Antimicrobial activity of antiproteinases
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Abstract
Low-molecular-mass neutrophil elastase inhibitors have been shown to be important in the control of lung inflammation. In addition to inhibiting the enzyme neutrophil elastase, these low-molecular-mass compounds (10 kDa) have been shown to have other activities. For example, secretory leucocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor/SKALP (skin-derived antileucoproteinase)/elafin have also been shown to have 'defensin'-like antimicrobial activities. Indeed, these inhibitors have antimicrobial properties in vitro against bacteria, fungi and, potentially, HIV. In addition, we have shown, using an adenovirus-mediated gene transfer overexpression strategy, that elafin is also active against Pseudomonas aeruginosa infection in mice in vivo. The mechanism of action is currently under investigation. In addition to these direct or indirect effects on microbes, it has been shown that lipopolysaccharide is able to up-regulate SLPI production in macrophages in vitro, and that the addition of recombinant SLPI to human monocytes or the transfecion of macrophages with SPLI can down-regulate pro-inflammatory mediators such as tumour necrosis factor.

Key words: adenovirus, elafin/elastase-specific inhibitor/SKALP, elastase, inflammation, SLPI.
Abbreviations used: APR, acute-phase response; IL-1 (etc.); LPS, lipopolysaccharide; SKALP, skin-derived antileucoproteinase; SLPI, secretory leucocyte proteinase inhibitor; TNF, tumour necrosis factor.

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Introduction

Antiproteases are important molecules that are involved in the acute-phase response (APR), which is the response of the body to the initiation and development of inflammation. The APR [1] is a very tightly regulated process which is initiated locally by inflammatory cells (such as monocytes, macrophages and neutrophils). These cells secrete products (cytokines) which in turn act at distant sites such as the liver, which is mainly responsible for switching on the APR. Antiprotease molecules are produced either locally at mucosal sites or systemically by the liver. Their primary function was thought until recently to be the prevention of the potential injurious effects of excess release of proteolytic enzymes from inflammatory cells. However, recently, new potential functions have been ascribed to these antiproteases.

These molecules seem to have developed in a parallel network, consisting of 'alarm' and 'systemic' inhibitors [2]. The first group is composed of secretory leucocyte proteinase inhibitor (SLPI) (also known as antileucoproteinase, mucus proteinase inhibitor or bronchial inhibitor; this inhibitor will henceforth be referred to as SLPI) and of the elastase-specific inhibitor, also known as elafin or skin-derived antileucoproteinase (SKALP) [3]. These molecules are synthesized and secreted locally at the site of injury. Interestingly, these molecules are produced in response to 'first-wave' cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF), and might therefore be part of a first wave of local, inducible defence by the antiprotease network. The concentration of these inhibitors is very low in the circulation [4,5] and they are not expressed in liver cells [6,7]. The second group is composed of systemic antiproteases such as α1-antichymotrypsin and α1-proteinase inhibitor (α1-PI), which are produced in abundance by the liver and secreted in high concentrations in the circulation as acute-phase reactants [8]. The liver produces these systemic antiproteases in response to 'secondary' cytokines, such as members of the IL-6 family, including IL-6 itself, leukaemia inhibitory factor and oncostatin M [1].

SLPI

Structure and function

SLPI is one of the two members of the antileucoproteinase superfamily of proteinase inhibitors (the other being elastase-specific inhibitor/elafin/SKALP; see below). The SLPI gene (2.65 kb in length [9,10]) is a non-polymorphic, stable gene that can be modulated at both the transcriptional and translational levels [11]. It is an 11.7 kDa protein, consisting of 107 amino acids and comprising two domains. It contains 16 cysteine residues, which form eight disulphide bridges. SLPI has been shown to inhibit human neutrophil elastase, cathepsin G, trypsin, chymotrypsin and chymase. Its major target is thought to be human neutrophil elastase, in view of its high affinity and kinetic constants (K, in the nanomolar range and k in the micromolar range) [12].

Cellular and histological distribution

SLPI is a molecule that is present constitutively in most mucosal sites (hence its alternative name mucus proteinase inhibitor). In lung tissues, it is produced in vitro by tracheal, bronchial, bronchiolar and type II alveolar cells, as well as by monocytes, alveolar macrophages and neutrophils [13,14]. It has also been shown to be produced in vivo by tracheal serous glands and bronchiolar Clara cells, and to be closely associated with elastin fibres in the alveolar interstitium [13]. Its roles in inflammatory cells, such as macrophages or neutrophils, are uncertain, but antibacterial or anti-inflammatory actions have been proposed (see below).

Elastase-specific inhibitor/elafin/SKALP

Structure and function

The neutrophil elastase inhibitor elafin (the other member of the antileucoproteinase superfamily of proteinase inhibitors; for a recent review, see [15]) was first identified as a 'non-SLPI' low-molecular-mass anti-elastase by Hochstrasser et al. [16] and Kramps and Klasen [17]. We further characterized it in the lung [18], and Wiedow et al. [19] and Molhuizen et al. identified it in the skin [20]. Its gene sequence shows that it is approx. 2.3 kb long, is composed of three exons and two introns, and contains typical 5' TATA and CAAT boxes. It is highly regulatable, as demonstrated by the presence of AP1 (activator protein 1) and nuclear factor-κB sites in the 5' untranslated region [21,22].

'Alarm signals', such as bacterial lipopoly-saccharide (LPS), IL-1, TNF, neutrophil elastase and defensins, are able to induce both SLPI and elafin. SLPI has also been shown to be down-regulatable by remodelling cytokines such as transforming growth factor-β [23–26]. At the
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Protein level, the elafin molecule is composed of 117 amino acids, including a hydrophobic signal peptide of 22 amino acids. Elafin is part of a four-disulphide core protein family which has recently been coined the "trappin family" [15]. Elafin can be divided into two domains: the C-terminal domain containing the antiproteinase active site, and the N-terminal domain containing characteristic VKGQ sequences. These sequences allow the elafin molecule to glue itself into polymers and bind other interstitial molecules, through transglutamination [15]. This feature could make elafin maximally effective as a tissue-bound inhibitor, as opposed to systemic inhibitors such as α1-proteinase inhibitor, which is present in high amounts in the circulation. SLPI has also been suggested to have a protective role locally, against neutrophilic damage, presumably because of its small size and negative charge. The elafin molecule shows 40% sequence identity with the SLPI molecule, and the active sites of the two inhibitors are very similar. In terms of its antiproteinase activities, elafin appears to be more specific in its spectrum of inhibition than SLPI: it has been shown to inhibit pig pancreatic elastase, human neutrophil elastase and proteinase-3 [15].

Cellular and histological distribution
Elafin was first demonstrated in the skin and in lung secretions, but is also present at mucosal sites in many tissues. Its purification from sputum, its presence in tracheal biopsies and bronchoalveolar lavage fluid from both normal subjects and patients [13], as well as its synthesis by Clara cell and type II cells, indicates a tracheo-bronchio-alveolar origin, as for SLPI. It is present at highest concentrations in sputum (approx. 10% of the concentration of SLPI).

α1-Proteinase inhibitor
Structure and function
α1-Proteinase inhibitor is a 52 kDa protein composed of 394 amino acids and three asparagine-linked complex carbohydrate side-chains. There are two major isotypes in serum [27]. It is the archetypical member of the serpin supergene family of proteinase inhibitors (other members include, to name a few, antithrombin III, α1-antichymotrypsin and plasminogen activator inhibitor-1 and -2). Although α1-proteinase inhibitor can inhibit a variety of enzymes, the kinetics of inhibition indicate that inhibition of human neutrophil elastase is one of its main functions. Severe deficiency of α1-proteinase inhibitor is associated with chronic liver disease in early childhood, and is often associated with pulmonary emphysema. A genetic deficiency of α1-proteinase inhibitor leads to progressive lung damage in early adult life for cigarette smokers [28] and accounts for about 3% of all patients with chronic obstructive airway disease [29]. The deficiency states are caused by mutations in the α1-proteinase inhibitor gene, with the two most common forms being the S and Z variants. The Z variant is the most problematic, and it arises from a glutamic acid-to-lysine substitution at amino acid position 342 in the mature protein [30]. The Z protein is synthesized at a normal rate, but it accumulates in the rough endoplasmic reticulum of hepatocytes; the clinical consequence of this is that approx. 10% of patients develop severe cirrhosis and liver failure. Lomas and colleagues [31] showed that hepatocyte cytotoxicity may derive from the ability of α1-proteinase inhibitor Z mutants to form intracellular polymers. Selective inhibitors of this polymerization are currently being studied [32]. The relevance, if any, of this phenomenon for the lung is also currently being assessed [33].

Synthesis
Plasma concentrations of α1-proteinase inhibitor increase 3–4-fold during the APR [1]. Although, on a molar basis, the liver is the major site of expression during the APR, lung epithelial cells can respond dramatically to oncostatin M, a cytokine of the IL-6 family, to synthesize α1-proteinase inhibitor and α1-antichymotrypsin [34,35].

Role of antiproteinases in innate immunity
Antiproteinases such as α1-proteinase inhibitor and the low-molecular-mass proteinase SLPI have recently been hypothesized to also be important in the regulation of innate immunity and in the control of excessive inflammation and septic shock. Indeed, α1-proteinase inhibitor has been shown to inhibit lethality in mice challenged with LPS and TNF, supporting the notion that the action of this proteinase inhibitor may be in prevention of the release of membrane-bound TNF [36,37].

It has also been shown in vitro that LPS is able to up-regulate SLPI production in macrophages [25] (which in turn is able to bind to
LPS). Furthermore, addition of recombinant SLPI to human monocytes or transfection of macrophages with SLPI or elafin down-regulates pro-inflammatory mediators such as TNF and matrix metalloproteinases upon stimulation with LPS, for example [25,39]. This suggests that these inhibitors may also function to interfere directly (by binding to LPS) or indirectly (by down-regulating NF-κB function, for example [40]) with LPS in a feedback fashion, and hence serve to limit the potential risks of sepsis and septic shock. Of note, SLPI also seem to be associated with a lack of responsiveness to LPS in vivo. Thus macrophages derived from a mouse line naturally resistant to LPS (C3H/HeJ, found to carry a mutation on the Toll-like receptor 4 gene) consistently expressed high levels of SLPI compared with C3H/HeN mice, which are sensitive to LPS [41].

**Direct antimicrobial activities**

In addition to their elastase-inhibitory properties, which were identified first (see above), and because of their biochemical characteristics (low-molecular-mass cationic peptides, heavily disulphide-bonded, present at mucosal sites), SLPI and elafin may also have direct antimicrobial activities, and may be good candidates as 'defensin-like' molecules. Indeed, SLPI and elafin have been shown to have antimicrobial properties in vivo against bacteria, fungi and, potentially, HIV [13,42,43].

Furthermore, we showed that elafin is also active in vivo against the lung bacterial pathogen *Pseudomonas aeruginosa*. Using adenovirus as a vector for elafin gene transfer administration (day 0) in C57/B16 mice, we administered bacteria intratracheally at day 5 and killed the animals 24 h later. A variety of parameters were measured at that time point (e.g. protein and albumin concentrations in bronchoalveolar lavage, bacterial count in bronchoalveolar lavage and in lung homogenates, etc.). This allowed us to show that treatment with adenovirus-elafin was able to protect murine lungs against pneumonia-like lesions [44].

The mechanism of the protection conferred by elafin in this model is still under investigation, but does not seem to involve its antiproteinase activity directly, since elafin contributed to less than 0.1% of the anti-elastase activity. Interestingly, a similar effect was noted by Cantin and Woods [45] in a rat model. It was noted that intratracheal administration of α1-proteinase inhibitor protein was also protective against *P. aeruginosa*.

**Therapeutic options and gene therapy**

Although still in its infancy compared with pharmacological methods of drug delivery, gene therapy, because of its less transient nature, might be advantageous. Indeed, adenovirus could be the vector of choice for many gene therapy applications. Although, historically, most of these applications have been directed to chronic pathologies such as cystic fibrosis and cancer, success has recently been achieved in animal models of lung infections and endotoxaemia in rodents using adenovirus vectors. In these studies, transgenes coded for molecules involved either in the priming of innate immunity [46,47] or in direct antimicrobial activities [44,48]. Because of the aforementioned characteristics of SLPI and elafin as 'alarm molecules' involved in the regulation of early events in the inflammatory process, overexpression of these proteinase inhibitors may be of benefit in combatting bacterial infections and their inflammatory sequelae.

**Conclusion**

Proteinase inhibitors form part of a major family of proteins (acute-phase proteins) that are upregulated either locally at mucosal tissues or systemically in the liver. Given their primary antienzymic properties, it was originally believed that the organism requires increased levels of these proteins to combat proteinases secreted in excess, either by invading micro-organisms or by the host itself.

Although this may still hold true, novel functions for proteinase inhibitors have recently emerged in relation to their role in innate immunity. These functions encompass direct antimicrobial activities against viruses and bacteria, as well as possible bacterial LPS-modulating activities. These latter activities may be of particular importance in pathophysiology and are the focus of our current work on the use of gene therapy vectors for antiproteinase augmentation. Translating these findings into the clinic and identifying the best vector for administration and the cohort of patients most likely to benefit will remain, undoubtedly, a major focus of studies in the foreseeable future.

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References

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