Stimulation of Specific Cytokines in Human Conjunctival Epithelial Cells by Defensins HNP1, HBD2, and HBD3

Jing Li,¹,² Hong Yuan Zhu,¹ and Roger W. Beuerman¹,²

PURPOSE. To investigate the effect of human defensins HNP1, HBD2, and HBD3 on human conjunctival epithelial cell cytokine secretion.

METHODS. HNP1, HBD2, and HBD3 were used to test cytotoxicity (1–50 μg/mL) and to stimulate (1–20 μg/mL) primary cultured and immortalized human conjunctival epithelial (IOBA-NHC) cells. Cytokine concentrations in the culture medium were measured by cytokine array and a multiplexed microbead analysis. Protein kinase activation was determined by Western blot analysis after defensin stimulation and with specific inhibitors.

RESULTS. HBD3, but not HNP1 or HBD2, killed more than 50% of IOBA-NHC cells at concentrations greater than 12.5 μg/mL. Only IL-6, IL-8, and RANTES were detected in the culture medium in the absence of defensins. All three cytokines increased in the presence of HNP1, HBD2, and HBD3 at concentrations of 5 to 20 μg/mL and between 2 and 8 hours and further accumulated at 24 hours Stimulation with HBD2 and HBD3 increased the secretion of IL-2 and MIP-1β in IOBA-NHC cells but only of MIP-1β in primary cultured cells. Activation of p42/44 mitogen-activated protein (MAP) kinase, Akt, and STAT3 was observed in primary and IOBA-NHC cells after defensin stimulation. Cytokine secretion was significantly decreased by the inhibition of p42/44 MAPK in IOBA-NHC cells.

CONCLUSIONS. HNP and HBD selectively increase the secretion of specific proinflammatory cytokines in conjunctival epithelial cells in a time- and concentration-dependent manner, suggesting a supporting role to the innate immune system of the ocular surface. (Invest Ophtalmol Vis Sci. 2009;50:644–653) DOI:10.1167/iovs.08-1838

Defensins are cysteine-rich, cationic, small antimicrobial peptides characterized by six cysteine residues forming three intramolecular disulfide bonds and various numbers of positively charged amino acids.¹ Mammalian defensins are classified into three subfamilies: α-, β-, and θ. In humans, six α-defensins, including four neutrophil defensins (HNP1–4), two intestinal Paneth cell defensins, and six β-defensins have been identified, though more defensin genes were found by computational search.²,³ HNP1 to HNP5 vary by only one amino acid and constitute the majority of neutrophil defensins. Among β-defensins, HBD1 to HBD3 are produced by keratino-cyes, mucosal epithelial cells of various tissues, and immune cells such as monocytes, dendritic cells, and macrophages.² HBD4 is found in testis and uterus, and the expression of HBD5 and HBD6 is restricted to epididymis.⁴

In addition to their antimicrobial activity, defensin peptides have been found to affect cell proliferation and death,⁵–⁸ wound healing,⁹–¹¹ and cell migration.¹²–¹⁵ The association between abnormal defensin expression and inflammatory diseases such as psoriasis, atopic dermatitis, Crohn disease, and cystic fibrosis are also recognized.¹⁶

Although defensin-stimulated cytokine secretion is reported, the effectiveness and specificity of different defensin peptides to elicit cytokine responses has not been examined systematically.¹³,¹⁷–¹⁹ It was reported in human bronchial epithelial cells that only HNP1, but not HBD2, stimulated an increase in IL-8 and IL-1β expression.¹⁸ In other studies, HBD2, HBD3, and HBD4 were shown to stimulate epidermal keratinocyte secretion of IL-6, IL-10, IF-10, MCP-1, MIP-3, and RANTES, whereas the effect of HNP was not reported.¹³ In human peripheral blood mononuclear cells, a wide spectrum of cytokines was reported to be upregulated by β-defensins.¹⁷ A comprehensive study is needed to understand the role of defensins in stimulating cytokine production in ocular surface epithelial cells.

Conjunctival epithelium of the ocular surface is bathed in tears that contain low concentrations of neutrophil defensins in normal conditions and become elevated after surgery.²⁰ Others have found the message for three β-defensins, HBD1, HBD2, and HBD3, in ocular surface epithelial cells.²¹ However, the protein form of any of the β-defensins has yet to be identified in tears, indicating a low concentration of the peptide at the ocular surface. HBD1 and HBD3 mRNA have been identified in normal human conjunctival and corneal epithelial cells.²² HBD3 has some unique properties: its broad-spectrum antimicrobial activity is less salt sensitive than other defensins,²²–²⁴ and it shows a wide variation of gene copy number.²⁶ Unlike that of HBD1 or HBD3, HBD2 expression was low or undetectable in normal corneal and conjunctival epithelial cells but increased in cells associated with inflammation such as dry eye syndrome.²¹ It was further identified that pathogens and proinflammatory cytokines such as IL-1 and TNFα stimulated the production of HBD2 in cultured corneal epithelial cells and in epithelial cells of other tissues.²¹ Previously, we showed that HNP1 and HBD2 are involved in fibroblast wound healing through the regulation of cell proliferation and extra-cellular matrix protein expression.²⁷ However, it remains unknown whether defensins also affect ocular surface epithelial cell cytokine secretion.

Here we report HNP1, HBD2, and HBD3 stimulated human conjunctival epithelial cell cytokine secretion. Cytotoxicity of the three defensins was determined in an immortalized human conjunctival epithelial cell line (IOBA-NHC cells). Proinflammatory cytokines in cell culture medium were initially screened after defensin stimulation by a membrane-based cytokine array containing 80 entries. The concentrations of 17 of those were quantified by multiplexed microbead analysis. The accumulation of cytokines over 24 hours of defensin stimulation was measured. Contributions of defensin-stimulated p42/44 mito-
gen-activated protein kinase (MAPK), Akt kinase, and signal transducer and activator of transcription signal 3 (Stat3) to increased cytokine secretion were determined. Similar effects of defensins were observed in primary cultured human conjunctival epithelial cells. To the best of our knowledge, this is the first report of the ability of defensins to stimulate conjunctival epithelial cell cytokine secretion.

**Materials and Methods**

**Cell Culture and Defensin Treatment**

Primary human conjunctival epithelial cells were isolated from cadaver conjunctival tissue obtained from Singapore Eye Bank. The protocol was approved by the institutional review board of Singapore Eye Research Institute and followed the tenets of the Declaration of Helsinki. Tissue was treated with antibiotic solution and was digested with dispase, as described previously. Isolated cells were cultured in serum-free keratinocyte medium (KSF; Invitrogen, Singapore) supplemented with bovine pituitary extract and epidermal growth factor. Cells were used at passages 2 to 3.

Immortalized normal human conjunctival epithelial cells (IOBA-NHC) were a gift from Yolanda Diebold at the University of Valladolid, Spain. These cells were cultured in a medium containing equal volumes of Dulbecco modified Eagle medium (DMEM) and F12, with an additional 2 ng/mL recombinant human EGF, 1 μg/mL bovine insulin, 0.1 μM chola toxin, 0.5 μg/mL hydrocortisone, and 10% FBS. HNP1, HBD2, and HBD3 were purchased from the Peptide Institute Inc. (Osaka, Japan). These were produced by solid-phase peptide synthesis technology with a purity of 99% or higher, as determined by high-performance liquid chromatography. The lyophilized peptide was dissolved in sterile PBS at a concentration of 1 mg/mL in glass vials and stored at −20°C. They were subsequently diluted as needed to the desired concentration in basal medium. The basal medium for IOBA-NHC cells was DMEM/F12 (1:1), and the basal medium for primary cells was KSF with bovine pituitary extract or epidermal growth factor.

**Cell Viability Analysis**

Viable IOBA-NHC cells, as determined by the trypan blue exclusion test, were plated on black 96-well tissue culture plates (Corning Life Sciences, Acton, MA) at a density of 1 × 10^4 cells/well. The number of live cells 24 hours after incubation with increasing concentrations of defensins was observed in several wells in each experiment. An aliquot of 1.2 mL culture medium was used to probe the preblocked cytokine array membrane overnight at 4°C. This was followed by incubation with biotin-conjugated primary antibody mixture and horseradish peroxide-conjugated streptavidin according to the protocol provided by the manufacturer. The final chemiluminescent signals were captured on x-ray film. To ensure maximal comparability, samples in each set (control, HNP1-treated, HBD2-treated) were incubated in parallel, and the resultant membranes were exposed to the same x-ray film.

**Multiplexed Cytokine Analysis**

Concentrations of the following cytokines were measured by multiplexed micro bead analysis (Bio-Rad, Singapore): IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 p70, IL-13, RANTES, eotaxin, GM-CSF, TNFa, IFN-γ, MIP-1α, MIP-1β, and MCP-1. Cell culture medium (50 μL/well) before and after treatment with HNP1, HBD2, or HBD3 was incubated with microbeads according to the protocol provided by the manufacturers. Beads were detected (Bio-Plex200; Bio-Rad) at the setting of 100 events per bead and high photomultiplier. Standards and samples were analyzed by a five-parameter logistic regression algorithm (SPL) provided in Bio-Rad software (Bio-Plex Manager 4.1) and were further corrected by the total number of cells from which the sample was obtained.

**Protein Kinase Activation**

The activation of protein kinases was determined by Western blot analysis of the phosphorylated kinase. Cells were cultured in basal medium for 4 hours, followed by defensin stimulation. Incubation was terminated by washing with cold PBS. Cells were lysed with buffer containing 50 mM Tris, 100 mM KCl, 1 mM EDTA, 1% NP40, 1 mM NaOAc, 100 mM NaF, and a protease inhibitor cocktail (Roche Diagnostics Asia Pacific, Singapore). Equal amounts of protein lysates were loaded on SDS-PAGE and subsequently transferred to 0.22-μm nitrocellulose paper. Antibodies against phosphorylated and nonphosphorylated p42/44 MAPK, Akt kinase, p38 MAPK, Stat1−5, Stat6, and p38/growth factor receptor were purchased from Cell Signaling Technology (Beverly, MA) and were used according to the manufacturer’s recommendation. Substrate reagent (Super Signal West Femto; Pierce, Rockford, IL) was used to detect the horseradish peroxidase-labeled secondary antibody, and the signal was captured on x-ray film.

**Inhibition of Protein Kinase Activation**

Cucurbitacin I (Calbiochem, La Jolla, CA), an inhibitor of Stat3, U0126 (Promega, Madison, WI), inhibitor of p42/44 MAPK and Wortmannin (Calbiochem), inhibitor of Akt, were added to the IOBA-NHC culture medium 2 hours before the addition of defensins. Cells were further incubated for 24 hours, and the culture medium was harvested for the measurement of IL-6 and IL-8 by multiplexed micro bead analysis.

**Statistical Analysis**

Student’s t-test or analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used to determine the difference between groups. P < 0.05 was accepted as significantly different. Data are presented as mean ± SD.

**Results**

**Effects of HNP1, HBD2, and HBD3 on Cell Viability**

The number of live cells 24 hours after incubation with increasing concentrations of HNP1, HBD2, and HBD3 was determined
Cytokines, as listed in Table 1. HNP1 and HBD2, at the concentration range of 6.25 μg/mL to 50 μg/mL. A maximal increase of 140% to 150% was observed at the concentration of 25 μg/mL for both defensins (n = 4; P < 0.05; HNP1- or HBD2-treated vs. control, t-test). HBD3 led to cell death at concentrations of 12.5 μg/mL and higher. The number of viable cells decreased to 25.3% of control in the presence of 50 μg/mL HBD3 at 24 hours (n = 2). At the concentration of 25 μg/mL, HBD3-related cell death was measured as early as 6 hours after incubation, and its rate increased to 59% at 16 hours (Fig. 1B).

### Defensin-Stimulated Cytokine Secretion

The effect of defensins on IOBA-NHC cell cytokine secretion was first screened using the antibody array that included 40 cytokines, as listed in Table 1. HNP1 and HBD2, at the concentration of 20 μg/mL in basal medium, were used to stimulate IOBA-NHC cells. The medium was harvested 24 hours later to probe the cytokine array. Because most of the cytokines could be revealed on x-ray film after several minutes’ exposure, we chose the exposure time of 1 minute for densitometry analysis of all samples (Fig. 2). The range of densitometry reading was 54.234 to 0.021. We arbitrarily defined the positive hits as those that had scanned densities higher than 10% of the averaged positive control spots on the same membrane. According to this criterion, in the absence of defensin stimulation, 10 cytokines were identified as positive hits in all three independent experiments, whereas 22 cytokines were negative through all experiments. More cytokines were determined as positive hits in the presence of HNP1 or HBD2 (Table 1).

We then compared the density of the spots for the same cytokines on control and defensin-treated membranes obtained from the same set of experiments, regardless of the density of each array spot relative to the positive controls. The average change (more than twofold) from three independent experiments is specified in Table 1.

To further verify the results obtained by the cytokine array, we measured the concentrations of IL-1α, IL-1β, TNFα, IL-6, IL-8, eotaxin, RANTES, MCP-1, MIP-1α, MIP-1β, IL-10, IL-12 p70, IFN-γ, IL-2, IL-4, IL-13, and GM-CSF in IOBA-NHC culture medium 24 hours after incubation with 5, 10, and 20 μg/mL HNP1, HBD2, and 1, 5, and 10 μg/mL HBD3 by multiplexed microbead cytokine analysis.

Three cytokines were consistently present in IOBA-NHC culture medium without stimulation: IL-6 (81.3 pg/mL/10^4

### Table 1. Summary of Cytokine Array Analysis

<table>
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<th>Cytokine</th>
<th>Sensitivity (pg/mL)</th>
<th>Control</th>
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<th>HBD2 Fold</th>
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Listed are all 40 cytokines included in the array. Sensitivity refers to the sensitivity of each antibody. Fold indicates average fold increase of the cytokine in defensin-treated samples compared with controls regardless of the relative density of each cytokine. +, positive appearance in one experiment; ++, maximal appearance given that array analysis was conducted three times; –, no appearance.
stimulated increases of IL-8 and RANTES were not obvious by array analysis. On the other hand, cytokine array data indicated HNP1 and HBD2 also stimulated increases of MIP-1α, IL-12 p70, GM-CSF, IL-1α, and IL-1β that were not confirmed by microbead analysis. IFN-γ, eotaxin, IL-13, and MCP-1, which were determined as negative by cytokine array, were also undetectable by multiplexed microbead analysis.

**Rapid Occurrence of Increased Cytokine Secretion after Defensin Stimulation**

We further analyzed the accumulation of IL-6, IL-8, RANTES, IL-2, and MIP-1β in IOBA-NHC cell culture medium at 2, 8, and 16 hours after stimulation with 5, 10, and 20 μg/mL HNP1 and HBD2 and 1, 5, and 10 μg/mL HBD3 (Fig. 4).

At 2 hours, MIP-1β was detected in samples treated with all three concentrations of HBD2 and HBD3. IL-2 was not detected in any of the HBD2- or HBD3-treated samples at 2 hours but was detected at 8 hours in all HBD2- and HBD3-treated samples.

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**FIGURE 1. Effect of HNP1, HBD2, and HBD3 on IOBA-NHC cell viability.** (A) Number of live IOBA-NHC cells 24 hours after the incubation of defensins. IOBA-NHC cells in 96-well plates were incubated with 50, 25, 12.5, 6.25, 3.125, or 0 μg/mL (control) of HNP1, HBD2, or HBD3 for 24 hours. The number of live cells was determined. Experiments with defensins at 50 μg/mL were repeated twice with duplicate wells; all other experiments were repeated four times with triplicate wells at each time. The graph shows the average of all experiments. *P < 0.05 when treated wells were compared with controls by unpaired t-test. (B) Numbers of live cells 6 and 16 hours after the stimulation of 25 μg/mL each of HNP1, HBD2, and HBD3. Cells were prepared as described. Reagent was added 2 hours before the termination of the incubation. The number of cells in untreated wells was 1 x 10^4 at 6 and 16 hours. For each condition, the experiments were repeated twice with triplicate wells of the same treatment at each time. Bar graph represents the average of the two experiments. *P < 0.05 when treated wells were compared with controls by unpaired t-test.

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**FIGURE 2. Inflammatory cytokine antibody array analysis.** Culture medium (1.2 mL) collected from 4 x 10^5 IOBA-NHC cells in six-well plates 24 hours after 0 (control), 20 μg/mL HNP1, or 20 μg/mL HBD2 stimulation was used to probe the arrays simultaneously, and the signals were captured on x-ray film. The image shown is one of the three sets of experiments. Each cytokine was spotted in duplicate. The spots for some of the cytokines are labeled. Pos, positive; neg, negative.
samples. Although detected at 2 hours, the concentration of RANTES showed a difference among control and defensin-treated samples (including HNP1, HBD2, and HBD3) after 8 hours of incubation. At 2 hours, HNP1- and HBD2-stimulated IL-8 secretion was higher than in controls, whereas HBD3-stimulated IL-8 secretion was not different from controls. At 8 hours, the difference among control and all three defensin-treated samples became significant. Similarly, the difference in IL-6 concentrations among control and HNP1-, HBD2-, and HBD3-treated samples was minimal at 2 hours and become significant at 8 hours. Overall, HNP1 and HBD2 stimulation led to increases in IL-6, IL-8, and RANTES at all three concentrations tested (5, 10, and 20 μg/mL) ($P < 0.05$, ANOVA LSD, treated vs. control, n = 3). HBD3 at 1 μg/mL and more caused significant increases in IL-6 and IL-8. However, only 5 and 10 μg/mL HBD3 caused significant increases in RANTES.

**HNP1-, HBD2-, and HBD3-Stimulated Activation of p42/44 MAP Kinase, Akt Kinase, and Stat3**

We next looked at IOBA-NHC cell protein kinase activation in response to 5, 10, or 20 μg/mL HNP1 or HBD2 or to 1, 5, or 10 μg/mL HBD3. Phosphorylation of p42/44 MAPK, Akt, and Stat3 was observed 10 to 30 minutes after stimulation (Fig. 5). The activation of p42/44 MAPK and Stat3 exhibited a concentration-dependent increase in the presence of all three defensins (Fig. 5A). The activation of Akt kinase by HBD2 showed a concentration-dependent increase. However, HNP1-stimulated Akt phosphorylation peaked at 10 μg/mL, whereas HBD3-stimulated Akt phosphorylation peaked at 1 μg/mL. No phosphorylated forms of EGF receptor, p38 MAPK, JUN kinase, NF-κB, Stat1,-2, or -5 were detected under the same conditions (data not shown).

When the activation of protein kinases was compared among the three defensins at the same concentration of 5 μg/mL, we found that HBD2 showed the most potent activation of all three kinases compared with HNP1 and HBD3, whereas HBD3 was the weakest among the three defensins (Figs. 5B, 5C).

**Contribution of p42/44 MAPK, Akt Kinase, and Stat3 Activation to Defensin-Stimulated Cytokine Secretion**

Inhibition of HBD2-stimulated p42/44 MAPK phosphorylation by 10 μM U0126, Akt kinase phosphorylation by 5 μM wortmannin, and Stat3 kinase phosphorylation by 1 μM cucurbitacin I in IOBA-NHC cells was confirmed by Western blot analysis (data not shown). To further understand the contribution of the above kinase activation to the observed increased cytokine secretion on defensin stimulation, IOBA-NHC cells were preincubated with U0126 at concentrations of 2.5, 5, and 10 μM, wortmannin at concentrations of 1, 5, and 10 μM, and cucurbitacin I at concentrations of 0.1, 1, and 10 μM 3 hours before the addition of 10 μg/mL HNP1 or HBD2. The culture medium was harvested 24 hours later for the determination of the levels of IL-6 and IL-8 (Fig. 6). Data showed that HNP1- and HBD2-stimulated cytokine secretion was most sensitive to the inhibition of p42/44 MAPK. Wortmannin-inhibited HNP1 stimulated IL-6 and IL-8 secretion only at the high concentration of 10 μM but was minimally effective on HBD2-stimulated cytokine secretion. The inhibition of Stat3 by cucurbitacin I did not affect the secretion of IL-6 or IL-8 stimulated by HNP1 or HBD2.

**Effect of HNP1, HBD2, and HBD3 on Primary Cultured Human Conjunctival Epithelial Cells**

To determine whether these phenomena were reproducible in primary cultured human conjunctival epithelial cells, we examined HNP1-, HBD2-, and HBD3-stimulated protein kinase activation and cytokine secretion in these cells.

Phosphorylation of p42/44 MAPK, Akt kinase, and Stat3 was observed in primary cultured conjunctival epithelial cells 30 minutes after stimulation with 10 μg/mL HNP1, HBD2, or HBD3 (Fig. 7). For the analysis of defensin-stimulated cytokine production, two batches of primary conjunctival epithelial cells isolated from two donors were stimulated with HNP1, HBD2, or HBD3 at concentrations of 10 μg/mL for 24 hours. The medium was collected to determine the concentrations of
IL-6, IL-8, RANTES, IL-2, MIP-1β/H9252, IL-1α/H9252, IFN-γ/H9251, IFN-α, MCP-1, and TNF-α using multiplexed microbeads. Consistent with data obtained from IOBA-NHC cells, increased levels of IL-6, IL-8, and RANTES were measured in primary cell samples stimulated by all three defensins. Increased MIP-1β secretion was also observed in primary cell samples stimulated with HBD2 and HBD3 (Fig. 8). Unlike IOBA-NHC cells, an average of 67.5 pg/mL MIP-1β/10⁴ cells was detected in primary cells in the absence of defensins. Although the cell number-corrected concentrations of IL-6, IL-8, and RANTES were similar between IOBA-NHC and primary cultured human conjunctival epithelial cells, the HBD2- and HBD3-stimulated MIP-1β concentrations were lower in primary cultured conjunctival epithelial cells. IL-2 was not detected in primary human conjunctival cell medium regardless of defensin stimulation.

**DISCUSSION**

In this study, we evaluated the roles of HNP1, HBD2, and HBD3 in modulating conjunctival epithelial cell cytokine production. In IOBA-NHC cells and primary cultured conjunctival epithelial cells, all three defensins increased the production of IL-6, IL-8, and RANTES, which were also the most abundant cytokines measured in these cells in the absence of stimulation. There was a difference between α- and β-defensins in the target of cytokines: MIP-1β was stimulated only by HBD2 and HBD3 but not by HNP1. Activation of p42/44 MAPK was necessary for HNP1- and HBD2-stimulated cytokine secretion in IOBA-NHC cells.

This is the first comprehensive study of α- and β-defensin-stimulated cytokine secretions in conjunctival epithelial cells. Unlike what was reported for human bronchial epithelial cells, we found that HBD2 also stimulated cytokine secretion in conjunctival epithelial cells.¹⁸ We also found that conjunctival...
epithelial cells were responsive to low concentrations of HNP1, HBD2, and HBD3 (1–5 μg/mL), which were not tested in studies using bronchial epithelial cells or keratinocytes. The low defensin concentration needed (1–5 μg/mL) to elicit a significant cytokine increase suggests close physiological relevance. Collectively, the results obtained from this study and others show that α- and β-defensins selectively stimulate cytokine secretion of epithelial cells. Our results also suggested that β-defensins may have a broader effect on cell cytokine production than α-defensins.

Although antibody array analysis revealed a number of cytokine changes after defensin stimulation in IOBA-NHC cells, as was similarly observed in human monocytes, quantitative analysis with multiplexed microbead analysis concluded that the results obtained from the antibody array were not entirely reliable. Unlike microbead analysis, the antibody array is better suited for the qualitative detection of cytokines. The sensitivity of antibodies immobilized on the membrane varies from 1 pg/mL to 100,000 pg/mL (Table 1). No internal standards were provided to ensure the linear binding of cytokines to the antibody. The actual sensitivity of each antibody was further compromised by the fact that all cytokine signals on one array were captured on the same x-ray film. Some cytokine signals might have been underdeveloped while others were saturated on the x-ray film. Although detection would be improved if the signal were captured and analyzed with equipment such as a charge-coupled device camera, quantitative analysis is recommended to confirm the findings obtained from the antibody array.

The findings on defensin-stimulated conjunctival epithelial cell cytokine secretion add another immune regulatory mechanism to the ocular surface. In the presence of pathogens and proinflammatory cytokines such as TNF-α and IL-1, the expression of epithelial cell β-defensins is increased. Increased α-defensin expression is also expected because neutrophils are attracted to the site of inflammation. It is generally believed that defensins directly participate in the killing of pathogens at a mucosal site as a fundamental component of innate immunity. By increasing epithelial cell secretion of IL-8, RANTES, and MIP-1β, neutrophils, monocytes, dendritic cells, and macrophages, among other cells, are recruited to the site of inflammation. These cells are also known to express α- or β-defensins. Although not identical, increased chemokine secretion was observed in bronchial epithelial cells and epidermal keratinocytes under β-defensin stimulation. Therefore, we speculate that defensin-stimulated cytokine secretion is a mechanism of self-amplification to ensure the sufficient local concentration of defensins for pathogen killing.
Despite numerous reports on the biological effects of defensins on eukaryotic cells, the molecular mechanisms underlying these activities are largely unknown. Little consensus was found regarding defensin-triggered intracellular signal transduction. Depending on target cells and the defensin subtype studied, the activation of p42/44 MAPK, Akt kinase, various Stat proteins, protein kinase C, and phospholipase C was found.\(^\text{15,28,29}\) In the present study, we found that HNP1, HBD2, and HBD3 all activated p42/44 MAPK, Akt kinase, and Stat3 but not EGF receptor, p38 MAPK, JUN kinase, NF-H9260, Stat1, -2, or -5. We also showed that the activation of p42/44 MAPK was primarily responsible for HNP1- and HBD2-stimulated IL-6 and IL-8 secretion in IOBA-NHC cells. Given that activation of the PI3 kinase and Akt kinase pathways is often linked to the activation of p42/44 MAPK, it is possible that the inhibition of HNP1-stimulated IL-6 and IL-8 secretion by high concentrations of wortmannin was caused by the indirect inhibition of p42/44 MAPK. It is likely that the activation of Akt and Stat3 has little effect on the HNP1- and HBD2-stimulated increased expression of IL-6 and IL-8.

The unique upregulation of MIP-1\(\beta\) and IL-2 in IOBA-NHC cells and MIP-1\(\beta\) in primary cultured conjunctival epithelial cells by HBD2 and HBD3 also indicated that another mechanism, most likely unique to \(\beta\)-defensins, exists. One possible mechanism is the direct entry of defensins through electrostatic interaction with the plasma membrane and even the nuclear membrane. All members of the defensin family bear positive net charges. HBD3 has 11 positively charged residues of 45 amino acids, HBD2 has 7 positively charged residues of 41 amino acids, and HNP1 has 3 positively charged residues of 30 amino acids. In fact, direct interaction and subsequent permeabilization of the pathogen cell membrane is one of the proposed mechanisms for the microbicidal activity of defensins.\(^\text{1}\) The absence of defensin receptors supports the possibility of a direct entry. Because \(\beta\)-defensins bear higher net charge...
positive charges than α-defensins, it is possible that they interact with different molecules once inside the cell and that this leads to different changes of cellular activity.

The high net positive charge of HBD3 is also likely responsible for its cytotoxicity. Additionally, based on the amino acid composition, HBD3 is the least hydrophobic peptide compared with HBD2 and HNP1. Our recent study using synthesized HBD3 analogs showed that cytotoxicity decreases with increasing hydrophobicity.\(^\text{30}\) The combined high net charge and low hydrophobicity of HBD3 make it possible to cause plasma membrane leakage and cell death at thresholds lower than those of HNP1 and HBD2. HBD3-activated p42/44 MAPK is not contradictory to the cell death observed in the presence of high concentrations of HBD3, if indeed HBD3-induced cytotoxicity is caused by permeabilization of the plasma membrane.

The results obtained from IOBA-NHC cells were reproduced in primary cultured human conjunctival epithelial cells. We did notice that the increased secretion of IL-2 in the presence of HBD2 and HBD3 was absent in primary conjunctival cells. IL-2 is a cytokine produced mainly by T cells. It is the only one affected by HBD2 and HBD3 among a group of predominantly adaptive immunity-related cytokines tested (including IL-12, IFN-γ, IL-2, -4, and -13). Epithelial cells are not known as significant cellular sources for IL-2; therefore, we believe the upregulation of IL-2 in IOBA-NHC cells represented an abnormality associated with the cell line. However, it also raised the possibility that defensins may have significant effect on T-cell cytokine secretion.

In conclusion, we present evidence for the first time that α- and β-defensins selectively stimulate cytokine production in conjunctival epithelial cells that is markedly suppressed by inhibitors of p42/44 MAPK. Based on the biological functions of the cytokines affected, it is possible that defensin-stimulated conjunctival epithelial cell cytokine production is a self-regulatory mechanism to ensure sufficient local concentrations of defensins for effective pathogen killing.

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**References**


