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Cytotoxicity of HBD3 for dendritic cells, normal human epidermal keratinocytes, hTERT keratinocytes, and primary oral gingival epithelial keratinocytes in cell culture conditions

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Abstract

Human β-defensin 3 (HBD3) is a prominent host defense peptide. In our recent work, we observed that HBD3 modulates pro-inflammatory agonist-induced chemokine and cytokine responses in human myeloid dendritic cells (DCs), often at 20.0 μM concentrations. Since HBD3 can be cytotoxic in some circumstances, it is necessary to assess its cytotoxicity for DCs, normal human epidermal keratinocytes (NHEKs), human telomerase reverse transcriptase (hTERT) keratinocytes, and primary oral gingival epithelial (GE) keratinocytes in different cell culture conditions. Cells, in serum free media with resazurin and in complete media with 10% fetal bovine serum and resazurin, were incubated with 5, 10, 20, and 40 μM HBD3. Cytotoxicity was determined by measuring metabolic conversion of resazurin to resorufin. The lethal dose 50 (LD_{50}, mean μM ± std err) values were determined from the median fluorescent intensities of test concentrations compared to live and killed cell controls. The LD_{50} value range of HBD3 was 18.2–35.9 μM in serum-free media for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes, and > 40.0 μM in complete media. Thus, HBD3 was cytotoxic at higher concentrations, which must be considered in future studies of HBD3-modulated chemokine and cytokine responses *in vitro*.

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Keywords

HBD3; defensins; cytotoxicity; dendritic cells; epidermal keratinocytes; hTERT keratinocytes; gingival epithelial GE keratinocytes

1. Introduction

Human β-defensin (HBD)3 is a dynamic host defense peptide. It is a small, 45 amino acid residue molecule with a strong cationic charge (+11) and monoisotopic mass of 5,157.7 Da (Liu et al., 2008). HBD3 is produced by a variety of cells and tissues of the oral cavity (Dunsche et al., 2002; Harder et al., 2001), respiratory tract (Devine, 2003; Harder et al., 2001; Ishimoto et al., 2006; Saito et al., 2012), gastrointestinal tract (Zilbauer et al., 2010), skin (Harder et al., 2001), urogenital tract (Harder et al., 2001), and lymphatic and circulatory systems (Tohidnezhad et al., 2011). HBD3 production can be induced by microbial products, prostaglandin D2, interferon (IFN)-γ, interleukin (IL)-1 and tumor necrosis factor (TNF)-α (Dhople et al., 2006; Joly et al., 2005; Kanda et al., 2010). Once produced, HBD3 has broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeasts, and enveloped viruses (Harder et al., 2001; Joly et al., 2004; Klotman and Chang, 2006; Liu et al., 2008; Saito et al., 2012).

HBD3 has a strong role in innate and adaptive immunity (Brogden et al., 2015). HBD3 enhances the barrier function of skin or mucosa by regulating permeability and membrane tight junctions in keratinocytes (Kiatsurayanon et al., 2014); stimulates the production of several chemokines from epithelial cells including macrophage inhibitory peptide (MIP) 3α, IFN-inducible protein (IP)-10, and IL-18 (Meisch et al., 2013); chemoattracts monocytes, macrophages, T cells, neutrophils, immature dendritic cells (DCs), and mast cells; promotes differentiation of human mesenchymal stem cells and osteoblasts (Kraus et al., 2012); and regulates complement activation (Prohaszka et al., 1997; van den Berg et al., 1998).

HBD3 also has potent immunomodulatory activity. *In vitro*, it can attenuate or enhance chemokine and cytokine production in response to a variety of pro-inflammatory stimuli (Rohrl et al., 2008; Semple and Dorin, 2012; Semple et al., 2011; Semple et al., 2010) including *Porphyromonas gingivalis* hemagglutinin B, which is the major virulence factor responsible for microbial attachment (Borgwardt et al., 2014; Harvey et al., 2013). At low concentrations, HBD3 attenuates pro-inflammatory agonist-induced chemokine and pro-inflammatory cytokine responses of DCs. At high concentrations and administered before or after a pro-inflammatory agonist, HBD3 enhances agonist-induced chemokine and pro-inflammatory cytokine responses of DCs (Borgwardt et al., 2014; Harvey et al., 2013). Some of these responses occur at 20.0 μM HBD3.

High concentrations of HBD3 are also produced in several diseases including oral squamous cell carcinoma, oral dysplasia, osteoarthritis, and ulcerative colitis (Fahlgren et al., 2004; Kawsar et al., 2009; Kesting et al., 2009; Varoga et al., 2009). Since 0.6–19.4 μM concentrations of HBD3 have been reported to be cytotoxic for eukaryotic cells (Lioi et al., 2012; Liu et al., 2008; Saito et al., 2012), it is important to determine the cytotoxicity of HBD3 in differing cell culture conditions. In this study, we assessed the cytotoxicity of

HBD3 for DCs, normal human epidermal keratinocytes (NHEKs), human telomerase reverse transcriptase (hTERT) keratinocytes, and primary oral gingival epithelial (GE) keratinocytes in serum free media with resazurin and in complete media with 10% fetal bovine serum and resazurin.

2. Material and Methods

2.1 HBD3 and solutions

A 400 μM stock solution of HBD3 (PeproTech, Rocky Hill, NJ) was prepared in 0.01 M sodium phosphate with 0.14 M NaCl, pH 7.2 (PBS) using pyrogen-free water (Lonza Walkersville, Inc., Walkersville, MD) and filtered (0.22 μm filter, Millipore, Billerica, MA). Twenty μl of the stock 400 μM HBD3 solution was then added to 180 μl of respective cell culture media in round bottom polypropylene plates (Costar 3879; Corning Inc., Corning, NY) and diluted 2-fold from 40 to 5 μM.

2.2 Cells and culture media

Human monocyte-derived immature myeloid DCs (ALLCELLS, Alamenda, CA) were grown in Lymphocyte Growth Medium-3 (LGM-3, Lonza Walkersville, Inc., Walkersville, MD) with 10% fetal bovine serum (ATCC, Manassas, VA).

NHEKs (No. 22179; Lonza Walkersville, Inc., Walkersville, MD) were grown in Keratinocyte Growth Medium (KGM-Gold™ Lonza Walkersville, Inc.) with 10% fetal bovine serum (ATCC, Manassas, VA).

Oral hTERT-immortalized human adenoid keratinocytes were obtained courtesy of Aloysius J. Klingelhutz (Department of Microbiology, The Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA) (Farwell et al., 2000). hTERT keratinocytes were grown in keratinocyte-serum free media with L-glutamine, human recombinant epidermal growth factor (EGF 1–53), bovine pituitary extract (BPE) (Gibco Life Sciences, Grand Island, NY) and 10% fetal bovine serum (ATCC, Manassas, VA).

GE keratinocytes prepared for a previous study and stored in liquid nitrogen were used in the present study (Joly et al., 2005). These cells were from healthy gingival tissue samples obtained from healthy non-smoking individuals who underwent crown lengthening or canine exposure procedures. Informed consent was obtained from these individuals per a reviewed and approved protocol from the University of Iowa Institutional Review Board. Concentrations of GE keratinocytes were determined and adjusted to contain 1.0×10^5 viable cells/ml LGM-3. Primary, first passage, cell lines GE369, GE370, and GE373 were used and grown in Keratinocyte-SFM with L-glutamine, human recombinant epidermal growth factor (EGF 1–53), and bovine pituitary extract (BPE) (Gibco Life Sciences, Grand Island, NY) and 10% fetal bovine serum (ATCC, Manassas, VA).

DCs, NHEKs, hTERT keratinocytes, and primary oral GE keratinocytes were first grown in their respective media with 10% fetal bovine serum (ATCC, Manassas, VA). Once the cell cultures were established, cells were then placed in their respective media with resazurin (Alamar Blue, Invitrogen Corp., Carlsbad, CA) (serum free media) or in their respective

media with 10% fetal bovine serum and resazurin (Alamar Blue, Invitrogen Corp., Carlsbad, CA) (complete media).

2.3 Flow cytometry

The identities of GE369 and GE373 keratinocytes were confirmed using flow cytometry as recently described (Poulsen et al., 2015). Keratinocytes resuspended in fresh media at 10^5 viable cells/ml were blocked with human IgG (Sigma, Saint Louis, MO) for 20 min, washed with stain buffer (BD Pharmingen, San Jose, CA), and resuspended in 100 μl stain buffer (BD Pharmingen, San Jose, CA) at 10^6 cells/ml before adding antibodies to surface markers CD24 (PE-Cy7-CD24; BD Pharmingen, San Jose, CA) and CD104 (FITC-CD104; BioLegend, San Diego, CA). After a 30 min incubation, cells were washed twice more, resuspended in fresh stain buffer, and stained using a near-IR LIVE/DEAD Cell Vitality Assay Kit (L10119, Molecular Probes, Eugene, OR) to differentiate between live and dead keratinocytes. Finally cells were prepared for intracellular staining by fixation (Fix Buffer 1; BD Pharmingen; San Jose, CA) and permeabilization (Perm/Wash Buffer 1; BD Pharmingen, San Jose, CA), and another wash step before resuspension in fresh Perm/Wash buffer. Intracellular cytokeratin (PE-anticytokeratin, reacting to cytokeratins 14, 15, 16, and 19; BD Pharmingen, San Jose, CA) and pancytokeratin (A647-pancytokeratin C11, reacting to cytokeratins 4, 5, 6, 8, 10, 13, and 18; Cell Signaling, Danvers, MA) were added to cells and allowed to incubate for 30 min before washing in stain buffer. Cells were resuspended in 600 μl fresh stain buffer at 10^5 cells/ml, and maintained at 4°C until analysis by flow cytometry. Marker binding was assessed in a LSR II Flow Cytometer (BD Biosciences, San Jose, CA in the University of Iowa Flow Cytometry Core Facility, Iowa City, IA). Isotype controls for each antibody were used to assess non-specific binding. Compensation controls were used to adjust for spectral overlap across dyes. Fluorescence-minus-one (FMO) controls were used to set gating parameters.

2.4 Cell viability

The viabilities of DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes were initially determined by propidium iodide (PI) staining. Thawed cells were suspended in their respective complete media; counted to determine the total and viable cell concentrations, the latter using immunofluorescence in the presence of 25 ng/ml PI to detect dead cells; and pelleted by centrifugation at 400 × *g* (Eppendorf 5810R centrifuge, Brinkmann Instruments, Inc., Westbury, NY) for 10 min at 24°C. Cells were suspended in their respective complete media and concentrations were adjusted to contain 1.0×10^5 viable cells per ml. 200 μl complete media containing 2.0×10^4 viable cells were put into each well of a 96-well microtiter plate (Costar 3595; Corning Inc., Corning, NY) and allowed to attach overnight at 37°C in 5% CO_2.

2.4 Cytotoxicity

At 24 h, the complete media was removed from each well and replaced with either the serum free media with resazurin and dilutions of HBD3 or the complete media with resazurin and dilutions of HBD3. Culture media with 1.0% sodium azide (Sigma Aldrich, St. Louis Mo)

was used for killed cell (KC) controls and culture media alone was used for live cell (LC) controls.

Cell cultures were then incubated at 37°C with 5% CO_2. At 0, 2, 4, 8, and 16 h post inoculation, the metabolic reduction of resazurin to resorufin was determined using an excitation wavelength of 544 nm and an emission wavelength of 590 nm (SpectraMax M2e Multi-Mode Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA). Percent cytotoxicity was defined as [the median fluorescence intensity (MFI) of resazurin in cell culture media of cells treated with dilutions of HBD3/MFI of resazurin in cell culture media of LC] × 100. The lethal dose 50 (LD_{50}) values were determined from the dose response curve where the 50 percent cytotoxicity intercepts with the HBD3 concentration on the x-axis.

All assays were performed in triplicate.

2.5 Statistical analysis

Differences in the LD_{50} values of HBD3 for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes in serum free media with resazurin and the complete media with resazurin were analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (Prism 6, GraphPad Software, Inc, La Jolla, CA). The cytotoxicities of 5, 10, 20, and 40 μM HBD3, the MFI data for live cells, killed cells, and treated cells were fitted to second order polynomial (quadratic) equations. Extra sum-of-square F-tests were used to compare each fitted model and to find whether there was a common model for every concentration. Statistical significance from the F-test indicated the presence of common fitted model, which means there were no differences in the cytotoxicity of HBD3 at different concentrations. Significance at the 95% confidence level ($\alpha = 0.05$) and 80% power were used in analysis.

3. Results

3.1 Cell viability

The viabilities of DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes were initially determined by PI staining and cell suspensions were adjusted to contain 1.0×10^5 viable cells per ml. At 16 h, untreated DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes remained viable throughout the entire incubation period in their respective serum free media with resazurin and complete media with resazurin. These cells served as non-inoculated live cell controls (labeled LC in Fig. 1 and Fig. 2). LC results were very consistent for each replication and MFI values were unique to the cell type and the assay conditions. Mean MFI ranged from 10,255.7 (253.2 std err) to 21,784.0 (253.2 std err). Mean MFI for LC of DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes were 25.7–69.5% higher in serum free media with resazurin mean MFI for LC of DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes in complete media with resazurin. This is not unusual. Fetal bovine serum is known to interfere with the metabolic reduction of resazurin to resorufin (Goegan et al., 1995). The effect is thought to be due to the binding of serum components to both resazurin to resorufin resulting in fluorescence spectral shifts and quenching.

3.2 HBD3 cytotoxicity

Graphs in Fig. 1 and Fig. 2 show the MFI values at 0, 2, 4, 8, and 16 h for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes exposed to 5, 10, 20, and 40 μM HBD3 in serum free media with resazurin and complete media with resazurin. Second order polynomial fitted equations showed no common fitted model in most of the cell types ($p < 0.05$) except in the group of DCs and GE370 keratinocytes in complete media with resazurin ($p = 0.054$ and $p = 0.96$, respectively). Thus, the cytotoxicity of HBD3 was dose dependent in all cell types in serum free media with resazurin and complete media with resazurin except for DCs and GE370 keratinocytes in complete media with resazurin. The LD_{50} value range of HBD3 was 18.2–35.9 μM for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes in serum free media with resazurin and > 40.0 μM in complete media with resazurin (Table 1). Using LD_{50} values of the complete media with resazurin as 40 μM, statistical differences in the cytotoxicity of HBD3 for hTERT keratinocytes and GE373 keratinocytes in serum free media with resazurin and the complete media with resazurin could be detected ($p = 0.02$, hTERT keratinocytes; $p < 0.01$, GE373 keratinocytes). This suggests differences among LD_{50} values in different media in only hTERT keratinocytes and GE373 keratinocytes. Using DCs as a comparison group, within the serum free media with resazurin groups, the differences in LD_{50} values of DCs and hTERT keratinocytes; and DCs and GE373 keratinocytes were statistically significant ($p = 0.03$ and $p < 0.05$, respectively).

There were differences in the LD_{50} values of GE keratinocytes, particularly GE369 and GE373 keratinocytes. GE369 keratinocytes had the highest LD_{50} value (34.26 μM) to HBD3 and GE373 keratinocytes had the lowest LD_{50} value (18.19 μM) to HBD3 (Table 2). Interestingly, there were also differences among these cells by flow cytometry. GE369 keratinocytes had the lowest percent staining patterns for cytokeratins 14, 15, 16, and 19 (61.0–69.2%), yet the highest percent staining pattern for CD104 B4 integrin (13.8–34.4%) (Table 2). In contrast, GE373 keratinocytes had high percent staining patterns for cytokeratins 14, 15, 16, and 19 (94.8–96.5%), yet low percent staining patterns for CD104 B4 integrin (3.6–4.5%).

4. Discussion

HBD3 is a very versatile host defense peptide. It has direct antimicrobial activities, regulates innate immune mechanisms, attenuates or enhances chemokine and pro-inflammatory cytokine responses, and enhances adaptive immune responses. HBD3 also has cytotoxicity against eukaryotic cells (Table 3). The extent of the overlap between the concentrations of HBD3 known to play roles in innate and adaptive immunity and its cytotoxicity are not well known, particularly at higher concentrations where HBD3 enhances chemokine and pro-inflammatory cytokine responses in cells, yet may also be cytotoxic. Here, we report that the cytotoxicity of HBD3 for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes varied with the cell type and the culture conditions. This demonstrates that HBD3 has some cytotoxicity at higher concentrations in cell culture conditions, which needs to be considered in future studies of HBD3-modulated chemokine and cytokine responses, *in vitro*. Also, there were some distinct cellular marker differences in GE369 and GE373 keratinocytes that

appeared to correlate with notable differences in their cytotoxicity to HBD3 in serum free media with resazurin (Tables 1 and 2). This is an interesting observation, and whether the phenotypes of these cells are related to their HBD3 cytotoxicity profiles is yet to be determined.

There are good examples of the overlap in the concentrations of HBD3 known to play roles in immunity vs. cytotoxicity. Cells exposed to lower concentrations of HBD3 (e.g., <0.2–1.9 μM) do not produce much of a chemokine and pro-inflammatory cytokine response, nor are these concentrations cytotoxic. Murine bone marrow derived macrophages treated with 1.0 μM HBD3 do not produce TNFα (Barabas et al., 2013) and 0.04 μM HBD3 has no toxicity against human periodontal ligament fibroblasts (Wang et al., 2011).

At higher concentrations, HBD3 can induce chemokine and pro-inflammatory cytokine responses and HBD3 cytotoxicity becomes variable, influenced by HBD3 concentration, cell type, and cell culture conditions. Keratinocytes treated with 0.2–7.8 μM HBD3 produce IL-18 (Niyonsaba et al., 2005); monocytes and macrophages treated with 3.9 μM HBD3 produce Gro-α, MDC, MCP-1, MIP-1α, MIP-1β and VEGF (Petrov et al., 2013); and keratinocytes treated with 5.8 μM HBD3 produce IL-6, IL-10, IP-10 (CXCL10), MCP-1 (CCL2), MIP-3α (CCL20), and RANTES (CCL5) (Niyonsaba et al., 2007). HBD3 in the range of 0.6–19.4 μM is not cytotoxic to rabbit erythrocytes (Liu et al., 2008), and less than 1% of erythrocytes are lysed (Bohling et al., 2006; Liu et al., 2008). Concentrations of HBD3 ranging from 2.4–9.7 μM are cytotoxic to human conjunctival epithelial cells (Liu et al., 2008; Zhou et al., 2011), THP1 human monocytic cells (Saito et al., 2012), peripheral blood mononuclear cells (Lioi et al., 2012), H441 human lung adenocarcinoma epithelial cells (Saito et al., 2012), and A549 human adenocarcinomic alveolar basal epithelial cells (Saito et al., 2012). At 7.8 μM, HBD3 is not cytotoxic to peripheral blood mononuclear cells, T cells, and osteosarcoma cells (Quinones-Mateu et al., 2003).

At the highest concentrations, HBD3 induces enhanced chemokine and pro-inflammatory cytokine responses and cytotoxicity becomes more pronounced. However, cells exposed to higher concentrations of HBD3 (e.g., 1.9–19.4 μM) produce higher amounts of chemokines and pro-inflammatory cytokines in a dose related fashion. HBD3 at 14.5 μM is cytotoxic to A549 epithelial cells (Saito et al., 2012) and 96.9 μM HBD3 is cytotoxic for human erythrocytes with up to 15% hemolysis (Harder et al., 2001). In the present study, the LD_{50} value range of HBD3 was 18.2–35.9 μM for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes in serum free media with resazurin and > 40.0 μM in complete media with resazurin.

The exact mechanism for HBD3-induced cell cytotoxicity is not well known. Evidence suggests that HBD3 may exert cytotoxicity by lipophilic and charge-charge interactions with phospholipids in the cell membranes (Lioi et al., 2012; Liu et al., 2008), leading to cellular necrosis rather than apoptosis. Lactate dehydrogenase (LDH) assays tend to reflect necrosis (Chan et al., 2013) and plasma membrane permeability with LDH release is observed after HBD3 treatment of THP1 human monocytic cells, H441 human lung adenocarcinoma epithelial cells, and A549 epithelial cells (Lioi et al., 2012; Saito et al., 2012).

HBD3 rapidly enters cells. Van Hemert et al. observed HBD3 in the cytoplasm of DCs min after it was added to cells in culture (Van Hemert et al., 2012), and Semple et al. showed that HBD3 rapidly enters toll-like receptor (TLR) 4-stimulated macrophages and targets TLR signaling pathways to inhibit the transcription of pro-inflammatory genes involved in the regulation of nuclear factor kappa B (Semple et al., 2011). Lioi et al. showed that HBD3 causes membrane perturbations in RAW264.7 macrophages incubated with TAMRA labeled HBD3 ($HBD3^{TAMRA}$), but not T or B cells (Lioi et al., 2012). After 10 min $HBD3^{TAMRA}$ was seen in the cell cytoplasm, but did not enter the nucleus. After 2 h, $HBD3^{TAMRA}$ continued to accumulate both on the cell surface and inside the cell. Lioi et al. extended these findings and HBD3 induced nonlethal perturbations in the cell membrane (Lioi et al., 2012). In flow cytometry, peripheral blood mononuclear cells (PBMCs) incubated with 3.9 μM HBD3 exhibit two different staining patterns using PI. A majority of the cells express low to intermediate levels of PI staining ($PI^{dim/intermediate}$) after treatment with HBD3 and a minority of cells had high levels of PI staining (PI^{bright}) after treatment with HBD3. $PI^{dim/intermediate}$ PBMCs had vesicles containing PI that did not gain access to nuclear DNA. PBMCs incubated with 11.6–15.5 μM HBD3 were PI^{bright} after treatment with HBD3, suggesting that high concentration of HBD3 causes severe membrane disruptions. Furthermore, PBMCs incubated with HBD3 produce surface expression of LAMP1, a membrane repair marker.

There are factors that influence HBD3-induced cell cytotoxicity. First, the presence of membrane cholesterol and polysaccharides may influence the cytotoxicity of HBD3 for cells. For example, Lioi et al used cyclodextrins to remove cholesterol from PBMC plasma membranes, which resulted in higher HBD3-induced cell cytotoxicity (Lioi et al., 2012). However, differences in free cholesterol membrane contents did not affect the sensitivity of monocytes to HBD3. Second, polysaccharide expression on cell surfaces may be important. PMBCs have a higher level of the negatively charged phospholipid on their outer leaflet, and pre-incubation of PMBCs with molecules that bind phospholipid protect PMBCs from HBD3-induced membrane damage (Lioi et al., 2012). The induction of polysaccharide expression in B and T cells also increases the susceptibility of cells to damage, suggesting that outer-membrane polysaccharide expression relates to susceptibility to HBD3 (Lioi et al., 2012). Third, cell repair mechanisms can influence HBD3-induced cell cytotoxicity. Expression of cell membrane repair marker LAMP1 (CD107a) in monocytes exposed to HBD3 increases significantly (Lioi et al., 2012). Also, the inhibition of cell repair by calcium chelater BAPTA-AM increases the amount of cell death, suggesting that calcium-dependent cell repair processes influence HBD3-induced cytotoxicity.

In conclusion, HBD3 has some cytotoxicity for DCs, NHEKs, hTERT keratinocytes, and primary oral GE keratinocytes. This activity is influenced by a number of factors including HBD3 concentration, cell type, and cell culture conditions. These factors need to be monitored closely in studies of HBD3-modulated chemokine and cytokine responses *in vitro*.

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Abbreviations

HBD3	Human β-defensin 3
DCs	dendritic cells
NHEKs	normal human epidermal keratinocytes
hTERT	human telomerase reverse transcriptase keratinocytes
GE	gingival epithelial keratinocytes
PBMCs	peripheral blood mononuclear cells
PI	propidium iodide
MFI	median fluorescence intensity
LD_{50}	lethal dose 50

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HIGHLIGHTS

- HBD3 is a very versatile host defense peptide.
- It has antimicrobial activities, regulates innate immune mechanisms, and regulates chemokine and cytokine responses.
- HBD3 also has cytotoxicity against eukaryotic cells
- We show that the cytotoxicity of HBD3 for DCs, NHEKs, hTERT keratinocytes, and GE keratinocytes varied with the cell type and the culture conditions.
- Thus HBD3 has some cytotoxicity, which needs to be considered in future studies of HBD3-modulated chemokine and cytokine responses.

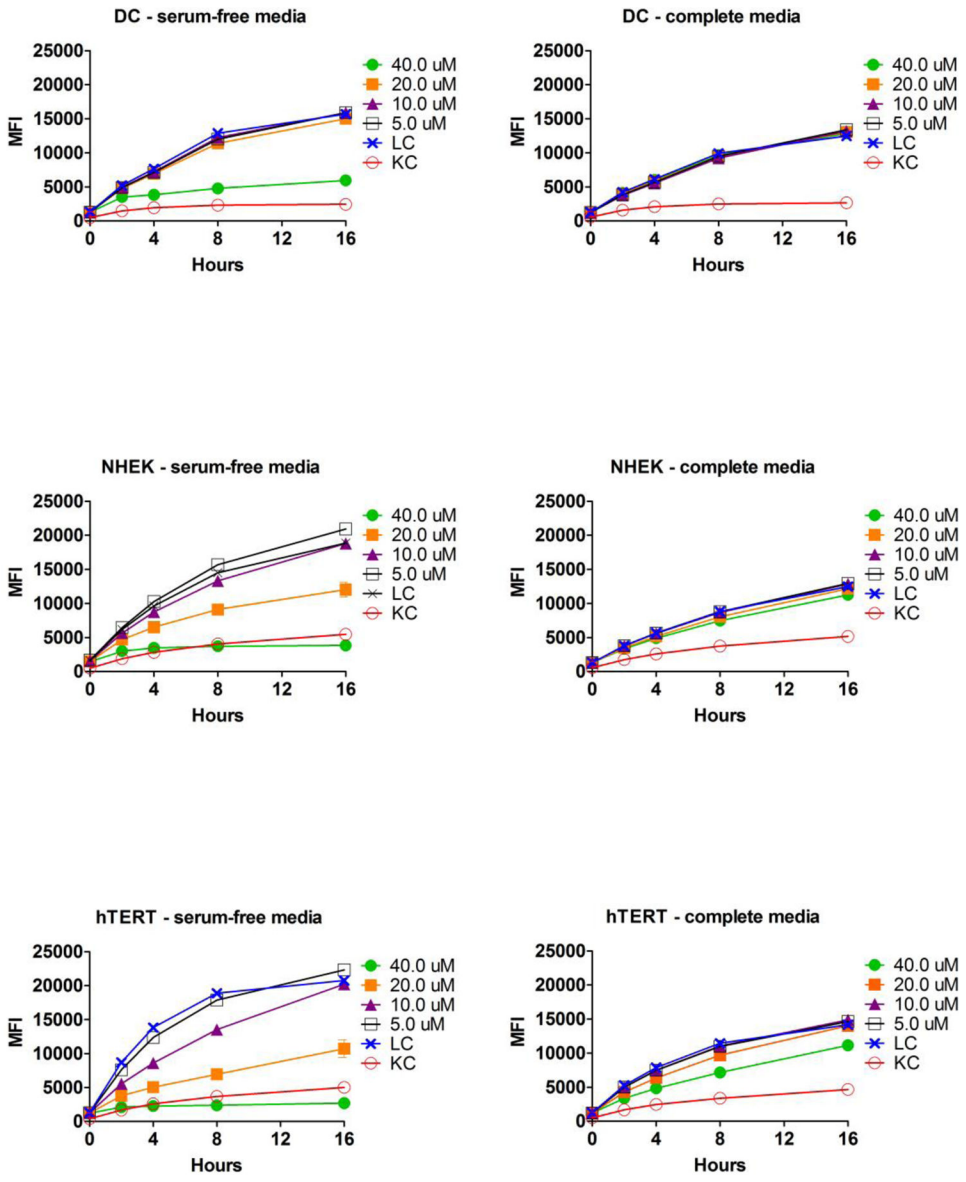

Figure 1.

Median fluorescent intensity (MFI) of DCs (a, b), NHEKs (c, d), and hTERT keratinocytes (e, f) at 5.0 to 40.0 μM HBD3 in serum-free media (a, c, e) and complete media (b, d, f) at 0, 2, 4, 8, and 16 h post exposure. All assays were performed in triplicate. Abbreviations: DCs = dendritic cells; NHEKs = normal human epidermal keratinocytes; LC = live cells; KC = killed cells.

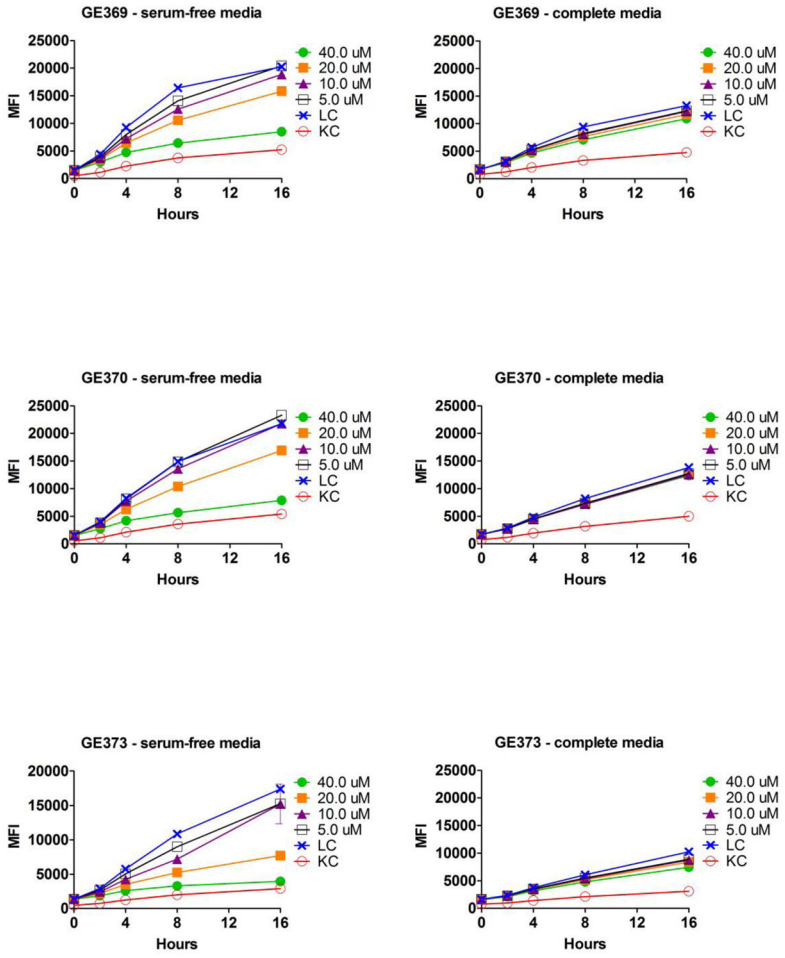

Figure 2.

Median fluorescent intensity (MFI) of GE369 keratinocytes (a, b), GE370 keratinocytes (c, d), and GE373 keratinocytes (e, f) at 5.0 to 40.0 μM HBD3 in serum-free media (a, c, e) and complete media (b, d, f) at 0, 2, 4, 8, and 16 h post exposure. All assays were performed in triplicate. Abbreviations: GE = gingival epithelial keratinocytes; LC = live cells; KC = killed cells.

Table 1

Lethal Dose 50 (LD_{50}) values of HBD3 for DCs and keratinocytes at 16 hours. The percent cytotoxicity was first determined as [the median fluorescence intensity (MFI) of resazurin in cell culture media of cells treated with dilutions of HBD3/MFI of resazurin in cell culture media of LC] × 100. The LD_{50} values were then determined from the dose response curve where the 50 percent cytotoxicity intercepts with the HBD3 concentration on the x-axis.

Cell types[a]	Complete media LD50 values of HBD3 (mean μM)[b]	Serum-free media LD50 values of HBD3 (mean μM ± std err)[b]
DCs	> 40.0	35.94 ± 0.47
NHEKs	> 40.0	25.84 ± 1.73
hTERT keratinocytes	> 40.0	21.56 ± 2.21
GE369 keratinocytes	> 40.0	34.26 ± 0.71
GE370 keratinocytes	> 40.0	33.37 ± 0.97
GE373 keratinocytes	> 40.0	18.19 ± 1.00

[a] DCs = dendritic cells; NHEKs = normal human epidermal keratinocytes; GE = gingival epithelial keratinocytes.

[b] LD_{50} values of HBD3 (mean μM ± std err) for DCs and keratinocytes from 3 replications.

Table 2

Characterization of gingival epithelial (GE) keratinocytes isolated from healthy gingival tissue samples and identified using antibodies to cell surface markers by flow cytometry procedures.

Keratinocytes[a]	CD83[b]	CD28[c]	Cytokeratin[d] (14,15,16,19)	Pancytokeratin[e] (4,5,6,8,10,13,18)	CD24[f]	CD104[g]
GE369	0	0	61.0–69.2%	3.6–12.7%	18.5–29.7%	13.8–34.4%
GE373	ND	ND	94.8–96.5%	4.9–6.3%	16.8–18.9%	3.6–4.5%

[a] GE = gingival epithelial keratinocytes.

[b] BV421 DC marker.

[c] BV510 T-cell marker.

[d] PE KER (keratinocyte cytoplasmic) marker.

[e] A647 KER (keratinocyte cytoplasmic) marker.

[f] PE-CY7 KER (keratinocyte surface) marker.

[g] FITC KER (keratinocyte surface) marker, B4 integrin.

Table 3

Reported *In vitro* studies of the cytotoxicity of HBD3.

HBD3 (μM)	Cell type	Endpoint	Toxicity	References
0.04	Human periodontal ligament fibroblasts	Cell viability	No	Wang et al., 2011
0.58–19.40	Rabbit erythrocytes	Hemolysis	No	Liu et al., 2008
2.42	Human conjunctival epithelial cells	50% metabolic kill	Yes	Liu et al., 2008 Zhou et al., 2011
2.42	THP1 human monocytic cells	LDH release	Yes	Saito et al., 2012
3.88	Peripheral blood mononuclear cells	Cell membrane permeation	Yes	Lioi et al., 2012
4.84	H441 human lung adenocarcinoma epithelial cells	LDH release	Yes	Saito et al., 2012
7.78	Peripheral blood mononuclear cells, T cells and osteosarcoma cells	MTT assay	No	Quinones-Mateu et al., 2003
9.69	A549 human adenocarcinomic alveolar basal epithelial cells	50% cell membrane permeation	Yes	Saito et al., 2012
14.54	A549 epithelial cells	LDH release	Yes	Saito et al., 2012
96.94	Human erythrocytes	15% hemolysis	Yes	Harder et al., 2001

LDH = lactate dehydrogenase; MTT assay = Thiazolyl blue-based colorimetric assay.