

EDUCATION ARTICLES

Endogenous Pulmonary Antibiotics

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Abstract

The human lung produces a variety of peptides and proteins which have intrinsic antimicrobial activity. In general these molecules have broad spectra of antimicrobial activity, kill micro-organisms rapidly, and evade resistance generated by pathogens. In recent years it has become increasingly apparent that the antimicrobial peptides (AMPs) simultaneously possess immunomodulatory functions, suggesting complex roles for these molecules in regulating the clearance of, and immune response to, invading pathogens. These collective properties have stimulated considerable interest in the potential clinical application of endogenous AMPs.

This article outlines the biology of AMPs, their pattern of expression in the lung, and their functions, with reference to both antimicrobial and immunomodulatory activity. We then consider the biological importance of AMPs, before concentrating on the potential to use AMPs to therapeutic effect. The principles discussed in the article apply to innate immune defence throughout the body, but particular emphasis is placed on AMPs in the lung and the potential application to pulmonary infection.

Introduction

The innate immune system provides the first-line of protection against invading microbes and in recent years the complexities of pulmonary innate immunity have become increasingly characterised. As a result it is now recognised that a variety of proteins and peptides with inherent antimicrobial and immunomodulatory activities contribute to pulmonary host defences. These antimicrobial peptides (AMPs) have been called “nature’s antibiotics”, as they have been described across the plant, insect and animal kingdoms.¹⁻⁴

Not surprisingly, significant interest has focused upon the potential for AMPs to be used therapeutically, driven in part by the observation that microbial resistance to AMPs is relatively rare,⁵ and by the discovery of additional potent immunomodulatory activities of these peptides.⁶ In contrast conventional antibiotics often result in toxicity and are associated with the emergence of resistance mechanisms in human pathogens.⁷⁻¹⁰

Dr Simpson was recipient of the Sir James Black Prize at the Scottish Society for Experimental Medicine Meeting, Aberdeen, in 1999. This article stems from an invitation, linked to the prize, to submit a review based broadly on work presented at the meeting.

This article will summarise the general properties of endogenous human AMPs and antimicrobial proteins. It will concentrate on their antimicrobial activity, but will also outline newly described functions that suggest diverse roles in host defence. With this background in mind we shall assess the biological importance of AMPs to the human host and then consider their therapeutic potential.

General properties

Cationic antimicrobial peptides are generally small (<10 kDa) peptide molecules with a positive charge and amphipathic structure.¹¹ The detailed biochemistry of the AMPs has been the subject of excellent reviews and will not be discussed further here.¹²⁻¹⁷ Several AMPs have been described in the human lung including alpha- and beta-defensins, and the cathelicidin LL-37. In addition the lung contains a variety of biologically active proteins with antimicrobial activities, including collectins (surfactant proteins -A and -D (SP-A and SP-D)), anti-elastases (elafin and secretory leukocyte protease inhibitor (SLPI)), lactoferrin, lysozyme and bactericidal/permeability-increasing protein (BPI) [Table I].

In the lung AMPs may be produced and secreted by submucosal glands, alveolar epithelial cells or phagocytic cells, such as resident alveolar macrophages or neutrophils recruited to the lung during infection.

A fascinating feature of the endogenous AMPs is that significant and widespread bacterial resistance has not developed despite their ubiquitous expression throughout the evolutionary tree. It is possible that the array of different antimicrobial peptides and proteins attacking in concert *in vivo* may negate the effectiveness of common resistance pathways. However, studies suggest that the ability of bacteria to develop resistance to a single AMP is limited, even after multiple passages in media containing 50% of the minimum inhibitory concentration of the AMP *in vitro*.⁵ Nevertheless, despite broad-spectrum antimicrobial profiles for most AMPs *in vitro*, organisms with inherent resistance have been described. These include *Burkholderia cepacia*, which is insensitive to AMPs by virtue of its unique outer membrane and *Staphylococcus*

Peptide / Protein	Source	Size	Antimicrobial activity	Selected additional effects
<i>α-Defensins</i>	E, N	3-5 kDa	Gram positive and Gram negative bacteria, yeast and fungi, and certain enveloped viruses.	Induction during inflammation; chemoattractants for immature dendritic cells, monocytes, mast cells and T lymphocytes; stimulate mast cell activation and degranulation; stimulation and release of neutrophil and monocyte chemokines; increase production of integrins involved in chemotactic responses; promotion of re-epithelialisation of wounds; enhance phagocytosis; adjuvant activity.
Human neutrophil peptide (HNP)-1, 2, 3, 4 Human <i>α</i> -defensin (HD)-5				
<i>β-Defensins</i>	D, E, M	3-5 kDa	Gram positive and Gram negative bacteria, and fungi.	
Human <i>β</i> -Defensins (HBD)-1, 2, 3, 4				
<i>Cathelicidin</i>	E, N	4,5 kDa	Gram positive and Gram negative bacteria, viruses and fungi.	Induction during inflammation; chemoattractant for monocytes, T lymphocytes, mast cells and neutrophils; stimulate activation and degranulation of mast cells; upregulation of neutrophil and monocyte chemokine expression; antiendotoxic activity; modulate cytokine expression; promote wound healing and angiogenesis; modulate D cell differentiation; signal to host cells of innate immunity through MAP kinase pathways.
LL-37 (hCAP-18)				
<i>Bactericidal/Permeability increasing protein (BPI)</i>	E, Eo, N	50 kDa	Gram negative bacteria.	Inhibition of CD-14 dependent cell activation by endotoxin; inhibition of angiogenesis; opsonic effects – enhances uptake of bacteria by phagocytes.
<i>Colliclins</i>				
SP-A and SP-D	E	SP-A 30-35 kDa SP-D 43 kDa	Bind bacteria (including mycobacteria), viruses and fungi.	Immunomodulators that regulate cellular recruitment.
<i>Anti-elastases</i>				
Elafin	E	10 kDa	Gram positive and Gram negative bacteria.	Inhibitor of human neutrophil elastase and proteinase 3; inhibition of NF- κ B; chemoattractant for neutrophils and macrophages.
SLPI	E, M, N	12 kDa	Gram positive and Gram negative bacteria, fungi and HIV-1.	Inhibitor of human neutrophil elastase, cathepsin G, trypsin, chymotrypsin and chymases; involvement in normal cutaneous wound healing; modifies macrophage function.
<i>Lactoferrin</i>	E, S	80 kDa	Gram positive and Gram negative bacteria, viruses, fungi and parasites.	Immunomodulatory; anti-tumour; anti-inflammatory; hypoferraemia; protease; protease inhibitor; ribonuclease; pro-coagulant; stimulates granulopoiesis; transcription factor; regulates iron absorption.
<i>Lysozyme</i>	E, M, N	14 kDa	Gram positive and Gram negative bacteria.	Lysis of bacterial cell wall.

Table 1 - Endogenous antimicrobial peptides and proteins described in the human lung
D – Dendritic cell, E – Epithelial cell, Eo – Eosinophil, M – Macrophage, N – Neutrophil, S – Serous cells of submucosal glands. For more details see references 106-116, in addition to those referenced in the text.

aureus, protected from defensins by a modification of membrane lipids.^{18, 19} In addition, membrane modifications resulting in inducible resistance have occasionally been described.²⁰⁻²³

Function

The mechanisms by which AMPs exert antimicrobial activity have been extensively studied. Although it would be misleading to suggest that all AMPs function in an identical manner, cationic residues are believed to interact with negatively charged structures of the microbial membrane, such as LPS, with resultant displacement of lipids, alteration of membrane structure and permeabilisation.²⁴⁻²⁷

It should be emphasised that AMPs have differing spectra of antimicrobial activity, and do not act in isolation. Peptide-specific synergy has been demonstrated between different classes of AMPs, and between AMPs and other antimicrobial proteins, including lysozyme and lactoferrin.^{28, 29} These interactions may ensure that an optimal antimicrobial effect is achieved, even with low concentrations of individual AMPs *in vivo*.

The mechanisms of action of other antimicrobial proteins found in the lung are varied, and are summarised in Table I. Bactericidal/permeability-increasing protein (BPI) binds to bacterial LPS with consequent immediate growth arrest.³⁰ Bacteria are probably then killed by damage to their inner membrane or through the synergistic activities of other antimicrobial peptides such as defensins and cathelicidins. In contrast SP-A and SP-D are believed to act primarily as opsonins which, through binding and aggregation, lead to enhanced bacterial clearance and killing by phagocytic cells.¹⁶ Lactoferrin sequesters iron (essential for the growth of all bacteria) and as a consequence halts microbial growth, but also has additional antimicrobial effects independent of its ability to bind iron.³¹ The potent antimicrobial protein lysozyme kills bacteria via enzymatic (by breaking the peptidoglycan chain of the bacterial cell wall)³² and non-enzymatic mechanisms.³² Finally, the antiproteases SLPI and elafin have been shown to have direct anti-microbial activity *in vitro*, but their mechanisms of action remain unclear.^{33,34} As for AMPs, synergistic enhancement of bacterial killing has also been reported for some of these antimicrobial proteins.³⁵

It is important to note that the antimicrobial activity of endogenous pulmonary antibiotics is not confined to bacteria (including mycobacteria such as *Mycobacterium tuberculosis*).³⁶ Some AMPs have antiviral activity. For example alpha defensins and SLPI are active against HIV, and cathelicidins against vaccinia virus.³⁷⁻³⁹ Antifungal activity has also been demonstrated for lactoferrin against *Candida albicans*, and for

SLPI against *C. albicans* and *Aspergillus fumigatus*.⁴⁰⁻⁵²

Importantly, in recent years it has become clear that AMPs have significant functions in addition to direct antimicrobial properties [Table I]. These newly described immunomodulatory properties have the potential to play fundamental roles in both the innate and adaptive immune systems, and are the subject of considerable current interest. The additional activities ascribed to certain peptides have led to the proposal that AMPs be renamed the cationic host defence peptides.⁴³ Immunomodulatory activities described include; anti-endotoxic activity, chemokine function, angiogenic potential, the enhancement of wound healing, the capacity to alter the transcription of numerous inflammatory genes in epithelial and monocytic cells, modulation of dendritic cell differentiation, and adjuvant properties.⁴⁴⁻⁵⁵ The mechanisms of action and the relevant receptors remain to be identified for most of these properties.

One of the major challenges ahead is to characterise the relative contribution of direct antimicrobial activity and immunomodulatory functions of AMPs in complex biological systems. Several immunomodulatory effects persist when physiological concentrations of AMPs are applied to complex media containing physiological concentrations of salt *in vitro*, whereas direct antimicrobial activity is generally lost under such conditions.^{35,56,57} Whether these principles apply when AMPs are expressed in, or applied to, complex organs like the infected lung remains to be determined. In the context of developing AMPs for clinical application the specific contribution of immunomodulatory and antimicrobial functions becomes particularly important. On one hand it is conceivable that beneficial effects on clearance of microbes by phagocytes may be achievable using doses lower than those associated with direct microbial killing. On the other, direct antimicrobial activity may be attended by a plethora of immunomodulatory activities, some of which may conceivably be detrimental to the host.

From a pragmatic clinical standpoint the debate surrounding specific functions of AMPs in complex biological systems generates two key questions – are the AMPs important physiologically, and can they be applied effectively and safely in clinical practice? These questions are the focus of the following sections.

Are AMPs physiologically important?

The constitutive and inducible expression patterns of AMPs at many key sites of potential host-pathogen interactions suggest that they contribute significantly to the innate immune response. Their importance can also be inferred by their *in vitro* activity against micro-organisms, which is both broad and effective.

However, proof of the importance of individual AMPs must come from *in vivo* studies. Experimental evidence from 'knockout' mice, transgenic mice, and gene therapy-treated animals have all confirmed the capacity of AMPs and antimicrobial proteins to impact significantly upon host defence against infection.

An illustration of the importance of AMPs *in vivo* comes from a study of group A *Streptococcus* (GAS), a pathogen sensitive to Cathelicidin-Related Antimicrobial Peptide (CRAMP), the murine homologue of the human cathelicidin LL-37.⁵⁸ The authors gave subcutaneous GAS to CRAMP 'knockout' mice (CRAMP^{-/-}), wild-type mice (CRAMP^{+/+}) and heterozygotes (CRAMP^{+/-}). The CRAMP^{-/-} mice developed much larger areas of infection than CRAMP^{+/+} littermates while heterozygotes tended to have lesions of intermediate size. Furthermore, the lesions increased more rapidly and persisted longer in CRAMP^{-/-} mice.⁵⁸ Another group studied mice deficient in the metalloproteinase matrilysin, which cleaves pro-peptides to release active alpha defensins in the murine intestine. These knockout mice showed increased susceptibility to orally administered bacteria.⁵⁹ Further studies using two strains of mice deficient in the homologue of human beta defensin 1, Defb1, showed that mutant mice harboured more *Staphylococcus aureus* in the bladder compared with controls, and had delayed clearance of *Haemophilus influenzae* from the lung.^{60,61} Deficiency of murine collectins has also been studied. Mice deficient in SP-A demonstrated decreased bacterial killing compared to wild type mice when infected by tracheal instillation with group B *Streptococcus*, *H. influenzae* or *P. aeruginosa*.^{62,63} Knockout mice have also been used to demonstrate roles for SP-A and SP-D in pulmonary clearance of influenza A and respiratory syncytial virus.⁶⁴⁻⁶⁷ Taking an alternative approach, studies using transgenic mice and gene therapy systems have also shown that augmentation of defensins and cathelicidins increases bacterial killing, as described in more detail later.^{68,69}

Further evidence for the importance of AMPs comes from the description of deficiencies of AMPs in association with human disease. Morbus Kostmann is a condition in which there is severe congenital neutropenia.⁷⁰ The condition was originally described as an autosomal recessive disorder in 1956.⁷¹ At the time all children with the disease died in infancy from bacterial infections (usually *Staphylococcal* or *Streptococcal*) resulting in omphalitis, otitis media, pneumonia or abscesses of the skin or liver.⁷² The disorder is characterised by maturation arrest at the promyelocyte to myelocyte stage.⁷³ Granulocyte levels have been restored in a few patients by giving daily injections of granulocyte-colony stimulating factor (G-CSF).⁷³ However maturation of granulocytes in G-CSF-treated patients is not entirely normal as these cells do not produce hCAP-18, the

precursor of LL-37. Patients still have problems with infections, especially chronic gingivitis and periodontitis, and require frequent courses of antibiotics.¹³ Western blot analysis revealed that LL-37 was missing both in granulocytes and in saliva from three patients with morbus Kostmann, whereas in healthy controls low levels were presumed to be deposited by homeostatic degranulation of neutrophils.⁷⁰ Although it is difficult to separate the effects of LL-37 deficiency from other aspects of this disease, one patient has been significantly improved by a bone-marrow transplant that restored LL-37.¹³

Functional implications for AMPs have also been derived from studies of human skin. In normal skin the concentrations of LL-37 and beta defensins such as HBD-2 are negligible, while they accumulate in inflammatory conditions such as psoriasis, and are decreased in patients with atopic dermatitis.⁷⁴ As LL-37 and HBD-2 show synergistic activity in killing *S. aureus*, a deficiency in the expression of these antimicrobial peptides may account for the susceptibility of patients with atopic dermatitis to this pathogen.⁷⁴ Another study has suggested that the susceptibility of patients with atopic dermatitis to eczema vaccinatum, a complication of infection with the vaccinia virus or smallpox, may be due to a deficiency of cathelicidin.³⁹

Finally a report examining the role of AMPs in the pathogenesis of bacillary dysentery has suggested that down-regulation of LL-37 and HBD-1 may be important.⁷⁵ The authors suggest that lysis of the *Shigella* bacterium by AMPs may contribute to release of bacterial plasmid DNA capable of suppressing local production of LL-37, thus leaving exposed epithelial cells susceptible to further bacterial invasion. This constitutes an attractive model of bacterial virulence, and suggests that absence of host AMP expression may be advantageous for pathogens.

Can AMPs be supplemented to therapeutic effect?

Several studies have been performed in animals to explore the potential benefits of AMPs in transgenic models, or using gene therapy.

Mice transgenic for the human alpha defensin HD-5 expressed, stored and processed HD-5 in the same pattern as humans, and were found to be markedly resistant to oral challenge with virulent *Salmonella typhimurium*.⁶⁸ Although synergy with murine AMPs and issues of peptide over-expression must be considered, this study illustrates the capacity for AMPs to supplement host defences therapeutically.

In another report, mice were injected either with tumour cells genetically engineered to express HBD-2 cDNA or with tumour cells that did not express this AMP. *Escherichia coli* were then injected into the tumour mass. Tumours were resected after 16 hours. Mice with tumours expressing HBD-2 had significantly

less intra-tumoural *E. coli*, demonstrating that enhancement of host defence by HBD-2 gene therapy may be possible.⁷⁶

Several studies have directed attention to the effects of augmenting specific antimicrobial peptides/proteins in the lung. Thus neutrophil defensins exerted significant antimicrobial activity against *E. coli* and *P. aeruginosa* in lung explants.⁷⁷ Furthermore, mice receiving an intra-tracheal instillation of adenovirus encoding LL-37 (Ad-LL-37) had a lower bacterial load and less pronounced inflammatory response after pulmonary challenge with *P. aeruginosa* than did control mice.⁶⁹ Intra-tracheal instillation of an adenovirus encoding elafin (Ad-elafin) has also been shown to protect mice significantly against injury mediated by *P. aeruginosa*.⁷⁸

Extending these principles, and with a view to therapeutic use in cystic fibrosis, a human bronchial xenograft model deficient in cystic fibrosis transmembrane conductance regulator (CFTR) has been developed to assess the effects of AMPs. The xenograft was exposed to Ad-LL-37 to examine antimicrobial activity against relevant pathogens.⁷⁹ Prior to administration of Ad-LL-37 the airway surface fluid failed to kill *P. aeruginosa* or *S. aureus*. Antimicrobial activity was restored by partial re-constitution of CFTR using adenovirus-mediated gene transfer, with no change in LL-37 levels. However, exposure to Ad-LL-37 alone, without changing CFTR expression, was also sufficient to restore microbicidal activity to normal. This provides further evidence that expression of AMPs does indeed have the potential to protect against bacterial infection when highly expressed, even in an unfavourable environment.

Additional *in vivo* reports have described the capacity of AMPs to act as adjuvants. In these studies AMPs were delivered with non-immunogenic antigens, including tumour antigens, either directly or in the form of fusion plasmid DNA vaccines. Mice given AMPs co-administered with antigen had altered adaptive immune responses, with enhanced production of antigen-specific antibodies, generation of antigen-specific T helper cells with significantly altered cytokine responses, proliferation of splenic cells, and increased resistance to challenge with specific tumours.⁵²⁻⁵⁴

Importantly, despite the proliferation of interest in AMPs as potential therapeutic agents, disappointingly few have emerged as candidates for clinical application. Several clinical trials have examined topical preparations of AMPs with smaller numbers assessing systemic administration, and it is notable that few have reached publication (the inference being that some commercially sponsored trials yielding negative results may not be submitted/accepted for publication), with fewer still showing unequivocal benefits.⁸⁰⁻⁸² It is worth noting that there are, and will undoubtedly be, difficulties in harnessing the beneficial

properties of AMPs in the absence of additional undesired effects, and they should not be seen as a panacea for the future. This is illustrated by the observation that some AMPs have been shown to exhibit pulmonary cytotoxicity and many of the AMPs are effective in animal models of infection only at high concentrations approaching the threshold of toxicity.⁸³

However, this rather gloomy assessment need not signal a bleak future for clinical application of AMPs. These molecules have been intensely studied for only a relatively short period, and it is perhaps not surprising that optimal routes of administration and tissue concentrations require refinement. Indeed, extremely encouraging results have emerged for particular AMPs and related antimicrobial proteins, perhaps best exemplified by reports suggesting beneficial effects of rBPI₂₁ in meningococcal sepsis. In a trial of children with fulminant meningococcaemia who were given rBPI₂₁ there was a reduction in mortality from a historical control figure of 20%, to 4%, and a trend towards improved outcome in all primary outcome variables.^{84, 85} These studies offer considerable promise for the future by suggesting that careful development of other AMPs may allow safe and efficacious application in human infections. In the specific context of lung disease colistin (which belongs to the family of bacterial products, the polymyxins) has found application in cystic fibrosis and ventilator-associated pneumonia.⁸⁶⁻⁸⁸ Furthermore, although the balance of *in vivo* evidence clearly demonstrates that AMPs can play an important role in host defence against infection, in many cases it remains unclear whether this is due primarily to direct antimicrobial activity or immunomodulatory effects. Thus current efforts to dissect out the antimicrobial, immunomodulatory and toxic effects of AMPs may prove to be highly rewarding, with the aim of developing AMPs as immune modifying therapeutics.

Conclusions

AMPs appear to constitute an important part of the innate immune system. A range of additional complex functions is being characterised and the next few years are likely to suggest more diverse roles for these molecules. How the lung regulates production of AMPs, and how these interact during exposure to pathogens requires further study. The clearer understanding of AMP biology anticipated in the coming years seems likely to suggest improved ways of manipulating AMPs to therapeutic advantage, either via exogenous application or by stimulation of endogenous production. These avenues continue to provide a source of optimism at a time when novel strategies for clearance of infection are urgently required.

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