Detection of Natural Peptide Antibiotics in Human Nasolacrimal Ducts

Friedrich P. Paulsen,1 Thomas Pufe,1 Ulrich Schaudig,2 Janka Held-Feindt,1 Jan Lehmann,3 Jens-Michael Schröder,4 and Bernhard N. Tillmann1

PURPOSE. To determine the expression and production of antimicrobial peptides by mucosal cells of the lacrimal passage in healthy and pathologic states.

METHODS. Detection of bactericidal-permeability-increasing protein (BPI), heparin-binding protein (CAP37), human cat-ionic antimicrobial protein (LL-37), human α-defensin 5 (HD5), human α-defensin 6 (HD6), human β-defensin 1 (HBD-1), and human β-defensin 2 (HBD-2) was performed by reverse transcription-polymerase chain reaction (RT-PCR). Intracellular deposition of lysozyme, lactoferrin, secretory phospholipase A2, human neutrophil defensins (HNP-1, -2, and -3), human β-defensin 1 (HBD-1), and human β-defensin 2 (HBD-2) was analyzed immunohistochemically. Samples were obtained from 15 patients by surgery and from 10 cadavers.

RESULTS. RT-PCR revealed BPI, CAP37, and HBD-1 mRNA in samples of healthy nasolacrimal duct epithelium. Additionally, HBD-2 mRNA was detected in epithelial samples from patients with dacryocystitis. Messenger RNAs for LL-37 and α-defensin 5 and 6 were absent in all samples investigated. Immunohistochemistry revealed lysozyme, lactoferrin, secretory phospholipase A2, and HNP-1, -2, and -3 to be present in all samples, whereas HBD-1 was present only in some of the healthy and inflamed samples. Immunoreactive HBD-2 peptide was visible only in some of the inflamed samples.

CONCLUSIONS. The data suggest that the human efferent tear ducts produce a broad spectrum of antimicrobial peptides. Under inflammatory conditions, changes in the expression pattern occurred, revealing induction of the human inducible defensin HBD-2 and in some cases downregulation of HBD-1 and CAP37. Antimicrobial peptides have a therapeutic potential in dacryocystitis, in that they have a broad spectrum of antimicrobial activity and accelerate epithelial healing. However, caution is appropriate, because defensins also promote fibrin formation and cell proliferation, which are key elements in scarring processes, such as dacryostenosis. (Invest Ophtalmol Vis Sci. 2001;42:2157–2163)

Dacryocystitis is the most frequent disease of the efferent lacrimal system. Its course may be either acute or chronic and slow to resolve, even with systemic antibiotic therapy.1

Similar to the cutis, all mucosal surfaces of the body are in constant interaction with environmental micro-organisms and hence are vulnerable to infection. The human mucosa has developed a variety of anti-infection strategies to prevent micro-organisms from entering through surfaces and to thwart attacks from micro-organisms that have already entered the epithelia.

The mucous membrane of the lacrimal sac and the nasolacrimal duct is a pseudostratified, columnar epithelium. Goblet cells are integrated in the epithelium as solitary cells or, particularly in the lacrimal sac, in a characteristic arrangement of several cell groups. Beneath the epithelium, the lamina propria consists of two strata: loose connective tissue containing many lymphocytes, sometimes arranged in follicles, as well as a cavernous body situated under the loose connective tissue.2,3 The arranged follicles were recently shown to be part of the organized mucosa-associated lymphoid tissue.4,5 Small seromucous glands are detected in addition to epithelial and goblet cells. These glands are situated in the region of the fundus of the lacrimal sac. Their excretory ducts penetrate the lining epithelium into the lumen of the sac.2

Recent evidence indicates that the surface of the efferent tear ducts is an integral part of the mucosal immune system.2,4–6 The protection of the lacrimal passage from microbial attack must therefore be of paramount importance for the survival of the individual and the preservation of the species.

During recent years, endogenous antimicrobial peptides, active against Gram-positive and Gram-negative bacteria, have attracted much interest in the ocular system as well.7–14 Moreover, some studies suggest that invertebrate epithelial cells can recognize micro-organisms and mount a rapid defense response through the production of various inducible antibiotic peptides as shown in Drosophila.15 This leads to a characteristic broad spectrum of antimicrobial activity against bacteria and fungi.16

In the present study, we sought to identify and characterize antimicrobial peptides released by the human tear duct mucosal cells in the healthy state and to investigate potential differences in the presence of dacryocystitis.

MATERIALS AND METHODS

Evidence of antimicrobial peptide production by the lining of the nasolacrimal duct and the lacrimal sac was sought by reverse transcription-polymerase chain reaction (RT-PCR), partly because of limited supplies of antibodies against antimicrobial peptides and the difficulty of obtaining pure human mucosal tissue.

OCULAR SAMPLE PREPARATION

Fifteen biopsy samples of lacrimal systems (4 male, 11 female; aged 5–73 years) obtained during surgical procedures and 10 lacrimal systems (5 male, 5 female; aged 53–88 years) obtained from cadavers donated to the Department of Anatomy, Christian Albrecht University of Kiel, Germany, were prepared. Material from surgical procedures was obtained with the permission of the medical ethics committee and used in accordance with the Declaration of Helsinki. All 15 patients had tear duct stenosis of different causes, with or without dacryocys-
### Table 1. Detection of Antimicrobial Peptides by RT-PCR in Surgically Obtained Biopsy Specimens of Nasolacrimal Ducts of Patients with Different Types of Tear Duct Stenosis, with or without Dacryocystitis

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Ad, additional inflammation; W, without inflammation; aDC, acute dacryocystitis; sDC, subacute dacryocystitis; cDC, chronic dacryocystitis; BPI, permeability-increasing protein; X, detection of antimicrobial peptide; -, absence of antimicrobial peptide.

### Cell Culture

The human Hakard epithelial cell line was obtained from the Department of Dermatology, Christian Albrecht University of Kiel and cultivated, as well as subcultivated (after trypsinization), in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum.

### Total RNA Purification and cDNA Synthesis

For RT-PCR, frozen samples (20 mg) were crushed in an agate mortar under liquid nitrogen. The RNA was isolated by the phenol guanidinium thiocyanate method and purified by isopropanol and repeated ethanol precipitation. Contaminating DNA was destroyed by digestion with RNase-free DNase I (20 minutes at 25°C; Boehringer-Mannheim, Mannheim, Germany). After inactivation of the DNase (15 minutes at 65°C), the RNA was generated with 1 μl (20 pmol) oligo(dT), primer (Amer sham Pharmacia Biotech, Upsalla, Sweden) and 0.8 μl superscript reverse transcriptase (RNase H; Gibco, Paisley, UK) for 60 minutes at 37°C, as described by Feindt et al.18

RNA was extracted from the culture cell lysates with a kit (RNeasy Total RNA; Qiagen, Chatsworth, CA) used according to the manufacturer’s instructions. cDNA was then generated with 1 μl (20 pmol) oligo(dT), primer (Amer sham Pharmacia Biotech) and 0.8 μl superscript reverse transcriptase (RNase H; Gibco) for 60 minutes at 37°C.18

### Reverse Transcription–Polymerase Chain Reaction

For PCR, 4 μl cDNA was incubated with 30.5 μl water, 4 μl 25 mM MgCl₂, 1 μl dNTP, 5 μl 10× PCR buffer, and 0.5 μl (2.5 U) platinum Taq DNA polymerase (Gibco) and the following primers (2.5 μl each containing 10 pmol): bactericidal-permeability-increasing (BPI) protein (forward primer: 5'-TTCACGTTCAGGCTTCCTGCTGCG-3'; reverse primer: 5'-CATCCACGGGAGGAGGTA-3', 841 bp); CAP37, heparin-binding protein (forward primer: 5'-AGCATGAGGAGGAATGGCTACGAC-3', reverse primer: 5'-GGTCTCCTGGGGTGTCACAGTCA-3', 235 bp); LL-37, human cationic antimicrobial protein (forward primer: 5'-ATCATGCGAGGCTTCAGC3'; reverse primer: 5'-GTCCCACTCAGGCCCTAC-3', 251 bp); HD5, human α-defensin 5 (forward primer: 5'-AGGCTCTGTCAGCTCATT-3', reverse primer: 5'-AGATTCCACACCCCGGAGA-3', 241 bp); HD6, human α-defensin 6 (forward primer: 5'-GTTCATCTGGTCAGCTCATT-3', reverse primer: 5'-CCATGCAGTGCGCTCCCAT-3', 269 bp); HBD-1, human β-defensin 1 (forward primer: 5'-TGGTCTGGAGCTGCTGCTCAGT-3', reverse primer: 5'-TATCTCTAAAGGAAATTCCTTTAT-3', 253 bp); and HBD-2, human β-defensin 2 (forward primer: 5'-CCAGCCTACAGCATGAGGTT-3', reverse primer: 5'-GGAGGTCCTTTACGTCACGGCA-3', 255 bp). Thirty-five cycles were performed with each primer pair (annealing temperature 60°C). A glycerolaldehyde-3-phosphate dehydrogenase (GAPDH)-specific intron-spanning primer pair (0.1 μM; 5'-CCACCGGGACGCTCCATCTC-3', 5'-ATGAGCCCCACCTTCTCCTCAT-3'), which yielded a 360-bp amplified product, served as the internal control for equal amounts of cDNA. All primers were synthesized by MWG Biotech AG, Ebersberg, Germany.

The positive control cDNA samples analyzed included one sample from human blood (BPI, CAP37, LL-37), one sample from cultured epithelial cells (HBD-1), and one sample from small intestine epithelium (HD5 and HD6). The cDNA was replaced with water for a negative control reaction.

### Immunohistochemistry

Immunohistochemical stains were performed with antibodies against lysozyme (1:200 in Tris-buffered saline [TBS], 60 minutes; A0099; Dako, Glostrup, Denmark), lactoferrin (1:150 in TBS, 60 minutes; A0186; Dako), secretory phospholipase A₂ (sPLA₂; 1:10 in TBS, 60 minutes; 05-143 mouse monoclonal sPLA₂; Upstate Biotechnology, Lake Placid, NY), human neutrophil defensins (HNPs-1, -2, and -3; 1:800 in TBS, 60 minutes; T1034 mouse monoclonal DEF-3; Bachem, Heidelberg, Germany), and human β-defensin 1 and 2 (both 1:500 in TBS, 60 minutes; courtesy of Thomas Ganz, Pulmonary Research Laboratory, Los Angeles, CA). These were applied by a peroxidase-labeled streptavidin-biotin technique, either with a microwave-heating pretreatment or using methods with trypsinization where appropriate. After they were counterstained with hemalun, the sections were finally dehydrated, immersed in xylene, and mounted (Aquatex; Boehringer-Mannheim). Two negative control sections were used in each case. One was incubated with the second antibody only, the other with the primary antibody only. Sections of human submandibular gland (lysozyme, lactoferrin), human jejunal mucosa (PARP), human spleen (DEF-3), and human skin (HBD-1) were demarcated as negative controls.

used for a positive control. All slides were examined by bright-field microscopy (Axiophot; Carl Zeiss; Oberkochen, Germany). For HBD-2, normal human skin was used as an additional negative control, because a positive control was not available.

**RESULTS**

**Reverse Transcription–Polymerase Chain Reaction**

RT-PCR of normal noninflamed and inflamed nasolacrimal duct samples for BPI revealed a product of 841 bp in all samples analyzed (Tables 1, 2; Fig. 1A). The negative control reaction (DNA-free water instead of cDNA) showed no PCR products. Internal control (GAPDH) revealed a product of 360 bp.

Products of 253 bp (CAP37) and 253 bp (HBD-1) revealed both peptides in normal noninflamed and inflamed nasolacrimal duct samples (Figs. 1A, 1B; Tables 1, 2). Positive control cultured epithelial cells also revealed corresponding products of 253 bp and 253 bp (Fig. 1B). The negative control reactions (DNA-free water instead of cDNA) showed no PCR products. The internal control revealed the expected 360-bp product.

Three mucosal samples revealed no expression of CAP37 (biopsy samples 5, 13, and 14; Table 1). Four mucosal samples (9, 11, 13, and 14; Table 1) showed no HBD-1 expression. It was notable that all the patients in these cases had acute or chronic dacryocystitis, and all the mucosal samples revealed expression of HBD-2 (Table 1).

RT-PCR of all cadaveric samples was negative for HBD-2 (Tables 1, 2), with one exception, revealing a weak HBD-2 product of 255 bp. In contrast, all the inflamed nasolacrimal duct samples obtained by surgery revealed a 255-bp product for HBD-2 (Fig. 1C, Table 1). A positive control was not available for HBD-2. The negative control showed no PCR products; the internal controls revealed the expected 360-bp product for GAPDH.

RT-PCR of all noninflamed and inflamed samples was negative for LL-37, as well as for HD5 and HD6. Human blood and small intestine samples showed appropriate PCR products (Tables 1, 2).

**Antimicrobial Peptide Immunostaining**

Lysozyme and lactoferrin were present in the epithelium of the lacrimal sac and the nasolacrimal duct and in the seromucous glands of the lacrimal sac. Lysozyme was produced by some of the epithelial cells, rendered visible as a red staining of the entire cytoplasm (Fig. 2A). Lactoferrin occurred in some of the epithelial cells as a fine granulation throughout the upper part of the cell (Fig. 2C). In seromucous glands, both antibodies stained the serous cells (Fig. 2B). sPA2 occurred only in epithelial cells of the epithelial lining, visible as a red staining of small intraepithelial vesicles (Fig. 2D). Production of lysozyme, lactoferrin and phospholipase A2 was detected in normal non-inflamed epithelium as well as in samples of inflamed epithelium.

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**Table 2. Detection of Antimicrobial Peptides by RT-PCR in Biopsy Specimens of Nasolacrimal Ducts from Cadavers**

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X, detection of antimicrobial peptide; -, absence of antimicrobial peptide.
Immunohistochemistry of the inflamed nasolacrimal duct samples with DEF-3 antibody specific for HNP showed strong positive staining of masses of neutrophils in the substantia propria and epithelium. However, all the normal noninflamed nasolacrimal duct samples also revealed red staining of scattered subepithelial and intraepithelial neutrophils (Fig. 3A). Moreover, epithelial cells of the lacrimal passage also showed a weak staining of the entire cytoplasm. The seromucous glands revealed staining of the ductular epithelial cells with DEF-3 antibody specific for HNP and high-power views demonstrated staining of intracellular granules within the lining epithelial cells of the ductules.

Immunoreactivity of HBD-1 was visible only in some of the healthy and inflamed samples. When present, it occurred as a fine granulation throughout the upper part of the epithelial cells (Fig. 3B). Immunoreactive HBD-2 peptide was visible only in some of the inflamed samples (Fig. 3C), rendered visible as a red staining of the entire cytoplasm of the epithelial cells (Fig. 3D).
DISCUSSION

In our study, we used RT-PCR and immunohistochemistry to study and compare antimicrobial peptides at work on the mucosal side of the lacrimal passage between cadavers and biopsy specimens from patients' inflamed mucosa. Our data suggest that the human nasolacrimal ducts produce a spectrum of antimicrobial peptides including the human inducible defensin HBD-2, which is produced after treatment of epithelial cells with proinflammatory cytokines or contact with pathogenic bacteria, such as the mucoid form of Pseudomonas aeruginosa.21-25

It appears that lysozyme, lactoferrin, and sPA2 are actually produced by the mucosal surface of the lacrimal sac and nasolacrimal duct, as well as the serous cells of seromucous glands. Lysozyme and lactoferrin are well known as constituents of tear protein.25 Lysozyme is a low-molecular-weight protein showing bacteriostatic and bactericidal activity. It is especially effective in cytolysis of Gram-positive organisms. Moreover, it enhances the antibacterial action of complement and T cells against Gram-negative bacteria.25 Lactoferrin is an iron-binding protein that reduces the amount of free iron available to bacteria. It provides both bacteriostatic and bactericidal protection26 and plays a role in primary antibody response, lymphocyte proliferation, cytokine production, natural killer (NK) cell activity and the regulation of complement activation.27 The calcium-dependent enzyme sPA2 has recently been revealed as an important tear component of host defense systems against many Gram-positive pathogens.28

This epithelial antimicrobial defense is supported by neutrophils present in large amounts inside the epithelium and the subepithelial connective tissue of the lacrimal sac and nasolacrimal duct.2,4,5 It has been speculated that this abundant occurrence of neutrophils could be based on the fact that the efferent tear ducts have to drain all antigens taken up by the ocular surface.4 The neutrophils are positive for α-defensins 1, 2, and 3, which have been shown to be amply present in these cells.27-29 α-Defensins 5 and 6 were not detected in the lacrimal passage. Therefore, on the information currently available, α-defensins 5 and 6 remain products of small intestine Paneth cells only.

In addition to α-defensins 1, 2, and 3, PCR analysis revealed BPI and CAP37 to be expressed in probes of the nasolacrimal epithelium. It seems that BPI and CAP37 are also released into the ocular surface fluids by resident or passing neutrophils as BPI—a 55-kDa basic protein found in azurophilic granules of polymorphonuclear leukocytes—28 and, more recently, on the surface of neutrophils,33 in eosinophils,34 in plasma,35 and in inflammatory fluids, BPI unfolds a highly cytotoxic action against many Gram-negative bacteria. CAP37, a human polymorphonuclear leukocyte granule-derived 37-kDa protein, has also been identified as possessing antimicrobial activity against Gram-negative bacteria.26 In addition, there are reports identifying antimicrobial peptides, such as α-defensins 1 and 2 and CAP37, as potent chemoattractants for T cells.37

Apart from α-defensins, mammals produce a second family of defensins, which, due to their structural similarity to α-defensins, are termed the β-defensin family. β-Defensins, which occur as ~4-kDa peptides containing 38 to 42 amino acids, are highly cationic, variably arginine-rich, and distributed in a greater variety of epithelia than α-defensins.38-40 The two β-defensins analyzed in the present study, HBD-1 and HBD-2, are thought to exert their antimicrobial activity by interacting with membranes of metabolically active bacteria, perhaps by forming pores and causing membrane disruption.41-44 Other possible roles could include promotion of nasolacrimal duct epithelial healing;42 monocyte;43 dendritic, and T-cell chemotaxis;41,42 synergistic activity with lysozyme and lactoferrin;44 and complement activation.48,49 Comparable to other mucosal sites,44-47,50-51 our PCR results revealed that HBD-1 is expressed in all healthy lacrimal systems, although we were unable to detect the peptide in all healthy samples analyzed by immunohistochemistry, perhaps because its concentration is below the limit of detection of our antibody. In the presence of inflammation, HBD-1 also appeared to be expressed in the lacrimal system. However, as shown in a recent study, early infections can reduce or turn off expression of antimicrobial peptides,52 which suggests that its production depends on the status of the local bacterial microflora. Our results underline this finding, because HBD-1 and CAP37 were sometimes not detected in acute dacryocystitis in particular (Table 1).

HBD-2, one of the only two known human inducible defensins, which is upregulated by contact with Gram-negative and Gram-positive bacteria as well as Candida albicans52,54 or generated in response to inflammatory cytokines during infection,14 is detected only in the lacrimal passage in the presence of bacterial dacryocystitis. The inducibility of HBD-2, and the fact that it is approximately 10 times as potent as HBD-126 and shows a wider antibacterial spectrum, makes HBD-2 a stronger candidate for antimicrobial defense in the efferent tear ducts, despite the salt content of tears, which approaches that of serum.53 Singh et al50 demonstrated that the antimicrobial activities of both HBD-1 and HBD-2 are reduced by NaCl. Thus, cytokine-induced HBD-2 production may be more important for antimicrobial defense than constitutive HBD-1 production.

Besides the surgically obtained samples from patients with dacryocystitis, one sample from a cadaver (Table 2) also revealed a PCR product for HBD-2. It is not known whether this was because of technical factors or, more likely, reflected a bacterial, viral, fungal, allergic, or sterile infection during the last days of the person’s life. Taken together, the present study suggests that both HBD-1 and -2 play important, although somewhat different, roles in the mucosal defense of the efferent tear ducts.

No expression was found for the peptide LL-37. It is not clear whether this was due to technical factors or reflects the absence of this inducible peptide in the nasolacrimal ducts. The latter is more likely, because there are no current publications available on LL-37 in ocular tissue.

Our data suggest that the human nasolacrimal ducts produce a spectrum of antimicrobial peptides. Such production of several antimicrobial peptides by different cells is more effective in antimicrobial defense, because the combined action of antimicrobial peptides leads to synergistic and additive killing effects.4 An understanding of the exact mechanism of production and regulation of antimicrobial peptides at the mucosal surface of the lacrimal passage will provide further insight into the occurrence of dacryocystitis, which often leads to residual functional impairment with epiphora. The factors controlling the production of nasolacrimal duct-associated antimicrobial peptides are unknown, and it is likely that some infection risk factors, such as old age, changes in hormonal status (postmenopausal women), a narrow bony channel, or immunodeficiency, are associated with downregulation of antimicrobial peptide production. Another hypothesis suggests that a pre-existing stenosis or narrowing of the lacrimal passage downregulates the production of antimicrobial substances. The normally constant flow of tears could be a positive feedback signal for production, which comes to a halt if tears are not drained into the nose. This does not, off course, explain why dacryocystitis never develops in some patients with epiphora due to postacral stenosis.

Moreover, it may be hypothesized that purified or recombinant antimicrobial peptides, especially inducible, may be ideal agents in the therapy of dacryocystitis when applied topically by injection through the lacrimal canaliculi directly to

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the site of infection. Nevertheless, there is little experience in the clinical use of cationic peptides, and this important aspect should be addressed in future investigations, especially because a few reports reveal that defensins could accelerate two key events involved in scarring processes of the lacrimal drainage passages, such as dacryostenosis: promotion of cellular proliferation 12–15 and formation of fibrin 16,57.

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References


On p. 1479 and in the Table of Contents, an author was inadvertently omitted from the manuscript. The author line should have read: Mineo Takagi,1,2 Hiroshi Oyamada,3 Haruki Abe,1 David S. Zee,4 Hiruma Hasebe,1 Atsushi Miki,1 Tomoaki Usui,1 Shigeru Hasegawa,1 and Takehiko Bando2,3.

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