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Defensins: More than Nature's Anti-bug Spray?

Erik de Leeuw*

Institute of Human Virology and Department of Biochemistry and Molecular Biology, Baltimore School of Medicine, University of Maryland, USA

Abstract

Human defensins were originally identified as natural antimicrobial peptides that protect the host from infections. More recently, novel biological functions of these peptides have been described, expanding their role as natural antimicrobials. Here, our current understanding of this expanding role of human α-defensins is reviewed, with emphasis on antibacterial killing, bacterial toxin neutralization and immunity.

Keywords: Defensin; Antibacterial; Immunity; Toxin

Abbreviations: HNP: Human Neutrophil Peptide; HD: Human Defensin; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid

Introduction

Antimicrobial peptides neutralize a broad range of microbes–an ancient but effective defense mechanism used by multicellular organisms throughout the animal and plant kingdom [1]. Most peptides with antimicrobial properties are positively charged and preferentially target microbial membranes which contains large amounts of negatively charged molecules. In general, cationic antimicrobial peptides are believed to cross the bacterial cell wall *via* a mechanism of self-promoted uptake, and subsequently cause lethality by permeabilizing the cytoplasmic membrane and inducing leakage of cellular contents [2-5]. The underlying molecular mechanisms for membrane permeabilization by antimicrobial peptides, however, remain poorly defined and additional alternative modes of action to kill bacteria may exist [3,6].

Defensins are a major group of small, cationic antimicrobial peptides, which constitute important components of the innate immune defense against microbial infection [7-10]. Human defensins are cysteine-rich, cationic peptides with molecular masses ranging from 3 to 5 kDa. Based on the connectivity of the six conserved cysteine residues and sequence homology, human defensins are classified into α and β families [11]. They primarily act as "natural antibiotics" against a wide range of microorganisms, including bacteria [12-14], viruses [15], and certain protozoa [16,17]. More recently, defensins have been recognized as potentially important modulators of adaptive immunity also [18-20]. Here, we will review our current understanding of the antibacterial activities of human α-defensins with emphasis on interactions with the microbial membrane, their anti-bacterial toxin properties, and their expanding role in host immunity.

Human α-Defensins

Human defensins were first described as natural peptide antibiotics in neutrophils, and were termed Human Neutrophil Peptide 1-3 or α-defensin 1-3 [21]. HNP1-3 is synthesized *in vivo* as pre-propeptides, consisting of a signal sequence, an N-terminal pro domain and the defensin domain. The pro domain is important for correct folding and trafficking of HNP1-3, and is proteolytically removed prior to storage by an as yet unknown enzyme [22]. They are stored in azurophil granules of neutrophils, and are released during phagocytosis to act against ingested microbes. The processed HNP1-3 peptides contain 29-30 amino acid residues and differ by only a single amino acid residue at the N-terminus [23]. A few years later, a fourth, much less abundant α-defensin was discovered in neutrophils, termed HNP-4, which shares only 30% sequence similarity with HNP1-3 [24-26]. More recently, two additional α-defensins were described, termed Human Defensin 5 and 6 [27,28]. HD-5 and HD-6 are stored in granules of specialized epithelial cells called Paneth cells in the small intestine [29]. In contrast to HNPs, HD-5 and HD-6 are secreted in response to bacterial stimulation as propeptides and processing occurs extra-cellularly [30].

Human defensins of the α family are characterized by a unique connectivity of the six conserved cysteine residues; Cys1-Cys6, Cys2-Cys4 and Cys3-Cys5. In addition, α-defensins contain a highly conserved Arg-Glu pair forming a salt bridge, which together with the three intra-molecular disulfide bonds stabilizes a conserved three-stranded β-sheet core structure [31-33]. Dimerization or even higher order structures have been suggested for certain defensins, although any functional consequences are largely not well understood [31,34].

Antibacterial Activity of α-defensins: The Microbial Membrane

Primarily, defensins act *in vivo* as antimicrobial "first responders" against predominantly bacterial threats. This notion is underscored by the presence of increased plasma levels of HNP1 in patients with septicemia or bacterial meningitis [35]. In addition, elevated concentrations of HNPs were reported in plasma, blood and body fluids, such as pleural fluid, bronchoalveolar lavage fluid, urine and cerebrospinal fluid from patients with a variety of infections, including bacterial and non-bacterial infections and pulmonary tuberculosis [36]. Also, lower than normal levels of HNPs or inactivation of the peptides have been linked to an increased risk of caries in the oral cavity [37], as well as infections of the airways, including cystic fibrosis [38,39]. A specific deficiency in HD-5 was observed in patients suffering from ileal Crohn's disease, a chronic inflammation of the gastrointestinal tract [40]. This observation has been underscored by a number of

***Corresponding author:** Erik de Leeuw, Institute of Human Virology and Department of Biochemistry and Molecular Biology, Baltimore School of Medicine, University of Maryland, USA, Tel: 410-706-1970; Fax: 410-706-7583, E-mail: edeleeuw2@som.umaryland.edu

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recent animal model studies. Reduced expression of certain α-defensins resulted in increased susceptibility to oral infection by *Listeria monocytogenes* compared to wild-type mice [41]. Similarly, mice that lack mature cryptdins (the murine orthologue for α-defensins) are more susceptible to ileal colonization by non-invasive *Escherichia coli* spp. [42]. Furthermore, Paneth cell expression of Human Defensin-5 rendered mice markedly resistant to oral, but not peritoneal challenge with a virulent strain of *Salmonella typhymurium* [43].

Exactly how defensins kill bacteria is not fully understood, despite their discovery more than three decades ago. The current prevailing view is that defensins and cationic antimicrobial peptides generally target the negatively charged bacterial membrane for destruction, causing leakage of the intracellular contents and ultimately, cell lysis and death. The importance of positive charge in bacterial killing by defensins is experimentally well supported [44-46]. These findings are further supported by observations that anionic residues in the pro-domain of defensins prevents their antibacterial activity, until enzymatic processing [30,47,48], although more recent evidence suggests that hydrophobicity of the pro-domain may be equally or more important [49]. A number of recent observations on the bacterial killing by defensins, however, could not fully be explained by the membrane disruption model. Firstly, this model assumes that stable formation of pores in the microbial membrane require peptide structure, oligomerization and structural amphiphilicity-separated clusters of hydrophobic and cationic residues. However, we and others have shown that a number of bacterial strains can be killed effectively independent of defensin structure [50,51]. Secondly, it has been noted for some time that α-defensins preferentially kill Gram-positive bacteria [12,52]. In contrast, β-defensins, while carrying more positive charges than α-defensins, kill Gram-negative bacteria more effectively, suggesting that cationicity alone is not the sole driving force for bacterial killing [45]. In light of this observed strain-selective killing, we recently showed that an HNP1 peptide composed entirely of unnatural D-amino acids show greatly reduced anti-bacterial activity against *Staphylococcus aureus* compared to the L-peptide [53]. Killing of *Escherichia coli* appeared comparable for both peptides, suggesting that differences in the composition of the bacterial cell wall between these strains could explain these findings. Recently, functional interactions of human and fungal defensins with Lipid II have been described [54,55]. Lipid II is an essential precursor of cell wall biosynthesis of Gram-positive species. Two additional fungal defensins, oryzeacin (from *Aspergillus oryzae*) and eurocin (from *Eurotium amstelodami*), as well as two invertebrate defensins, lucifensin (from the blowfly *Lucilia sericata*) and gallicin (from the mussel *Mytilus galloprovinciali*), were shown to bind Lipid II in that study [55]. More recently, the spectrum of defensins binding Lipid II was widened further to include Human β-Defensin-3 [56] and three oyster defensins [57].

In the case of *S. aureus*, the cell wall is primarily composed of a single, thick layer of peptidoglycan covered with (lipo) teichoic acid. The *E. coli* outer cell wall is composed of a thin layer of peptidoglycan, surrounded by an outer membrane largely composed of lipopolysaccharide (LPS) on the outside [58]. Cationic antimicrobial peptides can indeed associate with the negatively charged LPS or teichoic acid–an event thought to be important not only for antimicrobial selectivity, but also for the uptake across the bacterial cell wall [59-61]. Other structural components of the bacterial cell wall, such as peptidoglycan, may also play a similar role. Defensins have been shown to bind glycosylated proteins [53,62], and can be permanently retained on the Superdex resin made of highly cross-linked agarose covalently bound to dextran [63]. It is conceivable that certain defensins may be able to strongly interact with the peptidoglycan layer, thus attenuating the subsequent membrane permeabilization.

The ability of many cationic peptides to permeabilize model membranes correlates with their bactericidal activity, supporting the notion that membrane disruption is the killing mechanism [60]. However, for a number of cationic peptides, this correlation does not exist [64,65], and alternative mechanisms have been suggested [3,6]. These include membrane-independent mechanisms, where the antibacterial peptide exerts its function through intracellular targeting [3]. In fact, HNP1 has been shown to inhibit DNA, RNA and protein synthesis, as well as synthesis of periplasmic β-galactosidase in *E. coli* [66]. More recently, however, HNP1 was found to be non-membrane disruptive for both bacterial outer and cytoplasmic membrane [67]. A second alternative mechanism is the so-called carpet model of antimicrobial induced killing [3]. In this model, microbial membranes are disrupted by the formation of an extensive layer of peptide oriented parallel to the surface of the lipid bilayer. This model has not been extensively investigated in the case of defensins, however, given the extraordinarily high *in vivo* concentrations (mg/ml range) of HNP1-3 in neutrophil granules, as well as HD-5 in luminal crypts, certainly merits consideration.

Three recent studies on further expand the role of α-defensins in antibacterial innate host defense. Two studies reported novel defense mechanisms of host cells in *Mycobacterium* infection. *Mycobacterium tuberculosis* is an intracellular pathogen that targets macrophages. Macrophages do not express defensins, however, were shown recently to acquire neutrophil granules from dying neutrophils as a cooperative defense mechanism to combat this intracellular pathogen [68]. Interestingly, eosinophils were shown to express and produce α-defensins in a mycobacterial infection model, expanding the defensin host cellular source beyond neutrophils and Paneth cells [69]. Importantly, HD-5 was recently shown to regulate the composition of the intestinal flora in mice, and as a result to influence the mucosal immunological response [70]. These findings suggest an important, active role for defensins under conditions of immune balance, in addition to their anti-infectious response.

Antibacterial Activity of α-Defensins: Beyond the Microbial Membrane

Recently, the defensin spectrum of antibacterial activity has been widened significantly by the observation that defensins have the capacity to neutralize many secreted bacterial toxins.

The ability of defensins to neutralize secreted bacterial factors was demonstrated first for *S. aureus* staphylokinase [71], which in combination with host plasminogen exerts fibrinolytic activity [72]. HNPs appeared to inhibit staphylokinase by binding to a region important for its binding to plasminogen (71). Close thereafter, toxin neutralization by HNPs was shown for the anthrax lethal toxin, as well as their ability to protect mice against fatal infection with *B. anthracis* lethal factor [73]. Anthrax lethal toxin is a binary complex of lethal factor and protective antigen, two proteins secreted by *Bacillus anthracis* [74]. Lethal factor is a Zn^{2+}-dependent metalloprotease, which induces cell death in macrophages by cleaving essential cellular proteins. Defensins were shown to inhibit lethal factor in a non-competitive fashion, indicating that HNP1 binds to lethal factor away from its substrate binding site. In addition, HNP1 was shown to effectively kill germinating *B. anthracis* spores in neutrophils intracellularly [75]. Subsequently, HNP1-3 was shown to inhibit certain bacterial

ADP-ribosyltransferases, including diphtheria toxin and exotoxin A of *Pseudomonas aeruginosa* [76]. These toxin enzymes facilitate ADP-ribosylation of the host elongation factor 2, a modification that inhibits protein synthesis. Interestingly, HNP1-3 competitively inhibited against elongation factor 2, suggesting a different mode of inhibition to anthrax lethal factor [76]. Toxin B of *Clostridium difficile* was also reported to be inhibited by α-defensins [77]. Recently, neutrophil defensins were shown to protect erythrocytes against three cholesterol-dependent bacterial cytolysins, *B. anthracis* anthrolysin O, *Listeria monocytogenes* listerolysin O and *Streptococcus pneumoniae* pneumolysin, further expanding their role in toxin neutralization [78].

Role of Defensins in Adaptive Immunity: Receptor Studies

In recent years, studies on human defensins of both the α– and β-subfamilies have provided evidence for their role in innate and adaptive immune responses, expanding their role as mere antimicrobials [79]. Such functions include chemoattraction and immune cell activation and promotion of cell proliferation, often involving interactions with cellular receptors [18-21,80,81]. The capacity to chemoattractant monocytes was first described for Human Neutrophil peptides [81]. Subsequently, HNPs were shown to chemoattract different subsets of T lymphocytes and immature dendritic cells [82,83]. Similar functions were reported for β-defensins, which were shown to selectively chemoattract immature dendritic cells and memory T lymphocytes [19,84]. More recently, β-defensins were shown to act as endogenous ligands for Toll-like receptors on immature dendritic cells directly. This interaction mediated signaling for dendritic cell maturation and triggered a polarized immune response *in vivo* [18]. In the case of human β-defensin 2 (hBD2), the observed chemotaxis of immature dendritic cells and memory T cells was shown to result from directly binding the chemokine receptor CCR6 [19]. Subsequently, a murine β-defensin was shown to recruit tumor-infiltrating dendritic cell precursors through CCR6 also [85]. In contrast to these earlier studies, it was reported recently that β-defensins chemoattract mast cells and macrophages but not dendritic cells and lymphocytes, and that CCR6 was not involved [86]. Members of the β-defensin family have been further shown to interact with Toll-Like Receptor 4 [18] and the melanocortin 1 receptor [87], causing black coat color in domestic dogs. Recently, the interaction between Human beta-defensin 6 and the chemokine receptor CCR2 was described in molecular detail [88].

Specific receptors for the chemotactic activity of α-defensins have not been identified. Several studies, however, have shown that also for α-defensins this activity is blocked by pertussis toxin, indicating the involvement of $G_{i\alpha}$-coupled receptors [83,89]. Interestingly, defensins and chemokines share common structural features, both having a three-stranded anti-parallel β-sheet structure stabilized by a disulfide core [90]. Many chemokines like defensins, are cationic and interact with their cognate receptors mostly *via* electrostatic attractions [91]. In addition, several chemokines have reported antibiotic properties [92,93], however, a possible reason for this functional overlap remains to be explained.

In addition to their chemoattractant properties, defensins anti-viral capacity involves receptor-mediated interactions [15]. Here, we will briefly summarize defensin anti-HIV properties to illustrate some of the complexities defensin-viral-host cell interactions. As part of effective cervico-vaginal host defense, defensins act as key anti-HIV-1 molecules [94,95]. The inhibition of HIV replication was first reported for synthetic rodent α-defensins [96], and more recently, anti-HIV activities have been described for human α- and β-defensins [63,97-100]. To date, a plethora of distinct mechanisms of anti-HIV activity by defensins have been proposed, including direct interactions of defensins with the virus itself, as well as interactions of defensins with the host cell: (i) direct inactivation of HIV virions [101]; (ii) upregulation of chemokines [102]; (iii) downregulation of HIV co-receptors [103]; (iv) downregulation of CD4, a primary receptor for HIV [104]; (v) modulate protein kinase C signaling and viral replication [101]; (vi) inhibition of CD4-GP120 interactions [104]; (vii) inhibition of gp41-membrane fusion [105], and (viii) receptor-mediated induction of the HIV restriction factor APOBEC3g [106]. In contrast to these studies, it was reported recently that certain α-defensins enhance HIV infection *via* as yet unknown mechanisms [107,108], only adding to the complexity. This could be partly explained by the observation that defensins induce cell death of CD4+ T cells in the absence of serum [109]. Intriguingly, it was reported recently that HNP-1 potentiates HIV-1 anti-GP41 antibodies at sub-inhibitory concentrations, even in the presence of serum [110].

Small as they are, human defensins are versatile effectors of immunity beyond their antimicrobial capacity. Primarily, they protect the host against microbial challenge. In doing so, defensins have evolved to combat a surprisingly diverse range of microorganisms, ranging from many bacterial species [12], enveloped and non-enveloped viruses [15], and even certain protozoa [17]. The recent discovery of their capacity for neutralization of bacterial toxins only adds to their antibacterial versatility [111]. Defensins, in addition, seem to be important modulators of immunity. This is exemplified by their capacity to chemoattract and/or activate various immune cells, including monocytes, dendritic cells, macrophages and subsets of T lymphocytes [19,20,81,83,86]. This functional versatility may be explained in part by their promiscuity for binding to different molecular partners. Defensins reportedly interact with bacterial toxins [111], viral proteins [15], and a variety of host cellular receptors and proteins [112]. They also bind carbohydrates, nucleic acids, lipids and even self-associate, adding another layer of complexity [113]. In spite of immense progress in our understanding of defensin functional versatility, exactly how these peptides act at the molecular level remains not well defined, underscoring the notion that defensins are more than nature's anti-bug spray.

Conclusion

A great challenge to defensin research lies in their intrinsic promiscuity, described above. Our understanding of the functional ramifications of this promiscuity will be of key importance. Nevertheless, based on our current knowledge, it seems clear that a "one-fits-all"-model for any individual function of defensins may not hold. In the case of bacterial killing, for example, strain-selective killing has been reported for α- and β-defensins. However, individual α-defensins use seemingly different mechanisms against Gram-positive and Gram-negative strains. Thus, the bactericidal activity and membrane disintegration by defensins is more complex than previously believed, and may involve specific interactions with as yet unidentified cellular components. Breakthroughs in this field will provide critical insights into turning defensins into next-generation peptide antibiotics. Bacterial toxin neutralization, in particular, holds great promise, however, even more than for the bactericidal activity, will depend on detailed studies on individual toxins and defensins. Combined, we believe that studies in the foreseeable future in these

particular areas will facilitate the rational development of natural, defensin-based compounds into novel, next generation therapeutic agents for the treatment of pathogenic bacterial infections.

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