characteristics of cystic fibrosis in patients, including airway inflammation, mucus hypersecretion and obstruction, bacterial infection and structural lung damage [44–46]. Recent studies in

TABLE 1 Proteases with extracellular function associated with chronic lung diseases: mechanisms of action within the airway microenvironment, reporters and selected (commercially available) pharmacological inhibitors

Protease	Mechanism of action	Reporter	Inhibitors
Cysteine proteases			
Cathepsin B	Found in fibroblasts of IPF, contributes to myo-fibroblast differentiation *via* TGF-β1, Smad 2–3 pathway [19]	FRET reporter (Hu, unpublished observations)	VBY-376[#] [22] Leupeptin[#] E-64[¶][15]
Cathepsin K	Cleaves collagen, increased in fibroblasts of fibrotic tissue in IPF [16–18]	FRET reporter (Subramanian, unpublished observations)	CTSK inhibitor II[#] (Calbiochem) Cystatin C[#] [98]
Cathepsin L	Increased in BALF of cystic fibrosis patients, along with cathepsin B and cathepsin K [16]		Pentapeptide amide RKLLW-NH2
Cathepsin S	Lysosomal protease, unlike other cathepsins remains active at neutral pH [99]	FRET reporter [100]	Leupeptin[#] E-64[¶] [15]
Serine proteases			
Cathepsin-G	High affinity for nucleic acids, locates to NETs [28]		Chymostatin[#]
NSP-4	Recently identified fourth neutrophil-derived protease (along with cathepsin G, NE and proteinase 3) substrate unknown [29]		
Neutrophil elastase	Cleaves elastin, key risk factor for bronchiectasis in cystic fibrosis [30, 31]; induces IL-8, mucin secretion [33–40]	NEmo1, NEmo2 [47]	Sivelestat[#] Elastase inhibitor V[¶]
Proteinase-3	Degrades elastin [101]		
Metalloproteases			
ADAM-17 (TACE)	Initiates endothelial permeabilty and leukocyte recruitment *via* TNF generation [42, 43], involved in shedding TGF-α [48]		TAPI-1 [102]
ADAM-10	Causes experimental emphysema [44], possible sheddase for CD23 [45], involved in shedding TGF-α [48]		GI254023X[#] [103]
ADAM-33	Polymorphisms in ADAM-33 gene liked to asthma, COPD [46]	FRET probe [104]	Anti-ADAM-33 antibody
MMP-2	Cleaves type IV collagen, serum MMP-2 correlates with severity of pulmonary hypertension [105], cleaves serpin B6 and alpha-1 inhibitor-3 [77]		GM6001[#] [106] Actinonin
MMP-7	Possible marker for IPF (serum, BALF) [107]		GM6001[#] [106] Actinonin
MMP-8	Cleaves serpin A1, releasing elastase activity *in vivo* [79]		Actinonin
MMP-9 (gelatinase B)	Cleaves type IV and V collagen, recruits leukocytes [108]	FRET reporter (Halls, unpublished observations)	GM6001[#] [106] Actinonin
MMP-10	Possible marker for IPF (serum, BALF) [107]		GM6001[#] [106] Actinonin [107]
MMP-12 (macrophage elastase)	SNPs associated with lung function decline in COPD, asthma and cystic fibrosis [48, 49] Increased activity in smokers with COPD, asthma [36] and cystic fibrosis [37], associated with lung function decline in cystic fibrosis [48]	LaRee1, LaRee5 [86]	GM6001[#] [106] MMP408 Actinonin UK 370106
MMP-14	Processes SLPI [78]		Anti-MMP-14 antibody

Continued

TABLE 1 Continued

Protease	Mechanism of action	Reporter	Inhibitors
Antiproteases			
A1AT	Serpin, inhibits NE, cathepsin G, and proteinase 3 [64], deficiency causes emphysema and liver disease [65, 66], therapeutic potential for COPD [72]		
Elafin (trappin-2)	Inhibits proteases, anti-microbial, modulates immune response [51, 52]		
SLPI	Inhibits proteases, anti-microbial, modulates immune response [51, 52], therapeutic potential for cystic fibrosis [74]		

[#]: competitive inhibition; [¶]: covalent inhibition. IPF: idiopathic pulmonary fibrosis; TGF: transforming growth factor; BALF: bronchoalveolar lavage fluid; NET: neutrophil extracellular trap; NSP-4: neutrophil serine protease-4; NE: neutrophil elastase; IL: interleukin; NEmo: neutrophil elastase monitor; ADAM: A disintegrin and metalloproteinase; TNF: tumour necrosis factor; COPD: chronic obstructive pulmonary disease; MMP: metalloprotease; SNP: single nucleotide polymorphism; LaRee: lavage reporter; SLPI: secretory leukocyte protease inhibitor; A1AT: alpha-1 antitrypsin.

this cystic fibrosis model demonstrated that genetic deletion of NE reduces airway neutrophilia, mucin expression, goblet cell metaplasia and distal airspace enlargement without compromising anti-bacterial host defence *in vivo* [47]. In addition, whole-genome expression studies identified macrophage elastase (MMP-12) as a highly up-regulated gene in lungs from βENaC-Tg mice, and demonstrated that MMP-12 also contributes to structural lung damage *in vivo* [48]. The clinical relevance of this finding is supported by a genetic study showing that a functional single nucleotide polymorphism (rs2276109) in the MMP-12 promoter, previously associated with reduced lung function in COPD and asthma [49], was also associated with lung function decline in patients with cystic fibrosis [48]. More recent degradomic analysis of MMP-12 *in vivo* reveals its broad substrate variety. For instance, MMP-12 is potently anti-inflammatory in several *in vivo* models by cleaving and inactivating neutrophil chemokines [49], and terminating and complement factors, C4a and C5a [51, 52].

Taken together, these studies support important roles of elastases released from activated neutrophils and macrophages in the *in vivo* pathogenesis of multiple abnormalities in the cystic fibrosis lung, including inflammation, mucus hypersecretion and structural lung damage. These studies also demonstrate that the βENaC-Tg mouse will be a useful model for further studies of the roles of other emerging proteases including serine proteases, matrix metalloproteases and cysteine proteases [15, 25, 51], and their respective inhibitors, in the complex *in vivo* pathogenesis of cystic fibrosis and potentially other chronic inflammatory lung diseases.

Localising the activity to the membrane: A disintegrin and metalloproteinase (ADAM proteases)

The ADAM proteins constitute a major class of membrane-anchored multidomain proteinases that are responsible for the shedding of cell surface protein ectodomains, including the latent forms of growth factors, cytokines and receptors. As such, they are described as "signalling scissors". The ADAM family comprises ~34 members, 22 of which have been described in humans. Only 13 human ADAMs carry the zinc-binding motif (HExxHxxGxxH) required for proteolytic activity. Proteolytically active ADAMs can be regulated by induction of their gene transcription, by post-translational mechanisms upon stimulation with cytokines or bacterial toxins, or, in some cases, by cleavage of their ectodomain and subsequent release of a soluble form [52, 53]. Several members of proteolytically active ADAMs are involved in lung disease. ADAM17 is critical for generation of proinflammatory mediators, especially tumor necrosis factor (TNF)-α. The shedding of soluble activated TNF-α subsequently stimulates a cascade of events causing endothelial permeability and leukocyte recruitment. The overexpression of ADAM10 in lung epithelium causes the development of experimental emphysema [54]. ADAM10 has been identified as potential sheddase for CD23 and the soluble form of this mediator enhances the production of IgE [55]. Polymorphisms in the ADAM33 gene are linked with asthma or COPD [56] and BALF levels of the soluble form of this protease correlate with the severity of the disease [57]. Additionally, ADAM10 and 17 are critically involved in the shedding of several growth factors, including TGF-α, for example [58]. ADAMs are also implicated in host defence against infectious agents. ADAM10 functions as a receptor for *Staphylococcus aureus* toxin, and epithelial ADAM10 deficiency can prevent lethal *S. aureus* infection [59]. Finally, a key role has been proposed for ADAMs in airway remodelling associated with asthma [60]. Based on the above, it has been postulated that ADAM inhibition would decrease oedema formation, inflammatory cell recruitment, tissue remodelling, and reduction of *S. aureus* toxicity. Accordingly, the use of a selective ADAM17 inhibitor suppressed lipopolysaccharide-induced lung inflammation in mice [52]. From the above it is evident that ADAM proteases are implicated in acute and chronic inflammation processes but also in infection and regeneration. As inhibition of specific ADAMs might interfere with these processes, temporally and locally restricted and very specific inhibition strategies might be of interest for the treatment of lung disease.

Proteases and natural serine protease inhibitors: the dangerous liaisons

Besides an increase in protease expression and/or activation, deregulation of protease activity can arise from the loss of cognate protease inhibitors. Respiratory anti-proteases comprise serine protease inhibitors (serpins), tissue inhibitors of metalloproteinase, trappin-2/elafin, and secretory leukoprotease inhibitor (SLPI) [61]. These inhibitors not only neutralise protease activity, but also display anti-microbial and immune modulatory actions, which are highly relevant in chronic lung pathologies, such as in cystic fibrosis and COPD, where there is a combination of infection and exacerbated host responses [62, 63]. The clinical importance of protease inhibition is supported by studies demonstrating that inhibitor deficiencies result in structural lung damage. For instance, serpinopathies are a relatively newly defined group of disorders, where the underlying cause is the accumulation of misfolded protein, or lack of protein expression, in the affected tissue (figure 2). A1AT, a prototypical member of the serpin superfamily, is a specific inhibitor of NE, PR3 and catG [64]. Inherited A1AT deficiency, which causes familial emphysema and liver disease [65, 66], is a classic example of serpinopathy. A1AT deficiency is most often associated with the Z (Glu342Lys) mutation. Z-type A1AT forms oligomeric complexes; the intracellular retention of such complexes (gain-of-function defect) cause cellular damage [67, 68]. Strikingly, cigarette smoke-mediated oxidation can
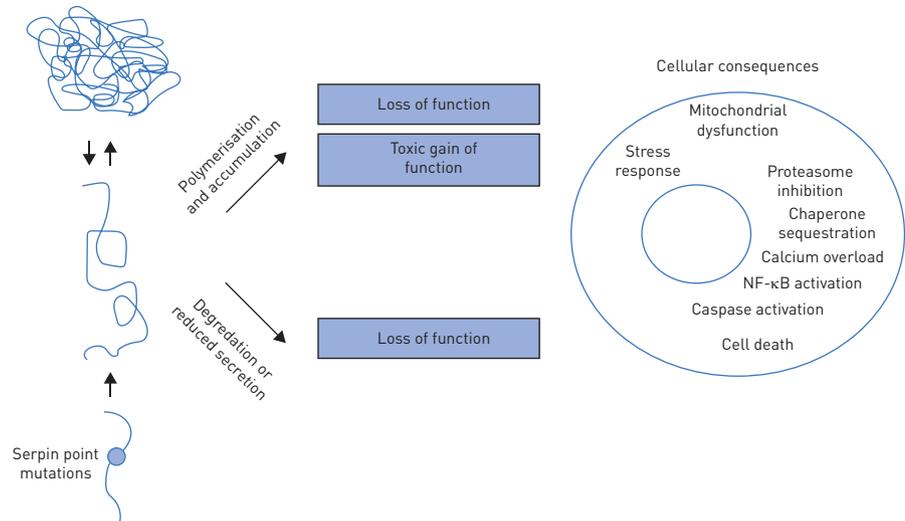
FIGURE 2 Mechanisms underlying serpinopathies. Point mutations in the gene encoding may result into misfolded protein and misfolded serpin polymerisation. Serpin polymers have no inhibitory functions towards serine proteases. Accumulation of serpin polymers has multiple cellular consequences. Other mutations can hinder protein expression. In either case, the loss of serpin results into uncontrolled proteolytic activity, thereby contributing to cell damage and the development of diseases.

enhance Z-A1AT polymerisation in human alveolar epithelial cells and induce endoplasmic reticulum-stress and NF-κB-mediated pro-inflammatory cytokine (TNF-α, IL-6 and monocyte chemoattractant protein 1) production [69, 70].

Hence, anti-protease supplements may reduce the deleterious effects of unregulated NE activity. Accordingly, the therapeutic potential of natural endogenous inhibitors of proteases has gained interest over the past decade, and their newly appreciated functions point to extended therapeutic uses. Thus, human plasma-purified A1AT is used in infusion in patients with A1AT deficiency to delay the progression of emphysema. Administration of A1AT also triggers the anti-inflammatory pathways necessary for resolution of inflammation and prevention of tissue damage [71]. Inhalation therapy offers another opportunity for easier, more efficient delivery of A1AT directly to the lungs. Inhaled A1AT indeed significantly ameliorates cigarette smoke-induced emphysema in the mouse [72]. In patients with cystic fibrosis, A1AT inhalation reduces airway inflammation [73]. Similarly, aerosolised recombinant SLPI reduces elastase activity in healthy subjects and those with cystic fibrosis [74]. Altogether, these data indicate the potential value of recombinant natural inhibitors in the treatment of chronic lung disease.

Noteworthy, while cognate inhibitors are essential for protease activity regulation, recent evidence demonstrates that reciprocally, protease inhibitors are also protease substrates. Isotopic labels of cellular and secreted proteins *in vitro* and *in vivo* [75] revealed that serpins are major substrates of MMPs [76]. In other words, serine protease activity is regulated by MMPs through serpin processing. Accordingly, MMP-2 cleaves serpin B6 and alpha-1 inhibitor III [77], while MMP-14 processes SLPI [78]. Such a regulation of inhibitors through protease processing has significant consequences in pathophysiology. For instance, during lung inflammation, the cleavage of serpinA1 by MMP-8 releases elastase activity *in vivo* [79]. In arthritis, a protective role for macrophage MMP-12 has been demonstrated: among other mechanisms, MMP-12 induces antithrombin III degradation and increased thrombin activation [80]. In acute skin inflammation, MMP-2 cleaves serpin G1, also known as complement C1 inhibitor. These findings have unveiled a mechanism, referred to as "metallo-serpin switch", by which MMP-2 triggers complement cascade activation [75]. Altogether, these data revealed that proteases pervasively influence the activity of other proteases, either directly or by cleaving intermediate proteases or protease inhibitors.

The protease web: is there a maze in the lung?
As the aforementioned examples illustrate, proteases can no longer be thought of acting in isolation. Thus, proteases in the same or different families activate protease zymogens, inactivate proteases by autodegradation *in trans*, cleave and release protease substrate binding domains termed exosites (resulting in altered protease specificity and substrates), and, as exemplified above, cleave and inhibit protease inhibitors. By mapping these interactions of all reported biochemical validated cleavages in proteases and inhibitors uploaded to the data base MEROPS and TopFIND [81, 82], graph theory was used to bioinformatically map the protease interactions of proteases termed the protease web [79]. Remarkably, at

the highly interconnected core of the protease web lie 255 highly connected proteases and inhibitors representing 50% of known proteases and inhibitors. Reachability analysis was performed, whereby the connections between proteases and inhibitors (paths) were mapped, and both the connections that are direct, *i.e.* 1:1, and indirect, *i.e.* further downstream reached by one or more intermediate connections. Thus, within this core, 158 of these proteins reached over 153 (60%) of the protease and inhibitors of the core, spanning all five human protease classes. The protease web is highly robust. Removal of one or more nodes did not "break" this highly interconnected core, nor did removal of paths. It was not until 40% of paths were removed did the web connectivity break down. Similarly it required removal of more than six proteases or inhibitors to destabilise the interconnected core. The connectivity will be shown to further increase as more protease and inhibitor interactions are uploaded to MEROPS and TopFIND by individual investigations upon publication of their data. The important implication of these analyses is that therapy must be designed with great care as blockade of one protease will almost inevitably have knock on effects on other proteases and activity *via* connections in the protease web, and may explain the failure of previous protease inhibitor clinical trials [83–85].

It is noteworthy that the aforementioned findings have also recently emerged from other fields of research in addition to pulmonology. Since, as described earlier, several, if not all, of these actors have been shown to be involved in lung pathophysiology, it is tempting to speculate that these mechanisms are highly relevant in lung disease, and further research in this direction is eagerly awaited.

Protease activity: a tool for diagnosis?

Because elevated protease activity is associated with lung disease, it can be postulated that quantitative measurement of such an activity could serve as a surrogate marker for diagnosis/prognosis in lung diseases. A significant number of fluorescent probes have been developed to directly measure protease activities. In the late 1970s, short peptide-based molecules had already been developed to feature an intrinsic fluorescent entity.

While commercially available and still heavily in use, sophisticated fluorescent reporters, often based on Foerster Resonance Energy Transfer (FRET), have been developed in recent years. Frequently, two fluorophores are employed as FRET donor and acceptor. This provides the possibility to measure FRET with a more sensitive readout in a ratiometric fashion, mostly independent from the amount of reporter present. Recent examples are FRET sensors for MMP12 (LaRee series) and neutrophil elastase (NEmo series) [86, 87], which have revealed elevated MMP-12 and NE activity. While such probes work extremely well with isolated enzymes in the test tube, their application to biological samples is much more difficult. This is partly due to the fact that most proteases are counteracted by protease inhibitors, also present in mouse or patient samples. Further, the zymogen forms of proteases require cleavage for activation by other proteases and such activation seems to vary locally. For instance, when investigating MMP-12 activity from mouse bronchoalveolar lavage samples by the soluble reporter LaRee1, very little activity was observed. However, the lipidated reporter LaRee1, located to the outer cell membrane of bronchoalveolar lavage macrophages, reported strong activity. This demonstrates that the enzyme activity resides predominantly on cell surfaces and suggests that the lung lumen is much less exposed [86]. Similarly, NE seems to be mostly blocked in its proteolytic activity in samples from lung lumen, but the lipidated reporter was cleaved readily on the surface of activated neutrophils [87]. FRET-based reporters have now made their way to patient samples and revealed, for instance, elevated strong increase in MMP-12 and NE activity on the surface of airway neutrophils and macrophages from βENaC-Tg mice and patients with cystic fibrosis, respectively [47, 48]. It is noteworthy that, very recently, different cathepsin-targeted imaging probes were developed and successfully used to monitor fibrotic disease progression in the murine model of bleomycin-induced pulmonary fibrosis and in IPF patients [88]. In the future, an extended array of reporters will help to characterise the protease activity landscape on cells isolated from patients and will hopefully assist physicians in the diagnosis of lung diseases, possibly predict disease course and aid the design of treatment strategies.

Hijacking protease activity for therapeutics

Dysregulation of MMP activity is a characteristic feature of organ fibrosis [89], such as that occurring in the lung [90] and malignant tumours [1, 91]. Recent progress in the field of nanotechnology has focused on the use of MMP-directed nanoparticles as diagnostic and therapeutic or theranostic devices [92–95]. Compared to commonly used drug and imaging formulations, nano-scaled drug carriers offer additional advantages, such as increasing the drug circulation time, minimising drug degradation, and overcoming first-pass drug metabolism [96]. Mesoporous silica nanoparticles, engineered to release their incorporated chemotherapeutic drug upon gate-opening by MMP-2/9 cleavable linkers, have recently been developed [97]. Efficient drug release was shown to be strictly dependent on the presence of MMP-9 in human lung tumour cell lines; moreover, drug release was highly effective from mesoporous silica nanoparticles in

mouse and human lung tumours. The successful application of therapeutic nanoparticles to *ex vivo* cultures of human lung tumours represents major progress in closing the translational gap in the development of novel MMP-targeted nanomedicines for lung tumour treatment. Thus, deregulation in protease activity in other lung diseases could be similarly hijacked for drug delivery and may represent a novel strategy for therapeutic intervention.

Conclusion

It is now clear that the traditional representation of proteases as mere extracellular matrix-degrading enzymes has shifted. The new paradigm allows a better understanding of how proteases are instrumental in the pathogenesis of a variety of chronic lung diseases. Protease deregulation during pathogenesis may occur through escape to its tight, spatio-temporal, self-limiting control. The wide variety of protease substrates adds a further complexity to this field. Indeed, the examples described in this review emphasise that the close interplay between proteases, substrates and inhibitors, which are interrelated in the protease interactome accounting for the net proteolytic activity in the lung, should be taken into consideration. As a result of the recent emergence of the aforementioned concepts, the understanding of the interplay and interdependency of these systems remains very scarce in the lung research field. Extrapolations of observations from different systems, together with translational approaches, combining degradomics data, murine models of lung disease and *ex vivo* observations from patients, should allow identification of an increasing number of instrumental proteases and protease inhibitors, in the perspective of integrative protease biology. Understanding in greater detail the complex molecular basis of regulation and function of protease activity will facilitate identification of new therapeutic targets as well as new diagnostic and prognostic tools for personalised medicine, and hopefully result in the development of new drugs to treat pulmonary diseases that result from inherent protease systems deregulation.

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