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Themed Section: Midkine

REVIEW

Midkine in host defence

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Midkine (MK) shares several features in common with antibacterial proteins of the innate immune system. These include growth factor properties, heparin-binding regions and effects on immune cells, such as recruitment and activation of neutrophils and macrophages. Indeed, recent research has demonstrated potent bactericidal and fungicidal activities of MK. This protein is constitutively expressed at relevant concentrations at barriers of the body, such as the skin and the large airways, where the body first encounters potential pathogens. The antibacterial properties of MK orthologues are preserved during evolution, as exemplified by *miple2* of *Drosophila*. In addition to retinoic acid, promoters of MK gene expression include factors present at sites of infection, reactive oxygen species, activation of the transcription factor NF-κB and hypoxia. In the light of the development of resistance in pathogenic bacteria to conventional antibiotics, MK is an interesting molecule that could serve as a template in developing novel therapeutic strategies against bacterial and fungal infections, either alone or in combination with conventional antibiotics.

LINKED ARTICLES
This article is part of a themed section on Midkine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-4

Abbreviations
ASL, airway surface liquid; BPI, bactericidal/permeability increasing protein; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; FAF, *Finegoldia magna* adhesion factor; HIF, hypoxia-inducible factor; RA, retinoic acid; ROS, reactive oxygen species; SufA, subtilase of *F. magna*; TA, teichoic acid; TLR, Toll-like receptor

Innate immunity

Virtually, all organisms have means to counteract bacterial infection. In invertebrates and plants, there is a less specific, readily available innate immune system that keeps the organism in a healthy state. With the appearance of bony fishes (*Osteichthyes*) during evolution, the adaptive immune system emerged, having high specificity (through the production of specific Igs) and a memory (memory B cells), best perhaps exemplified by vaccination. In humans, the immune system is therefore often divided into two parts, that is, the innate and the adaptive systems. Antibacterial proteins are an important arm of innate immunity, where other parts in humans include barriers, mucus, cells, such as macrophages and dendritic cells, sensing pathogen-associated molecular patterns, such as bacterial LPS and peptidoglycan via pathogen-recognition receptors, such as the Toll-like receptors (TLRs; receptor nomenclature follows Alexander *et al.*, 2013), effector cells (NK cells and granulocytes), complement, and the production of reactive oxygen species (ROS).

Sir Alexander Fleming was the first to describe an antibacterial protein that he named lysozyme (Fleming, 1922). This molecule is a muramidase that degrades peptidoglycans of the bacterial cell wall and is present at high concentration in secretions (tears, saliva, human milk and mucus) and preformed in cytoplasmic granules of neutrophils. Later, Hirsch showed that histones have antibacterial activity, and he also characterized bactericidal proteins present in neutrophils and described how these were released into the microbe-containing phagocytic vacuoles (Hirsch, 1958; Cohn and Hirsch, 1960). In 1975, Weiss and Elsbach isolated bactericidal/permeability increasing protein (BPI), an antibacterial protein present in azurophilic granules of neutrophils (Weiss *et al.*, 1975, 1978). Other important discoveries in the field of antibacterial proteins were the characterization of cecropins isolated from the haemolymph of the cecropia

moth larvae (*Hyalophora cecropia*), the magainins from the skin of the African clawed frog (*Xenopus laevis*), and the defensins present in azurophil granules of human neutrophil granules and expressed by epithelial cells (Steiner *et al.*, 1981; Zasloff, 1987; Lehrer *et al.*, 1991).

Antibacterial proteins show a high degree of redundancy and it is therefore difficult to rule out any one single molecule as being critically important for innate immunity. More than 2200 molecules derived from plants, bacteria, animals and men are currently listed as antimicrobial at the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) (Wang and Wang, 2004). However, their important roles in host defence have been exemplified in animal models of infectious disease (Nizet *et al.*, 2001). In humans, Kostmann's syndrome is a disease where affected individuals suffer from severe periodontitis. In the disease, there is an inherited deficiency in the antibacterial cathelicidin LL-37 and reduced concentrations of the α-defensins (human neutrophil proteins 1–3) (Pütsep *et al.*, 2002).

Antibacterial polypeptides

Antibacterial proteins, with few exceptions, are small (4–12 kD), with an overall positive net charge (being cationic) at physiological pH (Brogden, 2005). However, it is a very diverse group of molecules that cannot only be classified solely based on their amino acid sequence. One example is BPI with a size of 58 kD, consisting of one cationic and one anionic part (Weiss *et al.*, 1978). The molecules with antibacterial activities also include a small group of anionic peptides, containing glutamic and aspartic acid, linear cationic α-helical peptides with a hinge region that will adopt a α-helical structure in membranes, cationic peptides rich in a specific amino acid such as proline or arginine, and cationic peptides with conserved cysteine residues that will form disulphide bonds and stable β-sheets (Boman, 2003; Brogden, 2005). The last group includes the human α- and β-defensins and several chemokines with antibacterial properties, such as CXCL9, CCL20, and CCL28 (Cole *et al.*, 2001; Hoover *et al.*, 2002; Hieshima *et al.*, 2003). The protein midkine (MK), having two domains consisting of antiparallel β-sheets, also belongs to this group of antibacterial proteins.

Phylogenetic aspects of the bactericidal activity of MK

Orthologues of MK are found in many species, from insects to humans, although it is not found in the *Caenorhabditis elegans* genome, suggesting its origin among insects (Englund *et al.*, 2006). In *Drosophila*, MK and the closely related protein pleiotrophin exist as two genes, that is, *miple1* and *miple2*. These genes are expressed in restricted, non-overlapping patterns; *miple1* being expressed in the developing nervous system, whereas *miple2* is expressed during development of gut endoderm (Englund *et al.*, 2006). Using recombinant *miple2*, we found strong bactericidal activity (ED_{50} approximately 0.5 μM) against *Escherichia coli* but no activity against *Staphylococcus aureus* (Svensson *et al.*, 2010). Whether *miple1* and *miple2* indeed contribute to host defence in *Drosophila* remains to be investigated. Using peptide mapping, MK of amphibians (African clawed frog, *Xenopus laevis*) and fish (zebrafish, *Danio rerio*) all showed bactericidal activity in an order comparable to that seen in the corresponding regions of human MK, suggesting preserved antibacterial activity of MK orthologues during evolution (Svensson *et al.*, 2010). However, one should bear in mind that, on encountering bacteria, short peptide fragments are likely to behave differently from the larger protein with a complex three-dimensional structure, from which they are derived.

MK gene regulation in an inflammatory context

When first described, MK gene expression was shown to be dependent on the vitamin A derivative retinoic acid (RA) (Muramatsu, 2002), known to bind to several nuclear receptors, including RA receptors, retinoid X receptors and PPARβ and δ (Hall *et al.*, 2011). In addition to roles in organogenesis and differentiation, vision and metabolism, RA affects several aspects of immunity (Hall *et al.*, 2011). Several factors present during inflammation influence the generation of RA from vitamin A, for example, activation of TLR2, IL-4 and GM-CSF, which all promote RA synthesis, whereas PGE_2 inhibits RA synthesis (Manicassamy *et al.*, 2009; Yokota *et al.*, 2009). Thus, the expression of MK could be regulated at a transcriptional level through the altered levels of RA, available during inflammation.

NF-κB is a transcription factor that has key roles in activating cells, leading to the production of cytokines and a plethora of genes associated with both innate and adaptive immunity (Li and Verma, 2002; Ghosh and Hayden, 2008). Important inducers of NF-κB activity include ROS, TNF-α, IL-1β and LPS, the latter from the outer membrane of Gram-negative bacteria. In prostate cancer cells, TNF-α and IL-1β both induced expression of MK via the NF-κB pathway, suggesting that this could be a pathway activating MK expression in other cells as well (You *et al.*, 2008).

ROS, for example, superoxide, hydrogen peroxidase or hypochlorite, generated by the activation of membrane-bound oxidase systems present in granulocytes and epithelial cells, are an important part of innate immunity. ROS can activate the NF-κB pathway, and in an animal model, ROS was likely to be involved in MK expression (Hobo *et al.*, 2009). In this model, partial nephrectomy of mice caused expression of the ROS-generating NADH/NADPH oxidase-1, -2 and -4 via the renin–angiotensin system. In addition, the antioxidative reagent tempol reduced MK expression (Hobo *et al.*, 2009).

Infected tissues provide a hypoxic environment where the hypoxia-inducible factor (HIF) is an important transcriptional regulator of cellular stress responses caused by oxygen deprivation. HIF promotes the antibacterial functions of immune cells in response to microbial pathogens (Nizet and Johnson, 2009). In a mouse model, using hypoxia-susceptible CAST/EiJ mice, MK expression was induced in the airways by hypoxia (Reynolds *et al.*, 2004). Studies of MK expression, including site-directed mutagenesis, revealed that HIF-1α

enhanced MK transcription by acting on HIF-1α regulatory elements located in the MK gene promoter (Reynolds *et al.*, 2004). In another study, hypoxia increased MK expression in neutrophils, monocytes and endothelial cells (Weckbach *et al.*, 2012). MK also induces neovascularization, an activity absent in MK-deficient mice (Weckbach *et al.*, 2012). Taken together, the available evidence points to several factors present at sites of inflammation being able to enhance expression of MK, including RA, ROS, activators of the NF-κB pathway and hypoxia.

Bactericidal activity: mode of action

On a structural level, MK has two domains consisting of three antiparallel β-sheets, held together by a hinge region (Figure 1) (Iwasaki *et al.*, 1997). Other proteins with antibacterial properties are the β-defensins and the antibacterial chemokines, which share structural similarities with MK (Ganz, 2003; Yang *et al.*, 2003). Both antibacterial chemokines and β-defensins are cationic and have three antiparallel β-sheets that are stabilized by disulphide bonds between six cysteine residues, similar to the NH_2 terminal domain of MK. Additionally, the β-defensins have a short COOH terminal amphiphatic α-helix, containing antibacterial activity, which is also, to varying degrees, the case for antibacterial chemokines (Egesten *et al.*, 2007). In contrast, MK has an unordered COOH terminal with a high content of lysines that may attain an α-helical structure when inserted into a lipid bilayer, for example, the bacterial plasma membrane. Strong antibacterial activity of MK was mapped to the unordered COOH terminal tail and to the last β-sheet of the NH_2 terminal domain (Figure 1).

Another property that MK has in common with β-defensins and many antibacterial chemokines is that they oligomerize in solution, and in the case of MK, dimerization is promoted in the presence of transglutaminase (Iwasaki *et al.*, 1997; Kojima *et al.*, 1997). Oligomerization can provide a more efficient exposure of antibacterial residues to the target organism, as demonstrated for β-defensins (Hoover *et al.*, 2002). Another function could be to anchor oligomerized MK to glycosaminoglycans on the cell surface. This could be an economic way to retain an antibacterial gradient on the cell surface.

A common feature of antibacterial proteins is a heparin-binding motif (the Cardin–Weintraub motif) (Andersson *et al.*, 2004). Cardin and Weintraub (1989) proposed that the heparin-binding motifs were arranged in the pattern XBBBXXBX or XBBXBX (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids). Such consensus sites predict the arrangement of amino acids into either α-helices or β-strands. Additional consensus sequences, that is, XBBBXXBBBXXBBX and TXXBXXTBXXXTBB (where T defines a turn), were found in heparin-binding sites of growth factors (Sobel *et al.*, 1992; Hileman *et al.*, 1998). MK has Cardin–Weintraub motifs, and the heparin-binding properties of the molecule were identified early (Muramatsu, 2002). Most antibacterial proteins share

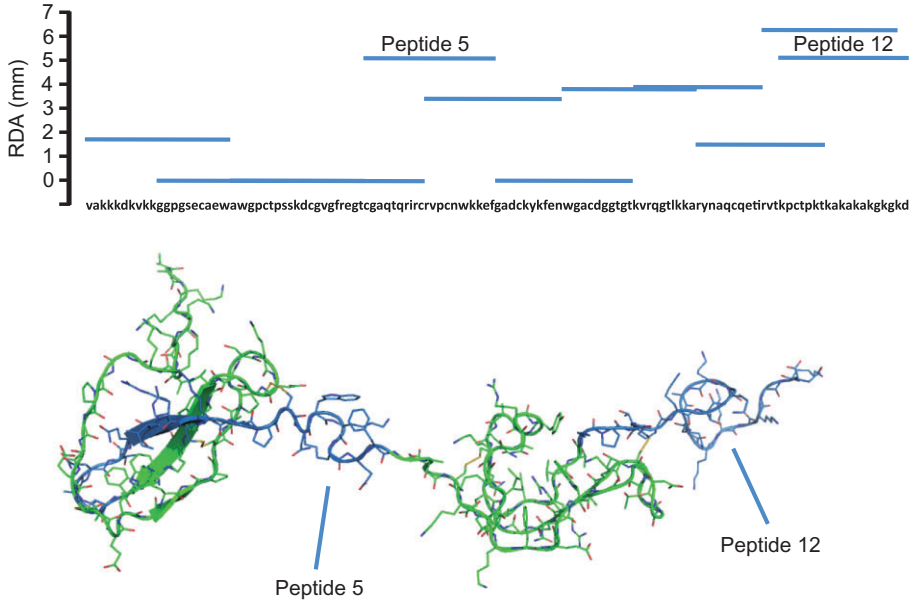

Figure 1

Distribution of bactericidal activity within the MK molecule. Using 20 amino acid long peptides and a radial diffusion assay (RDA) with *Escherichia coli*, the antibacterial activity was determined. In the assay, bacteria grow in solidified agar and peptides are added in wells that are punched out in the agar. After incubation, clearing zones around the wells can be measured, and the diameter corresponds to antibacterial activity. The primary sequence of MK is indicated and corresponding peptides are indicated (upper). The highest activities were recorded corresponding to peptide no. 5, associated with the NH_2 terminal domain and in the COOH terminal (e.g. peptide no. 12) as indicated (blue) in the model of MK (lower). (The figure is used with permission from Svensson *et al.*, 2010.)

the fundamental principle of arranging the amino acids in an amphipathic structure, where hydrophobic amino acids and cationic amino acids are intermingled so that the α-helix will have one side exposing hydrophobic amino acids and one side exposing cationic amino acids (Figure 2) (Zasloff, 2002). In the case of MK, mapping experiments suggested that the heparin-binding region of the molecules has a high antibacterial activity (Asai *et al.*, 1997; Svensson *et al.*, 2010). However, it should be noted that results obtained using peptides only 20 amino acids long peptides clearly cannot exactly reflect those obtained from the complete three-dimensional structure and activity of the holoprotein. Taken together, the positioning of cationic amino acid residues is likely to promote the membrane-disrupting properties of antibacterial proteins including MK.

The first step in the bacterial killing mediated by antibacterial proteins is the contact with the microbial membranes mediated by the electrostatic attraction between the positively charged antibacterial protein and the negatively charged outer membranes of bacteria, reflecting the negative phospholipid head groups including LPS on Gram-negative bacteria and teichoic acid (TA) on Gram-positive bacteria (Figure 2) (Brogden, 2005). Comparably, phosphomannans and other complex carbohydrates create a negatively charged surface on fungi (Chaffin *et al.*, 1998). In contrast, the outer membranes of eukaryotic cells are composed of lipids without net charge, and lipids with negative net charge are arranged in the membrane so that they face the cytoplasm (Zasloff, 2002). Once the antibacterial protein has gained access to the bacterial or fungal membrane, it will interact with the membrane and insert into the membrane, and either disrupt the membrane by organizing well-structured pores or disrupt the membrane in an un-ordered detergent-like fashion. This interaction is likely to be mediated by the amphipathic structure of the antibacterial proteins. Both ways will lead to leakage of intracellular contents and as a consequence the

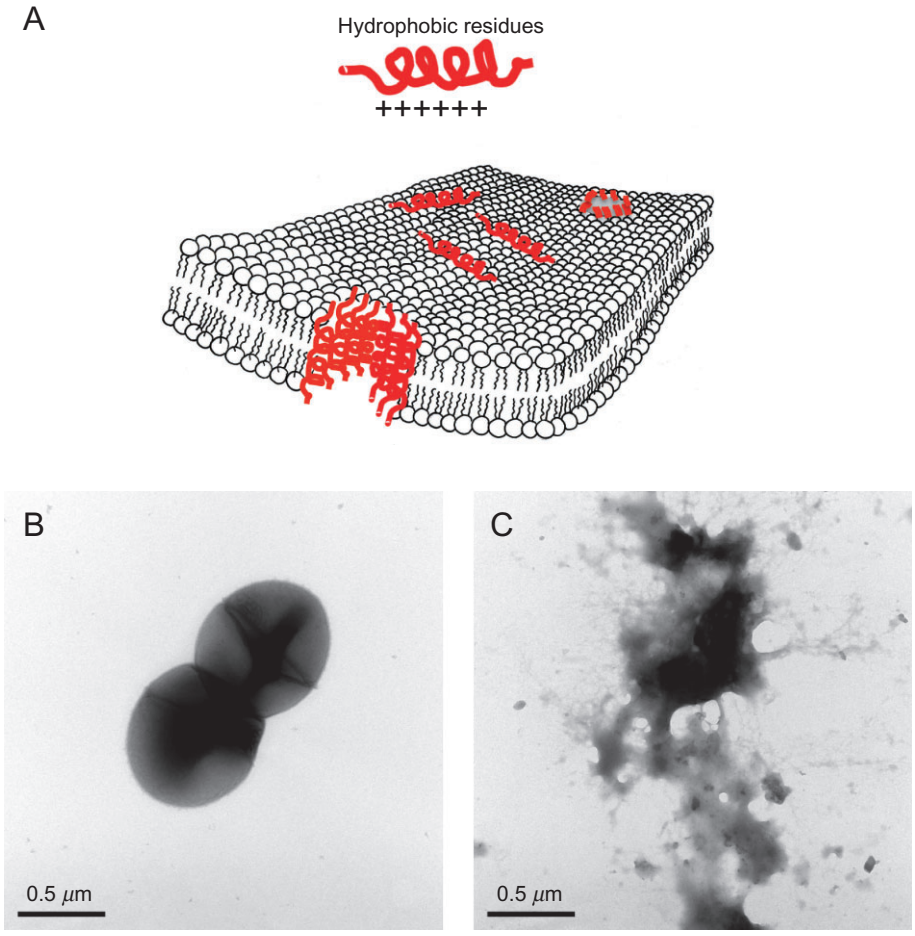

Figure 2

Possible mechanism involved in the membrane-disrupting activity of MK. (A) Many antibacterial proteins adapt an α-helical, amphiphatic structure that may also be the case for the COOH terminal tail of MK when inserted in the membrane. To obtain this structure, cationic and hydrophobic amino acid residues are intermingled so that upon helix formation, one side becomes hydrophobic and one cationic. Electrostatic forces attract the cationic antibacterial protein to the anionic plasma membrane of bacteria. Thereafter, the amphiphatic character of the antibacterial protein executes a detergent-like or pore-forming activity, resulting in loss of bacterial integrity (adapted from Brogden, 2005). (B, C) In the lower part of the figure, negative staining and transmission electron microscopy have been used to investigate bacteria (*Streptococcus pneumoniae*) incubated in buffer, showing intact bacteria (left) and disrupted bacteria after exposure to an antibacterial protein (right).

bacteria will die (Brogden, 2005). Although the bacterial membrane is thought to be the primary target, there are studies showing that antibacterial proteins have intracellular targets as well (Brogden, 2005). Antibacterial proteins can be translocated over the plasma membrane, into the cytoplasm where they can inhibit nucleic acid synthesis, protein synthesis and metabolic activities, thus amplifying their microbicidal activity (Cudic and Otvos, 2002).

Both Gram-positive (i.e. *Sta. aureus*, *Streptococcus pneumoniae* and *Str. pyogenes*) and Gram-negative (*Pseudomonas aeruginosa* and *E. coli*) bacterial species are highly susceptible to the bactericidal action of MK with typical ED_{50} values in the order of 0.3–0.5 μM (Svensson *et al.*, 2010; Frick *et al.*, 2011; Nordin *et al.*, 2013a). The Gram-negative bacteria, nontypeable *Haemophilus influenza,* is somewhat less sensitive, whereas *Burkholderia cepacia* was not affected at MK concentrations reaching 10–30 μM (S. L. Nordin, unpubl. obs.).

Several antibacterial proteins, for example, LL-37, bind and thereby neutralize the pro-inflammatory actions of LPS (Pulido *et al.*, 2012). LPS is bound in a complex with LPS-binding protein (LBP) together with CD14, which activates TLR4 resulting in activation of NF-κB. However, using LPS from *E. coli* and lipooligosaccharide from non-typeable *Ha. influenzae*, we have not been able to find such properties of MK (S. L. Nordin, unpubl. obs.).

Fungicidal activity of MK

The most common fungal pathogens include *Candida* spp., *Aspergillus* spp. and *Cryptococcus* spp. Fungi can cause both superficial and invasive diseases in humans, the latter mainly occurring in immunocompromised individuals including those with AIDS, during treatment with immunosuppressive agents and in states of disease with metastatic cancer. Some antibacterial proteins have antifungal properties, for example, angiogenin (RNAse 5 of the RNAse A family), the cathelicidin human cationic antimicrobial protein of 18 kD-derived peptide LL-37, the β-defensins, RNAse 8 and the complement fragment C3a (Harder *et al.*, 2001; Hooper *et al.*, 2003; Rudolph *et al.*, 2006; Schröder and Harder, 2006; Sonesson *et al.*, 2007). Most studies of antifungal activities of antibacterial proteins have been investigated *in vitro* using *Candida* spp as the test system. *Candida* has a complex cell wall consisting of a plasma membrane and a cell envelope constituted of β-glucan, chitin and mannoprotein, resulting in a surface with an overall negative charge (Shepherd, 1987). However, similar to the effect of antibacterial proteins in bacteria, a membrane-disrupting activity is also likely to be crucial for their fungicidal activity. As a consequence, antibacterial proteins would have to first saturate the negative charges of the cell wall or be subject to even stronger electrostatic and/or hydrophobic forces to reach and be inserted in the plasma membrane, executing their disrupting activity. Additional fungicidal mechanisms of MK are possible as has been demonstrated in the case of histatin 5 where the antifungal activity is dependent on internalization and inhibition of the respiratory chain in mitochondria (Pollock *et al.*, 1984; Helmerhorst *et al.*, 1999).

Why are eukaryotic cells protected against the membrane-disruptive properties of MK?

The cell surfaces of eukaryotic cells differ from that of prokaryotic cells. Both bacteria and fungi have cell walls composed of complex carbohydrates and lipids. The plasma membranes of eukaryotic cells and fungi contain sphingolipids and sterols, which bacteria lack. In the plasma membrane of yeast, the most abundant sterol is ergosterol, whereas eukaryotic cells contain cholesterol (Brogden, 2005). These differences make it possible for antibacterial proteins to differentiate between eukaryotic and prokaryotic cells, as eukaryotic cells have cholesterol-containing membranes that are more resistant to the disrupting activities of antibacterial proteins (Opekarová and Tanner, 2003) (Figure 3).

Effects of salt, pH and plasma on antibacterial actions

The antibacterial activity of many antibacterial proteins, for example, the human β-defensins, decreases in the presence of salt, a feature long believed to explain part of the impaired host defence in cystic fibrosis (CF) (Goldman *et al.*, 1997; Bals *et al.*, 1998; Guggino, 1999). In CF, mutations of the *CF transmembrane conductance regulator (CFTR)* result in impaired host defence functions of the airways and eventually acquisition

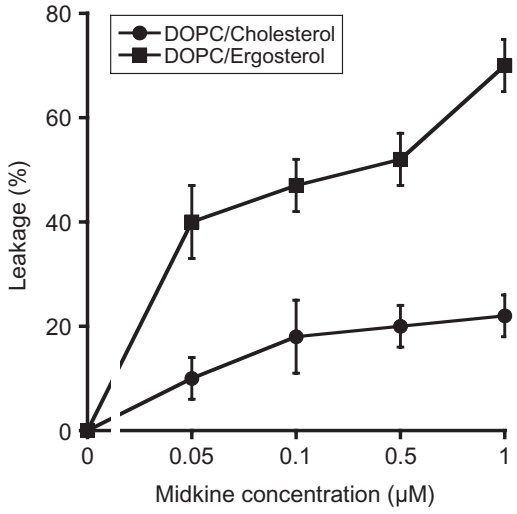

Figure 3

Cholesterol-containing lipid bilayers of eukaryotic cells are protected against the membrane-disrupting activity of MK. The lytic activity of MK was compared in an assay using micelles containing cholesterol (corresponding to eukaryotic plasma membranes) and ergosterol (corresponding to fungal plasma membranes). The lytic activity, reflected as leakage of a fluorescent dye, is higher in the case of ergosterol-containing membranes. The values represent mean (±SD) of three separate experiments. (The figure is used with permission from Nordin *et al.*, 2012.)

of chronic infection with *P. aeruginosa* (Smith *et al.*, 1996). Recently, it was shown that the antibacterial activity of lactoferrin and lysozyme, two major antibacterial proteins of airway surface liquid (ASL), the thin (approximately 5-μm-deep) liquid layer on airway epithelial surface, becomes reduced at lower pH, as found in ASL of patients with CF (Chen *et al.*, 2010; Pezzulo *et al.*, 2012). In the study by Pezzulo *et al.*, a porcine model of CF was investigated and the salt concentration of ASL was unaffected in *CFTR* –/– animals. In the case of MK, our results showed that the net charge of this molecule was mostly unaffected by pH values in the physiological range, but instead the charge on the bacterial membrane was neutralized due to protonation, thus weakening the disruptive properties of MK (Nordin *et al.*, 2013b). Because most antibacterial proteins kill bacteria by membrane disruption, it is likely that protonation of the bacterial membrane has a general, non-specific effect, impairing the antibacterial activity of most antibacterial proteins. Taken together, the effects of salt and pH are due to electrostatic screening and a charge neutralization of the membrane respectively.

Interestingly, we found that the antibacterial activity of MK was only slightly decreased in the presence of sodium chloride at physiological concentrations (NaCl at 140 mM) (Figure 4). However, shorter linear fragments of the holoprotein lost most of their antibacterial activity in the presence of salt, suggesting that the rigid structure held together by disulphide bonds contributed very significantly to the salt-resistant activity of the holoprotein (Svensson *et al.*, 2010).

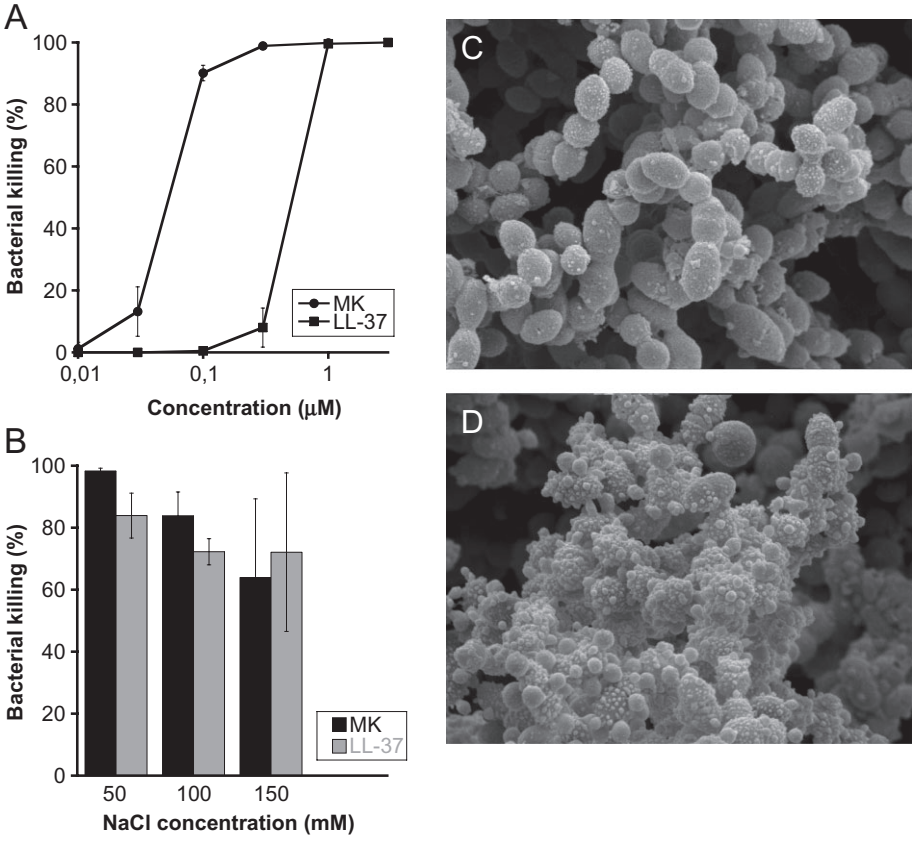

Figure 4

MK is bactericidal against *Streptococcus pneumoniae*. (A) Using a viable count assay, MK shows strong bactericidal activity against *Str. pneumoniae*, a Gram-positive bacterium that is the most common cause of community-acquired pneumonia. In the assay, bacteria were incubated with MK at the indicated concentrations for 60 min, plated, and the number of colonies counted and compared with the number of colonies after incubation in buffer alone. MK is almost 10-fold more potent than the 'classical' antibacterial peptide LL-37. The values represent mean (±SD) of three separate experiments. (B) MK retains most of its bactericidal activity in the presence of salt at physiological concentrations, that is, sodium chloride at 140 mM, as reflected by the viable counts assay described above. The values represent mean (±SD) of three separate experiments. (C, D) Using scanning electron microscopy, intact bacteria (*Str. pneumoniae*) (C) can be compared with bacteria that have been incubated with MK (D), the latter showing disturbed integrity and leakage of intracellular contents. (The figure is used with permission from Nordin *et al.*, 2012.)

Antiviral properties of MK

Interestingly, MK interferes with HIV-1 infection of cells

MK showed a dose-dependent inhibition of infection by T-lymphocyte and macrophage-tropic HIV-1 isolates (Callebaut *et al.*, 2001). The antiviral effect was not directed against the virus itself but rather interfered with binding of virus to the cell surface preceding its internalization. MK binds nucleolin at both high- and low-affinity sites, independent of heparan sulfate and chondroitin sulfate. After binding to cells, MK is internalized by an active process (Said *et al.*, 2002).

Relevant concentrations at the proper time and place to act as an "innate antibiotic"?

Even if many human proteins exert antibacterial properties, it is obvious that they have to be present at concentrations that can kill microbes at locations where the body is challenged by pathogens. The borders where the body encounters pathogens of the environment are lined with epithelial cells. Thus, to prevent or combat infection at an early stage, antibacterial proteins would have to be produced by epithelial cells or be available in close proximity. Another route for delivery is the recruitment of neutrophils containing large amounts of preformed antibacterial proteins that can be released at sites of inflammation (Borregaard, 2010).

Airways

Host defence functions of the airways include many components with different profiles at different levels. In the lungs, these include the mucociliary system of the bronchi, whereas in the bronchioles, mucus production is lost before the ciliated epithelium and the Clara cells appear, adding detoxifying and anti-oxidative functions. In the alveoli, alveolar macrophages and type 2 pneumocytes are important contributors to host defence, the latter producing the collectins surfactant protein A and D, in addition to surfactant. Recent work has demonstrated the important function in host defence of the thin (5 μm) periciliary liquid layer, that is, the ASL, covering the epithelial surface of epithelial cells in the bronchi and bronchioles (Chen *et al.*, 2010). The concentration and function of antimicrobial proteins is likely to be most important in the ASL where high and bactericidal concentrations could be reached, rather than in the mucin-rich sputum, the latter having a high content of anionic mucins and free DNA that are likely to impair the activity of antibacterial proteins. Determining the MK concentration in ASL, using an air liquid interface model with differentiated bronchial epithelial cells growing in contact with air, a MK concentration in the ASL of 0.7 μM could be calculated, which corresponds to a bactericidal level (Nordin *et al.*, 2012). Because RA is necessary to retain the integrity and function of the airways, the prerequisites for a constitutive expression of MK in the airways are available (Malpel *et al.*, 2000). MK indeed could be detected in induced sputum of healthy individuals by ELISA and Western blot (Nordin *et al.*, 2013a). MK expression was also detected in epithelial cells of normal lung tissue by *in situ* hybridization and immunohistochemistry (Nordin *et al.*, 2012, 2013a). However, in normal lung tissue, MK expression was only detected in bronchi of the large airways and in type 2 pneumocytes (Nordin *et al.*, 2012). In CF, a higher expression of MK was found, including in the epithelial cells of the small airways (Nordin *et al.*, 2012). In CF, several factors are present that may promote MK expression, including ROS, NF-κB activation and hypoxia (Reynolds *et al.*, 2004; You *et al.*, 2008; Hobo *et al.*, 2009). Interestingly, adult mice do not seem to have a constitutive expression of MK in the airways but do up-regulate this protein during hypoxia (Reynolds *et al.*, 2003). This may present problems when defining roles for MK in host defence using mouse models of human disease.

Skin

MK is constitutively expressed in human skin (Inazumi *et al.*, 1997). Using immunoelectron microscopy, we detected MK at several levels in skin from healthy donors, including association with the basal membrane (BM), at the nuclear membrane and in association with the plasma membrane (Frick *et al.*, 2011). Using a morphometric method, the concentration of MK was determined in the intercellular space. Skin was also infected with the commensal *Finegoldia magna*, and the pathogen *Str. pyogenes* (resembling erysipelas) *ex vivo*. MK concentrations of 1 μM (healthy skin) and around 3 μM (during infection) were found, corresponding to bactericidal concentrations (Frick *et al.*, 2011). The findings indeed suggest that MK could serve as an antibacterial protein in the skin.

Similar to MK, many innate antibiotics serve as growth factors

Many antibacterial proteins act as growth factors to promote tissue repair (e.g. promoting angiogenesis) and they can recruit and activate leukocytes. This pluripotency is characteristic of important antibacterial proteins such as the cathelicidins, the antibacterial chemokines and the defensins (Elsbach, 2003; Lai and Gallo, 2009). MK fits well into this picture, having a broad range of growth factor properties (Stoica *et al.*, 2002).

Pro-inflammatory properties of MK

In addition to growth factor properties, many antibacterial proteins promote chemotaxis and activation of leukocytes. The human cathelicidin LL-37 activates neutrophils, monocytes and T-cells by binding and activating the receptor formyl peptide receptor 2 (De Yang *et al.*, 2000). Furthermore, the chemokine CCL20, which binds and activates the

chemokine receptor CCR6, has antibacterial activity (Hoover *et al.*, 2002). The β-defensins 1 and 2 also bind to and activate CCR6. With time, antibacterial activity has proven to be a common theme among molecules with chemotactic properties. Chemokines comprise a large family of polypeptides that are key players in inflammation by regulating leukocyte trafficking and activation. They are divided into four groups, XC, CC, CXC and CX_3C chemokines, depending on the presence of conserved cysteine residues in their amino terminal region, providing a structure containing three antiparallel β-sheets. Several chemokines possess antibacterial properties, which are combined with the chemotactic properties and additional actions as growth factors (Yang *et al.*, 2003). Similarly, MK induces chemotaxis of human neutrophils and triggers mobilization of intracellular calcium in these cells (Takada *et al.*, 1997). The chemotactic activity of MK against neutrophils was confirmed in another study where it showed inflammation-dependent expression during synovitis. The mode of action of MK was found to be haptotactic; the substrate-bound form of MK was the active form (Takada *et al.*, 1997). In a mouse model of rheumatoid arthritis, MK knockout mice seldom developed the disease, whereas most wild-type mice did. In addition, MK has chemotactic activity against macrophages, an activity that plays roles in the formation of neointima (Horiba *et al.*, 2000; Hayashi *et al.*, 2001). These findings show that MK shares the features of being a growth factor in parallel with antibacterial properties and chemotactic activity, with most antibacterial proteins. MK binds and activates the anaplastic lymphoma kinase receptor, resulting in activation of NF-κB (Kuo *et al.*, 2007; Palmer *et al.*, 2009) and the binding of MK to this receptor may explain some of its pro-inflammatory properties.

Countermeasures by bacteria

Bacteria use a number of different strategies to avoid being killed by antibacterial proteins (Peschel and Sahl, 2006). These strategies are all aimed at counteracting the attachment and insertion of antibacterial proteins into the bacterial membrane. One strategy used by pathogenic bacteria is the release of proteases that can degrade and compromise the actions of antibacterial proteins (Potempa and Pike, 2009). This is exemplified by *F. magna*, an anaerobic Gram-positive coccus. This bacterium is both a member of the normal microbiota and an opportunistic pathogen causing several clinical conditions, such as soft-tissue infections, wound infections and bone/joint infections in immunocompromised hosts (Frick *et al.*, 2008). Most strains of *F. magna* express a subtilisin-like enzyme, subtilase of *F. magna* (SufA), which is associated with the bacterial surface (Karlsson *et al.*, 2007). It cleaves proteins at lysine and arginine residues, amino acid characteristic of the often cationic antibacterial proteins. We found that SufA degraded MK, generating fragments that were bactericidal against competing pathogens, that is, *Str. pyogenes* but leaving *F. magna* viable, thus promoting an ecological niche for itself (Frick *et al.*, 2011). *Str. pyogenes* is a highly virulent, Gram-positive pathogen causing both superficial and deep severe infections, such as pharyngitis, erysipelas, necrotizing fasciitis and septic shock (Cunningham, 2000). *Str. pyogenes* produces a potent cysteine protease that efficiently degraded MK (Frick *et al.*, 2011). *P. aeruginosa* is another important pathogen, particularly in chronic obstructive pulmonary disease (COPD) and CF. It releases an elastase and we found that it degrades MK, impairing the antibacterial activity against this bacterium (Nordin *et al.*, 2013b).

Inactivation of MK by bacterial proteins

In addition to the strategies described above, some bacteria release proteins that neutralize the activity of antibacterial proteins. These often have anionic stretches and have high affinity for the cationic antibacterial proteins. *F. magna* resides in the lower parts of the epidermal layer, where it binds to the protein BM-40, which is part of the BM, via the surface-associated protein *F. magna* adhesion factor (FAF) (Frick *et al.*, 2008). FAF can be released to the environment and we found that it binds MK with high affinity, neutralizing its antibacterial properties (Frick *et al.*, 2011). Another example is protein streptococcal inhibitor of complement of *Str. pyogenes* (Åkesson *et al.*, 1996). This is an unstructured 30 kD protein, produced and released in high amounts by *Str. pyogenes*. Initially, it was described as inhibiting complement activation. We found that this bacterial protein also binds and inactivates the antibacterial activity of MK (Frick *et al.*, 2011).

Surface alterations of bacteria as a means to circumvent antibacterial proteins

Gram-positive bacteria can reduce the negative charge on their membrane by modifying TA, and Gram-negative bacteria use the same strategy through modifying the LPS and thereby decreasing the electrostatic attraction between antibacterial proteins and the bacterial membrane. Why bacteria have not been more successful in developing resistance to antibacterial proteins, based on altering membrane charge, has been discussed and one possible reason for this failure is that to modify the membrane, the primary point of attack, is an expensive solution for the bacteria in terms of proliferative and competitive capacity (Zasloff, 2002).

MK in inflammatory and infectious diseases

MK is present in plasma of healthy individuals and increased levels are detected in several inflammatory and infectious conditions, for example, in sepsis and septic shock (Krzystek-Korpacka *et al.*, 2011). Among clinical characteristics associated with higher MK levels were sepsis-related hypoxia, cardiac failure and sepsis from Gram-positive bacteria. It is intriguing that MK levels increase in sepsis, and one

could speculate about potential roles in host defence. It seems unlikely that the increased levels of MK play an antibacterial role *per se*. Our own findings, that the antibacterial activity decreases in the presence of plasma, suggest that the execution of antibacterial properties for MK are limited to sites outside the blood circulation, for example, on mucosal surfaces and in the skin (Svensson *et al.*, 2010). Thus, MK may be bound to a carrier and delivered to sites of inflammation, or the increased levels of MK may reflect a systemic response including increased expression. An increased production of MK is also seen in meningitis where monocytes and other leukocytes contribute to the synthesis (Yoshida *et al.*, 2008).

Recently, we showed increased expression of MK in CF (Nordin *et al.*, 2013b). However, MK was partially degraded in sputum, possibly due to the elastase released by *P. aeruginosa* colonizing the airways of these patients.

Conclusions and perspectives

The regulation of MK expression in inflammatory contexts, its properties as an antimicrobial agent and modulation of its activities by host and bacterial proteins, for example, by proteases, demonstrate a novel area where pharmacological interference can tune innate immune mechanisms to prevent dys-regulated inflammation and eradicate infection. One of the big threats in clinical medicine today is the increasing bacterial resistance to conventional antibiotics. In addition, inflammatory bouts (exacerbations) triggered by bacteria and their released products are an important area where current pharmacological treatments have limited effects, for example, in COPD which will become the third leading cause of death by 2020 (Sethi and Murphy, 2008). In light of this, addition of innate antibiotics to the therapeutic arsenal is a possible strategy, either used alone or in combination with conventional antibiotics. Antibacterial proteins, for example, MK, serving as innate antibiotics are of great interest because of their possible use for these purposes. Several candidate drugs in this field are currently being tested at various levels. One advantage being that these agents are unlikely to cause allergic reactions, they have a rapid onset with respect to bactericidal activity, and it is unlikely that bacteria will develop novel resistance mechanisms. Thus, it is possible that we will have to return to innate antibiotics, for example, MK, to develop future antimicrobial treatments.

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Conflict of interest

All authors declare that they have no conflict of interest related to the content of this article.

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