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Midkine - a host defence protein

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Midkine - a host defence protein

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DOCTORAL DISSERTATION

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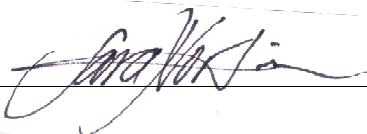
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Abstract:

Every day we encounter many potentially harmful microbes in our environment. The epithelial linings constitute an anatomical barrier protecting us and they also produce antimicrobial polypeptides (AMPs) that act as a chemical barrier. These AMPs are small and positively charged, killing a broad range of microbes rapidly. They can either be constitutively produced or rapidly induced when epithelial cells encounter pathogens or when an injury occurs.

Midkine (MK) is a heparin-binding growth factor of 123 amino acids. It is composed of two domains with three anti-parallel β-sheets in each domain and five disulphide bonds stabilize the structure.

We found that MK has both antibacterial as well as antifungal properties and exert these activities by disrupting the membranes of the microorganisms, resulting in leakage of intracellular contents. The antibacterial activity of MK is evolutionary conserved, originating in insects. The expression of MK is constitutive in the skin, but during inflammation the expression is increased and the concentrations found *in vivo* reach levels that are antibacterial. In the large airways, MK expression is also constitutive and may be responsible for a significant part of the antibacterial activity found in the air surface liquid covering the bronchial epithelium of the lungs. In patients with cystic fibrosis, a genetic disorder primarily affecting the ion-transport of the airway epithelium, the expression of MK is increased. These patients suffer from viscous mucus and chronic infections caused by bacteria, not least *Pseudomonas aeruginosa*. The antibacterial activity of MK was compromised because of the changed environment in the lungs, where both increased salt and a lowered pH decreased its antibacterial activity.

Taken together, MK is a potent antimicrobial peptide with activity against several bacterial and fungal species. The expression is constitutive in the skin and the large airways during healthy conditions and during inflammation the expression is increased. Since bacterial resistance against conventional antibiotics is increasing, AMPs such as MK, may provide templates for the development of novel therapeutics strategies to combat disease where microbes are either the primary cause or cause exacerbations of chronic diseases as seen in cystic fibrosis.

Key words: Antimicrobial polypeptides, Midkine, antibacterial, antifungal, innate immunity, epithelial surfaces

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Midkine - a host defence protein

Doctoral Thesis

by

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Cover image: Molecular representation of a model of full length midkine provided by Björn Walse, SARomics Biostructures AB.

To my family

Happiness is a state of mind, a choice and a way of living. It is not something to be achieved, it is something to be experienced.

-Steve Maraboli

Abstract

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Contents

List of papers

I. **Svensson SL**, Pasupuleti M, Walse B, Malmsten M, Mörgelin M, Sjögren C, Collin M, Schmidtchen A, Palmer R, Egesten A. Midkine and pleiotrophin have bactericidal properties – preserved antibacterial activity in a family of heparin-binding growth factors during evolution. *The Journal of Biological Chemistry*, 285: 16105-15, 2010.

II. Frick IM, **Nordin SL**, Baumgarten M, Mörgelin M, Sørensen OE, Olin AI, Egesten A. Constitutive and inflammation-dependent antimicrobial peptides produced by epithelium are differentially processed and inactivated by the commensal *Finegoldia magna* and the pathogen *Streptococcus pyogenes*. *The Journal of Immunology*, 187: 4300-4309, 2011.

III. **Nordin SL**, Sonesson A, Malmsten M, Mörgelin M, Egesten A. The epithelium-produced growth factor Midkine has fungicidal properties. *Journal of Antimicrobial Chemotherapy*, 67: 1927-1936, 2012.

IV. **Nordin SL**, Andersson C, Bjermer L, Bjartell A, Mörgelin M, Egesten A. Midkine is part of the antibacterial activity released at the surface of differentiated bronchial epithelial cells. *Journal of Innate Immunity*, 5(5): 519-530, 2013.

V. **Nordin SL***, Jovic S*, Kurut A, Andersson C, Gela A, Bjartell A, Mörgelin M, Olin A, Lund M, Egesten A. High expression of Midkine in the airways of patients with cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology,* 2013, in press (PMID: 23815177)

*Both authors contributed equally to this work

Abbreviations

ALI	Air-liquid interphase
ALK	Anaplastic lymphoma kinase
AMPs	Antimicrobial polypeptides
ASL	Air surface liquid
ATRA	*All-trans* retinoic acid
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
CFU	Colony-forming units
CLRs	C-type lectin receptors
ER	Endoplasmic reticulum
FAF	*Finegoldia magna* adhesion factor
GAGs	Glucosaminoglycans
hBD	Human β-defensin
hCAP-18	Human cationic antimicrobial protein
HIF-1α	Hypoxia-inducible factor-1
IFN	Interferon
IHC	Immunohistochemistry
IκB	Inhibitor of NF-κB
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIC	Minimal inhibitory concentration
MK	Midkine

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NLRs	Nucleotide-binding domain-like receptors
PAMPs	Pathogen-associated molecular patterns
PCL	Periciliary liquid
PRR	Pattern-recognition receptor
PTN	Pleiotrophin
PTPζ	Receptor-like tyrosine phosphatase-ζ
RA	Retinoic acid
RAR	Retinoic acid receptor
RDA	Radial diffusion assay
RXR	Retinoid X receptor
SIC	Streptococcal inhibitor of complement
SufA	Subtilase of *Finegoldia magna*
TLR	Toll-like receptor

Introduction

Every day we encounter countless number of microbes, both commensal bacteria normally colonizing our skin and mucosal surfaces, but also potential pathogens. Despite this we are rarely infected. Our immune system often rapidly eliminates the imminent threat of pathogens, but not always [1].

The defence system is comprised of an array of different mechanisms to protect against microorganisms. The first line of defence is a pure mechanical barrier, the keratinized epithelial cells of the skin and the mucosal lining of the gut and lungs, prevents pathogens from entering into our bodies. If these primary defences should be breeched, there is a great need for rapid and specialized defences [2, 3]. The immune system uses different strategies to protect us against infection and defend us once we are infected. The innate immune system produces antimicrobial polypeptides (AMPs), chemokines and cytokines for a non-specific and quick response. Adaptive immunity is highly specific, but instead requires longer time [4, 5]. Today, bacterial resistance against conventional antibiotics is rapidly increasing and there is a need for new pharmaceutical strategies. To take advantage of our own defence molecules and use AMPs as novel treatment would be one such strategy [5].

The main aim of this work was to determine the role of the heparin-binding growth factor midkine in the context of innate immunity. Is midkine antimicrobial, and what effect does it have in the skin and lungs during healthy conditions and during disease?

The immune system

The immune system is as old as life, without an effective defence against all the different microbes and pathogens in our surrounding we would not survive. In vertebrates, the immune system is comprised of two arms, the innate immune system and the adaptive immune system. These systems have different specialties but are interlinked [4, 6].

Innate immunity

Innate immunity is present in all living organisms. Vertebrates both have an innate and an adaptive immune system while invertebrates, fungi and plants rely exclusively on innate immunity and the production of antimicrobial polypeptides as

a defence, showing the importance of this system. The innate immune system is a rapid, non-specific defence. Since the number of bacteria can double every 20 minutes an efficient host defence must be faster than that [7-9].

The components of innate immunity include the skin and mucosal surfaces that forms a mechanical barrier but also the gastric acid of the stomach, pathogen removal by mucus and the motion of cilia in the lungs, the complement cascade, chemokines, cytokines and a chemical barrier. The chemical barrier consists of antimicrobial polypeptides (AMPs), which are effector molecules of the innate immune system and works as broad-spectrum antibiotics. AMPs can be constitutively expressed (by epithelial cells), rapidly induced upon stimulation (wounding, contact with pathogenic bacteria/bacterial products or cytokines) or brought to the site of invasion by mobile immune cells (e.g. neutrophils and leukocytes) [10-12].

The innate immune system recognizes microorganisms based on detection of conserved structures called pathogen-associated molecular patterns (PAMPs). PAMPs are shared by large groups of microorganisms and are usually essential for the survival of the microorganism and are therefor difficult for the microorganism to alter. Lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria, lipoteichoic acid (LTA) from the cell wall of gram-positive bacteria, DNA and flagellin from flagellated bacteria are examples of PAMPs [13, 14].
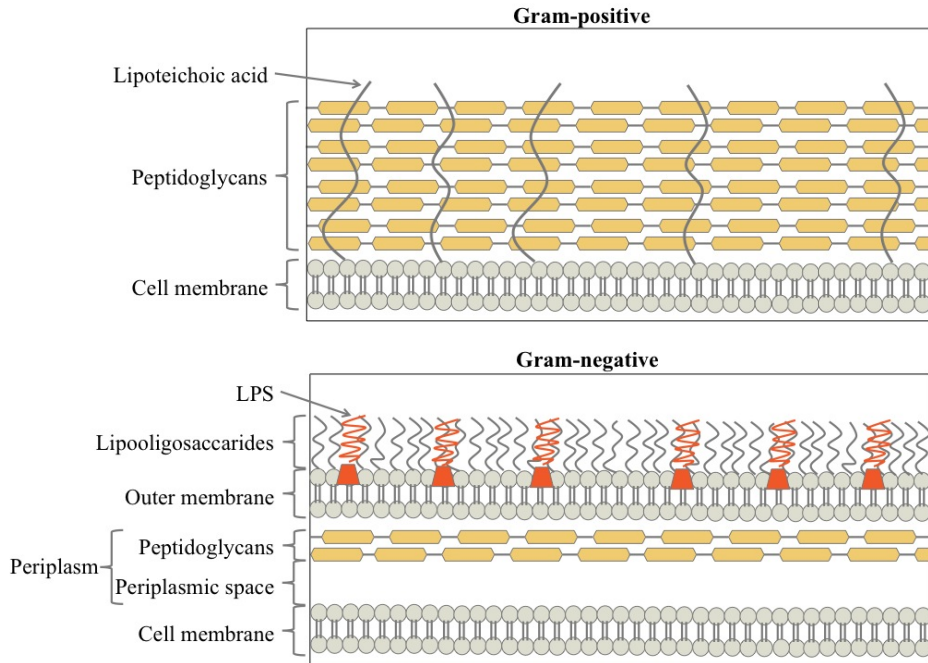
Figure 1. The structure of Gram-positive and Gram-negative bacteria. Schematic drawing of the structure of Gram-positive (upper panel) and Gram-negative bacteria (lower panel). Gram-positive bacteria have a thick peptidoglycan layer, while Gram-negative bacteria have a thinner peptidoglycan layer and an additional outer membrane creating a periplasm. The outer membrane contains different transmembrane proteins.

Innate immune cells involved in the initial microbial recognition (epithelial cells, dendritic cells, monocytes, natural killer cells and neutrophils) express pattern-recognition receptors (PRRs), which will recognize PAMPs. Different PRRs have distinct expression patterns and will react to specific PAMPs and activate different signalling pathways [14-17].

Many classes of PRRs have been identified, the best characterized PRRs in mammals are the Toll-like receptors (TLRs), which can be expressed on the cell surface or intracellularly in endosomes, lysosomes or in the endoplasmic reticulum (ER). TLRs are the homologue of the Toll-receptor first identified in fruit fly (*Drosophila melanogaster*) [16, 18]. Other PRRs are RIG-I like receptors (RLRs), C-type lectin receptors (CLRs) and nucleotide-binding domain-like receptors (NLRs). Together these receptors provide an extensive defence against a wide variety of ligands, both derived exogenously (from pathogens, virus, mycobacteria and fungi) but also endogenously derived from damaged tissue [19-21].

Binding of a ligand to the PRRs will result in the recruitment of adapter proteins and finally lead to the activation of the transcription factors NF-κB and MAPK signalling pathways. This will generate transcription of various genes with functions in innate immunity, such as pro-inflammatory cytokines, chemokines and major histocompatibility complex (MHC), which will direct and activate the adaptive immune system. Nitric oxide synthase and AMPs will also be induced, which directly kill pathogens [22-24].

Adaptive immunity

The adaptive immunity is mediated by lymphocytes (T- and B-lymphocytes), which present an enormous array of receptors, capable of recognizing antigen from all pathogens. Antigen-presenting cells (dendritic cells) activate T-cells by presenting antigen with the help of MHC. Chemokines produced by the innate immune system will attract and guide dendritic cells (DC-cells) to the site of pathogens invasion, where these cells will ingest and present pathogenic antigens, and thereby activating T-cells in the lymph nodes. The T-cells will also be guided by chemokines to the site of infection. B-cells can be activated by antigens and/or T-cells and as a response produce antibodies. The antibodies bind to any matching antigen, thereby inhibiting and marking the pathogens until phagocytes (macrophages and neutrophils) comes to engulf and kill the pathogens [4, 6, 25].

Since the adaptive immunity is a highly specific process, it is also a slower process than the innate immune system. It can take days or weeks before reaching full efficiency. On the other hand, an immunological memory will be created, which is a feature used with vaccines. The next time a pathogen is encountered, the adaptive immune system will immediately recognize it and start defending the host. The role of lymphocytes in adaptive immunity (recognition of specific antigens) is limited during the first encounter with a pathogen, but is instead very effective against microbes that are hard to eradicate and against microbes that have previously infected the host [1, 4].

Chemokines/Cytokines

The binding of a ligand to PRRs and their subsequent activation will lead to an immediate production of cytokines and chemokines. Cytokines are a diverse group of proteins, peptides and glycoproteins, which can modulate the immune system. Some act pro-inflammatory, recruiting immune cells to the site of infection, others work anti-inflammatory to promote healing and reduce inflammation once the infection has been cleared. Cytokines also modulates growth, survival and differentiation of different cell types. Depending on which PRRs that are activated (by pathogen or injury etc.) the pattern of cytokines and chemokines will differ [6, 26, 27]. Cytokines binds to specific receptors on target cells and can create cascades

and enhance or suppress the production of other cytokines and chemokines, thereby regulating the immune response. Recruited immune cells amplify the release of cytokines and chemokines, thereby supporting the innate immune response. Examples of cytokines are interferons (IFNs) and interleukins [28].

Chemokines are a large family of cytokines, which have chemotactic activities and regulate the movements of leukocytes to the site of microbial infection or injury. They also regulate the trafficking of leukocytes during normal conditions. Some chemokines attract and activate all kinds of leukocytes while others attract a specific subset of leukocytes, for example dendritic cells. The chemokines are retained locally on cell-surface proteoglycans and on the cell matrix, thus forming a chemical gradient surrounding the inflammation [29, 30].

Antimicrobial polypeptides

To date more than 2200 AMPs have been isolated from mammals, invertebrates, insects, plants and birds etc. [31]. AMPs represent an extensive collection of small, cationic peptides, which often have heparin-binding motifs (Cardin-Weintraub motifs) [32]. AMPs are found in all living organisms, showing their importance as effector molecules of innate immunity by providing a rapid defence against microorganisms. AMPs are effective against gram-positive and gram-negative bacteria but also against fungi and virus. They can be distributed in different ways, some AMPs are constitutively expressed and react immediately to microbial infestation or injury. Others are locally synthesized or released when epithelial cells are triggered, in response to pathogens, injury, pro-inflammatory cytokines or growth factors. AMPs can also be produced by immune-cells coming to the infected area [1, 5, 8].

AMPs are a diverse group of peptides but based on their amino acid composition, structure and charge they are divided into the following groups [33]:

- o Linear amphipathic α-helical peptides (e.g. LL-37 [34])
- o Peptides with β-sheets, stabilized by conserved cysteines forming disulphide bonds (e.g. defensins [35])
- o Peptides enriched in one or more amino acids such as proline or arginine (e.g. histatin [36])
- o Anionic peptides (e.g. dermcidin [37])

One important characteristic of many AMPs are their ability to arrange their hydrophobic and cationic amino acids in a certain order, resulting in an amphipathic structure (Figure 2), enabling them to get in close contact with bacterial membranes [38].
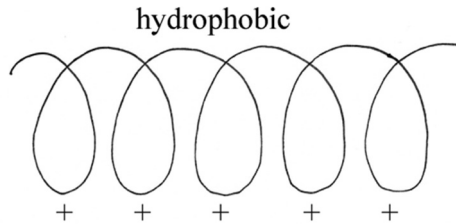
Figure 2. Amphipathic structure of AMPs. Proposed amphipathic structure of an AMP with the hydrofobic and positively charged amino acids arranged on different sides.

AMPs have been found on all sites of the body that are normally exposed to microbes, such as the mucosal linings and the skin. But they are also produced by different blood cells (e.g. neutrophils and eosinophils) [36, 39]. The concentrations of AMPs *in vivo* are typically much lower than the concentration needed to give bacterial killing *in vitro* (minimal inhibitory concentration, MIC). Nevertheless, AMPs still have an effect *in vivo*, either because they are able to work synergistically together with other AMPs or because AMPs accumulate locally at high concentration during inflammation [40].

In humans, the most known and studied AMPs are the defensins and the cathelicidins. Defensins are small and cationic polypeptides expressed in great amounts in humans. They are expressed in many different cells and tissues where bacterial infections are a threat, such as monocytes, macrophages, dendritic cells, keratinocytes and epithelial cells. All defensins are expressed as pro-peptides and are cleaved to become active. Structurally defensins are composed of three anti-parallel β-sheets stabilized by three disulphide bonds. They are classified into α-defensins, β-defensins and θ-defensins depending on their number of amino acids and how the cysteine residues are linked [41]. The θ-defensins is only expressed in rhesus macaques and baboons and not in humans due to a stop codon in the signal peptide [42, 43].

Six human α-defensins have been discovered in humans. The first were purified from granules in neutrophils and are called human neutrophil peptides (HNP1-4). They are constitutively produced, stored in granules and released during neutrophil activation. The other two, (HD5-6) are expressed by epithelial cells in the small intestine [44, 45].

Four β-defensins have been discovered (hBD 1-4) in humans. HBD-1 was first discovered from plasma [46] and the expression is constitutive in the urogenital and respiratory tract. HBD-2 and hBD-3 were isolated from psoriatic skin [47, 48] and are found in epithelial cells of the skin and respiratory tract. The expression is increased by bacterial infection and inflammatory factors. HBD-4 is expressed in neutrophils, lungs and kidneys [49].

20

Cathelicidins are found in all species and are expressed as pro-peptides. LL-37 is the only human cathelicidin found and it is stored as a precursor (hCAP-18). LL-37 displays a broad antimicrobial activity, as well as being chemotactic and promoting wound healing. LL-37 is expressed in neutrophils, lymphocytes, in keratinocytes and epithelial cells of the skin, gastrointestinal tract and airways. The expression is both constitutive and induced during infection, where LL-37 is stored as hCAP-18 in granules ready to be cleaved and activated [34, 50].

AMPs are often multifunctional (Figure 3), in addition to acting antimicrobial and inhibiting microbial growth, they also act as growth factors (promoting wound healing) and promoters of angiogenesis. Some AMPs also act as chemotactic agents (recruiting inflammatory cells) and/or inducing chemokine production. Thus, AMPs can act as a link between innate and adaptive immunity [40, 51].
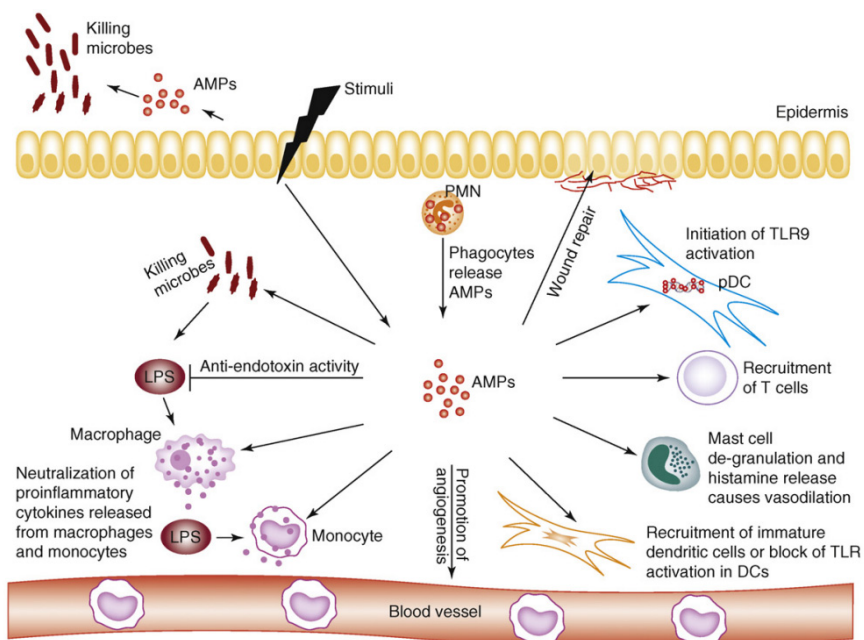
Figure 3. Multifunctionality of AMPs. AMPs are not just antimicrobial but have many additional features as well, recruiting immune cells, neutralizing endotoxins and pro-inflammatory cytokines as well as promoting angiogenesis and wound repair. Reprinted with permission from Elsevier, Trends in immunology, Lai and Gallo, 2009 [40].

History

Alexander Fleming was the first to describe an antibacterial protein which he named lysozyme in 1922 [52]. But it was not until the development of antibiotic-resistant bacteria in the early 1960's, that the interest in AMPs was renewed. Subsequently neutrophils were shown to kill bacteria with cationic peptides, without the adaptive

immune system, and these peptides were later named AMPs [53, 54]. In the 1980s the field expanded even more when the first α-helical AMPs, cecrospins, were isolated and purified from insects, magainins were isolated from amphibians and the first defensins were isolated from mammals [55-57]. Since then hundreds and yet hundreds of AMPs have been described and are found in all multicellular organisms and play an important role in the immune system [36, 58].

Mode of action

AMPs can roughly be organized into three groups depending on target [59, 60].

- o Membrane-active peptides, penetrating and disrupting the integrity of the microbial membrane.

- o Peptides acting on intracellular targets, inhibiting transcription, translation or other processes in the microbes.

- o Cell wall active peptides capable of binding components essential for the microbes (e.g. zinc, iron) or interfere with the cell wall synthesis.

AMPs have selectivity towards bacteria and fungi because of different compositions of the plasma membrane as compared to eukaryotic membranes. Bacterial membranes have lipids with negatively charged phospholipid head-groups on the outside, giving them an overall negative charge. Membranes of human cells, on the other hand, are composed of lipids with no net charge and the lipids with negative charge are facing into the cytoplasm. The membranes also differs between gram-positive and gram-negative bacteria, where gram-positive have a thick peptidoglycan layer with negatively charged lipoteichoic acid (LTA) and gram-negative bacteria have an outer plasma membrane with negatively charged lipopolysaccharides (LPS) covering the peptidoglycan layer [5, 11]. Human cells and fungi both have sterols in their membrane, cholesterol and ergosterol respectively, which bacteria do not. This enables the AMPs to discriminate between bacteria/fungi and host cells [61].

The first step in the disruption of membranes, and also for internalisation, is the ability of AMPs to get close and adhere to the bacterial membranes. Since most AMPs have a positive (cationic) net charge in physiological pH and an amphipathic structure, this will facilitate the electrostatic interaction with the negatively charged (anionic) bacterial membranes and allow the AMPs to come close and to aggregate on the bacterial surface [11].

There are a number of proposed mechanisms by which AMPs disrupts the bacterial membranes and the bacteria are killed because of leakage of intracellular content. But even before the bacterial membrane is disrupted the transmembrane potential, pH regulation and the osmotic pressure of the bacteria is destroyed, dysregulated or

inhibited by the AMPs. Regardless of mechanism, the AMPs must reach a threshold concentration on the surface of the membrane. This concentration varies depending on the peptide. The proposed mechanisms of action are [33, 62, 63] (see Figure 4)

a. Barrel-stave pore mechanism. The peptides form a pore (or channel) by vertical insertion in the membrane. The hydrophobic regions of the peptides are facing the lipid-containing membrane and the hydrophilic parts form the inside of the pore. More peptides can be recruited to the pore, which then grows in size and becomes more stable.

b. Carpet mechanism. Peptides accumulate parallel to the surface of the membrane and at a sufficiently high concentration they disrupt the membrane in a detergent-like manner.

c. Toroidal pore mechanism. Peptides are inserted vertically into the membrane and induce the lipids to form a local curve, resulting in a pore that partly consists of peptides and partly by the phospholipid head groups of the membrane.

d. Disordered toroidal pore mechanism. Just like the toroidal pore mechanism but the peptides form a less tight and organized structure and the pore lumen is lined with phospholipid head groups to a higher extent.
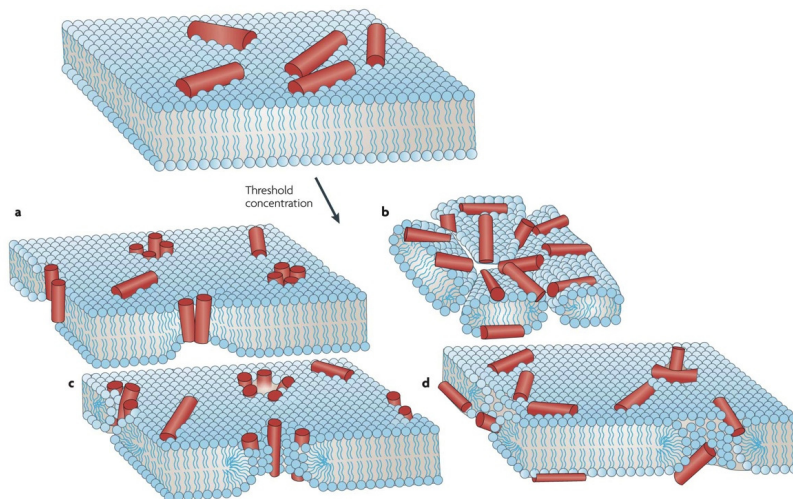
Figure 4. Proposed mechanisms of AMPs. To execute their membrane disrupting properties and thereby killing the bacteria AMPs must first reach a threshold concentration. The AMPs have been proposed to disrupt the membranes in different ways. **a.** Creating a Barrel-stave pore **b.** Carpet mechanism **c.** Toroidal pore **d.** Disordered toroidal pore. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Melo *et al* 2009 [62].

AMPs can also act on intracellular targets, where some peptides have the ability to traverse the bacterial membrane and enter into the cell where they can inhibit protein synthesis, nucleic-acid synthesis, cell wall synthesis or enzymatic activity. For example, histatins bind to a receptor on the membrane and are thereby actively taken up into the cell where they can disrupt the cell cycle, generate reactive oxygen species and inhibit the mitochondrial energy production [33, 64].

Bacterial defence against AMPs

The host-pathogen interactions have evolved over millions of years. The host have developed defence mechanisms against pathogens, but pathogens have also tried to develop resistance against the host defence.

Bacteria use a number of different strategies to avoid being killed by the AMPs produced by the host.

Altering cell surface charge. Since AMPs have to come in close contact with the bacteria to be able to insert into the membrane, one defence strategy of the bacteria is to reduce the negative charge of their membrane, thereby weakening the electrostatical forces attracting the cationic AMPs. Some gram-positive bacteria modify the negatively charged lipoteichoic acid (LTA) and gram-negative bacteria can alter their lipopolysaccharides (LPS) resulting in a less negative membrane [38].

Expression of efflux pumps. Bacteria can express efflux pumps on the surface that actively (requiring energy) exports AMPs from the bacteria. The efflux pumps will only recognize a limited number of substrates, which only enables the bacteria to avoid certain AMPs. Bacteria use the same mechanism in resistance against conventional antibiotics [65, 66].

Production of proteases and neutralizing proteins. Certain bacteria are able to produce proteases that will degrade AMPs and thereby impair or destroy their activity. AMPs with α-helical structures are often more susceptible for degradation of proteases while AMPs stabilized with disulphide bonds (e.g. defensins) are considerably more resistant to degradation. Other bacteria can produce protein that bind to the AMPs and neutralizes them. [38, 67]. Examples are the protease SIC, produced by the pathogenic *Streptococcus pyogenes* [68], the protease SufA [69] and the AMP-neutralizing protein FAF [70] from the opportunistic pathogen *Finegoldia magna*. Another mechanism by which bacteria might trap and inactivate AMPs is by degrading proteoglycans of the host epithelial cells and thereby releasing glycosaminoglycans (GAGs) that can bind and inhibit AMPs [65, 71].

Modification of host cellular processes. Certain bacteria can inhibit the production of AMPs by evading the initial recognition of innate immunity or actively downregulate specific signalling pathways. Examples are *Shigella dysenteriae*, a

gastrointestinal pathogen, which can downregulate LL-37 and hBD-1 on a transcriptional level by releasing a RNA plasmid [65, 72].

The reasons why bacteria have not been more successful in resisting AMPs are because the alterations of the membrane are energetically costly for the bacteria. AMPs are also a very diverse group of polypeptides without shared conserved epitopes that proteases can use to recognize them [5]. Since many AMPs are expressed at the same time and work synergistically, proteases will only affect a subset of AMPs and still leave the bacteria vulnerable [11].

Midkine

Midkine (MK) is, together with pleiotrophin (PTN), a small family of heparin-binding growth factors where they are the only two members [73]. MK is comprised of 123 amino acids (without signalling peptide), has a molecular weight of 13.4 kDa and a high isoelectric point of 10.4 [74, 75]. MK was first discovered as a gene induced by retinoic acid in murine carcinoma cells [76, 77] and in parallel a chicken homologue was purified [78].

MK is a multifunctional protein (see Figure 5), acting as:

- o Growth factor. MK is important during development and is tightly regulated, in mice the expression is high during mid-gestation where the epithelial-mesenchymal interactions create the lungs, pancreas and small intestine [79]. MK also stimulates the migration of embryonic neurons and promotes the differentiation of neurons [78]. High expression of MK is often seen in many cancers due to its anti-apoptotic features [80]. MK is also involved in tissue repair and regeneration, for example in wound healing [81, 82].

- o Pro-inflammatory agent. MK is expressed during inflammation and act pro-inflammatory by activating and recruiting neutrophils and macrophages [83, 84].

- o An antimicrobial agent. MK has shown an antimicrobial activity against both gram-positive and gram-negative bacteria and also fungi [75, 85]. Shown in **Paper I and III**.

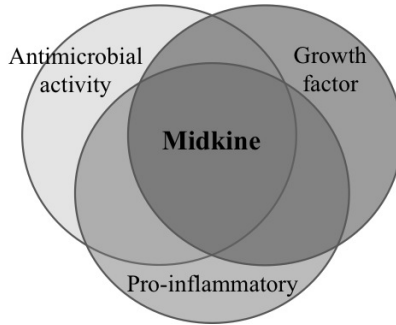This multifunctionality is a typical feature of AMPs.

Figure 5. Multifunctionality of MK. MK exert multiple functions, acting as a growth factor, a pro-inflammatory agent and also beeing antimicrobial against both gram-positive and gram-negative bacteria as well as fungi.

The expression of MK is high during development in mice and is then downregulated in many tissues. In the adult, MK is expressed in the skin, mucosal surfaces of the lungs and gut, kidneys, bladder and spleen. Most organs and cell types have the ability to secrete MK during normal conditions [86-88]. The expression is increased during inflammation, tissue repair, ischemic conditions and hypoxic conditions [84, 89-92]. Circulating MK is elevated in different autoimmune chronic inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, suggesting the use of MK as a potential biomarker [4, 89, 93].

Structure

MK is comprised of two domains, one located in the N-terminal part and the other located in the C-terminal (Figure 6). Each domain consists of three anti-parallel β-sheets and the domains are linked with a flexible, highly conserved hinge region. The domains are stabilized with, in total, five disulphide bonds where three disulphide bonds are located in the N-terminal part and two in the C-terminal. The end of the C-terminal is a long and unordered tail rich in cationic amino acids (e.g. lysine) [94, 95]. The stability of MK to acid and high temperatures are considered to depend on the stabile structure with many disulphide bonds [96].

All ten cysteines in vertebrate MK is conserved, and the C-terminal domain is evolutionary the most conserved part [97]. Homologues of MK have been reported from many different species, for example mice, fishes, frogs and insects. The mouse MK and the human MK have a sequence identity of 87% [98]. *Drosophila melanogaster* (fruit fly) do not express MK, but instead have Miple-1 and Miple-2, a combination of MK and PTN, which lack two cysteine residues in the N-terminal part. No homologues of MK have been reported in the genome of *Caenorhabditis elegans*, suggesting an origin among insects [99].

NH2-terminal   COOH-terminal
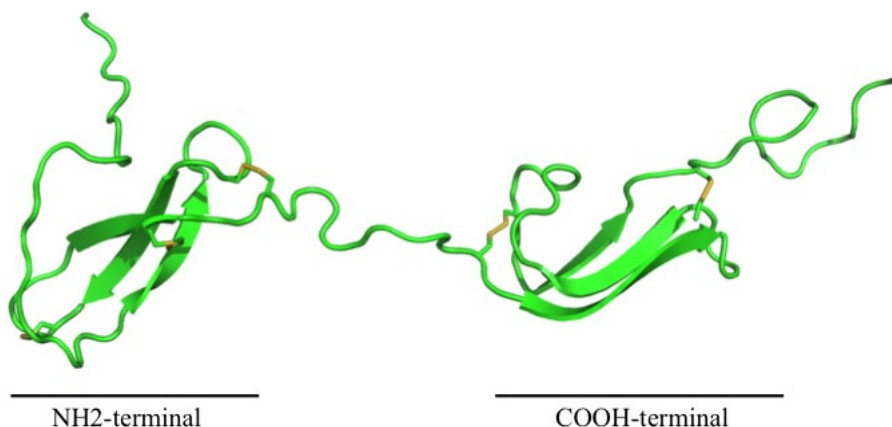
Figure 6. Structure of MK. MK consists of two domains with three anti-parallell β-sheets in each domain, and the structure is stabilized by five disulphide bonds. This work was originally published in Journal of Biological Chemistry by Svensson *et al*, 2010 [75].

The structure of MK resembles β-defensins, both being cationic with anti-parallel β-sheets and being stabilized by disulphide bonds [41, 94] where MK looks like a tandem-defensin. MK tends to spontaneously form dimers (or oligomers) and the dimers are stabilized by transglutaminases (Ca^{2+}-dependent enzymes) and this dimerization seems to be important for MK activity [100].

Regulation

The expression of MK was already at discovery shown to be dependent on retinoic acid (RA), a derivative of vitamin A [77]. In the promoter region of MK there is a retinoic acid (RA)-responsive element [101]. Vitamin A is obtained from the diet in the form of *all-trans* retinol or β-carotene, which is further processed to *all-trans* retinoic acid (ATRA) and stored in the liver and fat [102]. ATRA binds to different nuclear receptors; the retinoic acid receptor (RAR) and retinoid X receptors (RXR) and in some cases PPARβ [103, 104]. Vitamin A is essential for the life of all vertebrates and has many important functions in embryonic growth and development, boosting the immune system (enhancing cytotoxicity and B- and T-cell activation and proliferation) and it is essential for the vision. Another important function of ATRA is the differentiation and maintenance of epithelial surfaces such as the lungs [102, 105] where MK is expressed [87, 106]. The amount of accessible ATRA during inflammation is affected by immune cells (e.g. dendritic cells), which can induce the enzymes metabolizing vitamin A to ATRA. Activation of TLR2 and other factors present during inflammation can also influence the generation of ATRA, while for example prostaglandins inhibit the ATRA synthesis. Thus the level of available ATRA during inflammation, and thereby the expression of genes

with RA-responsive elements such as MK, can be regulated by different factors present during inflammation [103, 107].

NF-κB is a transcription factor activated during infection. Normally NF-κB is bound to an inhibitory protein, inhibitor of NF-κB (IκB) but stimulation through TLRs (pathogens or injury) or cytokines of innate immunity, will result in the release of NF-κB. It will be transported to the nucleus of the cell, form dimers and bind to DNA containing their target sequence. This will result in the production of cytokines, chemokines and other proteins important for the immune response [108]. MK was shown to be induced by the NF-κB signalling pathways in prostate cancer cells, suggesting that MK have a NF-κB responsive element in the promotor, which will activate MK also in other cell types [109].

MK expression has been shown, in mice, to be induced by hypoxia-inducible factor-1 (HIF-1α) during hypoxia [92]. Infected tissues often present an environment that is unfriendly for the pathogens, but where the immune system can still function. These sites are often hypoxic. HIF-1α is rapidly induced in hypoxia and give rise to expression of genes needed for the survival of neutrophils and other cells in the tissue during hypoxia but also increased bacterial killing, protease production, angiogenesis and wound healing [110-112].

Altogether, MK expression is enhanced by several factors present during inflammation.

Signaling

MK binds to a variety of receptors and affect many different cell types, probably in a receptor complex consisting of many molecules. The receptors that are best characterized in this complex are receptor-like tyrosine phosphatase-ζ (PTPζ) [113], lipoprotein receptor-related protein (LRP) [114], α4β1-integrin and α6β1-integrin [115]. Other receptors have also been proposed, such as the anaplastic lymphoma kinase (ALK) [82, 116], which can be recruited to the receptor complex and cause activation of NF-κB. This suggests that different complexes of receptors can give rise to different down-stream signalling and thereby distinctive responses [116].

Defence on skin and mucosal surfaces

The immune system has evolved alongside the commensals, where AMPs keep the fine balance between host and microbiota and fine-tune the composition of the microbiota. Pathogens will therefor have a harder time attaching to the mucosal linings because that niche is already occupied [7, 117]. Inflammation and injury will facilitate crossing of pathogens through the mucosal/epithelial barrier and reduce the commensal flora by invading their niche. Pathogens can also produce effector

molecules that diminish the affect of the AMPs [118]. The microbiota is controlled by the host, for example by supplying nutrients to favour the growth of specific commensals and by producing different AMPs that modify and balance the composition of the microbiota. The microbiota, in turn, also affect the immune system, creating a somewhat protected niche by producing proteases and neutralizing proteins that inhibit AMPs [117, 119].

Skin

The skin is one of the largest human organs and it is always in contact with the environment and the potential pathogens therein, but infection rarely occurs. The skin itself is a mechanical barrier where the top layer, the stratum corneum, consists of tightly packed keratinocytes. The skin surface is constantly renewed and the outermost layer is continuously shed [120]. The skin is cool, acidic and dry and it is overall an unfriendly environment for microbes [121]. The skin is also covered with a specific microbiota [122], and the content is affected by many factors such as age, climate, gender and location on the skin. The microbiota is divided into two groups, resident microbes (commensals, which are re-established after disturbance) and transient microbes from the environment, which are not permanent but persist temporarily on the skin. Both resident and transient microbes can be opportunistic pathogens [123]. The microbiota occupies the skin, thus competing over space and nutrients with potential pathogens and have the ability to produce bacteriocidins (antimicrobial polypeptides produced by bacteria) to kill pathogens competing for the space [3, 124, 125]. The host controls both the microbiota, and potential pathogens, by the production of bacteriostatic and antibacterial molecules, for example psoriasin and RNase7 to keep a balance [10, 126]. Should the pathogens gain entry, in for example a wound, AMPs, such as β-defensin, will act as a soluble barrier inhibiting infection, either constitutively expressed or induced [127-129]. Keratinocytes are the main producer of AMPs in skin, but during inflammation the recruited immune cells express the majority of AMPs [125]. MK is constitutively expressed in human skin [130] at concentrations of about 1 μM corresponding to levels needed for antibacterial activity [86].

Gastrointestinal tract

The stomach contains low pH and gastric acid designed to digest food but also to kill bacteria. The gastrointestinal tract is covered with a mucosal layer of enterocytes, which absorb nutrients by microvilli with the help of digestive enzymes and transporters, but also act as a barrier with tight junctions between the cells preventing entry of potential pathogens [131]. The enterocytes also produce AMPs to protect against pathogens and the expression can be either constitutive or induced by infection [132]. Another specialized cell type in the gut are the goblet cells that secrete mucins, which act as a protective layer above the epithelial layer and

facilitate the intestinal motility, which removes bacteria in faeces [133]. Paneths cells, in the base of the crypts, regulate the composition of the intestinal microbiota and defend against pathogens by producing AMPs such as α-defensins [134].

Just as the skin, the gut contains a microbiota of commensal microorganisms (in the number of 10^{13}-10^{14}) consisting of up to 500 different species such as *lactobacillus* spp, *enterococcus* and *clostridium* spp. The composition is individual and is affected by diet, medications, stress and diseases. The huge commensal microbiota serves a very important role by preventing bacterial colonization (competing over space, nutrients and producing antimicrobial compounds), digesting nutrients the host cannot otherwise digest and also by modulating and maintaining the immunity on the mucosal surface (e.g. inducing development or recruitment of immune cells) [133, 135, 136].

MK is expressed in gastrointestinal cancers and also during the healing of gastric ulcers [137, 138].

Airways

The ambient air contains 10^2-10^5 colony-forming units (CFU) of bacteria per m^3, meaning that with every breath, potential pathogens are inhaled [139]. The nose acts as a filter and the sneeze-reflex will rapidly remove potentially dangerous material from ever entering the airways [140].

Further down, the epithelium of the airway is important for the defence against the pathogens that enter the airways and consists of ciliated epithelial cells and secretory cells. Together, the cells provide a barrier and are connected by cell-cell junctions (e.g. tight junctions) making them impermeable [141]. The surface of the epithelium is covered with periciliary liquid (PCL) covering the cilia and a mucus layer, together called air surface liquid (ASL) (Figure 7) which is 5-20 µm deep in healthy individuals.
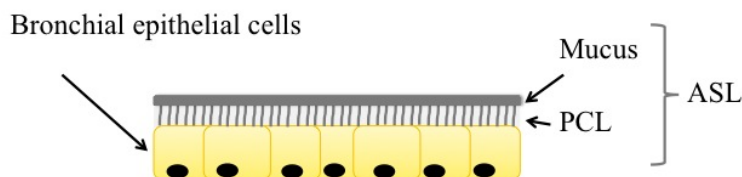
Figure 7. Airway surface liquid (ASL). The ASL covering the lungs are composed of a mucus-layer and a periciliary liquid layer (covering the cilia) with antibacterial activity.

The PCL have a low viscosity, allowing the cilia to move and their tips are in contact with the mucus layer, resulting in movement of the ASL [142]. This mucociliary clearance removes about 90% of all inhaled particles and microbes, which are trapped in the mucus. The epithelial cells regulate the volume and content of the

ASL by ion transport [141]. The ASL is a complex mix of mucins, ions and water but also contain secreted proteins and peptides, of which many are AMPs (e.g. defensins, LL-37 and lysozyme) and concentrate an efficient antimicrobial defence under the mucus layer. Thereby, the ASL makes up an important part of the immune defence in the lungs [143, 144]. The airway epithelial cells also have PRRs, by which they recognize microbes and activate the production of cytokines, AMPs and other components of innate immunity [145].

Respiratory diseases

Respiratory infections, particularly pneumonia, are still one of the most important causes of death both in adults and children. *Streptococcus pneumoniae*, *Haemophilus influenza*, *Moraxella catarrhalis* and *Staphylococcus aureus* are the most common bacteria in respiratory infections [146]. In addition, there are also other respiratory diseases such as chronic obstructive pulmonary disease (COPD), which is a very common lung disease where chronic bronchitis and emphysema destroys the lungs [147]. Cystic fibrosis is a genetic disorder, described below, that primarily affects the lung and we focus on this disease in paper V.

Cystic fibrosis

Cystic fibrosis (CF) is a life-threatening hereditary airway disease, where mutations in the *CF-transmembrane conductor regulator* (*CFTR*) gene result in dysfunction or absence of a chloride ion transport protein normally situated in the membrane on the apical side of certain epithelial cells. The incidence of CF is 1 case per 2500 births. [148-151]. The *CFTR*-gene is 180,000 base pair long and over 1,000 different mutations have been found in different regions, resulting in different severity of CF. The most common mutation is called ΔF508, where a phenylalanine is missing at position 508. The ion transport protein is normally synthesised but recognized as misfolded and thereby degraded before it can reach the cell-surface [152].

In comparison with normal airway mucus, CF is characterized by the production of large volumes of sputum with altered properties. Sputum has an altered composition compared to mucus, with abnormal mucins and a presence of macromolecules such as DNA, actin, lipids and proteoglycans resulting in a thick and sticky mucus layer. In addition, the mucociliary clearance is impaired, the airways are obstructed and the immune system is dysregulated. As a consequence, patients with CF acquire bacterial infections in their lungs. Many different bacterial species can be involved, but *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most common [153]. The infection is non-resolving leading to chronic infections with accumulation of effector cells, for example neutrophils, further damaging the epithelia by the production of proteases [154-156].

The abnormal electrolyte transport over the epithelia has been proposed to affect the host defence in many ways. One theory is that the salt-concentration of the ASL is increased, resulting in diminished or abolished action of AMPs such as hBD-1 and hBD-2 [157-159]. The depth of the periciliary liquid (PCL) has been proposed to be eliminated or reduced, and/or the abnormal amount and composition of the mucus is altered (thickened and viscous), which will lead to impaired mucociliary clearance [151, 160, 161]. It has been shown, in a porcine model, that the difference without *CFTR* was a lower pH of the ASL, and the lower pH reduces the antibacterial effect of AMPs [162].

The chronic airway infection, the exaggerated inflammatory response and the airway obstruction lead to decreased lung function and a progressive disease. The life expectancy of patients with CF has increased over the years due to improved therapies and physical therapy and in 2008 the life expectancy was 37 years [151, 163].

Applications

The bacterial resistance to conventional antibiotics are increasing and there is a need for new strategies. AMPs have a broad-spectrum activity against microbes and their control of inflammation and aiding in wound healing makes them promising candidates as therapeutics [5]. Unlike conventional antibiotics use of AMPs could leave the normal microbiota intact and only kill the pathogens [63]. AMPs can also enhance the efficiency of existing antibiotics, possibly by facilitating access into the bacterial cell through the membrane [164].

Some aspects need to be evaluated more, their interactions with immune cells and activities as growth factors need to be tightly controlled to avoid side effects [165].

In nature the microbes are exposed to a wide variety of AMPs of different structure and function, which reduce the selection pressure that give rise to the development of resistance. Microbes also encounter different AMPs within the host from time to time [166]. Since different AMPs act on many different targets they are less prone to induce resistance and changes needed to be done by the microbes (change of membrane etc.) are very costly [63].

The most tested form of distribution for AMPs have so far been topical applications, to avoid the adverse side effects that can occur with systemic distribution. Another way of distribution would be an aerosol formula, which would give a topical application in the lungs [165].

Instead of using synthetic AMPs, one way to utilise them as therapy, would be to start the endogenous production of AMPs either by topic or systemic stimulation locally at sites of infection. This would mean avoiding the difficulty of manufacturing and delivering the synthetic peptides to their target area [167].

Vitamin D3 (VD3) is for example a potent inducer of human cathelicidin (LL-37) [168] and retinoic acid is a powerful inducer of MK [101]. To minimize the risk of resistance a combination of antibiotics and/or peptides could be used [7].

Aims

The aims of this thesis were to characterize the expression and activity of the heparin-binding growth factor Midkine (MK) in host defence. The specific aims of the thesis were:

- To investigate the antibacterial activity of MK *in vitro*, against an array of gram-positive and gram-negative bacteria and to study how this activity is affected by salt and if the activity is exerted through membrane disruption or intracellular impairment.

- To determine MK expression in normal skin and inflamed skin and see how commensals and pathogenic bacteria can use proteases and other defence molecules to affect MK and other chemokines.

- To examine if MK exert an antifungal activity and how the protein can distinguish between host cells and fungi.

- To investigate the expression of MK in the airways and determine which part of MK exert the antibacterial activity. *In vitro*, to study if MK is released into the lumen of the airways and if it influence the antibacterial activity of the ASL against common airway pathogens.

- To determine the expression of MK in patients with CF and examine how MK is affected *in vitro* by conditions specific for CF (proteases, pH, salt).

Methods

Antimicrobial assays

Two different methods were primarily used to evaluate the antimicrobial activity, viable count and radial diffusion assay (RDA).

Viable Count

A viable count is a direct counting method where only viable bacteria are counted. In the assay, bacteria are grown to mid-logarithmic growth phase and incubated with peptide or buffer for one hour. The samples are diluted and plated on agar-plates, which are incubated over night. The surviving bacteria are allowed to grow and the colonies are then counted and compared to the control (Figure 8).

This assay is work-intense but the advantage is that bacteria and the peptide are in direct contact with each other and the sensitivity is high because a single surviving colony can be seen and the direct killing is measured. It also allows inspection and identification of the bacteria to verify that no contaminants are present. Disadvantages are that clumps of bacteria can look like one colony and the solution needs to be homogenous for the dilution steps to work properly.
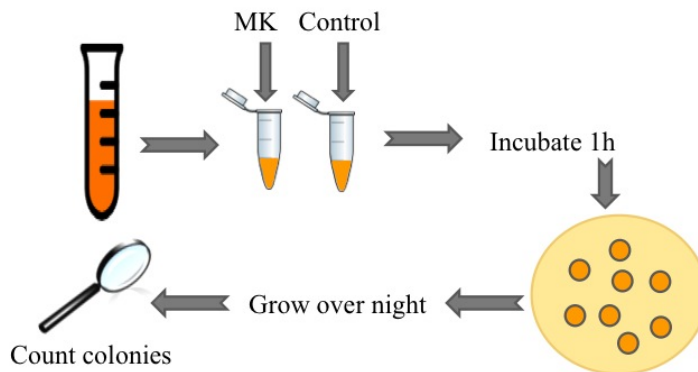

Figure 8. Viable count. Schematic presentation of the viable count assay. Bacteria are grown to mid-logarithmic growth phase, peptide or buffer is added to the solution of bacteria and incubated for 1 hour. The samples are then diluted and plated on agar plates. The plates are incubated over night and the colonies can then be counted and compared to the control.

RDA

In the radial diffusion assay (RDA) bacteria are grown to mid-logarithmic growth phase and added to an agarose solution, which are then moulded in plates. Holes are punched in the agarose (with bacteria) and peptides or buffer alone are added into the holes and allowed to diffuse for three hours. The agarose is then covered with a nutritious overlay gel. The plates are incubated over night, the bacteria will grow and the peptides will potentially inhibit the growth. The growth inhibition will be seen as clearing zones, which can be measured and compared to the control (Figure 9). This assay is good for screening of many peptides, but the peptides cannot be to big because then they will not be able to diffuse into the agarose. The peptides are present in the agarose gel during the growth of the bacteria, which make it impossible to determine if the activity is bactericidal or bacteriostatic.
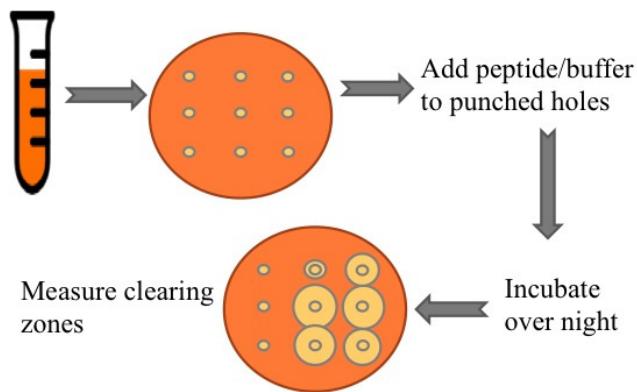
Figure 9. Radial diffusion assay. Schematic presentation of radial diffusion assay (RDA). Bacteria are grown to mid-logarithmic growth phase, added to agarose and moulded in plates. Holes are punched in the agarose-solution and peptide or buffer is added to the hole and allowed to diffuse. The plates are incubated and activity is seen as clearing zones where bacteria have not been able to grow.

Cell culture in air-liquid interphase

Air-liquid interphase (ALI) is a cell culturing technique where cells become differentiated and produce an air-surface liquid (ASL) with mucins. Primary bronchial epithelial cells are composed of a mix of cell types, such as goblet cells and ciliated and non-ciliated columnar epithelial cells, thus providing a closer resemblance to the airways *in vivo* than monolayer cell cultures. When the cells are confluent the medium is removed from the apical side and the cells are exposed to the ambient air (see Figure 10). The medium on the basolateral side is enriched with *all-trans* retinoic acid (ATRA), which will induce differentiation of the cells. Some cells will become secretory goblet cells producing mucins and others will become ciliated cells [169-171]. ATRA is known to be vital for the differentiation of airway

epithelial to columnar epithelium, mucus-production, production of surfactant proteins and to maintain the epithelial cells in a differentiated state [172].
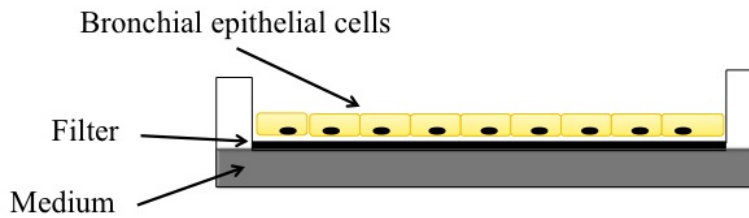
Figure 10. ALI cell culture. Primary bronchial epithelial cells cultured in an air-liquid interphase (ALI) culture where the cells are in contact with the ambient air from the apical side.

Using ALI cell culture technique with human bronchial epithelial cells, the concentration of MK in the *in vitro* ASL was measured to 0.7 µM which is a concentration sufficiently high for antibacterial activity [87].

Present investigations

Paper I

Midkine and pleiotrophin have bactericidal properties – preserved antibacterial activity in a family of heparin-binding growth factors during evolution

MK and PTN are a family of heparin-binding growth factors. They are both cationic proteins of 123 and 136 amino acids respectively, and they are comprised of two domains with three anti-parallel β-sheets stabilized by disulphide bonds. AMPs of innate immunity often have several functions, such as being growth factors and acting pro- or anti-inflammatory [173]. MK and PTN share many characteristics of AMPs, MK is upregulated during different states of inflammation [89, 174] and they have a structural resemblance to β-defensins although they comprise their own family. Consequently, in paper I, we decided to investigate the antibacterial activity of MK and PTN.

By using a viable count assay where different concentrations of MK or PTN was incubated with different strains of bacteria, we could determine that both MK and PTN had an antimicrobial activity *in vitro* against both the gram-positive *S. aureus* and *S. pyogenes* and also against the gram-negative *E. coli* and *Ps. aeruginosa*. The concentrations of MK and PTN required for complete bacterial killing were lower than 1 µM for gram-positive and 1 µM for gram-negative. MK was more potent than PTN. Since salt often inhibit the activity of many AMPs [175], different physiological concentrations of sodium chloride was added to the viable count assay but only a slight decrease in bacterial killing was seen.

Using transmission electron microscopy, MK and PTN were found to cause blebbing and leakage of intracellular content, suggesting membrane-disruptive properties.
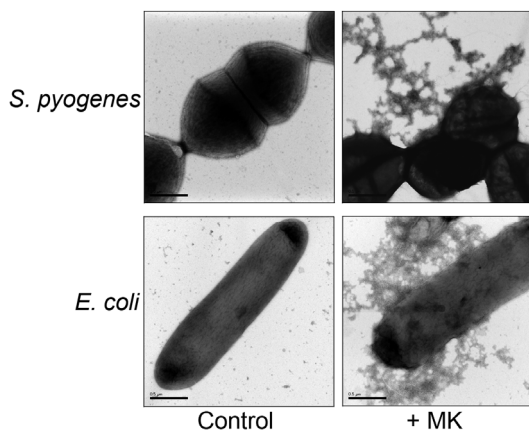
Figure 11. MK have membrane-disrupting properties. Electron microscopy revealed that MK cause membrane disruptions, blebbing and leakage of intracellular content of both gram positive *S. pyogenes* and gram negative *E. coli*. This work was originally published in Journal of Biological Chemistry by Svensson *et al*, 2010 [75].

To verify the finding of membrane disruption, a liposome leakage induction assay was also performed, where model lipid bilayers were formed to micelles and filled with fluorescent dye (carboxy-fluoroscein). These micelles were incubated with MK and PTN with and without addition of sodium chloride. MK and PTN both caused leakage of the fluorescent dye even at low concentrations and the addition of sodium chloride did not affect the disruption.

A comparison of MK and PTN showed an identity of 45% and a similarity of 61%. All cysteine residues are conserved and the secondary structure of the two proteins is very similar. To investigate which part of the full-length proteins that had antibacterial activity, short overlapping peptides (20 amino acids long) were synthesised and used in a radial diffusion assay with *E. coli*. The most active fragments were found in the last β-sheet of the N-terminal domain and in the tail of the C-terminal domain.

MK and PTN have orthologues in many species, from insects to humans. It is however, not found in *Caenorhabditis elegans* genome, suggesting an origin among insects. A phylogenetic tree showed that MK and PTN have a common ancestor but during evolution of vertebrates they are separated and belong to different clades.

To investigate the possible conservation of the antibacterial activity a sequence alignment of MK and PTN orthologues in the amphibian *Xenopus laevis* and the fish *Danio rerio* were aligned with the human MK and PTN. The ten cysteines are conserved, suggesting that the structure is also conserved among the species. Highly cationic regions also showed a high similarity. The same overlapping peptides were synthesized of *X. laevis* and *D. rerio* and showed antibacterial activity in the same

regions. In the case of *Drosophila melanogaster*, we had access to one of the two proteins of MK and PTN called Miple-2, in full-length, which showed antibacterial activity against the gram-negative *E. coli*.

In conclusion, the present study showed that MK and PTN have antibacterial activity and exert that activity through membrane disruption. The activity was relatively unaffected by sodium chloride, which is a quite unique feature of AMPs and the activity was evolutionary preserved.

Paper II

Constitutive and inflammation-dependent antimicrobial peptides produced by epithelium are differentially processed and inactivated by the commensal *Finegoldia magna* and the pathogen *Streptococcus pyogenes*

The first line of defence against microbial infestation is the epithelial lining of the skin, which function as an anatomical barrier. At infection, the cells will increase the production of AMPs, for example human β-defensin 2 (hBD-2) and human β-defensin 3 (hBD-3) but the cells also continuously produce AMPs such as the antimicrobial chemokine BRAK/CXCL14 and MK, which is expressed in the epidermal layer of the skin [47, 48, 75, 130, 176]. The skin is also colonized by bacteria of the normal microbiota thus preventing potential pathogens from getting access to the skin and they also produce bacteriocidins that can kill/inhibit neighbouring bacteria as well as pathogens. There is a fine-tuned balance between the microbiota and the constitutive host production of AMPs [125]. The anaerobic gram-positive coccus, *Finegoldia magna*, is found on the skin as part of the normal microbiota. *F. magna* is an opportunistic bacteria, which could give rise to wound infections and soft tissue infections [177]. It is found in the basal parts of the epidermal cell layer where it binds to the basal membrane through the surface-associated *F. magna* adhesion factor (FAF). It also expresses a protease, subtilase of *F. magna* (SufA) and both SufA and FAF could be released to the environment, where they cleave AMPs and thereby inactivate them or neutralize their action [69, 70, 178].

The gram-positive *Streptococcus pyogenes* is a virulent pathogen causing both superficial and deep infections (pharyngitis, necrotizing fasciitis and septic shock). *S. pyogenes* release the cysteine-protease SpeB that degrade AMPs and other host defence molecules. In addition, *S. pyogenes* release protein SIC, which inhibits complement activation and also inactivate AMPs. These proteins facilitate the colonization and infection of the bacteria by avoiding recognition and inhibition by the immune system [68, 179, 180]. Since MK and BRAK are found at the same locations as these bacteria, in paper II, we wanted to investigate the effect of commensal bacteria and pathogenic bacteria on the antibacterial activity of MK and BRAK.

Using immunohistochemistry, MK and BRAK were located in the epidermis of healthy skin. This was compared with biopsies from a wound edge, four days post-injury, where MK showed a more intense and broad expression but the expression of BRAK was diminished. A cDNA microarray analysis confirmed that the MK-gene was upregulated (approximately 3-fold), while the BRAK-gene was downregulated (approximately 2-fold) in the wound-area. MK is likely to be upregulated during inflammation due to the NF-κB –responding element in its promotor region [109]. The subcellular location was determined using immunoelectron microscopy where bound specific MK- or BRAK-antibodies were detected with secondary antibodies conjugated with colloidal gold-particles and showed them both associated with the basal membrane and the plasma membrane of keratinocytes in the healthy skin. In the wound, only a weak expression of BRAK was seen.

To assess whether MK and BRAK had different antibacterial activity against the commensal *F. magna* and the pathogenic *S. pyogenes* a viable count was conducted. MK displayed high antibacterial activity and were more potent in killing *S. pyogenes* while BRAK had an overall lower activity but was more potent against *F. magna*. As a comparison, hBD-2 and hBD-3 was used, and especially hBD-3 showed high antibacterial activity against both bacterial species.

To investigate the action of bacterial proteases (SpeB from *S. pyogenes* and SufA from *F. magna*), the proteases were incubated together with MK, BRAK, hBD-2 or hBD-3 and the fragments were visualised with SDS-PAGE. SpeB rapidly cleaved all polypeptides almost completely. SufA degraded MK to smaller fragment whereof two were stable over time, and completely degraded BRAK, but no degradation was seen with hBD-2 or hBD-3. The antibacterial activity of the fragments generated by SufA after 1 hour incubation were still able to kill *S. pyogenes* but their activity against *F. magna* was significantly reduced. Electron microscopy revealed membrane protrusions and leakage of intracellular content in the presence of the full-length polypeptides but to a lesser extent for *F. magna* when incubated with the SufA-generated fragments.

F. magna expresses FAF on the surface, and *S. pyogenes* express protein SIC, of which both can be released to the surroundings. Both FAF and SIC could inhibit the antibacterial activity of MK and BRAK, but FAF was more efficient. Electron microscopy with skin biopsies *ex vivo* infested with *F. magna* and biopsies from *S. pyogenes* infected skin showed a co-localization of MK and BRAK with all proteins (SpeB, SufA, FAF and SIC) on the surface of the bacteria.

SufA seems to be more specific than SpeB and could provide a shielded habitat in the skin for the commensal *F. magna* during healthy conditions by degrading the AMPs constitutively produced, but leaving them viable to affect pathogenic

bacteria. During inflammation, SufA has no effect on the induced AMPs (hBD-2 and hBD-3).

SpeB, produced by the pathogenic *S. pyogenes* on the other hand, is more un-specific and can cleave many AMPs, thereby enabling the infestation of *S. pyogenes* in the host. FAF and SIC enables the bacteria to inhibit AMPs at a distance.

The concentrations of MK and BRAK in the biopsies were determined by counting the gold-probe density /μm^2 for the specific antibodies and comparing with normal IgG antibodies [181-184]. MK is increased during inflammation and the concentrations are antibacterial already in healthy skin, while the level of BRAK is reduced during inflammation.

Table 1. Concentrations of MK and BRAK in skin. The density of gold probes in biopsies from healthy skin, skin *ex vivo* infected with *F. magna* and skin infected with *S. pyogenes* was quantified and from that, concentrations of MK and BRAK were calculated. Table modified from Frick et al, 2011 [86]

Protein (µM)	Healthy skin	*F. magna* infected skin (*ex vivo*)	*S.pyogenes* infected skin
MK	1.2	3.2	2.9
BRAK/CXCL14	9.2	0.7	0.6

In conclusion, MK and BRAK are expressed in skin during healthy conditions. During inflammation the expression of MK is increased while the expression of BRAK is decreased. MK and BRAK, as well as hBD-2 and hBD-3, show antibacterial activity against both bacteria. The commensal *F. magna* produce SufA and FAF that cleave and inhibit the action of MK and BRAK but have a smaller effect on the induced AMPs during inflammation. Thus enabling *F. magna* to protect a niche from constitutively produced AMPs during normal conditions. The pathogenic *S. pyogenes* produce SpeB and SIC which cleave and destroy the AMPs more efficiently and thus enables the bacteria to counteract the innate immune system to some extent.

Paper III

The epithelium-produced growth factor Midkine has fungicidal properties

Candida spp. are common in the normal microbiota of the skin and mucosal surfaces, but can also cause both superficial and invasive infections. *Candida albicans* and *Candida parapsilosis* are among the most common species found in humans. The plasma membrane of fungi differs from membranes of human cells in the content of sterols, where fungi contain ergosterol and human cells contain

cholesterol [61, 185, 186]. Since MK has been found constitutively expressed in skin [86, 130] and showed antibacterial activity, the aim of paper III was to examine the possible antifungal activity of MK, if it co-localizes with fungi and how it bind and recognize fungal membranes.

Using polyclonal MK-antibodies in immunohistochemistry, MK was detected in the epidermal layer of biopsies both from healthy controls and from patients with fungal dermatitis. MK was also expressed in the outermost stratum corneum, at the same location as the fungal infection. *In vitro*, in a viable count assay, MK showed antifungal activity against *C. albicans* and to a less extent against *C. parapsilosis*, but was more efficient than LL-37 in both cases. At sodium chloride concentrations corresponding to those found in skin, the activity was almost unaffected. As the sodium chloride concentrations were increased, the activity declined. The initial binding of MK is probably affected because the positively charged ions of the salt disturb the electrostatic interactions between the cationic MK and the negatively charged fungal membrane.

To evaluate if MK affect the fungal membranes, scanning electron microscopy of *C. albicans* incubated with MK or in buffer alone was performed. The result showed partial membrane disruption, blebbing and protrusions, some fungi had a rugged appearance but some looked intact even though the parallel viable count showed that all fungi were killed (97 and 99% respectively). The fungi could be stabilized by their thick outer envelope [187], looking intact although the plasma membranes integrity is destroyed. To verify the membrane disruption, immunoelectron microscopy was performed, where *C. albicans* were incubated with MK or in buffer. The redundant MK was washed away and the pellets of fungi was embedded in epon, incubated with polyclonal MK-antibodies and then a gold-conjugated secondary antibody. MK was located on the fungal plasma membrane but also found intracellularly, suggesting that MK could have intracellular targets as well as affecting the membrane.

To validate that MK affect the fungal plasma membranes and to see how MK affect membranes of human cells, a liposome leakage induction assay was performed. Liposomes containing ergosterol and cholesterol respectively where filled with fluorescent dye and incubated with MK at different concentrations. MK disrupted the ergosterol-containing liposomes and did so to a much higher extent than the liposomes containing cholesterol.

Taken together, MK is antifungal and membrane-disruption seems to be important for the antifungal activity, but MK are also found inside the fungi and could have intracellular targets. Cholesterol stabilizes the host cell membranes and MK preferentially disrupts fungal membranes rather than host cells.

Paper IV

Midkine is part of the antibacterial activity released at the surface of differentiated bronchial epithelial cells

The airways use many different strategies to keep us healthy, among these they present an anatomical barrier of epithelial cells. The mucociliary clearance is another, with mucus that traps pathogens, and cilia moving the air surface liquid to the trachea, which is then swallowed and ends up the stomach. Surfactant proteins are also produced, which bind to microbes, opsonize them and thereby promote the killing of the microbes by phagocytic cells. AMPs are produced constitutively and resides in the periciliary liquid (a part of the air surface liquid) covering the epithelial cells, but they are also induced by infection [188, 189].

Streptococcus pneumoniae are very common airway bacteria and normally resides in the upper respiratory tract, but are also the most common cause of pneumonia and can cause sepsis. Pneumococcal sepsis is the cause of 1,2 million infant deaths per year in developing countries. *S. pneumoniae* is a gram-positive bacteria with 91 different capsular serotypes known, with somewhat different structures [190].

Since retinoic acid has been shown to be important for the differentiation and maintenance of the airway epithelia [172] and MK is upregulated by retinoic acid [101], our aim in paper IV, was to examine the expression of MK in the airways and further examine the antibacterial activity of MK against airway pathogens. We also wanted to investigate if MK is produced by airway epithelia and could be found in ASL *in vitro*.

In order to see if MK is expressed in airways, *in situ* hybridisation and immunohistochemistry (IHC) of lung biopsies from healthy donors where used. The *in situ* hybridisation showed MK gene expression in the columnar epithelial cells of the bronchi and the IHC showed the same expression pattern of the MK protein. Both techniques also localized expression of MK (gene and protein) in some sub-epithelial cells. In the alveoli MK was detected in type 2 pneumocytes, which produce surfactant proteins, and also co-localised with surfactant protein A (SP-A) as showed with fluorescent double staining of MK and SP-A.

To further investigate where MK is located, immunoelectron microscopy was used, where MK-specific antibodies were visualized with secondary antibodies conjugated with gold-particles. The result confirmed the IHC and *in situ*, and detected MK in many different sections associated with the bronchial epithelium, both in the lumen of the airways on cilia, the mucus and the cell surface. MK was also detected between the cells and basolaterally at the basal membrane. In the alveoli MK was found on the surface of pneumocytes and in granules.

Next, we investigated the antibacterial activity of MK in a viable count assay, against two serotypes of *S. pneumoniae* and MK showed high activity against both strains and retained most of the activity in the presence of sodium chloride. Bacteria were incubated with MK or buffer alone and the morphology was investigated with scanning electron microscopy. The bacteria incubated with MK showed a rugged appearance and blebbing of the membrane as a result of lost membrane integrity. Further, the antibacterial activity of the different domains of MK was investigated and the C-terminal domain including the tail portion showed the highest activity although both domains had activity. The tail-portion of the C-terminal domain consists of many positively charged lysine residues, thereby increasing the net charge, which is important during the initial attraction to the bacteria.

To further look at the role MK might play in the host defence of the airways we investigated the airway surface liquid *in vitro* using an air-liquid interphase (ALI) cell culture model. Primary bronchial epithelial cells were cultured on transmembrane inserts and exposed to air on the apical side. The medium was enriched with retinoic acid, which will make the cells differentiate to columnar epithelial cells of which some will become ciliated and others will become secretary cells producing mucins. The cells produce an ASL, which was collected. The mucus was removed from the rinsing fluid and MK was removed from one part by immunoprecipitation, where the fluid was incubated with MK-antibodies that bind MK, and then the antibodies were removed with Protein-G sepharose. The rinsing fluid without MK was then used in a viable count assay against *S. pneumoniae* and compared with rinsing fluid incubated with control antibodies. The antibacterial activity was significantly lower in the sample where MK had been removed, suggesting MK constitute a significant part of the antibacterial activity of periciliary liquid (as part of the air surface liquid) *in vitro*. The rinsing fluid resulted in blebbing of the bacterial membrane showed with electron microscopy. Assuming that the ASL is 20 µm thick, the MK concentrations found in the rinsing fluid would be approximately 0.7 µM, which is a concentration high enough to be antibacterial.

In conclusion, MK is expressed on many levels in the airways, especially in association with the bronchial epithelial cells but also in the alveoli. MK display an antibacterial activity against *S. pneumoniae*, and the C-terminal half, including the tail, possess the highest activity. MK is found in rinsing fluid resembling the air surface liquid, *in vitro*, where MK constitute a significant part of the antibacterial activity.

Paper V

High expression of Midkine in the airways of patients with cystic fibrosis

In cystic fibrosis (CF) the airway immune defence is compromised and patients suffer from thick mucus and chronic infections of bacteria, especially *Pseudomonas aeruginosa*. The origin of CF is mutations of the *CFTR*-gene giving rise to an abnormal transport of electrolytes across epithelial cells mainly in the airways. Different hypothesis have been made as to what causes the initial defect in innate immunity; increased salt concentrations or reduced pH inhibiting the effects of AMPs or reduced depth of the air surface liquid and/or defect composition of the mucus leading to diminished mucociliary clearance, obstruction of the airways and inflammation. *Ps. aeruginosa* produce elastases, which damage the epithelium and the chronic infection leads to an accumulation of immune effector cells, such as neutrophils, further causing damage by producing proteases [152, 162].

Finally, in paper V, we wanted to investigate how MK is expressed in the airways of patients with CF and how the antibacterial activity is affected by salt, pH and proteolysis of elastases from neutrophils and *Ps. aeruginosa*.

Using *in situ* hybridization and immunohistochemistry, the expression of MK (gene- and protein expression respectively) were analysed in lung tissue from transplanted lungs of patients with end-stage CF. The results showed strong MK expression in both the large and small airways and a smaller expression in the alveoli. Double staining with fluorescent dye showed co-localization of MK and surfactant-protein A in the alveoli, and the double staining also revealed the expression of MK in neutrophils and mast cells.

MK was incubated together with different strains of *Ps. aeruginosa* and showed a dose-dependent antibacterial activity, the activity was high against the laboratory strain (PA01) and one of the clinical isolates (032) and the other two clinical isolates (335 and 022A) needed a higher concentration of MK. Bacteria incubated with MK was visualised using scanning electron microscopy and negative staining and showed membrane protrusions and leakage of intracellular content, further confirmed by flow cytometry where MK caused disruptions of the bacterial membrane, seen by the internalization of propidium iodide (PI).

The addition of salt in the viable count assay and a decrease in pH caused the antibacterial activity to drop. The net charge of MK was calculated at different pH values and sodium chloride concentrations. The calculations showed that the net charge of MK is quite constant in the pH-range used in the viable count experiments, suggesting that the decreased antibacterial activity is due to changes of the net charge on bacterial membranes and electrostatic shielding by the added salt ions.

MK was found in sputum from CF patients (0.7 nM) and to further characterize MK and comparing it to healthy controls (induced sputum) a western blot was made. Faint bands of the full-length protein were seen both in the control and the sputum from CF patients. The sputum from CF patients also showed smaller fragments of MK, which was not seen in the control, indicating that MK is degraded in the sputum. Two likely candidates that can degrade MK are elastases from neutrophils and *Ps. aeruginosa*, which are present in the CF airways. MK was incubated with these elastases and visualized on a SDS-PAGE. Neutrophil elastases degraded MK and showed three fragments after one hour incubation, but after 18 hours only one faint band was seen. With elastase from *Ps. aeruginosa*, most of the full-length protein was intact after one hour and after 18 hours most MK was degraded to smaller fragments. The antibacterial activity was investigated in a viable count showing that the degradation of MK with neutrophil elastase resulted in substantial decrease of the activity and the 18-hour incubation had no activity at all. MK incubated for 1 hour with *Ps. aeruginosa* elastase showed no decrease in activity, but after 18 hours incubation the activity was diminished. These results correlate well with the western blot.

In conclusion, we showed that MK is expressed in the airways of CF patients both in the large and the small airways. The antibacterial activity is compromised because of the changed microenvironment in the diseased lungs where both increased salt and low pH decreased the antibacterial activity of MK *in vitro*. In addition, elastases from neutrophils and *Ps. aeruginosa* degraded MK completely or to some extent respectively.

Conclusions of the thesis

- Midkine (MK) is antibacterial against both Gram-positive and Gram-negative bacteria and exerts the activity through membrane disruption resulting in leakage of intracellular contents. The effect of physiological salt concentrations is minor, which is a quite unique feature of AMPs. Orthologues of MK are found in many species, but seems to originate in insects, and the antibacterial activity is conserved during evolution.

- MK is constitutively expressed in healthy skin and the expression is increased during inflammation. The commensal *Finegoldia magna* produces proteases that cleave and inhibit the action of MK thus possibly creating a niche for itself in the microenvironment of the skin. In contrast, the pathogen *Streptococcus pyogenes* produces proteases that cleave and destroy the action of many different AMPs, including MK, promoting the invasive infections characteristic of this bacterium.

- MK has antifungal activity against *Candida* spp. The activity is exerted through membrane disruption but MK is also found inside the fungi, suggesting intracellular targets as well. The host membranes are stabilized by cholesterol and MK preferentially disrupts ergosterol-containing membranes that are characteristic of fungi.

- In the airways, MK is expressed in association with the bronchial epithelial cells but is also found in alveoli. MK is antibacterial against common airway pathogens and the C-terminal part exerts the highest activity. *In vitro*, MK is released into the periciliary liquid and constitutes a significant part of its antibacterial activity.

- MK is expressed in both large and small airways of patients with cystic fibrosis. The antibacterial activity of MK is altered due to changes in the diseased lungs, where increased salt, pH and increased release of proteases from bacteria and neutrophils may decrease the activity *in vitro*.

Populärvetenskaplig sammanfattning

Vi utsätts varje dag för en stor mängd bakterier som skulle kunna göra oss sjuka, men för det mesta håller vi oss ändå friska. Epitelcellerna på hudens yta och på lungornas slemhinna bildar det första försvaret mot sjukdomsframkallande bakterier, dels genom att de utgör en fysisk barriär men också genom att de kan producera små bakteriedödande substanser, så kallade antimikrobiella polypeptider (AMPs). En del AMPs produceras kontinuerligt och utgör ett färdigt skydd medan andra snabbt börjar produceras och frisätts när epitelceller kommer i kontakt med bakterier eller då en skada uppstår. Den bakteriedödande effekten är snabb och bakterierna hinner ofta dödas innan det långsammare specifika immunförsvaret, med bland annat produktion av antikroppar, hunnit bli aktiverat.

Midkine (MK) är en tillväxtstimulerande molekyl som bland annat har betydelse för anläggningen av kroppens organ under fosterstadiet. Den har också påvisats i huden och har flera olika funktioner i kroppen hos den vuxna individen. Om man studerar MK-molekylen i detalj består den av två veckade regioner som hålls på plats av överbryggande svavelatomer. I detta avseende liknar MK människans β-defensiner vilka är väl karakteriserade AMPs.

I denna avhandling har vi kunnat visa att MK har en avdödande aktivitet mot många olika typer av bakterier och svampar samt att dessa dör genom att MK förstör deras ytterhölje (membran). Membranen på våra celler skiljer sig från membranen som omger bakterier och svampar. AMPs attraheras till bakteriers membran eftersom dessa är mera negativt laddade jämfört med våra. Våra cellers membran är också stabiliserade eftersom de innehåller kolesterol medan svampar innehåller den liknande molekylen ergosterol. Detta gör det möjligt för AMPs att döda bakterier och svampar utan att påverka våra celler.

MK finns i många olika arter, exempelvis hos möss, fiskar och grodor. MK har också hittats i insekter där den verkar ha sitt ursprung och den bakteriedödande aktiviteten är bevarad hos samtliga av dessa arter. Vi har visat att MK finns i frisk hud och vid inflammation ökar produktionen. Vissa bakterier som normalt förekommer på vår hud inaktiverar delvis MK genom att producera ämnen som klipper sönder det (proteaser). Sjukdomsframkallande bakterier har effektivare proteaser som helt och hållet förstör aktiviteten hos många AMPs, inklusive MK.

I friska lungor skyddar lagret av epitelceller oss från att bli sjuka och AMPs ligger som ett kemiskt skyddande lager i ett tunt vätskelager ovanpå och mellan cellerna. Vi har visat att MK finns i lungorna och fungerar bakteriedödande mot de bakterier

som är den vanligaste orsaken till lunginflammation (så kallade pneumokocker). I en cellodlings-modell där cellerna har kontakt med luft på ovansidan och får samma egenskaper som cellerna i lungorna bildas ett tunt vätskelager. Denna vätska är bakteriedödande och vi har visat att MK står för en stor del av denna aktivitet.

Cystisk fibros är en genetisk sjukdom som ger upphov till tjockt slem och kronisk infektion med bakterier i lungorna. Olika teorier finns om varför dessa patienter har så svåra bakteriella infektioner som immunsystemet inte klarar av att få bukt med. Teorierna är att salthalten är förhöjd eller att pH är för lågt i vätskan som täcker epitelcellerna i lungorna. Andra bidragande faktorer kan vara att bakterierna och det stora inflödet av immunceller resulterar i höga koncentrationer av proteaser som bryter ner AMPs som därmed inte kan döda bakterierna lika bra. Vi har undersökt lungvävnad från patienter med cystisk fibros (som genomgått lungtransplantation) och visat att produktionen av MK är förhöjd i deras lungor. Aktiviteten hos MK är sannolikt förändrad eftersom både hög salthalt, lågt pH och proteaser minskar den bakteriedödande aktiviteten.

Sammanfattningsvis finns MK i huden och i lungorna, ytor som är kontinuerligt exponerade för bakterier och svampar vilka kan skapa infektioner. MK är effektiv på att döda både olika sorters bakterier och svampar. Vid kroppens svar på infektion (inflammation) ökar produktionen men vid vissa sjukdomstillstånd verkar MK delvis ha förlorat sin aktivitet på grund av ändrade förhållanden i vävnaden. Att återställa aktiviteten för MK vid dessa tillstånd skulle kunna vara en ny behandling vid svåra infektioner.

Bakteriedödande kroppsegna peptider, som MK, kan utgöra en bas för utveckling av nya strategier för behandling av infektioner exempelvis genom att applicera dessa på infekterad hud. En annan strategi skulle kunna vara att stimulera en ökad produktion så att kroppen själv på ett effektivare sätt kan bekämpa infektionen. Nya strategier mot infektioner är viktiga då den bakteriella motståndskraften mot konventionella antibiotika snabbt ökar.

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