

Expression of Human Beta-Defensins in Intraocular Tissues

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PURPOSE. Defensins are naturally occurring antimicrobial peptides. Recently the authors published evidence of defensin production by the human ocular surface. A study was undertaken to look for intraocular defensins that may account for unexplained antimicrobial activity of intraocular fluids.

METHODS. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on human postmortem ciliary body samples for beta defensins-1 (HBD-1) and beta defensin-2 (HBD-2), and alpha defensins 5 and 6. Induction of defensins by cytokines was analyzed in cultured human ciliary body epithelial (CBE) and retinal pigment epithelial (RPE) cells. Polyclonal antibodies were used to immunoblot aqueous and vitreous to detect HBD-1 and HBD-2 and to estimate their concentration.

RESULTS. RT-PCR revealed constitutive HBD-1 message in ciliary body. HBD-2 and alpha defensin 5 and 6 messages were absent. HBD-2 message was induced by cytokine stimulation of both CBE and RPE cells. Immunoblots of vitreous and aqueous stained positively for HBD-1 but not HBD-2. The estimated aqueous concentration of HBD-1 was less than 16 ng/ml.

CONCLUSIONS. This study demonstrates that HBD-1 is constitutively present in the aqueous and vitreous, probably at sub-bacteriocidal concentrations. HBD-2 was absent from aqueous, but cytokine stimulation studies suggest that it may be generated in response to inflammatory cytokines during infections. HBD-2 has a wider antibacterial spectrum, is 10-fold more potent, and may play a more significant role in antimicrobial defense than HBD-1. The use of defensins therapeutically may be indicated; however, caution is required because defensins also promote cell proliferation and fibrin formation, which are 2 key elements in ocular scarring processes such as proliferative vitreoretinopathy. (*Invest Ophthalmol Vis Sci.* 2000;41:3026-3031)

The eyes, being constantly exposed to environmental pathogens, are particularly vulnerable to infection. The protection of the eyes from microbial attack is of paramount importance, because overwhelming infection of the cornea or intraocular contents has a devastating effect on vision. The eye is provided with multiple defense mechanisms, many of which operate via the tears on the ocular surface and via the aqueous humor inside the eye.

Defensins are naturally occurring peptides that are considered to be among the earliest developed molecular effectors of innate immunity.¹ They are highly conserved molecules present in many animal classes (mammals, birds, insects, and amphibians).² Defensins have a wide range of antimicrobial activity encompassing Gram-positive and Gram-negative bacteria, fungi, and viruses (including human immunodeficiency virus and herpes simplex virus).³⁻⁵ They are also believed to

accelerate wound healing by virtue of their mitogenic effect on epithelial cells and fibroblasts.^{6,7}

Two families of mammalian defensins, alpha and beta, have been described. In humans, alpha defensins are largely present in polymorphonuclear (PMN) cells (alpha defensins 1 through 4)^{8,9} and in the small intestinal Paneth cells (alpha defensins 5 and 6).¹⁰⁻¹² Beta defensins have a wider cellular distribution than alpha defensins, with human beta defensin-1 (HBD-1) expressed by the pancreas, kidney, and respiratory epithelium¹³ and human beta defensin-2 (HBD-2) recently demonstrated in the skin.¹⁴

Recent work has shown that the ocular surface expresses a spectrum of antimicrobial defensins.¹⁵⁻¹⁷ Evidence of HBD-1 and HBD-2 production was found in the cornea and conjunctiva, and HBD-1 (but not HBD-2) was found in the lacrimal gland. Alpha defensins 1 through 4 are also likely to contribute to ocular surface defense, being released by PMN cells within the ocular mucosa and tears.^{15,16} Evidence of alpha defensin 5 and 6 production was not found on the ocular surface.

The physiological blood aqueous and blood retinal barriers ensure a very limited immune cell traffic within the aqueous and vitreous humors. This should make the intraocular contents particularly vulnerable to infection. However, intraocular infection, despite the vast numbers of operations performed (cataract extractions), remains a surprisingly rare event. The postoperative infection rate after cataract surgery ranges from 0.022% to 1.42%,¹⁸⁻²⁰ well below the 7.5% seen in other types of surgery,²¹ even though bacterial contamination

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of intraocular lenses is in the order of 26%²² and contamination of anterior chamber aspirates from eyes that have undergone uncomplicated extracapsular cataract extraction is in the range of 29% to 43%.^{23,24} This led us to postulate that this antimicrobial effect is related to the production of intraocular defensins. This theory is supported by work in other species, which has revealed the presence of a biologically active antibacterial factor in rabbit aqueous humor.²⁵ This factor was found to be a peptide of approximately 8 kDa in size (about double that of defensin). It is possible that the peptide isolated in this rabbit study was a defensin.²⁶ A study was, therefore, undertaken in human eyes to look for intraocular evidence of the known human alpha and beta defensins.

METHODS

Evidence of defensin production by intraocular tissues and the presence of defensin protein in intraocular fluids was sought by a variety of methods, governed partly by limited supplies of anti-HBD-1 and -HBD-2 antibodies and by the difficulty in obtaining pure human intraocular tissue and fluid samples.

Ocular Sample Preparation

Seven samples of human ciliary body were taken from cadaveric eyes donated and consented for transplantation and research and processed within 48 hours postmortem for total RNA purification so as to maximize the yield of viable mRNA.

Sixteen aqueous humor samples were obtained, with informed consent, from patients undergoing cataract surgery, and 7 vitreous humor samples were obtained from patients undergoing pars plana vitrectomy for a variety of pathologies including 5 cases of macular hole, 1 epiretinal membrane, and 1 retinal detachment. Vitreous samples were taken in preference from patients undergoing vitrectomy for pathologies where there is minimal contamination of the vitreous with cells not usually present in the healthy vitreous. Therefore, cases such as extensive retinal detachment, diabetic vitreous hemorrhage, trauma, and uveitis were excluded from the study. The vitreous samples were sonicated on ice to reduce viscosity, before blotting.

An immortalized human nonpigmented ciliary body epithelial cell line (CBE), which has previously been described,²⁷ was kindly donated by Miguel Coca-Prados, PhD (Professor of Research, Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT). Two cell lines of human retinal pigment epithelium (RPE) were established from separate cadaveric donors.

At all times the Helsinki agreement as it relates to vitreous and aqueous donors and the relatives of the donor cadavers was observed and complied with.

Cell Culture and Cytokine Stimulation

The human CBE cell line was cultured in Primaria tissue culture flasks (25 cm² from Falcon; Becton-Dickinson, Oxford, UK) using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). The 2 human RPE cell lines were cultured in growth medium consisting of Eagle's minimal essential medium supplemented with 10% FBS, single-strength nonessential amino acids, 1 mM sodium pyruvate, 4 mM L-glutamine, and gentamicin (100 µg/ml). Cells were grown to

near confluence in a 5% CO₂ incubator at 37°C. The growth medium was replaced with serum-free medium and culture continued for a further 18 hours. Cells were then stimulated for 24 hours with either interleukin (IL)-1β, tumor necrosis factor (TNF)-α, or IL-1β plus TNF-α (PeproTech E.C. Ltd., London, UK) at a final concentration of 10 ng/ml. Control cultures were run simultaneously without cytokine stimulation. Cells were harvested with trypsin EDTA and lysed in buffer containing guanidinium isothiocyanate and β-mercaptoethanol to denature proteins.

Total RNA Purification and cDNA Synthesis

RNA was extracted from the culture cell lysates and from the postmortem ciliary body samples with the RNeasy Total RNA Kit (Qiagen, Chatsworth, CA) as per manufacturer's instructions. cDNA was then synthesized directly from total RNA using the "Ready to go" T-Primed First Strand Kit (Pharmacia Biotech, St. Albans, Herts, UK) as per manufacturer's instructions.

Adequate cDNA synthesis in each sample was confirmed by including primers to amplify Hypoxanthine Phospho Ribosyl Transferase cDNA (HPRT, a low level message produced by all living cells).

Polymerase Chain Reaction

The primers used to amplify HBD-1 and HBD-2 and alpha defensin 5 and 6 cDNA were designed on the basis of published genomic DNA and mRNA sequences (GenBank database accession Nos. X92744, U50930, U50931, Z71389, M96679, M96682, and M97925): HBD-1 primer sequence, 5'-CCC AGT TCC TGA AAT CCT GA-3' and 5'-CAG GTG CCT TGA ATT TTG GT-3'; HBD-2 primer sequence, 5'-CCA GCC ATC AGC CAT GAG GGT-3' and 5'-GGA GCC CTT TCT GAA TCC GCA-3'; alpha defensin-5 primer sequence, 5'-ATC CAC TCC TGC TCT CCC TC-3' and 5'-AGC AGA GTC TGT AGA GGC GG-3'; alpha defensin-6 primer sequence, 5'-AAC CCT CAC CAT CCT CAC TG-3' and 5'-TCT GCA ATG GCA AGT GAA AG-3' (forward primers italicized). All primers were intron-spanning, thus making contaminant products derived from genomic DNA readily detectable.

cDNA (0.5 µl) was added to a mixture (final volume 25 µl) that contained 0.5 µl dNTP mix (10 mM), 0.25 µl 1% Tween-20, 0.5 µl of the 3' and 5' primers for the relevant defensin (20 µM each), 1 µl of ELONGASE Enzyme Mix (*Taq* and *Pyrococcus species* GB-D thermostable DNA polymerases; Life Technologies, Paisley, UK), 5.0 µl 5× Buffer B and 15.75 µl diethyl pyrocarbonate treated water.

Positive control cDNA samples analyzed included 1 sample from cultured corneal epithelial cells (HBD-1 and HBD-2), 1 sample from lacrimal gland (HBD-1 only), and 1 sample from small intestine epithelium (defensins 5 and 6). The cDNA was replaced with water for the negative control reaction. PCR amplification was performed with an automated thermal cycler for 30 cycles using an annealing temperature of 55°C.

Immunoblotting

Seven vitreous and 8 aqueous humor samples were blotted directly onto Immobilon-P polyvinylidene difluoride membranes (Millipore [U.K.] Ltd., Watford, UK). The blots were fixed for 3 hours with 10% buffered formalin, washed briefly in

Tris-buffered saline (TBS; 50 mM Tris + 150 mM NaCl, pH 7.6), and blocked for 30 minutes in 5% nonfat milk powder in TBS. Rabbit anti-HBD-1 serum²⁸ was used as the primary antibody, which was diluted 1:1000 in TBS containing 0.05% Tween-20 (TBST), 5% normal swine serum, and sodium azide. The blots were incubated overnight in the primary antibody at room temperature. The secondary antibody was swine anti-rabbit serum (DAKO Ltd., Cambridge, UK), and the tertiary antibody was rabbit APAAP (alkaline phosphatase anti-alkaline phosphatase; Sigma Chemical, Poole, Dorset, UK). Both antibodies were diluted 1:100 in TBST and incubated for 90 minutes at room temperature; the blots were washed 3 times in TBST between each antibody application. After the final incubation the blots were washed 5 times in TBST and once in water. The blots were placed inside a clear plastic wallet and covered with approximately 1 ml of chemiluminescent substrate (Lumi-Phos 530; Boehringer-Mannheim, Lewes, East Sussex, UK), a piece of Blue Sensitive x-ray film (Genetic Research Instrumentation, Dunmow, Essex, UK) was placed on top and exposed for 1 hour. Positive and negative control samples were cell culture supernatant containing recombinant HBD-1 and culture medium, respectively. Control duplicate blots were processed with the rabbit preimmune serum in place of the rabbit anti-HBD-1 serum for comparison.

Four aqueous humor samples were immunoblotted and stained, as above, using a polyclonal anti-HBD-2 antibody.^{28,29}

In an attempt to establish whether aqueous HBD-1 concentrations correlated with the reported in vitro bacteriocidal HBD-1 concentrations,²⁹ semiquantitative immunoblots were performed to estimate the concentration of HBD-1 in 4 separate aqueous humor samples. Serial dilutions of the 4 separate aqueous humor samples and serial dilutions of pure recombinant HBD-1 peptide²⁸ were blotted and immunostained as described above. The staining patterns of the known peptide standards and the unknown aqueous serial dilutions were compared. An estimate of the HBD-1 concentration in the neat aqueous was then calculated.

RESULTS

Reverse transcription-polymerase chain reaction (RT-PCR) of ciliary body samples for HBD-1 revealed a product at 215 bp in all 7 samples analyzed. Adequate cDNA yields were confirmed by including primers for HPRT in the PCR reactions. Positive control cultured corneal epithelial cells and lacrimal gland also revealed a corresponding product at 215 bp. The negative control reaction with water replacing cDNA showed no PCR products (Fig. 1).

RT-PCR of all the ciliary body samples was negative for HBD-2 and alpha defensins 5 and 6. Positive control cornea and small intestine samples showed appropriate PCR products.

Both RPE and CBE cell culture lines showed inducible (but not constitutive) expression of HBD-2 after stimulation with IL-1 β , but not TNF- α alone (Fig. 2). IL-1 β plus TNF- α showed a more prominent band than IL-1 β alone. Unstimulated control cultures did not show any HBD-2 message (Fig. 2).

All the fresh postmortem ciliary body samples (Fig. 1) and 1 of the cultured RPE cell lines showed constitutive expression of HBD-1 before treatment with cytokines, but this band did not increase in intensity after cytokine stimulation. The other

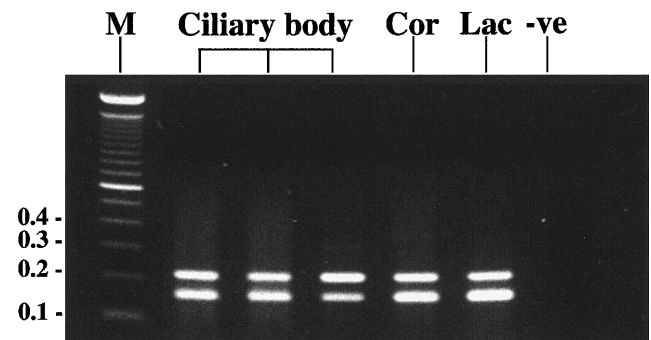


FIGURE 1. Analysis of HBD-1 expression by RT-PCR. RT-PCRs all show an HBD-1 band at 215 bp and a low molecular weight HPRT band confirming adequate cDNA levels in each reaction. M, 100-bp ladder; Ciliary body, postmortem ciliary body samples; Cor, positive control cultured corneal epithelial cells; Lac, positive control lacrimal gland cells; -ve, negative control reaction with H₂O replacing cDNA.

RPE cell line and the CBE cell line did not show HBD-1 expression, either before or after cytokine stimulation.

Dot immunoblots of all 7 vitreous and all 8 aqueous humor samples tested showed positive staining for HBD-1 (Fig. 3). Positive control cell culture supernatant containing recombinant HBD-1 and negative control culture medium were clearly positive and negative, respectively. Also, control duplicate blots processed with the rabbit preimmune serum in place of the rabbit anti-HBD-1 serum were all negative. Dot immunoblots of aqueous for HBD-2 were all negative (data not shown).

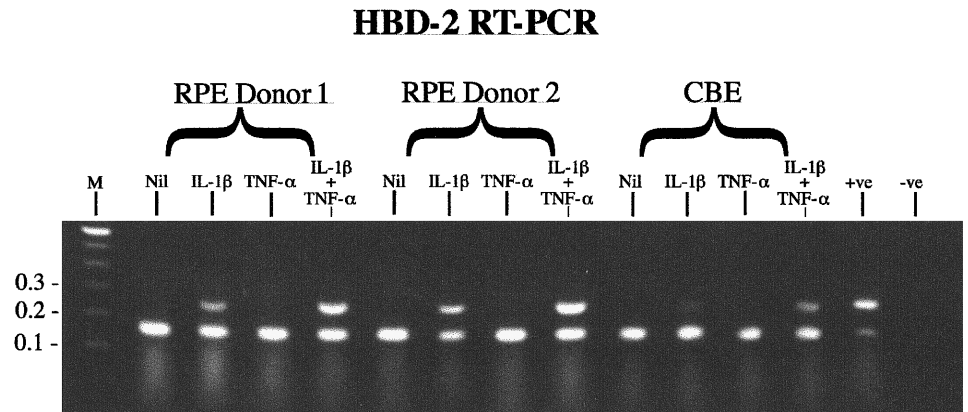
Immunoblotting of serial dilutions of the control pure recombinant HBD-1 peptide showed that even at the lowest serial dilution concentration of 8 ng/ml the staining intensity was greater than that of aqueous diluted 1:2. This suggests that the HBD-1 concentration in aqueous is less than 16 ng/ml (data not shown).

DISCUSSION

Our data suggest that HBD-1 is present at low concentrations (<16 ng/ml) inside the human eye and that the ciliary body and RPE contain the mRNA necessary for its constitutive manufacture and possible secretion into the aqueous humor and vitreous. In vitro luminescence assays with *Escherichia coli* DH5 α have shown that at the salt concentration of 150 mM found in the aqueous, the bacteriocidal concentration of HBD-1 would need to be approximately 12 μ g/ml (salt inhibits the antimicrobial effect of beta defensins).²⁹ We estimated the aqueous HBD-1 concentration to be less than 16 ng/ml, suggesting that the HBD-1 concentration in the aqueous is well below the reported in vitro bacteriocidal concentration.

The sub-bacteriocidal HBD-1 concentration found in our study mirrors results in other tissues.²⁹⁻³¹ However, despite their low concentrations, defensins (including HBD-1) may have significant antimicrobial effects. The high positive charge of defensins may result in their localization to negatively charged ocular mucous and epithelia, and this microenvironmental concentrating effect could elevate the local defensin concentration to effective antimicrobial levels.²⁹ Definite evidence of the function of HBD-1 in the eye remains obscure. Other possible roles could include promotion of ocular surface

FIGURE 2. RT-PCR analysis of HBD-2 expression by cultured RPE and CBE cells, after cytokine stimulation. M, 100-bp ladder; Nil, cells unstimulated by cytokines. Other lanes show cells stimulated with IL-1 β , TNF- α , or both IL-1 β and TNF- α , at a final concentration of 10 ng/ml. The RT-PCRs show an HBD-2 band at 255 bp in RPE and CBE cells stimulated with IL-1 β or IL-1 β and TNF- α . A low molecular weight HPRT band confirms adequate cDNA levels in each reaction. +ve, positive control cultured corneal epithelial cells; -ve, negative control reaction with H₂O replacing cDNA.



healing,^{6,7} dendritic and T-cell chemotaxis,³² synergistic activity with lysozyme and lactoferrin,³⁰ and complement activation.^{33,34}

Evidence of intraocular alpha defensins 1 through 4 production was not sought by RT-PCR in this study, because all tissues containing blood would be positive because of the alpha defensins 1 through 4 derived from PMN cells. Tissues containing masses of PMN cells are known to become saturated with alpha defensins 1 through 4, which even penetrates structures such as the blood-brain barrier.³⁵ Therefore breakdown in the blood-ocular barriers during endophthalmitis or surgery, with the accumulation of intraocular PMN cells would probably lead to elevated intraocular alpha defensin 1 through 4 levels, adding to the innate antimicrobial response. However, beta defensins are not produced by leukocytes, and their de-

tection by RT-PCR in a tissue sample implies their production by the cells of the tissue and not by the blood cells contained within it.

There was no constitutive expression of HBD-1 in the cultured CBE cell line and 1 of the RPE cell lines. The other RPE cell line and all the fresh postmortem ciliary body samples constitutively expressed HBD-1. This may be related to the downregulation of certain genes after multiple cell culture passages.

The data on HBD-2 suggested that HBD-2 is not constitutively expressed inside the human eye, confirmed by both aqueous immunoblots and by ciliary body and RPE RT-PCR. However, cytokine stimulation studies suggested that HBD-2 could be generated inside the eye in response to inflammatory cytokines. Whether the concentration of the HBD-2 produced by this mechanism is bacteriocidal or bacteriostatic remains to be discovered. Further studies of aqueous from infected or inflamed eyes are required to establish whether antimicrobial concentrations of HBD-2 are achieved in eyes challenged by inflammatory stimuli.

The inducibility of HBD-2 but not HBD-1 in cultured CBE and RPE was to be expected because HBD-2 is known to be inducible by inflammatory cytokines in other tissues, whereas HBD-1 is not.^{13,14,29} This may be because the 5' flanking region of the genomic HBD-2 sequence contains consensus binding sequences for nuclear factor- κ B (NF- κ B), which is implicated in transcriptional responses to inflammatory cytokines,³⁶⁻³⁸ suggesting that HBD-2 is transcriptionally regulated by immunologic stimuli such as IL-1 β . However, the HBD-1 genomic sequence lacks the transcription factor regulatory elements for NF- κ B, making it likely that HBD-1 is not transcriptionally regulated by inflammatory agents.^{28,29,39-41}

The inducibility of HBD-2, and the fact that it is approximately 10-fold more potent than HBD-1²⁹ with a wider antibacterial spectrum, makes HBD-2 a stronger candidate for antimicrobial defense in the eye, despite the high salt content of the ocular fluids. Cytokine-induced HBD-2 production may be more important for antimicrobial defense than the constitutive HBD-1 production.

In the future purified or recombinant defensins may be useful therapeutic agents in the eye because they could be applied directly to the site of infection on the ocular surface or injected into the aqueous and vitreous during infections. Of particular clinical interest is that defensins have a broad spec-

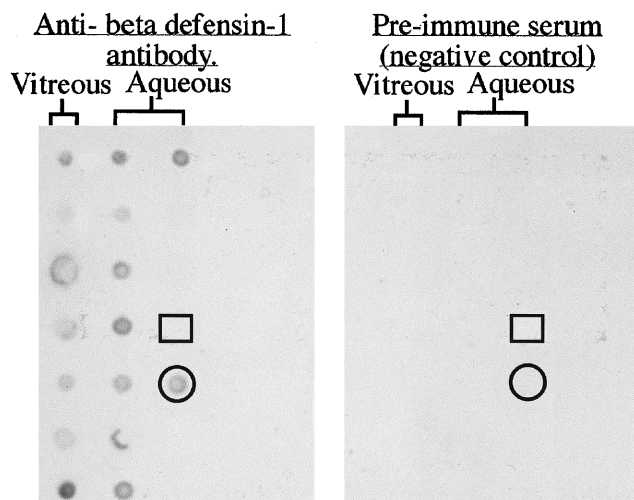


FIGURE 3. Dot immunoblots of 7 vitreous and 8 aqueous humor samples showing positive staining for HBD-1 with rabbit anti-beta defensin-1 antibody. The sites of application of the positive control recombinant HBD-1 samples are indicated by *circles*. The sites of application of the negative control culture medium samples are indicated by *boxes*. In the anti-beta defensin-1 antibody blot the positive and negative controls are clearly positive and negative, respectively. Control duplicate blots of aqueous, vitreous, and control samples, processed with the rabbit preimmune serum in place of the rabbit anti-beta defensin-1 antibody are all negative. APAAP plus chemiluminescent substrate.

trum of activity, by their unique mechanism of action may be less susceptible to the development of bacterial resistance, and appear to be nonantigenic. This contrasts with most current antibiotics, which have a comparatively limited spectrum of activity (e.g., Gram-negative bacteria only), interfere with healing due to ocular toxicity, and sometimes produce allergic reactions. However, cautious work in the area of the clinical use of defensins is required, because defensins could also have negative effects inside the eye. For example, by promoting cellular proliferation^{6,7} and fibrin formation^{42,43} defensins could accelerate 2 key events in ocular scarring processes such as proliferative vitreoretinopathy (PVR).^{44,45} Interestingly, the inflammatory cytokines that stimulated RPE cells to produce HBD-2 in our study, and RPE cells themselves, have also been found in the vitreous and proliferative membranes of eyes with PVR.⁴⁶⁻⁴⁸

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