FUNCTIONAL DUALISM OF ANTIMICROBIAL HOST DEFENCE PEPTIDES

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Abstract: Antimicrobial host defence peptides are central to innate immunity and many possess direct antimicrobial actions on bacteria as well as indirect immunomodulatory functions on human leukocytes. Different variants of the bifunctional *Helicobacter pylori* peptide, Hp(2-20), were synthesised and inhibition zone assays and chemiluminescence systems were employed for determination of direct antimicrobial action and superoxide release (immunomodulatory) from human neutrophils, respectively. α-helically stabilised peptides displayed increased antimicrobial action, while destabilised peptides were impaired. Only native Hp(2-20) had the capability to induce superoxide production from neutrophils, this representing a sequence-specific feature. The well known chemokine interleukin-8 (IL-8) contains a C-terminal part with many similarities to α-helical antimicrobial peptides. The C-terminal part of IL-8 was synthesised and displayed antibacterial activity but was incapable of inducing neutrophil superoxide production and chemotaxis, prominent activities of the native protein. IL-8 could thus be viewed as a bifunctional molecule with the two effects residing in different parts of the molecule. A prominent example of a human host defence peptide exhibiting functional dualism is LL-37 that permeabilises microbial membranes. LL-37 also selectively permeabilised apoptotic human leukocytes leaving viable leukocytes intact, as measured by flow cytometry. The activity was reminiscent of its antimicrobial activity; it was rapid, independent of known surface receptors and/or active cell signalling. Selectivity was probably related to changes in membrane composition of apoptotic cells. Permeabilisation of apoptotic leukocytes by LL-37 was accompanied by leakage of cytoplasmic and intragranular molecules that may shift the balance between pro- and anti-inflammatory signals and by this be of importance for the termination of acute inflammation. LL-37 also interacted with different receptors present on viable leukocytes. Neutrophil- and monocyte NADPH-oxidase activation by LL-37 was studied and shown to depend on the FPRL1 receptor. Also, the rise of intracellular Ca^{2+} triggered by LL-37 was FPRL1 dependent, but the peptide was a rather weak FPRL1 agonist. However, L-selectin shedding from neutrophils was independent of FPRL1, suggesting the presence of another receptor on neutrophils for LL-37. The dual action of host defence peptides makes them especially important for handling infections; fighting the pathogen directly as well as indirectly by alarming the immune system.

Keywords: antimicrobial, apoptosis, cathelicidin, host defence peptide, interleukin 8, LL-37, permeabilisation

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This thesis is based on the following papers referred to in the text by their roman numerals:

I. Fu H, Björstad Å, Dahlgren C, Bylund J. **A bactericidal cecropin-A peptide with a stabilized alpha-helical structure possess an increased killing capacity but no proinflammatory activity.** *Inflammation. 2004. 28(6):337-43.*

II. Björstad Å, Fu H, Karlsson A, Dahlgren C, Bylund J. **Interleukin-8-derived peptide has antibacterial activity.** *Antimicrobial Agents and Chemotherapy 2005. 49(9):3889-95.*

III. Björstad Å, Askarieh G, Brown KL, Christenson K, Forsman H, Önnheim K, Li H, Teneberg S, Maier O, Hoekstra D, Dahlgren C, Davidson DJ, Bylund J. **The host defence peptide LL-37 selectively permeabilises apoptotic leukocytes.** *Antimicrobial Agents and Chemotherapy 2009. 53(3). In Press.*

IV. Björstad Å, Önnheim K, Karlsson J, Rabiet MJ, Dahlgren C and Bylund J. **LL-37 mediated activation of human leukocytes - characterisation of receptor usage.** *Manuscript.*

Till Hans, Elias och Arvid

TABLE OF CONTENTS

ABBREVIATIONS

CF	Cystic fibrosis
CRAMP	Cathelicidin-related antimicrobial peptide
CXCL	CXC chemokine ligand
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
FPRL1	Formyl-peptide receptor-like 1
HBD	Human β-defensin
hCAP-18	Human cationic antimicrobial protein 18
HD	Human defensin
HNP	Human neutrophil peptide
IL	Interleukin
LPS	Lipopolysaccharide
PS	Phosphatidylserine
SCTE	Stratum corneum tryptic enzyme
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

INNATE IMMUNITY

On a daily basis the human body is challenged by a wide variety of microbes but despite this, actual disease is the exception. The system that initially protects us from microbial invasion is called innate immunity, which as the name suggests is present from birth. Basically, the function of innate immunity is to recognise the microbes and eliminate and/or impede their spread until the slower-acting adaptive immune system has had sufficient time to mobilise. The components that constitute the innate immune system are evolutionary old mechanisms that carry out the defence against microbial pathogens in most organisms including plants, fungi, insects and humans (12). In more primitive organisms innate immunity is the sole defence system, and in such organisms antimicrobial peptides are of prime importance. In higher animals innate immunity relies not only on antimicrobial peptides, but also on a highly developed inflammatory process and the presence of specialised defence cells. The innate immune system provides an immediate defence against infectious agents and control infection before the onset of adaptive immunity. However, the system lacks the long-lasting effects, flexibility and diversity that characterise the adaptive immune system. There are of course multiple overlaps and a constant cross-talk between the innate and adaptive immune systems, thus the boundaries between them are in many cases not strictly defined. Antimicrobial peptides are striking examples of such cross-talk, first recognised only for their role in direct killing of microbes, but increasingly recognised as signalling molecules with an ability to affect both the inflammatory process as well as adaptive immunity.

Inflammation

Introduction

When microbes have entered the tissue, recognition of various microbial molecules will initiate acute inflammation, a process whereby a variety of leukocytes leave circulation and migrate towards the infected area. Historically inflammation has been characterised by five cardinal signs: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function). These states are caused by increased vascular permeability, local accumulation of immune cells and the substances they release into the surrounding tissue. The process is highly regulated and involves a number of different innate immune cells e.g., mast cells, natural killer (NK) cells, and phagocytes such as monocytes/macrophages and neutrophils. In addition, inflammation involves a complex network of cytokines that control blood vessel permeability and guides the leukocytes through the tissues.

Cytokines with chemotactic activity are known as chemokines and are primarily produced by epithelial cells upon sensing microbial intrusion. The chemokines diffuse from the site of production and form a concentration

gradient that guides extravasated leukocytes through the tissues. A prominent example of such a chemokine is interleukin-8 (IL-8), which is crucial for the accumulation of neutrophils during the early stages of inflammation. In addition to chemokines, other molecules function as chemoattractans, e.g., bacterial-derived substances such as formylated peptides and activated components of the complement system (C5a). The accumulated cells (at the infectious site) are often armed with highly toxic substances, and if these substances reach the extracellular surroundings they will not only harm microbes, but also the host tissue. In addition, assembled leukocytes release additional pro-inflammatory cytokines and chemokines, creating a positive amplification loop of infiltrating inflammatory cells and release of, often toxic, inflammatory mediators. Thus the inflammatory response needs to be delicately balanced – powerful enough to eradicate invading microbes, yet mild enough to minimise tissue damage and avoid the development of chronic inflammation.

Neutrophils

Neutrophils are the most numerous leukocytes in the human innate immune system and are critical components of inflammatory reactions. These cells mature in the bone marrow before being released into circulation where they make up some 60-70% of leukocytes. The recruitment of neutrophils from the blood stream, their migration to and activities at inflammatory foci as well as the manner in which these cells die are all of fundamental importance for the regulation of inflammation. The neutrophil cytoplasm contains a large number of membrane enclosed storage organelles; granules, formed mainly during cellular differentiation in the bone marrow (14). The granules are formed in a strictly ordered fashion starting with the azurophil granules followed by two other granule types, the specific and gelatinase granules. In addition to the different granule types, neutrophils also contain secretory vesicles which are formed by endocytosis late in neutrophil maturation, and several not yet characterised storage organelles of unknown origin (33, 86, 87). Each organelle type is created within a certain time span of neutrophil maturation and contain different matrix proteins and specific membrane receptors that are synthesised during the period of formation.

It has recently been proposed that *de novo* protein synthesis, once thought to occur only during neutrophil maturation in the bone marrow, can take place as the neutrophil enters the tissue to fight an ongoing infection (15). Protein synthesis is however minimal compared to other leukocytes thus neutrophils largely depend on preformed molecules stored in the various granules. The segregation of granule proteins into different compartments confers neutrophils the ability to display different effector molecules (as well as receptors) at specific time-points, but it also provides the cell with the ability to segregate proteins that simply cannot co-exist from a safety point of view. Catalytically active proteases from one granule type may, upon extracellular secretion or delivery to a phagosome (containing engulfed microbes), encounter proform proteins/peptides from another granule type and transform the pro-proteins/peptides into active effector molecules (109).

After maturation in the bone marrow, neutrophils are released into the blood where they only survive for a matter of days (34, 115). In the bloodstream they circulate in a resting state awaiting signals that indicate microbial infection or inflammation. The signals needed for the neutrophil to respond to such a state are mediated by the endothelium lining the blood vessels that upon activation up-regulate adhesion molecules on the surface facing the bloodstream. Neutrophil recruitment starts with an interaction between the endothelial adhesion molecules and the neutophil adhesion molecules. One conspicuous example of a neutrophil adhesion molecule is L-selectin that is cleaved as activation progresses with concomitant expression of other adhesion molecules, allowing tighter interaction with the endothelial cells (111). This process is followed by transmigration of neutrophils across the endothelium, thus entering the tissue. Once in the tissue, the neutrophils start to "crawl" toward the source of inflammation, guided by a gradient of increasing concentrations of chemoattractants; e.g. IL-8 and/or bacterial products, a process known as chemotaxis (31).

When the inflammatory site is reached, neutrophils will adhere to microbes or cell debris, an event followed by phagocytosis of the material. Once ingested into the phagosome, granule mobilisation with subsequent membrane fusion and deposition of the antimicrobial content into the phagosome will occur, forming a phagolysosome in order for killing/degradation of the prey. The killing activity can be oxygen-dependent relying on the activation of the NADPH-oxidase and production of toxic oxygen radicals (53). However, as neutrophils are a prominent source of antimicrobial molecules besides reactive oxygen species, a considerable part of the antimicrobial activity in neutrophil phagolysosomes is oxygen-independent, and this activity relies on antimicrobial peptides as well as proteins (64, 78, 135).

Neutrophils are short-lived cells and will not stay viable for prolonged times in the tissue. Since the cells are so well equipped with potential tissue-destructing substances, the manner in which they die and are subsequently removed from the system is also of importance. The physiological way to die is by programmed cell death, apoptosis. Apoptotic cells are non-functional, but maintain an intact cell membrane that prevents uncontrolled leakage of potentially harmful intracellular molecules, thus minimising inflammatory damage (46). This is opposed to the more pathological type of cell death, necrosis, in which the cells have leaky membranes freely permeable to intracellular content. Apoptotic cells are more negatively charged as compared to viable cells due to, in part, the externalisation of the phospholipid phosphatidylserine (PS). In viable cells, an energy-dependent process preserves the uneven distribution of phospholipids such that the negatively charged PS is maintained on the inner side of the double-membrane. In a non-functional, apoptotic cell the phospholipid sorting is stopped, resulting in exposure of PS also on the outer leaflet (68). PS exposure not only makes the membrane of apoptotic cells more negatively

charged, but also serves as an important "eat me" signal that is recognised by active phagocytes, typically resident macrophages that engulf the dead cells and remove them from the tissue in an efficient manner (39). This secondary clearance of apoptotic neutrophils by macrophages is associated with the release of anti-inflammatory cytokines from the macrophages dampening the inflammatory response (95). However, neutrophils that have entered apoptosis following phagocytosis of bacteria can, as opposed to the anti-inflammatory effect described above, increase the production of pro-inflammatory cytokines in macrophages (136), further showing that apoptotic neutrophils may guide inflammation in either direction.

Human cells may under some other circumstances, besides apoptosis, externalise PS. Cancer cells have slightly more negative charge on the plasma membrane compared to normal cells. This charge difference is in part derived from higher amounts of PS present on the plasma membrane side facing the exterior (85). Several publications have shown that cationic peptides have increased affinity and capacity to kill cancer cells as compared to normal cells (24, 29, 114, 120, 137). Cells infected by viruses also externalise their PS as an early marker following infection (105).

Antimicrobial peptides

Introduction

Antimicrobial peptides are widespread in nature, having been found in prokaryotes as well as in a wide range of eukaryotes like plants, insects and mammals. In many invertebrates these peptides are the only defence mechanism present, while in mammals they constitute an important part of the early, innate immune response. In mammals, antimicrobial peptides are primarily produced by cell types first encountering invading microbes; i.e. leukocytes as well as cells lining epithelial surfaces (44). The first mammalian peptides with microbicidal properties were isolated from rabbit macrophages some 25 years ago (102). They turned out to be members of a large family of antimicrobial peptides, the defensins, later found also in humans (103). We now know that, in general, each species from the animal and plant kingdom are armed with 15 – 40 different antimicrobial peptides (13). During the last few years the roles of these antimicrobial peptides in the immune response of higher organisms has been partly revised. Originally depicted as strictly antimicrobial, they are today also ascribed a growing number of immunoregulatory properties and the primary function of these peptides *in vivo* in higher organisms has not yet been settled (8, 16-18, 21, 38, 44, 80, 91, 98, 103, 125, 129, 133). The terminology concerning antimicrobial peptides has accordingly shifted toward the term host defence peptides, more properly reflecting their multi-functionality *in vivo* i.e. directly microbicidal as well as immunomodulatory. Studies of the immunoregulatory properties of antimicrobial peptides are in their infancy and there is still much to learn.

Common Features

Many antimicrobial peptides share several basic properties; they are relatively small (generally no longer than 50 amino acids with a molecular weight of less than 10 kDa), they have a net positive charge (cationic) with pI values above 7, and they are amphipathic with distinct hydrophilic and hydrophobic areas within the molecule. These physicochemical properties are of importance for their direct antibacterial nature (**Paper I**). The antibacterial activity is widely believed to be dependent on direct interaction with the bacterial cell membrane, ultimately resulting in pore formation and lysis of the bacterium (as discussed in the following paragraph). The positive net charge is probably of importance for the early interaction of a peptide with the negatively charged bacterial membrane, also when targeting of intracellular structures is the important effector function rather than pore formation and direct lysis of the microbe (16, 89).

The primary, secondary as well as tertiary structure of antimicrobial peptides varies greatly, but based on the secondary structure four different groups of antimicrobial peptides can be identified. The most extensively studied group of peptides are the α-helical peptides, including, among others, human LL-37 (Figure 1), cecropins, magainins and the recently described human interleukin 8 (IL-8) derived peptide. The stability of the α-helical structure is generally of importance for the direct antimicrobial activity such that a more stable α-helix is usually more potently antimicrobial (**Paper I**, 51, 66). Human defensins, including human neutrophil peptide 1 HNP 1 (Figure 1), belong to the second group of antimicrobial peptides, the cysteine-rich peptides, and are grouped together based on the presence of one to four disulfide bridges between cysteine residues. Yet another group of peptides are the β-sheet peptides which form a β-hairpin structure and contain one or two disulfide bridges. In this group several hemipteran derived peptides and human lactoferricin B (Figure 1) can be found. Some antimicrobial peptides are composed of an unusually high amount of one specific amino acid; for instance indolicidin (a member of the cathelicidin family) is rich in tryptophan while PR-39 (a porcine cathelicidin) is rich in arginine and proline. Certain rare antimicrobial peptides contain modified amino acids (89).

There are two main classes of antimicrobial peptides found in man; the cathelicidins and the defensins. The former is solely represented by the cathelicidin precursor human cationic antimicrobial protein 18 (hCAP-18), which is found primarily in neutrophil granules (107). The defensins (α-form) are also found in neutrophil granules (40), and both defensins and cathelicidins will be discussed in more detail below.

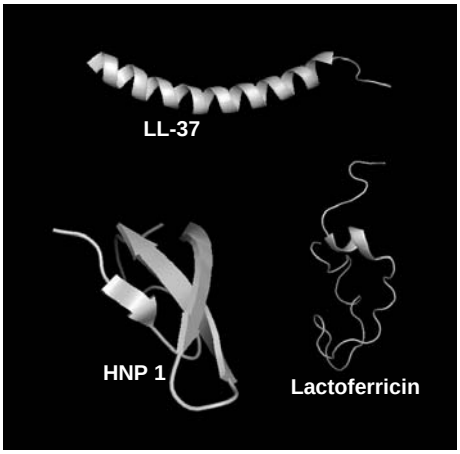


Figure 1. Examples of antimicrobial peptides belonging to three different structural classes; α-helices (LL-37), cysteine-rich peptides (HNP 1) and β-hairpin peptides (lactoferricin).

Microbicidal Mode of Action

The mode of antimicrobial action of host defence peptides has not been determined conclusively, but the leading hypothesis is that these peptides are membrane-active with the ability to break the integrity of bacterial cell membranes more or less selectively over mammalian membranes (104). Bacterial membranes are composed of different phospholipids as compared to eukaryotic cell membranes, the bacterial membranes being more negatively charged than the eukaryote counterpart. The presences of bacterial membrane structures like lipopolysaccharide (LPS); the outer membrane component of Gram-negative bacteria, is of importance for the overall negative charge of bacterial membranes. This negative charge is believed to be a determinant for the interaction with cationic peptides, and gives the peptides some kind of selectivity for the microbial membranes (69). Eukaryotic cell membranes will under some circumstances adopt a more negatively charged membrane (e.g. apoptotic cells and cancer cells as mentioned above), and this property might make them more susceptible to the membrane perturbing actions of cationic antimicrobial peptides.

The bacterial membrane disrupting mechanism is usually described by either of two different models; the barrel stave model and the carpet model (89). In the barrel stave model amphipathic peptides are inserted into the bacterial membrane, interacting with each other and the membrane, thus forming a pore. In the carpet model, the peptides are not inserted into the bacterial membrane, but instead break up the membrane in micelles with the hydrophobic phospholipid chains facing inward. The peptides cover the outside of the micelles. In both cases the bacterial membrane is destabilised and bacterial death follows lysis (89). The amphipathicity of the peptides is

a very important feature in both models, facilitating interaction between peptides as well as between peptides and membranes.

For many years membrane disruption was thought to be the only mechanism of microbial killing, but several antibacterial peptides completely lyse bacteria only at higher concentrations than those that are needed for antimicrobial activity (32). Some peptides have consequently been shown to use limited membrane disruption primarily for entering bacteria and as means for reaching intracellular targets involved in critical cellular processes such as DNA and protein synthesis (32). It should be noted that, at high concentrations, virtually all antimicrobial host defence peptides are cytotoxic to a whole range of eukaryotic cell types, at least when their activities are determined *in vitro* (17).

DEFENSINS

Introduction

The defensin family of antimicrobial peptides is widely spread in nature and found in mammals, plants and insects. In vertebrates three defensin subfamilies have been identified: α-, β and θ-defensins (θ-defensins only in non-human primates) that differ mainly in the organisation of three disulfide bonds, a feature also characterising the whole family (64). In humans both α- and β-forms are found. The two subfamilies differ in size; the human α-defensins are 29-33 amino acids long, while the β-defensins consists of 35-72 amino acids (16). The human α-defensins have disulfide bonds linking cysteines 1 and 6, cysteines 2 and 4 and cysteines 3 and 5, whereas the β-defensins have disulfide connections between cysteines 1 and 5, cysteines 2 and 4 and cysteines 3 and 6 (103). All vertebrate defensins have several properties in common: a positive net charge and a turn-linked β-strand structure (Firgure 1) being the two most prominent features (103).

There are 6 different human α-defensins; 4 were first isolated from neutrophils and are thus called human neutrophil peptide (HNP) 1-4, and they have today been shown to co-localise with several peripheral blood leukocytes including monocytes, NK cells, macrophages, B cells and T cells beside being present in neutrophils (major source; 55, 80, 103). Remarkably, murine neutrophils totally lack α-defensins (37), however α-defensins are present in other cell types of the mouse, not least in the mouse intestine where they are called cryptdins. Transgenic mice deficient in matrilysin which is required for production of mature intestinal cryptdins, were more susceptible to infections with *Salmonella typhimurium*, showing the important role for defensins *in vivo* (121). At a later time point, two additional human α-defensins generated by Paneth cells of the intestine were identified: human defensin (HD) 5 and HD 6 (83).

Human β-defensin (HBD) 1-4, are found in keratinocytes, epithelial cells, monocytes, dendritic cells (DCs) and mast cells (not HBD 1) (80). The human genome contains more than 20 β-defensin homologues based on sequence similarity to HBD 1-4 (96), but so far only HBD 1-3 have been isolated on the protein level hence they are the most studied peptides within this group (16).

Antimicrobial Activity

The killing-spectrum of human defensins includes bacteria, fungi and viruses. They kill fungi by inducing a release of fungal adenosine triphosphate (ATP) which binds to purinergic receptors and subsequently activates cytotoxic pathways in the fungus (36). Viruses are killed by the binding of defensins to the viral envelopes, so non-enveloped viruses are generally unaffected by the defensin activity (52). Bacterial killing by human defensins is thought to occur by membrane disruption, but other

mechanisms have been suggested as complementary to this mechanism. HNP 1 has been proposed to kill bacteria by inhibiting DNA and protein synthesis (122). Another theory concerning the killing mechanism involves the capability of defensins to activate bacterial autolytic cell wall enzymes that mimics the action of β-lactam antibiotics (82). The direct microbial killing capacity of defensins is promoted by low-salt buffers, and at mM concentrations of divalent cations Ca^{2+} and Mg^{2+} or 100-200 mM NaCl, the capacity is severely impaired (5, 45, 63). This could partially be circumvented by increasing the peptide concentration and the high concentration of HNP 1-3 in azurophil granules ensures a direct antimicrobial role for them in phagolysosomes of neutrophils upon phagocytosis. The activity is however partly counteracted by the acidic pH found in the phagolysosome (65).

Immunomodulating Effects

Human defensins have been ascribed several immunomodulatory activities in addition to the direct microbicidal effects. Some of these activities will be discussed here, focusing on effects on innate immunity. There are several reviews covering the effects of defensins on adaptive immunity (16, 80, 103, 125).

α-defensins have been shown to be chemotactic for several cell types of the immune system including monocytes, T cells and immature DCs of human as well as murine origin (25, 64, 112, 126). The chemotactic activity on both T cells and DCs was shown to be dependent on G-protein coupled receptors as both activities could be inhibited by the addition of pertussis toxin (126). HBD 1-2 are chemotactic for immature DCs and memory T cells (128), whereas only HBD 2 is chemotactic for primed neutrophils (75). All these responses depend on the HBD interaction with the chemokine receptor CCR6 (75, 128). HBD 3 is chemotactic for monocytes, but this effect is not dependent on CCR6 (43). Besides functioning as direct chemotactic agents, α-defensins may also indirectly contribute to massive accumulation of inflammatory cells by increasing transcription and translation of the IL-8 gene in lung epithelial cells (117). This property might be exclusive to α-defensins as HBD 2 is incapable of inducing IL-8 expression in bronchial epithelial cells (94). Other effects exerted by α-defensins on the airway epithelium involve proliferation and repair of injured epithelium and, at higher concentration, cytotoxic actions on epithelial cells (1). Neutrophil derived α-defensins from several species, including humans, have the capability to function as potent mast cell activators with resulting histamine release dependent on a G-protein coupled receptor exposed on mast cells. Granule products released concomitantly with histamine are also potent neutrophil chemoattractants, increasing the infiltration of neutrophils to the site of release (10). Thus the α-defensins are highly pro-inflammatory by attracting inflammatory cells, both in a direct- as well as in an indirect manner.

Addition of neutrophil-derived α-defensins to resting monocytes *in vitro* did not affect cytokine production. If defensins were added simultaneously with *Staphylococcus aureus* or phorbol myristate acetate, the expression of tumour necrosis factor-α (TNF-α) and IL-1β was up-regulated, while the expression of IL-10 was down-regulated; the effect observed thus being pro-inflammatory (22). HNPs can also function as potent mitogens on fibroblasts and epithelial cells *in vitro* at concentrations expected to be found *in vivo* during wound healing, suggesting an important function in wound healing and resolution of injury-induced inflammation (70). Evidently, β-defensins do not share these mitogenic properties and could not increase proliferation of epithelial cells or fibroblasts (72). However, recently HBD 2 was shown to promote intestinal wound healing independent of proliferation of epithelial cells (81).

Among the other immunomodulatory functions reported for HNP 1, it has the capability to bind to and inhibit C1q hemolytic activity, thus preventing activation of the classical complement pathway and in this demonstrating a possible anti-inflammatory role *in vivo* (116). In an *in vitro* based system, cultured mouse macrophage phagocytosis of latex beads was studied, and when HNP 1 was added to the system, an enhancement of phagocytosis was observed. The authors did not draw any conclusions concerning whether the effect observed was due to opsonisation as opposed to direct effects on the macrophages (49). However, a recent study demonstrated that HNP 1-3 can enhance macrophage phagocytosis of IgG-opsonised *S. aureus* by increasing the expression of Fcγ-receptors I and II on the macrophage surface, suggesting direct effects of defensins on macrophages as being responsible (106). HNP 1 and HBD 2 also have the capability to bind to LPS and neutralise its activating effects on a macrophage cell line with subsequent inhibition of TNF-α production (100). For HNP 1 and HBD 2 the observed effect was quite modest, but for several other cationic antimicrobial peptides the inhibition is extensive. HNP 1 and HBD 2 function by blocking the interaction between LPS and LPS binding protein, an interaction necessary for LPS binding to the cell surface receptor CD14 (100).

The human α-defensin genes encoding HNP 1 and 3 show polymorphisms in both copy number and location of tandem repeats on the chromosome. RNA levels found in leukocytes correspond to the copy number of the genes. However, the functional consequences of these polymorphisms have not been established. Polymorphisms in the gene coding for HBD 1 have been shown to correlate with the susceptibility to chronic obstructive pulmonary disease (55). A clinical symptom hinting to the importance of HNPs is specific granule deficiency, a rare disease characterised by lack of both the specific- and azurophilic granules of neutrophils combined with a marked decrease in defensin expression. The patients suffer from severe infections and defective neutrophil-mediated microbial killing (42). The specific role of the defensin shortage in microbial defence is hard to establish since other components, including antimicrobials and

immunomodulators, of the specific granules are also depleted in this disease (42).

HD 5-6 are involved in inflammatory bowel diseases, as illustrated by the fact that a deficiency of HD 5 is associated with Crohn's disease (56). The disease is, in part, caused by the colonisation of certain intestinal bacteria that may initiate and maintain mucosal inflammation. Patients with Crohn's disease also have reduced antibacterial activity in their intestinal mucosal extracts due to the deficiency in defensins (56). A murine animal model support the impact of defensins in Crohn's disease as decreased expression of the peptides induced changes in the intestine that were comparable with those seen in Crohn's disease (56, 119). β-defensins have also been implicated as playing an important role in this disease as individuals with a reduced copy number of the HBD 2 gene have a significantly higher risk of developing Crohn's disease (41).

CATHELICIDINS

Introduction

The cathelicidins form a large family of antimicrobial peptides found in all mammalian species examined so far; including human, cow, pig, rabbit, sheep, mouse, monkey and horse. They have also been identified in birds and fish (123, 133) and are grouped together as a family based on a conserved pro-region (the cathelin domain) of about 120 amino acid residues, which is not found in the mature, active peptides. The possible function of the cathelin domain has remained unknown until recently when it was discovered that the cathelin domain of the human cathelicidin resembles proteins of the cysteine protease inhibitor family and was able to inhibit the protease activity of cathepsin L, and as a consequence limit tissue damage (132). This domain also exhibited antimicrobial actions on its own, distinct from the LL-37 sequence described in detail below, implying that also the cathelin domains possess biological activity. The composition of the mature, antimicrobial, carboxy-terminal domain varies greatly and the mature peptides may belong to any of the different structural groups of antimicrobial peptides mentioned above (134). Cathelicidins are expressed in epithelial cells as well as in neutrophils and macrophages (8). Only one human cathelicidin has been isolated; hCAP-18 (unprocessed form), or LL-37 (mature, processed, antibacterial form). It was first described in 1995 independently by several groups of researchers (3, 30, 61). The amino acid sequence of LL-37 was predicted when screening a human bone marrow cDNA library using a probe derived from the pig cathelicidin PR-39 gene (3). Following this, LL-37 was isolated from bone marrow neutrophils undergoing differentiation. Since then, LL-37 protein has been found in epithelial cells lining the testis, skin, gastrointestinal and respiratory tract and in other inflammatory cells including NK cells, T cells and B cells (2, 8). LL-37 is a 37 amino acids long amphipathic, α-helical molecule (Figure 1); the two first amino acids being leucine residues, hence the name LL-37.

Human neutrophils are the main source of LL-37 and the gene for hCAP-18 is transcribed during the differentiation of precursor cells in the bone marrow; the cathelicidin is then stored in its unprocessed pro-peptide form in specific granules (8, 107). The cells contain large amounts of hCAP-18 and the concentration can be as high as 630 $\mu g/10^9$ neutrophils (108), and up to 5 μg/ml in the bulk of different body fluids like blood plasma, airway surface fluid, wound fluid and blister fluids formed during infection or inflammation (8). The local concentration in damaged tissue can greatly exceed the bulk concentrations given above (79). In activated neutrophils the pro-form is cleaved enzymatically, by proteinase 3, to its active form (LL-37) upon degranulation towards the extracellular compartment (109). Proteinase 3 is a serine protease stored in the azurophil granules and the arrangement of storing an enzyme and its substrate in different compartments ensures that the active product, in this case LL-37, is not formed until it is required (97). In neutrophils, the mature form is not

readily formed in the phagosome/phagolysosome, but is rather generated by extracellular cleavage (109).

Antimicrobial Activity of LL-37

The antimicrobial activity of LL-37 is effective against bacteria, fungi and viruses. The killing mechanism is dependent on the membrane active properties of the peptide, which has the ability to interact with both the inner and the outer membrane of Gram-negative bacteria. The mechanisms of action on Gram-positive species are not known in detail, but LL-37 is active also against these bacteria at low concentrations (8). The antimicrobial activity of LL-37 is abolished at salt and protein concentrations resembling physiological levels seen in blood/plasma (27, 118). This is a property shared with the majority of, but not all antimicrobial peptides. It is also one of the reasons to why there are different opinions about the precise *in vivo* function of many antimicrobial peptides. In contrast to the direct antimicrobial effects, many peptides maintain their immunomodulatory functions under physiological conditions and in some cases the modulatory action may even be potentiated by the presence of serum. A recent publication has shown that LL-37 may influence bacterial fitness also by preventing the formation of biofilms by *Pseudomonas aeruginosa* in an *in vitro* based system. The observed effect was due to decreased bacterial attachment, stimulation of bacterial motility and down-regulation of genes responsible for biofilm formation, demonstrating yet another way by which LL-37 can contribute to host defence (84).

Effects on Innate Immunity by LL-37

In addition to the direct effects of LL-37 on microbes, achieved through interaction with the microbial cell membrane or by inhibiting the formation of biofilms, the peptide also exerts more indirect effects in combating invading pathogens. During the last 8 years there has been a wealth of papers describing LL-37 mediated effects on the innate immune system, as well as on the adaptive immune response. Several excellent reviews dealing with the effects on the adaptive immune system are available for the interested reader (16, 17, 129), and below is a summary of the LL-37 induced effects on the innate immunity.

The first report describing LL-37-provoked, non-cytotoxic effects on mammalian cells, came in the early 2000 and it was shown that the peptide was chemotactic for several cell types of the immune system, including neutrophils, monocytes and T cells (35). The chemotactic effect was dependent on the binding of LL-37 to formyl-peptide receptor-like 1 (FPRL1), a cell surface receptor belonging to the family of seven transmembrane G-protein coupled chemoattractant receptors. This chemotactic activity was intact in the presence of serum, a component known to abolish the antibacterial and cytotoxic effects of LL-37 as mentioned earlier (35). LL-37 is also chemotactic for mast cells (isolated

from rats) using G-protein coupled receptor and a phospholipase C signalling pathway (73) and capable of inducing histamine release from these cells (76). In addition, it was recently shown that LL-37 shifts mast cell function towards innate immunity in the presence of bacterial products, assessed as Toll-like receptor (TLR) expression in, and release of cytokines from mast cells (130). The receptor(s) responsible for mast cell activity is, however, not identical to FPRL1 (73, 76, 130), suggesting that there are multiple receptors responsible for mediating the immunomodulatory effects of LL-37. LL-37 is certainly able to activate FPRL1, but the affinity is rather low and in order to activate primary human neutrophils through this receptor, prior priming that exposes increased FPRL1 levels on the surface, is required (**Paper IV**). Primed neutrophils (and monocytes) exposed to high concentrations of LL-37 utilise FPRL1 to produce reactive oxygen species, whereas another activation feature, L-selectin shedding, occurred totally independent of FPRL1 (**Paper IV**), indicating the existence of (at least) two functional receptors on these cells.

LL-37 can induce the release of chemokines from different leukocytes and from epithelial cells of the bronchi. The peptide can also promote the activation of extracellular signal-regulated kinase (ERK) 1/2 and p38 by a non-G-protein coupled receptor (19, 98). Release of IL-8 from keratinocytes is achieved with both the L- and D-form of LL-37, a process that could be inhibited by a epidermal growth factor receptor tyrosine kinase inhibitor, results that further strengthen the suggestion that different receptors are involved in the recognition of LL-37 (20). LL-37 was recently shown to inhibit the replication of HIV-1 in peripheral blood mononuclear cells and T cells by a mechanism independent of direct effects on the virion (11). The effect was also independent on FPRL1 ligation, an activity previously known to be capable of inhibiting HIV-1 replication, suggesting a yet unknown signalling pathway for the observed action (11). The observed effect is thus an antimicrobial action mediated by the infected host cell, rather than a direct inhibitory function on the virion.

The fact that LL-37 mediates a release of IL-8 from epithelial cells would increase the recruitment of neutrophils, that in turn could release even more LL-37 in a positive feedback loop (18). Besides up-regulating the production of chemokines, LL-37 efficiently increases the surface expression of several chemokine receptors on cells of the immune system, resulting in increased cell migration towards the infection/inflammatory site. This effect has also been shown *in vivo* in a mouse model (98). In addition, LL-37 is capable of potently reducing the pro-inflammatory activities of bacterial endotoxin/LPS (and other bacterial products) at physiological concentrations of salt (113). This effect is not solely dependent on direct binding to the target (93, 101) and emphasises a complex immunomodulatory role of this peptide.

Two recent studies demonstrated that LL-37 appeared to function as an inhibitor of human neutrophil apoptosis (9, 71), an effect regarded as being

pro-inflammatory as it prolongs the lifespan of the phagocytes. Interestingly, in one of the papers the authors noted an increased population of cells characterised as necrotic as compared to untreated cells (9) and this finding has now been extended in **Paper III**. We investigated whether the membrane changes following apoptosis, including the externalisation of PS, could give rise to a specific LL-37-induced permeabilisation of apoptotic cells as compared to viable cells. A mixed population of viable and apoptotic neutrophils were labelled for assessment of PS exposure (Annexin V) and membrane integrity (7-AAD; Figure 2). When LL-37 was added, a very specific permeabilisation of the apoptotic cells was noted (Figure 2). The observed effect was apparently analogous to the direct antimicrobial effect of LL-37 rather than the immunomodulatory, mostly receptor dependent effects in that it was rapid, independent of cell signalling and could be inhibited by serum components (**Paper III**). The specific permeabilising activity of LL-37 was also recorded for apoptotic NK cells, suggesting that the observed mechanism is independent of cell type. We reasoned that the specificity could be due to the presence of PS in apoptotic membranes, conferring negative surface charge and thus in a way mimicking microbial membranes.

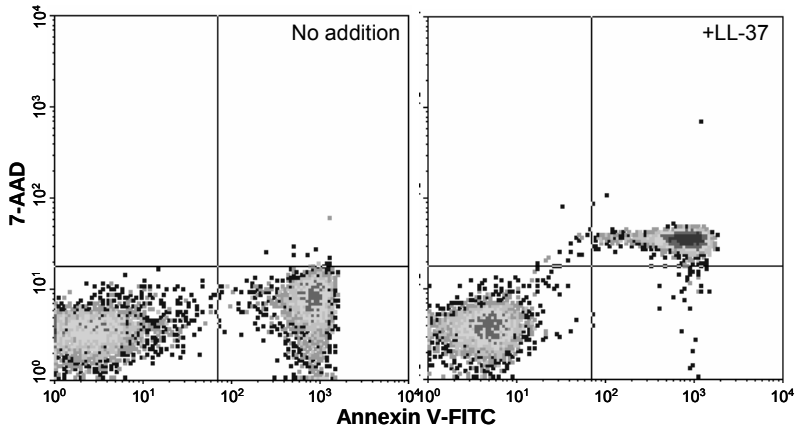


Figure 2. Neutrophils incubated over night will display a viable population (lower left quadrant) and an apoptotic population (lower right quadrant) as shown in the left density plot. When LL-37 is added to this mixed population, only the apoptotic cells will be permeabilised, thus staining positive for 7-AAD (upper right qudrant) as depicted in the right density plot.

However, the observed specificity of the permeabilisation was not directly dependent on PS, suggesting that some other membrane alteration is important. Actual permeabilisation of apoptotic neutrophils was shown also by measuring the release of intracellular and intragranular proteins (**Paper III**) which could influence the balance between anti- and proinflammatory responses and also be of importance for termination of the acute inflammatory response. However, the clearance of these permeabilised apoptotic cells by macrophages has actually been found to be equal to or even greater as compared to the clearance of intact apoptotic neutrophils (Hsin-Ni Li *et al.*, manuscript in preparation), perhaps limiting the damage of these leaky apoptotic cells.

LL-37 in Skin Defence

Several antimicrobial peptides, including LL-37, have been implicated as important for an effective and successful defence of the skin, and animal models have strengthened this hypothesis (77). The LL-37 found in the skin could initially be produced locally by epithelial cells, but when neutrophils are recruited to an inflammatory site these cells will heavily contribute to the LL-37 present at the site. Increased or decreased levels of LL-37 have been demonstrated in several inflammatory skin diseases, including psoriasis and atopic dermatitis (74, 79). The amount of LL-37 is higher in psoriasis than in atopic dermatitis, perhaps explaining why atopic dermatitis patients are more prone to skin infections than patients suffering from psoriasis. The LL-37 level in psoriatic skin has been estimated to be as high as 304 μM (> 1000 μg/ml) (79). However, LL-37 is not the only antimicrobial peptide found in elevated levels in psoriatic skin. The number of gene copies for β-defensins is increased in psoriasis patients (48).

In skin, the physiological conditions (absence of serum and pH around 5) are such that direct antimicrobial action of LL-37 could be anticipated to occur, and antimicrobial peptides are thought to be important for maintaining the skin free from infection. Besides the direct effect on microbes, the more indirectly microbicidal immunomodulatory action of LL-37 is also of importance for maintaining the barrier against potentially harmful microorganisms. Upon a threatening infection or skin injury, epithelial cells can increase the production of antimicrobial host defence peptides. If decreased levels of LL-37 in the skin result in infections, as in atopic dermatitis, too high levels of the peptide or alternative processing may be equally bad, resulting in inflammatory pathogenesis. The mature LL-37 peptide found in human epidermis is formed when hCAP-18 is cleaved by proteinase 3, elastase or stratum corneum tryptic enzyme (SCTE) (109, 124). LL-37 can then be further proteolytically processed by SCTE into shorter peptide fragments. A recent study demonstrated that LL-37 is expressed at very high levels in the facial skin of patients suffering from the inflammatory skin disease rosacea (123). The SCTE-processed LL-37 fragments also differed from those found in healthy individuals, resulting from an up-regulation of SCTE in the epidermis. Injecting rosacea-specific

peptides into the skin of mice increased the inflammation in mouse skin, and the same outcome was observed when adding SCTE or increasing the activity of the protease in mice (123). This study clearly shows the important role for LL-37 in skin inflammatory states and that increased levels of LL-37 and LL-37-derived peptide fragments can exert too potent pro-inflammatory effects resulting in chronic inflammation. Yet another intriguing finding on the role of LL-37 in psoriasis was recently presented, showing that the peptide can interact with immunologically inert self-DNA to form condensed structures (60). These complexes were shown to be highly immunoreactive and potently triggered interferon production, a hallmark of psoriasis, from plasmacytoid DCs using mainly TLR9 that normally recognise non-self DNA exclusively. Whether this novel mechanism applies also to other autoimmune and/or inflammatory conditions remains to be determined, but it is an interesting phenomenon especially when put in context of the permeabilisation of apoptotic neutrophils by LL-37 as described in **Paper III**. Upon the permeabilisation of apoptotic cells by LL-37, DNA will be free to leak out from the apoptotic cells with the potential formation of highly reactive self DNA:LL-37 complexes.

In addition, LL-37 has been implicated in wound healing and when secreted from epithelial or inflammatory cells at wound sites, the peptide stimulates the proliferation of endothelial cells and angiogenesis, processes which are crucial for successful tissue repair and wound healing. LL-37 has been shown to directly activate the endothelial cells at wound surfaces via FPRL1 (57). Also, in an *in vitro* based, organ cultured skin wound system, re-epithelialisation and wound closure could be inhibited by the addition of LL-37-directed antibodies (47). The level of LL-37 increases in healthy skin in response to injury, whereas no change is seen in chronic non-healing ulcers.

LL-37 Deficiencies and Animal Models

In morbus Kostmann, a severe disorder caused by a recessive mutation in the gene encoding the mitochondrial protein HAX1 (54), patients suffer from severe congenital neutropenia and arrest in the bone marrow of neutrophil maturation at the promyelocyte/myelocyte level. Patients treated with granulocyte colony-stimulating factor are improved and their neutrophils mature. The mature patient cells are, however, devoid of LL-37, and patients suffer from frequent, severe infections and periodontal disease (13, 88). Also the saliva of patients with morbus Kostmann lacks LL-37, suggesting an important role for LL-37 *in vivo* and a plausible explanation to the periodontal disease. Whether this effect is dependent upon direct antimicrobial actions or indirect, immunomodulatory actions has not yet been determined (13, 88).

Many of the mammalian species equipped with cathelicidins express more than one gene encoded form, mouse and human being exceptions.

Cathelicidin-related antimicrobial peptide (CRAMP) is the only cathelicidin found in mice, and consequently this animal has been extensively used as an *in vivo* model for studying the effects of LL-37 and cathelicidins. CRAMP shows several similarities to LL-37; they are encoded by similar genes, they are both α-helical, and have comparable antimicrobial spectrums as well as tissue distributions. The CRAMP knock-out mouse has no obvious phenotype when housed under aseptic barrier-controlled conditions, but has a reduced capacity to handle skin infections caused by group A streptococci (77). CRAMP deficient mice also have an impaired defence against microbial infections in the urinary tract, as well as a defective innate immune response in the intestine against colonisation with bacterial pathogens (26, 50).

A model using airway epithelium from cystic fibrosis (CF) patients that was subsequently grown as bronchial xenografts in mice showed that the airway surface fluid from CF patients failed to kill the bacteria included in the study. When the level of LL-37 was increased in the airway surface fluid, also the fluid from CF patients successfully eradicated the bacteria (6). Overexpression of LL-37 in the murine lung augmented the defence against infection with *P. aeruginosa* and when LL-37 was expressed systemically, the survival rate of the animals increased in the presence of intravenous injections with LPS or *Escherichia coli* (7).

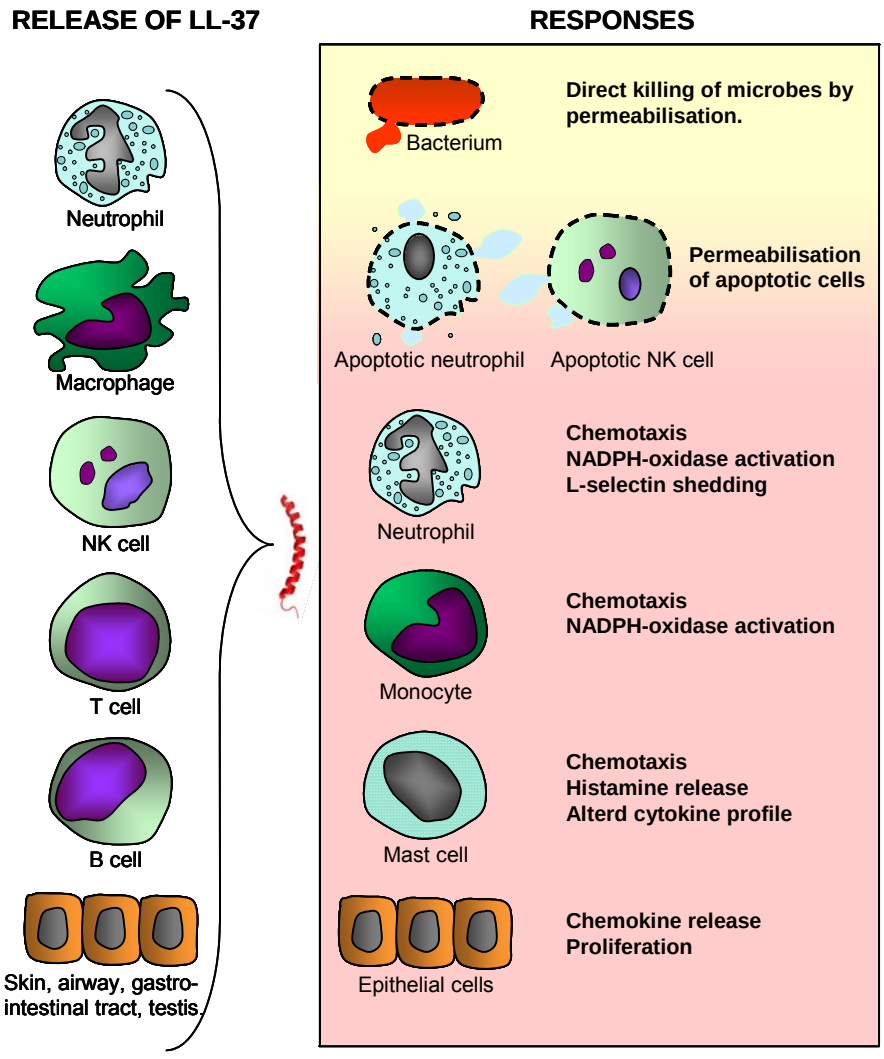


Figure 3. Examples of LL-37 effects on membrane integrity and innate immunity. Details are given in the text.

FUNCTIONAL DUALISM

Introduction

The functional dualism of the major antimicrobial host defence peptides in man has been described above; both defensins and LL-37 display indirect killing of microbes through their immunomodulatory functions as well as direct antimicrobial activities through their membrane perturbing action. The chemokines, cytokines with chemotactic properties, are another class of molecules with corresponding dual actions and there are similarities between chemokines and antimicrobial peptides, especially the defensins. Consequently it has been shown that many chemokines display modest antimicrobial activity, resembling the activity of defensins (127), while some antimicrobial peptides bind to chemokine receptors and induce a chemotactic response (110). Carboxy-terminal-derived antibacterial peptides from other chemokines, besides the IL-8 derived peptide described in detail below, have been described, including peptides derived from CXC chemokine ligand 4 (CXCL4), CXCL7, CXCL9, CXCL10 and CXCL11 (125). Also some full-length CXC-chemokines exhibit antibacterial activities in addition to their immunomodulatory properties (28, 127). The use of G-protein coupled receptors by antimicrobial peptides/defensins to induce chemotaxis of various leukocytes have been suggested as yet an indicator of the similarities and functional overlap that exist between these two players in immunity, as the same receptors are used by CXC-chemokines (125).

The tertiary structure is similar between defensins and chemokines, and the defensins have been called “microchemokines” (80). However, they display no homology at the amino acid level and their evolutionary relationship, if any, is unclear. It is plausible that some antimicrobial peptides/host defence peptides have evolved from deletion products of CXC-chemokines (125). The immunomodulatory and the antimicrobial part of molecules displaying these dual actions can often be structurally separated. This feature is observed for the porcine cathelicidin PR-39 (23) as well as for the human cathelicidin LL-37 in which the antimicrobial part (against skin pathogens including bacteria, viruses and fungi) is distinct from the immunomodulating part (assessed as the capability of LL-37 to induce keratinocytes to produce IL-8; 20). Amino-terminal truncated forms of LL-37 were most potent as antimicrobials while full-length LL-37 displayed the highest potency for inducing IL-8 production (20). Indeed, shorter fragments of LL-37 have been found *in vivo*, e.g., in inflammatory skin; the importance of these alternative cleavage products is still unclear (123).

The primary amino acid sequence of an antimicrobial peptide can often be changed with maintenance of antimicrobial activity, as long as important features of the peptide like amphipathicity and α-helical structure are retained. However, the majority of the immunomodulatory actions of the peptide will most likely disappear under the same conditions (**Paper I**).

This, of course, results from the fact that the immunomodulatory actions are mainly dependent on highly specific receptor interactions that will not allow any tampering on the primary sequence of the peptide, while the direct antimicrobial mode of action is less specific (**Paper I**).

The Chemokine IL-8 as a Dual Function Molecule

The CXC subfamily of chemokines consists of a number of different monocyte and granulocyte activating proteins including CXCL8, also termed IL-8 (92). This interleukin is generated and secreted by several different types of inflammatory cells as well as by epithelial cells and fibroblasts. IL-8 is not only a very potent endogenous monocyte/neutrophil chemoattractant but also an effective secretagogue and activator of the phagocyte NADPH-oxidase, inducing a release of a large number of proteases and reactive oxygen metabolites (4, 59). IL-8 was previously believed to be stored in the secretory vesicles of neutrophils (58), but recent work in our lab has shown that IL-8 is stored in a compartment different from the classical granules and the known non-classical organelles (86). Furthermore, newly synthesised IL-8 is partly subject to constitutive release and partly present in an organelle that was refractory to secretion even using potent secretory stimulation (86). The full-length form of IL-8 has several important effects on the innate immune response, the chemotactic activity exerted on neutrophils being its most prominent feature. The activities of IL-8 on cells of the immune response are accomplished by binding to cell surface G-protein coupled receptors CXCR1 and 2. In addition to the chemotactic effect, IL-8 may also activate the neutrophil NADPH-oxidase with resulting production of reactive oxygen species, working as a pro-inflammatory agent. IL-8 is thus an important regulator of the innate immune response, and it has been shown that it can remain in inflammatory tissue for days in an *in vivo* situation (90).

In **Paper II**, we describe a novel antimicrobial peptide derived from the carboxy-terminus of IL-8. This chemokine is a homodimeric protein, each part consisting of 72-77 amino acids arranged in three β-sheets and one carboxy-terminal α-helix. Upon closer examination of the α-helical portion of IL-8, we noticed that it shared many properties with antimicrobial peptides: the small size (18 amino acids), the α-helical structure and the division of amino acids into distinct hydrophobic and hydrophilic parts in an amphipathic arrangement. To investigate possible antimicrobial activity, a synthetic peptide corresponding to this region was tested against different bacterial strains in a radial diffusion system. Indeed, the peptide had antimicrobial activity against several bacterial species, including *E. coli*, *Klebsiella pneumoniae* and *Streptococcus pyogenes*. The peptide could be generated by *in vitro* acid hydrolysis of full-length recombinant chemokine, but lacked the pro-inflammatory activities exerted on neutrophils by full-length IL-8. The IL-8 sequence contains several sites suitable for proteolytic cleavage by common cellular proteases, indicating that the antimicrobial

fragment could be generated *in vivo* and maybe contributes to direct microbial killing (**Paper II**).

The *in vivo* relevance of the antimicrobial IL-8 peptide remains to be determined, and it has not yet been confirmed that it is formed *in vivo*. The α-helical part of IL-8 in the full-length chemokine is most probably shielded or hidden by other parts of the molecule, thus making the interaction with the membrane of the target microbe impossible, but it should be noted that also the full-length form of IL-8 has been reported to display virtually the same antimicrobial action as the IL-8 peptide (131). We have not been able to confirm any antimicrobial activity of recombinant full-length IL-8 and when tested in a liposome system constructed to mimic bacterial membranes; the full-length form displayed virtually no lytic activity at all while the IL-8 derived peptide resulted in more than 50% release of the intra-liposomal calcein dye at the same concentration (Figure 4). The discrepancy between our results and those showing antibacterial activity of full-length IL-8 (131) could potentially be due to the use of different assays or assay conditions.

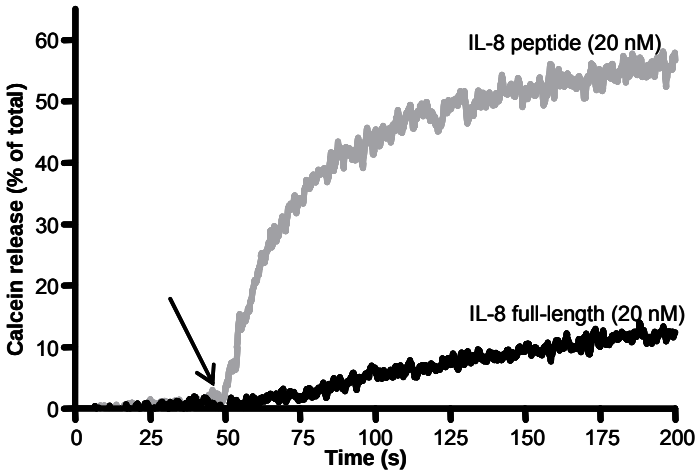


Figure 4. Liposomes synthesised to resemble bacterial membranes were challenged with the IL-8 peptide or full-length IL-8. Leakage of an intraliposomal dye was recorded. The peptide was much more potent in permeabilising the liposomes as compared to the full-length protein.

The granulocyte chemotactic protein 2 (GCP-2 or CXCL6) is a CXC chemokine produced by macrophages and epithelial cells that activates neutrophils and also has antibacterial properties in its full-length form (67). The organisation of the protein highly resembles that of IL-8 with three β-sheets and a carboxy-terminal located α-helix. However, for this protein the antibacterial activity relies on the whole protein, and fragments comprised of the β-sheets or the α-helix had reduced antibacterial activity as compared to the full-length form (67). This suggests that several different structural motifs can be responsible for the observed antimicrobial activity of chemokines.

As mentioned above, the pro-inflammatory activities of full-length IL-8 were absent in the peptide (**Paper II**). This was not so surprising since three amino acids at the amino-terminal, the so called ELR-motif (glutamic acid – leucine – arginine), have been shown to be essential for receptor binding (90). Thus the IL-8 peptide should not be thought of as a host defence peptide since no immunomodulatory activities are known. However, the CXC-chemokine IL-8 should be regarded as a "host defence protein" with dual functions, possessing immunomodulatory activities in its full-length form and, when cleaved, having the ability to kill microbes directly.

CONCLUDING REMARKS

The assays commonly used for investigating antimicrobial properties of peptides and for analysing immunomodulatory properties are *in vitro* systems. These experimental conditions do not necessarily correspond to the environmental conditions encountered *in vivo*; e.g., the ionic composition, as well as the presence of serum proteins, proteases and anionic polysaccharides may differ and as a consequence the functional properties of the peptides may be altered. In addition, the concentration of the peptides encountered in an *in vivo* situation is also important when making predictions as to whether these peptides are primarily antimicrobial, immunomodulatory or both. Often much higher concentrations are used for *in vitro* antimicrobial assays than those actually encountered physiologically. On the other hand, *in vitro* based studies do not consider possible synergistic actions between antimicrobial peptides or proteins or the contributing effects of growth factors or cytokines that can be found at certain locations *in vivo*. This is illustrated by the fact that α-defensins are sensitive to physiological concentrations of divalent cations and LL-37 looses its microbicidal activity against *S. aureus* and *S. typhimurium* in the presence of physiological salt concentrations (19, 63). The antimicrobial activity of host defence peptides is often inhibited by serum components, such as serpins, α2-macroglobulin and complement factors, whereas the immunomodulatory functions remain intact under these conditions (116). The antimicrobial activity of LL-37 is also reduced in the presence of various serum proteins including apoplipoprotein A1 and HDL (**Paper III**, 62, 118).

It is worth noticing that antimicrobial host defence peptides are often able to induce chemotaxis, angiogenesis and cytokine production under physiological salt conditions and in the presence of serum. There are difficulties distinguishing between direct (antimicrobial) and indirect (stimulation of immunity) host defence mechanisms *in vivo* (16). However, in one study a synthetic, modified antimicrobial peptide, devoid of direct antimicrobial activity, was still able to protect mice against certain bacterial infections, an effect most probably achieved through its immunomodulatory properties leading to an enhanced immune response (99). The peptide concentrations needed for immunoregulatory activities is also generally lower than the concentrations needed for direct antimicrobial activity. Together, these facts suggest that the primary function for antimicrobial host defence peptides *in vivo* is likely immunomodulatory rather than directly antimicrobial.

A direct antimicrobial role for host defence peptides may however be obtained inside the phagosomes of neutrophils where direct killing of microbes by HNP 1-3 can be anticipated. In the dermis/epidermal layer, and in urine, the conditions with respect to protein and salt contents probably allow for direct antimicrobial activity. Accordingly, the same antimicrobial host defence peptide could have different roles depending on where in the

body they are present (16). The fact that antimicrobial peptides are ancient and widely spread in nature indicates that they serve several important functions in the immune response, and loss of these peptides would impair the organism. Despite much interest in mammalian antimicrobial peptides, the exact nature of their *in vivo* function(s) is lacking and whether they are most important as direct antimicrobial effectors or immunomodulators is still debated. Future studies will hopefully bring clarity to these matters.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Varje dag utsätts våra kroppar för bakterier och virus (mikrober) som hotar att ta sig in i kroppen och orsaka sjukdom, men endast undantagsvis blir vi faktiskt sjuka. Detta beror på att vi utvecklat ett effektivt svar på dessa attacker; immunförsvaret. Hos enkla organismer, t.ex. encelliga organismer som amöbor, utgörs försvaret av lösliga substanser (proteiner) som kan frisättas vid behov. Hos mer komplicerade organismer (inklusive människan) består detta försvar av flera olika delar med fysiska barriärer (hud, slemhinnor), lösliga komponenter och celler (exempelvis vita blodkroppar) som viktiga delar. Den fysiska barriären stänger i ett oskadat tillstånd ute det stora flertalet av sjukdomsframkallande mikrober. Exempel på proteiners funktioner i immunförsvaret är förmåga att kalla till sig och aktivera vita blodkroppar så att dessa kan bekämpa infektionen. Andra proteiner eller peptider (korta proteiner) kan direkt döda och oskadliggöra bakterier genom att göra hål i deras cellmembran; s.k. permeabilisering, och på så sätt hjälpa till med att stoppa infektionen. Dessa peptider brukar med ett gemensamt namn kallas antimikrobiella peptider. De vita blodkropparna utgör en mycket viktig del i försvaret, och det finns många olika typer. Jag har särskilt använt mig av en typ som är viktig för att hålla en hotande infektion i schack under ett tidigt skede; neutrofilen. Vanligtvis far neutrofilen runt i vår blodbana i ett icke-aktivt tillstånd. I blodbanan är neutrofilen den allra vanligaste förekommande celltypen och utgör 60-70% av alla vita blodkroppar.

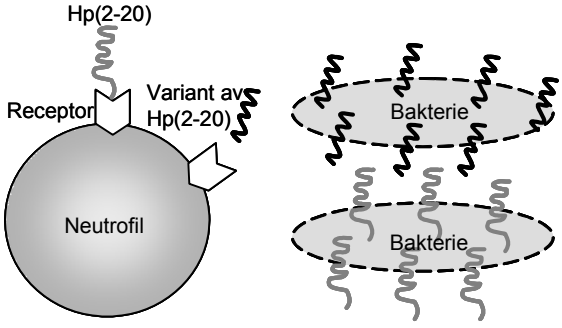
Då man exempelvis skär sig i fingret uppstår det en skada i den fysiska barriären där mikrober kommer att kunna ta sig in i våra kroppar. I såområdet kommer det att frisättas olika substanser, både från den skadade vävnaden och från eventuella bakterier som tagit sig in. Dessa substanser kommer att varsla de vita blodkropparna inklusive neutrofilen att något har hänt ute i vävnaden. Neutrofilen kommer då att aktiveras och tar sig ut i vävnaden genom att klämma sig ut ur blodkärlet och krypa fram till skadan där cellen aktiveras ytterligare. Neutrofilen är en s.k. professionell fagocyt vars främsta uppgift i kroppen är att fagocytera (äta upp) skräp och främmande material (bakterier) och på så sätt oskadliggöra dessa. För att göra detta är neutrofilen laddad med en massa små blåsor som innehåller alla proteiner den behöver. Många utav dessa proteiner är givetvis toxiska för att kunna användas i avdödningen av bakterier, och de kan också vara giftiga och skadliga för våra egna celler och vävnader om de frisätts okontrollerat till omgivningen. För att undvika detta sker avdödningen efter det att mikroben fagocyterats och inneslutits i en blåsa inuti neutrofilen. Till den blåsan frigörs sedan de proteiner som avdödar mikroben. Efter att neutrofilen fagocyterat och dödat mikroben har den fullgjort sina uppgifter och måste städas bort på ett sådant sätt att omgivningen påverkas minimalt. Därför kommer en neutrofil som fagocyterat att genomgå programmerad celldöd, eller apoptos, och cellskelettet fagocyteras sedan i sin tur av en annan vit blodkropp, oftast makrofagen. En annan typ av celldöd som ger upphov till större, skadligare effekter på omgivningen är nekros. En

nekrotisk cell har inte längre ett intakt cellmembran varför intracellulära beståndsdelar, inklusive toxiska proteiner, kommer att kunna läcka ut i omgivningen och där orsaka skada.

Om inte det tidiga immunförsvaret beskrivet ovan är tillräckligt för att kontrollera infektionen, kommer en ytterligare aktivering av det specifika immunförsvaret att ske med resulterande cellaktivering och specifik igenkänning och eliminering av infektionsorsakande agens som följd.

I mitt avhandlingsarbete har jag studerat de antimikrobiella peptiderna. Dessa korta proteiner uppmärksammades först för att de effektivt kan avdöda bakterier enligt beskrivning ovan, men idag vet man att de också har andra funktioner i immunsystemet. Många utav våra antimikrobiella peptider har förmåga att kalla till sig och aktivera vita blodkroppar, stimulera sårläkning osv. och de beskrivs idag som ”försvarspeptider” (host defence peptides) med dubbla funktioner (functional dualism).

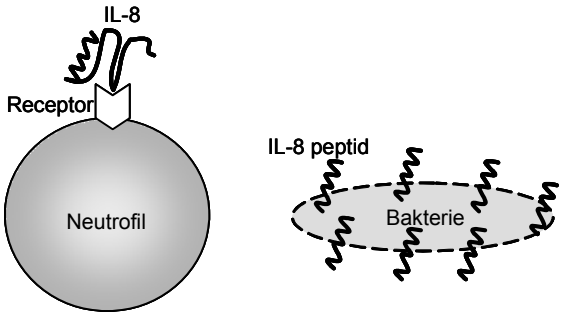
I. I det första arbetet tittade vi på en antimikrobiell peptid från magsårsbakterien *Helicobacter pylori*, Hp(2-20). Den här peptiden tillverkades tillsammans med varianter av Hp(2-20). Varianterna innehöll små förändringar som resulterade i att peptidens struktur antingen blev störd eller stabiliserad. Vi tittade på peptidernas förmåga att döda bakterier och såg att de stabiliserade peptiderna dödade bakterier ännu bättre än vad Hp(2-20) gjorde, medan de destabiliserade varianterna var sämre på det. Vi undersökte också hur väl peptiderna kunde aktivera neutrofiler och såg att endast Hp(2-20) kunde göra detta. Av de här resultaten drog vi slutsatserna att man kan göra en peptid mer potent i att avdöda bakterier genom att stabilisera den struktur som krävs för avdödning, men att denna stabilisering inte ger någon ökad aktivering av immunceller. Även små förändringar riskerar att göra peptiden verkningslös i att aktivera celler. Detta beror på att cellaktiveringen går via en mycket specifik interaktion mellan den struktur på cellen som känner igen peptiden (receptorn) och peptiden. Den direkta avdödningen kräver således en mindre specifik struktur på peptiden än vad cellaktiveringen gör.



Endast Hp(2-20) kan aktivera celler (specifik interaktion).

Både Hp(2-20) och stabila varianter kan döda bakterier.

II. I det andra arbetet identifierade vi en ny antimikrobiell peptid som härstammade från det större proteinet IL-8. IL-8 är en molekyl som är mycket viktig för att kalla till sig vita blodkroppar, inklusive neutrofiler, till en infektion. Vi såg att den ena änden på IL-8 hade en sådan struktur att den liknade en antimikrobiell peptid och lät tillverka den delen. Peptiden visade sig ha antibakteriella effekter precis som vi antagit. Den bakteriedödande effekten fanns inte hos fullängds IL-8. Peptiden saknade dock den förmåga att aktivera neutrofiler, t.ex. att kalla till sig dessa celler, som fullängds IL-8 har. IL-8 kan ses som en bifunktionell molekyl som då den klyvs kan verka även bakterieavdödande utöver de cellaktiverande egenskaper den har.

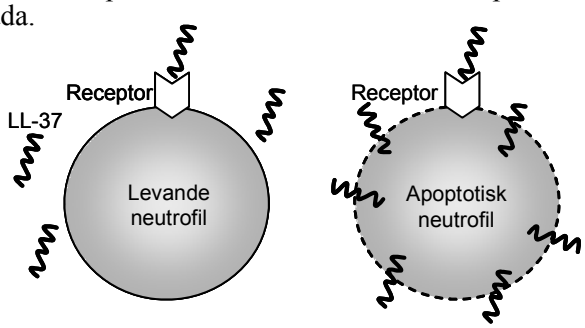


Endast IL-8 kan aktivera neutrofiler.

Endast IL-8 peptiden avdödar bakterier.

III. I arbete tre har vi undersökt den antimikrobiella försvarspeptiden LL-37. LL-37 återfinns i de celler i kroppen som först stöter på invaderande bakterier; de vita blodkropparna, inklusive neutrofiler, samt i celler som utgör den fysiska barriären och möter yttre miljö dvs. hud och slemhinnor. Då bakterier hotar att bryta sig igenom den fysiska barriär vi har mot infektioner, kommer LL-37 att frisättas från dessa celler och ut i omgivningen. Där kan LL-37 fungera genom att antingen direkt döda bakterien genom permeabilisering, eller fungera mer indirekt genom att kalla dit och aktivera celler från immunsystemet som kan hjälpa till i bekämpningen av bakterien. Det tredje arbetet beskriver en helt ny typ av permeabilisering som LL-37 uppvisar, utöver den tidigare kända permeabiliseringen av bakteriers membran. Det är sedan tidigare känt att celler som genomgått apoptos har annorlunda cellmembran jämfört med celler som fortfarande lever, bland annat kommer deras cellmembran att bli mer negativt laddade. Apoptotiska celler kommer då att likna bakteriers cellmembran som är mer negativt laddade än våra levande cellers membran. Som en följd av detta ville vi undersöka om LL-37 kunde permeabilisera apoptotiska celler på samma sätt som bakterier permeabiliseras, och samtidigt lämna levande celler intakta. När LL-37 sattes till en blandad population med levande och apoptotiska neutrofiler blev endast de apoptotiska cellerna permeabiliserade. Den här effekten berodde inte på receptorer eller på signalering i neutrofilen. Permeabilisering av apoptotiska celler torde kunna medföra ett farligt läckage av toxiska substanser ut i

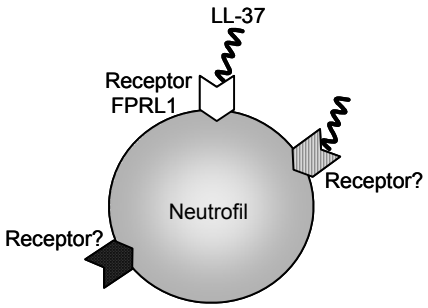
omgivningen och på så sätt bidra till att skapa inflammation och vävnadsskada.



Levande neutrofilers membran påverkas inte av LL-37.

Apoptotiska neutrofiler (celler) permeabiliseras av LL-37.

IV. I det avslutande arbetet har vi undersökt LL-37s effekter på levande neutrofiler och vilka receptorer LL-37 binder till för att aktivera celler. Vi fann att LL-37 band till FPRL1, en sedan tidigare känd receptor för peptiden på neutrofiler, och aktiverade cellerna genom denna receptor. Utöver FPRL1 fann vi ytterligare minst en receptor på neutrofiler för LL-37. Denna/dessa receptorers identitet är fortfarande okänd, men ansvarar för en del av den aktivering LL-37 kan ge upphov till i en neutrofil.



LL-37 kan aktivera neutrofiler via minst två olika receptorer; FPRL1 och ytterligare en/flera okända receptorer.

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