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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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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.

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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*.

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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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 5157.7 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