Antimicrobial peptides in human corneal tissue of patients with fungal keratitis

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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. In this study, our aim was to profile a spectrum of AMPs in corneal tissue from patients with FK during the active phase of 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 PCR.

Results  Messenger RNA expression (mRNA) for all AMPs was shown to be significantly upregulated in FK samples. The 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 with 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. These results will help to identify HBD sequence and their expression in corneal infections.20

INTRODUCTION

Fungal keratitis (FK) is the most common cause of corneal blindness in developing countries. 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.2–4 The principal risk factor for FK in more than 70% of these cases was reported to be vegetative ocular injury.1 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, the incidence of FK cases is also increasing and mainly associated with contact lens use and ocular trauma.7 In the UK, FK incidence increased from 4.5 cases per year (between 1994 and 2006) to 14 cases per year (between 2007 and 2014).5 In the midwestern region of the USA, 16% of cases with infective keratitis (between 1999 and 2013) were identified as FK with poor healing and major complications.6 The socio-economic impact of this disease has been significantly high, because it predominantly affects individuals in the 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 antifungal agents have further compounded the problem of FK.11–15 Therefore, to seek alternative therapies, current research in this field has been mainly focussed on the understanding of mechanisms by which host immunity responds to fungi and yeast.

Antimicrobial peptides (AMPs) are naturally occurring host defence proteins with broad-spectrum antifungal 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 (OS)19 and profiled the range of AMPs (human beta-defensin (HBD)-1, -2, -3, -9 and cathelicidin (LL-37)) at the human OS and their expression in corneal infections.20–22 We also elucidated the signalling 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 known25–27 through animal studies, but the profile of human AMPs at the OS 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 the 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, Bhubaneswar, India. Informed consent was obtained from all patients prior to collection of samples, which included scrapes/impression cytology (IC) from patients with active FK, corneal button (CBs) (part of) from patients that were
subjected to therapeutic penetrating keratoplasty (PK) for FK 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 FK.

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 scrapings were taken using a sterile surgical blade (#15) on Bard-Parker handle under aseptic conditions.28 The procedure was performed under a slit lamp after instillation of 0.5% proparacaine hydrochloride. After wiping the discharge, epithelial cells surrounding the ulcer were gently scraped and placed in proparacaine hydrochloride. After wiping the discharge, epithelium was performed under a slit lamp after instillation of 0.5% enzyme control samples were also prepared.

Sample collection
► Patients able to give informed consent.
► Patients diagnosed clinically as suffering from FK and confirmed by standard microbiology and/or histopathology techniques.

Laboratory science

Patient sample collection
Corneal tissue in RLT buffer was homogenised using TissueRuptor (Qiagen, Germany) for 60 s on ice. Total RNA was isolated from CB, IC samples, and corneal scrapes using RNeasy Mini Kit (catalog No. 74 104; Qiagen, Germany) according to manufacturers’ instructions, including the optional DNase step. Isolated RNA was quantitated using BioSpectrophotometer (Eppendorf, Germany). Two hundred nanograms of total RNA was reverse transcribed to complementary DNA (cDNA) using Eurogentec Reverse Transcription Core Kit (RT-RTCK-03, Eurogentec, Belgium) as per manufacturers’ instructions. No RT–enzyme control samples were also prepared.

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Quantitative real-time PCR
Quantitative real-time PCR (qPCR) was conducted for selected AMPs (Table 1) using TaqMan probe chemistry. The limited amounts of RNA obtainable from small samples of tissue informed the decision to select only representative AMPs from each of the four families of AMPs known to be expressed at the OS. TaqMan assays specific to AMPs, hypoxanthine-guanine phosphoribosyltransferase 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, the cDNA template was initially diluted 1:5 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 10 μL of 2× mastermix, 1 μL of 20× TaqMan assay, 5 μL of diluted cDNA and 4 μL 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. CT values were acquired using MxPro Software version 5.0 and further analysed by delta-delta CT comparative method.29

Results
As shown in table 2, a total of 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 postmedical 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 and 30 from the day of presentation and those from the healed group between 6 and 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 and 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 the healed group. As shown in table 2, final diagnosis was based on the positive growth of fungi in cultures from corneal scrapings and/or on histopathology evaluation. Of n=23 cases of FK, 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).

Table 1 List of TaqMan probes

| Gene name | Assay ID |
|-----------|---------|
| DEFBl (HBD-1) | Hs00608345_m1 |
| DEFB4 (HBD-2) | Hs00823638_m1 |
| DEFBl03 (HBD-3) | Hs00218678_m1 |
| DEFBl09 (HBD-9) | Hs00760065_g1 |
| S100A7 | Hs00961622_m1 |
| CAMP (LL-37) | Hs00189038_m1 |
| HPRIT1 | 4 325 801 |

DEFB, defensin beta; HBD, human beta-defensin; HPRIT, hypoxanthine-guanine phosphoribosyltransferase 1; S100A7, S100 calcium binding protein A7.
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 with those in controls. Levels of mRNA expression are denoted as mean±SD.

HBD-1 and -2 mRNAs 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 with 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 with active FK.

Table 2

| 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                 | 11 m       | 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                 | 6 m        | 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 | IC     | Resolved keratitis/ND | NA | NA | NA |
| 6.  | 30/F    | Corneal ulcer      | TCI              | Aspergillus spp. | Scrape | Failed to resolve/Y  | 30 d       | Fungal corneal ulcer | NG     |
| 7.  | 30/F    | Corneal ulcer      | TCI              | Aspergillus spp. | IC     | Failed to resolve/Y  | 30 d       | Fungal corneal ulcer | NG     |
| 8.  | 22/F    | Corneal ulcer      | 2x1.5            | NG      | Scrape      | Partially resolved/Y | 30 d       | 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  | 18 d       | Fungal corneal ulcer | NG     |
| 11. | 27/F    | Microbial keratitis | 3.5x2            | Fusarium spp. | IC     | LFU                  | NA | NA | NA |
| 12. | 31/M    | Corneal ulcer with hypopyon | TCI | Aspergillus spp. | IC     | Resolved keratitis/Y | 6 m        | Corneal scar | NG     |
| 13. | 75/M    | Fungal keratitis   | 5x2              | Acremonium spp. | CB     | Failed to resolve/Y  | 30 d       | Fungal corneal ulcer | NG     |
| 14. | 39/M    | Microbial keratitis | 5.5              | Fusarium spp. | CB     | Failed to resolve/Y  | 28 d       | Fungal corneal ulcer | Y      |
| 15. | 44/M    | Perforated corneal ulcer | TCI | ND      | CB          | Perforated corneal ulcer/Y | 0 d       | Fungal corneal ulcer | Y      |
| 16. | 59/M    | Fungal keratitis   | TCI              | Fusarium spp. | CB     | Large corneal ulcer/Y | 1 d        | Fungal corneal ulcer | Y      |
| 17. | 48/M    | Microbial keratitis | 3x4              | Aspergillus spp. | CB     | Large corneal ulcer/Y | 6 d        | Fungal corneal ulcer | NG     |
| 18. | 46/M    | Microbial keratitis | 3x2              | NG      | CB          | Large corneal ulcer/Y | 4 d        | Fungal corneal ulcer | Y      |
| 19. | 54/M    | Fungal keratitis   | 6x5              | Aspergillus spp. | CB     | Large corneal ulcer/Y | 4 d        | Fungal corneal ulcer | Y      |
| 20. | 58/F    | Microbial keratitis | 3.5x3            | Unid. dematiaceous fungus | CB | Impending perforation/Y | 2 d        | Fungal corneal ulcer | Y      |
| 21. | 56/M    | Fungal keratitis   | 10x8             | Fusarium spp. | CB     | Near total infiltrate/Y | 9 d        | Fungal corneal ulcer | NG     |
| 22. | 44/F    | Microbial keratitis | 7x6              | NG      | CB          | No response/Y        | 5 d        | Fungal corneal ulcer | NG     |
| 23. | 44/M    | Perforated corneal ulcer | TCI | ND      | CB          | Perforated corneal ulcer/Y | 0 d       | Fungal corneal ulcer | Y      |
| 24. | 37/F    | Fungal keratitis   | 3.5x2.5          | Fusarium spp. | CB     | Resolved keratitis/Y | 7 m        | Corneal scar | NG     |
| 25. | 52/M    | Fungal keratitis   | TCI              | Burkholderia spp. | CB     | Resolved keratitis/Y | 10 m       | Corneal scar | NG     |
| 26. | 26/M    | Microbial keratitis | Bx5              | Pseudoallescheria boydii | CB | Resolved keratitis/Y | 10 m       | Corneal scar | NG     |

CB, corneal button; CB-C/S, growth of fungus in culture from corneal button samples; d/m/yrs, days/months/years; IC, impression cytology; Inf, infiltrate; LFU, lost to follow-up; M/F, male/female; NG, no growth; NA, not applicable; ND, not done; Pres to Ker, duration between the date of first presentation and date of keratoplasty; Scr-C/S, growth of fungus in culture from scraping samples; TCI, total corneal infiltrate; unid, unidentified; Y, yes.
The levels of HBD-3 and S100A7 mRNAs were elevated in 11/20 FK samples, whereas HBD9 mRNA was upregulated (>two-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 with controls. Whereas in healed samples, mRNA levels for S100A7 were reduced to 0.19±0.34-fold (p=0.009) compared with 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. It is acknowledged that the number of control and ‘healed’ samples were relatively low, but the comparative trends in differential expression are quite clear.

**DISCUSSION**

AMPs have attracted special attention as potential therapy for microbial infections due to their unique mode of action compared to available antimicrobial therapies. In corneal epithelial cells, expression of HBD-2, -3 and LL-37 were shown to be significantly elevated in response to infection with *Fusarium solani* and *Candida albicans*, respectively. Elevated levels of cytokines and other innate immune receptors were previously demonstrated in human corneal specimen from patients with *Aspergillus flavus* and *F. solani* infection. Moreover, increased susceptibility to corneal infections by *A. fumigatus, F. solani* and *C. albicans* was previously demonstrated in mice-deficient in cathelin-related antimicrobial peptide (CRAMP) and murine β-defensins (mBD-1 to -4).

In this study, we demonstrated an increased pattern of AMPs (HBD-1, -2, -3, -9, S100A7 and LL-37) expression in corneal specimen during the active phase of infection. Notably, in healed specimens, mRNA of all AMPs was found to be at a basal level. In our previous study, HBD1 expression was found to increase by threefold in active bacterial keratitis samples compared to the 12-fold increase in FK shown in this study. This suggests that the HBD family of molecules, particularly 1 and 2, are key players in FK. 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. Moreover, in an animal model of *C. albicans* keratitis, CRAMP and β-defensins (mBD-1 and -2) have demonstrated a variable expression at the onset of disease but returned to their role of human AMPs in microbial keratitis. In corneal epithelial cells, expression of HBD-2, -3 and LL-37 were shown to be significantly elevated in response to infection with *F. solani* and *C. albicans*, respectively. Elevated levels of cytokines and other innate immune receptors were previously demonstrated in human corneal specimen from patients with *A. flavus* and *F. solani* infection. Moreover, increased susceptibility to corneal infections by *A. fumigatus, F. solani* and *C. albicans* was previously demonstrated in mice-deficient in cathelin-related antimicrobial peptide (CRAMP) and murine β-defensins (mBD-1 to -4).

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normal level upon healing at day 7 postinfection. In F. solani keratitis mouse model, mBD-3, -4, -14 and CRAMP were shown to be significantly increased by day 3 postinfection, which then started to decrease with reduction in disease severity reaching to baseline upon healing. From the aforementioned 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 OS.

Previously we have demonstrated a significant downregulation of HB9D expression in specimen from patients with bacterial keratitis, acanthamoeba keratitis, viral keratitis and dry-eye disease. Interestingly, in the current study, we have noted an elevated pattern of HB9D expression in active FK samples. This unique response of HB9D during fungal infection suggests a potential anti-fungal function of this AMP. We have generated the recombinant protein of HB9D using Escherichia coli expression system and demonstrated that both recombinant HB9D 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 HB9D against disease-causing pathogens (unpublished observations).

S100A7 was first isolated from the skin of patients with psoriasis; hence, it is also known as psorin.

AMPs are potent chemoattractants and capable of eliciting adaptive immunity. 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 keratocytode- derived chemokine (KC, a neutrophil chemokine) in corneal tissue. Similarly, in C. albicans keratitis model, deletion of CRAMP was also shown to increase yeast burden, neutrophil recruitment and levels of interleukin (IL)-1β and MIP-2. In stark contrast to keratitis model, severity of C. albicans infection in oral mucosa in mBD-1 knockout mice has been associated with low neutrophil recruitment and reduced levels of IL-1β, KC, IL-17A and IL-17P. 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.

Our results corroborate previous studies on AMP regulation during fungal infection in animals. This further implicates a pivotal role for AMPs in OS defence 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. Although 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.

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