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Defensin: a multifunctional molecule lives up to its versatile name

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Human neutrophil proteins 1, 2 and 3 (HNP1–3) were originally identified as endogenous antibiotics that can kill microbial pathogens immediately after the onset of the host innate immune response. Recent studies revealed that these peptides perform additional, previously unexpected functions, notably the neutralization of certain secreted bacterial toxins. In this Opinion article, a brief overview of the well-established functions of HNP1–3 is given and novel biological activities of HNP1–3 are described, with emphasis on neutralization of secreted bacterial toxins. We propose that toxin neutralization represents a novel biological function of HNP1–3 in host defense.

Human neutrophil proteins

Defensins are a family of small cationic vertebrate peptides with a dominant β-sheet structure and three intramolecular disulfide bridges [1]. They are divided into three subfamilies, α-, β- and θ-defensins, based on their cysteine pairing and structure [1]. In humans, six α-defensins have been identified. Four of them, human neutrophil proteins (HNP)1–4, are mainly produced by granulocytes [2] and certain leukocytes [3]. HNP1–3 share identical amino acid sequences except for the first N-terminal residue [4]. The other two α-defensins, HD-5 and HD-6, are produced by Paneth cells in the small intestine [2]. Intriguingly, mouse neutrophils lack homologs of HNP1–3 [5].

Originally, HNP1–3 were identified as natural peptide antibiotics that display microbicidal activities against various bacteria, fungi and viruses [6] but recently, additional functions were ascribed to HNP1–3. They function as 'danger' signals at concentrations far below those required for their antimicrobial action [7] and they are involved in wound healing by promoting cell proliferation [8]. Most recently, a novel biological function of HNP1–3 was elucidated, namely the neutralization of toxins secreted by bacterial pathogens (Figure 1). These toxins include lethal factor from *Bacillus anthracis*, *Corynebacterium diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A [9,10]. Moreover, the fibrinolytic activities of staphylokinase, the major virulence factor from *Staphylococcus aureus*, are blocked by HNP1–3 [11,12]. Here, we propose that the neutralization of secreted bacterial toxins is a novel biological activity of HNP1–3.

Defensin as an antimicrobial peptide

Neutrophils are the first cells that are recruited to sites of infection. At these inflammatory sites, neutrophils serve as professional phagocytes, which rapidly engulf and kill microorganisms by oxygen-dependent and oxygen-independent mechanisms [13]. Neutrophil antimicrobial peptides such as defensins and cathelicidins are considered to be a central part of oxygen-independent bactericidal effector mechanisms.

Without doubt, HNP1–3 express antimicrobial activities in distinct niches such as the phagosomes of human neutrophils, where bacteria are exposed to high (mg ml^{-1}) concentrations of HNPs [1,14]. Yet, their direct microbicidal activities in extracellular locations remain unclear. Frequently, antimicrobial activities of defensins were studied *in vitro* under artificial laboratory conditions comprising low ionic strength and in the absence of serum components. HNP1–3, however, display poor microbicidal activities in the presence of serum or physiological salt concentration [1,2,15,16]. This weak antimicrobial activity under physiologically relevant conditions is observed with many other cationic peptides including the cathelicidin LL-37 [17]. This perplexity has prompted scientists to search for novel functions of cationic peptides under more physiological conditions.

Defensin as an endogenous danger signal

It was suggested that HNP1–3 display their antimicrobial activities by permeabilizing the lipid bilayer similar to other lytic peptides [18]. Interestingly, the overall dimensions, positive charge, β-sheet structure and disulfide bonds of HNP3 as elucidated by X-ray crystallography are reminiscent of various snake, scorpion and spider toxins, which function by binding to specific receptors rather than by permeabilizing the cell membrane [19]. Over the past few years, an accumulation of evidence suggests that HNP1–3 bind to distinct receptors [7,20] and promote local innate inflammatory and systemic adaptive immune responses by serving as chemoattractants and activators of immune cells. HNP1–3 are chemotactic for T cells and dendritic cells by signaling through G(iα) protein-coupled receptors [7]. Moreover, HNP1–3 enhance secretion of antigen-specific antibodies and distinct cytokines by immune cells [21,22]. Accordingly, Oppenheim and Yang [23] suggested redesignating these peptides 'alarmins' because they are rapidly released in response to danger signals to alert the immune system.

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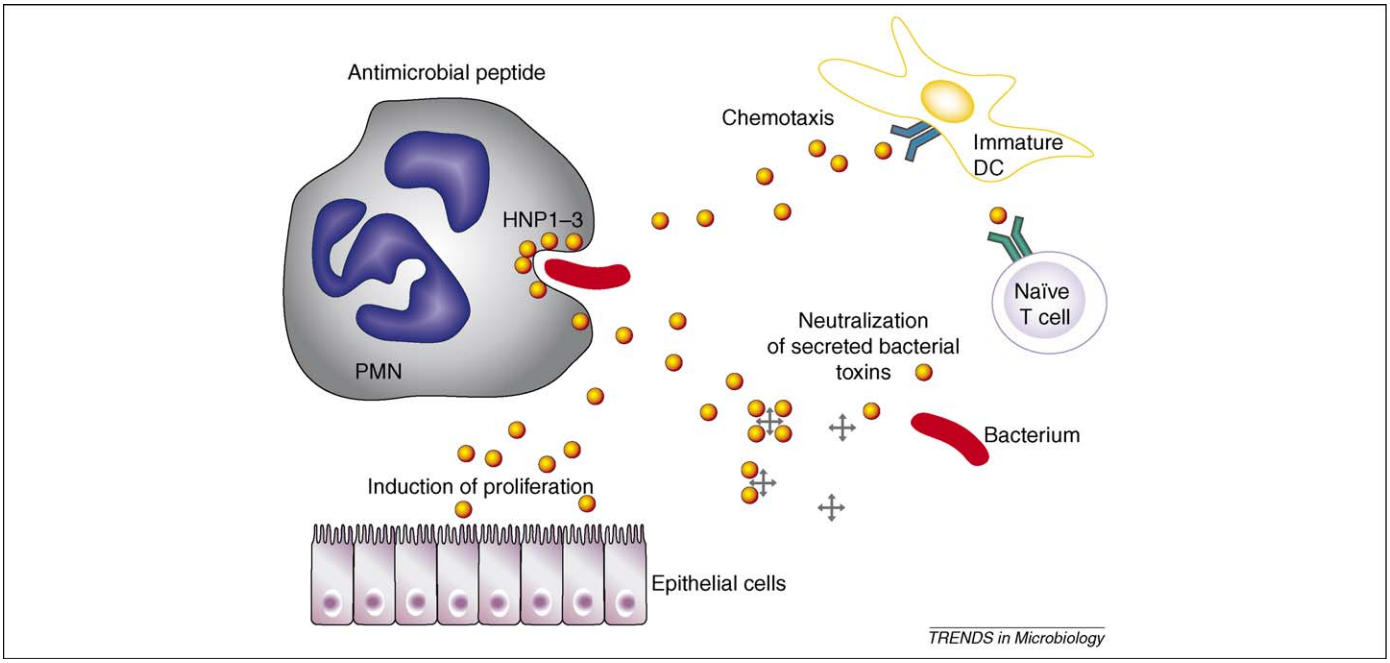

Figure 1. Divergent functions of HNP1–3 in antibacterial defense. HNP1–3 were initially identified as antimicrobial peptides. When polymorphonuclear cells (PMN) ingest bacteria, they end up in phagosomes and, subsequently, granules that contain abundant HNP1–3 are fused with the vacuole [1]. In this setting, bacteria are exposed to a high concentration of HNP1–3 and as a result, bacterial cell membranes are permeabilized. In extracellular regions, HNP1–3 display a range of other activities. HNP1–3 are chemotactic for dendritic cells (DC) and T cells [7] and can induce pulmonary epithelial cell proliferation [8] and neutralize secreted bacterial toxins [9,10].

Defensin as a global bacterial toxin inhibitor

Numerous bacterial pathogens secrete effectors into the host environment to avoid destruction by the immune system. Although many bacterial effectors are named toxins on the basis of their *in vitro* cell-killing activities, in biochemical terms, most of them are actually enzymes. A novel function of HNP1–3, namely the neutralization of secreted bacterial enzymes, was recently reported [9,10]. Under physiological conditions, HNP1–3 inhibit the cytocidal activity of anthrax lethal factor (LF) [9] and of certain bacterial ADP-ribosyltransferases (ART), in particular, diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) [10] (Figure 2).

LF is a metalloprotease that cleaves the N terminus of certain mitogen-activated protein kinase (MAPK) kinases (MKKs) and is essential for anthrax pathogenesis [24]. It was demonstrated that HNP1–3 function as potent noncompetitive inhibitors of LF by binding to a region that is remote from the active center of LF. HNP1 inhibits cleavage of MKK and restores impaired MAPK signaling in anthrax lethal toxin (LeTx)-treated macrophages [9]. Moreover, it rescues murine macrophages from *B. anthracis*-induced cytotoxicity and protects mice against the fatal consequences of LeTx. The unique structure of HNP1, which is determined by intramolecular disulfide bridges, was identified as a crucial requirement for this inhibitory activity because reduced linear HNP1 lost its anti-LF activity [9].

Mono ADP-ribosylation is a covalent chemical modification process in which an ADP-ribosyltransferase transfers the ADP-ribose moiety of NAD^+ to a target protein with nicotinamide release [25]. This reaction is not only a component of host cell intracellular signaling but is also a widely used mechanism of bacterial pathogenesis [26]. Because mammalian ART-1 can modify HNP1 [27], it is tempting to examine whether bacterial ARTs have any effect on HNP1–3. Unexpectedly, instead of serving as a substrate of bacterial ARTs, HNP1–3 operated as an inhibitor of the DT group of bacterial ARTs [10]. DT and ETA catalyze the ADP-ribosylation of host elongation factor 2 (EF2), which results in inhibition of protein synthesis and, finally, cell death [28]. In the presence of HNP1, ADP-ribosylation of EF2 was efficiently prevented; thus, HNPs

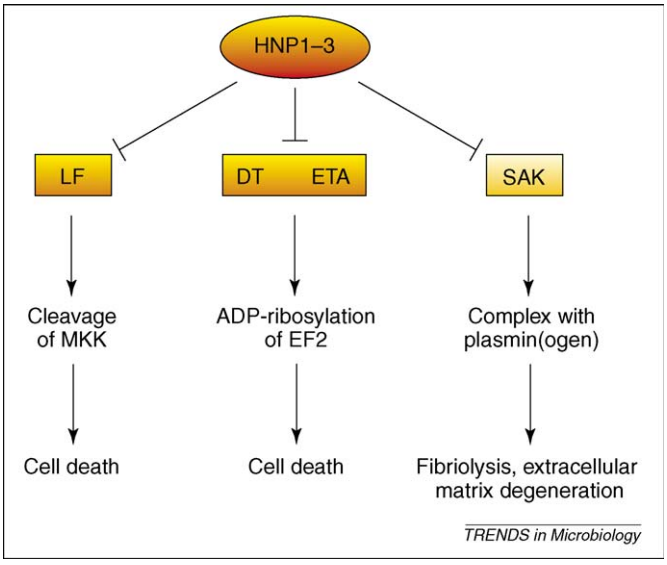

Figure 2. Mode of action of bacterial toxins and their inhibition by HNP1–3. Anthrax lethal factor (LF) is the major toxin of *Bacillus anthracis*. Once LF is delivered to the cytosol, this Zn^{2+}-dependent metalloprotease cleaves certain MKKs, leading to death of the host cells [24]. Diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) are mono-ADP-ribosyltransferases. They transfer ADP-ribose to host elongation factor (EF) to inhibit protein synthesis in host cells [37]. Staphylokinase (SAK) from *Staphylococcus aureus* is a secreted protein that forms a complex with plasminogen, leading to conformational changes and changes in plasmin(ogen) specificity [38].

protect cells from DT- or ETA-mediated death [10]. In contrast to the mode of inhibition against anthrax LF, in the case of DT and ETA, HNP1 behaved as a competitive inhibitor against EF2 and an uncompetitive inhibitor against NAD^+. Mammalian ART-1 has been shown to transfer ADP-ribose to HNP1 [27]. As a corollary, HNP1 would be anticipated to recognize and bind to the EF2 docking site within both DT and ETA. Therefore, it is not surprising that HNP1 is a competitive inhibitor against the EF2 substrate for the toxins.

In keeping with these findings, a recent report revealed that HNP1–3 neutralize fibrinolytic activities exerted by staphylokinase (SAK) from *Staphylococcus aureus* [11,12]. Although SAK itself is not an enzyme, in combination with host plasmin(ogen), it functions as an enzyme [29]. HNP1–3 seem to achieve inhibition by binding to the region of the SAK molecule located around the Lys74 position, which is important for its interaction with plasminogen. It remains to be clarified whether unique HNP1–3 binding pockets exist in such functionally distinct bacterial toxins as LF, DT, ETA and SAK.

HNP1–3 are the most abundant azurophilic granule peptides and constitute 30–50% of the granule proteins [30]. The concentration of HNP1–3 in plasma under normal physiological conditions is $\sim$40 ng ml^{-1} but can rise to 0.9–170 $\mu g\ ml^{-1}$ during severe infections [31]. Because recruited human neutrophils release abundant HNP1–3 locally at sites of infection, local concentrations of HNP1–3 at sites of inflammation are expected to be even higher [32]. It would be interesting to determine whether HNP1–3 efficiently neutralize secreted bacterial toxins in the *in vivo* setting.

Defensin and host enzymes

The findings that HNP1–3 inhibit bacterial enzymes raise the question as to whether they can also inhibit host enzymes. It is known that mammalian protein kinase C (PKC) is inhibited by HNP1–3 [33]. However, the physiological meaning of this inhibition has so far remained mysterious. It is known that defensins inhibit a variety of viruses [34]. Recently, it was shown that HNP1–3 inhibit HIV-1 replication by interference with PKC signaling in CD4+ T cells [35]. The involvement of PKC signaling in HNP1-mediated HIV-1 inhibition was cell-specific because the anti-HIV activity of a known PKC inhibitor (Go6976) mimicked HNP1 in primary CD4+ T cells and transformed T cells but not in HeLa cells [35]. Although numerous questions remain to be solved regarding the inhibition of host enzymes by HNP1–3, the example described suggests a supportive role in host defense.

Concluding remarks and future perspectives

HNP1–3 are abundant and widely distributed peptides involved in host defense. Although they were initially identified as natural antibiotics with direct microbicidal activities, emerging evidence suggests additional biological functions. We suggest that HNP1–3 inhibit distinct secreted bacterial enzymes with potent toxic activity. Based on their structure, defensins can be divided into three subfamilies: α-, β- and θ-defensins. It will be of interest to investigate whether α-defensins of other animals or other defensin families also function as enzyme inhibitors, although the amino acid sequences of defensins differ markedly from one family to the other and between humans and other species. The first evidence for the neutralizing activities of θ-defensins was recently presented [36].

Elucidation of the multifunctional activities of HNP1–3 in infectious diseases has implications for a deeper understanding of pathogenesis and the development of novel intervention strategies. HNP1–3 kill a variety of microbial pathogens, they attract and activate leukocytes and they neutralize bacterial toxins. Because HNP1–3 are naturally occurring endogenous peptides that express multiple complementary host defense mechanisms, they provide a promising basis for the rational design of a novel class of anti-infectives. After the original discovery of their microbicidal activities, defensins were baptized with a rather versatile name for a unifunctional effector molecule. In recognizing the diverse biological activities of defensins, it is obvious that they are truly multifunctional effector molecules and, thus, have lived up to the name given to them.

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References

1 Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 3, 710–720
2 Lehrer, R.I. *et al.* (1993) Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* 11, 105–128
3 Agerberth, B. *et al.* (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* 96, 3086–3093
4 Selsted, M.E. *et al.* (1985) Primary structures of three human neutrophil defensins. *J. Clin. Invest.* 76, 1436–1439
5 Eisenhauer, P.B. and Lehrer, R.I. (1992) Mouse neutrophils lack defensins. *Infect. Immun.* 60, 3446–3447
6 Ganz, T. *et al.* (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* 76, 1427–1435
7 Yang, D. *et al.* (2000) Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J. Leukoc. Biol.* 68, 9–14
8 Aarbiou, J. *et al.* (2004) Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression *in vitro*. *Am. J. Respir. Cell Mol. Biol.* 30, 193–201
9 Kim, C. *et al.* (2005) Human α-defensins neutralize anthrax lethal toxin and protect against its fatal consequences. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4830–4835
10 Kim, C. *et al.* Human α-defensins neutralize toxins of the mono-ADP-ribosyltransferase family. Biochem. J. (in press). DOI:10.1042/BJ20060425 (www.biochemj.org).
11 Jin, T. *et al.* (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J. Immunol.* 172, 1169–1176
12 Bokarewa, M. and Tarkowski, A. (2004) Human α-defensins neutralize fibrinolytic activity exerted by staphylokinase. *Thromb. Haemost.* 91, 991–999
13 Gudmundsson, G.H. and Agerberth, B. (1999) Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. *J. Immunol. Methods* 232, 45–54
14 Ganz, T. (1987) Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect. Immun.* 55, 568–571
15 Bowdish, D.M. *et al.* (2005) A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr. Protein Pept. Sci.* 6, 35–51
16 Yang, D. *et al.* (2002) Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol.* 23, 291–296
17 Bowdish, D.M. *et al.* (2005) Impact of LL-37 on anti-infective immunity. *J. Leukoc. Biol.* 77, 451–459

18 Lehrer, R.I. *et al.* (1989) Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. *J. Clin. Invest.* 84, 553–561
19 Hill, C.P. *et al.* (1991) Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* 251, 1481–1485
20 Nassar, T. *et al.* (2002) Human α-defensin regulates smooth muscle cell contraction: a role for low-density lipoprotein receptor-related protein/α2-macroglobulin receptor. *Blood* 100, 4026–4032
21 Lillard, J.W., Jr *et al.* (1999) Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc. Natl. Acad. Sci. U.S.A.* 96, 651–656
22 Tani, K. *et al.* (2000) Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. *Int. Immunol.* 12, 691–700
23 Oppenheim, J.J. and Yang, D. (2005) Alarmins: chemotactic activators of immune responses. *Curr. Opin. Immunol.* 17, 359–365
24 Collier, R.J. and Young, J.A. (2003) Anthrax toxin. *Annu. Rev. Cell Dev. Biol.* 19, 45–70
25 Okazaki, I.J. and Moss, J. (1996) Mono-ADP-ribosylation: a reversible posttranslational modification of proteins. *Adv. Pharmacol.* 35, 247–280
26 Corda, D. and Di Girolamo, M. (2002) Mono-ADP-ribosylation: a tool for modulating immune response and cell signaling. *Sci. STKE* PE53
27 Paone, G. *et al.* (2002) ADP ribosylation of human neutrophil peptide-1 regulates its biological properties. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8231–8235
28 Yates, S.P. *et al.* (2006) Stealth and mimicry by deadly bacterial toxins. *Trends Biochem. Sci.* 31, 123–133
29 Gladysheva, I.P. *et al.* (2003) Coevolutionary patterns in plasminogen activation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9168–9172
30 Ganz, T. *et al.* (1990) Defensins. *Eur. J. Haematol.* 44, 1–8
31 Panyutich, A.V. *et al.* (1993) Plasma defensin concentrations are elevated in patients with septicemia or bacterial meningitis. *J. Lab. Clin. Med.* 122, 202–207
32 Ganz, T. and Lehrer, R.I. (1994) Defensins. *Curr. Opin. Immunol.* 6, 584–589
33 Charp, P.A. *et al.* (1988) Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. *Biochem. Pharmacol.* 37, 951–956
34 Klotman, M.E. and Chang, T.L. (2006) Defensins in innate antiviral immunity. *Nat. Rev. Immunol.* 6, 447–456
35 Chang, T.L. *et al.* (2005) Dual role of α-defensin-1 in anti-HIV-1 innate immunity. *J. Clin. Invest.* 115, 765–773
36 Wang, W. *et al*. Retrocyclins kill bacilli and germinating spores of *Bacillus anthracis* and inactivate anthrax lethal toxin. *J. Biol. Chem*. (in press). DOI:10.1074/jbc.M603614200 (http://www.jbc.org)
37 Collier, R.J. (2001) Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon* 39, 1793–1803
38 Parry, M.A. *et al.* (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends Biochem. Sci.* 25, 53–59