Antimicrobial peptides in human corneal tissue of fungal keratitis patients

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Precis

HBD-1 and -2 were consistently expressed in fungal keratitis samples. While HBD-3, -9, S100A7, and LL-37 showed variable expression pattern. The results indicate the therapeutic potential of recombinant or linear AMPs against fungal pathogens.

ABSTRACT

Background: Fungal keratitis (FK) is the leading cause of unilateral blindness in the developing world. Antimicrobial peptides (AMPs) have been shown to play an important role on human ocular surface (OS) during bacterial, viral, and protozoan infections. Here, our aim was to profile a spectrum of AMPs in corneal tissue from FK patients with active infection and after healing.

Methods: OS samples were collected from patients at presentation by impression cytology and scraping. Corneal button specimens were collected from patients undergoing therapeutic penetrating keratoplasty for management of severe FK or healed keratitis. Gene expression of human beta-defensin (HBD)-1, -2, -3 and -9, S100A7, and LL-37 was determined by quantitative real-time polymerase chain reaction (qPCR).

Results: Messenger RNA expression (mRNA) for all AMPs was shown to be significantly upregulated in FK samples. Levels of HBD-1 and -2 mRNA were found to be elevated in 18/20 FK samples. Whereas mRNA for HBD-3 and S100A7 was upregulated in 11/20 and HBD9 was increased in 15/20 FK samples. LL-37 mRNA showed moderate upregulation in 7/20 FK samples compared to controls. In healed scar samples, mRNA of all AMPs was found to be low and matching the levels in controls.

Conclusion: AMP expression is a consistent feature of FK, but not all AMPs are equally expressed. HBD-1 and -2 are most consistently expressed and LL-37 the least, suggesting some specificity of AMP expression related to FK. ~~This information will be useful in developing strategies~~The outcomes will form the basis to use HBDs sequence as a template for designing FK-specific peptide therapeutics ~~for use of AMPs in treating FK~~.

INTRODUCTION

Fungal keratitis (FK) is the commonest cause of corneal blindness in developing world. The prevalence of FK has been linked to geographical climate[1]. In India, it was estimated that up to 35% of patients that present with infective keratitis were culture positive for mycotic organisms[2 3]. The principal risk factor for FK in more than 70% of these cases was reported to be vegetative ocular injury[3]. Increasing reports of FK cases from countries with temperate conditions have added to the infection-related incidence of blindness globally[4-6]. In developed countries, incidence of FK cases is also increasing and mainly associated with contact-lens use and ocular trauma[7]. In the United Kingdom, FK incidence increased from 4.5 cases per year (between 1994 to 2006) to 14 cases per year (between 2007 to 2014)[5]. In the midwestern region of the United States, 16% cases of infective keratitis (between 1999 to 2013) were identified as FK with poor healing and major complications[8]. The socio-economic impact of this disease has been significantly high because it predominantly affects individuals in working age group[9-11]. Clinical features of FK often overlap with bacterial keratitis and frequently these are difficult to diagnose[12]. Poor penetration and lack of effective anti-fungal agents has further compounded the problem of FK[13-15]. Therefore, to seek alternative therapies, current research in this field has been mainly focused on understanding of mechanisms by which host immunity responds to fungi and yeast.

Antimicrobial peptides (AMPs) are naturally occurring host defence proteins with broad-spectrum antimicrobial activity against bacteria, fungi and viruses[16-18]. They play an important role in innate immunity and are known to be expressed at epithelial surfaces of the human body. We were the first group to provide evidence for the presence of AMPs at the ocular surface[19] and profiled the range of AMPs (human beta-defensin (HBD)-1 to 3, HBD-9 and cathelicidin (LL-37)) at the human ocular surface and their expression in corneal infections[20-22]. We also elucidated the signaling mechanisms involved in RNase-7 and HBD-9 secretion from human corneal epithelial cells[23 24].

Antifungal activity of AMPs (such as defensins and cathelicidin) is well known[25-27] through animal studies, but the profile of human AMPs at the ocular surface in response to fungal infections has not been elucidated. In this study, we profiled the gene expression of well-characterised human AMPs in corneal specimens during active phase of infection and after healing.

MATERIALS AND METHODS

Research was conducted in accordance with the tenets of the Declaration of Helsinki. Study was approved by the Institutional Review Board of Hyderabad Eye Research Foundation (**Ethics code: 2016-13-CD-13**), L.V. Prasad Eye Institute (LVPEI), Bhubaneswar, India. Informed consent was obtained from all patients prior to collection of samples, which included scrapes/ impression cytology from patients with active fungal keratitis, corneal buttons (part of) from patients that were subjected to therapeutic penetrating keratoplasty (tPK) for fungal keratitis not responding to medical management and corneal tissue from patients with non-inflammatory corneal scar that underwent optical penetrating keratoplasty (controls).

Study design:

A prospective consecutive case series of patients with fungal keratitis

Inclusion criteria

- Patients diagnosed clinically as suffering from FK and confirmed by standard microbiology and/or histopathology techniques.
- Patients with active FK who had received no treatment or were unresponsive to treatment.
- Patients of 18 years of age or older.
- Patients able to give informed consent.

Exclusion criteria

- Patients with mixed fungal and bacterial keratitis

- Patients on topical or systemic steroid treatment
- Patients on immunosuppressive treatment or known to be immunosuppressed
- Patients presenting with non-infectious causes of ocular inflammation
- Patients with viral keratitis

Sample collection:

Corneal scrapes were collected using standard techniques with sterile Bard Parker blade number15[28]. Impression cytology (IC) was performed to collect superficial layers of corneal epithelium using cellulose acetate ester discs, as previously reported[20]. Part of corneal button (CB) from patients undergoing therapeutic or optical PK was collected in 600 μL RNAlater solution (Qiagen, Germany) on ice and then transferred to buffer RLT prior to storage at -80^{o}C.

Total RNA isolation and Reverse transcription:

Corneal tissue in RLT buffer was homogenized using a tissue ruptor (Qiagen, Germany) for 60 seconds on ice. Total RNA was isolated from CB, IC samples, and corneal scrapes using RNeasy Mini Kit (catalog No. 74104; Qiagen, Germany) according to manufacturers' instructions, including the optional DNase step. Isolated RNA was quantitated using Biospectrophotometer (Eppendorf, Germany). 200ng total RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using Eurogentec Reverse Transcription Core Kit (RT-RTCK-03, Eurogentec, Belgium) as per manufacturers' instructions. No RT-enzyme control samples were also prepared.

Quantitative real-time polymerase chain reaction (qPCR):

Quantitative PCR was conducted for selected AMPs (Table 1) using Taqman probe chemistry. Taqman assays specific to AMPs, hypoxanthine-guanine phosphoribosyltransferase [HPRT] and appropriate controls were run in duplicate in a 96-well plate in the Mx3005p real-time PCR instrument (Agilent technologies, Milton Keynes, UK). Briefly, template cDNA was initially diluted to 1 in 5 using nuclease-free water. A 20 μL reaction mix was prepared for each well as per

instructions for Taqman Gene Expression mastermix (Applied Biosystems, Waltham, MA). Each reaction mixture comprised of 10 μL of 2x mastermix, 1 μL of 20x taqman assay, 5 μL of diluted cDNA and 4μL of nuclease-free water. All probes used in this study were template specific. However, to rule out any genomic amplification, appropriate no-RT controls were also included against each gene probe. C_T values were acquired using MxPro Software version 5.0 and further analysed by delta-delta C_T comparative method[29].

Statistical analysis:

The qPCR data was subjected to statistical analysis using Prism version 8.1 software (GraphPad software, San Diego, CA) with alpha set at $p \leq 0.05$. Due to differences in the group size, we performed unpaired Welch's unequal variances *t*-test for control versus active and active versus healed groups.

RESULTS

As shown in table 2, a total 26 samples were collected. These included a part of CB (n=17), scrapings (n=5) and IC (n=4). Of CB specimens, 3 were grouped as 'controls' (patients' that required optical PK for management of non-inflammatory corneal scar); 11 were grouped as 'active FK' (patients' with severe FK that required tPK) and 3 were grouped as 'healed' keratitis (patients' that required PK post-medical management for FK). Table 2 mentions the day of collection of corneal button samples (Column no.8). CB tissue of active FK was collected between day 1 to 30 from the day of presentation and those from the healed group between 6-12 months from the day of presentation. Scraping and IC samples (5+4) were also included under the group of 'active FK' and were collected between day 0 to 3 from the day of presentation, before commencement of antifungal therapy. In total, we studied n=3 in controls; n=20 in active FK and n=3 in healed group. As shown in table 2, final diagnosis was based on positive growth of fungi in cultures from corneal scrapings and/or on histopathology evaluation. Of n=23 cases of fungal keratitis, 3 samples showed no growth on culture,

however, they were later confirmed as fungal by aetiology on histological evaluation. In addition, 2/23 patients presented with a perforation or an impending perforation were not scraped but later confirmed as FK, on histopathology. A range of fungi were identified but fusarium species was the most common (Table 2).

Variable expression of AMPs in active FK and healed samples

HBD-1, -2, and -9 and LL-37 were shown to be constitutively expressed in all control samples, whereas mRNAs for HBD-3 and S100A7 were expressed at a very low level in controls. As shown in figure 1, all AMPs were significantly increased during active FK. Notably, in healed samples, their mRNA expression was found to be at a similar level to those in controls. Levels of mRNA expression is denoted as mean ± standard deviation.

HBD-1 and -2 mRNA were significantly increased in 18/20 active FK samples. HBD-1 was upregulated by 12.03 ± 9.06 fold ($p<0.0001$) and HBD-2 was elevated by 254.7 ± 335.82 fold ($p=0.003$) in FK compared to controls. However, in healed samples, these levels were significantly reduced with HBD-1 at 2.19 ± 1.72 fold ($p=0.0003$) and HBD-2 at 0.23 ± 0.28 fold ($p=0.003$) compared to active FK.

The level of HBD-3 and S100A7 mRNAs were elevated in 11/20 FK samples, whereas HBD9 mRNA was upregulated (> 2-fold) in 15/20 FK samples. HBD-3 was increased by 5.54 ± 8.28 fold ($p=0.007$) and HBD-9 was elevated by 31.83 ± 51.15 fold ($p=0.018$) in FK. Similar to other defensins, HBD-3 and HBD-9 also showed baseline expression in healed samples with levels at 0.01 ± 0.02 fold ($p=0.007$) and 0.51 ± 0.40 fold ($p=0.013$), respectively. S100A7 mRNA expression was shown to be increased by 61.89 ± 95.73 fold ($p=0.009$) in FK samples compared to controls. Whereas in healed samples, mRNA levels for S100A7 were reduced to 0.19 ± 0.34 fold ($p=0.009$) compared to FK.

Of all AMPs, LL-37 mRNA was moderately increased in FK showing elevated expression (4.05 ± 7.41 fold; *p=0.047*) only in 7/20 samples. In healed samples, LL-37 was expressed at low levels (1.16 ± 1.01 fold; *not significant*) matching the mRNA levels in controls.

DISCUSSION

AMPs have attracted special attention as potential therapy for microbial infections due to their unique mode of action compared to available antimicrobial therapies[30]. In the last two decades, we and others have extensively demonstrated an essential role of human AMPs in microbial keratitis[17 19-23 31-33]. In corneal epithelial cells, expression of HBD-2, HBD-3 and LL-37 were shown to be significantly elevated in response to infection with *Fusarium solani*[34] and *Candida albicans*[35], respectively. Elevated levels of cytokines and other innate immune receptors was previously demonstrated in human corneal specimen from patients with *Aspergillus flavus* and *Fusarium solani* infection [36]. Moreover, increased susceptibility to corneal infections by *Aspergillus fumigatus, Fusarium solani* and *Candida albicans* was previously demonstrated in mice deficient in cathelin-related antimicrobial peptide (CRAMP) and murine β-defensins (mBD-1 to -4)[37 38].

In this study, we demonstrated an increased pattern of AMPs (HBD-1 to -3, HBD-9, S100A7 and LL-37) expression in corneal specimen during active infection. Notably, in healed specimen, mRNA of all AMPs was found to be at a basal level. A similar phenomenon has been noted in our previous demonstration of HBD-3 and HBD-9 mRNA levels in OS specimen collected during active bacterial keratitis and following complete healing[31]. Moreover, in an animal model of *Candida albicans* keratitis, CRAMP and β-defensins (mBD-1 and -2) have demonstrated a variable expression at the onset of disease but returned to their normal level upon healing at day 7 post-infection[38]. In *Fusarium solani* keratitis mouse model, mBD-3, mBD-4, mBD-14 and CRAMP were shown to be significantly increased by day 3 post-infection, which then started to decrease with reduction in disease severity reaching to baseline upon healing[37]. From above studies, it could be inferred that rapid normalisation of AMPs expression following healing occurs and is likely to be of importance considering the fact that high levels of AMPs could elicit toxic responses on ocular surface.

Previously we have demonstrated a significant downregulation of HBD9 expression in specimen from patients with bacterial keratitis, acanthamoeba keratitis, viral keratitis and dry-eye disease[20]. Interestingly, in current study, we have noted an elevated pattern of HBD9 expression in active FK samples. This unique response of HBD9 during fungal infection suggest a potential anti-fungal function of this AMP. We have generated the recombinant protein of HBD9 using *E. coli* expression system and demonstrated that both recombinant HBD9 protein and its full-length synthetic linear peptide were unstable, which was attributed to its proline rich C-terminus. Failure to keep the protein or its linear peptide in solution has thus far prevented us to test the antimicrobial efficacy of HBD9 against disease-causing pathogens (unpublished observations).

S100A7 was first isolated from skin of Psoriasis patients hence it is also known as Psoriasin[39]. S100A7 has been shown to express constitutively in different regions of anterior segment of the eye[40]. Interestingly, cysteine-reduced form of S100A7 protein has been previously shown to exhibit potent antifungal activity against dermatophytes and filamentous fungi[41]. Whereas both native and reduced forms of S100A7 showed activity against the yeast, *C. albicans*[41]. A previous study has demonstrated the increased levels of S100A7 in vaginal biopsies from patients with *C. albicans* vulvovaginitis[42]. Similar increased levels of S100A7 were also noted in this study. Further research using gene knockout experiments would highlight the importance of S100A7 in host defense to fungal infections at the ocular surface.

Although FK is slowly progressive compared to bacterial keratitis, the rate of corneal perforation has been reported to be high in FK cases[43]. AMPs are potent chemo attractants and capable of eliciting adaptive immunity[30]. Thus, in addition to direct killing of microbes, increased level of AMPs during FK could potentially increase neutrophil infiltration, which might contribute towards tissue damage, melting, and scarring. A recent report has demonstrated that deficiency of CRAMP and mBD-3 and -4 increased the susceptibility to *F. solani* keratitis and led to excessive infiltration of neutrophils which was attributed to high levels of KC (a neutrophil chemokine) in corneal tissue[37]. Similarly, in *C.*

albicans keratitis model, deletion of CRAMP was also shown to increase yeast burden, neutrophil recruitment and levels of IL-1β and MIP-2[44]. In stark contrast to keratitis model, severity of *C. albicans* infection in oral mucosa in mBD-1 knockout mice have been associated with low neutrophil recruitment and reduced levels of IL-1β, KC, IL-17A and IL-17F[45]. In mice cornea, the specific function of neutrophil derived calprotectin (S100A8/A9) in clearance of *A. fumigatus* has been demonstrated. Unlike cationic AMPs, calprotectin was shown to exhibit antifungal activity *via* chelation of zinc and manganese which retards *A. fumigatus* growth[46].

Our results corroborate previous studies on AMP regulation during fungal infection in animals. This further implicates a pivotal role for AMPs in OS defense against fungal pathogens. However, their diverse function in modulation of neutrophil infiltration and inflammation in cornea and other mucosal sites during infection still remains unclear. Though this is the first study on AMPs in human FK further studies are highly warranted to understand the mechanisms of immune activation of AMPs during FK. The limitation of this study was that it only involved assessment of gene expression of AMPs in active and healed groups. A follow-up study addressing the cellular source of these AMPs in active FK samples will further enhance the understanding of function of key AMPs towards fungi/yeast.

ACKNOWLEDGEMENT

Funding supported by the Fight for Sight small grant award (Ref: 5007/08) to HSD and IM.

Authors wish to express their gratitude to Dr. Srikant Kumar Sahu, Dr. Smruti Rekha Priyadarshini, Dr. Sanchita Mitra and Aparajita Mallick for their clinical, laboratory, and technical support.

FINANCIAL DISCLOSURE

IM: None

DM: None

DSG: None

MRB: None

MR: None

AAS: None

SD: None

HSD: Honoraria and Travel expenses from Dompe, Croma, Santen, Allergan, Thea. Shares in NuVision Biotherapies and Glaxo Smithkline.

RM: None

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Figure legend

Figure 1. Gene expression of antimicrobial peptides in corneal tissue from patients with fungal keratitis. Relative fold change of A) HBD1, B) HBD2, C) HBD3, D) HBD9, E) S100A7 and F) LL-37 in control, active FK and healed groups. Data points represent individual patients in each group. Student's t-test with Welch's correction was performed to compare control vs active FK and active FK vs healed with $p \leq 0.05$ denotes statistical significance.

Tables

Table 1. List of TaqMan probes

Gene name	*Assay ID*
DEFB1 (HBD-1)	Hs00608345_m1
DEFB4 (HBD-2)	Hs00823638_m1
DEFB103(HBD-3)	Hs00218678_m1
DEFB109(HBD-9)	Hs002760065_g1
S100A7	Hs00961622_m1
CAMP (LL-37)	Hs00189038_m1
HPRT1	4325801

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486 **Table 2.** Clinical diagnosis and laboratory results
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No.	*Age /Sex*	*Clinical diagnosis*	*Size of inf.(mm)*	*Scr-C/S*	*Sample used*	*Outcome / Keratoplasty*	*Pres to Ker*	*Histopathology findings*	*CB-C/S*
1.	36 /M	Corneal scar	NA	NA	CB	NA/Y	11m	Vascularised corneal scar	NA
2.	38 /F	Corneal scar, post trauma	NA	NA	CB	NA/Y	5 yrs	Non inflammatory corneal scar	NA
3.	19 /M	Corneal scar since childhood	NA	NA	CB	NA/Y	6m	Non inflammatory corneal scar	NA
4.	86 /M	Microbial Keratitis	3.5x2.5	unid. hyaline fungus	Scrape	Resolved keratitis /ND	NA	NA	NA
5.	86 /M	Microbial Keratitis	3.5x2.5	unid. hyaline fungus	Imp	Resolved keratitis/ ND	NA	NA	NA
6.	30 /F	Corneal ulcer	TCI	*Aspergillus spp.*	Scrape	Failed to resolve/ Y	30d	Fungal corneal ulcer	NG
7.	30 /F	Corneal ulcer	TCI	*Aspergillus spp.*	Imp	Failed to resolve/ Y	30d	Fungal corneal ulcer	NG
8.	22 /F	Corneal ulcer	2x1.5	NG	Scrape	Partially resolved /Y	30d	Fungal keratitis with yeast like spores	NG
9.	36 /M	Fungal Keratitis	4x4.5	*Fusarium spp.*	Scrape	Resolved keratitis /ND	NA	NA	NA
10.	69 /M	Perforated Corneal ulcer with hypopyon	TCI	*Lasiodiplodia spp.*	Scrape	Failed to resolve/ Y	18d	Fungal corneal ulcer	NG
11.	27 /F	Microbial Keratitis	3.5x2	*Fusarium spp.*	Imp	LFU	NA	NA	NA
12.	31 /M	Corneal ulcer with hypopyon	TCI	*Aspergillus spp.*	Imp	Resolved keratitis/ Y	6m	Corneal scar	NG
13.	75 /M	Fungal Keratitis	5x2	*Acremonium spp.*	CB	Failed to resolve/ Y	30d	Fungal Corneal ulcer	NG
14.	39 /M	Microbial Keratitis	5.5	*Fusarium spp.*	CB	Failed to resolve/ Y	28d	Fungal Corneal ulcer	Y
15.	44 /M	Perforated corneal	TCI	ND	CB	Perforated corneal	0d	Fungal Corneal ulcer	Y

		ulcer				ulcer/Y			
16.	59 /M	Fungal Keratitis	TCI	*Fusarium spp.*	CB	Large corneal ulcer/Y	1d	Fungal Corneal ulcer	Y
17.	48 /M	Microbial Keratitis	3x4	*Aspergillus spp.*	CB	Large corneal ulcer/Y	6d	Fungal Corneal ulcer	NG
18.	46 /M	Microbial Keratitis	3x2	NG	CB	Large corneal ulcer/Y	4d	Fungal Corneal ulcer	Y
19.	54 /M	Fungal Keratitis	6x5	*Aspergillus spp.*	CB	Large corneal ulcer/Y	4d	Fungal Corneal ulcer	Y
20.	58 /F	Microbial Keratitis	3.5x3	*Unid. Dematiaceous Fungus*	CB	Impending perforation / Y	2d	Fungal Corneal ulcer	Y
21.	56 /M	Fungal Keratitis	10x8	*Fusarium spp.*	CB	Near total infiltrate/ Y	9d	Fungal Corneal ulcer	NG
22.	44 /F	Microbial Keratitis	7x6	NG	CB	No response/ Y	5d	Fungal Corneal ulcer	NG
23.	44 /M	Perforated corneal ulcer	TCI	ND	CB	Perforated corneal ulcer/Y	0d	Fungal Corneal ulcer	Y
24.	37 /F	Fungal Keratitis	3.5x2.5	*Fusarium spp.*	CB	Resolved keratitis/ Y	7m	Corneal scar	NG
25.	52 /M	Fungal Keratitis	TCI	*Burkholderia spp.*	CB	Resolved keratitis/ Y	10m	Corneal scar	NG
26.	26 /M	Microbial keratitis	8x5	*pseudo allescheria boydii*	CB	Resolved keratitis/ Y	10m	Corneal scar	NG

Abbreviations: Inf- infiltrate; M/F- male/female; CB-corneal button; Y-yes; Pres to Ker- duration between the date of first presentation and date of keratoplasty; d/m/yrs-days/months/years; TCI- total corneal infiltrate; NG-no growth; NA- not applicable; ND-not done; unid: unidentified; LFU-lost to follow up; Scr-C/S: Growth of fungus in culture from scraping samples; CB-C/S: Growth of fungus in culture from corneal button samples, Imp-Impression cytology.