Defensins and Other Antimicrobial Peptides at the Ocular Surface

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Abstract

Although constantly exposed to the environment and "foreign bodies" such as contact lenses and unwashed fingertips, the ocular surface succumbs to infection relatively infrequently. This is, in large part, due to a very active and robust innate immune response mounted at the ocular surface. Studies over the past 20 years have revealed that small peptides with antimicrobial activity are a major component of the human innate immune response system. The ocular surface is no exception, with peptides of the defensin and cathelicidin families being detected in the tear film and secreted by corneal and conjunctival epithelial cells. There is also much evidence to suggest that the role of some antimicrobial peptides is not restricted to direct killing of pathogens, but, rather, that they function in various aspects of the immune response, including recruitment of immune cells, and through actions on dendritic cells provide a link to adaptive immunity. A role in wound healing is also supported. In this article, the properties, mechanisms of actions and functional roles of antimicrobial peptides are reviewed, with particular emphasis on the potential multifunctional roles of defensins and LL-37 (the only known human cathelicidin) at the ocular surface.

Keywords

antimicrobial peptide; cationic peptide; defensin; immune response; innate immunity; LL-37; microbicidal peptide; peptide; wound healing

I. INTRODUCTION

The body's first line of defense against invading pathogens is the innate immune response. This response encompasses a variety of mechanisms, some of which (e.g., skin and mucous membranes) are designed to stop organisms from entering the body in the first place. Others, such as the presence of polymorphonuclear neutrophils in the tear film, are designed to stop an organism in its tracks if it does happen to breach the outer defenses, and, at the same time, to signal to the T lymphocytes of the adaptive immune system to prepare for action. The innate immune response utilizes a variety of different substances to kill invading organisms. These include enzymes of the respiratory burst, whose activity generates lethal reactive oxygen species within the phagocytic vacuole of neutrophils and secreted enzymes, such as lyzozyme and phospholipase A2, which are present in a variety of body fluids, including the tear film. [1] The innate and adaptive arms of the immune system have been reviewed recently by Dempsey et al[2] and Beutler.[3]

Studies over the past 20 years have revealed that the innate immune system also uses a variety of small peptides (defined as having less than 100 amino acids) to ward off unwanted invaders. The majority of these peptides have a positive charge due to an excess of positively charged amino acids, such as arginine and lysine; hence, they are frequently referred to as *cationic antimicrobial peptides*.

The first published report indicating the existence of such peptides in animal species appeared in 1963, but it was not until the early 1980s that the first peptides were identified.[4] By that time, it was well established that plants use peptides as a major mechanism of defense.[5] In 1981, Steiner et al[6] described the sequence of two peptides, later called *cecropins*, that they had isolated from the moth *Hyalophora cecropia* the previous year.[7] In 1983, Selsted et al[8] described the first mammalian peptides, which were found in rabbit macrophages. In 1985, the first human antimicrobial peptides, which the authors referred to as "defensins" were isolated. [9,10] Subsequent studies have shown that antimicrobial peptides are a common feature of the immune system of many species. A database maintained by the University of Trieste (http://www.bbcm.units.it/~tossi/pag1.htm) contains a list of over 800 antimicrobial peptides (and some proteins) from species as widely different as amoeba, plants, penguins, and humans. Various attempts have been made to classify the peptides. Most commonly, they are assigned to groups based upon secondary structural features, with three to four different groups being recognized: linear α-helical peptides (e.g., LL-37), peptides with β-strands linked by disulphide bridges (e.g., defensins), loop peptides (e.g., bactenecin), and those with a high proportion of specific amino acids (e.g., histatins).[11–13] As discussed in more detail below, these peptides exhibit potent antimicrobial activity against bacteria, fungi, and some viruses, chiefly by disrupting the microbial membrane.

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Initial interest in these peptides focused on their antimicrobial activity; however, it soon became apparent that some of the peptides are also able to modulate various mammalian cell functions. This has led to the concept that, at least in mammalian systems, cationic antimicrobial peptides

are multifunctional molecules.[14–18] As discussed below, the peptides provide protection against invading organisms by direct microbicidal activity and by modulating the activity of a variety of other components of the immune system. Additionally, a growing number of studies document the participation of antimicrobial peptides in activities not directly related to the immune response.

In this review, the expression, mode of action, and functional roles of antimicrobial peptides are reviewed with emphasis on their microbicidal effect and potential other functions at the ocular surface. Because the defensins and the cathelicidin LL-37 are the most studied, they are the best understood of mammalian cationic antimicrobial peptides; therefore, their role forms the focal point of this review. For details on other antimicrobial peptide families, particularly those of lower species and plants, and anionic peptides with antimicrobial activity, the reader is referred to various review articles.[5,12,19–23]

II. DEFENSINS AT THE OCULAR SURFACE

A. Defensins: An Overview

Classic mammalian defensins are 29 to 45 amino acids in length and are characterized by the presence of six cysteine residues that interact to form three disulphide bonds and a β-sheet structure. They can be classified into two groups, referred to as α and β, depending upon the location and connectivity of the cysteines. For α-defensins, the pattern of connectivity is C1-C6, C2-C4 and C3-C5, whereas for β-defensins, the arrangement is C1-C5, C2-C4 and C3-C6.[24] A third novel defensin class, referred to as θ (also minidefensins), has been identified in rhesus macaque leucocytes.[25–27] The 18 amino acid θ-defensins are unique in that they are formed by splicing of two truncated α-defensins to create a circular structure. Six genes for human θ-defensins (termed retrocyclins) have been identified, but premature stop codons prevent their translation.[28]

In humans, six α and four β-defensins have been identified and characterized. The six α-defensins are human neutrophil peptide (**HNP**) 1 through 4 and human defensin (**HD**) 5 and 6. As their name suggests, HNP 1–4 are found in neutrophils, where they reach high concentrations within azurophil granules.[9,10,29] They have also been found in monocytes and lymphocytes.[30] HD5 and HD6 are present in the granules of Paneth cells, which are specialized host defense cells of the intestine.[31,32] HD-5 has also been localized to the female reproductive tract.[33] The α-defensins are expressed constitutively and are encoded as 90–100 amino acid prepropeptides.[34,35] HNP 1–4 are synthesised, processed to the mature product, and packaged into azurophil granules as the neutrophils are developing in the bone marrow.[36,37] HD-5 and -6 are stored as proforms, then are processed during or after release from the Paneth cell granules.[24]

The four β-defensins are named human β-defensin (**hBD**) 1–4 and are expressed chiefly by epithelial tissues, although expression by immune cells (monocytes, macrophages and dendritic cells) has been reported.[38] Like α-defensins, β-defensins are encoded as larger precursor molecules; however, they are not stored in cytoplasmic granules.[24] hBD-1, which was first isolated from human plasma,[39] is constitutively expressed by a variety of epithelia, including airway epithelia,[40] urogenital tissues,[41] nasolacrimal duct,[42] and mammary gland.[43] hBD-2 and hBD-3 are inducible in many epithelia by bacterial products and cytokines.[44–46] The expression of hBD-4 is more limited, with testes and epididymis having the highest levels.[47,48] The expression of this peptide is inducible, although not by the cytokines reported to upregulate hBD-2 and hBD-3.[47] In 2002, Schutte et al[49] reported the discovery of 28 novel human β-defensin genes (**DEFB**) identified with use of a computational method. While some of these novel genes mapped to chromosome 8 in regions close to the known α-and β-defensin genes, others mapped to chromosomes 6 and 20. A number of these novel genes are now under

investigation. Yamaguchi et al[48] cloned two of the new genes, DEFB-5 and -6, and found that their products, named hBD-5 and -6, respectively, were specifically expressed in the epididymis. Recent cloning of five more of these novel genes (DEFB25–29, on chromosome 20) also shows that they are preferentially expressed by the male genital tract.[50] Prematanachai et al[51] studied the expression of DEFB-5 through 14 in gingival keratinocytes and found that DEFB-7, -9, -11, and -12 were constitutively expressed, whereas DEFB-8, and -14 were inducible by cytokines and infection. hBD-5 and the product of DEFB-9 have also been found to be expressed in the lung.[52]

B. Defensins of the Human Ocular Surface

The existence of defensins at the human ocular surface was first reported in 1998.[53–55] Gottsch et al[53] observed that α-defensin mRNA (the in situ hybridization probe they used was previously found to identify HNP-1 and -3[34]) and protein (the antibody used detected HNP-1, -2 and -3) was detectable in human corneal stroma in cases of rejected transplants and post-infectious keratitis but not normal cornea. Haynes et al[55,56] also observed positive immunoreactivity for HNP1–3 in inflamed conjunctiva and samples of normal tear film. Recently, Zhou et al[57] confirmed the presence of HNP1–3 in tear film and, using liquid chromatography-mass spectrometry, they determined the levels of HNP-1 and -2 to be in the range 0.2–1 μg/ml. In each of these studies, the authors presumed that inflammatory cells, primarily neutrophils, were the source of α-defensins. Haynes et al[55,56] also used RT-PCR to look for expression of the α-defensins HD-5 and HD-6; however, no evidence for the production of these defensins was found in any of the ocular samples they tested.

Both cornea and conjunctival epithelial cells express β-defensins. hBD-1 is constitutively expressed,[54–56,58–61] whereas the expression of hBD-2 is variable.[54,59–61] Indeed, we[60] found this defensin to be expressed by the corneal epithelium in only two of eight cadaveric corneas tested (Figure 1). However, hBD-2 expression is known to be inducible.[44] McNamara et al[58] showed that hBD-2 expression by SV40-immortalized human corneal epithelial cells was upregulated by bacterial lipopolysaccharide by a pathway involving tyrosine kinase and p38 mitogen-activated protein (**MAP**) kinase activation.[62] A later study also showed nuclear factor-κB (**NFκB**) to be involved.[63] Studies of bacterial induction of hBD-2 expression in several cell types corroborate the involvement of MAP kinase and NFκB.[64–68] Also, Birchler et al[68] and Wang et al[70] observed that upregulation of hBD-2 expression by the bacterial products lipoprotein and lipotechoic acid is mediated via Toll-like receptor 2.

We have shown that hBD-2 is upregulated in regenerating corneal epithelium in an in vitro organ culture model of corneal epithelial wound healing.[71] Additionally, we have shown that hBD-2 expression by corneal[60] (Figure 2) and conjunctival epithelial cells[61] is upregulated by the proinflammatory cytokines interleukin-1 (**IL-1**) and tumor necrosis factor (**TNF**)-α. In the case of corneal epithelial cells, the effects of IL-1 on hBD-2 expression were mediated via p38MAP kinase, JNK and NFκB.[60] We have also observed that corneal (Figure 1) and conjunctival epithelial cells (Figure 3) express hBD-3. Some studies have indicated that expression of this defensin is inducible[45,46,72] by TNFα and interferon-γ; however, we did not observe increased hBD-3 expression in either corneal (McDermott et al, unpublished observation) or conjunctival epithelial cells[61] following cytokine treatment. We have also used RT-PCR to investigate the expression of three other β-defensins, hBD-4, -5 and -6, but did not find evidence of their production by cornea or conjunctival epithelial cells (McDermott, Proske & Huang, unpublished observation). β-Defensins have not been detected in the tear film.[57]

Because inflammation is a significant component of all forms of dry eye,[73] we were interested to know what effect this condition had on the expression of β-defensins by conjunctival epithelial cells. We observed that conjunctival epithelium from patients with symptomatic mild to moderate dry eye expressed hBD-2, whereas samples from subjects without dry eye did not

(Figure 3).[61] There was no difference in the expression of hBD-1 and hBD-3 between the two groups. Kawasaki et al[74] also noted increased expression of DEFB4 (formerly known as DEFB2), the gene for hBD-2, in conjunctival epithelial cells of patients with Sjogren syndrome, who have a very severe form of dry eye resulting from a systemic autoimmune condition. Based on the fact that hBD-2 expression can be upregulated by proinflammatory cytokines[61] and that such cytokines are increased in dry eye,[75,76] we hypothesized that increased hBD-2 expression is the result of enhanced inflammatory cytokine activity at the ocular surface in this condition. As discussed below, upregulated hBD-2 expression likely gives additional antimicrobial protection to the compromised ocular surface, and, through the nonmicrobicidal activities mediated by the peptide, may have other, although not necessarily beneficial, effects.

As summarized in Table 1, current data show that the epithelia of the human ocular surface normally express hBD-1 and hBD-3, whereas hBD-2 expression can be upregulated with appropriate stimuli, such as infection and inflammatory cytokines. Additionally, α-defensins HNP 1 through 3 are normally present in the tear film, and, during inflammation, they may be released in the cornea by infiltrating inflammatory cells.

C. Defensins at the Ocular Surface in Non-Human Species

Expression of β-defensins is not unique to human ocular surface epithelia. In 1997, Stolzenberg et al[77] reported that bovine conjunctiva expressed lingual antimicrobial peptide (**LAP**), a peptide so named because it was first identified in tongue lesions.[78] We have confirmed Stolzenberg's observation and shown that tracheal antimicrobial peptide (**TAP**), the first β-defensin to be identified[79] and the bovine homologue of hBD-2, is also expressed by bovine conjunctival epithelium.[80] Additionally, we found that whereas bovine corneal epithelium constitutively expressed LAP, TAP was significantly expressed only after injury. Other studies in our laboratory have shown that rBD-1 and rBD-2, the rat homologues of hBD-1 and hBD-2, respectively,[81] are expressed by rat cornea and conjunctiva and that rBD-2 expression is upregulated after corneal injury in vivo.[82] Interestingly, Gottsch et al[53] reported that IL-1α induced LAP expression in bovine keratocytes cultured in the presence of serum. We repeated their experiment using cultured human corneal fibroblasts and primers specific for human β-defensins, but we found no such response (McDermott and Redfern, unpublished observation).

III. LL-37 AND OTHER ANTIMICROBIAL PEPTIDES AT THE OCULAR SURFACE

To date, defensins have been the most studied of the human antimicrobial peptides; however, several others have been identified and are now the subject of intense investigation. In particular, the 37 amino acid linear peptide LL-37 has gained much attention. This peptide is derived by cleavage of a larger precursor referred to as *human cationic antimicrobial protein 18* (**hCAP18**), which is the only known human member of a family of antimicrobial peptides called the *cathelicidins* (for recent reviews, see references [19] and [83]). LL-37/hCAP18 was first cloned from bone marrow[85,86] and is expressed by immune and inflammatory cells[30,87,88] and epithelial tissue.[89–91] LL-37 is released from its parent molecule by extracellular cleavage with proteinase 3.[92] It has potent antibacterial activity and, in keeping with the observation that antimicrobial peptides are multifunctional molecules, exerts a variety of effects on mammalian cells.[19,83,93] We used RT-PCR and immunoblotting to determine if ocular surface epithelial cells express LL-37. Low expression (mRNA and protein) of this antimicrobial peptide was detected in human corneal epithelial cells, and this was upregulated by injury and exposure to IL-1β.[94] We also detected the peptide in cultured human conjunctival epithelial cells (Huang and McDermott, unpublished observation) and have shown that it stimulates corneal epithelial cell migration and kills ocular pathogens.[95] These preliminary findings strongly suggest that LL-37 is an important ocular surface defense peptide.

Several other molecules may also fulfill this role. Cationic antimicrobial protein 37 (**CAP37**, azurocidin, heparin binding protein), a protein first isolated from human neutrophil granules, [96] has been found to be expressed by rabbit corneal epithelium, stromal fibroblasts, and bulbar conjunctiva in response to infection with *Staphylococcus aureus*.[97] The authors also reported that IL-1β and TNFα induced CAP37 expression in immortalized human corneal epithelial cells. Strictly speaking, being 222 amino acids in length, CAP37 cannot be called a peptide; nevertheless, it has features in common with its smaller relatives, including similarities in the mechanism of antimicrobial activity (such as sensitivity to ionic strength, see below). CAP37 has also been shown to modulate various mammalian cell functions,[98] including stimulation of human corneal epithelial cell migration, proliferation, and regulation of gene expression. [99,100]

A further candidate for an ocular surface antimicrobial peptide is the 43 amino acid peptide Thymosin-β4 (Tβ-4). This peptide is a member of the thymosin family of G-actin sequestering peptides and has been shown to have additional biological effects, including chemotaxis, angiogenesis, and inhibition of inflammation,[101] and, most recently, antimicrobial activity. [102] Sosne et al have shown that exogenously applied Tβ-4 stimulated corneal epithelial healing and cytokine production after debridement with alcohol.[103] Similarly, they found that Tβ-4 promoted wound healing after alkali injury, but, in this case, cytokine production was decreased.[104] They have also shown that Tβ-4 stimulates migration of human corneal and conjunctival epithelial cells.[103,105] Katz et al[106] reported that Tβ-4 is a component of the normal human tear film, and recently Sosne et al[107] reported that Tβ-4 levels increased in a mouse model of wound healing. Thus, Tβ-4 bears the hallmarks of a multifunctional peptide with antimicrobial activity, although, as an anionic peptide, its mechanism of action must be distinct from that of the cationic peptides, such as the defensins.

Other human peptides with known antimicrobial activity include histatins,[108] human natriuretic peptides,[109] dermcidin,[110] hepcidin/liver-expressed antimicrobial peptide (**LEAP**)-1,[111,112] LEAP-2,[113] HE2α and HE2β1,[114] and the following chemokines of the CXC and CC families: CXCL9, 10, 11[115]; CXCL4, CTAP-3, CCL5[102]; CCL28[116]; CCL20.[117,118] Of these, corneal and/or conjunctival mRNA expression has been reported for histatins and CCL28[119] and LEAP-1 and -2, and dermcidin.[120] Histatin 5 has been detected in tear film[121] and CCL20 mRNA was found to be markedly upregulated in human corneal fibroblasts in response to IL-1α.[122] For most of these peptides, studies are required to show that translation and protein production does actually occur. Once complete, the results of such studies are likely to confirm that, in addition to defensins and LL-37, the ocular surface is equipped with a wide range of antimicrobial peptides.

IV. ANTIMICROBIAL ROLE OF DEFENSINS AND OTHER PEPTIDES AT THE OCULAR SURFACE

A. Mechanism of Antimicrobial Activity

As signified by their name, antimicrobial peptides have been identified based on their ability to kill or prevent the growth of microorganisms. While the individual peptides vary in their ability to kill different organisms, as a group they are active against both Gram positive and Gram negative bacteria, some fungi, and some viruses. As noted earlier, over 800 different antimicrobial peptides have been described; the vast majority are cationic and can be classified into several different groups based on their structure. Despite this significant diversity, it is generally accepted that the cationic peptides exert their antimicrobial activity in a similar fashion, although the details of this are poorly understood. By virtue of their positive charge, the peptides interact electrostatically with negatively charged components, particularly phospholipids, of microbial cell membranes, leading to increased permeability of the cell

membrane and death. The relative selectivity of the peptides for microorganisms versus eukaryotic cells has been attributed to the low membrane potential, fewer negatively charged phospholipids, and high cholesterol levels of the latter.[123,124] Several models have been proposed to describe the peptide-membrane interactions. For a more in-depth review than is given below, the reader is referred to articles by van't Hof et al[12] and Shai.[125]

In the barrel-stave model, peptide monomers associate with the membrane and then assemble together to form a stable transmembrane pore. The pore size increases as more peptide monomers are added, and leakage of intracellular contents through the pores eventually leads to cell death. The α-defensin HNP-2 has been reported to form stable pores with a maximum diameter of 2.5 nm, possibly representing a hexamer of defensin dimers.[126]

In the carpet model, the microbial membrane is first coated in a "carpet" of peptide; then, when the point of saturation is reached, the membrane collapses, creating "worm holes," leading to lysis of the organism. The carpet model has been used to describe the mechanism of action of many antimicrobial peptides, including human β-defensins[127] and LL-37.[128]

In the final model, the aggregate channel model, the peptides cluster into unstructured aggregates within the membrane, which causes the transient formation of channels through the membrane. These channels allow for the leakage of ions and, additionally, allow the peptides to cross the membrane and reach intracellular targets, where they exert their killing effect. The histatins are believed to reach their intracellular target, the mitochondria, in this way.[129]

By utilizing one of the approaches outlined above, antimicrobial peptides are able to kill Gram negative and positive bacteria and some fungi. Antiviral activity, particularly against some enveloped viruses, may be mediated by similar mechanisms[130,131]; however, other modes of action also appear to be involved. Indeed, HNP-1 and HD-5 have been observed to inhibit adenoviral infection in vitro,[132,133] and, as adenovirus strains do not have an envelope, a mechanism other than membrane perturbation must be involved. Defensins have been shown to be active against herpes simplex virus (**HSV**) and human immunodeficiency virus (**HIV**). [130,131,134] Recent studies suggest multiple modes of anti-HSV and -HIV activity, including inhibition of viral entry by masking of essential glycoproteins on the viral envelope or via a chemokine-like effect of the defensin, which results in downregulation of the coreceptors used by the viruses to gain entry to the host cell.[135–138]

In vitro studies show defensins and other peptides to have antimicrobial activity at concentrations in the micromolar range, with minimum inhibitory concentrations (**MIC**) of 1–100 μg/ml, depending upon peptide and organism.[23,139,140] Within the confines of neutrophil granules, it is estimated that α-defensin concentrations can reach 1–10mg/ml, a level more than adequate for antimicrobial activity.[139] The levels of α-defensins in body fluids is low, typically less than that required for antimicrobial activity, but they rise markedly in inflammation and infection to concentrations within the active range.[141–143] Whether antimicrobial peptides secreted by epithelial surfaces can reach such high levels has been debated, but recent studies show that in some tissues it is possible. Liu et al reported that following stimulation with IL-1, levels of hBD-2 in organotypic epidermal cultures reached 3.5 to 16μM.[144] Also, Oren et al observed that in human epidermis, hBD-2 is packed into lamellar bodies and localized to intercellular spaces, which would help increase local concentrations.[145] Indeed, it had previously been suggested that high levels may actually be achieved in vivo by virtue of the peptides being concentrated at their sites of secretion.[146] hCAP18, the parent molecule of LL-37, is present in plasma at a concentration of 1.2 μg/ml, but, interestingly, it is found in seminal plasma at seventy times this level.[147]

Although it has been extensively shown that defensins and other peptides have potent antimicrobial activity in vitro, conclusive proof of their activity in vivo is not as easily obtained.

Nevertheless, a small number of studies do show that antimicrobial peptides are indeed important components of the innate immune system. Inactivation of the gene for matrilysin, an enzyme required for processing mouse intestinal α-defensins to their active form, increased the susceptibility of the animals to infection with *Salmonella typhimurium*.[148] Mice lacking the gene Cnlp, which codes for CRAMP, the mouse homologue of LL-37, were found to be much more susceptible to skin infection caused by group A streptococcus.[149] Knocking out the mouse β-defensin-1 gene led to delayed clearance of *Haemophilus influenzae* from the lung[150] and an increase in *Staphylococcus* colonization in the bladder.[151] Most recently, Salzmann et al reported that transgenic mice expressing the gene for human intestinal α-defensin HD-5 were much more resistant to an oral challenge with *S. typhimurium* than their wild-type counterparts.[152]

Other evidence supporting a role of antimicrobial peptides in vivo comes from a study of the severe recessive disorder *morbus Kostmann*.[153] In this condition, an unknown mutation results in the failure of promyelocytes to develop into myelocytes, leaving patients with very few neutrophils. Although patients can now be treated with recombinant granulocyte-monocyte colony-stimulating factor to boost neutrophil numbers, they are still prone to recurrent infections, and, in particular, have severe oral infections. Putsep et al reported that such patients have normal neutrophils in terms of respiratory burst activity and lactoferrin production but that LL-37 is missing from both the neutrophils and saliva.[153]

B. Antimicrobial Activity at the Ocular Surface

Several studies show that antimicrobial peptides are effective against ocular surface pathogens in vitro. A variety of peptides have been tested, including defensins, magainins (antimicrobial peptides originally isolated from the African clawed frog[154]) and cecropins. Table 2 summarizes the peptides and the organisms against which they are effective.

These examples clearly demonstrate that antimicrobial peptides can kill a variety of known ocular pathogens in vitro. However, it remains to be determined if this is actually the case with the peptides that are present at the ocular surface in vivo. One question is whether or not the peptides reach high enough concentrations to have antimicrobial activity. Zhou et al reported tear fluid levels of HNP-1 and -2 to be in the range 0.2–1 μg/ml, and that these rose to 5–15 μg/ml in patients after ocular surgery, so reaching the necessary levels for microbicidal activity, at least against some organisms.[57]

Based on measurements of hBD-1 and hBD-2 secretion by cultured human corneal epithelial cells, we have estimated that human corneal epithelium is capable of producing microgram quantities of hBD-1 and hBD-2, but we do not know the actual concentrations.[60] As is the case with skin,[145] accumulation of the peptide in intercellular spaces may mean that very high concentrations are, indeed, achieved. Interestingly, several studies have shown that antimicrobial peptides can interact with each other and with other antimicrobial substances, such as lysozyme, in an additive or synergistic fashion.[161–163] Therefore, the micromolar concentrations of peptide required for in vitro activity may not be required at the ocular surface, because there may be synergistic interactions between the several peptides present at the surface and also with components of the tear film, such as lysozyme.

An additional factor that must be considered when addressing the question of whether or not antimicrobial peptides are active at the ocular surface is the possibility that components of the tear film may compromise their activity. Indeed, Rich et al[164] reported that the activity of the synthetic rabbit defensin NP-3a against *P. aeruginosa* was reduced in the presence of human tears in vitro, and we have observed similar effects on the activity of hBD-2.[165] The tear film contains a significant amount of sodium chloride,[166] and it is known that the antimicrobial activity of many peptides is sensitive to physiological salt concentrations, presumably because

the salt interferes with the initial electrostatic interactions between peptide and microbial membrane.[139] Thus, any peptide secreted onto the ocular surface by the epithelial cells, present in the tear film or exogenously applied (see Section VI.B), may be rendered ineffective by the salt in the surrounding environment. Interestingly, hBD-3, which we find to be constitutively expressed by the ocular surface epithelia, [60,61] is the defensin least sensitive to the effect of salt,[45] a characteristic attributed to its having a higher net positive charge and an increased affinity to form dimers compared to hBD-1 and-2.[167] It should also be noted that the effects of salt are dependent upon the concentration of peptide, with higher levels being minimally affected.[168] Furthermore, it has been observed that synergistic interactions between peptides were able to overcome the detrimental effects of salt.[168] Thus, while a high enough concentration of peptide at the ocular surface may circumvent the actions of salt, such a concentration may not be necessary, due to synergistic interactions between the different peptides present.

In fact, having synergistic interactions rather than high peptide concentrations may be the better option, as both defensins and LL-37 are cytotoxic to some mammalian cells at high concentrations. α-Defensins have been shown to be cytotoxic to various tumor cell lines,[169] MRC-5 fibroblasts and primary cultured human umbilical vein endothelial cells,[170] A549 lung epithelial cells,[170,171] and alveolar macrophages.[172] hBD-1 was found to be toxic to NIH-3T3 fibroblasts,[173] and LL-37 was toxic to lymphocytes and peripheral blood leukocytes. [174] Cytotoxicity was concentration-dependent, and, typically, it first became apparent at antimicrobial peptide concentrations of approximately 25 μg/ml, although this varied with the cell types being tested.

The mechanism of cytotoxicity has similarities to that of antimicrobial activity and involves an electrostatic (and initially reversible) interaction with the cell membrane, followed by pore formation and possible entry of the peptide into the target cell and DNA damage.[175] However, while antimicrobial effects are apparent in minutes, cytotoxic effects take several hours to develop. Thus, it is possible that prolonged and excessive neutrophil infiltration and/or epithelial expression of hBD-2 and LL-37 may raise the concentration of antimicrobial peptides to a level that may actually cause damage to the ocular surface.

The tear film is a complex mixture of over 500 different proteins,[176] some of which have been shown to be capable of inactivating some antimicrobial peptides. For example, α2-macroglobulin and serpins (serine protease inhibitors) both bind to and inactivate defensins. [177,178] Although these proteins have been detected in tear film,[179] their concentrations may not reach a level high enough to effectively impair peptide antimicrobial activity. But, if appropriate concentrations are achieved, these inhibitors may represent one mechanism for regulating the activity of the antimicrobial peptides and thus preventing unwanted cytotoxicity.

Given that antimicrobial peptides are active against ocular surface pathogens in vitro, that mechanisms exist to circumvent some of the potential interference from the tear film, and that the peptides are active in vivo (see section IV.A), the defensins, LL-37 and other peptides present at the ocular surface are likely to play a major role in defending the ocular surface from infection. Based on the previously described studies of peptide expression, it appears likely that hBD-1 and hBD-3 provide the main baseline defense against infection. Then, after injury, inflammation, or infection, hBD-2 and LL-37 are additionally expressed, and neutrophil infiltration causes an increase in α-defensins. As these antimicrobial peptides have differing spectra of activity[44]—for example, hBD-3 is particularly effective against *Staphylococcus aureus*,[45] whereas hBD-2 has potent anti-pseudomonal activity[44]—and this enhances the ability of the ocular surface to ward off a wide range of pathogens.

V. NONMICROBICIDAL ROLES OF DEFENSINS AND OTHER ANTIMICROBIAL PEPTIDES AT THE OCULAR SURFACE

Defensins and other antimicrobial peptides were first identified through their ability to kill microorganisms; however, it is now recognized that many also have the capacity to modulate mammalian cell behavior. Indeed, the list of peptide nonmicrobicidal activities grows ever longer and more varied, with effects as disparate as defensin inhibition of adrenocorticotrophin activity (formerly, some defensins were also referred to as *corticostatins*),[180] defensin regulation of smooth muscle cell contraction,[181] and LL-37-induced angiogenesis.[182] Because they occur via mechanisms completely independent of that responsible for antimicrobial activity (discussed below), these activities typically occur at peptide concentrations much lower than those required for effective antimicrobial activity and are not sensitive to the effects of salt.

Most attention has been paid to the effects of antimicrobial peptides, particularly those of defensins and LL-37, on immune cell functions. Defensins are chemotactic for T cells, monocytes, immature dendritic cells, neutrophils, and mast cells.[183–188] They have also been shown to modulate monocyte and epithelial cell cytokine and chemokine production[189–191] and stimulate histamine release from mast cells.[192,193] Also, murine β-defensin-2 (mDF2β) stimulates dendritic cell activation and maturation.[194] LL-37 has been found to exert very similar effects. This peptide is chemotactic for monocytes, T cells, neutrophils, and mast cells.[195,196] It stimulates mast cell histamine release,[193] modulates chemokine and chemokine receptor gene expression in macrophages, and stimulates airway epithelial cell release of the neutrophil chemoattractant IL-8.[193,196,197] It has also been observed that LL-37 modifies dendritic cell differentiation—cells derived in the presence of LL-37 had altered characteristics, including increased endocytic capacity and secretion of T helper (**Th**)-1 cell-inducing cytokines and were able to generate an enhanced Th1 response.[198]

For both defensins and LL-37, strong evidence exists to suggest that their activities are mediated via specific cell surface receptors. The G-protein coupled receptor chemokine receptor 6 (**CCR6**) was found to mediate hBD-2-induced chemotaxis of immature dendritic cells and T cells.[185] Toll-like receptor 4 mediates mDF2β effects on mouse dendritic cells,[194] whereas hBD-2 effects on mast cells appeared to involve a G-protein linked receptor but not CCR6.[187,193] The G-protein coupled receptor formyl peptide receptor-like 1 (**FPRL-1**) mediates LL-37-stimulated chemotaxis of neutrophils and monocytes[195] but not mast cells.[196] LL-37 has also been shown to transactivate the epidermal growth factor receptor (**EGFR**), possibly by first activating a metalloproteinase, which cleaves membrane-anchored EGFR ligands, which, in turn, bind to and activate the receptor.[199] An interaction of LL-37 with the purinergic receptor P2X7, which leads to IL-1β release by monocytes, has also recently been observed.[200]

That defensins and LL-37 can directly and indirectly, via mast cell products and induction of chemokine secretion, attract various immune cells implies that in vivo these peptides can help mobilize the cellular components of the immune system in response to pathogen entry. Defensins may also participate in the immune response by causing neutrophil activation[201] and modulating the activity of the classical complement pathway by binding to complement C1q.[202,203]

The aforementioned ability of defensins to inhibit adrenocorticotrophin activity,[180] and, therefore, reduce production of anti-inflammatory glucocorticoids, may also be a mechanism by which these peptides can enhance the immune response. Additionally, LL-37 is able to bind and neutralize lipopolysaccharide and lipotechoic acid, and, thus, reduce the inflammatory response associated with these molecules.[86,197,204]

That defensins are chemoattractive for dendritic cells and that defensins and LL-37 can activate these cells has led to the suggestion that these peptides also provide a link between the innate and adaptive immune systems.[185,186,198] Furthermore, some studies suggest that defensins can promote both humoral and cellular antigen-specific immune responses.[205] Thus, in addition to their direct microbicidal role, antimicrobial peptides may participate in the immune response by recruiting and activating immune cells, regulating aspects of the inflammatory response, and promoting adaptive immunity. For more details on the evidence supporting the various roles of defensins and LL-37 in innate and adaptive immunity, the reader is referred to a recent review by Yang et al.[18]

The non-microbicidal roles of the peptides do not end with effects on the immune system. There are several lines of evidence to suggest that antimicrobial peptides may also be involved in wound healing. Defensins have been shown to stimulate proliferation of fibroblasts and epithelial cells.[206,–211] Human α-defensins have been shown to induce wound closure in a culture model using NCI-H292 airway epithelial cells.[212] Here the peptides stimulated both cell proliferation and migration via pathways involving MAP kinase to accelerate wound healing. HNP-1 has also been shown to enhance fibroblast expression of proα1(I) collagen but suppress that of matrix metalloproteinase-1.[213] Whereas, increased hBD-1 expression is associated with keratinocyte differentiation,[214] LL-37 has been shown to be angiogenic, acting via FPRL-1 to stimulate vascular endothelial cell proliferation and promote the development of vessel-like structures.[182] LL-37 has also been implicated as an important factor in re-epithelialization of skin wounds, where it was suggested to stimulate cell proliferation.[215] Although not conclusive, together these experimental findings do provide evidence for direct participation of antimicrobial peptides in wound healing responses.

Which, if any, of these nonmicrobicidal activities do antimicrobial peptides exert at the ocular surface, and are certain peptides more likely to mediate these effects? The second part of the question will be addressed first, as it is more easily answered. Antimicrobial peptides that first appear or whose expression increases under specific circumstances are the most likely to be involved in activities independent of antimicrobial activity. That is not to say that these peptides do not participate in killing of microorganisms, simply that in addition to this activity, they may have other actions. Thus, as hBD-2 and LL-37 are upregulated in regenerating corneal epithelium and in response to inflammatory cytokines,[60,61,71,95] and given that α-defensins will be present only when neutrophil infiltration occurs, these are the most likely to show non-microbicidal activities. The non-microbicidal actions in which these antimicrobial peptides participate at the ocular surface can, for the most part, only be extrapolated from studies in nonocular tissues. For example, neutrophils are known to infiltrate the cornea after injury[216–219]; therefore, through their chemotactic properties, hBD-2 and LL-37[188,195] may assist this recruitment. α-Defensins coming from the newly recruited neutrophils may, in turn, help mediate influx of additional neutrophils, perhaps by stimulating release of IL-8.[191]

Notably, we have found that defensins stimulate IL-8 secretion by cultured corneal fibroblasts.[220] Recently, α-defensins have been shown to enhance the expression of the mucins MUC5B and MUC5AC by lung epithelial cells[212]; therefore, they may be able to stimulate the production of the MUC5AC by conjunctival goblet cells.[221] Also, hBD-2 and LL-37 may stimulate the immature dendritic cells[185,186,198] recently found to be resident in the corneal stroma[222] and thus promote activation of the adaptive immune system.

hBD-2, LL-37 and α-defensins may also be involved in ocular surface wound healing. We[71] have observed that hBD-2 is upregulated in regenerating corneal epithelium in an in vitro organ culture model of corneal epithelial wound healing (Figure 4). As IL-1 and TNFα are upregulated after injury,[223–225] they are likely to be responsible for upregulated hBD-2 expression in our wounding model. In support of our in vitro observations, hBD-2 expression

was found to be increased compared to normal controls in corneal epithelial cells from a patient with a severe corneal abrasion.[58] Also, we have shown that corneal epithelial expression of rBD-2 (the rat homologue of hBD-2) is upregulated in an in vivo rat model of corneal scrape injury.[82] We also observed that LL-37 expression was increased in regenerating epithelium in our in vitro culture model.[95]

If defensins and/or LL-37 are important for corneal wound healing, then they would be expected to enhance one or more of the events that occur during the process. We have begun to address this, using in vitro assays to study corneal epithelial cell migration and proliferation, both important components of corneal wound healing. We find that while defensins alone cannot stimulate cell migration, both hBD-2 and HNP-1 can enhance fibronectin-stimulated migration.[220] This is of particular significance, as it is fibronectin across which the epithelial cells must migrate in order for wound closure to occur.[226,227] On the other hand, LL-37 alone can stimulate cell migration and also enhance fibronectin-induced migration.[95] We have also observed that hBD-2 and HNP-1 can stimulate human corneal epithelial cell proliferation (McDermott and Proske, unpublished observation). These preliminary data do support a role for antimicrobial peptides in corneal epithelial wound healing; however, it remains to be determined if this is the case in vivo. Indeed, many factors that can modulate corneal epithelial cell migration and proliferation have been identified through in vitro experimentation,[228–230] but none have yet been conclusively established as a regulator of wound healing in vivo.

Not all of the potential nonmicrobicidal effects of the antimicrobial peptides may be beneficial to the ocular surface. Excessive recruitment of immune cells may lead to uncontrolled inflammation and cause ocular surface damage. For example, defensin and LL-37 stimulation of mast cell chemotaxis and degranulation may be particularly harmful. Also, LL-37-mediated angiogenesis would certainly not be a good thing for the cornea, although it is not clear that this peptide can initiate growth of vessels in areas where they were previously nonexistent.

Figure 5 summarizes some of the major effects that defensins and LL-37 may have at the ocular surface, using the cornea as a model.

VI. POTENTIAL OF ANTIMICROBIAL PEPTIDES AS OCULAR THERAPEUTICS

A. Current Status of Antimicrobial Peptide Development

To date, in the development of the cationic peptides for clinical use, the emphasis has been on their potential as antimicrobial agents. However, as more is learned and understood of the roles of these peptides in regulating acquired immunity and wound healing, investigation of these potential uses may become prominent. The emergence of organisms resistant to the drugs fashioned to eradicate them is the driving force behind efforts to discover new classes of antimicrobials. Achieving this goal is of utmost importance, as resistance to traditional pharmaceuticals develops and spreads rapidly, and some organisms now exist for which we have no adequate treatment. Thus, the discovery of antimicrobial peptides generated great excitement, as it brought forth a new class of molecule to be investigated. In particular, antimicrobial peptides are thought of as very attractive drug candidates, because they have a broad spectrum of activity and kill very rapidly, and because the likelihood that organisms will become resistant to them is believed to be low.[22,123,231]

As discussed by Zasloff,[22] resistance may be unlikely to develop because the peptides target membranes; thus, resistance would necessitate a complete overhaul of the microbial membrane —"a 'costly' solution for most microbial species." Also, development of specific proteases to degrade the peptides would be complex, and, as the strike against the microbe usually consists of multiple structurally different peptides, elimination of just one peptide may not be a good enough defense. However, while it may indeed be more difficult for an organism to counter

an attack by a peptide, it is not impossible, and there are a growing number of literature reports documenting ways in which organisms circumvent peptide action.[232] For example, the enteropathogen Shigella causes downregulation of hBD-1 and LL-37 expression.[233] Some pathogens express phosphorylcholine on their cell surface; this mimics mammalian cell membrane lipids, therefore decreasing killing.[234] Other organisms produce proteinases that can degrade and inactivate peptides,[235] and, recently, it was reported that *S. aureus* produces staphylokinase, which is capable of forming a complex with and, therefore, inactivating α-defensins.[236]

As discussed by van't Hof et al, difficulties associated with utilizing peptides as therapeutics include sensitivity to ionic strength, susceptibility to proteolysis, cytotoxicity, and cost of production.[12] Despite this, a number of biotechnology/pharmaceutical companies are evaluating peptides from several sources for potential pharmaceutical use in a variety of clinical settings. Some peptides under development for pharmaceutical use are listed in Table 3, with notation of their stage of development and applications. To date, the peptide that advanced furthest in pharmaceutical development was pexiganan (Locilex™), a magainin. However, in a somewhat controversial decision, FDA denied approval, not for safety reasons or lack of efficacy, but because the peptide was no more effective than existing treatments.[237] At the present time, there are no peptides in development with specific ocular applications; however, an internet search revealed that the biotechnology company Xoma had considered one of their bactericidal/permeability increasing protein[238] derivatives (I-PREX™) as a possible treatment for infected corneal ulcers.

B. Possible Uses of Antimicrobial Peptides in Eye Care

Exogenous antimicrobial peptides could be employed in several ways to improve/maintain the health of the ocular surface. As discussed in more detail below, one important modality would be as "stand-alone" topical antimicrobial drugs for the treatment and/or prophylaxis of ocular infections. Secondly, peptides may have a place as combined agents that are antimicrobial and can promote wound healing, which would be useful for treatment of injuries or of an ocular surface compromised by severe dry eye or diabetes, for example. Administration via a contact lens with the therapeutic antimicrobial peptide attached[239] may be a useful alternative to the eye drop, as it may help overcome some of the dilution and flushing effects of the tear film.

The peptides might also be used as disinfectants in contact lens cleaning/storage solutions. Cecropin D5C, a synthetic insect antimicrobial peptide, has been shown to augment the antimicrobial activity of contact lens solutions against *P. aeruginosa*.[240]

Schwab et al tested defensin antimicrobial activity against *S. aureus, S. pneumoniae*, and *P. aeruginosa* at different temperatures in the corneal storage medium Optisol™.[241] Because the peptides were effective at 4°C, the authors concluded that defensins exhibit promise as preservative agents for corneal storage. Interestingly, in a related study, the bovine antimicrobial peptide BNP-1 helped preserve endothelial cells in corneas stored in Optisol.[242] Thus, the peptides, when added to corneal storage media, may have dual benefits.

Two papers report in vivo studies on the ability of topical antimicrobial peptides to prevent ocular surface infections. Nos-Barbera et al showed that rabbits infected by intrastromal injection with *P. aeruginosa*, then treated with synthetic peptides containing partial sequences from cecropin A (an insect peptide) and melittin (from bee venom) had reduced inflammatory signs and less bacterial damage than vehicle-treated controls.[243] Mannis extensively tested the effect of a synthetic peptide named COL-1 (designed using computational drug design software) in a rabbit model of *P. aeruginosa* keratitis.[244] Although the peptide showed potent antimicrobial activity in vitro, no clinical benefit was observed in the in vivo situation. In fact, the author reported that "rabbits treated with the peptide demonstrated more inflammation than

controls." He suggested that a possible reason for the lack of activity in vivo could be that the tear film components are a likely source of interference. As previously mentioned, tears have been shown to reduce peptide effectiveness in vitro;[164,165] therefore, understanding how the peptides interact with tear film components is significant for successful development of antimicrobial peptides as topical ocular pharmaceuticals.

As previously noted, synergistic interactions exist between some of the peptides,[161–163] so it may be that while one single peptide is not sufficient, two peptides applied together may be beneficial. Synergistic interactions with standard antibiotics have also been noted,[245,246] so antimicrobial peptides may be utilized for augmentation of existing pharmaceuticals rather than as stand-alone pharmaceuticals.

Another approach would be to manipulate endogenous expression of the peptides, rather than adding them exogenously.[247] This has been termed *immunomodulation*. For example, hBD-2 expression is known to be inducible, and this peptide is particularly effective against *P. aeruginosa*.[44] Thus, it may be possible to upregulate hBD-2 expression in the corneal epithelium of patients at high risk of developing pseudomonas keratitis.

VII. SUMMARY

Current data show that the corneal and conjunctival epithelia express three β-defensins and LL-37, and the tear film contains α-defensins; thus, the ocular surface is well equipped with these important multifunctional effectors of innate immunity. hBD-1 and -3 are constitutively expressed, whereas hBD-2 and LL-37 are upregulated in response to stimuli such as injury and inflammation. In addition to affording protection by directly killing invading organisms, these peptides probably help regulate the ocular surface immune response by recruiting immune cells to a site of injury or infection and modulating the behavior and activity of these cells once they have arrived. Some of the antimicrobial peptides may also help restore the ocular surface after injury by promoting epithelial cell migration and proliferation. Finally, although very much still in the research and development stage, antimicrobial peptides may ultimately find a place as topical ocular pharmaceuticals and/or additives to eye care solutions and corneal preservation media.

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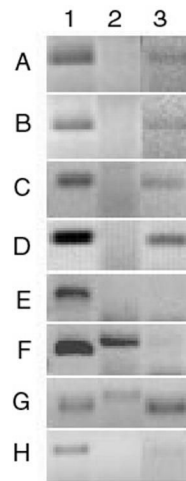
Figure 1.
β-Defensin expression by human corneal epithelium. RT-PCR was performed using RNA isolated from epithelium scraped from eight cadaver corneas (A–H). All samples expressed hBD-1. Two samples (F and G) expressed hBD-2 and five (A–D, G) expressed hBD-3. 1 = hBD-1, 2 = hBD-2, 3 = hBD-3. (Reprinted from McDermott AM, Redfern RL, Zhang B, et al. Defensin expression by the cornea: multiple signaling pathways mediate IL-1β stimulation of hBD-2 expression by human corneal epithelial cells. Invest Ophthalmol Vis Sci, 2003; 44:1859-65, with permission of Association for Research in Vision and Ophthalmology.)
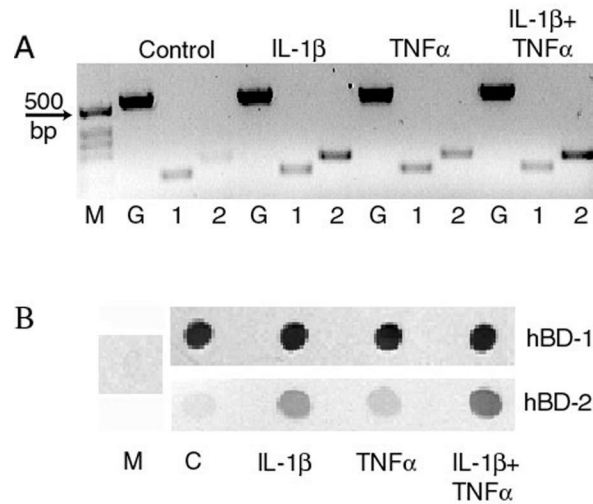
Figure 2.
Effect of cytokines on β-defensin expression by primary cultured corneal epithelial cells. Cells were treated with 10ng/ml IL-1β, TNFα or both for 24 hours then RT-PCR (panel A) was used to study defensin mRNA expression and immunoblotting (panel B) was used to study peptide secretion in to the culture media. Panel A: M = size markers, G = housekeeping gene glyceraldehyde-3-phospahte dehydrogenase, 1 = hBD-1, 2 = hBD-2. Panel B: M = culture media that had not been in contact with cells, C = supernatant from control cells. Expression of hBD-1 is constitutive whereas hBD-2 is upregulated by IL-1β and TNFα. (Reprinted from McDermott AM, Redfern RL, Zhang B, et al. Defensin expression by the cornea: multiple signaling pathways mediate IL-1β stimulation of hBD-2 expression by human corneal epithelial cells. Invest Ophthalmol Vis Sci, 2003;44:1859-65, with permission of Association for Research in Vision and Ophthalmology.)
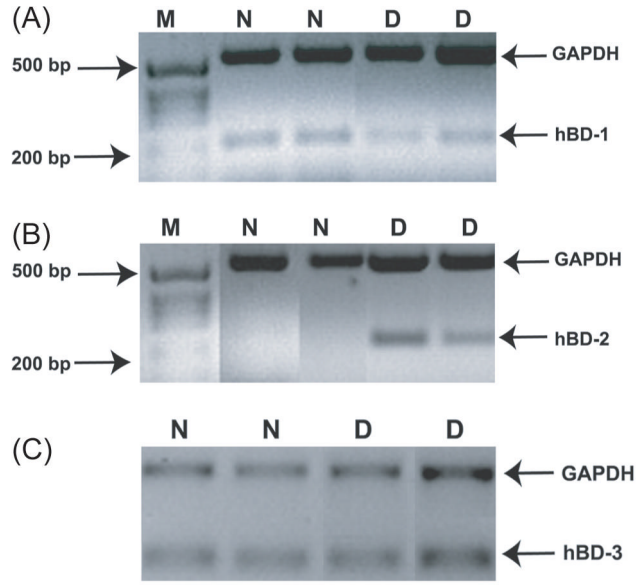
Figure 3.
Expression of human β-defensin in conjunctival epithelium: relevance to dry eye disease. Cell samples were obtained by conjunctival impression cytology from normal subjects (N) and patients with moderately dry eyes (D). RT-PCR was used to investigate expression of hBD-1 (A), hBD-2 (B) and hBD-3 (C). M = size marker. hBD-1 and hBD-3 were constitutively expressed, whereas hBD-2 was only detected in samples from patients with dry eye. (Reprinted from Narayanan S, Miller WL, McDermott AM. Proinflammatory cytokines and Pseudomonas aeruginosa upregulate human β-defensin-2 in conjunctival epithelial cells : relevance to dry eye disease. Invest Ophthalmol Vis Sci 2003;44:3795-801, with permission of Association for Research in Vision and Ophthalmology.)
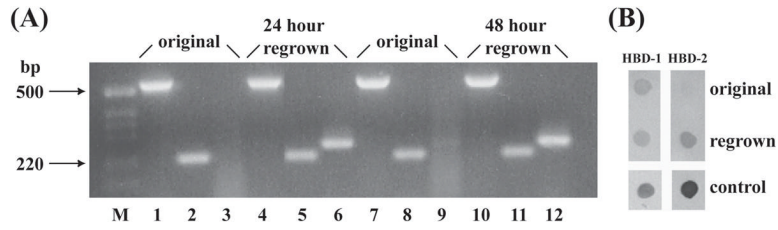
Figure 4.
Expression of β-defensins in regenerating corneal epithelium. The corneal epithelium
("original") was scraped from cadaver corneas, then the corneas were placed in to organ culture
and regenerated epithelial cells ("regrown") collected 24 or 48 hours later. RT-PCR (A) and
immunoblotting (B) were used to study defensin expression in the samples. A: M = size marker;
Lanes 1,4,7,10 are products for the housekeeping gene glyceraldehyde-3-phosphate
dehydrogenase; Lanes 2,5,8,11. are products for hBD-1; Lanes 3,6,9,12 are products for
hBD-2.B : Control are samples of synthetic defensin peptide (25ng/dot). hBD-1 is
constitutively expressed but hBD-2 is only present in regrown samples. (Reprinted from
McDermott AM, Redfern RL, Zhang B. Human β-defensin 2 is upregulated during re-
epithelialization of the cornea. Curr Eye Res 2001;22:64-7, with permission of Current Eye
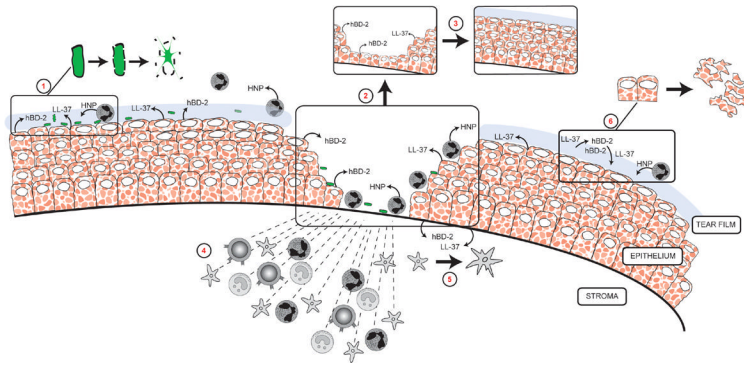Research.)

Figure 5.
Potential roles for defensins and LL-37 in the cornea. Epithelial hBD-2 and LL-37 expression
is increased in response to injury, inflammation and infection. HNP (α-defensins) are detected
in normal tear film and would be in present in greater amounts after injury and during infection
due to neutrophil infiltration. These peptides may then participate in the following: direct
killing of pathogens (1); corneal epithelial wound healing by stimulating migration (2) and
proliferation (3); recruitment of immune cells (4); promotion of adaptive immunity by
activation of dendritic cells (5); cytotoxicity to ocular surface cells if in excess (6). hBD-1 and
hBD-3 (not shown) are believed to provide a baseline defense against infection.

Table 1

Summary of Antimicrobial Peptides Present at the Human, Bovine and Rat Ocular Surface

Species, location and peptide		Comment
Human		
Corneal epithelium:	hBD-1	Constitutively expressed[55, 56, 59, 60]
	hBD-2	Variably expressed, upregulated by proinflammatory cytokines, infection, injury[55,56,58–60, 71]
	hBD-3	Constitutively expressed[60]
	CAP37	222 amino acid protein expressed in response to *S. aureus* and upregulated by proinflammatory cytokines[97]
	LL-37	Upregulated by injury and IL-1β[94]
Conjunctival epithelium:	hBD-1	Constitutively expressed[54–56, 59, 61]
	hBD-2	Variably expressed, upregulated by proinflammatory cytokines and in dry eye patients[55, 56, 59, 61, 74]
	hBD-3	Constitutively expressed[61]
Corneal stroma:	HNP-1, -3	Present in cases of transplant rejection and post-infectious keratitis but not normal cornea, infiltrating neutrophils presumed to be the source[53]
Tear film:	HNP-1, -2, -3	Present in normal tear film,[55–57] increased after ocular surface surgical procedure,[57] neutrophils presumed to be the source
Other potential antimicrobial peptides at human ocular surface:	Thymosin β-4	Expressed by corneal epithelium,[107] recently shown to have antimicrobial activity[102]
	Histatin 5	Known antifungal activity, detected in tear film,[121] corneal and conjunctival epithelia[119*]
	CCL28	Antimicrobial chemokine[116] detected in corneal and conjunctival epithelium[119*]
	CCL20	Antimicrobial chemokine[117–118] detected in IL-1α stimulated corneal fibroblasts[122]
	LEAP-1, -2	mRNA detected in corneal and conjunctival epithelia[120*]
	Dermcidin	mRNA detected in corneal and conjunctival epithelia[120*]
Bovine		
Corneal epithelium:	LAP	Present and upregulated after injury[80*]
	TAP	Only significantly expressed after injury[80*]
Conjunctival epithelium:	LAP	Constitutively expressed[77*]
	TAP	Constitutively expressed[80*]
Rat		
Corneal epithelium:	rBD-1	Constitutively expressed[82*]
	rBD-2	Only significantly expressed after injury[82*]

*
Indicates study in which only mRNA expression has been studied. hBD = human β-defensin; CAP37 = cationic antimicrobial protein 37; HNP = human neutrophil peptide; LEAP = liver expressed antimicrobial peptide; LAP = lingual antimicrobial peptide; TAP = tracheal antimicrobial peptide; rBD = rat β-defensin.

Table 2

Ocular Pathogens Susceptible to Antimicrobial Peptides In Vitro

Peptide	Organism	Reference number
NP-1 (rabbit α-defensin)	*S. aureus, P.aeruginosa, S. pneumoniae, α*-hemolytic streptococcus, *C. albicans, M. morangii*	155
Magainins (MSI-103, MSI-94)	*Acanthamoeba polyphaga*	156
Shiva-11 (synthetic cecropin)	*P. aeruginosa, S. aureus, S. pneumoniae, C. albicans*	157
Protamine	*P. aeruginosa, S. marcescens, S. aureus, S. intermedius*	158
Melittin	*P. aeruginosa, S. marcescens, S. aureus, S. intermedius*	158
Cecropin A	*P. aeruginosa, S. marcescens, S. aureus, S. intermedius*	158
LL-37	*P. aeruginosa, HSV-1, Adenovirus (Ad3, 5, 8, 19)*	95, 159
Defensins: HNP-1, hBD-1, hBD-2	*Adenovirus (Ad3, 5, 19)*	160
hBD-2	*P. aeurginosa*	McDermott & Huang unpublished

Table 3

Summary of Antimicrobial Peptides in Clinical Development

Company	Peptide–Name	Peptide–Origin	Application	Development Stage
Genaera (formerly Magainin)	Pexiganan (Locilex)	22aa magainin	Infected diabetic foot ulcers	Denied FDA approval 1999
Intrabiotics	Isegan	Protegrin	(a) Pneumonia in mechanically ventilated patients	Phase III—stopped June 2004 for safety reasons
			(b) Lung infection in cystic fibrosis	Phase IIa
Micrologix	MBI-226	No information	Catheter related blood stream infection	Phase III
	MBI-594	No information	Acne	Phase IIb
Demgen (formerly Periodontix)	P113D	Histatin	Lung infection in cystic fibrosis November 2002	Orphan drug designation
	P113	Histatin	Oral candadiasis in HIV	Phase I/II
Xoma	XMP-629	9aa fragment of BPI[*]	Acne	Phase II
Entomed	ETD-151	Heliothis virescens	Antifungal	Preclinical
	ETD-P1263	Insect defensin	MRSA	Preclinical
AmPharma	No information	No information	(a) Systemic antibiotic/antifungal	Preclinical
			(b) Incorporation in to surgical devices to prevent post-surgery infection	

[*] BPI is bactericidal/permeability increasing protein, a 50–55kDa protein of neutrophils[238]