Defensin Expression by the Cornea: Multiple Signalling Pathways Mediate IL-1β Stimulation of hBD-2 Expression by Human Corneal Epithelial Cells

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Purpose. To investigate the expression of human β-defensins (hBDs) by human corneal epithelium and determine the effects of proinflammatory cytokines on expression of human β-defensin (hBD)-2 by human corneal epithelial cells (HCECs) in culture.

Methods. RNA was extracted from corneal epithelial cells scraped from cadaveric corneas and from cultured HCECs, and RT-PCR was performed to detect hBD-1, -2, and -3 mRNA. To study the effects of proinflammatory cytokines on expression of defensin, HCECs were cultured and then exposed to interleukin (IL)-1β or tumor necrosis factor (TNF)-α for up to 36 hours, with a range of concentrations (0.01–100 ng/mL). In some experiments, cells were pretreated with various cell signaling pathway inhibitors before the addition of IL-1β. At the end of the incubations, the cells were harvested for RT-PCR and the culture media collected for the detection by immunoblot analysis of secreted defensin peptide.

Results. All epithelial tissue collected from cadaveric corneas expressed mRNA for hBD-1. hBD-2 was detectable in two of eight donors corneas, whereas hBD-3 was detected in five. All primary cultures of HCECs expressed hBD-1 and -3. A faint band for hBD-2 was detectable in three of eight cultures. Cultures of simian virus (SV)40-transformed HCECs always expressed hBD-1 and -3, but did not express hBD-2 under control conditions. IL-1β and TNFα each stimulated the expression of hBD-2 in HCECs and were more effective in combination than alone. The effects of IL-1β were concentration- (maximal at 10 ng/mL) and time-dependent (maximal at 12 hours and 24 hours for hBD-2 mRNA expression and protein secretion, respectively). The upregulation of hBD-2 mRNA persisted for at least 24 hours after removal of IL-1β. The NFκB inhibitors pyrrolidinedithiocarbamate (PDTC; 100 μM), caffeic acid phenethyl ester (CAPE; 90 μM), and MG-132 (25 μM), blocked IL-1β–stimulated expression of hBD-2. The p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 (5 μM) and the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125 (25 μM) partially blocked (by 47% and 59%, respectively) the effect of IL-1β. However, PD98059, an ERK inhibitor, had no effect. Genistein (50 μM) and dexamethasone (1 μM) also partially blocked (by 26% and 28%, respectively) the effect of IL-1β.

Conclusions. Human corneal epithelium expresses hBD-1 and -3. hBD-2 is not typically present, but its expression can be stimulated by proinflammatory cytokines such as IL-1β, acting through mitogen-activated protein (MAP) kinase and nuclear factor (NF)-κB pathways. Because IL-1 is known to be increased at the ocular surface after injury, the current observations provide a mechanism to explain the previous finding that hBD-2 is upregulated in regenerating corneal epithelium. Cytokine stimulation of hBD-2 expression most likely provides additional protection against infection and raises the possibility that this defensin in particular may be involved in the wound-healing response, per se. (*Invest Ophthalmol Vis Sci.* 2003;44:1859–1865) DOI:10.1167/iovs.02-0787

Defensins are cationic antimicrobial peptides characterized by the presence of six cysteine residues linked to form three disulfide bridges. Two forms of human defensin, α and β, are recognized, depending on the location and connectivity of the cysteines. α-Defensins have been localized to neutrophils and Paneth cells of the intestine, whereas β-defensins are expressed by many epithelia.[1,2] To date, six human β-defensins (hBD-1 through -6), have been identified.[3–8] hBD-1 is constitutively expressed, whereas hBD-2 and -3 are inducible by cytokines and bacterial products. hBD-4 appears to have a more limited distribution than hBD-1,- 2, or -3; In addition, its expression can be upregulated by bacterial infection but not by inflammatory factors that upregulate hBD-2 and -3.[7] The most recently identified family members are hBD-5 and -6, which have been localized to the epididymis.[8]

Defensins have a broad spectrum of antimicrobial activity, being effective against many Gram-positive and -negative bacteria, some fungi, and enveloped viruses.[1,2] The antimicrobial activity of defensins has been attributed to permeabilization of microbial membranes and subsequent release of cellular contents. Exactly how this is achieved has yet to be determined, but two models have been suggested: one in which defensin monomers assemble to form pores within the microbial membrane[9] and a second in which defensins disrupt the membrane by electrostatic interactions with the polar head groups of the bilayer.[10] In addition to their antimicrobial effects, defensins have been shown to modulate a variety of cellular activities including, chemotaxis of T cells, dendritic cells,[11,12] and monocytes[13]; stimulation of epithelial cell and fibroblast proliferation[14]; stimulation of cytokine production[15,16]; and stimulation of histamine release from mast cells.[17,18] These effects, which typically occur at defensin concentrations much lower than those required for antimicrobial activity, suggest that defensins not only participate in the innate immune response system by virtue of their ability to kill microbes, but also that they can act as regulatory factors.

Previous studies have shown that human corneal epithelial cells (HCECs) express hBD-1 constitutively and that the expression of hBD-2 is variable[19,20] and can be upregulated by bac-

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terial products.[21] Work in our laboratory has shown that whereas hBD-1 is constitutively expressed, expression of hBD-2 is upregulated during reepithelialization of wounded corneas in organ culture.[22] We have speculated that the upregulation of hBD-2 after injury may give added protection to the ocular surface at a time when it is particularly vulnerable to infection and that hBD-2 may, through its nonmicrobicidal activities, contribute to the wound-healing process, per se.

In the present study, we examined the expression of the recently identified hBD-3, by HCECs. Also we began to investigate the mechanism underlying upregulation of hBD-2 during wound healing. Studies by others have shown that proinflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α increase expression of hBD-2 in a variety of cell types.[23–28] Proinflammatory cytokines are known to increase during wound healing (Ayliffe W, Espaillat A, Foster CS, Lee SJ, ARVO Abstract 3325, 1993)[29–31] thus providing a stimulus by which hBD-2 may be upregulated after injury. Therefore, here we have investigated the effect of exposure to proinflammatory cytokines on expression hBD-2 by corneal epithelial cells.

Materials and Methods

Chemicals

Unless otherwise stated, tissue culture media and associated reagents were obtained from Invitrogen (Carlsbad, CA). Recombinant human cytokines IL-1α, IL-1β, and TNFα were obtained from R&D Systems (Minneapolis, MN). Caffeic acid phenethyl ester (CAPE), SP600125, and PD98059 were from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO). Antibodies to hBD-1 and -2 were a gift from Tomas Ganz (University of California, Los Angeles). Enzyme-conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA).

Corneal Tissue

Human corneas unsuitable for transplantation were obtained from eye banks within 3 to 5 days of death. The tissue was obtained in accordance with the guidelines of the Declaration of Helsinki regarding research involving human tissue. The average donor age was 68 $\pm$ 2 years. Corneas were used for cell culture (described later), or epithelial tissue was collected by scraping and then snap frozen in liquid nitrogen and stored at -80°C until RNA extraction and analysis of defensin expression by RT-PCR.

Cell Culture

Primary cultures of HCECs were prepared from single or pairs of corneas using a method based on that described by Maldonado and Furcht.[32] Briefly, after removal of the sclera, the cornea was placed epithelial side down in a Petri dish containing dispase II diluted to a final concentration of 1.2 U/mL in serum-free medium (KGM; Clonetics, Walkersville, MD, or EpiLife; Cascade Biologics, Portland, OR). After a 3-hour incubation at 37°C, the cornea was transferred to a dish containing a small amount of DMEM supplemented with 10% FBS. The epithelial layer was gently scraped free with a no. 15 scalpel blade. The cell suspension was then transferred to a 15-mL tube and centrifuged at 1000*g* for 2 minutes. The supernatant was aspirated and 1 mL of serum-free medium added to the pellet. The cells were resuspended by passing several times through a 22-gauge needle fitted to a 1-mL syringe. The cell suspension was transferred to a flask coated with a mixture of fibronectin and collagen (FNC; BRFF, Baltimore, MD) containing 5 mL of serum-free medium.

SV40-transformed HCECs[33] were a gift from Kaoru Araki-Sasaki (Tane Memorial Eye Hospital, Osaka, Japan). The cells were maintained in SHEM (DMEM-Ham's F12, 1:1 by volume) supplemented with 10% FBS and gentamicin (30 μg/mL).

All cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. When confluent, cells were passed using standard trypsin-EDTA methods. Primary cultured HCECs of passages 1 to 2 and SV40-transformed HCECs of passages 25 to 35 were used in the experiments.

Cytokine Treatment of HCECs

Cells were trypsinized from their growth flasks and passed into six-well culture plates or 25 cm^2 flasks. When SV40-transformed HCECs were 80% confluent, the growth medium was aspirated, the cells were washed twice with PBS, fresh serum-free SHEM was added, and the cells were left to incubate overnight. This procedure was omitted for the primary cultured cells, because they were maintained in serum-free medium.

In initial experiments, cells were exposed to 10 ng/mL IL-1α, IL-1β, TNFα, or combinations of IL-1β and TNFα for 24 hours. The culture media were collected for immunoblot analysis, and the cells were collected for RT-PCR. These studies established that IL-1α and -1β had identical effects on defensin expression and that IL-1 was more effective than TNFα. A more detailed investigation of the effects of IL-1β on defensin expression was then conducted. HCECs were treated with 10 ng/mL IL-1β for 3 to 36 hours or with 0.01 to 100 ng/mL IL-1β for 24 hours. At the end of the experiments, the cells were collected for RT-PCR and the culture media for immunoblot analysis. In a washout experiment, SV40-transformed HCECs were treated with 10 ng/mL IL-1β for 6 hours, the cells were washed three times with PBS, and fresh serum-free medium added. Cells were collected for RT-PCR at 12, 24, 36, and 48 hours after removal of the IL-1β. In all experiments control cells were treated with serum-free medium only.

To study the role of intracellular signaling pathways, various inhibitors were added to the cells 30 minutes before the addition of 10 ng/mL IL-1β. Three hours after addition of the cytokine, the cells were collected for RT-PCR. In all experiments, control cells were treated with serum-free medium containing 0.1% dimethyl sulfoxide (DMSO), the vehicle for diluting most of the inhibitors. The inhibitors and their final concentrations used were 50 μM genistein, 1 μM dexamethasone, 5 μM SB203580, 25 μM SP600125, 10 μM PD98059, 100 μM pyrrolidinedithiocarbamate (PDTC), 25 μM MG132, and 90 μM CAPE.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from samples using a kit (RNeasy; Qiagen, Valencia, CA). RNA at 250 ng was used in each reaction in one-step RT-PCR (Superscript I kit; Invitrogen). Reverse transcription was performed at 50°C for 60 minutes. After denaturation of the enzyme (94°C, 5 minutes) amplification of cDNA was performed for 40 cycles as follows: denaturation 94°C for 50 seconds, annealing 62°C for 30 seconds, and extension 72°C for 1 minute. The constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to ensure that equal amounts of RNA were added. All primers (Invitrogen) were used at a final concentration of 25 pmol/reaction. The primer sequences and product sizes were as follows: GAPDH[21] forward 5′-GTGAAGGTCGGAGTCAACGGATTT-3′, reverse 5′-CACAGTCTTCTGGGTGGCAGTGAT-3′, 555 bp; hBD-1[19] forward 5′-CCCAGTTCCTGAAATCCTGA-3′, reverse 5′-CAGGTGCCTTGAATTTTGGT-3′, 215 bp; hBD-2[21] forward 5′-CCAGCCATCAGCCATGAGGGT-3′, reverse 5′-GGAGCCCTTTCTGAATCCGCA-3′, 257 bp; and hBD-3[5] forward 5′-AGCCTAGCAGCTATGAGGATC-3′, reverse 5′-CTTCGGCAGCATTTTGCGCCA-3′, 206 bp.

Products generated with these primers were sequenced (Seqwright, Houston, TX) to confirm their identities. The RT-PCR products were analyzed by gel electrophoresis on 1.3% agarose gels stained with ethidium bromide. A digital image of the gel was captured using a gel documentation system (AlphaImager; Alpha Innotech, San Leandro, CA).

Immunoblot Analysis

Culture supernatant was collected at the end of the incubation, centrifuged to remove any cells, and immediately frozen and stored at

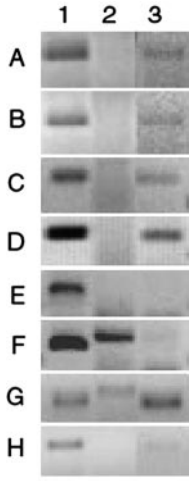

FIGURE 1. Defensin expression by human corneal epithelium. RT-PCR for three human β-defensins was performed using RNA isolated from epithelium scraped from eight cadaveric corneas (**A**–**H**). All epithelial tissue expressed mRNA for hBD-1. Two samples (**F**, **G**) showed significant expression of hBD2 and five (**A**–**D**, **G**) of hBD-3. *Lane 1*: hBD-1; *lane 2*: hBD-2; *lane 3*: hBD-3.

−80°C until analysis. For immunoblot analysis, 100 μL of culture supernatant was directly applied to a membrane (Immobilon P; Amersham Biosciences, Piscataway, NJ) by vacuum, using a dot-blot apparatus. In addition, 100 μL of either recombinant human (r)hBD-1 peptide (gift of Tomas Ganz) or synthetic hBD-2 peptide (Peninsula Laboratories, Belmont, CA) at concentrations of 32, 64, 128, and 256 ng/mL was applied to the membrane as peptide standards. The membrane was fixed by incubating with 10% formalin for 2 hours at room temperature. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline (TBS) containing 5% nonfat powdered milk for 30 minutes at room temperature. The membrane was then incubated overnight at room temperature with either rabbit anti-human hBD-1 or rabbit anti-human hBD-2 diluted 1:1000 in TBS containing 5% nonfat powdered milk, 5% goat serum, 0.05% Tween 20, and 0.02% sodium azide. After the membrane was washed, it was incubated for 1 hour at room temperature with a second antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:5000 with 5% nonfat powdered milk. Immunoreactivity was visualized with a peroxidase substrate kit (TMB; Vector, Burlingame, CA), and the results were documented by capturing a digital image of the stained membrane with the gel documentation system (AlphaImager; Alpha Innotech). A standard curve was generated by plotting the density of the peptide standard versus concentration and was used to determine the amount of hBD-1 or -2 peptide in the samples.

RESULTS

Expression of β-Defensins by HCECs

Figure 1 shows the typical pattern of defensin expression by epithelium scraped from the human cornea. Of samples collected from eight different donors, all expressed hBD-1 mRNA. Two of the eight corneas expressed hBD-2 mRNA, and five expressed hBD-3 mRNA. All cultures of primary and SV40-transformed HCECs tested (data not shown) expressed mRNAs for hBD-1 and -3. Five of eight cultures of primary HCECs did not express mRNA for hBD-2, whereas in the remaining three a faint band was detected. mRNA for hBD-2 was not detected in any cultures of SV40-transformed HCECs. Thus, HCECs typically express mRNA for hBD-1 and -3 but not hBD-2.

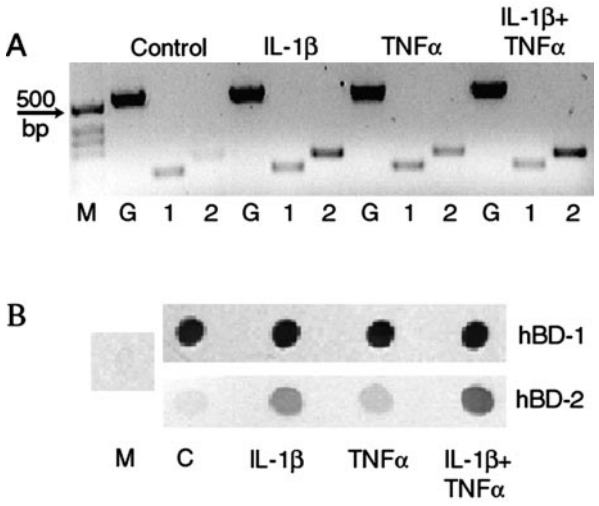

FIGURE 2. Effect of IL-1β and TNFα on defensin expression by primary cultured HCECs. RT-PCR was used to study the profile of defensin expression by primary cultured HCECs. (**A**) RT-PCR and (**B**) immunoblot analysis showing the effects on expression of hBD-1 and -2 of treating the cells with 10 ng/mL IL-1β, TNFα, or a combination of both for 24 hours. Data are representative results from one experiment repeated three times. (**A**) *Lane M*, markers; *lane G*, GAPDH; *lane 1*, hBD-1; *lane 2*: hBD-2. (**B**) M, culture media that had not been in contact with cells; C, culture supernatant from control cells.

Effect of Proinflammatory Cytokines on Defensin Expression by HCECs

IL-1 and TNFα. Although absent (or expressed only in small amounts) in untreated primary cultured HCECs, hBD-2 mRNA was readily detectable after treatment with IL-1α (not shown), IL-1β, TNFα, or a combination of IL-1β and TNFα (Fig. 2A). The combination of both cytokines was more effective than either cytokine alone. Semiquantitative analysis compared with expression of GAPDH indicated that there was no significant effect of cytokine treatment on expression of hBD-1 (hBD-1:GAPDH band density ratios were 0.183, 0.186, 0.184, and 0.175 for control, IL-1β, TNFα, and IL-1β+TNFα treated cells, respectively). The effects of IL-1β and TNFα on the expression of hBD-3 were also examined. The results (data not shown) were equivocal, with three of seven experiments indicating an increase in expression. Immunostaining (Fig. 2B) of the culture media by dot blot indicated that both hBD-1 and -2 were secreted into the culture media. The results were the same as for mRNA, with secretion of hBD-1 being unchanged by exposure to cytokines, whereas secretion of hBD-2 was upregulated. Table 1 shows the amount of each defensin se-

TABLE 1. Amount of hBD-1 and -2 Secreted into Culture Media by HCECs over 24 Hours under Different Treatment Conditions

Defensin (ng/mL)	Control	IL-1β	TNFα	IL-1β + TNFα
hBD-1	423 ± 59	437 ± 49	447 ± 56	461 ± 58
hBD-2	5.3 ± 4.6	77.1 ± 15.9*	29.2 ± 5.1	161.3 ± 3.0*

Data shown are mean ± SD of three experiments. One-way ANOVA (post hoc multiple comparison: Scheffé) was used to test for differences between the amount of defensin secreted by control and cytokine-treated cells. Although increased secretion was observed with TNFα treatment, it did not reach statistical significance.

* $P \leq 0.01$ compared with the control. The combined treatment of IL-1β and TNFα caused a significant ($P \leq 0.001$) increase in hBD-2 secretion compared with either cytokine alone.

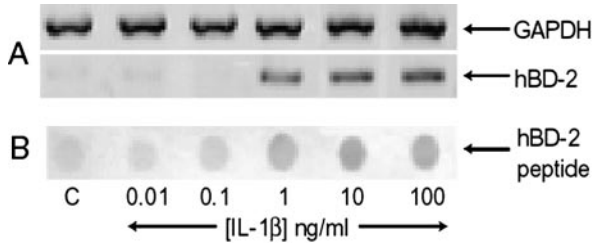

FIGURE 3. Effect of different concentrations of IL-1β on hBD-2 expression. HCECs were incubated with serum-free medium alone (**C**) or with media containing increasing concentrations of IL-1β for 24 hours. The expression of hBD-2 by the cells was determined by RT-PCR (**A**). The secretion of hBD-2 peptide in to the media was detected by immunoblot analysis (**B**). The data shown are from a representative experiment repeated three times with SV40 HCECs. Identical results were observed when primary cultured cells were used.

creted under the different conditions, as calculated from standard curves generated using known amounts of defensin peptide.

IL-1β–Stimulated Upregulation of hBD-2 . As shown in Figure 3, IL-1β stimulated expression of hBD-2 mRNA and protein secretion in SV40-transformed HCECs in a concentration-dependent manner. Expression of hBD-2 mRNA was detectable after a 24-hour incubation with as little as 1 ng/mL IL-1β (Fig. 3A). Semiquantitative analysis in comparison with GAPDH indicated that the maximum was obtained at 10 ng/mL. Immunoblot analysis studies showed that the secretion of hBD-2 peptide into the culture medium was only readily detectable after treatment with at least 1 ng/mL IL-1β (Fig. 3B). Figure 4 shows the time course of upregulation of hBD-2 mRNA expression and peptide secretion. hBD-2 mRNA was detectable after a 3-hour incubation with IL-1β, and semiquantitative analysis in comparison with GAPDH showed the effect reached maximum at 12 hours of incubation (Fig. 4A). As expected, there was a delay in the secretion of hBD-2 peptide into the culture medium compared with the time course of mRNA upregulation (Fig. 4B). Secretion of hBD-2 peptide could be detected in the media after 12 hours of incubation with 10 ng/mL IL-1β and was at its maximum at 24 hours. Immunoreactivity for hBD-2 was still present at 36 hours.

Expression of hBD-2 mRNA after Removal of IL-1β. To determine how long upregulation of hBD-2 mRNA is maintained after exposure to IL-1β, experiments were conducted in which SV40-transformed HCECs were exposed to IL-1β for 6

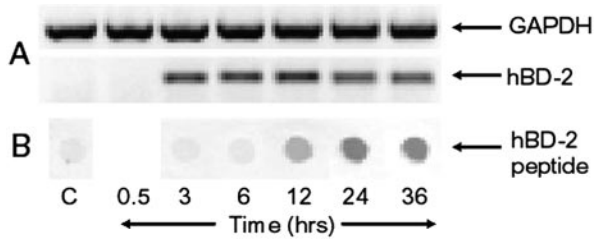

FIGURE 4. Effect of different lengths of incubation with IL-1β on expression of hBD-2. HCECs were incubated with 10 ng/mL IL-1β for 30 minutes to 36 hours. The expression of hBD-2 mRNA was determined by RT-PCR (**A**). The secretion of hBD-2 peptide into the medium was detected by immunoblot analysis (**B**). Untreated control cultures were included at each time point. There were no differences between any of these samples, and therefore only the control for the first time point (30 minutes for RT-PCR, 3 hours for immunoblot analysis) are shown (**C**). The data are from a representative experiment repeated three times using SV40-transformed HCECs. Identical results were observed when primary cultured cells were used.

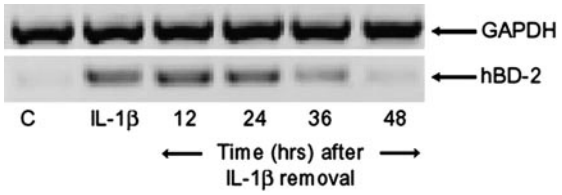

FIGURE 5. Effect of removing IL-1β after 6 hours of incubation on expression of hBD-2. SV40-transformed HCECs were incubated with 10 ng/mL IL-1β for 6 hours, at which point the cells were washed to remove the IL-1β. RT-PCR was used to determine hBD-2 mRNA expression 12, 24, 36, and 48 hours after the removal of IL-1β. Untreated control cells were included at every time point. There were no differences between any of these samples and therefore only the control collected at 6 hours is shown (**C**). IL-1β, cells treated with IL-1β for 6 hours. The data are from a representative experiment repeated twice.

hours, after which the cytokine was removed by washing. As shown in Figure 5, hBD-2 mRNA was readily detected 12 and 24 hours after removal of the cytokine. After 24 hours, the expression of hBD-2 decreased, but some mRNA was still detectable 36 hours after removal of IL-1β.

Inhibitors of Intracellular Signaling Pathways. To investigate the intracellular signaling pathways involved in mediating stimulation by IL-1β of hBD-2 expression, experiments were conducted in the presence of inhibitors of various pathways. The effects of the nuclear factor (NF)-κB inhibitors PDTC, MG132, and CAPE were most profound, with each blocking the effects of IL-1β on expression of hBD-2 mRNA (Fig. 6A). Semiquantitative analysis compared with GAPDH indicated that PDTC, MG132, and CAPE inhibited the effect of IL-1β by 87%, 83%, and 86%, respectively (mean result of three to four experiments). Inhibitors of the three mitogen-activated protein (MAP) kinase pathways were also tested (Fig. 6B). SB203580 (a p38MAP kinase inhibitor) and SP600125 (a c-Jun NH2-terminal kinase [JNK] inhibitor) both partially blocked

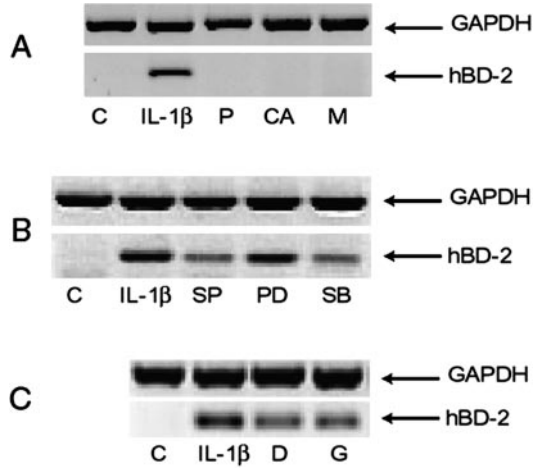

FIGURE 6. Effect of various intracellular signaling inhibitors on IL-1β stimulation of hBD-2 expression. Inhibitors were added 30 minutes before the addition of 10 ng/mL IL-1β to determine which pathway(s) is involved in IL-1β stimulation of expression of hBD-2 mRNA in SV40-transformed HCECs. (**A**) Effects of preincubation with inhibitors of NFκB. (**B**) Effects of inhibitors of the three MAP kinase pathways. SB, SB203580 (p38MAP kinase inhibitor); SP, SP600125 (JNK inhibitor); PD PD98059 (ERK inhibitor). (**C**) The effects of the glucocorticoid dexamethasone (*lane D*) and the tyrosine kinase inhibitor genistein (*lane G*). (**A–C**) *Lane C*: untreated control cells. The data are from a representative experiment repeated three to five times for each inhibitor. Incubation of cells with any of the inhibitors alone did not induce the expression of hBD-2 (not shown).

IL-1β–stimulated hBD-2 expression resulting in 47% and 59% inhibition, respectively (mean result of four to five experiments). When a combination of both of these inhibitors was used (not shown) attenuation of IL-1β–stimulated hBD-2 expression reached 72% (mean result of two experiments). PD98059, an ERK inhibitor, inhibited the effect of IL-1β by only 6.5% (mean result of three experiments). Genistein (a tyrosine kinase inhibitor) and dexamethasone (an anti-inflammatory glucocorticoid) were both partially effective (Fig. 6C), attenuating the effect of IL-1β by 26% and 28%, respectively (mean result of four experiments). No additional effect of dexamethasone was observed if the incubation time before addition of IL-1β was extended to 24 hours (data not shown).

Discussion

We used RT-PCR to study the expression of three human β-defensins by HCECs. As may be expected, some variability between samples obtained from different donors was observed, but the overall pattern in epithelial tissue scraped from cadaveric corneas and in primary cultured and SV40-transformed HCECs was that hBD-1 and -3 mRNA are present but hBD-2 mRNA is absent. A number of studies have previously reported that hBD-1 is constitutively expressed by the corneal epithelium, whereas hBD-2 is variably expressed if at all,[19–21] findings that are in keeping with ours. Before our study, the expression of hBD-3 has not been studied in the corneal epithelium. We conclude that hBD-1 and -3 provide a baseline defense to protect the cornea from infection. It is notable that hBD-3 is the only human β-defensin shown to have antimicrobial activity that is insensitive to salt concentration,[5]; therefore, it may be particularly beneficial at the ocular surface, where the salt content of tears may interfere with the activity of the other salt-sensitive defensins.

A previous study in our laboratory showed that although human corneal epithelium does not typically express hBD-2, this defensin can be readily detected in regenerating epithelium in an in vitro culture model of wound healing.[22] We have recently confirmed the importance of this in vitro observation by showing that rBD-2, the rat homologue of hBD-2,[34] is upregulated during wound healing in vivo (McDermott AM, Proske RJ, Woo HM, Campbell S, Murphy CJ, ARVO Abstract 4198, 2002). Because previous studies in other cell types had shown that expression of hBD-2 can be stimulated by proinflammatory cytokines,[23–28] we reasoned that the increase in levels of such cytokines at the ocular surface after injury may be responsible for stimulating expression of hBD-2 during corneal epithelial regeneration in our culture model. To determine whether proinflammatory cytokines can stimulate the expression of hBD-2 in HCECs we exposed cells in culture to IL-1, TNFα, or a combination of both. Our results showed that both IL-1 and TNFα induced the expression of hBD-2 by HCECs, and that the combination of both cytokines was more effective than either alone. In keeping with the observations of others, IL-1 and TNFα had no effect on the expression of hBD-1.[24,26,27] Cytokine-mediated upregulation of hBD-3 expression has been observed in some epithelial cells.[5,6] In our experiments the effects of cytokine treatment on hBD-3 expression were variable, with significant upregulation being seen in only three of seven cultures. Quantitative RT-PCR studies will be useful in clarifying the effects of cytokines on HCEC hBD-3 expression.

The effects of IL-1β on HCEC expression of hBD-2 were rapid (within 3 hours) and sustained (36 hours). Prolonged upregulation of hBD-2 mRNA expression has been observed in some cell types,[23,28] but in others the expression is more transient, returning to baseline by 8 hours.[24] This suggests that some cells have a greater capacity for producing hBD-2 and so would be better protected against infection. We observed that only a short exposure (6 hours) to IL-1β was needed to upregulate hBD-2 mRNA for a further 36 hours. Thus, even a relatively brief exposure of the cornea to inflammatory cytokines is sufficient to upregulate hBD-2 for a sustained period, thus ensuring protection after the initial inflammatory response subsides. The levels of IL-1 are elevated for up to 1 week after injury.[29] We would expect levels of hBD-2 to persist for as long as IL-1 is elevated and, based on our current data, at least 24 to 36 hours longer. The longest time point examined in our in vitro wounding model[22] was 48 hours, however, in an in vivo model we observed that mRNA for rBD-2 was still upregulated 1 week after injury (McDermott AM, Proske RJ, Woo HM, Campbell S, Murphy CJ, ARVO Abstract 4198, 2002).

We found that the amount of hBD-2 secreted from cytokine-treated HCECs over a 24 hour period was markedly less than the amount of hBD-1 secreted. Although, owing to differences in sample preparation and techniques, it is not possible to compare results between studies directly, O'Neil et al.[26,27] also observed that intestinal epithelial cells produced more hBD-1 than -2. It has been reported that the antimicrobial activity of hBD-2 is typically 10 times greater than that of hBD-1[23]; therefore, it is possible that the differences in the amounts of defensins produced by HCECs relates to their relative biological activity. Based on the incubation volume (3 mL) used in our experiments, we can say that 1.4 μg of hBD-1 and 0.5 μg of hBD-2 were secreted per flask of approximately 4 million cells. The number of epithelial cells present on the cornea is estimated to be 3 to 4 million (Bergmanson JPG, personal communication, June 2002), and therefore, theoretically at least, the same level of defensin secretion could be achieved in vivo. However, the constant flushing of the ocular surface by tears probably means that the actual concentration of defensins is low—probably significantly below the micromolar concentrations required for antimicrobial activity in vitro.[4–7,35] In other studies in which lower-than-expected defensin concentrations were observed,[23,25] the authors rationalized their findings by suggesting that localization of the peptides to nearby surfaces would have a concentrating effect, thus increasing their concentration to effective levels. This may also be the case in the corneal epithelium. Additionally, defensins are known to have synergistic or additive effects with other antimicrobial peptides and endogenous "antibiotics" such as lysozyme.[7,36–38] Thus, the micromolar concentrations of defensins needed to attain antimicrobial activity in vitro, may not be required at the ocular surface in vivo, because defensins may synergize with other peptides that are present such as LL-37 (Huang L, McDermott AM, unpublished observation, 2002) or CAP-37.[39]

Cloning of the hBD-2 gene has revealed that it is unique among defensin genes, having three binding sites for the transcription factor NFκB.[40] Consensus binding sites for several other transcription factors, including activator protein (AP)-1 and nuclear factor for IL-6 are also present.[41,42] This suggests that hBD-2 gene expression may be modulated via a number of different pathways. Indeed, whereas some studies support the involvement of NFκB in both cytokine and bacteria stimulated upregulation of hBD-2 expression[26,43–45] (Maltseva I, McNamara N, Fleiszig SMJ, Basbaum C, ARVO Abstract 3195, 2002) there is evidence in other cells that NFκB is not involved.[46] As reviewed by Martin and Wesche,[47] binding of IL-1 to its receptor results in the formation of a "signalosome" composed of MyD88/IRAK and TRAF6, which triggers activation of TAK1, which in turn may activate the NFκB pathway or MAP kinase pathways that lead to activation of AP-1. To determine the signal transduction pathway involved in mediating IL-1β stimulation of hBD-2 expression in HCECs we used known inhibitors to block various pathways. Inhibitors of

NFκB activation (PDTC, CAPE, and MG132) completely blocked the effects of IL-1β, indicating that activation of this transcription factor is important for upregulation of hBD-2 in HCECs. We also observed that SB203580 (a p38MAP kinase inhibitor) and SP600125 (a JNK inhibitor) both partially inhibited the effect of IL-1β on hBD-2 expression. In contrast, PD98059, an inhibitor of the third MAP kinase pathway, the extracellular signal-regulated kinase (ERK) pathway, did not attenuate the effects of IL-1β.

Therefore, our results show that p38MAP kinase and JNK are involved in mediating IL-1β–upregulated hBD-2 expression in HCECs. These findings are in contrast to those of Moon et al.[48] who observed that IL-1α–stimulated hBD-2 expression in middle ear epithelial cells was dependent on the ERK pathway and did not involve p38 MAP kinase. However, they are supported by other studies in which bacterial stimulation of hBD-2 expression was found to involve p38MAP kinase and JNK.[46] (McNamara NA, Evans DJ, Van R, Fleiszig SMJ, ARVO Abstract 2068, 1999). That NFκB inhibitors were able to block the effects of IL-1β in HCECs suggests that activation of this transcription factor is sufficient for upregulation of hBD-2 expression and that AP-1 activation through MAP kinase pathways is not involved. The combination of the MAP kinase inhibitors SB203580 and SP600125 was more effective than either inhibitor alone, but did not completely block IL-1β stimulation of hBD-2 expression. Thus, our data suggest that the effects of IL-1β are mediated by two pathways, each resulting in activation of NFκB. One pathway may involve the "direct" activation of NFκB through IκB kinase β[47] whereas the other involves upstream signaling by p38 MAP kinase and JNK.

Genistein was also found to attenuate IL-1β stimulation of hBD-2 expression, indicating the involvement of tyrosine kinase activity. The residual effect of IL-1β on hBD-2 expression after the addition of both SB203580 and SP600125 approximates the inhibitory effect of genistein; therefore, it is a possibility that upstream signaling through tyrosine kinases contributes to the non–MAP-kinase–mediated activation of NFκB. IL-1β–stimulated hBD-2 expression in HCECs was also partially attenuated by the glucocorticoid dexamethasone. Duits et al.[49] observed that dexamethasone attenuates bacterial induced hBD-3 but not hBD-2 expression in bronchial epithelial cells. Terai et al. (Terai K, Sano Y, Adachi W, Matsumoto A, Kinoshita S, ARVO Abstract 3147, 2001) reported that dexamethasone decreases hBD-1 expression by HCECs, but they did not study its effects on hBD-2. In contrast to these studies, we did not observe any effect of dexamethasone on the expression of any defensin other than hBD-2 (data not shown). This may have been due to differences in the cell types under study[49] or to differences in the treatment conditions. Current evidence suggests that glucocorticoid effects on gene transcription are largely due to interference with NFκB and AP-1.[50] As previously mentioned, sites for both of these transcription factors are present on the hBD-2 gene,[40–42] and thus interference with their activation may account for the ability of dexamethasone to inhibit hBD-2 expression. The treatment with dexamethasone for 24 hours resulted in a 28% decrease in IL-1β–stimulated hBD-2 expression. This is a substantial decrease and suggests that the use of topical ocular preparations containing dexamethasone could contribute to reduced antimicrobial peptide activity at the ocular surface.

Comparing our studies with IL-1β with those using *P. aeruginosa* as stimulant[21] (McNamara NA, Evans DJ, Van R, Fleiszig SMJ, ARVO Abstract 2068, 1999; (Maltseva I, McNamara N, Fleiszig SMJ, Basbaum C, ARVO Abstract 3195, 2002) both cytokine and bacterium appear to act through the same pathways in HCECs, namely tyrosine kinases, p38 MAP kinase, and NFκB. It is therefore conceivable that rather than having a direct effect, *P. aeruginosa* stimulation of hBD-2 expression is mediated through an increase in production of IL-1 which then acts in an autocrine fashion. In keeping with this, CD14 and TLR4, receptors for lipopolysaccharide, a major component of the outer membrane of *P. aeruginosa*, have been identified in corneal epithelial cells and their activation led to increased secretion of cytokines including IL-1.[51] However, it remains to be determined whether *P. aeruginosa* stimulated cytokine production occurs before the appearance of hBD-2 or if HCECs also express TLR2, a toll receptor previously shown to mediate the induction of hBD-2 in response to bacterial products.[52]

Overall our results show that proinflammatory cytokines have the capacity to upregulate the expression of hBD-2 in HCECs, thus providing a mechanism to explain our previous observation that hBD-2 is upregulated in regenerating corneal epithelium in organ culture.[22] Our inhibitor studies indicate that NFκB mediates IL-1β–stimulated hBD-2 expression in HCECs and that tyrosine kinases, p38 MAP kinase, and JNK are involved in the upstream signaling pathways leading to NFκB activation. Our data indicate that typically the corneal epithelium expresses hBD-1 and -3, which presumably provide the baseline defense against infection. The additional expression of hBD-2 that would result during inflammation and injury due to elevated levels of cytokines, presumably widens the spectrum of antimicrobial activity, probably helps regulate the ocular surface immune response, and may directly affect the wound-healing process, per se.

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