ANTIMICROBIAL PEPTIDES EXPRESSION BY OCULAR SURFACE CELLS IN RESPONSE TO ACANTHAMOEBA CASTELLANII: AN IN VITRO STUDY

Ahmad Muneer Otri, Imran Mohammed, Asiya Abedin, Zhiyi Cao, Andrew Hopkinson, Noorjahan Panjwani, Harminder Singh Dua

Antimicrobial Peptides Expression By Ocular Surface Cells In Response To *Acanthamoeba Castellanii*: An *In Vitro* Study

Authors: [1]Ahmad Muneer Otri, [1]Imran Mohammed, [1]Asiya Abedin, [2]Zhiyi Cao, [1]Andrew Hopkinson, [2]Noorjahan Panjwani, [1]Harminder S Dua.

Affiliation: 1. Division of ophthalmology and visual sciences, University of Nottingham, England and 2. New England Eye Centre, Tufts University School of Medicine, Boston, MA, USA

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Corresponding Author

Harminder S Dua

Division of Ophthalmology and Visual Sciences

B Floor, Eye ENT Centre

Queens Medical Centre, Nottingham University Hospitals NHS Trust

Derby Road, Nottingham. NG7 2UH

England. UK.

Harminder.dua@nottingham.ac.uk

Telephone +44 115 9709796

Fax +44 115 9709963

Abstract

Aims: Antimicrobial peptides (AMPs) are natural effectors of the innate immune response. Much work has been done to study their response and effects on bacterial and viral infection. Little if any information is available in relation to protozoal infections. Our aim was to comprehensively study the gene expression of the ocular AMPs in human corneal limbal epithelial cells (HCLE) stimulated with *Acanthamoeba castellanii (AC)*.

Methods: Human corneal limbal epithelial cells were exposed to *AC* at different time points, up to 9 hours, the genomic profile of the AMPs were analysed at these time point using real time PCR. HCLE cells not infected with AC were used as controls.

Results: Seven of the 8 studied AMPs showed statistically significant upregulation in gene expression. Human beta Defensin 3 (hBD3) showed a very significant 10 fold upregulation in the exposed cells and Ribonuclease-7 (RNase-7) showed a very early and consistent increase. Human beta Defensin 1 (hBD1) was the only downregulated AMP.

Conclusions: The study data suggests a possible role of the AMPs in combating the amoebic infection at the ocular surface. Using AMPs singly or in combination is a promising avenue for further exploration in the treatment of the sight threatening *Acanthamoeba* keratitis.

Introduction

The ocular surface comprises of cornea, conjunctiva, and tear film. Like other mucosal surfaces, it is in direct contact with the environment and exposed to environmental pathogens such as bacteria, viruses, fungi, and protozoa. It also maintains a population of commensal organisms, which have the potential to cause opportunistic disease. To combat these threats, the ocular surface has developed innate and adaptive immune mechanisms.[1] One important component of the innate response is the repertoire of antimicrobial peptides (AMPs). AMPs are ubiquitous natural effectors of the host defence system and are conserved across the plant and animal kingdom with broad spectrum microbicidal activity and cell signalling functions. They can be considered to represent the eukaryotic analogues of antibiotics.[2, 3]

At the ocular surface the epithelial cells and neutrophils are a major source of AMPs which include beta Defensins (BD) 1 to 4 & 109, Liver expressed antimicrobial peptide 1 (LEAP) 1 (also known as Hepcidin) and LEAP 2, Cathelicidin (LL37) [4], and Ribonuclease-7 (RNase-7) (unpublished observation). *Acanthamoeba* is a ubiquitous free-living genus of amoeba that can survive in diverse conditions and has been isolated from tap water, seawater, soil, dust and air. The life cycle of *Acanthamoeba* has two stages: a replicative trophozoite stage that under adverse conditions such as extreme temperature, starvation, or osmolarity changes can develop into a dormant cyst stage. [5, 6]

Acanthamoeba is notorious as a cause of serious corneal infection particularly amongst wearers of soft contact lenses. The infection is sporadic but two outbreaks have been recently reported in Chicago and Singapore.[7, 8] The mechanism by

which this infection occurs is directly related to the ability of *Acanthamoeba* trophozoites to adhere to mannose glycoproteins on the corneal epithelium via the amoeba's mannose-binding receptor.[9-11] This binding induces production of a cytolytic factor, mannose-induced protein (MIP133), by the *Acanthamoeba*.[12] Subsequently, a number of matrix metalloproteases are activated, killing corneal epithelial cells, and penetrating the cornea by dissolution of the basement membrane and the underlying collagen of the Bowman's zone and stroma. [11, 13, 14]

Although there are several studies on the gene expression of AMPs in corneal epithelial cells challenged with microbial stimuli, there is no work on AMPs expression by any human cell type in response to *Acanthamoeba*. In this study we characterized the profile of AMPs gene expression by a human corneal limbal epithelial cells exposed to live *Acanthamoeba castellanii*.

Methods

Human cell culture

Telomerase reverse transcriptase-immortalized human corneal limbal epithelial cells (HCLE) were used in this study. This cell line has been shown to be very similar to the native corneal epithelium in relation to cornea specific keratin and mucin expression and other parameters. [15-17] According to previously described protocols [15], HCLE were maintained in keratinocyte serum-free media (K-SFM; Invitrogen, Carlsbad, CA) supplemented with 0.2 ng/ml epidermal growth factor (EGF), 25 µg/ml bovine pituitary extract (BPE) and 0.4 mM CaCl2 at 37 ˚C in a 5% CO2 humidified incubator. The HCLE were subcultured in 6-well cell culture plates (9.5 cm^2; BD, Franklin Lakes, NJ) until confluency was reached and then starved

overnight in growth supplement and BPE free media before exposure to *Acanthamoeba.*

Isolation and culture of *Acanthamoeba castellanii* trophozoites

Acanthamoeba castellanii (AC) trophozoites: An *Acanthamoeba* strain derived from an infected human cornea (MEEI 0184; *A. Castellanii,* genotype T4) was used throughout this study. The parasites were axenically cultured in a proteose peptone-yeast extract-glucose (PYG) medium prior to the exposure/stimulation studies.[18]

***Acanthamoeba* exposure/stimulation of HCLE**

The final density of the *Acanthamoeba* used was 2 x 10^5 amoebae/ ml. 1.5 ml of amoebae suspension was used for each 60 mm dish. Amoebae from PYG media were washed and suspended in the following media: [1 ml of Non-essential amino acid, 10 ml of 4% BSA and 89 ml of Eagle's minimal essential medium, (EMEM) with L-Glutamine] prior to stimulating HCLE cells. The confluent HCLE cells were incubated with *A. castellanii* trophozoites and samples were collected at 1, 3, 6 and 9 hours. Control samples were obtained from non stimulated HCLE. At each time point, the culture media was removed and 700 µl of RLT buffer (Qiagen) was added t each well for 15 minutes until the cells were fully solubilised and the lysate was collected and stored at -80 C. Additionally, the suspension of *Acanthamoeba* culture (without cells) was collected separately and spun down for 2 mins at 16,000 rpm. 700 µl of RLT buffer (Qiagen) was added and the samples were kept at -80^o C for further analysis. All tests were done in triplicate and each of the triplicate set of tests was on cells of the same passage. The morphology of the immortalized cells used in

the current study was very similar to that of the primary cultures of human corneal epithelium.

Isolation of total RNA and cDNA synthesis.

Total RNA was extracted from RLT lysate using the RNeasy Mini kit, (according to manufacturer's instructions; Qiagen). Reverse Transcription into cDNA of 4 μg of template RNA from cultured cells was carried out according to the manufacturer's instructions (Quantitect Reverse Transcription kit, Qiagen).

Quantitative real-time PCR (qPCR)

QPCR analysis was performed to measure the relative gene expression of the following AMPs: beta Defensins (hBD1, hBD2, hBD3, and BDEF109), LL37, LEAP1, 2 and RNase-7 mRNA

Pre-optimised Real-time qPCR reactions were run on a 96-well plate (Applied Biosystems) in the Mx3005p real-time PCR system (Stratagene, Agilent Technologies, UK). The data obtained from the machine were further analysed to calculate the relative gene expression of AMPs mRNA levels

Taqman assays (Applied Biosystems, Europe) were used for all the AMPs genes and the endogenous control [Hypoxanthine-guanine phosphoribosyltransferase (HPRT)].

The details of genes and Taqman IDs are given in table 1.

Table1- Details of genes and accession numbers used in the study. (Applied Biosystems, Europe)

Gene Symbol	Taqman assay ID	Accession number
HPRT	4333768F	NM_000194.1
DEFB1/Hs00174765_m1	Defensin, beta1/HBD1	NM_005218.3
DEFB4/Hs00823638_m1	Defensin,beta4/HBD2	NM_004942.2
DEFB103A/Hs00218678_m1	Defensin,beta103A/HBD3	NM_018661.2
CAMP/Hs00189038_m1	Cathelicidin AMP	NM_004345.3
HAMP/Hs00221783_m1	Hepcidin AMP (LEAP 1)	NM_021175.2
LEAP2/Hs00364834_m1	Liver expressed AMP2	NM_052971.2
Hs00261482_m1	RNase7	NM_032572.3
Hs02760065_g1	DEF109	NM_001037380

Statistical analysis

The qPCR data was statistically analyzed on the SPSS 16.0 ver (IBM, Chicago, IL) software with significance set at $P< 0.05$. Using the Student's *t*-test, the mRNA expression in treated samples with those obtained in untreated controls was compared. All data was represented as mean and standard error of two independent experiment performed in triplicate.

Results

AMPs expression in *Acanthamoeba*-exposed HCLE showed a statistically significant upregulation in 7 of the 8 studied AMPs. hBD1 was the only AMP which showed variable decrease in the gene expression. It showed reduced expression at all time points with the downregulation at the 6 hour time point (0.6 fold) reaching statistical significance (P= 0.038, Figure 1- A).

hBD2 gene expression increased with time with a dramatic and significant four folds increase at 9 hours (P= 0.036, Figure 1- B).

hBD3 gene expression showed a significant upregulation at 3, 6, and 9 hours (7 folds, 7.5 folds, and 10 folds, respectively) (P= 0.044, P= 0.00050, P= 0.017 respectively, Figure 1- C).

DEF109 gene showed a trend towards decreased expression for the first 6 hours but this did not reach statistical significance. It increased and reached a significantly higher level at the 9 hour time point (1.5 fold, p= 0.002, Figure 1- D)

LL37 gene expression varied slightly over the time course with a slight but significant increase at the 9 hours time point (p= 0.003, Figure 1- E)

LEAP1 gene expression was variable at the earlier time points but was significantly unregulated at 9 hours (5 folds increase, p= 0.001, figure 1- F).

LEAP2 gene expression showed a significant down regulation at the 3 hours time point ($p = 0.002$, Figure 1g) and then a statistically significant up-regulation at 6 and 9 hours (2 and 3.5 folds increase, $p= 0.033$, $p= 0.0001$ respectively, Figure 1- G).

RNase 7 gene expression showed significant upregulation at all time points from 1 to 9 hours ($p= 0.04$, $p= 0.03$, $p= 0.003$, $p= 0.006$; Figure 1h), with the highest expression (3.5 fold) at 9 hours. (Figure 1- H)

Quantitative RT-PCR of cDNA obtained from lysate of *A castellani* organisms that were maintained in culture for 24 hours did not show any AMP expression.

Discussion

AMPs are a component of the innate immune defence and as such respond early to any external challenge with microbes. These responses are known to occur within a few hours of the microbe interacting mainly with the toll like receptors of the surface cells.[19] Hence we decided to study the responses in the early time points following infection. We did extend the time course study to 24 hours but most host cells had lysed and meaningful information could not be obtained (data is not presented).

The upregulation of AMPs and other epithelial-derived components of innate immunity, e.g. inflammatory cytokines, is thought to be a key component of an effective defence against infection at the ocular surface.[20] AMPs contribute to the host defence by direct action on microbes and also by augmenting other defence pathways via host cell stimulation.[21] It is also demonstrated that AMPs can work synergistically with more than one AMP working together in mounting a host defence response. Two or more AMPs acting together may have equally potent antimicrobial

effects at smaller concentrations. This strategy may be a way of overcoming undesirable side effects of AMPs on host cells seen at higher concentrations.[22]

The anti-amoebic effect of specific AMP (Magainins) has been previously established [23, 24]. Schuster et al showed that Magainin peptide in combination with silver nitrate and/or other antimicrobial agents have in vitro activity against *Aanthamoeba trophozoites and cysts*.[23] Sacramento et al have recently shown increased permeabilisation of *A. castellanii* with peptides of α-helical or β-sheeted AMPs. Some of these peptides were hydrolysed by *Acanthamoeba* culture supernatants suggesting enzymatic digestion. They suggested use of more specific peptides that are resistant to proteolysis produced by the organism or the combined use of the AMPs with specific protease inhibitors.[25] Our study is therefore of relevance as the potential to treat *Acanthamoeba* corneal infections with AMPs has been demonstrated.

Our *in vitro* study demonstrated statistically significant upregulation of 7 AMPs out of 8 studied following interaction of epithelial cells with *A. castellanii*.

Human hBD3 has independent broad spectrum *in vitro* antimicrobial activity against gram negative bacteria, gram negative bacteria, fungi and some viruses. This action is least affected by salt found in tear film on the ocular surface compared with the other AMPs.[21, 26] In this study hBD3 was the most significant, almost doubling the gene expression at three hours and showing a ten fold increase at 9 hours. This AMP had the maximum increased expression of all AMPs studied and the increase

also started at an early time point. This was followed by LEAP1, hBD2 and RNAse-7. At the 9 hour time point hBD3 expression was two and a half times more than hBD2 and RNAse-7 and two times that of LEAP1. This upregulation could suggest a specific anti-amoebic role of the hBD3 gene on the ocular surface.

LEAP1 (Hepcidin) showed significant up regulation only at the 9 hour time point. This AMP was initially noted for its antimicrobial effects and has been linked to iron metabolism.[27] Its expression was predominantly detected in the liver and to a much lower extent, in the heart. It is now recognized for iron homeostasis and through this mechanism may contribute to the innate immunity by restricting iron availability to microbes [28] like lactoferrin which is a known constituent of tears.[29] LEAP1 has demonstrable *in vitro* antibacterial activity against Gram-positive and Gram-negative bacteria as well as the yeasts. [30]

Like LEAP1, hBD2 also showed up-regulation of the gene expression at 9 hours. This AMP's expression is upregulated by proinflammatory cytokines such as TNF-α and IL -1β.[31] AT the ocular surface, hBD2 is known to be inducible in response to Gram positive and Gram negative bacteria.[22] It has potent antimicrobial effect against *Pseudomonas aeruginosa* and *Staphylococcus. aureus*.[21]

Human hBD1 is constitutively expressed on the ocular surface and its expression does not change much with bacterial infection.[21, 32] In this study, with *Acanthamoeba* too, it showed downregulation at all time points. This could be

explained by the non-inducible nature of this AMP on the ocular surface [22] which could indicate a limited antimicrobial role of the hBD1 in infectious diseases.

LL37 has good activity against both Gram negative and Gram positive bacteria. Additionally, it has antiviral effects when tested *in vitro*.[21, 33] However, in response to *Acanthamoeba*, its upregulation was not as much as has been reported with bacteria and may represent a differential response to protozoan infection.

The expression of DEFB109 showed significant up-regulation in *in vitro* samples at 9 hours with variable downregulation at early stages. We have previously shown down-regulation of DEFB109 gene expression in patients with *Acanthamoeba* keratitis.[32] This downregulation could be related to the fact that all the patients with *Acanthamoeba* keratitis were contact lens wearers. Interaction of hydrophilic contact lenses with epithelial cells *in vitro* has demonstrated a downregulation in expression of hBD2 following exposure to *Pseudomonas aureginosa*. [34] Unlike in samples taken from patients' eyes, *in vitro* exposure to *Acanthamoeba* showed upregulation of DEFB109 suggesting that expression of this AMP is susceptible to external influences.

LEAP2 showed early downregulation at 3 hours then up-regulation at 6 and 9 hours, though comparatively less than other AMPs. Although, chicken Liver Expressed Antimicrobial Peptide-2 (cLEAP-2) is known to have killing activities against Salmonella spp and Gram positive bacteria, [35] It is well documented that human LEAP-2 is not microbicidal peptide. The secondary structure of this AMP has a negative effect on its antibacterial activity in humans.[36]

RNase-7 exhibits broad spectrum antimicrobial activity in vitro against several potentially pathogens like Gram-positive bacteria, Gram-negative bacteria and the yeast *Candida albicans*. Furthermore, RNase-7 shows extremely high activity against vancomycin-resistant *Enterococcus faecium*. [37] In our study, RNase-7 gene expression revealed remarkable early upregulation in HCLE in response to *A. castellani.* This continued to increase gradually. This finding could suggest a possible role of this highly cationic AMP in the anti amoeba activity.

There was no AMPs gene expression in samples taken from lysate of *Acanthamoeba* alone which confirmed the specificity of the TaqMan probes against the human AMPs genes in the HCLE.

This study provides a comprehensive profile of the ocular surface AMP gene expression in response to *A. castellani* infection. The profile differs from that seen following bacterial infection and illustrates the differential expression in response to different organisms. It provides the foundation upon which further studies to elucidate anti *Acanthamoeba* activity of specific AMPs. *Acanthamoeba* infection of the cornea is a serious and sight threatening condition with not licensed drug available for its treatment. Dilute disinfectant agents such as PHMB and chlorhexidine [38] are employed in a non-specific manner. The potential of using AMPs singly or in combination to combat this infection is a promising avenue for further exploration.

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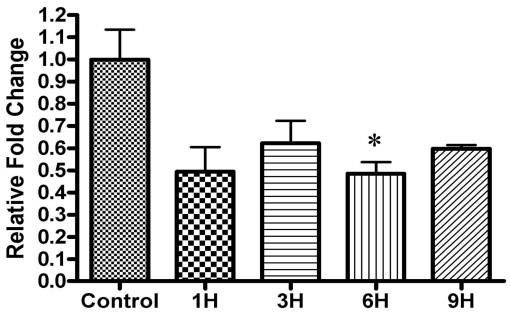
Figure legends

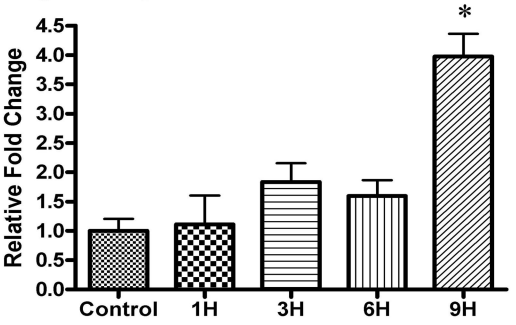
Figure1- The AMPs gene expression in HCLE stimulated with AC

(*= P value is less than 0.05, **= P value is less than 0.01, ***= P value is less than 0.001)

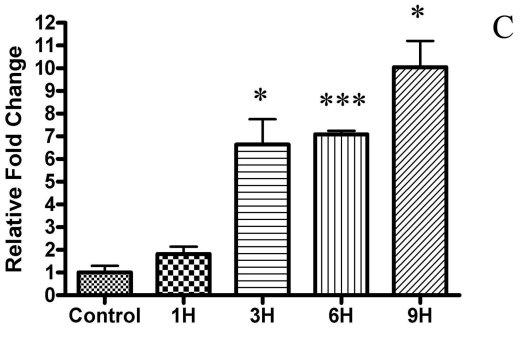
hBD1 gene expression in HCLE stimulated with AC



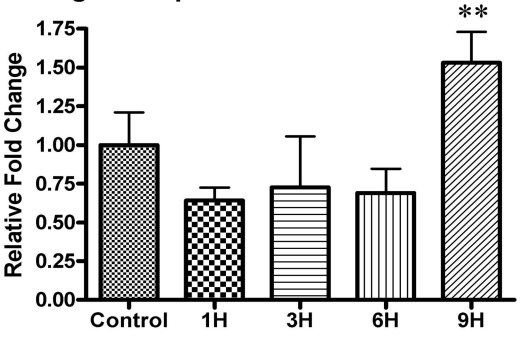
hBD2 gene expression in HCLE stimulated with AC



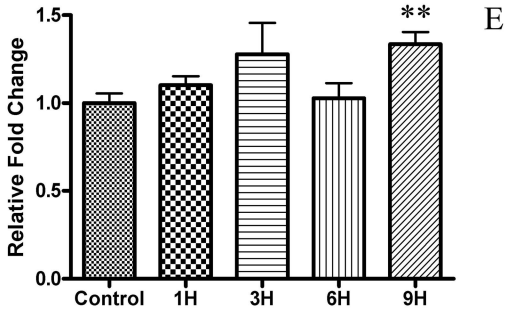
hBD3 gene expression in HCLE stimulated with AC



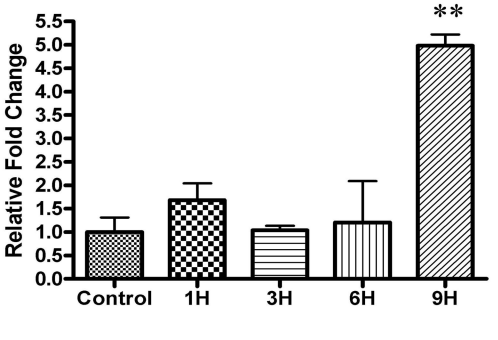
DEFB109 gene expression in HCLE stimulated with AC



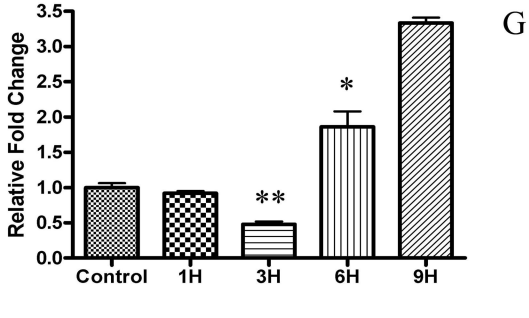
LL37 gene expression in HCLE stimulated with AC



LEAP1 gene expression in HCLE stimulated with AC



LEAP2 gene expression in HCLE stimulated with AC



RNase7 gene expression in HCLE stimulated with PA