Variable Expression of Human beta Defensins 3 and 9 at the Human Ocular Surface in Infectious Keratitis

Ahmad Muneer Otri, Imran Mohammed, Moubamed A. Al-Aqaba, Usama Fares, Chen Peng, Andrew Hopkinson, and Harminder S. Dua

PURPOSE. The authors have previously reported the presence of the antimicrobial peptides human beta defensin (hBD) 3 and hBD9 in the ocular surface (OS). These play an important role in infection and inflammation. In the present study, the authors studied the gene expression levels of hBD3 and hBD9 in healthy subjects and during and after healing of infectious keratitis.

METHODS. Human OS specimens were obtained by impression cytology from healthy controls and patients with *Acanthamoeba* and Gram-negative and -positive bacterial keratitis (BK), both during active infection and after healing. The gene expression levels of hBD3 and hBD9 were determined using quantitative real-time polymerase chain reaction (RT-PCR).

RESULTS. hBD3 and hBD9 were constitutively expressed in all healthy controls. During acute *Acanthamoeba* keratitis (AK), hBD3 levels were markedly increased and then returned close to normal levels after healing. In BK, hBD3 gene expression was moderately increased and then decreased after healing. In contrast to hBD3, hBD9 was significantly downregulated in both AK and Gram-positive BK, whereas it showed an insignificant decrease in Gram-negative BK. After healing, the expression showed upregulation except in Gram-positive BK, where it continued to decline.

CONCLUSIONS. This is the first study that demonstrates the gene expression of hBD3 and hBD9 in response to infection. It illustrates that not all antimicrobial peptides (AMPs) behave in a similar manner. Some are upregulated and some are downregulated, suggesting a diverse role of AMP in infection and inflammation. The results point to a role of AMP-mediated host defense in *Acanthamoeba* keratitis as well. (Invest Ophthalmol Vis Sci. 2012;53:757–761) DOI:10.1167/iovs.11-8467

The ocular surface (OS) consists of cornea, conjunctiva, limbus, and tear film. Like other mucosal surfaces, it is exposed to environmental pathogens such as bacteria, viruses, fungi, and protozoa. *Acanthamoeba* keratitis (AK) is a sight-threatening infection for which contact lens wear is a risk factor. *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) are Gram-negative and Gram-positive bacteria, respectively. They are common causes of bacterial keratitis (BK) in all age groups. Antimicrobial peptides (AMPs) are small, cationic host defense peptides secreted by a variety of cell types with a broad spectrum of activity against bacteria, fungi, and enveloped viruses. At the OS, AMPs are expressed strongly by neutrophils and epithelial cells and play a key role in combating infection that may result from environmental challenges such as dust, trauma, or contact lens wear. AMPs are widely present on the OS and include human beta defensin (hBD) 1 to 3 and hBD9 (gene analog of defensin beta 109); liver-expressed antimicrobial peptides (LEAP) LEAP-1 (also known as hepcidin) and LEAP-2; cathelicidin (LL37) and RNase-7 (RNase-7). Because of their potent antimicrobial activity, defensins are the most studied AMPs in humans.

hBD9 is an AMP at the human OS recently discovered by our group. In contrast to most other AMPs, it shows reduced ex vivo gene expression in patients with infectious keratitis and dry eye disease compared with healthy controls. In our previous study, we showed variable hBD9 gene expression in response to the activation of innate immune effectors (Toll-like receptors [TLRs]), nucleotide oligomerization domains (NODs), and interleukin-1 receptor (IL-1R). Moreover, in another study, we demonstrated that compared with controls, the in vitro gene expression of hBD9 was variably downregulated at early time points and then upregulated at later time points in human corneal limbal epithelial cells (HCLEs) cocultured with *Acanthamoeba castellani* (AC).

hBD3 has an independent broad spectrum in vitro antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, and some viruses. This action is least affected by salt found in tear film compared with other AMPs. In a recent study, we showed that the in vitro expression of hBD3 had increased about 10-fold in HCLEs challenged with AC compared with controls. This increase was the highest among the AMPs studied. Therefore, hBD3 and hBD9 may play significant roles in infection control and onset. Studying their roles during microbial infection may lead to an understanding of mechanisms by which infectious organisms overcome host defenses to cause infection.

Based on the results of our previous work and to improve our understanding of the downregulation of hBD9 in response to infection or inflammation, we sought to further investigate the ex vivo gene expression of hBD9 and hBD3 at the ocular surface in patients with different eye infections before and after treatment compared with healthy controls.

MATERIALS AND METHODS

Research was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the local ethics committee and the Research and Development Department of the National Health Service, Nottingham University Hospitals, NHS Trust (number, 757
Sample Collection

Impression cytology (IC) samples of OS epithelium (conjunctiva and part cornea) were collected from healthy controls (with no recent history of ocular infection) and from patients during the acute stage of infection (within the initial 48 hours before the initiation of topical treatment) and from the same patients 4 to 6 weeks after complete healing. Complete healing of the ulcer was always associated with complete cessation of all treatment. At that stage, the main clinical criteria were complete healing of the corneal epithelial defect, total resolution of the corneal infiltrate, no AC activity, and a white uninflamed eye. IC was performed with cellulose ester discs, as previously reported. Briefly, cellulose ester discs of pore size 0.45 μm (Millipore Corporation, Bedford, MA) were used. Each filter disc with a diameter of 13 mm was cut into halves, which were applied to the upper OS under topical anesthesia (proxymetacaine hydrochloride 0.5% minims; Bausch and Lomb, Surrey, UK). The discs were applied to cover the upper bulbar conjunctiva and adjacent limbus and peripheral cornea, at a site distal to the actual ulcer or abscess, for 20 to 30 seconds and were gently peeled off and stored in RNA later buffer (Qiagen, Crawley, UK) at −80°C until RNA extraction. To obtain the maximum yield of RNA, each area was sampled twice using separate filters applied sequentially to the same site. The second filter allowed us to sample the cells in the deeper layers as well. Cells from both filters were then combined in one tube. Corneal scraping from the ulcer was then performed, and the samples were subjected to microbiologic examination and culture to isolate the causative organisms.

Extraction of RNA and Complementary DNA Synthesis

All samples were stored in stabilization reagent (RNAlater; Qiagen, Crawley, UK) at −80°C centigrade until analysis. Briefly, after the samples were thawed on ice, the filters were transferred to a separate tube with RLT buffer (RNasey minikit; Qiagen). The residual reagent buffer in the original tube was centrifuged for 5 minutes at 21,000g. RLT buffer then was then added to the pellet after the supernatant was discarded. The second tubes were left at room temperature for approximately 10 minutes and then were vortexed for 5 minutes and centrifuged for 30 seconds at 21,000g. The filter was removed from the second tube, and samples in both tubes were combined in one tube. Subsequently, total RNA was isolated and homogenized (RNasey minikit and QIAshredder columns; Qiagen) according to the manufacturer’s instructions. Briefly, the cell lysate was homogenized with a spin column and mixed with 70% ethanol in equal volume (1:1). The mixture was then applied onto spin columns and centrifuged at 21,000g for 15 seconds. The filtrate was discarded, and the spin column was washed with buffer RW1 and then with buffer RPE by centrifugation. Total RNA was eluted in RNase-free water and quantified (Nanodrop Spectrophotometer; Thermo Fisher Scientific, Loughborough, UK). Samples (see Results) that showed poor RNA yield (<45 ng/μL) after heating were excluded from our study. The quality of the total RNA extracted was assessed (Nanodrop Spectrophotometer; Thermo Fisher Scientific). Subsequently, reverse transcription into complementary DNA (cDNA) of 500 ng template RNA was carried out according to the manufacturer’s instructions (Quantitect Reverse Transcription Kit; Qiagen). Total RNA (500 ng) was reverse transcribed into cDNA with a reverse transcription kit and was then mixed with genomic DNA wipeout buffer (Qiagen) on ice, and the volume was adjusted with water. The mixture was incubated at 42°C for 5 minutes and then placed on ice. The reverse transcription enzyme mix (Quantitect reverse transcriptase, Quantitect RT buffer, and RT primer mix; Qiagen) was prepared on ice and mixed with total RNA mixture. The final mixture was then incubated at 42°C for 30 minutes, followed by reverse transcriptase deactivation at 95°C for 3 minutes. Samples were stored at −20°C until further analysis. RNA and cDNA in each IC sample were analyzed individually and processed for RT-PCR.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR analysis was used to measure the relative gene expression of hBD3 and hBD9 according to methods established in our department and reported previously.7 Briefly, preoptimized gene expression assays (TaqMan; Applied Biosystems Europe, Göteborg, Sweden) were used to study each defensin gene, and the endogenous control (hypoxanthineguanine phosphoribosyltransferase [HPRT]). RT-PCR reactions were run on a 96-well plate (Applied Biosystems Europe) in the Mx3005p real-time PCR system (Stratagene, Agilent Technologies, Milton Keynes, UK). Each plate was run with the genes of interest and HPRT in triplicate.

For each sample, the RT-PCR experimental setup was carried out as detailed in the manufacturer’s protocol (Applied Biosystems Europe). Initially, template cDNA was diluted to 1:2 using nuclease-free water to perform the RT-PCR in triplicate. Each reaction was prepared to 20 μL, final reaction volume with 10 μL of 2% gene expression master mix, 1 μL of 20% assay (primers and probe mix; TaqMan; Applied Biosystems), 5 μL diluted cDNA, and 4 μL nuclease-free water. Appropriate negative (nontemplate control and reverse transcriptase control) and positive (human reference RNA; Applied Biosystems Europe) controls were also run in each experiment. Raw data were acquired (MxPro software, version 4.01; Stratagene) on the computer linked to the RT-PCR machine. The data were then transferred to a spreadsheet (Excel; Microsoft, Redmond, WA). Detailed information about the genes used in this study is given in Table 1.

Statistical Analysis

Using the 2−∆∆CT formula, the RT-PCR data were statistically analyzed (Prism, version 5.00 for Windows; GraphPad Software, San Diego, CA) with significance set at P < 0.05. The resultant data were statistically analyzed by the Kruskal-Wallis nonparametric statistical analysis test assuming a non-Gaussian distribution of data. Dunn’s test was also used to compare multiple groups.

RESULTS

In total, 60 IC samples were taken; 24 were from healthy controls, 18 during active infection and 18 after complete resolution. Because control samples were run with each batch of infected samples, the controls were obtained from the same eight healthy volunteers. Eighteen samples from 18 patients were collected during different types of active ocular infection, and 18 more samples were taken from the same patients after complete healing. Of the 18 patients studied, 11 had central ulcers and seven had peripheral ulcers. Five samples (two AK, two Gram-negative [one Klebsiella spp., one P. aeruginosa], and one Gram-positive [S. aureus] bacterial infections) were

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**Table 1. Genes Used in the Study**

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<thead>
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<th>Gene Symbol</th>
<th>Assay (TaqMan) ID</th>
<th>Accession Number</th>
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<tr>
<td>HPRT</td>
<td>4335768F</td>
<td>NM_000194.1</td>
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<td>NM_018661.2</td>
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<td>Hs02760065_g1</td>
<td>DEFB109 (hBD9)</td>
<td>NM_001037380</td>
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excluded from the study because of poor post-treatment RNA yields. Details are provided in Table 2.

Disease Diagnosis
The diagnosis was validated by microbiologic examination in all cases, except for three cases of AK, for which the diagnosis was based on the clinical manifestations and distinct double wall cysts on in vivo confocal microscopy (IVCM) as has been previously reported. Details of the isolated organisms are elaborated in Table 2.

RNA Quality Assessment
The UV light absorbance of the RNA and the contaminants at the 280-, 260-, and the 230-nm wavelengths were assessed using spectrometry (NanoDrop Spectrophotometer; Thermo Fisher Scientific). All samples showed good RNA quality with values >2.0 of the A260/A280 ratio and >1.7 of the A260/A230 ratio.

Gene Analysis
The final RT-PCR analysis was carried out for hBD3 and hBD9 expression in a total of 24 samples from healthy controls, 18 samples from active keratitis, and 13 samples after healing (after excluding five cases with low RNA yield).

Relative Gene Expression of hBD3
hBD3 showed low levels of gene expression in all control subjects with normal eyes. Its expression was upregulated in all types of acute infectious keratitis, was significantly increased compared with controls \( (P < 0.05) \) during acute AK (approximately 120-fold), and returned to almost the level of the control after healing (Fig. 1A). Although it was not statistically significant, there was an overall 4-fold and 10-fold increase in the gene expression of hBD3 during Gram-negative and Gram-positive bacterial keratitis, respectively (Figs. 1B, 1C). This expression showed downregulation after treatment (which was significant in Gram-positive bacterial infection). It almost returned to the initial level of healthy subjects after the healing of Gram-negative BK (Fig. 1B). Interestingly, the gene expression of hBD3 showed further reduction compared with levels in healthy controls after treatment of Gram-positive infection (Fig. 1C).

Relative Gene Expression of hBD9
As shown in Figure 2A, hBD9 expression in patients with acute AK was significantly downregulated (50-fold, \( P < 0.05 \)), which then increased after healing but not to the level observed in healthy controls.

In addition, in Gram-negative bacterial keratitis, hBD9 expression modestly decreased during infection and returned to near control levels after complete healing (Fig. 2B). In Gram-positive bacterial keratitis, there was a significant 5-fold down-regulation in gene expression during active infection \( (P < 0.05) \), which showed a further decrease after healing \( (P < 0.05) \) (Fig. 2C).

DISCUSSION
Key AMPs that are expressed at the human OS include defensins and cathelicidin (LL37). Human beta defensins are cationic host defense peptides with potent antimicrobial activity at physiological concentrations. In addition, they can act as chemoattractants and play a role in cell signaling and wound healing.9,15

The antimicrobial activity of hBD3, but not hBD9, is well established.10,16 Indeed, hBD3 has in vitro bactericidal activity against \( P. aeruginosa \) and \( S. aureus \). This effect is salt independent, which implicates an active in vivo antimicrobial potency in the presence of tears.13,17 In addition, hBD3 has been linked to the initiation of the adaptive immune response through its ability to activate dendritic cells in the cornea and conjunctiva.9

These multiple roles of hBD3 at the OS are consistent with its upregulation in all cases of acute infectious keratitis, both bacterial and \( Acanthamoeba \), demonstrated in this study. When compared with healthy controls, it showed a statistically significant 122-fold increase in acute \( Acanthamoeba \) keratitis, whereas it showed only 4-fold and 10-fold upregulation in Gram-negative and Gram-positive bacterial keratitis, respectively. The expression decreased rapidly in all the studied groups after healing, returning to almost normal level (controls). Such a decrease in AMP gene expression after the initial upregulation during the acute stage of infection has been previously reported for murine fungal keratitis.18

The significant ex vivo upregulation of hBD3 in patients with acute AK is in keeping with our previous report in which we showed that hBD3 gene expression was the most significant among the studied AMPs in samples of human corneal epithelial cells treated in vitro with \( A. castellanii \) trophozoites, showing a 10-fold increase.12 This consistent and significant ex vivo and in vitro upregulation of hBD3 during \( Acanthamoeba \) infections could suggest a specific antiamebic role of this gene on the ocular surface. The potential role of AMPs as antiamebic treatment was previously reported.19–21 Feldman et al. have shown that the in vitro combination of magainin (a host defense AMP) with silver nitrate is active against \( Acanthamoeba \) trophozoites and cysts. Recently, Sacramento et al. reported
that beta defensin induces permeabilization and relative resistance against Acanthamoeba castellanii protease activity, suggesting a possible in vitro antiamebic activity of AMPs.

Another important finding of this study was the significant downregulation of hBD9 gene expression during acute microbial infections (especially AK, a 40-fold decrease). This would support the hypothesis that Acanthamoeba specifically targets this gene to invade the corneal epithelium and bind to the mannose receptors as a first step in AK pathogenesis. The ability of Acanthamoeba to inhibit this gene could be one of the factors related to its virulence. However, the mechanism by which Acanthamoeba dampens the defensin-mediated host defense is unknown. It has been shown that the binding to the mannose glycoprotein on the corneal epithelium by the 136-kDa mannose binding protein of the trophozoites can release several proteases that interfere with the host immune response. Most AMPs are sensitive to proteases, and Acanthamoeba is known to secrete large amounts of proteases.

The degradation role of secretory proteinase produced from Acanthamoeba on host defense proteins (immunoglobulin, interleukin-1) and its participation in Acanthamoeba virulence has been previously described. This study has demonstrated that Acanthamoeba can also suppress defensin expression at the gene level.

Downregulation of human beta defensin genes in response to inflammatory or infectious stimuli has previously been demonstrated. The absence of defensin-2 gene expression in the limbal region of mice with allergic conjunctivitis compared with control has been shown by Ikeda et al. Additionally, Premratanachai et al. have reported the downregulation of hBD9 after in vitro stimulation of gingival keratinocytes with Candida albicans. Similarly, Yuan et al. have demonstrated the initial downregulation of both hBD1 and hBD2 in the murine OS with fungal keratitis. Recently, an initial increase in hBD9 mRNA levels followed by a significant downregulation in response to stimulation provided by pathogen-associated molecular patterns and inflammatory cytokines have also been reported.
Similar to AK, this study demonstrated a modest downregulation of hBD9 during acute Gram-negative infection, but this was significantly downregulated in Gram-positive bacterial keratitis. After healing, gene expression levels returned almost to control levels in the Gram-negative bacterial group but remained downregulated in the Gram-positive bacterial group. This variation in return to normal level could be a time-related phenomenon, or it may suggest continuing subclinical activation at the cellular level. The reduced level of hBD9 during active bacterial keratitis has already been shown. Whether the reduced expression is a cause or an effect of the infection is unclear. It remains to be seen what relationship, if any, exists between this defensin and the cytokines, which are known to be a part of the intracellular signaling cascade.

Conversely, Mohammed et al. found that the HCECs treated with lipopolysaccharide (TLR4 agonist) and flagellin (TLR5 agonist) of the Gram-negative bacteria and Pam3CSK4 (cell wall protein) from Gram-positive bacteria, induced hBD9 mRNA expression. This suggests that hBD9 response can vary in relation to different pathogenic organisms and between in vitro and in vivo conditions, indicating a significant role of AMPs at the OS.

This is the first study to demonstrate the variable gene expression pattern of hBD3 and hBD9 at the OS in infected corneas and after healing compared with healthy controls, and it clearly indicates the importance of AMP-mediated host defense in different OS infections, especially vision-threatening AK. Further studies to explore the potential antiamebic activity of hBD3 and the key role of hBD9 in AK pathogenesis would help our understanding of the pathogenesis of corneal infections.

References