Structure-dependent functional properties of human defensin 5

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Abstract The mucosal epithelium secretes a variety of antimicrobial peptides that act as part of the innate immune system to protect against invading microbes. Here, we describe the functional properties of human defensin (HD) 5, the major antimicrobial peptide produced by Paneth cells in the ileum, in relation to its structure. The antimicrobial activity of HD-5 against *Escherichia coli* proved to be independent of its structure, whereas the unstructured peptide showed greatly reduced antimicrobial activity against *Staphylococcus aureus*. We find that HD-5 binds to the cell membrane of intestinal epithelial cells and induced secretion of the chemokine interleukin (IL)-8 in a concentration- and structure-dependent fashion. Incubation of HD-5 in the presence of tumor necrosis factor alpha further increased IL-8 secretion synergistically, suggesting that HD-5 may act as a regulator of the intestinal inflammatory response.
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Keywords: Human defensin 5; Intestinal epithelium; Interleukin 8; TNF-α

1. Introduction

Epithelial cells of the mucosa form a barrier between the gut lumen and underlying host tissues. In addition to this barrier function, epithelial cells perform a key role in host innate and adaptive responses, secreting of a wide range of immunomodulatory molecules [1,2]. Paneth cells are specialized ileal epithelial cells located at the crypt base in close vicinity of multipotent stem cells and fulfill a crucial role in innate immunity. They are a source of several antimicrobial enzymes such as lysozyme and group IIA phospholipase A2 (PLA2) as well as the antimicrobial peptides human defensin 5 and 6 (HD-5 and HD-6), which are stored in secretory granules [3,4].

Defensins are small, cationic peptides with a characteristic β-sheet-rich structure stabilized by three internal disulfide bonds [5]. Based on the connectivity of the six cysteine residues, human defensins are classified into α and β subfamilies [6–8]. In humans, six α-defensins have been described: HD-5 and HD-6 and the human neutrophil peptides 1–4 (HNP1–4) expressed predominantly in neutrophils and natural killer cells [9]. Both the α- and β-defensin gene families evolved from an ancestral β-defensin gene and distinct clusters of both families are found adjacent on the chromosomal maps of all mammals expressing α- and β-defensins [10,11].

Murine intestinal epithelium expresses at least 20 isoforms of α-defensins, termed cryptdins [12,13]. Procryptdins are processed to mature peptides by the matrix metalloproteinase matrilysin, which is co-expressed within the Paneth cell secretory granules [14]. In contrast, HD-5 is stored in its pro-form and is further processed to the mature peptide after secretion. Pro-forms of HD-5 can be processed in vitro by trypsin, which is also expressed in Paneth cells [15]. In addition to their antimicrobial properties, defensins have chemokine-like activities [16]. Members of both α- and β-defensins act as chemotactic attractants for human monocytes and subsets of dendritic cells and T cells [17,18], however, their effect on intestinal epithelial cells is not very well characterized. Here, we examine the antibacterial activity and the interaction with intestinal epithelial cells of HD-5 in relation to its structure.

2. Materials and methods

2.1. Materials

Chemicals used for solid phase peptide synthesis were obtained as described [19]. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were from Microbiologics (St. Cloud, MN). Caco-2 cell line was obtained from the American Type Culture Collection (Manassas, VA).

2.2. Solid phase peptide synthesis

Chemical synthesis of HD-5 and HD-5Abu, a linear, unstructured form of HD-5 in which the six cysteine residues are replaced by isosteric α-aminobutyric acid (Abu) was carried out as described [19]. Folding of HD-5 was carried out as described [19]. The molecular mass of the peptides was verified by electrospray ionization mass spectrometry (ESI-MS) as described previously [19]. 5-Carboxyltetramethylrhodamine (Molecular Probes, Eugene, OR) was coupled to HD-5 as follows: 2.0 mg HD-5 was dissolved in 1.0 ml 0.1 M $NaHCO_3$, pH 8.3, 0.2 ml of 5-carboxyltetramethylrhodamine (10 mg/ml in DMSO) and 40 μl di-isopropyl-ethylamine (DIEA) were then added. After stirring for 2 h at room temperature, the reaction mixture was filtered and purified by reverse phase HPLC. The molecular mass was verified by ESI-MS as described above.

2.3. Antibacterial activity assay

The antibacterial activity of HD-5 and HD-5Abu against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 was carried out in a 96-well turbidimetric assay as described previously [20].

2.4. Evaluation of IL-8 secretion by Caco-2 cells

Subconfluent monolayers of Caco-2 cells were maintained in RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical,

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Abbreviations: HD, human defensin; HNP, human neutrophil peptide; IL, interleukin; TNF, tumor necrosis factor

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Winchester, VA), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1× nonessential amino acids, 1 mM sodium pyruvate and 5% penicillin/streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2. Caco-2 cells were used between passages 35 and 42. Caco-2 cells were plated at a density of 4×10^4 cells/cm^2 in a 96-well plate 48 h before use. The cells were gently washed twice with serum-free medium and incubated for a further 18 h in serum-free medium containing the peptides at a final concentration of 50 or 100 μg/ml. In the presence of serum, the induction of IL-8 secretion was not observed (not shown). Human recombinant tumor necrosis factor alpha (Sigma; 100 ng/ml) was included during incubation with the peptides (100 μg/ml) as indicated. The culture supernatant was collected for measurement of IL-8 using the Luminex-100 system (Bio-rad Laboratories).

2.5. Confocal Microscopy

Caco-2 cells (10^4 cells) were cultured on glass cover slips as described above for 24–48 h. The cells were washed twice in serum-free medium and incubated with rhodamine-HD5 (10 μg/ml) for 3 h. After incubation, cells were washed twice with Hanks' balanced salt solution (HBSS). The localization of rhodamine-HD5 on Caco-2 cells was visualized using a Zeiss Laser Scanning Microscope (LSM) 510 system (Carl Zeiss MicroImaging Inc., Thornwood, NY). Fluorescence was excited using a helium–neon laser (543 nm). Emission was passed through a 560 nm long-pass filter prior to acquisition. Optical sections were 1 μm thick.

3. Results

3.1. Chemical synthesis of HD-5 peptides

The HD-5 structure involves three intra-molecular disulfide bonds [19]. To determine the structure of HD-5 in relation to its function, a HD-5 derivative peptide was synthesized, in which the six cysteine residues were replaced with L-α-aminobutyric acid (HD-5Abu), thus preventing the formation of disulfide linkage while leaving the peptide sequence otherwise unaltered. Folded and purified HD-5 and purified HD-5Abu were analyzed on C18 RP-HPLC (Fig. 1A and B). HD-5 was less hydrophobic than HD-5Abu, as indicated by their relative retention time on C18 RP-HPLC. The molecular mass of both peptides was confirmed by ESI-MS (Fig. 1C and D). The observed molecular masses of 3582.0 ± 0.5 Da for HD-5 and 3480.7 ± 0.3 Da for HD-5Abu agree with the calculated average isotopic values of 3582.2 and 3480.2 Da, respectively.

3.2. Antimicrobial activity of HD-5 peptides

The antimicrobial activity of both peptides was examined against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 (Fig. 2A). As described previously [20], HD-5 efficiently killed both bacterial strains and, at comparable peptide concentration, proved more toxic towards *S. aureus* as compared to *E. coli*. At the highest peptide concentration tested (125 μg/ml), *S. aureus* appeared unable to recover from the 2 h incubation with HD-5. HD-5Abu was comparable to HD-5 in antimicrobial activity towards *E. coli*, and was even slightly more efficient in killing at higher concentrations. Surprisingly, killing of *S. aureus* by HD-5Abu was 4–5 orders of magnitude less efficient than killing by HD-5 at comparable peptide concentration.

To evaluate the salt dependence of HD-5 bacterial killing, the antimicrobial assay was performed at increasing sodium chloride concentrations (Fig. 2B). For these experiments, a fixed peptide concentration of 100 μg/ml was used against

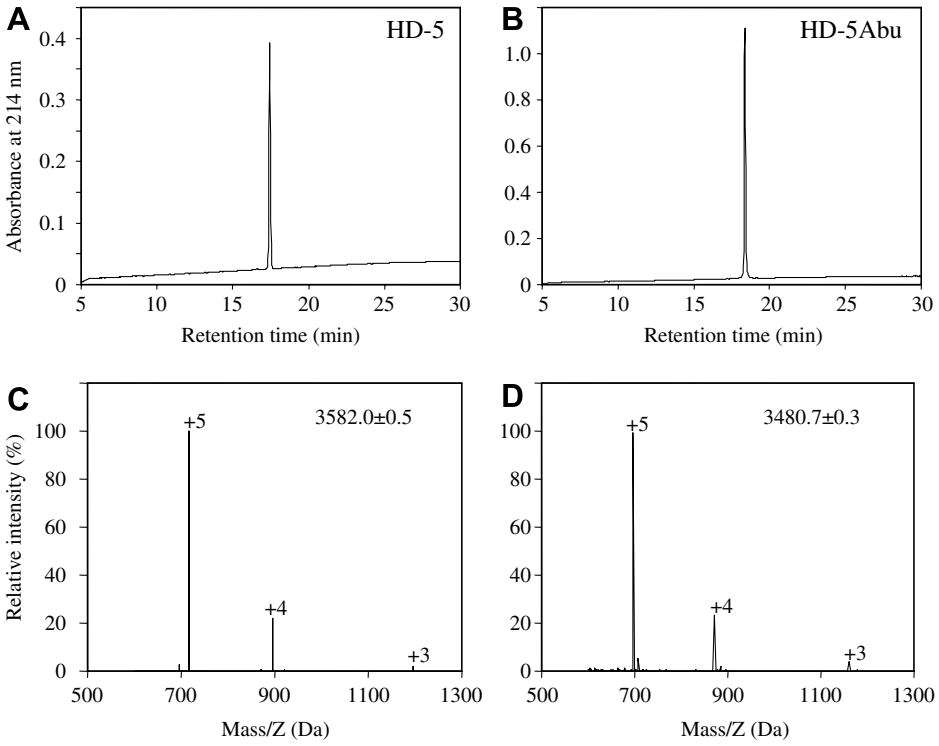

Fig. 1. Folded and purified HD-5 and HD-5Abu analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The HPLC analysis was carried out at 40 °C using a linear gradient of 15–60% (solvent A: water + 0.1% TFA; solvent B: acetonitrile + 0.1% TFA) at a flow rate of 1 ml/min over 30 min. The determined molecular masses were within experimental error of the expected values based on calculations of the average isotopic compositions.