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Antimicrobial peptides are degraded by the cytosolic proteases of human erythrocytes

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ABSTRACT

Well-studied and promising antimicrobial peptides (AMPs), with potent bactericidal activity, *in vitro*, have yet to have a significant impact in human medicine beyond topical applications. We previously showed that interactions of AMPs with concentrated human erythrocytes inhibit many of them, and suggested that screens and assays should be done in their presence to mimic host cell inhibition. Here, we use AMPs to characterize the activity of proteases that are associated with human erythrocytes. The representative AMPs, ARVA and indolicidin, are degraded significantly during incubation with dilute, washed erythrocytes and yield a variety of degradation products, suggesting significant exopeptidase activity. Comparison of these fragments with those obtained from incubation with serum shows that the proteolytic activity associated with cells yields unique products that are not explained by residual serum proteases. By separately testing the membrane and cytosolic fractions, we show that erythrocyte proteolytic activity is found only in the cytosol. Finally, we incubated a diverse cross-section of natural and synthetic linear AMPs with human erythrocyte cytosolic extracts and observed degradation of all of them. These results show that, in addition to cell binding, proteolysis can also contribute significantly to host cell inhibition of AMPs *in vitro* and possibly also *in vivo*.

1. Introduction

Peptide drug candidates possess several characteristics that complicate their development as therapeutics. A non-exhaustive list includes bioavailability [1], cytotoxicity [2], rapid metabolism *via* degradation [3] and systemic clearance [4]. Despite these issues, a number of peptide drugs, including the HIV fusion inhibitor enfuvirtide [5] and the peptide hormones insulin and glucagon, used in the control of diabetes mellitus [6], play important roles in the clinical management and treatment of disease. Further, as of 2015, > 100 peptide therapeutics were in clinical trials for wide spectrum of clinical conditions, a number that is expected to increase in the future [7]. It has also been shown that cell-penetrating peptides (CPPs) and other peptide delivery vehicles may be used to deliver macromolecules such as oligonucleotides [8] and promising small molecules to their intracellular targets [9]. Results like these combined with a vast available sequence space will continue to drive peptide-based biomedical research.

One of the more promising classes of peptide drugs are the antimicrobial peptides (AMPs), which are key effectors of innate immunity for organisms in many clades of the animal kingdom [10]. Since their discovery several decades ago [11,12], researchers have studied whether their potent antimicrobial and immunomodulatory effects could be harnessed and amplified for applications in clinical medicine. Despite the vast amount of research that has been conducted and the several thousand active sequences, both natural and synthetic, that have been discovered [13], progress thus far is not encouraging, as there are few FDA approved antimicrobial peptides [14]. As opposed to traditional antibiotics that often target specific enzymes and biosynthetic processes, AMPs usually exert their antimicrobial effects through interactions with and destabilization of the bacterial cytoplasmic membrane [2]. In favor of AMPs, this less specific mechanism often conveys broad-spectrum antimicrobial activity and is not as susceptible to the emergence of resistant phenotypes [15]. Conversely, this lack of specificity can lead to off-target effects, including the potential to harm symbiotic microflora, cytotoxicity to eukaryotic cells, and loss of activity under physiological conditions, complicating their transition into clinical medicine [16].

Recently, we investigated a critical barrier to the clinical activity of antimicrobial peptides: interactions with host cells, modelled with concentrated human erythrocytes [17]. While it is well established that eukaryotic cells may be permeabilized by certain AMPs, we sought to test if, for at least some AMPs, *interactions* with host cells can inhibit

Abbreviations: RBC, red blood cell; AMP, antimicrobial peptide

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their activity against prokaryotes when both cell types are present, at least when AMPs encounter human cells prior to bacteria. By comparing the behavior of the L and D amino acid isomers of the synthetic, 12-residue peptide, ARVA [18], along with several other well-studied AMPs, we demonstrated that host cell interactions, including binding, have the potential to be major contributors to AMP activity loss *in vivo*. We concluded that the processes of screening for, and characterization of, potentially clinically active AMPs could be improved by consistently including concentrated host cells in these assays, creating an environment more representative of that encountered *in vivo*. Continuation of this work has revealed that even peptides that do not strongly associate with eukaryotic membranes may be subject to diminished antimicrobial efficacy in the presence of host cells. Here, we show that proteolysis can also be a significant factor in AMP activity loss in our suggested model system of human erythrocytes plus AMPs and bacteria. This activity cannot be attributed to serum proteases, which are the most common concern for AMPs and peptide drugs in general. Instead, we demonstrate that proteolytic degradation of peptides in our system was caused by unique enzymes directly associated with the cytosol of human erythrocytes.

While the presence of proteolytic activity associated with RBCs is well established, it has not been examined in the context of antimicrobial peptides, or peptide therapeutics in general. We show here that these proteases are able to degrade antimicrobial peptides with seemingly low sequence specificity, as evidenced by the variety of fragments produced. Further, we show definitively that this activity is directly associated with the erythrocytes themselves and is not a residual component of serum. The proteolytic activity is initially sequestered in the cytoplasm and thus may be innocuous to many potential peptide therapeutics. But given the propensity for AMPs to induce hemolysis, even at low levels, these proteases have the potential to interfere with activity in *in vitro* model systems and perhaps also *in vivo*. Finally, we show that a wide variety of AMP sequence variants, including synthetic and natural AMPs, are susceptible to degradation by this proteolytic activity, although the degradation rates vary substantially.

2. Materials and methods

2.1. Peptides

All peptides used in this study were synthesized using solid-phase FMOC chemistry and purified to > 95% by Bio-synthesis Inc. (Lewisville, TX). Peptides were dissolved in 0.025% acetic acid solution and concentrations were determined by absorbance at 280 nm, if possible. In the absence of tryptophan or tyrosine residues, concentrations were determined by measured weight of peptide and volume of solvent.

2.2. Red blood cell and serum preparation

Human red blood cells and serum were purchased from Interstate Bloodbank (Memphis, TN). Red blood cells were from O + donors and were collected in a citrate phosphate dextrose (CPD) anticoagulant solution. Upon receipt, the cells were washed three times with sterile PBS and stored at 4 °C at a density of 5×10^9 cells/mL until use. Before experiments were performed, cells were diluted to a working concentration of 1.33×10^8 cells/mL and washed three additional times, collecting and storing the supernatant with each wash. Serum was devoid of clotting factors (OTC) and was stored at − 20 °C until use. It was sterile filtered with a 0.22 μm vacuum filter to remove any particulates and precipitates before dilution to 2% of the initial concentration in PBS.

2.3. Preparation of red blood cell cytosolic extracts and membrane ghosts

Red blood cell ghosts were prepared per the method published by Steck and Kant [19]. Approximately 1 mL of washed, packed RBCs were added to a 50-mL centrifuge tube and 40 mL of cold, 5 mM phosphate buffer (pH 8.0) was added to lyse the cells. The lysed cells were rocked while incubating on ice for 30 min and then centrifuged at 18,000 × *g* to separate the membrane fraction from the cytosolic components. At this point, the supernatant was collected to serve as the cytosolic extract for analysis. The membrane fractions were subjected to two additional rounds of washing with cold, 5 mM phosphate buffer and centrifugation at 18,000 × *g*. The membrane fractions were then resuspended in warm PBS (pH 8.0) and incubated with rocking for 45 min at 37 °C to reseal the membranes. The resealed membranes were washed three times with room temperature PBS and centrifuged at 18,000 × *g* following each wash. Both cytosolic and membrane fractions were stored at 4 °C until use.

2.4. Peptide degradation experiments with cells

For each time point, a washed cell suspension was mixed with peptide to give final concentrations of 20 μM peptide and 1.0×10^8 cells/mL. All experiments contained 5 μM FMOC-aspartic acid as an internal HPLC standard. The mixtures were incubated at 37 °C with agitation. At the appropriate time points, the mixtures were centrifuged at 1000 × *g* to pellet the RBCs and the supernatant was removed for analysis by HPLC.

2.5. Peptide degradation with erythrocyte ghosts and cytosolic extracts

Membrane ghosts or cytosolic extracts were prepared as above. To match the cell-based experiments, peptide and cytosol or ghosts were mixed to give 20 μM peptide and a final concentration of cytosol/ghosts equivalent to 1.0×10^8 cells/mL. The mixtures were incubated at 37 °C with agitation. At the appropriate time points, the mixtures were analyzed *via* HPLC.

2.6. Peptide degradation with serum

Serum was diluted to 2% in PBS to match the dilution of the cells used in other experiments. As with other experiments, the final peptide concentration was 20 μM. The mixtures were incubated at 37 °C with agitation. At the appropriate time points, the mixtures were analyzed *via* HPLC.

2.7. HPLC analysis of peptide degradation

Analysis of peptides and degradation products was performed using reversed-phase chromatography. The stationary phase was a 100 mm × 4.6 mm C-18 column from Kromasil (Bohus, Sweden). The mobile phase was composed of a gradient of distilled water (+ 0.1% trifluoroacetic acid) and acetonitrile (+ 0.1% trifluoroacetic acid) with a flow rate of 1 mL/min. Where possible, peptide and peptide fragments were analyzed using tryptophan fluorescence (285ex/340em). In the absence of tryptophan residues, peptide was analyzed by absorbance at 220 nm.

2.8. Fragment collection and mass spectrometry

HPLC was used to collect potential peptide fragments eluted during analysis. Each potential fragment was subjected to MALDI-TOF mass spectrometry using α-Cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Masses observed in this analysis were compared to a list of all potential cleavage products. An error of less 0.5 Da was considered acceptable for peptide identification.

3. Results

We previously reported that even a few minutes of preincubation of