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Functional intersection of Human Defensin 5 with the TNF receptor pathway

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ABSTRACT

Defensins are cationic antimicrobial peptides that contribute to regulation of host cell function also. Here, we report on the regulation of cell death by Human Defensin 5, the major antimicrobial peptide of ileal Paneth cells. We find that Human Defensin 5-mediated cellular effects depend on functional expression of Tumor Necrosis Factor receptors and downstream mediators of TNF signaling. Our data indicate the involvement of interactions between Human Defensin 5 and the extra-cellular domain of Tumor Necrosis Factor receptor 1. Human Defensin-5 also induces apoptosis intrinsically by targeting the mitochondrial membrane.

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1. Introduction

Defensins are endogenous peptides produced by certain leukocytes and epithelial cells [1–3]. These peptides demonstrate antimicrobial, antiviral, toxin-neutralizing and immuno-modulatory properties [3–7]. They are small, cationic, beta-sheet, tri-disulphide peptides [8,9] and, in humans, comprise genetically distinct alpha and beta subfamilies that evolved from a common ancestral gene [10,11]. In humans, six α-defensins have been identified to date, termed Human Neutrophil Peptide 1–4 and Human Defensin 5 and -6. Human Neutrophil Peptides (HNP1-3) differ from each other by an N- terminal amino acid residue and were originally isolated from azurophilic granules of neutrophils [12,13]. HNP4, dissimilar to HNP1-3 and much less abundant, was found later in neutrophils [14–16], followed by the discovery of the two enteric α-defensins (HD-5 and HD-6), expressed in Paneth cell of the small intestine [17,18].

Defensins also act in adaptive immunity by serving as chemoattractants and activators of immune cells [2,19]. For example, HNP-2 and hBD2 are chemotactic to $CD4^+/CD45RA^+$ naive T cells, $CD8^+$ T cells, immature dendritic cells and monocytes/macrophages [20–22]. For hBD2, chemotaxis of immature dendritic cells and memory T cells results from its direct binding and activation of the chemokine receptor CCR6 who's only known chemokine-ligand is MIP-3α [23]. Members of the β-defensin family have been further shown to interact with Toll-Like Receptor 4 [24] and more recently the melanocortin 1 receptor, causing black coat color in domestic dogs [25]. For most defensins, however, their cognate receptors in adaptive immune responses have yet to be identified. The only member of the human α-defensin family to date with reported receptor interactions is HNP1. Interaction of HNP-1 with the purinergic receptor P2Y on airway epithelial cells has been reported [26]. In addition, HNP-1 was shown to inhibit monocyte differentiation, partly due to interactions with the P2Y6 receptor [27]. More recently, the activation of macrophages by HNP-1 was shown to be insensitive to pertussis toxin, as well as independent of purinergic receptors, Toll-like Receptor and CD18 signaling [28] suggesting these effects may be cell type dependent.

We previously reported that HD-5 signaling is MAPK- and NF-κB-dependent and that HD-5 signals together with TNFα in a synergistic fashion [29,30]. Here, we report on the involvement of TNFα receptors on HD-5-mediated cellular effects. We first examined the effects of HD-5 on murine cells lacking TNF receptor genes (TNFR1 and 2 knockout). More detailed involvement of receptors was examined in cells that express human TNF receptors carrying deletions or mutations in their extracellular domains. Cell viability, apoptosis, signaling, caspase activation and mitochondrial membrane activity was measured in cells deleted for TNF receptor downstream effectors RIP1 kinase and caspase-8.

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2. Material and methods

2.1. Solid phase peptide synthesis

Chemical synthesis of HD-5 was carried out as described [31]. FAM-HD-5, a fluorescein-labeled form of HD-5 was prepared as follows: N-hydroxysuccinimide-activated FAM was added to HD-5 peptide at 3:1 molar ratio in 50% Di-Methyl Formamide (DMF), 0.1 M HEPES pH 7.3 at a final peptide concentration of 8 mg/ml. The reaction was carried out for 2 h and labeled peptide was repurified by HPLC. The molecular mass of the peptides was verified by electrospray ionization mass spectrometry (ESI-MS) as described [31].

2.2. Cell culture

The Jurkat and the capsase-8 deficient Jurkat cell line I9.2 were obtained from the American Type and Tissue Culture (ATTC). The RIP1-deficient Jurkat cell line termed TM2 [32] was generously provided by Dr. Ting (Mount Sinai Hospital, New York). Wild-type and TNF receptor double knockout murine macrophages [33] were generously provided by Dr. Aggarwal (University of Texas MD Anderson Cancer Center). TNF receptor 1 and 2 double knockout murine macrophages expressing the extra-cellular domain of TNFR1 fused to FAS (TNFR1-FAS), the extra-cellular domain of TNFR1 with the first cysteine-rich domain deleted (ΔTNFR1-FAS) and the extra-cellular domain with the cysteine-rich domain of TNFR2 replacing that of TNFR1 ($CRD1_{TNFR2}$-TNFR1-FAS) were generously provided by Dr. Scheurich (University of Stuttgart, Germany [34]). Jurkat TM2 cells were passaged in Iscove's modified Dulbecco's medium with 10% iron- supplemented calf serum, 0.25 mg/ml xanthine, 0.1 mM hypoxanthine, and 5 μg/ml mycophenolic acid. For all experiments, the wild-type and KO12 murine macrophages and the Jurkat cell lines were maintained in RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical, Winchester, VA), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1 × non-essential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2. The TNFR1-FAS, ΔTNFR1-FAS and $CRD1_{TNFR2}$-TNFR1-FAS cells were maintained with 5% FBS RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical, Winchester, VA), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1 × non-essential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2.

2.3. Cell viability

The effect of defensin peptides on cell viability was assessed by measuring the mitochondrial activity using MTS assays according to the manufacturer's instructions (Cell Titer 96 proliferation assay, Promega). The cells were cultured in 96-well plates in the presence of serum, washed once in culture medium without serum and subsequently exposed to the peptides at the indicated concentration for 16 h. The number of viable cells was determined by measuring the absorbance at 450 nm on a microplate reader (Molecular Devices).

2.4. Evaluation of cytokine secretion

Jurkat cells were gently washed in serum-free medium and subsequently plated in 96-well plates at 1×10^6 cells/ml in serum-free medium. Cells were incubated for 16 h in the presence of HD-5 peptide at indicated concentrations. Culture supernatants were collected for measurement of IL-8 using the Luminex-100 system (Bio-Rad).

2.5. Apoptotic assays

Caspase-8 and caspase-9 activity in cell lysates were measured using the fluorogenic Ac-VETD-AMC peptide substrate (Sigma) or the caspase-9 colorimetric kit (Invitrogen) according to manufacturer's instructions. For these experiments, cells were cultured on 6-well tissue culture plates. Cells were washed with serum-free medium and incubated for 16 h in serum-free medium containing the peptides at final concentrations as indicated. Following incubation, cells were washed with PBS and lysed in 200 μl of lysis buffer (20 mM HEPES, 50 mM NaCl, pH 7.2 10 mM DTT containing 1% CHAPS, 1 mM EDTA, 2 mM PMSF, leupeptin (10 μg/ml; Sigma) and pepstatin A (10 μg/ml; Sigma) for 30 min on ice. After centrifugation (7000×*g*, 10 min), the protein concentration of the supernatant was determined using the BCA Protein assay reagent (Bio-Rad). Subsequently, 20 μg of each sample was diluted to a final volume of 200 μl in assay buffer (20 mM HEPES, 50 mM NaCl, pH 7.2 10 mM DTT, 0.1% CHAPS containing either the caspase-8 or caspase-9 substrate) in a 96-well plate. For caspase-8, fluorescence was determined (excitation 405 nm, emission 538 nm) with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). For caspase-9 activity, absorbance at 450 nm was measured.

2.6. Flow cytometry

Apoptosis in Jurkat cells was determined using the Annexin-FITC apoptosis kit from Calbiochem according to manufacturer protocol. Jurkat, Jurkat I9.2 or Jurkat TM2 cells (2×10^5 cells/ml) were exposed to peptide in serum-free medium for 16 h and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences).

2.7. Confocal microscopy

Jurkat cells or murine macrophages were cultured on collagen-coated cover glass slips (Mylteni Biotech) as described above for 24 in the presence of serum. The cells were gently washed twice in serum-free medium and incubated at 4 °C for 90 min. Subsequently, cells were exposed to FAM-HD5 (10 μg/ml) and either fixed immediately with 4% formaldehyde or further incubated at 37 °C for 30 min before fixation. The localization and visualization of FAM-HD5 on the cells was analyzed using fluorescence confocal microscopy (Zeiss).

2.8. Cell membrane integrity and ATP release assays

Membrane integrity and ATP release was determined using the mitochondrial ToxGlo assay (Promega) according to manufacturer's instructions. For these experiments, cells were cultured on 96-well tissue culture plates, gently washed with serum-free medium and subsequently incubated for 16 h in serum-free medium containing the peptides at final concentrations as indicated.

3. Results

3.1. Interaction of HD-5 with cells of the host

To study the functional interaction between HD-5 and TNF receptor-mediated events, we first determined the effects of functional expression of TNF receptors on the ability of HD-5 to induce cell death (Fig. 1). Cells expressing the TNF receptor 1 and 2 or cells that do not express these receptors (KO12) were exposed to HD-5.