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The role of the multifunctional peptide LL-37 in host defense

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TABLE OF CONTENT

1. ABSTRACT

Neutrophil granules contain several antimicrobial peptides (AMPs) that are important effector molecules of innate immunity. In mammals, the main families of these peptides are the cathelicidins and defensins. Several defensins have been characterized in humans, while there is only one human cathelicidin, designated LL-37. This peptide is stored in specific granules of neutrophils in an inactive proform, which is processed extracellularly to the mature active peptide LL-37 and the propart cathelin after neutrophil degranulation. Apart from exhibiting a broad antimicrobial spectra, it is now evident that LL-37 possesses several additional functions that are related to host defense. Examples of such functions are chemotactic, endotoxin neutralizing, angiogenic and wound healing activities. These effects of LL-37 reveal a role as a mediator between innate and adaptive immunity. This review is giving an overview of the different immunological effects exerted by LL-37 and the physiological significance of these functions in immunity.

2. INTRODUCTION

The granules of neutrophils contain several antimicrobial peptides (AMPs) that possess microbicidal activities. These peptides are secreted upon exocytosis to kill extracellular pathogens or act in phagosomes to destroy ingested bacteria. Together with bactericidal radicals and proteins in neutrophil granules, AMPs comprise a diverse array of defense molecules that constitute a substantial part of our innate immune system (1). Apart from the direct microbicidal activity of AMPs, there is growing evidence that these peptides also exhibit immunumodulatory activities. Examples of such activities are recruitment of inflammatory cells, stimulation of epithelial proinflammatary factors, antiendotoxin effects, stimulation or inhibition of apoptosis, modulation of dendritic cell (DC) function and stimulation of wound healing (reviewed in 2-4). These new insights strongly indicate the importance of AMPs in the interplay between innate and adaptive immunity. Although AMPs exhibit high diversity in structure, they share some common features such as a

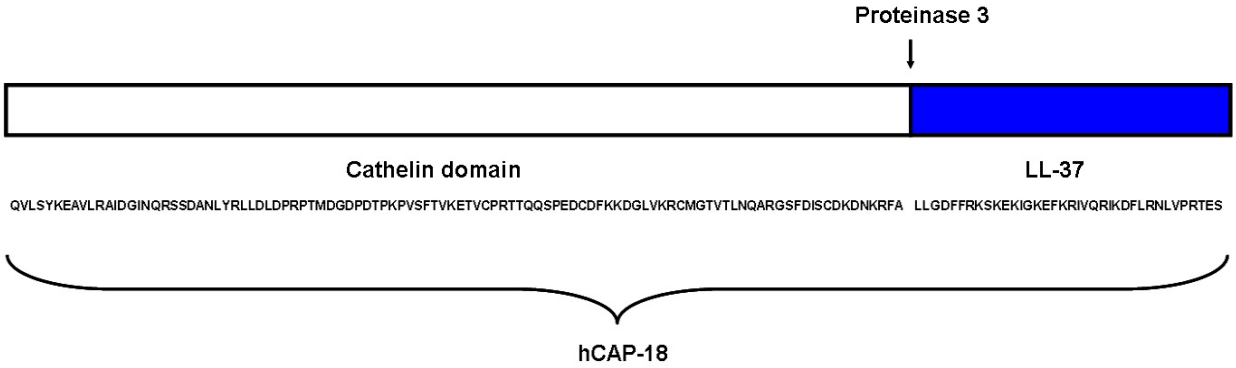

Figure 1. The primary structure of hCAP-18; the proform of LL-37 with the cathelin domain and the C-terminal antimicrobial domain (LL-37).

positive net charge, a size between 30-45 amino acid residues and an amphipatic character (5). The mammalian repertoire of antimicrobial peptides is mainly the defensins and the cathelicidins; the defensins have a characteristic beta-sheet fold with a framework of six disulphide link cysteines (6), whereas the cathelicidin peptides are diverse in structure but share a conserved anionic N-terminal propart (cathelin) (7).

3. STRUCTURE AND PROCESSING OF LL-37

LL-37 is the only cathelicidin peptide identified in humans, found predominantly in neutrophils and various epithelial linings (8, 9), but also present in monocytes, NK-cells, lymphocytes and mastcells (10, 11). The name LL-37 derives from the first two leucine (L) residues at the N-terminus and the number of amino acids (37) constituting the peptide. However, LL-37 was originally predicted from a cDNA clone isolated from a human bone-marrow library as a 39-residue peptide named FALL-39. This putative peptide was based on a typical processing site located after two basic residues (12). Later, the mature active peptide was isolated from degranulated neutrophils and found to be two residues shorter at the N-terminus, hence the name was corrected to LL-37 (9). hCAP-18 is the name of the inactive proform of LL-37 and has an approximate mass of 18 kDa (Figure 1). This inactive proprotein is stored in specific granules of neutrophils at a molar concentration as high as lactoferrin (40 μmol or 630 μg/10^9 cells), constituting a substantial part of the proteins in specific granules of neutrophils (13, 14). Three known proteases in neutrophils (elastase, cathepsin G and proteinase 3) have been shown to cleave hCAP-18 *in vitro*. However, only the serine protease, proteinase 3, has been found to cleave the two-domain protein into the mature peptide and the cathelin part in exocytosed material from neutrophils (15). Proteinase 3 is stored in peroxidase positive granules of the neutrophils, well separated from hCAP-18 in specific granules. These two proteins are associated either in phagolysozymes formed during the phagocytic process or extracellularly upon degranulation. Despite that both hCAP-18 and proteinase 3 have been detected in phagolysozymes, detailed analyses indicate that the processing of hCAP-18 occurs only outside the neutrophils (15). This suggests that the environmental conditions are important for the activity of proteinase 3 and hence processing of hCAP-18. Although proteinase 3 has been reported to be the only neutrophil-derived protease important in processing hCAP-18 recent data suggests that also kallikrein (16) and gastricsin (17) can generate peptides of different sizes of hCAP-18. Shorter forms of LL-37 (RK-31, KS-30, LL-29, KS-22 and KR-20) have been detected in sweat (18) or skin (16) whereas in seminal plasma, a longer form (ALL-38) (17) is generated at low pH, suggesting that additional antimicrobial peptides are generated from hCAP-18 by postsecretory processing. Interestingly, these derivatives of hCAP-18 possess different antimicrobial and immunomodulatory properties compared to LL-37, broadening the functional spectra of one gene product.

4. FUNCTIONS OF LL-37 IN IMMUNITY

4.1 Antimicrobial and cytotoxic activities

4.1.1. *In vitro*

LL-37 was characterized by Gudmundsson *et al.* in 1996 (9), and since then detailed data about its direct antimicrobial properties has appeared in the literature. The majority of these studies are performed *in vitro* using the synthetic peptide that has to be considered when interpreting the physiological role of LL-37. The reported antimicrobial activities of LL-37 vary in several studies, indicating that the activity is sensitive to differences in the assay conditions, such as salt, pH and the phase of bacterial growth. However, under most laboratory conditions, LL-37 has a broad range of activity against both gram-negative and gram-positive bacteria. Among the microbial strains that have been analyzed; *Escherichia coli, Salmonella typhimurium* and *Neisseria gonorrhoeae* have been found to be most sensitive with MIC values of 0.6-7.6 μg/ml (0.1-2 μM) (19), 0.4-3.6 μg/ml (0.1-0.8 μM) (19) and 0.9 μg/ml (0.2 μM) (20), respectively. Furthermore, LL-37 exhibits potent activity against *Pseudomonas aeruginosa* with a MIC value of 0.9-5.7 μg/ml (0.2-1 μM) (19) and group A Streptococcus with a MIC value of 5-72 μg/ml (1-16 μM) (21), whereas low activity has been detected against the yeast *Candida albicans* (MIC >250 μg/ml or 56 μM) (19). LL-37 has also been shown to possess some antiviral activity against herpes simplex virus (22) and vaccinia

virus (23), making it a promising broad spectrum antimicrobial agent for therapeutic use. A correlation between the antimicrobial activity and the structure of LL-37 has been observed, where a higher extent of alpha-helical conformation is beneficial for its ability to kill both gram-negative and gram-positive bacteria (24). Environmental factors that promote the alpha-helical conformation of LL-37 are physiological pH and anions, such as HCO_3^-, SO_4^{2-}, or $CF_3CO_2^-$. It was also found that LL-37 adopts an alpha-helical structure in salt solutions mimicking plasma-, intracellular- and interstitial-fluid, suggesting optimal antimicrobial activity *in vivo*. Like most other antimicrobial peptides, LL-37 kills microbes by disrupting their membrane, resulting in lysis. By using solid-state NMR data and differential scanning calorimetry, Henzler Wildman and colleagues proposed a mechanism of the killing process, where LL-37 covers the surface of the membrane, resulting in toroidal pore formation with subsequent leakage of the cytoplasmic content (25). In addition to kill microbes, LL-37 can also destroy eukaryotic cells. This cytotoxicity of LL-37 has been observed *in vitro* to different blood cells, including both erythrocytes (26) and leukocytes, where T-cells are most sensitive (24). However, the cytotoxic concentrations (>58 μg/ml or 13 μM) are in general higher than the concentrations required for elimination of micro-organisms, revealing a cell-selective killing mechanism. This selectivity can be explained by the fact that LL-37 has stronger affinity to microbes than eukaryotic host cells, due to differences in composition and charge of cell membranes (reviewed in 27). The harmful cytotoxic effects of LL-37 are substantially attenuated by serum components (24), such as apolipoprotein A but also other lipoproteins, which have been shown to bind to LL-37 (28, 29). This binding mechanism suggests an important role for serum lipoproteins in protecting human cells from damage caused by LL-37.

4.1.2. *In vivo*

The concentration of LL-37 in human serum is low, whereas hCAP-18 is present at higher levels (1.2 μg/ml or 0.3 μM) under normal conditions (14). However, the concentration of the mature LL-37 peptide increases in close vicinity to degranulating neutrophils (15). Furthermore, the levels of LL-37 can reach dramatically higher levels during inflammatory conditions, i.e. in psoriatic lesions with estimated concentration as high as 1366 μg/ml (304 μM) (30). Thus, the MIC values of LL-37 found *in vitro* are compatible with physiological concentrations during infectious and inflammatory disorders. Rather than being a soloplayer, LL-37 acts in concert with other antimicrobial components, i.e. beta-defensin 2 (30), lysozyme and lactoferrin (31), to exert optimal killing capacity. Since the reported MIC values are determined for LL-37 alone, there might be an underestimation of the antimicrobial activity during *in vivo* conditions, where synergistic effect to other antimicrobial components has to be considered. The role of LL-37 in disease has been demonstrated in several reports. Mice deficient in the gene encoding mouse cathelicidin related antimicrobial peptide (mCRAMP), the singel mouse cathelicidin, have been utilized to investigate the relevance of cathelicidin expression in infections. Since mCRAMP is similar to LL-37 in structure, tissue distribution and antimicrobial activity, the mCRAMP knockout model is a useful model for studying the function of the human cathelicidin. Interestingly, it has been found that the $mCRAMP^{-/-}$ mice were more susceptible to Group A Streptococcus, herpes simplex virus and vaccinia virus infections in skin than the wildtype mice (23, 32, 33), indicating a pivotal role of mCRAMP/LL-37 in antimicrobial host defense. The *in vivo*-role of LL-37 in preventing infections is also supported by an observation made in patients with morbus Kostmann (34). This disease is characterized by congenital neutropenia, which can be treated by GM-CSF (granulocyte-macrophage colony-stimulating factor) administration. Despite that the treatment with GM-CSF restore the neutrophil level, the patients still suffer from recurrent periodontitis (35). Intriguingly, it was found that this group of patients lack LL-37 in their neutrophils in the saliva, which may contribute to their increased susceptibility to oral infections (34). An additional strategy to determine the role of LL-37 in certain diseases is to overexpress LL-37. As example, an adenovirus vector was utilized to transfer the LL-37-gene systemically or into trachea of mice (36). Furthermore, the vector was inserted into human bronchial xenograft, derived from patients with cystic fibrosis (37). The overexpression of LL-37 resulted in an augmented bacterial killing in all these cases, further supporting a protective function of LL-37.

4.2. Chemotactic activity

4.2.1. Direct chemotactic activity

Recent data shows that LL-37 is involved in a variety of additional functions in host defense, including chemotaxis, suggesting a pivotal role of LL-37 as a link between innate and adaptive immunity. Detailed evidence shows that LL-37 is chemotactic *in vitro*. However, it must be stressed that *in vivo* experiments are required to confirm the relevance of chemotaxis. Formyl Peptide Receptor Like-1 (FPRL-1) is the only receptor that is found to activate direct migration of immunological cells, such as monocytes, neutrophils and T-cells, to a site of infection. The activation of this G-coupled receptor is dose-dependent, with maximum activity at concentrations of 0.5-50 μg/ml (0.1-11 μM) (38, 39). Intriguingly, activation of FPRL-1 requires relatively high concentrations of LL-37 compared to other classical chemoattractant agents, suggesting a low-affinity peptide-receptor interaction. This implies that cellular FPRL-1 mediated recruitment by LL-37 *in vivo* may be active only when a threshold concentration of the peptide is reached following upregulation of the LL-37 gene in epithelial cells or after massive release from invading neutrophils. Notably, the direct chemotactic activity of LL-37 is not affected by serum (38), as the microbicidal activity is (24). This discrepancy may be due to the fact that the chemotactic activity is receptor-mediated and not dependent on a peptide-membrane interaction. Presumably, the part of the LL-37 peptide that activates the FPRL-1 is not hidden or changed by association of serum components, indicating that recruitment of leukocytes by LL-37 is an important biological mechanism in the blood stream.

4.2.1. Indirect chemotactic activity

By stimulation of chemokine-production of several cells, such as epithelial cells, LL-37 exerts indirect chemotaxis of leukocytes. This effect is mediated via Epidermal Growth Factor Receptor (EGFR), which is present on airway epithelial cells and is transactivated by LL-37 at more than 10 μg/ml (2 μM) via metalloproteinase-mediated cleavage of membrane anchored EGFR ligands. Downstream signaling involves the MAPK/ERK pathway, leading to release of the potent chemoattractant IL-8 (40). Similar results were obtained for monocytes, showing that LL-37 mediates an IL-8-inducing effect via a G-protein coupled independent receptor activating ERK and p38 pathways (41). For this activation, a higher concentration of LL-37 was required (50 μg/ml or 11 μM). Interestingly, the presence of GM-CSF increased the magnitude of this activation, and significantly decreased the threshold amount for LL-37, suggesting a synergistic effect *in vivo*. Recently, also the purinergic receptor P2X7 was found to interact with LL-37, a receptor that is predominantly expressed on monocytes, macrophages and DCs. LL-37-stimulation via P2X7 of LPS-primed monocytes induced processing and release of the potent cytokine IL-1 beta (42). This proinflammatory cytokine upregulates adhesion molecules on endothelial cells and hence promote migration of leukocytes to the affected tissue. Recently, it was shown that LL-37 upregulates adhesion molecules on endothelial cells, promoting recruitment of inflammatory cells to the vessel. This action might affect the inflammatory process in atherosclerotic lesions, where LL-37 is present (43).

4.3. Endotoxin binding properties

Some AMPs, including LL-37, have the capacity to neutralize bacterial endotoxins, such as lipopolysaccharide (LPS), at physiologically relevant concentrations (more than 1 μg/ml or 0.2 μM) (19, 44, 45). Upon gram-negative bacterial cell death, LPS is released and is able to activate immunological cells to produce and release proinflammatory cytokines. This event is initiated by binding of LPS to LPS-binding protein (LBP), followed by a second binding of the LPS-LBP complex to CD14. Subsequently, this complex interacts with toll-like receptor 4 (TLR4), which activates a downstream signal cascade in the cell (46). During bacterial infections, the levels of LPS may increase to such concentrations that the cytokine release and the inflammatory responses cause septic shock (47). However, the LPS-binding property of LL-37 may dampen the inflammation, ameliorating the shock. The first study reporting high affinity binding of LL-37 to LPS was published already one decade ago (48). Later, it was shown that LL-37 also blocks the ability of LPS to exert its effect via CD14 on macrophages (44, 49) and DCs (50), thereby reducing the production of proinflammatory cytokines, such as TNF alpha and IL-6. The main mechanism by which LL-37 is thought to neutralize LPS-induced responses is the direct binding of LL-37 to LPS, inhibiting LPS interaction with LBP and/or CD14 (51). Another mechanism have also been suggested, where LL-37 binds directly to CD14, inhibiting LPS to associate with its receptor (51). Furthermore, recent work shows that LL-37 not only blocks the effect of LPS, but also other bacterial components, such as flagellin, lipoteichoic acid and non capped lipoarabinomannan (49, 50). The antiendotoxic effect of LL-37 has been confirmed *in vivo* using rat as a model system (52, 53), making it a promising candidate for treatment of endotoxin shock or sepsis.

4.4. Effect on apoptosis

The action on LL-37 in apoptosis has recently been highlighted. The results indicate that LL-37 exhibits divergent effects on apoptotic cell death of different cell types. LL-37 promotes apoptosis in various T-cells, smooth muscle cells and epithelial cells (54-57), while it inhibits cell death in neutrophils (55, 58). A consequence of the inhibition of apoptosis of neutrophils may be a survival strategy, leading to an increase of viable neutrophils at the site of infection, which is beneficial for the host during bacterial invasion. It has been proposed that the underlying mechanism for the inhibition of apoptosis by LL-37 is mediated via FPRL-1 and P2X7 receptors, resulting in the inhibition of caspase 3 activity (55, 58). Noteworthy, this inhibition is mediated at a concentration as low as 0.01-5 μg/ml LL-37 (58), supporting physiological relevance. As mentioned above, LL-37 exhibits apoptosis-inducing activity on airway epithelial cells as observed both *in vitro* and *in vivo,* a cytotoxic effect that is suppressed by serum (54). Furthermore, the C-terminal part of LL-37 can specifically induce apoptosis in oral squamous carcinoma cells but not in normal gingival fibroblast or keratinocytes, suggesting an anti-tumorigenic role of LL-37 (59). In contrast, LL-37 has been detected at high levels in breast cancer cells (60). In this report, a growth promoting effect is suggested, opposing the hypothesis that LL-37 exhibits antitumor effect. The role of LL-37 in tumor development is very limited and further experiments are required to establish a function of LL-37 in cancer. Also the mechanism behind the inducing effect by LL-37 in apoptosis is not fully understood, although recent data has brought some insight of the pathways. Aarbiou and colleagues found that LL-37 induces cytochrome C-release and DNA fragmentation, but not caspase activation, in cell death of T-cells and lung epithelial cells (56). On the other hand, it was recently shown that caspase 3 is involved in LL-37-induced apoptosis in vascular smooth muscle cells and epithelial cells (54, 57).

4.5. Additional immunomodulatory functions

Apart from the functions of LL-37 described above, LL-37 has been shown to induce histamine release from mast cells in a dose-dependent manner, with an effective dose of 1 μg/ml (0.2 μM). This effect was inhibited by pertussis toxin and a phospholipase C inhibitor (U-73122), suggesting a G-protein coupled receptor- and phospholipase C-dependent pathway to be involved in this process (61). A role of LL-37 to bridge innate and adaptive immunity was gained further support in a study, where it was found that LL-37 can modulate DC functions and differentiation (62). By enhancing the process of DC endocytosis and upregulation of phagocytic receptors, an increase in microbial clearance and antigen presentation of DCs was observed. The mechanism for this effect is not yet defined although there are some indications that a G-coupled receptor is involved. Furthermore, LL-37 has been

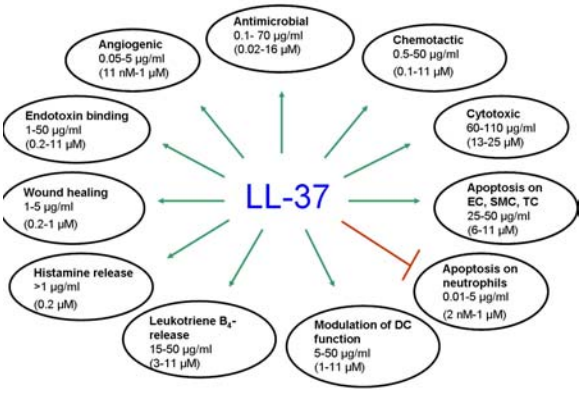

Figure 2. Approximate concentrations of different activities of LL-37. EC: Epithelial cells; SMC: Smooth muscle cells; TC: T-cells; DC: Dentritic cells.

found to be internalized by DCs (63), where it may exert these effects. An additional important function of LL-37 connected to host defense is its ability to directly stimulate wound closure in skin (64) and in the airway (65). LL-37 can also indirectly promote tissue repair by inducing angiogenesis as demonstrated both *in vitro* and *in vivo* (66). Experimental data shows that the angiogenic activity is mediated via FPRL-1-receptor at a concentration of 50 ng/ml or 11nM. Interestingly, it was recently observed in psoriasis that LL-37 can drive autoimmunity by binding to self-DNA. The complex of LL-37 and self-DNA is sensed by plasmacytoid DCs as a danger signal (67). Thus, by breaking innate tolerance, LL-37 is not always beneficial in host defense.

5. PERSPECTIVE

The complete picture of the role of LL-37 is not yet clear. However, there is no doubt that LL-37 is an important central molecule in host defense (Figure 2). In addition to be an antimicrobial peptide, LL-37 has been categorized as an "alarmin" because of its ability to promote local inflammation and respond to danger signals by alerting the adaptive immune system (68). The broad spectra of antimicrobial activities together with the immunomodulatory and regulatory properties make LL-37 a promising therapeutic agent for diseases connected to infection and/or inflammation. However, there are still issues related to mechanisms behind the action of this peptide and human physiological responses that have to be elucidated before a drug can be developed. Attempts to produce analogues and variants of LL-37 with lower cytotoxic effects and a better selectivity of the antimicrobial/immunomodulatory activities have been made in various structure-function studies (69, 70). In order to use these peptides in anti-infectious and/or anti-inflammatory therapy, the production cost of synthetic peptides has to be overcome. A more promising strategy may be to stimulate the synthesis or release of LL-37 at tissue sites by factors such as vitamin D (71), butyrate (72) or leukotriene B4 (73). However, clinical trials are required before these components can be accepted as anti-infectious drugs.

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Abbreviations: AMPs: Antimicrobial peptides, DCs: Dendritic cells, EGFR: Epidermal Growth Factor Receptor, FPRL1: Formyl Peptide receptor like-1, GM-CSF: granulocyte-macrophage colony-stimulating factor, hCAP-18: human cationic antimicrobial peptide 18, LBP: LPS-binding protein, LPS: Lipopolysaccharide, mCRAMP: mouse cathelicidin related antimicrobial peptide, TNF: Tumor necrosis factor, TLR: Toll-like receptor

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