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PO Box 117
221 00 Lund
+46 46-222 00 00

NOVEL ENDOGENOUS ANTIMICROBIAL PEPTIDES

Emma Andersson Nordahl

Institutionen för kliniska vetenskaper, Lund
Avdelningen för dermatologi och venereologi,
Lunds universitet

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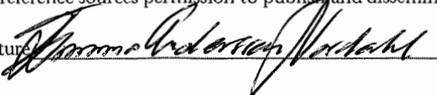
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Professor Dan Hultmark
Institutionen för molekylärbiologi,
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Abstract Antimicrobial peptides serve as a first line of defence against invading microorganisms and are an essential part of our fast innate immune system. They are ancient molecules found in all classes of life. Antimicrobial peptides rapidly kill a broad spectrum of microbes and are immunomodulatory, i.e. having additional actions influencing inflammation and other innate immune responses. Results presented in this thesis demonstrate that proteases of common human pathogens degrade and inactivate the antimicrobial peptide LL-37, probably a strategy for bacteria to circumvent the action of antimicrobial peptides. Likewise, heavily sulphated glycosaminoglycans like dermatan sulphate and heparin were shown to bind to and inactivate LL-37. Furthermore, we demonstrate that structural characteristics associated with heparin affinity (cationicity and amphipathicity) may confer antimicrobial properties to any given peptide. Heparin-binding consensus sequences were proven to be active against Gram-positive bacteria, Gram-negative bacteria and the fungus <i>Candida albicans</i> . Similar results were obtained with synthetic peptides derived from heparinbinding sequences within endogenous proteins. In addition, novel antimicrobial activity and heparin-binding capacity were discovered for the anaphylatoxin C3a, generated during activation of the complement system, and the inactive derivative of C3a (C3a _{desArg}), as well as shorter synthetic peptides from the molecule. Novel antimicrobial activity was also shown for the heparin-binding and cell-binding domain 5 of high molecular weight kininogen, a substrate in the intrinsic pathway of coagulation. Interestingly, the peptide HKH20 (His ⁴⁷⁹ -His ⁴⁹⁸) from this domain was active in high salt, and highly resistant to degradation by various bacterial proteases. The understanding that heparin binding is a property of many antimicrobial peptides may represent a powerful tool in the discovery of novel endogenous antimicrobial peptides from complex biological mixtures.			
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NOVEL ENDOGENOUS ANTIMICROBIAL PEPTIDES

Emma Andersson Nordahl

Department of Clinical Sciences, Lund
Section of Dermatology and Venereology,
Lund University



LUND UNIVERSITY
Faculty of Medicine

Lund 2009

Emma Andersson Nordahl

Phone: +46 (0)736 00 12 34

Fax: +46 (0)40 41 09 21

E-mail: emma.nordahl@nykraft.nu

Front image: Electron micrograph of *Pseudomonas aeruginosa* bacteria (upper left) subjected to C3 (lower left), C3a (upper right) and C3a_{desArg} (lower right).

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**With love to my
angel baby girl Isa**

ABSTRACT

Antimicrobial peptides serve as a first line of defence against invading microorganisms and are an essential part of our fast innate immune system. They are ancient molecules found in all classes of life. Antimicrobial peptides rapidly kill a broad spectrum of microbes and are immunomodulatory, i.e. having additional actions influencing inflammation and other innate immune responses. Results presented in this thesis demonstrate that proteases of common human pathogens degrade and inactivate the antimicrobial peptide LL-37, probably a strategy for bacteria to circumvent the action of antimicrobial peptides. Likewise, heavily sulphated glycosaminoglycans like dermatan sulphate and heparin were shown to bind to and inactivate LL-37. Furthermore, we demonstrate that structural characteristics associated with heparin affinity (cationicity and amphipathicity) may confer antimicrobial properties to any given peptide. Heparin-binding consensus sequences were proven to be active against Gram-positive bacteria, Gram-negative bacteria and the fungus *Candida albicans*. Similar results were obtained with synthetic peptides derived from heparin-binding sequences within endogenous proteins. In addition, novel antimicrobial activity and heparin-binding capacity were discovered for the anaphylatoxin C3a, generated during activation of the complement system, and the inactive derivative of C3a (C3a_{desArg}), as well as shorter synthetic peptides from the molecule. Novel antimicrobial activity was also shown for the heparin-binding and cell-binding domain 5 of high molecular weight kininogen, a substrate in the intrinsic pathway of coagulation. Interestingly, the peptide HKH20 (His⁴⁷⁹-His⁴⁹⁸) from this domain was active in high salt, and highly resistant to degradation by various bacterial proteases. The understanding that heparin binding is a property of many antimicrobial peptides may represent a powerful tool in the discovery of novel endogenous antimicrobial peptides from complex biological mixtures.

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ABBREVIATIONS

AMP	antimicrobial peptide
BK	bradykinin
BPI	bactericidal/permeability-increasing protein
C3, 4, 5	complement factor 3 - 5
<i>CAMP</i>	cathelicidin antimicrobial peptide
CRAMP	cathelin-related antimicrobial peptide
CS	chondroitin sulphate
D3, 5	domain 3, 5
DS	dermatan sulphate
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FIC	fractional inhibitory concentration
FPRL1	formyl peptide receptor-like 1
GAG	glycosaminoglycan
HBP	heparin-binding protein
hBD-1, 2, 3, 4	human β -defensin 1 - 4
hCAP-18	human cationic antimicrobial protein-18
HD-5, 6	human defensin 5, 6 (α -defensins)
HDP	host defence peptide
HMWK	high molecular weight kininogen
HNP-1, 2, 3, 4	human neutrophil peptide 1 - 4 (α -defensins)
HRG	histidine-rich glycoprotein
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAC	membrane attack complex

MASP	mannose binding lectin-associated serine protease
MBL	mannose binding lectin
MEC	minimal effective concentration
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
PAMPs	pathogen associated molecular patterns
PDGF	platelet-derived growth factor
PMN	polymorphonuclear leukocytes (neutrophils)
PRRs	pattern recognition receptor
rD5	recombinant domain 5
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLPI	secretory leukoprotease inhibitor
TGF	transforming growth factor
TLR	toll-like receptor

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I - IV):

- I. Artur Schmidtchen, Inga-Maria Frick, **Emma Andersson**, Hans Tapper and Lars Björck (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Molecular Microbiology* **46**, 157-168.
- II. **Emma Andersson**, Victoria Rydengård, Andreas Sonesson, Matthias Mörgelin, Lars Björck and Artur Schmidtchen (2004). Antimicrobial activities of heparin-binding peptides. *European Journal of Biochemistry* **271**, 1219-1226.
- III. **Emma Andersson Nordahl**, Victoria Rydengård, Patrik Nyberg, D. Patric Nitsche, Matthias Mörgelin, Martin Malmsten, Lars Björck, and Artur Schmidtchen (2004). Activation of the complement system generates antibacterial peptides. *Proceedings of the National Academy of Sciences U S A* **101**, 16879-16884.
- IV. **Emma Andersson Nordahl**, Victoria Rydengård, Matthias Mörgelin, and Artur Schmidtchen (2005). Domain 5 from high molecular weight kininogen is antibacterial. *The Journal of Biological Chemistry* **280**, 34832-34839.

PREFACE

My Personal Aspect of Life Science

During my Masters, I studied antibiotic resistance in a common human pathogen, Streptococcus pyogenes. I noticed that resistance against conventional antibiotics was easily developed. Without treatment these bacteria may cause severe infections in the host like strep throat, scarlet fever and acute rheumatic fever. Invasive infections may result in streptococcal toxic shock syndrome. Since antibiotic resistance among a wide range of bacteria is increasing at an alarming rate, society urgently needs to find new ways to combat these microbes.

During a project, I came across the interesting and fascinating field of antimicrobial peptides. These ancient peptides, found in all classes of life, are known to be important agents in the first line of defence against invading bacteria. What is their mode of action? May antimicrobial peptides be a solution in the efforts to find new approaches to fight pathogenic bacteria and fungi? Is it possible to design drugs based on the principles of these peptides?

In 2002, I got the opportunity to start my PhD-studies in the Artur Schmidtchen group at the section of dermatology, Lund University. About the same time his research was entering the world of antimicrobial peptides and I felt fortunate to start working with the group at such an early stage in this field of research. The main question we wanted to answer was – Why do most wounds stay free from all surrounding bacteria and heal, whereas some wounds, especially among elderly people and diabetes patients become extensively colonized with several types of bacteria and are not able to heal?

Bacterial proteases common in chronic wounds were found to degrade the AMP, LL-37 (paper I). In the same study we also realized that LL-37 is bound to and inactivated by disaccharides like heparin and dermatan sulphate. My PhD-research then continued to focus on using the heparin-binding capacity of AMPs to find novel ones. We discovered that many AMPs may be produced via degradations of different plasma proteins during normal wound healing (paper II, IV), with probably a key role in keeping the acute wound clean and free from microbes. Importantly, we also found that potent AMPs are made during activation of the complement system (paper III).

The 11th of July 2005 my daughter, Isa, was borne. I went on parental leave thinking that this will be a nice break from my PhD-studies. Isa was a gorgeous and happy child and we had a wonderful time together. When she was four months of age the most terrible thing came into our lives. Isa was diagnosed with infant acute lymphatic leukaemia. Our entire world was completely destroyed. Ten months of treatments and hospital care ended when Isa died on the 16th of September 2006. My husband and I went from the greatest happiness to the deepest of sorrows in a moment. Importantly, Isa taught me some essential things about life that I will always bear with me; the importance of a positive attitude to life, a bearable philosophy, and the importance of believing in the beauty and joy of tomorrow to be able to live fully today.

Nowadays, I often think about this in connection with medical science. In our western society medical science has sometimes become more of a religion. Clinical doctors keep trying to decide about life and death, even if they almost never succeed in this. It is important to see that unexpected situations appear all the time, both positive and negative ones. Personally, I do not think that it is beneficial for anybody to portray the worst scenario in a clinical situation and to let children and families feel terrible about things that may never occur? In real life, we all have to deal with the things that actually occur anyway, so what is the point of taking the worst thing, including sorrows and anxiety, out in advance.

As researchers, we must all remember that we do not have all the answers. Why not, in concert with medical science, use the most powerful tool we all have - the strength of our positive thoughts and feelings? I think it must be easier for everybody involved to give people strength, hope, and fighting spirit, rather than doing the opposite. Is it not inhibitive for research to believe that we have the only solution to a problem, especially if we do not completely understand the background of it? It is very important to do research and to try to find answers, but also to be humble to the fact that the answer might be of an entirely different kind than we have first imagined. I am completely sure that we will get even better clinical results if we allow ourselves to believe in the strength of the individual and support those who want to and have decided to feel good and to be well.



INTRODUCTION

Background

Rapid defence mechanisms are essential to prevent invasion and colonization by pathogens in response to a disrupted barrier, such as a mucosal layer or the skin. This defence is promoted by cooperations between the fast innate and the slower adaptive immune systems. The recognition of microbes by innate immunity is mainly due to binding of pathogen-associated molecular patterns (PAMPs), such as polysaccharides and peptidoglycans, by Toll-like receptors (TLRs) on either plasma cells or epithelial cells. This sensing is followed by an efficient killing of microorganisms by recruitment of inflammatory leukocytes or local synthesis of bactericidal substances by epithelial cells. In either scenario, the response includes mobilization and/or production of antimicrobial peptides (AMPs). As part of innate immunity, AMPs contribute to an important first line of defence against invading microorganisms. They provide a rapid and non-specific response.

AMPs produced by bacteria are called bacteriocins and are classified into two groups, based on the inclusion or exclusion of an unusual amino acid called lanthionine. The bacteria *Lactococcus lactis* produce a lantibiotic called, nisin, to combat other surrounding microbes. Nisin was actually one of the first AMPs to be recognized¹. Nisin is extremely potent in action against different Gram-positive bacteria with minimal inhibitory concentrations (MICs) at only nanomolar amounts. Without any significant development of resistance, nisin has been commonly used for over 50 years as a food preservative.

As early as the mid fifties, Hirsch^{2,3} presented evidence for bactericidal substances in extracts from phagocytic granules of rabbit polymorphonuclear leukocytes (PMN). Seven years later, this finding was correlated with the discovery of Zeya and Spitznagel, who identified antimicrobial activity in cationic proteins from rabbit neutrophilic lysosomes^{4,6}. However, these peptides were not successfully purified

until 1984⁷. In 1975, bactericidal/permeability-increasing protein was identified and purified from rabbit granulocytes⁸ and the same year it was shown that a number of different cationic proteins are responsible for the oxygen independent, heat stable killing of bacteria in human polymorphonuclear leukocytes⁹. Some of these cationic peptides were eventually isolated, characterized, and named defensins¹⁰.

The AMP group was however first properly described in the silk moth, *Hyalophora cecropia*, in the early seventies by Hans G. Boman and associates. The immune system of this insect was investigated by injecting bacteria in the *cecropia* pupae and isolating the hemolymph. After several years of hard work, the primary structures of the first two AMPs were reported, cecropin A and B¹¹. This field of research expanded further with Michael Zasloff's discovery and isolation of two potent AMPs (magainins) from the skin of the African clawed frog *Xenopus laevis*¹².

Today we know that all multicellular organisms, thus far investigated, express a blend of AMPs which are distributed at biological boundaries likely to be exposed to the surrounding microflora¹³⁻¹⁶. In higher animals AMPs are produced both locally in epithelial cells, and systemically carried in granules of myeloid cells. They are both inducibly and constitutively expressed, depending on the site of expression¹⁷.

Skin Epithelium

The skin accounts for approximately 16% of the total weight of the human body and is thereby our largest organ. It presents a physical, chemical, as well as an immunological barrier to infection, and displays a substantial innate immune capacity, including AMPs and antimicrobial proteins¹⁸. Other important functions include gas exchange, nutrient absorption, water conservation, regulation of body temperature, and synthesis of vitamin D¹⁹.

Anatomy

Two different layers of the skin are defined and they are collectively called the cutis. The outer layer is epithelial and of ectodermal embryonic origin, called the epidermis. The epidermis is attached to and supported by connective tissue in the mesenchymal dermis, of mesodermal origin. Beneath the dermis is the subcutis or hypodermis. This region is rich in adipose tissue (fat) as an energy reservoir.

The skin is often exposed to harmful assaults like ultraviolet radiation from the sun, scratches, and wounds. It is therefore important for the epidermis to be able to constantly undergo renewal to replace old cells and repair tissue damage. This renewal is dependent on keratinocyte stem cells found in the lower epidermis and is performed by the process of homeostasis²⁰.

The Epidermis

The thickness of the epidermis averages 0.1 mm, with only 0.02 mm on the face and up to 1 - 5 mm on the soles of the feet. Despite the thin nature of the epidermis it is composed of many layers of cells and it is subdivided into five strata (figure 1).

The keratinocyte is the predominant cell type within the epidermis. These cells gradually move from the basal layer towards the surface of the skin. During the migration, the cells change shape, composition, and become filled with keratins, the major structural proteins of skin, hair, and nails. The terminal differentiation, called keratinization, takes place at the granular layer and the cells transform into flat horny skin cells called corneocytes, where the cytoplasm of the cells have disappeared and the nuclei have been digested. The *Stratum corneum* corneocytes are dead cells that provide an impenetrable physical barrier that protects the underlying viable layers. This barrier is continuously replenished as inner layers migrate outwards, and dead cells are sloughed off from the skin surface¹⁹.

Lipids of glucosyl ceramides, cholesterol, cholesterol esters, and long-chain fatty acids are released by lamellar bodies (keratinosomes) of keratinocytes in *Stratum granulosum* into the intercellular space as the *Stratum corneum* is formed. The lipids form multiple layers between the corneocytes. This lipid “mortar” is important for the barrier function of the skin, and the fatty acids contained may be necessary for the acidic pH (4 to 5.5) at the surface of the skin^{19,21}. The lamellar bodies have also been described to release the AMP human β -defensin-2 (hBD-2) into the intercorneocyte space²².

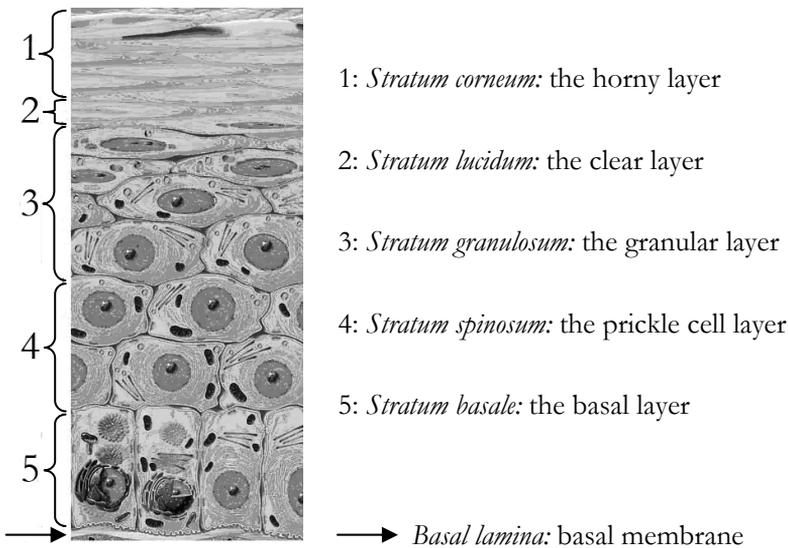


Figure 1. Schematic drawing of the layered epidermis of human skin.

Other important cell types within the epidermis are the pigment producing melanocytes, the dendritic immune cells of the skin and mucosa, (Langerhans cells), and the basal Merkel cells. Melanin is the pigment of skin and hair produced by melanosomes in the melanocytes. Melanosomes are transferred to dividing keratinocytes at the basal layer and melanin serves to protect the nuclei from damaging UV-radiation. The Langerhans cells are important mediators in adaptive

immunity and are found in all layers of epidermis. In response to infection local Langerhans' cells will take up and process microbial antigens to become functional antigen-presenting cells¹⁹. Merkel cells are oval cells found in the *Stratum basale*. Their precise function is still unclear, but they are associated with the sense of touch and with sensory nerve endings.

The Dermis

The dermis is divided into two functional layers, papillary dermis and reticular dermis. The papillary zone is found closest beneath the *Basal lamina* with a depth of between 0.3-0.4 mm, depending on factors like age and anatomical location. The papillary region contains a vascular network to serve the avascular epidermis with nutrients and to regulate body temperature. In sensitive skin areas there are free sensory nerve endings, such as Meissners corpuscles. In the papillary dermis the collagen fibre bundles are thin and poorly organized²³. The reticular dermis, located beneath the papillary region, is built up by dense connective tissue to give the skin elasticity and strength. It contains well-organized fibre bundles of thick collagen, the elastic protein elastin, and proteoglycans. These are all secreted by fibroblasts, the most common cell type of the dermis. The reticular dermis also houses hair follicles, sweat glands, and sebaceous glands²³.

Proteoglycans are heavily glycosylated glycoproteins with a core protein and one or more covalently attached glycosaminoglycan (GAG) chains. These GAG-chains are long and linear carbohydrate polymers, with a negative charge under physiological condition, due to sulphate and uronic acid side groups. Dermatan sulphate (DS), previously called chondroitin sulphate-B (CS-B), and heparan sulphate (HS) are two GAGs found in dermis proteoglycans. Heparin is a member of the heparan sulphate family of complex sugars and is released from mast cells at sites of tissue injury. The interesting findings that these GAGs bind and inactivate the cationic AMPs, α -defensin (HNP-1)²⁴ and LL-37²⁵, were the starting point of the work for this thesis.

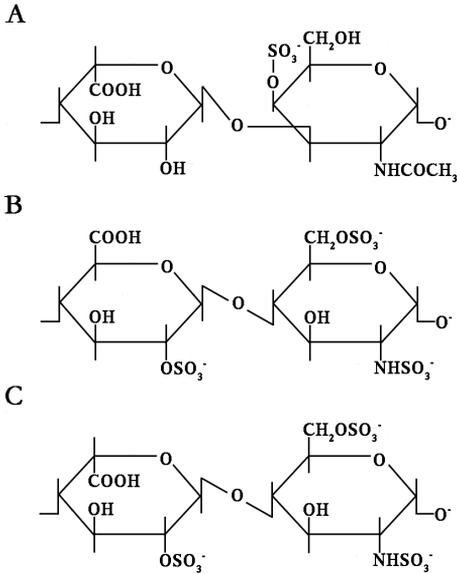


Figure 2. Chemical structures of single units of two glycosaminoglycans (GAGs) found in the human dermis (A) Dermatan sulphate (DS) and (B) Heparan sulphate (HS). This compared with a single unit of (C) the heparin molecule found in and released from mast cells.

Wound Healing

Wound healing is a complex and dynamic process whereby the body regenerates itself and repair tissue damage. The process requires cooperation of a number of different cell types, such as platelets, different leukocytes, and ultimately fibroblasts and keratinocytes. Wound healing encompasses three overlapping phases including inflammation, proliferation, and tissue remodelling.

Inflammatory Phase

Tissue injury causes disruption of blood vessels and blood constituents are released into the wounded area. Platelets aggregate to form the primary hemostatic plug. In order to stop extensive blood loss, platelets stimulate activation of plasma coagulation factors. Prothrombin is activated to thrombin which then converts fibrinogen to fibrin to generate a stable clot of fibrin, fibronectin and the GAG hyaluronic acid²⁶. This clot serves as a provisional extracellular matrix (ECM). It protects the wound and is the main structural support until collagen fibres are restored in the area.

Aggregated thrombocytes are triggered to stimulate an inflammatory response. They secrete potent chemoattractants for inflammatory cells, such as platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β)²⁷, factors that activates local fibroblasts and endothelial cells and vasoconstrictors²⁸.

The inflammatory phase is characterized by a massive degradation of injured ECM components. Neutrophils clean the wound from bacteria, and macrophages phagocytose matrix and cell debris, including consumed neutrophils. Monocytes and macrophages are essential during repair, both in cleaning the wound from debris, but also in the production of growth factors necessary for matrix formation and angiogenesis (TGF- α , TGF- β and interleukin-1), making these cells important in the transition between inflammation and tissue re-epithelialization during wound healing²⁶. The absence of macrophages significantly impairs wound healing²⁹.

Proliferative Phase

The proliferative phase is characterized by activation of local fibroblasts, granulation, contraction, and epithelialization.

Growth factors, particularly PDGF and TGF- β 1, together with ECM molecules stimulate fibroblasts at the wound margin to express integrin receptors, to proliferate and to migrate over the provisional ECM in the wounded area. Fibroblasts begin to enter the wound area about two or three days after injury, and this migration marks the start of the proliferative phase, even before the inflammatory phase has ended. In three to five days, these cells will become the predominant cell type in non-infected wounds.

Fibroblasts are responsible for ECM materials like collagen (type I and III), fibronectin, and proteoglycans to be deposited in the area and thereby the provisional matrix is gradually replaced by a collagenous ECM. Angiogenesis, which provides the area with nutrients and oxygen, fibroblast proliferation, and the

expression of the appropriate integrin receptors that bind fibronectin and fibrin, are essential steps in the formation of granulation tissue (new stroma)^{26,29}.

Wound contraction is a phenomenon in which the marginal skin is pulled toward the wound. About a week after the injury, fibroblasts start to differentiate into myofibroblasts and the wound begins to contract. Myofibroblasts are responsible for contraction and they contain the same kind of actin as in smooth muscle cells. Actin is linked across the cell membrane to fibronectin and collagen in the ECM. When the actin in myofibroblasts contracts the wound edges are pulled together. The contraction stage ends when myofibroblasts stop contracting and go into apoptosis³⁰. Without any new formation of tissue, the contraction process dramatically decreases the size of the wound and speeds up the closure of the wound resulting in a smaller scar than that by epithelialization alone²⁸.

During epithelialization, keratinocytes migrate across the wound to re-establish the epidermis. Formation of actin filaments and pseudopodia makes keratinocytes able to move from the basal membrane at the edge of the wound²⁹. Keratinocytes and fibroblasts secrete laminin and type IV collagen to restore the injured basal lamina. The keratinocytes then change shape in the newly formed *Stratum basale* and become columnar and start to divide to establish a new epidermis²⁸.

Remodelling Phase

Tissue remodelling or scar maturation starts when collagen production is equal to degradation. The balance is regulated by matrix metalloproteinases (MMPs), such as collagenases, gelatinases, and stromelysins, which are responsible for the degradation of ECM components²⁸. During maturation, type III collagen is gradually replaced by the stronger type I, and disordered collagen fibres are organized and cross-linked to increase the tensile strength of the wound. The ultimate strength of wounded skin reaches about 80% of that of normal skin. The remodelling phase may last for a year or more depending of the nature of the wound³¹.

Immune Systems

An Overview

Immunity is the ability of the host to resist infection or disease. The vertebrate immune system is divided into one innate and one adaptive part. Innate immunity is the first and oldest defence of the body and refers to everything that is already present. This includes physical barriers and epithelial cells, neutrophils and macrophages as well as their effectors, the complement system, proteins with direct antimicrobial effects, and AMPs. This immune system is fast and unspecific in action. On the other hand, adaptive immunity or acquired immunity is slow; a primary immune response takes up to seven days and a secondary response takes about three days³². The adaptive immune response evolved ~450 million years ago, in the period between the jawless and jawed fishes³³. It is specific and inducible in action and can discriminate between self and non-self. The adaptive immunity is able to remember pathogens that the organism has come across earlier. It comprises lymphocytes with the B-cell derived repertoire of antigen specific antibodies, and activated B- and T-cells as immunological memory. During evolution, natural selection has made adaptive immunity a common feature of all vertebrates.

In the early 20th century, there was much controversy between the two fields of immunology, ‘cellular’ and ‘humoral’. The conflict was to some extent resolved and the importance of both fields was emphasized in 1908, when the Nobel Prize in Physiology and Medicine was shared between Metchnikoff ‘cellularist’, and Ehrlich, ‘humoralist’, for the cooperative interactions between innate and adaptive immunity³⁴. This cooperation is well illustrated in the ‘classical’ pathway of the complement system where antigen-antibody complexes trigger activation of the system, ultimately leading to microbial killing either by the membrane attack complex (MAC) or by phagocytosis of C3b-opsonized bacteria. Another example from the ‘classical’ pathway is our own finding that factor C3a and its inactive derivative C3a_{desArg} exert strong direct antimicrobial activity³⁵. Dendritic cells

constitute yet another important link between the two immune systems. They contribute to innate immunity by expressing TLRs and by their phagocytic activity, and to adaptive immunity by presenting antigens to naïve T-cells via the major histocompatibility complex (MHC) class II³⁶.

Innate Immunity

Innate immunity is a rapid and universal form of immunity; the majority of organisms survive with only this type of defence system. The innate immune system is ancient in origin and found in all studied multicellular organisms. Mechanisms of innate immunity are dynamic during evolution, because microbes impose selective pressures on the host resulting in survival of the fittest.

The starting point for research in innate immunity must be attributed to Ilya Metchnikoff, who in 1884 announced his theory of phagocytosis. He had witnessed the engulfment of particulate dyes and fungal spores by ‘wandering cells’ in invertebrates³⁷.

Innate immunity comprises both constitutive and inducible mechanisms. Included in constitutive defences are physical barriers, like the skin epithelium and different types of mucosal epithelia, antimicrobial proteins, and AMPs — the subject of this thesis. The inducible mechanisms in innate immunity differ from those in adaptive immunity. They are rather non-specific and use conserved molecular patterns to recognize pathogens, such as bacterial lipopolysaccharides (LPS) and lipoteichoic acids (LTA)³². The induced innate defence is rapid, requiring only minutes to hours. It includes the activation of phagocytes, such as PMNs and macrophages, other leukocytes, such as mast cells, the lectin pathway of complement, and signalling pathways that result in transcription of different genes involved in immune response, like cytokines and other inflammatory mediators^{32,38}. Some AMPs are also inducible in nature like hBD-2, which is induced from epithelial cells by interleukin-1 (IL-1)³⁹.

Recognition of Microbes

The inducible innate defence recognizes microbes by non-clonally distributed receptors that sense certain molecules exclusively found on the surface of microbes and not on self-tissues. These molecules are called pathogen associated molecular patterns (PAMPs) and the receptors are collectively named pattern recognition receptors (PRRs)³⁶. One important type of PRRs is the TLRs⁴⁰. The *TLR* family of genes are evolutionary very old and are conserved in both invertebrates and vertebrates^{41,42}. The receptors were called Toll-like because of the homology to the first receptor found in this class, the receptor Toll described in *Drosophila*. The *Toll* pathway was found to regulate the synthesis of an antifungal peptide, drosomycin^{43,44}. The vertebrate TLRs are found on the surface of epithelial cells, macrophages, dendritic cells, and PMNs.

The Complement System

The complement system is an evolutionary old and significant part of innate immunity. It is activated either directly or indirectly by microbes. The system includes about 35 different proteins and their activation proceeds in a cascade fashion eventually resulting in bacterial opsonisation by C3b. This is followed by either phagocytosis or the assembly of a pore-forming MAC on the bacterial surface, both leading to lysis and destruction of bacteria. The system can be activated via three pathways that differ only in their formation of the C3 convertases in the initiation of the cascade⁴⁵.

They are called the classical, alternative and lectin pathway (figure 3). The classical pathway is triggered by antigen-antibody complexes and activation of C1qrs leads to activation of the classical C3 convertase, C4bC2a. In the absence of antigen-antibody complexes, an alternative spontaneous conversion of C3 to C3b is triggered by different surfaces. On the host cell surface, inhibitory proteins and sialic acid immediately stop the complement cascade. On foreign surfaces, like bacteria,

inhibitory substances are not present and C3b binds Factor B, forming an alternative C3 convertase (C3bBb) and more C3b are converted from C3. An alternative pathway positive feedback loop thereby amplifies the initially small amounts of C3b. The lectin pathway is started when mannanose binding lectin (MBL) binds to bacterial surfaces with mannanose containing polysaccharides. The surface binding of MBL generates an association between two MBL-associated serine proteases, (MASP-1 and MASP-2, which are homologous to C1r and C1s in the classical pathway) and the active MBL/MASP-1/MASP-2 complex activates C4 and C2⁴⁵ to generate the classical C3 convertase.

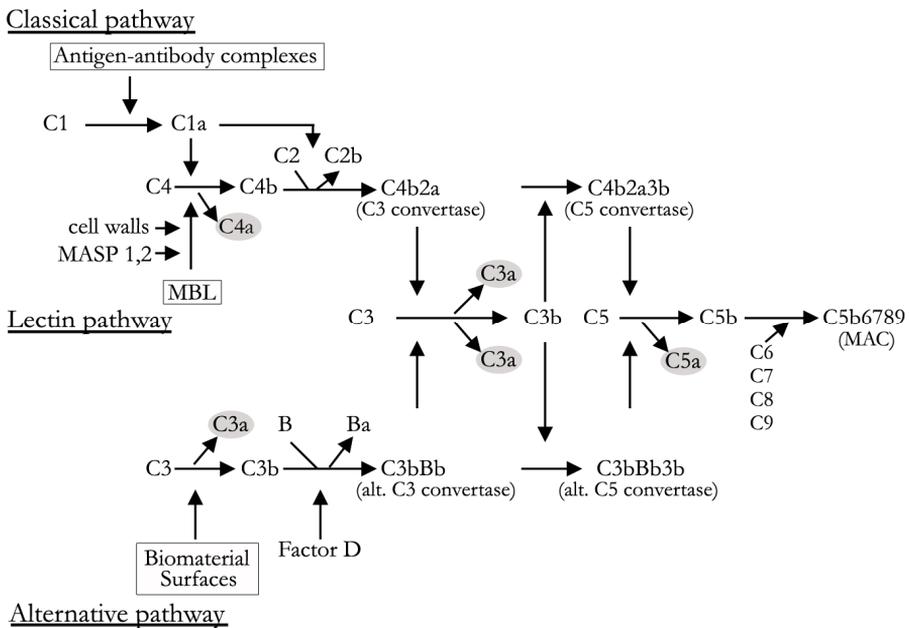


Figure 3. Overview of the complement cascade. The three initiation pathways are underlined and the release of the anaphylatoxins is shown in grey. The starting material of each pathway is presented in a rectangle. MBL = mannanose binding lectin, MASP = mannanose binding lectin associated serine protease and MAC = membrane attack complex.

Peptides with anaphylatoxic activity are released during the complement cascade, C3a, C4a and C5a. These peptides are proinflammatory agents. They bind to receptors on basophils and mast cells and thereby mediate degranulation and histamine release. They promote enhanced vascular permeability and contraction of smooth muscle cells. The C5a peptides also mediate chemotaxis during inflammation with attraction of leukocytes to the site of infection. In addition, C5a triggers leukocyte activation, and generation of cytotoxic oxygen radicals.

The complement factor C3a is a cationic peptide (pI 11.3) of 77 amino acids with a molecular weight of 9.083 kDa (figure 4). The secondary structure of C3a contains four α -helical regions and six cysteine residues that are responsible for the formation of three intrachain disulphide bridges. The folding of the compact C3a molecule is mainly dependent on these disulphide bonds. The plasma concentration of C3 is 5 - 11 μ M and during inflammation additional amounts of C3 are generated by monocytes and keratinocytes^{46,47}. C3a may therefore be generated in micromolar concentrations at the site of complement activation. In sepsis patients levels of up to 0.5 μ M of C3a has been measured in blood⁴⁸ and in patients suffering from acute media otitis concentrations of approximately 0.7 μ M C3a are found in ear secretions⁴⁹. C3a may be present in significant amounts in blood, but its anaphylatoxin activity is under strict regulation by the serum protease carboxypeptidase N, which cleaves off the C-terminal arginine residue generating the inactive peptide C3a_{desArg}⁵⁰. The primary structures of C3a from different species are distinct, but some common characteristics are believed to be essential for maintaining function. The three disulphide bridges that stabilize the structure are for instance important. The binding site of C3a to its receptor is located in the far C-terminal LGLAR peptide, which are also highly conserved between species. This explains the site of action of the regulating protease. It is important to note that many of the amino acid replacements in C3a that occur between species are conservative, meaning that they maintain characteristics like side-chain size, polarity and shape, which minimize the 3D consequences of the change^{50,51}.

An additional important activity of C3a is presented in this thesis. The peptide is proven to be a potent antimicrobial agent^{35,51,52}. Its antimicrobial activity is highly conserved during evolution from invertebrates to humans and it seems that this activity is more connected to the 3D structure of the molecule than to the primary structure, that shows significant sequence variations⁵¹. Interestingly, evolutionary pressures on functionality and structure must have been imposed on C3a to preserve critical features important for antimicrobial activity side by side with the development of the other immunologically important activities of this peptide.

SVQLTEKRM¹⁰ KVGKYPKELR²⁰ KCCEDGMREN³⁰ PMRFSCQRRT⁴⁰
 RFISLGEACK⁵⁰ KVFLDCCNYI⁶⁰ TELRRQHARA⁷⁰ SHLGLAR⁷⁷

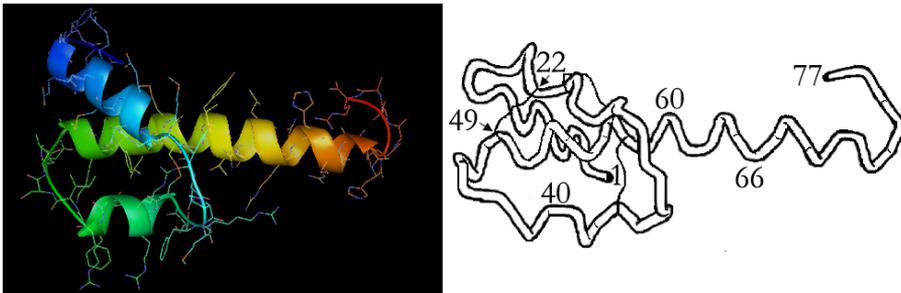


Figure 4. Amino acid sequence and molecular models of C3a. Underlined is the CNY21 peptide, the smallest C3a peptide known to possess nearly full anaphylatoxic activity⁵³. Left: α -helical regions in the molecule are indicated. Right: Three intrachain disulphide bridges (C22-C49, C23-C56, and C36-C57) and numbered amino acid residues are shown from N-terminal S1 to C-terminal R77.

Antimicrobial Peptides

AMPs are ancient and essential components of the fast acting innate immune system in probably all classes of life³². During evolution these peptides have obtained high efficiency in spite of highly mutable target microbes with a fast generation time. This suggests an important co-evolution between hosts and

pathogens. About 1400 AMPs are now defined in a variety of life forms (<http://aps.unmc.edu/AP/main.php>)⁵⁴, including plants⁵⁵, insects⁵⁶, amphibians⁵⁷, fishes⁵⁸, birds⁵⁹, and mammals⁶⁰. AMPs are also found in prokaryotes as a defence against competing local microbes^{1,61}.

AMPs comprise a diverse spectrum of activity against various targets such as bacteria, fungi⁶², protozoa⁶³, enveloped viruses^{64,65}, and even tumour cells^{66,67}. Some AMPs display broad-spectrum activity against Gram-positive bacteria, Gram-negative bacteria, and fungi⁶⁸, whereas other peptides are specialists, like cecropin P1⁶⁹ showing activity only against Gram-negative bacteria. AMPs are primarily constitutively expressed, but at special sites in the body the expression may be induced in response to certain stimuli like cytokines, bacteria, and PAMPs. The killing of microorganisms and the destruction of bacterial plasma membranes by human AMPs occurs at micromolar concentrations, but fortunately normal human host cells are relatively resistant at those concentrations⁷⁰. Synergistic effects are found between different AMPs, but also between AMPs and larger antimicrobial proteins like lysozyme⁷¹. Synergy means that the activity is increased more than additively by the combination of factors.

Host Defence Peptides

It has been increasingly evident that many AMPs possess complementary abilities, besides their direct antimicrobial effects, in modulating the innate and inflammatory responses⁷²⁻⁷⁴ (figure 5). AMPs are found to influence inflammation, affect chemotaxis and accumulation of leukocytes at inflammatory sites⁷⁵⁻⁷⁷, enhance phagocytosis, neutralize the effects of LPS, stimulate release of prostaglandin, promote angiogenesis^{78,79} and induce wound healing³². Other biological activities of AMPs include promotion of apoptosis⁸⁰⁻⁸², antiangiogenesis, inhibition of proteases, as well as stimulation of cell growth. Many AMPs show rather weak or no direct antimicrobial activity in physiological high salt conditions (see below), but in the same environment are both potent and selective immunomodulatory agents. LL-37

has been demonstrated to trigger multiple signalling pathways, including the classical Mitogen-activated protein (MAP) kinase pathway, by using a specific intracellular receptor resulting in induction of different chemokines⁸³. Cathelicidins (LL-37, BMAP-27 and indolicidin) are also found to suppress the effects of LPS, leading to reduced production of proinflammatory cytokines like TNF- α ⁸⁴⁻⁸⁶. By neutralizing LPS, cathelicidins may protect the host from endotoxaemia and toxic shock. LL-37 and BMAP-27 both reduce substantially various LPS-induced chemokines⁸⁶, indicating a role for cathelicidins in maintaining homeostasis during endotoxin challenge by balancing the pro- and anti-inflammatory responses. Because of the many immunomodulatory effects of antimicrobial peptides these molecules are currently frequently referred to as host defence peptides (HDP)⁸⁷.

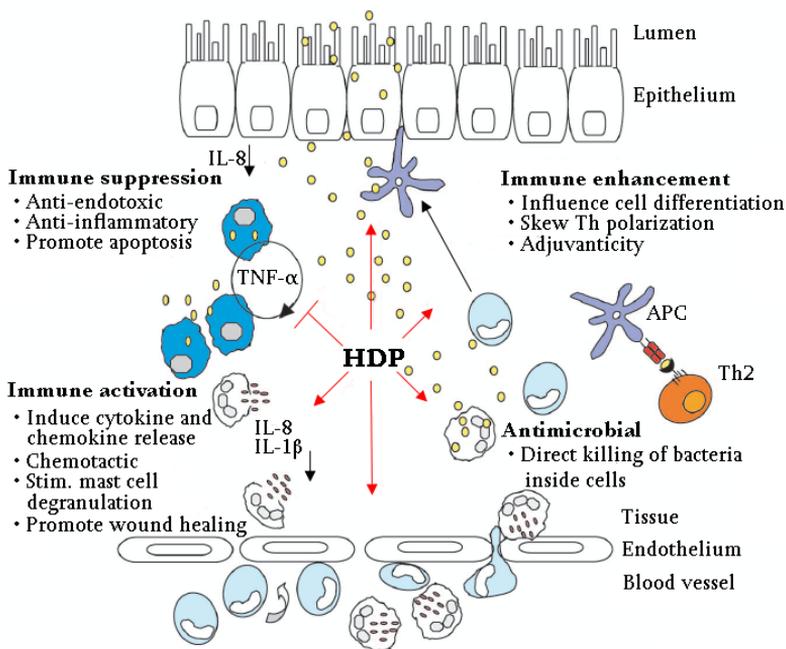


Figure 5. Multifunctionality of AMPs or host defence peptides (HDPs). Interestingly, AMPs/HDPs maintain homeostasis by simultaneously promote pro- and anti-inflammatory immune responses. Small circles represent bacteria or bacterial products. Schematic view modified from Brown and Hancock 2006⁸⁸

Structure

Despite diverse origins of AMPs, they have several biophysical parameters in common. Small size, cationicity, amphipathicity and hydrophobicity are probably important factors for the efficacy of AMPs. AMPs are relatively short polypeptides (< 10 kDa), generally composed of between 12 and 60 amino acid residues. Their net charge is positive (+2 to +9) with about 50% hydrophobic amino acids and an excess of basic residues (arginine, lysine and/or histidine). The secondary structure is often of amphipathic nature^{32,89}, consisting of both a significant non-polar and a polar part. Amphipathicity of a peptide means that there is a spatial separation between hydrophobic and hydrophilic amino acid side chains in the molecule. Amphipathicity can be determined by calculating the hydrophobic moment, which is the vector sum of the hydrophobicities of each amino acid residue perpendicular to the axis of the helix. The mean hydrophobic moment per residue is used to compare amphipathicity between peptides of different length⁹⁰. A more descriptive way to present amphipathicity of an α -helical peptide is to show a helical wheel projection⁹¹, where polar and non-polar amino acids are distributed into discrete sectors of the wheel (figure 6). Many AMPs are salt-sensitive and therefore inactive in the presence of physiological salt. This sensitivity is proposed to be due to instability of the α -helical structure in addition to strong electrostatic interactions in high salt conditions⁹². Capping of the N- and C-terminal ends of salt-sensitive AMPs is a way to stabilize the helical structure, and the result indeed results in salt-resistant antimicrobial peptides⁹². However, studies have shown that increasing helicity and helix-stability of antimicrobial peptides may not only increase the antimicrobial activity but also the toxicity of these peptides against eukaryotic cells⁹³.

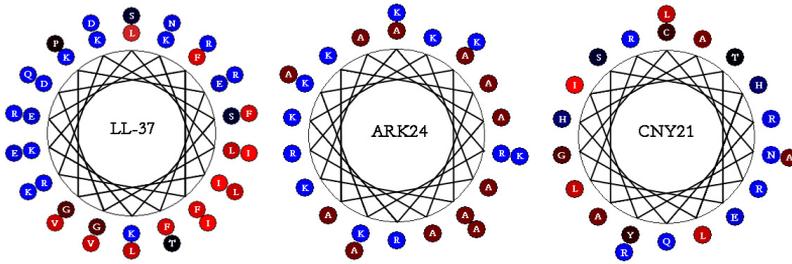


Figure 6. Helical wheel projection of three antimicrobial peptides. The cathelicidin LL-37, the heparin-binding consensus sequence ARK24 and the C3a-derived peptide CNY21. Red indicates hydrophobic (non-polar) residues and blue hydrophilic (polar) residues, dark red indicates less hydrophobicity and black indicates intermediate residues.

Classes of Antimicrobial Peptides

AMPs are a very diverse group of molecules and there are different ways to categorize these peptides. Here I have chosen a subgrouping according to the primary and secondary structure and with regard to the origin of the peptides. Most of the AMPs fit into one out of four major subgroups⁹⁴; linear cationic α -helical peptides; cationic peptides with β -strands stabilized by disulphide bonds; cationic peptides enriched for certain amino acid residues; cationic peptide fragments of larger proteins. An additional very small subgroup consists of anionic peptides.

The subgroup of **linear cationic α -helical peptides** is the most studied group of AMPs. These linear peptides do not contain disulphide bridges and cysteine residues and typically consist of less than 40 amino acid residues⁹⁵. Many of these AMPs are unstructured, extended or linear in aqueous solutions, but are able to adopt an α -helical conformation in hydrophobic environments, like in the closeness of membrane lipid bilayer structures^{89,95}. The extent of α -helicity is well correlated with the antimicrobial activity of these peptides. Increased α -helical content gives

increased antimicrobial effects⁹⁶. AMPs with this secondary structure include for example cecropins^{11,97,98}, magainins¹², dermaseptins⁹⁹, buforin II⁹⁶ and LL-37^{100,101}.

The AMPs in the subgroup of **cationic peptides with β -strands stabilized by disulphide bonds** often have several antiparallel β -strands and the structures are stabilized by up to six disulphide bridges¹⁰². This is a highly diverse group of peptides and conventional multiple sequence alignments (MSA), in the N- to C-terminal orientation, found no consensus motifs with respect to the primary structure. However, by using stereospecific MSA, a general pattern was discovered among all antimicrobial peptides that are stabilized by disulphide bridges¹⁰³. This characteristic cysteine motif, called the γ -core motif, includes 8 to 16 amino acid residues and a conserved GXC or CXG motif within the sequence¹⁰³. Other characteristics of the γ -core motif are a net positive charge (+0.5 to +7) with basic amino acids polarized along the axis of the motif; amphipathicity; and a stabilizing function of at least one disulphide bridge¹⁰³. Peptides in this subgroup include for instance, protegrin from pigs, tachyplesins from horseshoe crabs¹⁰⁴ and the different defensins, α -defensins¹⁰⁵, β -defensins¹⁰⁶, rhesus θ -defensin¹⁰⁷ and insect defensins¹⁰⁸.

AMPs containing one or more amino acids that are overrepresented belong to the subgroup **cationic peptides enriched for certain amino acid residues**. The predominant residues are often proline/arginine, histidine, or tryptophan. These AMPs are variable in structure and not well characterized. Examples are PR-39 from pigs, with an overrepresentation of proline and arginine; the human histatins, with a large amount of histidine residues¹⁰⁹; the bovine indolicidin, with the highest tryptophan content of any known protein residues.

The fourth subgroup is based on the origin of the peptide and the members are **cationic peptide fragments of larger proteins**. All novel antimicrobial peptides presented in this thesis are representatives of this subgroup. Examples of peptides are lactoferricins from lactoferrin^{110,111}, casocidin-I from bovine casein¹¹²,

hemocidins from apohemoproteins like hemoglobin, myoglobin and cytochrome c^{113,114}, C3a from C3³⁵, degradation products of high molecular weight kininogen (HMWK)^{115,116} and antimicrobial and heparin-binding domains from many different endogenous proteins¹¹⁷. These peptide fragments show strong antimicrobial effects and share biophysical parameters with conventional AMPs, as described above, but their actual importance in innate immunity needs to be further investigated. Interestingly, the main bactericidal action of human seminal plasma is due to peptides generated by proteolytical degradation of semenogelin I and II¹¹⁸. Semenogelins, and the peptides derived thereof, are rich in histidin residues, and could therefore also fit in the subgroup of peptides enriched for certain amino acids. Semenogelin peptides, in common with most other histidin-rich polypeptides, exhibit zinc-dependent antimicrobial effects¹¹⁸.

A small additional subgroup of AMPs is identified as **anionic peptides**. Three of these peptides are present in human surfactant extracts, bronchoalveolar lavage fluid, and in airway epithelial cells. They are very small in size, rich in glutamic and aspartic acids, and require zinc as a cofactor for antimicrobial activity⁹⁴. These peptides are totally different from the well-characterized cationic AMPs and they are thought to be a part of the innate pulmonary defence system. Collectively, they are called surfactant-associated anionic peptides (SAAP)¹¹⁹. Other anionic AMPs are maximin H5 isolated from the toad *Bombina maxima*¹²⁰ and dermcidin from human sweat glands¹²¹. The modes of action of these anionic AMPs have not been completely revealed.

Many AMPs are unstructured in aqueous solution and are folded into an amphipathic formation when they come in contact with a membrane. This is true for AMPs forming α -helices, but also for peptides enriched for certain amino acid residues, which often fold into more extended structures. Disulphide stabilized β -sheet or β -hairpin peptides are regularly prefolded in their secondary and tertiary structure in solution. Independent of the 3D arrangement, and when the folding of

the peptides takes place, the final structure contains some hydrophobic parts and some charged hydrophilic parts that enable peptide interactions with bacterial plasma membranes¹²².

Main Families

Mammals have two main families of AMPs; the cathelicidins and the defensins¹⁶. Cathelicidins are made as inactive precursors and the antimicrobial peptide becomes active when it is proteolytically cleaved off from the C-terminal end of the protein. Mammalian defensins are instead stored in their mature active forms and their structure is based on a β -sheet core stabilized by three disulphide bridges.

Cathelicidins

Cathelicidins are synthesized as prepropeptides. They have a general structure of a signal peptide in the far N-terminal end, a very conserved proregion in the middle, and a highly variable C-terminal part, containing the antimicrobial peptide. Cathelicidins are processed in two steps. First, the signal peptide is cleaved off and second, the mature AMP is activated by release. These peptides are stored in cells in the propeptide form. The conserved proregion is called the cathelin-like region because of the high sequence identity with the pig *cath*epsin *L* inhibitor, cathelin. The genes encoding cathelicidins are evolutionary conserved and generally include four exons. The first encodes the signal peptide, the second and third encode the cathelin-like region, and the fourth exon encodes the processing site and the antimicrobial domain.¹²³ Cathelicidins are mainly produced in the bone marrow and stored in granules of neutrophils¹²⁴, but human LL-37 is also found to be expressed in monocytes, macrophages¹²⁵, B-cells and T-cells⁷⁵. However, some cathelicidins are also identified in epithelial cells, like keratinocytes in inflamed skin¹⁰⁰ and in the epithelia of the human lung^{100,126}, indicating a local role for these molecules. Examples of mammalian cathelicidins are proline-arginine-rich PR-39 and protegrins from pigs, CRAMP from mice, bovine BMAP-27, BMAP-28, Bac5 and

Bac7¹²³. In addition, cathelicidins are also found in a few other species like fish¹²⁷ and chicken¹²⁸.

The only human cathelicidin protein is hCAP-18 (human cationic antimicrobial protein-18, 18 kDa), with its active AMP, LL-37 (figure 7) – a peptide of 37 amino acids (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES). *CAMP* (cathelin antimicrobial peptide) is the gene encoding hCAP-18 and it is located on chromosome 3p21.3¹²⁹. hCAP-18/LL-37 is found at high concentrations (40 μ M or \sim 630 μ g/ 10^9 cells)¹³⁰ in its unprocessed form in the peroxidase-negative granules of human neutrophils¹²⁹. The propeptide is processed upon degranulation by the serine protease proteinase 3 from azurophilic granules of neutrophils, either in the phagolysosome or in the extracellular environment, and the active LL-37 peptide is cleaved off and released¹³¹. hCAP-18/LL-37 is also present in high concentrations in seminal plasma¹³². Interestingly, when seminal plasma enters the vagina after sexual intercourse, the progastricsin becomes activated by the acidic milieu and the proteolytic enzyme gasticsin is able to process hCAP-18 and release the active AMP¹³³. This is probably a way to lower the risk of infection following sexual intercourse.

Besides their antimicrobial effects, cathelicidins have a number of additional functions as HDPs. Two receptors are for example found to be involved in cell signalling mediated by LL-37 in leukocytes. LL-37 interacts with the G protein-coupled receptor known as formyl peptide receptor-like 1 (FPRL1), a signalling pathway leading to chemoattraction of neutrophils, monocytes, T-cells⁷⁶, and eosinophils¹³⁴, and the purinergic P2X₇ receptor, known to mediate LL-37 induced processing and release of interleukin-1 beta¹³⁵. LL-37 has also been demonstrated to activate and release histamine from mast cells⁷⁶ and to inhibit HIV-1 replication. The HIV-1 inhibitory effect was also shown to be independent of FPRL1 signalling¹³⁶.

Defensins

Like cathelicidins, the defensins are synthesized as prepropeptides, which are then processed to various extents to release the active peptides. Three subfamilies of defensins are known in mammals; α -defensins, β -defensins and θ -defensins. All three subfamilies have features in common including, rather short peptides (18 - 45 amino acids, 2 - 6 kDa) with a net positive charge (+1 to +11), and three intramolecular disulphide bridges with tertiary structures dominated by turn-linked β -strands. The spacing between the cysteines and location of the disulphide bonds differ between α -defensins and β -defensins¹³⁷ (figure 7). It was assumed that the disulphide bond and the tertiary structure were essential for the antimicrobial activity of the defensin peptides, but now data indicates that protection from proteolysis is the main function of the intramolecular bridges. It is however shown that substitution of the cysteines completely abolishes the chemotactic activity of human β -defensin 3 (hBD-3), but the antimicrobial activity remains unaffected in the absence of disulphide bridges¹³⁸. Despite the differing secondary structure of α -defensins and β -defensins, their tertiary (3D) structure is very similar (figure 7)¹⁷. The genes encoding α -defensins, *defa*, and the most studied β -defensins, *def β* , are found in the same region of chromosome 8 (8p23) in a 450 kb segment, and it is suggested that the α -defensin gene family has evolved from a common ancestral β -defensin gene¹³⁹.

Six different α -defensins are identified in humans, human neutrophil peptides 1 - 4 (HNP 1 - 4) and human defensins 5, 6 (HD-5, 6). Four of them (HNP 1-4) are expressed systemically and stored in azurophilic granules of neutrophils¹⁴⁰. Interestingly, half of the protein content of these granules is actually comprised of HNP-1, -2 and -3, whereas HNP-4 occurs in lower concentrations¹⁴¹. Recently, expression of HNP1-3 has also been found in human monocytes¹⁴² and natural killer cells¹⁴³. HD-5 and HD-6 are primarily expressed in the human intestinal Paneth cells¹⁴⁴, where the processing of the propeptide (proHD-5) is mediated by Paneth cell trypsin¹⁴⁵. However, HD-5 has also been found in vagina, cervix, and Fallopian tubes in the human female reproductive tract¹⁴⁶.

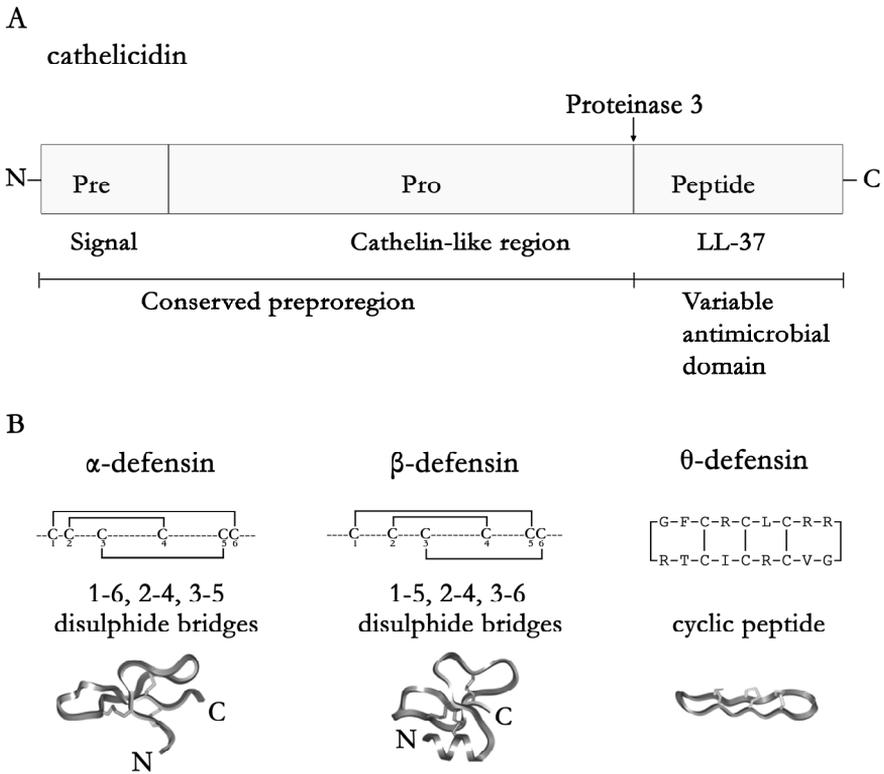


Figure 7. Representatives of the main families of mammalian AMPs, cathelicidins (A) and defensins (B). (A) Schematic drawing of the human cathelicidin prepropeptide hCAP-18 with the C-terminal AMP LL-37. (B) The processed peptides of defensins with disulphide bridges. α -defensin (left), β -defensin (middle) and θ -defensin (right).

In 1993 a set of defensin-like peptides from bovine PMNs called β -defensins were discovered¹³⁷. The increasing family of vertebrate β -defensins has at the moment ~60 members, including 43 from mammals, 6 from birds and 11 (β -defensins-like peptides) from marsupials and reptiles¹⁴⁷. Although about 30 human β -defensin genes have been identified with bioinformatics¹⁴⁸, only four peptides (hBD-1,

hBD-2, hBD-3, and hBD-4) have so far been isolated. In contrast to α -defensins β -defensins are generally expressed locally in epithelial cells. hBD-1 was the first human β -defensin to be found. Its gene is constitutively expressed by several epithelia directly exposed to the surrounding microbial flora (e.g. lung, salivary gland and kidney)¹⁴⁹. hBD-1 is present in plasma and some truncated forms are found in urine, but the peptide was first purified from hemofiltrate from kidney dialysis patients. hBD-2 and hBD-3 were later isolated from the scales of psoriatic skin¹⁷. The expression of both hBD-2 and 3 are induced by inflammatory stimuli (such as TNF- α)¹⁵⁰. By using bioinformatics including the Basic Local Alignment Search Tool (BLAST), novel genes in the 8p23 cluster were found (hBD-4-6)^{151,152}. The fourth β -defensin to be isolated was hBD-4, with the highest expression level found in the testis and in the gastric antrum¹⁵³. In 2002, also by the use of bioinformatics, three new β -defensin gene clusters (at 6p12, 20q11.1 and 20p13), containing 28 novel human β -defensin genes, were discovered¹⁴⁸. However, isolation of these β -defensins has not yet been reported.

θ -defensins, the third subfamily of defensins, are cyclic peptides that arise from two precursor peptides containing nine amino acid residues each. They are only found as pseudogenes in humans and in New World monkeys, but are fully expressed in several species of Old World monkeys (like Rhesus macaque and Pigtail macaque) and in the ape orangutans. The human θ -defensin mRNA is not translated due to a premature stop codon in the transcripts¹⁵⁴. In Rhesus macaque θ -defensin is only detected in neutrophils and monocytes.¹⁰⁷ The θ -defensins antiviral properties are much more powerful than their antibacterial and antifungal effects. Interestingly, the θ -defensins retrocyclin 1 and especially retrocyclin-2 show striking activity against human immunodeficiency virus (HIV-1), herpes simplex virus-1 (HSV-1), and herpes simplex virus-2 (HSV-2).

Mode of Action

AMPs can be divided into membrane or non-membrane active⁹⁴. Most linear cationic α -helical AMPs are suggested to form pores in and disrupt the anionic bacterial plasma membrane¹⁵⁵, but alternative mechanisms, involving interaction with intracellular targets exists^{156,157}.

Membrane Active AMPs

Amphipathicity in the molecule, with amino acid residues segregated into distinct hydrophobic or cationic regions, is a crucial secondary structure in most AMPs. Regardless of the mechanism of the final killing of the bacteria, AMPs have to interact with the bacterial plasma membrane, either to cross the membrane or to rupture it. The precise nature of this interaction between amphipathic α -helical AMPs and biological membranes is not completely understood, although extensively studied and much debated.

Nevertheless, it has been shown that peptide-lipid interactions, rather than receptor-mediated recognition, are important in the function of many membrane active AMPs¹⁵⁸. Three specific steps in the membrane action are identified: attraction, attachment, and peptide insertion. Attraction is mainly due to electrostatic bonding. Cationic AMPs are thought to be attracted to the anionic outer envelope (LPS or phospholipids) of Gram-negative bacteria and to the teichoic acids of Gram-positive bacteria. The peptides have to pass through the layers of LPS in Gram-negative bacteria and polysaccharides, teichoic acids, and/or lipoteichoic acids in Gram-positive bacteria to be able to attach and interact with the outer membrane or the cytoplasmic membrane, respectively. How this is mediated for different AMPs is not fully elucidated. However, when the α -helical peptides reach the membrane bilayer they embed themselves, due to hydrophobic interactions, into the region of the lipid head groups. This embedded state of peptides is called surface state (S state). AMPs in this state are orientated parallel to the membrane and are

functionally inactive, but the lipid bilayer is stretched and membrane thinning is mediated. The thinning effect is specific to the AMP and is directly proportional to the concentration of peptide¹⁵⁹. When the peptide concentration is increased and the correct peptide-to-lipid molar ratio is reached for the specific combination of AMP and membrane, a transition from S state to the pore-forming peptide insertion state (I state) will occur. Peptides in the I state are orientated perpendicularly in the membrane and are able to form transmembrane pores¹⁵⁹, leading to destruction of the transmembrane electrochemical potential and pH gradient, an altered osmotic regulation, inhibited respiration, and eventually complete collapse of the membrane and cell death. Four different (three of them are presented in figure 8) models have been proposed to describe membrane permeabilization by amphipathic α -helical peptides: the “aggregate” model, the barrel-stave model, the toroid-pore model, and the carpet model^{94,160}

The aggregate model proposes that AMPs cross the membrane as peptide-lipid complexes and the peptides in these complexes form irregular aggregates within the membrane without adopting any particular orientation¹⁶¹.

In **the barrel-stave model** the formation of transmembrane pores is made by bundles of peptides. AMPs assemble as multimers on the surface of the membrane (S state) and in the I state the peptides are inserted into the hydrophobic core of the membrane lipid bilayer. The hydrophilic side of the α -helical peptides form the interior of the transmembrane pore and the hydrophobic side align the lipid core. Additional peptide monomers are then recruited and attached to the bundle leading to an increase in the size of the pore⁹⁴. The fungal peptide alamethicin is the best studied AMP that uses the barrel-stave type of pore¹⁶⁰.

The toroid-pore model describes the formation of pores or channels in the membrane by monomers of peptides. The polar or hydrophilic side of the peptides associate with the polar heads of the lipids. When the α -helical peptides insert into the

membrane they induce the lipids to tilt and connect the two layers of the bilayer membrane, thereby forming a bend continuously through the pore. Both the lipid head groups and the inserted AMPs make a hydrophilic lining all through the pore. This model differs from the barrel-stave model in the continuous association between the peptides and the lipid head groups, and also in the I state of the peptides. Magainins, protegrins, and LL-37 induce this type of pore in bacterial membranes⁹⁴.

According to **the carpet model** the AMPs bind as monomers with their hydrophobic sides against the surface of the lipid bilayer and their hydrophilic sides facing the solvent. The peptides will cover the membrane as a carpet and permeabilization will occur only after a threshold peptide-to-lipid molar ratio has been reached¹⁶². In order to form a carpet of cationic peptides, the presence of negatively charged lipids (acidic phospholipids) is important because this minimizes the repulsive force between all positively charged AMPs¹⁶⁰. In this model, AMPs are neither inserted into the hydrophobic core of the bilayer nor assembled in bundles. At high concentration of peptides, when the transition into I state has occurred, the AMPs disrupt the membrane in a detergent-like manner, which eventually leads to the formation of micelles^{94,163}. As an intermediate stage in this model, toroid-pores or wormholes are formed and low molecular weight molecules are able to leak out of the microbe prior to total membrane collapse¹⁶². The carpet model describes the action of for example dermaseptin S¹⁶⁴, cecropin¹⁶⁵, and melittin¹⁶⁶.

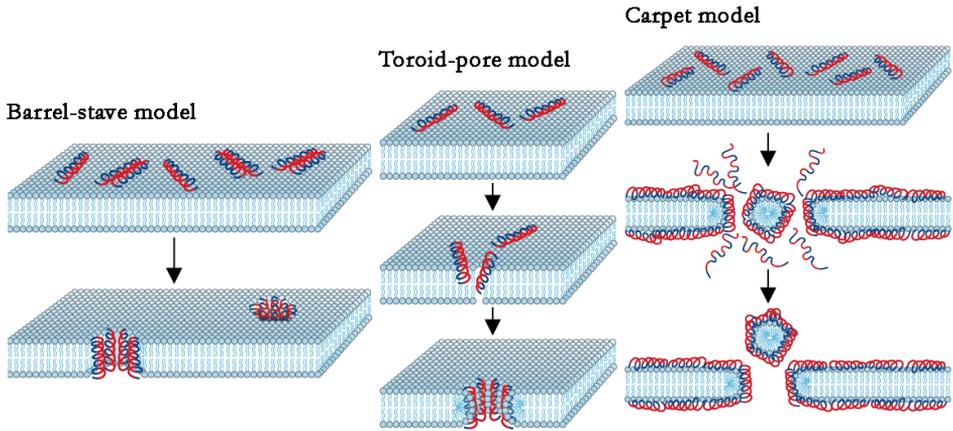


Figure 8. Mode of action of membrane active AMPs. Barrel-stave model, toroid-pore model, and carpet model are shown. See text for details. Schematic illustration modified from Brogden 2005⁹⁴.

Non-Membrane Active AMPs

Instead of membrane rupture some AMPs use intracellular targets that activate different pathways and lead to bacterial killing. Receptor-mediated activity has been reported for some *Drosophila* AMPs⁷⁰. Non-membrane active AMPs, which do not activate receptors on the surface of microbes, have to cross the plasma membrane and they have developed a variety of mechanisms to do this. These AMPs are either bactericidal or bacteriostatic in the cytoplasm. They can modify membrane septum formation, bind DNA (buforin II and tachyplesin), inhibit cell-wall synthesis (the lantibiotic mersacidin), or inhibit enzymatic activity (histatins). Dermaseptin, pleurocidin, PR-39, indolicidin, and HNP-1 are AMPs that inhibit DNA, RNA, and protein synthesis. PR-39 and indolicidin can also induce a very elongated morphology of the microbe, indicating that cell division in these cells is prevented⁹⁴. Some cationic amphipathic peptides are able to bypass the receptor step and directly activate G-proteins in mast cells, leading to degranulation and histamine release.

Venom peptides, neuropeptides, and hormones are also included in this family of peptides¹⁶⁷.

Membrane Specificity for AMPs

Most α -helical peptides are rather harmless to normal host cells. The toxicity to microbes is due to the high amount of anionic lipids in their membranes, a considerable electrical potential gradient over the bilayer, and a lack of supportive lipids, like the steroid-alcohol cholesterol, in the membrane. The majority of AMPs that have a high net positive charge and contain basic amino acids spread all over the hydrophilic side of the α -helix, are non-haemolytic and non-toxic to mammalian cells. On the other hand, AMPs that have a low net positive charge and a very high helix content are more harmful to both bacterial and mammalian cells. The negatively charged bacterial plasma membranes have a high content of acidic phospholipids, whereas the membranes of normal mammalian cells are neutral and predominantly composed of zwitterionic phosphatidylcholine and sphingomyelin phospholipids. Highly positively charged peptides preferentially act against the negatively charged bacterial membrane and have low or no affinity to zwitterionic mammalian membranes¹⁶². However, it should be mentioned that certain AMPs are potentially toxic to mammalian cells including, the bee AMP melittin¹⁶⁸, the frog AMP temporin L¹⁶⁹, the scorpion charybdotoxin, and the wasp venom mastoparan¹²².

Determination of Antimicrobial Activity

Generally, the antimicrobial activity of an antimicrobial agent, including antibiotics and AMPs, is presented as a MIC-value. Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a specific microorganism after overnight incubation. The MIC-value is usually determined by using either agar or broth dilution assay¹⁷⁰. MICs are presented in mg/l or M (mol/litre).

Synergy among AMPs

The biological activities of antibiotics or antimicrobial peptides are generally examined in isolation. However, in nature different AMPs and larger antimicrobial proteins affect microbes simultaneously, and they will inevitably interact with each other despite differences in their spectra of activity. Studies suggest that this interaction between AMPs is important for the final antimicrobial activity and may lower the amount of a single peptide required to enhance antimicrobial activity. The term synergy means that the resulting antimicrobial activity of peptides interacting with each other is more than the sum of their individual antimicrobial activities. The fractional inhibitory concentration (FIC) is an interaction coefficient indicating whether the combined inhibitory effect of drugs is synergistic, additive, or antagonistic¹⁷¹. To calculate FIC for the combination of AMP A and B, the following formula is used; $FIC_{AB} = FIC_A + FIC_B$. $FIC_A = MIC_{AB} / MIC_A$ and $FIC_B = MIC_{AB} / MIC_B$, where MIC_{AB} is the MIC of the combination A + B, MIC_A is the MIC of AMP A alone and MIC_B is the MIC of AMP B alone. Interpretations of the FIC index varies in different studies, but generally FIC values ≤ 0.5 show synergy, FIC = 1 shows additive effects, and FIC ≥ 4 show antagonistic effects.

Peptides may also show synergy in their antimicrobial mechanism. The magainin family of peptides from the African clawed toad (*Xenopus laevis*) magainin 2, and PGLa are examples of such peptides. Both form pores in the cytoplasmic membrane leading to membrane disruption. The magainin 2 peptide forms pores at a rather slow rate, but the pores formed are relatively long-lived. In contrast, PGLa is able to form pores very rapidly, but they are rather unstable and thereby short-lived. When magainin 2 and PGLa work in synergy, they form complexes that have the advantage of each individual peptide: both fast pore formation and increased pore stability¹⁷². Other examples are the two-component bacteriocins, where each component shows very little or no antimicrobial effect, but acting in combination their effects increase dramatically¹⁷³.

Bacterial Resistance Strategies

Various microbes are not equally susceptible to AMP activity, and it seems like the ability to circumvent AMP killing is a selective quality of a number of common and significant human pathogens. Some pathogens like *Staphylococcus aureus* and *Salmonella* spp. are generally very resistant to AMPs compared with other similar organisms¹⁷⁴. As an interesting connection, *S. aureus* is also known to be the most common cause of human wound infections and abscesses in deep tissues and *Salmonella* spp. are the major cause of chronic systemic infections in otherwise healthy individuals. On the other hand, microbes that are more susceptible to AMPs, for instance *Escherichia coli*, may cause a local infection at the mucosal surface but are generally not able to infect deep tissues unless the patient is in some way immunosuppressed. Recently, a broad diversity of bacterial strategies to resist AMP killing have been identified¹⁷⁵. Some bacterial species modify the normal anionic cell surface in various ways, and thereby alter the cell surface charge. The teichoic acids in the cell wall of Gram-positive bacteria and the lipopolysaccharides in the outer membrane of Gram-negative bacteria are modified in these AMP resistant strains. The modifications include for instance introduction of NH_3^+ in different ways. The substitutions make the surface less negatively charged and it will repulse, rather than attract cationic AMPs¹⁷⁵.

Another resistance strategy is binding of and/or blocking AMPs which leads to neutralization and inactivation. These AMP-inhibitory proteins can be either surface-associated or secreted¹⁷⁵. An example is the *S. aureus* staphylokinase, which induces secretion of α -defensins from human PMN, forms a complex with them and causes near complete inhibition of their bactericidal effect¹⁷⁶. Microbes may also induce release of host molecules that bind and inactivate AMPs. Some pathogens secrete proteases that are able to degrade negatively charged proteoglycans attached to the surface of host epithelial cells. For instance, proteases from *S. pyogenes*, *E. faecalis*, and *P. aeruginosa* degrade the proteoglycan decorin, and thereby release the GAG dermatan sulphate (DS). Released DS are then able to bind and inactivate

α -defensins²⁴. In paper I in this thesis, we demonstrate that the GAGs DS and chondroitin sulphate (CS-E) is able to bind and inactivate the AMP LL-37²⁵. In the same paper, we show that proteases from common pathogenic bacteria (*P. aeruginosa*, *E. faecalis*, *P. mirabilis* and *S. pyogenes*) degrade LL-37²⁵. Proteolytic degradation is yet another strategy for microbes to resist the action of host antimicrobial peptides. Other examples are the metalloprotease aureolysin from *S. aureus* that also cleaves and inactivates LL-37¹⁷⁷ and proteases from *E. coli* and *S. aureus* that inactivate bovine Lactoferricin B¹⁷⁸.

Active energy-dependent efflux systems represent significant mechanisms for resistance against conventional antibiotics, but may also contribute to the resistance of some microbes against cationic AMPs of the innate immune system. For example, *Neisseria gonorrhoeae* express an efflux system that ejects hydrophobic antibacterial agents and thereby gives increased resistance to antibiotics like penicillin and erythromycin. It has been demonstrated that gonococcal resistance to the AMPs protegrin-1 and LL-37 is altered by over expression of the same genes that control this efflux system¹⁷⁵.

Even though it seems like there is a selective pressure for resistance against AMPs, many bacterial species remain sensitive. Why? Probably, there must be a balance between the bacterial gain in being resistant and the bacterial metabolic cost for a mutation to take place and to retain it. Some mutations may just be metabolically too expensive for the bacteria.

Antimicrobial Proteins

Recently, an increasing number of cationic proteins considerably larger than the classical AMPs have been shown to be directly antimicrobial. There is an informal limit of approximately 50 amino acids in length separating the smaller peptides from the larger proteins. Alexander Fleming was the first one to observe and described the activity of an antimicrobial polypeptide, lysozyme from human respiratory

secretions¹⁷⁹. Other proteins with antimicrobial activity are found in airway secretions, including lactoferrin and Secretory Leucoprotease Inhibitor (SLPI). In this environment, lysozyme and lactoferrin are the most abundant, with concentrations of between 0.1-1 mg/ml¹⁸⁰.

Many different proteins with antimicrobial properties are now known. Some examples are; the two iron binding proteins, transferrin from plasma⁷¹ and lactoferrin isolated from respiratory secretions, breast milk, tears, gastrointestinal fluid, cervicovaginal mucus, seminal fluid, and secondary granules of PMNs¹⁸¹, the gastric protein pepsinogen C⁷¹, cathepsin G - a major protease released by activated neutrophils¹⁸², the liver derived plasma protein histidine-rich glycoprotein (HRG)¹⁸³, the bactericidal/permeability increasing protein (BPI)¹⁸⁴, and the heparin binding protein (HBP)^{183,185}, both isolated from azurophilic granules of human PMN.

Many proteins with a previously well-defined and established function have recently been described to also possess potent antimicrobial effects. Some interesting findings include the neuropeptides substance P and neuropeptide Y¹⁸⁶⁻¹⁸⁸, peptide hormones^{187,189}, the complement anaphylatoxins C3a^{35,52}, and C4a⁵¹. Activation of the intrinsic pathway of coagulation via the contact system is found to release proteins and peptides with potent antimicrobial effects, such as bradykinin (BK)^{186,187} and domain 3 (D3)¹¹⁵. In addition, in paper IV we describe antimicrobial activities of domain 5 (D5)¹¹⁶ from HMWK. Many chemokines^{190,191} and several different growth factors¹⁹² are very similar to AMPs in size, charge, amphipathicity, and heparin-binding abilities and are also shown to be antimicrobial¹⁹².

The antimicrobial effect is not the only and perhaps not the main activity of these proteins, but their complexity of functions is poorly understood. Possibly, the antimicrobial effect is the original function of a certain protein before more specific and specialized functions were developed during evolution, as in the case of the complement factor C3a⁵¹. Nevertheless, the antimicrobial properties may still be of

great importance in a particular environment under special circumstances and in concert with the more specific actions of the protein. Interestingly, the antimicrobial domain is usually traced to a cationic, amphipathic, and heparin-binding sequence in the larger protein and in many cases peptides released by acid hydrolysis of the larger protein have potent antimicrobial effects, like for instance lactoferricins and kaliciocins from lactoferrin⁸⁹ and buforin I from histone H2A¹⁹³.

Antimicrobial Defences of the Skin

Human skin epidermis cells are able to produce many different innate immunity molecules^{194,195}. Healthy keratinocytes and sweat glands together constitutively produce lysozyme, SLPI, RNase 7¹⁹⁶, dermcidin¹⁹⁷, cathepsin D¹⁹⁸, and LL-37¹⁹⁹. It has also been demonstrated that LL-37 from skin and sweat is further processed into smaller peptides with increased antimicrobial activity^{200,201}, probably enabling a shift in the activity of LL-37 from being largely immunomodulatory toward being pure antimicrobial. A local production of the AMPs hBD-2, hBD-3, and LL-37 is induced during inflammation in skin keratinocytes. This induction may come from the action of pro-inflammatory cytokines^{39,202} as well as growth factors²⁰³. In response to microbial stimuli, Langerhans cells in the skin secrete inflammatory mediators like IL-1, which then are able to induce the expression of different AMPs in keratinocytes³⁹. Damaging the skin is actually enough to induce AMPs at relevant concentrations. This is mediated by heparin-binding epidermal growth factors (EGF) that are released from keratinocytes. These growth factors bind and activate epidermal growth factor receptors (EGFR), leading to expression of different AMP genes and production of for example hBD-3²⁰⁴. Keratinocytes also produce and secrete an 11 kDa protein called psoriasin in response to the Gram-negative bacteria *E. coli*. Psoriasin is found to be the main mediator of *E. coli* killing in human skin²⁰⁵. It has also been revealed that ultraviolet light (UVB) from the sun indirectly induces expression of the *CAMP* and *defβ2* genes in human keratinocytes. Vitamin D is formed in the skin by UV-light and it has been demonstrated *in vivo* that vitamin D

induces expression of the *CAMP* gene and upregulation of the production of hCAP-18 as a result^{206,207}. Other cell types in the skin that produce LL-37 are neutrophils²⁰⁸ and mast cells²⁰⁹.

Antimicrobial Peptides in Clinical Medicine

The importance of AMPs in innate immunity is well established and different clinical studies have blamed defective AMP activity for impaired bacterial clearance. Many AMPs are known to be salt-dependent, and fail to work properly in high salt conditions. The airway surface fluid from patients with cystic fibrosis (CF) has elevated levels of Na⁺ and Cl⁻, caused by mutations in a gene encoding a phosphorylation-regulated Cl⁻-channel found in normal airway epithelia²¹⁰. This increase in ionic strength is a key factor contributing to deficient antimicrobial activity in this fluid²¹¹. HBD-1-3 are salt-dependent AMPs and are thereby inactivated in these patients²¹². Another interesting finding, is that hBD-2 and 3 are proteolytically degraded by the cysteine proteases cathepsins B, L, and S, which are found in high amounts in CF airway fluid²¹³. A recent study highlights that LL-37 is also inactivated in CF patients, either as a result of LL-37 interaction with DNA, filamentous actin, and cell debris, or interaction between LL-37 and the Gram-negative bacterial endotoxin LPS, which all are abundant in CF sputum. It is also interesting to note that LL-37 activity could be enhanced by addition of LBP, which competes with LL-37 for binding to LPS²¹⁴.

Deficiency or Abundance

Different medical conditions are connected with deficiency or overproduction of AMPs, leading to certain physical consequences for the patients. Patients suffering from specific granule deficiency (SGD) lack α -defensins and patients with Chediak-Higashi syndrome (CHS) have a severe deficiency of both cathepsin G and elastase in their PMNs. These patients frequently suffer from serious bacterial infections and the lack of important AMPs in these diseases is thought to contribute

to defective microbial killing²¹⁵. Another example is the disorder morbus Kostmann, where patients have a congenital deficiency of neutrophils. Treatment with recombinant granulocyte-colony stimulating factor restores the amount of neutrophils, but despite normal levels, these neutrophils are found to be deficient in the cathelicidin LL-37 and have only reduced amounts of the α -defensins HNP1-3. There was a total lack of LL-37 in plasma and saliva, which could explain why all people with morbus Kostmann syndrome also suffer from frequent oral bacterial infections and severe periodontal disease²¹⁶. LL-37 and hBD-2 are also downregulated in keratinocytes in atopic dermatitis skin lesion, whereas LL-37 and hBD-2 are instead overexpressed in psoriatic lesions^{217,218}. Psoriatic skin is also known to express a blend of other AMPs, including psoriasin²¹⁹. This may explain why about 30% of the patients suffering from atopic dermatitis have microbial infections of the skin, compared with only 7% of the psoriasisics²²⁰. Recently, it has also been proposed that LL-37 in fact is a central trigger of the pathogenesis and autoimmunity of psoriasis. LL-37 is found to bind to self-DNA and this complex is then able to translocate into endosomes of plasmacytoid dendritic cells and signal through TLR-9, leading to induce an interferon- α response. TLR-9 is an intracellular receptor that is usually only activated by viral or microbial DNA. The location of TLR-9 inside cells is a safeguard against autoimmunity and the recognition of self-DNA in normal healthy skin. By binding self-DNA, LL-37 breaks this self tolerance and yields a similar response as viral or microbial DNA, with an induction of interferons which then initiate innate and adaptive immunity²²¹.

AMPs as Future Therapeutics

Antimicrobial peptides are promising agents in our search for new and potent therapeutics for topical or systemic administration. They are interesting candidates against pathogenic microbes, but also as immunomodulatory agents. Their broad spectra activity with a rapid action, the low potential for pathogen resistance on repeated exposure, and the low toxicity for eukaryotic cells are attractive features.

Many AMPs are immunomodulatory (figure 5), but have only limited immunogenicity³². Their small size makes them easy to synthesize even though the substantial expense of peptide synthesis must still be overcome.

There are studies where synthetic host defence peptides (HDPs) have been constructed without any direct antimicrobial activities, and these peptides were still shown to efficiently protect against bacterial infection⁸³. These results indicate that direct antimicrobial activity is not a requirement for protection against infection. However, knowledge of all the *in vivo* interactions and all the immunomodulatory effects of AMPs must be further elucidated in order to assess safety before being administered systemically. A database, www.innatedb.com, is now available presenting all known interactions and signalling pathways involved in the mammalian innate immune response²²². As more research is carried out, this database of knowledge will grow bigger and will probably be an important tool in understanding the complexity of actions of AMPs/HDPs. With increased knowledge and understanding, there is a great potential for a therapeutic use of these immunomodulatory and anti-infective AMPs/HDPs. These cationic peptides will probably circumvent issues of antimicrobial resistance because they do not eliminate microbes directly.

AMPs of evolutionary distant origins are found to be functionally very similar, and the knowledge and understanding of the design of lower invertebrate peptides will be of great help in the development of new antibiotics, host defence and / or immunomodulatory agents in humans. A good example is plectasin from the black cup fungus *Pseudoplectania nigrella*. Plectasin is the first defensin isolated from the fungi kingdom and it shows homology with plant and insect defensins. This AMP has a great therapeutic potential and tests on mice have shown very promising results²²³, with strong activity against many multiresistant strains primarily among Gram-positive bacteria.

Drugs derived from AMPs are already in clinical trials. Pexiganan (MacroChem Corporation, NY), a drug derived from the magainin family¹² of AMPs are used as topical treatment for diabetic foot infections, and omiganan pentahydrochloride (MBI-226)²²⁴ (Cutanea Life Sciences, PA), an analog of the bovine cathelicidin indolicidin, will be used clinically for the prevention of infections in catheters. Promising clinical Phase III trials with omiganan are also underway for the treatment of the chronic dermatologic disorder, Rosacea.

Another area of research with potential therapeutic value is the development of therapeutics that will stimulate expression of certain AMPs and thereby augment endogenous antimicrobial activity.

PRESENT INVESTIGATION

Aims of the Thesis

The general aim was to study antimicrobial peptides present in wounds during inflammation and healing.

We specifically wanted to:

- Investigate the stability of LL-37 in the presence of chronic or acute wound fluids.
- Study the effects of common bacterial proteases against LL-37.
- Investigate whether LL-37 is bound to various GAGs, and if so study the effect on LL-37 of this binding.
- Test our hypothesis that motifs associated with heparin-binding may confer antimicrobial properties to any given peptide.
- Identify novel endogenous antimicrobial peptides from heparin-binding motifs that may be generated by proteolysis during inflammation.
- Investigate if the cationic complement factor, C3a is a heparin-binding peptide. In that case may this anaphylatoxin also possess potent antimicrobial activity?
- Elucidate whether the heparin-binding and cell-binding D5 of HMWK is antimicrobial. Identify shorter antimicrobial peptides within the D5 sequence.

Results and Discussion

Paper I

Here we show that proteases of common human pathogens degrade and inactivate the AMP LL-37. The metalloproteinase, elastase (LasB or pseudolysin) from *Pseudomonas aeruginosa*, was found to hydrolyze LL-37. The generated fragments were analyzed by LC-MS TOF and MS-MS TOF. *P. aeruginosa* elastase cleaves LL-37 in the central part, where the antimicrobial activity has been ascribed²²⁵, and it preferentially cleaved at sites with hydrophobic residues (Ile, Leu, Phe) in the P1' position. This finding corresponds well with the P1' specificities reported for the thermolysin-like family of proteases, to which *P. aeruginosa* elastase belongs²²⁶. Fragments of LL-37 generated within 30 min of incubation with *P. aeruginosa* elastase exerted only residual antimicrobial activity. Furthermore, the inhibition of LL-37 mediated killing was also tested. A non-elastase producing strain of *P. aeruginosa* was incubated together with LL-37 with and without added *P. aeruginosa* elastase, the suspensions were plated and after overnight incubation the colony forming units were counted. A clear survival advantage was noticed for bacteria in the presence of increasing amounts of *P. aeruginosa* elastase. Growth media from *Enterococcus faecalis* and *Proteus mirabilis* contains *E. faecalis* gelatinase and the *P. mirabilis* metalloproteinase ZapA, respectively. These growth mediums and also the purified *Streptococcus pyogenes* cysteinyl protease (streptococcal pyrogenic exotoxin B (SpeB)) were also able to degrade LL-37, as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Inhibitors of the proteases were able to block this degradation of LL-37.

We showed that human chronic wound fluids degrade LL-37, whereas acute wound fluids do not. It is known that chronic wounds, in contrast to acute wounds, are largely colonized with bacteria. Our data indicate that the bacterial proteases present in chronic ulcers may be responsible for the inactivation and degradation of LL-37. To test this further, the following *ex vivo* experiments were performed. An elastase

producing *P. aeruginosa* isolate and a non-elastase producing *P. aeruginosa* isolate were separately added into sterile wound fluid, grown to stationary phase and the mixtures were incubated with LL-37 for increasing periods of time. The *P. aeruginosa* elastase producing isolate induced degradation of LL-37 leading to enhanced bacterial survival. The resistance of the LL-37-region of the proprotein (hCAP18) against *P. aeruginosa* elastase was also tested. HCAP18 was incubated with conditioned media from the elastase-positive and elastase-negative isolates of *P. aeruginosa*. The results were visualized by SDS-PAGE and western blotting, using polyclonal antibodies against LL-37. Interestingly, degradation was shown after only 10 min of incubation at 37°C in the samples containing the protease. Previously, it was reported that α -defensins bind to GAGs and this binding inhibited the antimicrobial action of the peptide²⁴. In this study we wanted to see if the same was true for LL-37. Indeed LL-37 was bound to and inactivated by DS (CS-B) (figure 2a) and the closely related CS-E. The binding not only blocked the antimicrobial activity of LL-37, it also protected the peptide from degradation by *P. aeruginosa* elastase.

In summary, the proteolytic degradation of LL-37 by bacterial proteases presented in this paper may be an important bacterial virulence mechanism. It may provide a way for microbes to circumvent the host immune system, both the direct antimicrobial activity of LL-37 but also all the other immunomodulatory actions of this peptide. The role of GAGs as inhibitors of LL-37 activity, but also their role as protectors of LL-37 against bacterial proteolytic degradation, remains to be elucidated.

Paper II

As we demonstrated in paper I, LL-37 is bound to DS and CS-E, GAGs very similar in structure to heparin. The biophysical parameters common for many otherwise very different AMPs are cationicity, amphipathicity and α -helicity. These characteristics are also common among heparin-binding peptides. In this study we

wanted to see more generally, if peptides with structural motifs associated with heparin affinity also hold antimicrobial activity.

In paper II, we demonstrate that synthetic peptides derived from heparin-binding motifs of endogenous proteins, all involved in or present in certain ways during inflammation, are indeed antimicrobial. Heparin-binding sequences from laminin, fibronectin, von Willebrand factor, protein C inhibitor, vitronectin, and complement factor C3 were synthesized. Both viable count analysis (bactericidal activity) and radial diffusion assay (inhibition of growth) were used to analyse antimicrobial activity. The microorganisms tested were *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus mirabilis* and also the fungi *Candida albicans*. Heparin-binding activity was confirmed or determined by using a slot binding technique with radiolabelled heparin. In 1989, Cardin and Weintraub²²⁷ proposed consensus sequences for heparin-binding peptides, XBBBXXBX or XBBXBX (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids). Antimicrobial activity was also tested for synthetic peptides of different lengths, with amino acids organised in the Cardin and Weintraub patterns [AKKARA]₁₋₄ and [ARKKAAKA]₁₋₃. The larger peptides (AKK24 and ARK24) were shown to exert potent activities, similar to the effects of LL-37. *P. aeruginosa* was incubated with some of the peptides at LD₅₀ and the bacteria were then fixed, stained, and analysed by electron microscopy. Morphological differences compared to the control were demonstrated with local perturbations and breaks along the bacterial plasma membrane. In this study, we reveal that heparin-binding motifs of endogenous proteins exhibit antimicrobial activities. In addition, the Cardin and Weintraub motifs and also the laminin-derived peptides RLR22 and the shorter variant PGR11, showed that too short peptides were not as active as larger variants, probably due to their inability to form a stable α -helix.

During the inflammatory phase in normal wound healing, there is a massive degradation of the damaged tissue before the regeneration starts. ECM proteins and

different blood and plasma proteins are highly degraded, and our findings indicate a novel antimicrobial role for this cell debris. In general, our finding that many heparin-binding peptides also possess antimicrobial properties may ease the search for novel AMPs in complex biological mixtures.

Paper III

During complement activation C3a may be generated in blood in micromolar concentrations, but it is strictly regulated and controlled by inactivation by carboxypeptidase N. The carboxypeptidase cleaves off the C-terminal arginine residue generating the C3a_{desArg} peptide⁵⁰. The structural features of factor C3a resemble those of many AMPs. C3a is cationic (pI 11.3), contain four α -helices, and as described in paper II, possesses heparin-binding sequences¹¹⁷. This prompted us to investigate the antimicrobial effects of the complete C3a. In paper III, we describe the antimicrobial activity of the anaphylatoxin C3a and also of the inactive C3a_{desArg} peptide, indicating an important additional function for these complement peptides. Even after inactivation of the anaphylatoxin C3a, the resulting C3a_{desArg} peptide may contribute to innate immunity by its direct antimicrobial activity. By using radial diffusion assay (RDA), the minimal effective concentrations (MEC) for C3a were determined at 0.70 μ M and 0.75 μ M against *P. aeruginosa* and *E. coli* respectively. This could be compared with a MEC of 1.0 μ M LL-37 for *E. coli*.

Earlier studies have described four helical regions of C3a⁵⁰. We ordered synthetic peptides spanning the four helices to investigate whether these epitopes were carrying the antimicrobial activity. A peptide known to exert full anaphylatoxic activity (CNY21), the inactive peptide (CNY20), and finally the smallest peptide with any activity (LGL5)⁵⁰, were also synthesized. RDA and viable count analysis revealed that the complete C3a molecule (77 aa), but also the synthetic peptides are active against *E. coli*, *P. aeruginosa*, and *E. faecalis*. Interestingly, C3a showed potent antimicrobial activity also in physiological salt conditions. Heparin-binding capacities of the peptides were analysed by slot-binding-assay with radiolabelled

heparin. Fluorescence and electron microscopy demonstrated that the C3a and the there of derived peptides bind to and induce breaks in the bacterial plasma membrane. C3a was also shown to induce membrane leakage of liposomes. Furthermore, one of the C3a-derived peptides (CNY21), was found to suppress infection by *S. pyogenes* in mice. By using western blot analysis with an antibody against one of the synthetic C3a peptides (LGE27), we found that acute wound fluid incubated together with lysed neutrophils generated C3a-like peptides. We also showed that shorter degradation products of C3a are generated by neutrophil elastase, where at least one of them is stable. This peptide was sequenced and synthesized and found to exert similar antimicrobial effects as the CNY21 peptide. Paper III presents an important and interesting link between two key branches of innate immunity, the complement system and antimicrobial peptides.

Paper IV

In paper IV we show that recombinant D5 (rD5) of HMWK is antimicrobial. HMWK is a 120 kDa glycoprotein found in plasma (~80 µg/ml) and in α -granules of platelets. It is present during normal wound healing and inflammation.

By analysing synthetic overlapping peptides within the D5 sequence, we wanted to reveal the location of the bactericidal activity. The antimicrobial part was found in the His-Gly-Lys-rich subdomain (Gly⁴⁷⁵-Lys⁵⁰²) of D5, the same region which also shows strong Zn²⁺-independent binding of heparin.²²⁸ All three peptides (GGH20, HKH20, and GKH17) from this region exerted potent activity against *E. coli* in RDA. The most potent peptide against *P. aeruginosa* and *E. faecalis* in RDA, HKH20 (His⁴⁷⁹-His⁴⁹⁸), was also shown to efficiently kill *E. coli*, *P. aeruginosa*, and *E. faecalis* in viable count analysis. Using RDA, the MEC of HKH20 against *P. aeruginosa* was estimated to be 0.4 µM. The interaction between HKH20 and bacteria was studied by fluorescence microscopy and electron microscopy. By using fluorescent dye labelled HKH20, microscopy revealed that HKH20 was attached to the *P. aeruginosa* surface, and this binding was blocked by heparin. The binding of heparin to rD5

and HKH20 was confirmed in the slot-binding-assay with radiolabelled heparin. Electron microscopy demonstrated that HKH20 caused local perturbations and breaks along the *P. aeruginosa* plasma membrane, but also that intracellular material was found extracellularly. Importantly, HKH20 was found to be antimicrobial in physiological salt conditions and in the presence of plasma. The results of assays for hemolysis, LDH-release, and MITT-assay showed no lytic effects of HKH20 against eukaryotic cells. Interestingly, in contrast to LL-37 (paper I) and α -defensin²⁴, HKH20 was found to be very stable and not degraded by *P. aeruginosa* elastase. Since HMWK is not antimicrobial by itself, we also wanted to know if D5-peptides might be generated after proteolytic cleavage of HMWK. Proteolytic degradation of HMWK was carried out *in vitro* with human neutrophil elastase, lysed PMN, as well as the bacterial *P. aeruginosa* elastase. This indeed generated an antimicrobial blend, as demonstrated by RDA against *E. coli*. Analysis with SDS-PAGE revealed that HMWK was indeed degraded into multiple fragments and both *P. aeruginosa* elastase and neutrophil extracts yielded smaller peptides. Western blot analysis demonstrated that some of the generated peptides were derived from the His-Gly-Lys-rich subdomain of D5 and thereby recognized by an anti-HKH20 antibody. One of the peptides generated after digestion by *P. aeruginosa* elastase was analysed by Edman degradation and MALDI-TOF. The peptide generated was LDD40 (Leu⁴⁶¹-Gly⁵⁰⁰). A synthetic peptide LDD40 was found to be antimicrobial against *E. coli* and *E. faecalis*. Although proteolytic degradations were only studied *in vitro*, this result indicates a release of antimicrobial D5 peptides during inflammation and proteolysis. During activation of coagulation via the intrinsic pathway (contact activation), plasma kallikrein releases the inflammatory mediator bradykinin and D3-derived AMPs from HMWK¹¹⁵. Proteolytic cleavage and the release of bradykinin causes a conformational change in the resulting two chain HMWK (called HMWKa), exposing the heparin-binding and cell-binding D5²²⁹. In summary, paper IV reveals a previously unknown antimicrobial activity of D5 in HMWK.

Conclusions

- Wound fluid from chronic skin ulcers degrades LL-37, whereas wound fluid from acute wounds does not.
- Common human pathogens, *P. aeruginosa*, *E. faecalis*, *P. mirabilis*, and *S. pyogenes*, produce proteases that inactivate and degrade LL-37. This indicates an important bacterial virulence mechanism.
- GAGs, like dermatan sulphate and heparin, bind LL-37 and thereby inhibit the antimicrobial activity. However, the binding can also protect the peptide from proteolytic degradation by bacterial proteases.
- There is an association between antibacterial activity of a peptide and its ability to bind heparin. Consensus sequences for heparin-binding peptides show potent antimicrobial activity against *E. faecalis*, *P. aeruginosa*, *E. coli*, and *P. mirabilis*, but also against the fungi *C. albicans*.
- Various matrix, plasma, and blood proteins that are known to be degraded during inflammation carry antimicrobial motifs.
- Novel antimicrobial properties have been established for both the anaphylatoxin C3a and its inactive variant C3a_{desArg}, peptides generated during complement activation.
- Domain 5 of HMWK is antimicrobial and antimicrobial sequences within D5 have been characterized.
- A synthesized D5 peptide HKH20, shows broad antimicrobial activity with a strong effect also in the presence of physiological salt and plasma. Furthermore, HKH20 is not degraded by *P. aeruginosa* elastase.

Concluding Remarks

A healthy, balanced system has very low levels of antimicrobial peptides (AMPs). After injury and during inflammation production of AMPs is increased in several ways. AMPs are generated in response to infection by signalling through receptors like TLRs and EGFR²³⁰, via recruited neutrophils, and via complement activation (paper III). Interestingly, the anaphylatoxic and antimicrobial C3a peptide may also be generated via activation of the intrinsic pathway of the coagulation system (factor Xa and XIa)²³¹. It has also been shown that the trauma alone in a non-infected wound may induce expression of AMPs via transactivation of the EGFR²⁰⁴. During normal wound healing, the inflammation phase is characterized by massive degradation of the damaged ECM, generating fragments of different matrix, blood, and plasma proteins. Many of the degraded proteins contain heparin-binding and antimicrobial sequences that may be released (paper II, IV), indicating a novel role for innate immunity in this mixture of inflammatory peptides. After the generation of protein fragments, the degradation process continues until the area is cleared and the healing process enters the proliferative phase with reepithelialisation. If there is a prolonged period of high inflammation, the balance in the system could be lost. Instead of being an important part of healing, the inflammation is now devastating for the host. All the protein fragments containing potential antimicrobial peptides will be totally degraded and their potential activities lost. Bacteria are still colonizing the wound and bacterial proteases are able to inhibit and degrade AMPs like α -defensin²⁴ and LL-37 (paper I). Proteoglycans from the epidermis and dermis are also broken down and GAGs, such as dermatan sulphate and heparan sulphate, are released. These GAGs may possibly bind and inhibit the action of α -defensin²⁴ and LL-37 (paper I). Instead of the normal healing process, bacteria are now in control of the area. Although very simplified, this is probably the scenario in many chronic infective processes such as chronic skin ulcers.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ett bra immunförsvar är viktigt för att förhindra sjukdom vid angrepp av bakterier och virus. Kroppens försvar kan delas upp i ett snabbt medfött och ett långsammare förvärvat immunsystem. Det förvärvade är baserat på antikroppar som sedan fungerar som minnesmolekyler vid framtida infektioner. Det kan ta veckor innan det förvärvade immunförsvaret har sin fulla verkan. Det medfödda immunförsvaret aktiveras däremot direkt som svar på en infektion. Till det medfödda immunförsvaret hör dels fysiska barriärer så som huden och slemhinnor eller ogästvänliga miljöer som magsäckens saltsyra, dels olika sorter av vita blodkroppar. Ett exempel på vita blodkroppar är neutrofiler som kan äta upp (fagocytera) inkräktande bakterier eller skicka ut inflammatoriska ämnen (cytokiner) som på olika sätt leder till att inkräktaren oskadliggörs.

En annan viktig del av det medfödda och snabba immunförsvaret är antimikrobiella peptider, vilket är ämnet för denna avhandling. En peptid är ett kort protein och antimikrobiella peptider är oftast inte längre än 60 aminosyror (de byggstenar som bygger upp alla protein). De är direkt bakterie-, svamp- eller virusdödande. Vidare är de positivt laddade med en amfipatisk struktur, d.v.s. de har en vattenskyende och en vattenlöslig sida. Deras struktur samt den positiva laddningen gör att de har förmåga att binda till, tränga igenom och ta i sönder de negativt laddade bakteriemembranen. Andra antimikrobiella peptider verkar inne i bakterien, där de stör livsviktiga processer som t.ex. förmågan att producera proteiner. Antimikrobiella peptider är evolutionärt sett väldigt gamla och återfinns inom alla klasser av liv; djur, växter, svampar, men även bland bakterier, såsom ett försvar mot konkurrerande bakterier. Antimikrobiella peptider blev först kända för deras direkta bakteriedödande effekt, men numera har man sett att deras verkan är mycket mer komplex än så och att de spelar en mängd olika roller i vårt immunförsvar (de är s.k. immunomodulatoriska) för att skydda oss mot eller bli av med en befintlig infektion. Idag känner man till ca 1400 antimikrobiella peptider och med mer forskning

upptäcks fler hela tiden. Bland annat har ett flertal peptider med andra, sedan tidigare kända, funktioner nyligen visat sig även vara antimikrobiella, som exempel kan nämnas vissa tillväxtfaktorer och komplement faktorn C3a (delarbete III).

Antimikrobiella peptider är bra kandidater för att utveckla nya mediciner som kan användas istället för eller som komplement till konventionell antibiotika, vilket är viktigt i dessa tider då antibiotikaresistensen hos våra vanliga sjukdomsbakterier ökar lavinartat. Bra egenskaper är att antimikrobiella peptider har effekt mot många olika typer av bakterier samt vissa virus och verkar inte utveckla resistens på samma sätt som konventionell antibiotika. Intressant är t.ex. en antimikrobiell peptid (retrocyclin 2) från rhesus apa som visat sig ha en mycket påfallande effekt mot HIV-virus, och lovande mediciner håller på att utvecklas. Det pågår några kliniska prövningar med läkemedelskandidater som är baserade på antimikrobiella peptider. Det handlar då främst om krämer som appliceras lokalt vid hud och sårvård. Däremot måste den immunomodulatoriska effekten av peptiderna klarläggas helt och hållet innan man vågar använda antimikrobiella peptider i terapier systemiskt i kroppen.

I **delarbete I** har vi sett att några av våra vanligaste bakterier släpper ut olika proteiner som bryter ner den antimikrobiella peptiden LL-37. LL-37 är en peptid som finns i hög koncentration i de vita blodkroppar som kallas neutrofiler. Det är bl.a. dessa som rekryteras i stor utsträckning vid inflammation vid t.ex. sårläggning. Bakterierna har alltså på detta sätt utvecklat en strategi för att undkomma verkan av LL-37. Vi har även visat att de bakterier som producerar ett sådant protein överlever behandling med LL-37 i mycket högre grad än de som inte bildar detta protein. Kroniska sår (t.ex. liggsår och diabetessår) är till skillnad från akuta (t.ex. vanliga skärsår och rivsår) fullpackade med bakterier. Normal sårläggning börjar med en inflammationsfas, då bl.a. neutrofiler strömmar till och den skadade vävnaden bryts ner för att vid nästa fas i sårläggningen kunna ersättas med frisk vävnad. I akuta sår hålls bakterierna i schack av kroppens snabba immunförsvar, medan

läkningsprocessen i kroniska sår istället har fastnat i en inflammatorisk fas och bakterierna har i många fall tagit överhanden. Det är sedan tidigare känt att olika bakterieproteiner även kan bryta ner strukturer i huden och därigenom frisätta negativt laddade sockerkedjor s.k. polysackarider (t.ex. dermatansulfat). I detta delarbete har vi visat att hudens eget dermatansulfat, frisatt av bakterieproteiner eller långvarig inflammation, binder till och förhindrar verkan av LL-37. Intressant är att bindningen skyddar LL-37 från nerbrytning av bakterieproteiner.

I **delarbete II** har vi tittat vidare på det faktum att negativt laddade polysackarider binder till positivt laddade antimikrobiella peptider. Förutom dermatansulfat i huden så finns det en del snarlika polysackarider i människan, den mest kända är heparin som också är välkänd för sin blodförtunnande effekt. Eftersom LL-37 binder heparin så kan man kalla den för en heparinbindande peptid. Många heparinbindande peptider och/eller heparinbindande delar av ett större protein är kända sedan tidigare. Vår frågeställning i detta delarbete var av generell karaktär. Är en heparinbindande peptids form och struktur densamma eller snarlik den form och struktur som karaktäriserar en antimikrobiell peptid? Är i så fall de flesta heparinbindande peptider antimikrobiella?

1989 presenterades generella utseenden av peptider, vilka ger dessa optimal förmåga att binda till heparin. Peptider med detta ”principutseende” testades i försök med bakterier och peptiderna var verkligen starkt bakteriedödande. Det framkom också att peptiderna inte bör vara alltför korta, förmodligen beroende på att de då inte kan in ta den mest fördelaktiga 3D-strukturen för att kunna interagera med bakteriemembranen. Syntetiska peptider från olika kända heparinbindande protein som på olika sätt finns närvarande vid inflammation och sårhäkning testades också. Även dessa peptider visade sig i försök vara starkt bakteriedödande. Inflammationen vid sårhäkning kännetecknas av kraftig nedbrytning av vävnad och de proteiner som vi undersökt blir alla sönderdelade till småbitar. Det är därför troligt att många heparinbindande peptider med antimikrobiell funktion finns närvarande vid

sårsläkningen och har där tillsammans med andra försvarsmekanismer den viktiga uppgiften att hålla såret fritt från bakterier. Upptäckten att många heparinbindande peptider också är antimikrobiella är viktigt, då det gör det möjligt att lättare hitta nya antimikrobiella peptider genom att använda sig av deras förmåga att binda till heparin.

I **delarbete III** har vi visat en tidigare okänd antimikrobiell verkan av peptiden C3a i komplementsystemet. Komplementsystemet är en del av vårt medfödda försvar som aktiveras vid infektion. Namnet kommer av att det är ett komplement till immunförsvaret och dess uppgift är att oskadliggöra inkräktande bakterier. C3a är en 77 aminosyror lång peptid som frisätts vid aktivering av komplementsystemet. Den är känd för att dra igång kraftig inflammation och om pådraget blir för stort kan resultatet likna en allergisk chockreaktion. Den inflammatoriska verkan av C3a regleras i kroppen genom att peptiden inaktiveras då en aminosyra i ena änden klipps av, peptiden kallas då C3a_{desArg}. I detta delarbete har vi visat att även C3a är heparinbindande peptid som mycket effektivt dödar bakterier. En intressant och viktig upptäckt är att också C3a_{desArg} är kraftigt antibakteriell, eftersom det är denna variant av C3a som finns tillgänglig under en längre period vid inflammation och sårsläkning. På omslaget av avhandlingen finns bilder tagna med hjälp av ett elektronmikroskop föreställande pseudomonasbakterier före (uppe till vänster) och efter behandling med C3a (uppe till höger) och C3a_{desArg} (nere till höger). Verkan av kortare syntetiska peptider från C3a molekylen har också testats och även dessa har stark antibakteriell effekt. En av dessa peptider blev även injicerad i möss infekterade med streptokocker. Man såg då en långsammare spridning av bakterier i mössen jämfört med då man injicerat en överksam peptidvariant.

I **delarbete IV** har vi studerat antibakteriell verkan hos en del av proteinet kininogen. Även kininogen har en viktig roll vid inflammation, men också vid aktivering av blodkoagulationen vid sårsläkning. Den del av kininogen som vi studerat kallas domän 5 (D5) och är sedan tidigare känd för att kunna binda till

heparin. Vi har i detta delarbete visat att hela D5 (ca 130 aminosyror) har förmåga att döda bakterier. För att vidare kartlägga var i proteinet den antimikrobiella effekten av D5 finns så har vi i bakterieförsök testat korta syntetiska peptider längs med D5 molekyl. Det visade sig vara den senare delen och den mest positivt laddade biten av D5 som har den bakteriedödande verkan. Vidare tittade vi lite närmare på den mest verksamma av de syntetiska peptiderna, HKH20. Den var både heparinbindande och hade en antibakteriell effekt mot alla de tre bakterietyper vi testade. Bäst effekt hade peptiden mot pseudomonasbakterier, då endast ytterst låga koncentrationer av peptiden behövdes för att döda mer än 90 % av bakterierna, på endast 5 minuter. På bilden nedan visas pseudomonasbakterier som blivit behandlade med HKH20 i två olika koncentrationer. Viktigt är också att HKH20 visade sig vara okänslig mot nedbrytning av de bakterieproteiner som vi tidigare sett bryta ner andra antimikrobiella peptider t.ex. LL-37 (delarbete I). Vi har också med olika metoder studerat HKH20s verkan på mänskliga celler. Trots att HKH20 är effektiv för att döda bakterier så verkar peptiden vara ofarlig för våra mänskliga celler. Detta gör peptiden intressant för fortsatt forskning i jakten på bra antimikrobiella peptider som verksamma ämnen i nya antibakteriella läkemedel.



Bilder av *Pseudomonas aeruginosa* bakterier tagna med elektronmikroskop. Obehandlade bakterier (vänster), samt bakterier behandlade med olika koncentrationer av HKH20 (mitten och höger).

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