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Neutralization of *Pseudomonas auruginosa* Exotoxin A by human neutrophil peptide 1

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ABSTRACT

Pseudomonas aeruginosa produces a large number of virulence factors, including the extracellular protein, Exotoxin A (ETA). Human Neutrophil Peptide 1 (HNP1) neutralizes the Exotoxin A. HNP1 belongs to the family of α-defensins, small effector peptides of the innate immune system that combat against microbial infections. Neutralization of bacterial toxins such as ETA by HNP1 is a novel biological function in addition to direct killing of bacteria. In this study, we report on the interaction between HNP-1 and Exotoxin A at the molecular level to allow for the design and development of potent antibacterial peptides as alternatives to classical antibiotics.

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1. Introduction

Human defensins constitute a family of innate immune effector peptides with the ability to act against a variety of microbial pathogens, including bacteria, fungi and viruses [1,2]. In addition to their antimicrobial activities, defensins play a significant role in adaptive and innate immunity. Defensins chemoattract a variety of host cells [3–5] and have the ability to modulate host cell cytokine responses [6,7]. In humans, defensins have been classified based on a difference in disulfide linkage of conserved cysteines in two families, termed α and β. Both families have likely evolved from a common ancestral β-defensin gene [8,9], and in spite of variation in primary amino acid sequence, share similar tertiary structures [10–12]. Defensins of the α-family are expressed predominantly in specialized intestinal epithelial cells called Paneth cells in the case of Human Defensin 5 and 6 (HD-5 and HD-6) or in neutrophils (termed human neutrophil peptides or HNPs) [13,14]. HNPs are located inside of azurophilic granules that fuse with endosomes containing potential pathogens, but can also be detected outside of the cell. It was assumed that the antibacterial properties of these peptides relied on their ability to disrupt prokaryotic membrane structures due to their cationic properties, however in recent years, additional and alternative mechanisms-of-action have been revealed. Defensins, including HNP-1 were shown to bind to the cell wall precursor Lipid II as a more specific way of targeting the bacterial membrane [15–17]. In addition, HNP-1 was shown to have the ability to neutralize bacterial toxins that are actively secreted by pathogens, including diphtheria toxin, anthrax lethal factor and *Pseudomonas aeruginosa* exotoxin A or ExoA [18,19]. Here, we describe the interaction between Human Neutrophil peptide-1 or HNP-1 with ExoA in molecular detail. .

2. Material and methods

2.1. Solid phase peptide synthesis

Chemical synthesis of HNP-1 and HNP-1 single alanine mutants was carried out as described [20]. The molecular mass of the peptides was verified by electrospray ionization mass spectrometry (ESI-MS) as described [21]. *Pseudomonas aeruginosa* Exotoxin A was purchased from Calbiochem, Inc.

2.2. Cell culture

Subconfluent monolayers of HeLa cells 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 x nonessential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2. HeLa cells were used between passages 18–35 and were plated at a density of 4×10^5 cells per cm^2.

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2.3. Cell survival assay

HeLa cells were cultured in 96-well plates in the presence of serum. Cells were gently washed twice with serum-free medium and incubated for a further 6 h in serum-free medium containing the HNP-1 peptides at a final concentration of 5 μg/ml in the absence or presence of 100 ng/ml of Exotoxin A. The effect of defensin peptides on HeLa 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 number of viable cells was determined using a standard curve of serially diluted untreated cells in each experiment by measuring the absorbance at 450 nm on a microplate reader (Molecular Devices). Data presented are the average of three experiments.

2.4. Surface plasmon resonance binding

Experiments were performed on a BIAcore T100 System (BIAcore, Inc., Piscataway, NJ), unless stated otherwise, at 25 °C in 10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20, pH 7.4 (±3 mM EDTA). Pa Exotoxin A was immobilized on a CM5 sensor chip at a level of ~250 response units (RU) by the amine-coupling protocol. The HNP-1 analytes (125 nM−1.9 nM) were introduced into the flow-cells at 30 μl/min in the running buffer. Association and dissociation were assessed for 5 and 10 min, respectively. Resonance signals were corrected for nonspecific binding by subtracting the background of the control flow-cell. After each analysis, the sensor chip surfaces were regenerated with 50 mM KOH and equilibrated with the buffer before next injection. Binding isotherms were analyzed with BIAevaluation software.

3. Results

3.1. HNP-1 neutralizes exotoxin A-induced cell death

To determine the capacity of HNP-1 to neutralize the toxic effects of ExoA, we first determined the killing of HeLa cells by the toxin protein. HelA cells were exposed to ExoA in increasing concentration ranging from 50 to 500 ng/ml (Fig. 1A). At a concentration of 100 ng/ml of the toxin, ~40% of the cells survived and cell survival did not significantly alter at higher concentrations of the toxin tested. We therefore used 100 ng/ml as the concentration of ExoA in all subsequent experiments. Next, we exposed HeLa cells to increasing concentrations of wild-type HNP-1 ranging from 2 to 8 μM in the presence of 100 ng/ml of ExoA (Fig. 1B). At increasing concentrations of HNP-1, we observed an increase of cell survival from ~40% in the absence of HNP-1 to ~60% in the presence of peptide. This increase was dose-dependent up to a concentration of ~5 μM and subsequently decreased as higher peptide concentrations. This decrease was due to the observation that the HNP-1 peptide alone showed cell killing at concentrations of 8 μM and higher (data not shown). Together, these data show that HNP-1 functionally neutralizes ExoA *in vitro*.

To gain insight in the interaction between HNP-1 and ExoA in detail, we determined the effect of single alanine substitutions in the HNP-1 sequence on the ExoA neutralizing capacity. We first determined the effect on HeLa cell survival following incubation of HNP-1 single alanine mutants at a concentration of 5 μM, since at this concentration, no significant cell killing by wild-type HNP-1 was observed (Fig. 2A). As shown in Fig. 2A, single alanine substitutions in the HNP-1 sequence mostly had no significance on the direct killing of HeLa cells by these peptides. Notable exceptions were the E13A, Q22A and G23A substitutions. Alanines at these

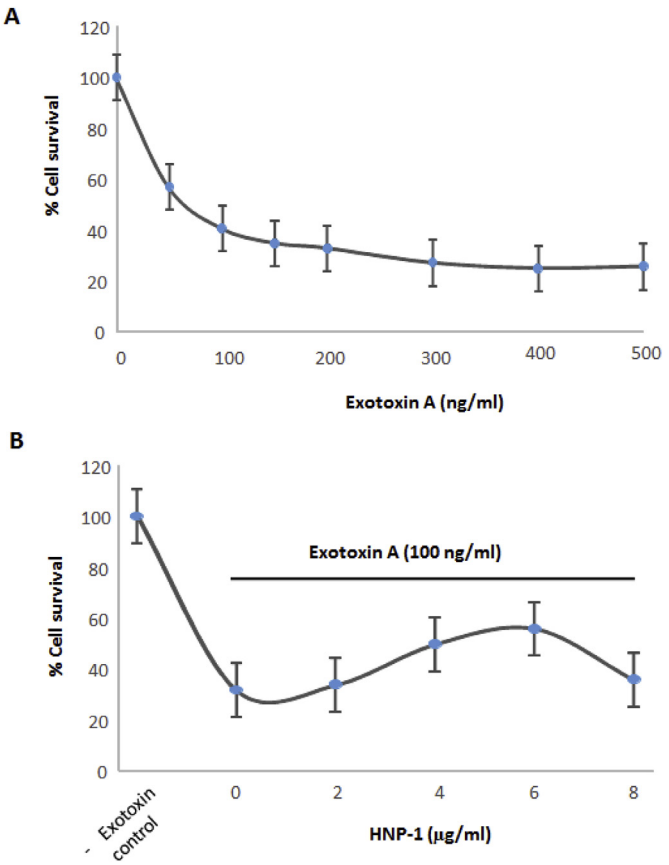

Fig. 1. Neutralization of P. aeruginosa Exotoxin A by HNP-1. Panel A: Percent cell survival of HeLa cells exposed to ExoA at the indicated concentrations. Cells were incubated for 16 h and survival was determined by MTS assay.
Panel B: Percent cell survival of HeLa cells exposed to HNP-1 at the indicated concentrations in the absence or presence of 100
ng/ml of ExoA. Cells were incubated for 16
h and survival was determined by MTS assay. For both assays, untreated cells were set at 100% and data presented are average of three experiments.

positions did cause significant cell killing, indicating that these residues are involved in the cytotoxic profile of HNP-1 itself. We next determined the ability of single alanine mutants to rescue ExoA-mediated cell killing (Fig. 2B). As before, wild-type HNP-1 increased the percentage of cell survival from ~40 to ~60%. Out of all the peptides tested, only the E13A and the Q22A substitutions results in rescue of cell survival similar to the wild-type peptide (Fig. 2B).

The increased levels of cell survival observed with specific HNP-1 mutants could be due to altered cytotoxicity of these peptides. Alternatively, these effects could be mediated trough altered interaction with ExoA. To distinguish between these two possibilities, we determine the binding of HNP-1 peptides to ExoA by surface plasmon resonance (Table 1). Wild-type HNP-1 peptide bound to ExoA with a binding affinity of ~40 nM. Compared to the wild-type, the E13A mutation did not significantly alter binding to ExoA, suggesting that this position is mostly important for direct cytotoxicity of the peptide itself. In contrast, changing glutamine at position 22 to alanine increased the binding affinity to ExoA two-fold, suggesting that this residue is important in the interaction between the defensing and the toxin. In addition to E22, several more residues showed significant changes in binding affinity to ExoA, notably, I6, Y16 and W26 (Table 1).

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