A Pocket Guide to Explorations of the Defensin Field

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Abstract: Antimicrobial peptides are among the most ancient effectors of host defense. Intersecting lines of research demonstrate that life forms as diverse as plants, insects, and vertebrates employ antimicrobial peptides to kill or neutralize a wide variety of microbial species. Defensins, of which there are three structural sub-families, constitute a major category of host defense peptides in vertebrates. Presented here is a brief history of the emergence of the defensin field with an emphasis on the role of these peptides in mammalian innate immunity.

Key Words: Defensins, host defense, antimicrobial, peptides.

INTRODUCTION

The theme of this volume of Current Pharmaceutical Design is focused on the role of defensins in oral health and disease. The editor has asked me to provide an introduction to the defensin research field, and the six outstanding reviews that follow, by tracking its emergence over the past 20-plus years. Let me begin this admittedly personalized retrospective with a descriptive definition for the uninitiated: *defensins* comprise three structural families (termed α, β, and θ-defensins) of host defense peptides that participate in innate and acquired immune functions of vertebrates ranging from snakes to humans (Fig. **1**). It has now been 24 years since the publication of the first mammalian defensin amino acid sequences [1], and 22 years since the term *defensin* first appeared in print [2, 3] wherein human defensins were first described. With the discovery that defensins are expressed by all vertebrates examined, and that structurally similar peptides are found in insects and plants, the field now includes investigations of the role of defensins in most multicellular life forms.

The first vertebrate defensins isolated and characterized were α-defensins expressed in phagocytic leukocytes (Fig. **1**). Investigators had labored for decades to understand how ingested microbes were lysed in the phagocytic vacuole. In the late 1950's, James Hirsch demonstrated that neutrophil extracts contained one or more substances (termed *phagocytin*) that killed bacteria *in vitro* [4]. Shortly thereafter, Zeya and Spitznagel analyzed the antibacterial activities and compositions of more highly purified protein preparations obtained from the cytoplasmic granules of guinea pig and rabbit granulocytes [5, 6]. Then, in the late 1960's, the field experienced an abrupt hiatus due a new, almost feverish focus in characterizing the newly detected "respiratory oxidase" of neutrophils. However, a number of investigators, including Robert Lehrer at UCLA, persisted in their studies of the *oxygen-independent* microbicidal apparatus of neutrophils, some of which focused on the further examination of the contents of the cytoplasmic granules of professional phagocytes [7-11].

It was my good fortune to join the Lehrer laboratory in 1980 where the antimicrobial properties of small cationic proteins produced by lung macrophages in immune-stimulated animals had been under investigation for some time. It was quite evident that these small cationic proteins were potent microbicides *in vitro* and that they rapidly permeabilized bacteria and yeast, and disrupted macromolecular synthesis. The advent of new HPLC methods and early-generation automated Edman sequencing facilitated my entry into the field as we determined the primary structures of the first two α-defensins: macrophage cationic peptides (MCP) 1 and 2 [1]. Shortly thereafter, six homologous peptides were isolated from rabbit neutrophils and characterized [12, 13]. The rabbit granulocyte peptides were all 33 or 34 amino acids in length, arginine-rich, and contained a conserved tridisulfide backbone. At about that time, Tomas Ganz, a recently minted pulmonologist joined the Lehrer lab. Tom surmised that human neutrophils would be armed with similar peptides and undertook a fresh look at the contents of the azurophil granules. Those studies confirmed the presence of human orthologs of the rabbit peptides, termed human neutrophil peptides (HNP) 1-3. The HNP's which localized to the neutrophil granules, were microbicidal against bacteria, fungi, and certain enveloped viruses [2]. Sylvia Harwig and I solved the α-defensin disulfide motif in HNP-2, allowing for first three-dimensional modeling of the peptides [14]. The first experimentally derived structures were determined by Arthur Pardi's lab who deduced the α-defensins fold by 2D-NMR [15-17], and the HNP-3 crystal structure was determined by Chris Hill in David Eisenberg's lab [18]. These early structural studies were all performed with peptides purified from rabbit or human neutrophils. Subsequently, defensin production by heterologous expression systems and solid-phase synthesis greatly accelerated the structural studies of these peptides. The paper by Pazgier *et al.* in this volume highlights technologic advances in defensin production methodologies, and the acquisition of high resolution structural data on members of the α, β, and θ-defensin subfamilies.

By the late 1980's, many studies were underway to characterize α-defensin gene structure, patterns of expression, and mechanisms of action [19-22]. At this point the existing data suggested that α-defensins were expressed almost exclusively in granulocytic leukocytes, and it appeared that the peptides exerted their antimicrobial effects in the phagolysosomal vacuole. Indeed, the cytotoxic activities of several α-defensins suggested that extracellular release of the peptides would be detrimental to the host. This perspective was fundamentally overturned when Andre Ouellette reported that α-defensins are expressed and constitutively secreted by Paneth cells in the mouse small intestine [23, 24]. In a collaboration of the Ouellette and Selsted labs, it was shown that numerous microbicidal α-defensins were produced in mouse Paneth cells [25, 26] and were readily isolated from the intestinal lumen [26], demonstrating an extracellular role of epithelium-derived α-defensins. Of particular interest was the observation that secretion of Paneth cell defensins was stimulated by gram positive and gram negative bacteria or antigens derived there from, but fungal species did not elicit this response [27]. The targeted disruption of the gene encoding matrilysin, the enzyme responsible for activation of mouse Paneth cell α-defensins, produced a mouse lacking mature intestinal α-defensins. The genetically altered mice had markedly increased susceptibility to enteric bacterial pathogens [28], providing strong evidence for a role for epithelial α-defensins in mucosal host defense.

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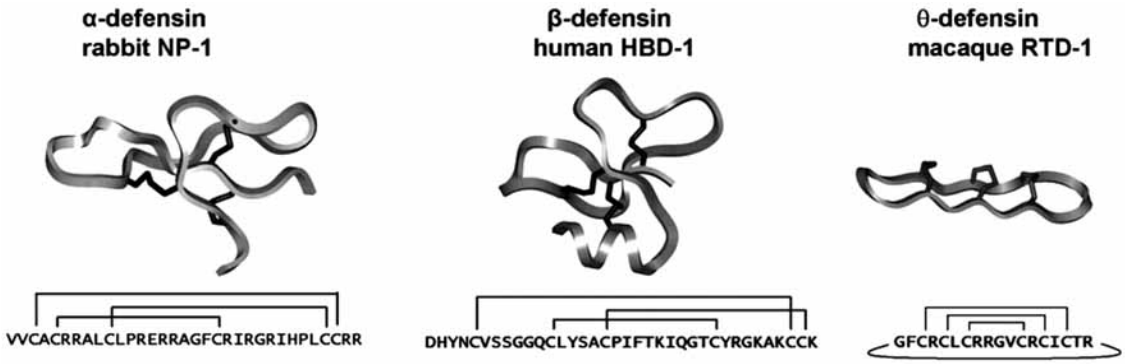

Fig. (1). Defensin peptides.

Ribbon diagrams of representative members of α, β, and θ-defensin peptide families are shown with schematics of their covalent structures and disulfide bonding patterns (adapted from reference [42] with permission).

In the early 1990's, a second family of defensins emerged from studies carried out on bovine tissues in the labs of Charles Bevins and the Selsted group. The Bevins lab isolated and characterized bovine tracheal antimicrobial peptide (TAP) [29], the first member of the β-defensin peptide family, and this was followed by the report of thirteen homologous peptides from the granules of bovine neutrophils [30]. Like α-defensins, the bovine β-defensins were tridisulfide, cationic peptides that were microbicidal against bacteria, fungi, and certain viruses. Interestingly, the β-defensin disulfide motif (1-5, 2-4, 3-6) turned out to be different from that found in the α-defensins (1-6, 2-4, 3-5) [31]. Despite the differing disulfide patterns and cysteine linear spacing, the α– and β-defensin backbones are nearly superimposible [32]; (see Pazgier *et al*. in this volume).

Soon after bovine β-defensins were described came the discovery by Bensch *et al*. of a human ortholog present in human plasma [33]. Bob Lehrer and colleagues demonstrated that HBD-1 is expressed in the epithelium of many human tissues [34], and Tom Ganz isolated several isoforms of HBD-1 from human urine [35]. The different isoforms possessed modest antibacterial activities *in vitro*. These studies set the stage for the isolation of HBD-2 and HBD-3 from psoriatic skin [36, 37]. Like HBD-1, these β-defensins are expressed in numerous epithelial sites as well as in some leukocytes [38]. However, unlike HBD-1 which is expressed constitutively, gene expression of HBD-2 and -3 (and several other human β-defensins analyzed at the transcriptional level) are induced by a variety of proinflammatory stimuli. Among the sites of β-defensin expression was mucosal epithelium in the oral cavity [39, 40]. In this volume, Chung *et al*. provide a review of the role and regulation of human β-defensin expressed by gingival epithelium. Abiko and Saitoh then review the role of α- and β-defensins in human saliva. In Chapter 3, Komatsuzawa *et al*. review the microbicidal properties of α- and β-defensins against oral bacteria, including oral cariogenic and periodontopathogenic species.

As the millennium was coming to a close, one more defensin subfamily was revealed: the θ-defensins. In studies of rhesus macaque leukocytes, my colleague Yi-Quan Tang purified a potently microbicidal peptide from neutrophil granules. The peptide contained three disulfides, but only 18 amino acids (as determined by amino acid analysis and mass spectroscopy), so this molecule was too small to be an α- or β-defensin. The peptide lacked a free N-terminus that would enable sequencing, so Yi-Quan determined the primary structure of the peptide the old fashioned way - by aligning proteolytic fragments. To our amazement, the peptide backbone was found to be cyclic (Fig. **1**), with the three disulfides stabilizing a β-sheet connected by two hairpins at the ends. The symbol θ was chosen to designate this class of defensin because of the covalent architecture described in the backbone of this peptide, and rhesus theta defensin-1 (RTD-1) was the prototype [41]. Next door, cloning experiments carried out by Jun Yuan disclosed that RTD-1 was biosynthesized in neutrophil precursors by an improbable route: nonapeptides from α-defensin-like precursors were excised and spliced together, head-to-tail, to produce the cyclic molecule, thus providing the first example of a macrocyclic peptide in animals [41, 42]. The Lehrer group, which had independently purified macaque θ-defensins, showed that binary pairing of nonapeptides derived from unique θ-defensin precursors could generate different mature peptides, and three of the predicted six θ-defensins were isolated from bone marrow [43] and peripheral blood [44] from rhesus monkeys. Analysis of the θ-defensin precursor genes demonstrated that θ-defensin genes are closely related to those encoding α-defensins, differing only by the fact that the primary translation product is truncated about twenty amino acids upstream of the usual α-defensin carboxyl terminus. To date there are no published data on the post-translational processing machinery responsible for the excision/ligation steps that produce θ-defensins.

Surveys of prosimian and primate tissues and/or gene data bases disclosed that θ-defensins are expressed in cells of several Old World monkeys, in at least one Lesser Ape (*H. syndactylus*) and one Great Ape (orangutan), but not in other New World monkeys, great apes or humans [45]. The lack of peptide expression in humans, chimpanzees, and gorillas is the result of a mutation that introduced a premature stop codon in the signal sequence in exon 2. It is apparent from these studies that θ-defensin peptide expression appeared after the emergence of prosimians and began disappearing in ancestors of orangutans since the genome of this primate contains both active and silenced θ-defensin genes, and the mutated codon is highly conserved [46].

Several laboratories have recently demonstrated that θ-defensins have antiviral properties *in vitro* against HIV-1 [47, 48] and herpes simplex virus [49], and that this effect is mediated by binding of the cyclic peptide to carbohydrate moieties on the virus (e.g., HIV gp120) and/or target cells (e.g., CD4); [50-52]. Moreover, compared to many α- and β-defensins studied previously, we have found that θ-defensins possess very little toxicity to primary or cultured mammalian cells, and are exceedingly well tolerated by rodents when administered systemically (unpublished data). These findings have raised the possibility that θ-defensins might be useful templates for antiviral drug development.

Given that there are several hundred publications documenting the antimicrobial properties of α, β, and θ-defensins *in vitro* or *in vivo*, it is not surprising that the physiologic role of the peptides was presumed for years to be predominantly antimicrobial. Indeed evidence supporting this view is reiterated in several of the chapters in this volume. However, there is also a large body of data implicating defensins in more complex immune responses. Studies dating back

to the late 1980's, demonstrated that human α-defensins were chemotactic for monocytes [53]. In their review in this volume, Yang *et al*. present a concise overview of the multiple roles defensins may play in mobilizing and activating phagocytes, mast cells, T lymphocytes, and dendritic cells, as well as the induction of numerous proinflammatory and regulatory cytokines. As discussed, these responses may be critical for informing the adaptive arm of the immune system that a *bona fide* pathogen has been encountered by the host, providing an important signal for eliciting appropriate Th1 and Th2 responses. A role for defensins in bridging innate and adaptive responses also has implications for eliciting anti-tumor immunity in lymphoid neoplasias, as demonstrated in mouse models [54, 55]. In this volume, Meyers and Harder review studies on the expression of defensins in squamous cell carcinoma of the oral cavity and other cancers, and present intriguing theories on the role of defensins and other antimicrobial peptides as tumor cell cytotoxins and markers of cancer progression.

The current state of defensin research is one of rapid growth, as increasing numbers of investigators delineate diverse biologic roles for these peptides in immune responses to microbes and neoplastic cells. More than two decades ago, our ignorance of these roles elicited the (paraphrased) comment from a grant reviewer that *defensins were interesting molecules in search of a function*. Indeed we are now in possession of a large body of evidence implicating defensins in *numerous* biological functions, and the physiologic relevance of these roles has been validated in a number of mouse models. Reflexively, the skeptic might ask: how can defensins participate in such a variety of biological processes? One explanation is provided by studies demonstrating that defensins evolved very rapidly during evolution [56-58] as vertebrate species encountered unique external and internal (i.e., commensal) microbial ecosystems. There is abundant evidence that α-defensins evolved from preexisting β-defensins, and that θ-defensins are the binary ligation products of truncated α-defensin precursors. Thus it is apparent that α, β, and θ-defensins have a common β-defensin ancestor. Mutation and repeated gene duplication within the defensin subfamilies have generated diverse gene products that equip the host to recognize and neutralize pathogens, recruit adaptive elements of the immune response, and possibly respond to oncogenic transformation. As our knowledge of defensin biology continues to grow, many of the questions posed by the authors in the articles that follow will surely be answered.

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