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Antimicrobial activity and mechanism of PDC213, an endogenous peptide from human milk

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ABSTRACT

Human milk has always been considered an ideal source of elemental nutrients to both preterm and full term infants in order to optimally develop the infant's tissues and organs. Recently, hundreds of endogenous milk peptides were identified in human milk. These peptides exhibited angiotensin-converting enzyme inhibition, immunomodulation, or antimicrobial activity. Here, we report the antimicrobial activity and mechanism of a novel type of human antimicrobial peptide (AMP), termed PDC213 (peptide derived from β-Casein 213-226 aa). PDC213 is an endogenous peptide and is present at higher levels in preterm milk than in full term milk. The inhibitory concentration curve and disk diffusion tests showed that PDC213 had obvious antimicrobial against *S. aureus* and *Y. enterocolitica*, the common nosocomial pathogens in neonatal intensive care units (NICUs). Fluorescent dye methods, electron microscopy experiments and DNA-binding activity assays further indicated that PDC213 can permeabilize bacterial membranes and cell walls rather than bind intracellular DNA to kill bacteria. Together, our results suggest that PDC213 is a novel type of AMP that warrants further investigation.

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1. Introduction

Breast milk has always been considered an ideal source of basic elemental nutrients and is finely attuned to infant growth requirements [1]. It is well established that breastfeeding, compared to formula feeding, is associated with better long- and short-term outcomes, such as neurological and behavioral outcomes [2]. More detailed examination has revealed that milk represents a more functional ensemble, with benefits to both infants and mothers beyond its simple nutritional benefit [3]. Specifically, the antimicrobial and immunomodulatory components of breast milk are deemed to compensate for the insufficiencies of the neonatal immune system and impair the translocation of infectious pathogens across the gastrointestinal tract. For premature infants, breast milk is an important source of optimal nutrients and protection against infection [4].

In recent decades, many short chain and low molecular weight peptides have attracted increasing interest [5]. These peptides are significantly efficient at inhibiting bacterial growth and are usually termed antimicrobial peptide (AMP). AMPs constitute a group of peptides with similar structure characteristics and biochemical properties [6,7]. AMPs can act directly to protect human against a wide variety of microbial infections, including viral, fungal and protozoan infections [6]. Human milk contains hundreds of naturally occurring antimicrobial peptides, which are important in the prevention of inflammatory diseases and childhood infections, such as allergies and colitis. For example, a lactoferrin-derived peptide showed both antimicrobial and immunomodulatory activities in vitro and in experimental animal models [8]. Currently, antimicrobial peptides from human milk show promise to serve an important role in future medicine. Therefore, experimentation to find new antibacterial peptides is important.

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Endogenous peptides are produced from corresponding proteins through the action of proteases that are naturally present in milk [9]. In past studies, endogenous peptides from human milk have been considered as basic elemental nutrients, and little has been studied from a functional viewpoint. Over 400 endogenous peptides originating from human milk have been identified before [10]. The majority of peptides are derived from β-casein, and exhibit various functions, including antimicrobial, immunomodulatory, antihypertensive, antithrombotic and opioid activities [11]. The constitution of endogenous milk peptides may gradually change to meet the growth and development requirements of infants [12]. The human milk peptides from mothers delivering preterm and at term are significantly different. However, little is known on the function of these endogenous peptides, and it is essential to explore the activity and mechanism of endogenous peptides.

PDC213 is present at high levels in human milk from women delivering preterm infants, according to previous research [13]. Considering that endogenous peptides from milk exhibit antimicrobial activity as a whole, we speculated that PDC213 has a similar function. Here, we revealed that PDC213 had the capacity to kill *S. aureus* and *Y. enterocolitica*in vitro; both are common nosocomial pathogens in neonatal intensive care units (NICUs). Furthermore, we found that PDC213 can effectively permeabilize the bacterial membranes to function, but not for intracellular targets such as DNA-binding against pathogens. Our research is meaningfully to understand the mode of action for PDC213 antimicrobial activity and its mechanism.

2. Materials and methods

2.1. Materials

The chemical synthetic peptide PDC213 was synthesized from Science Peptide Biological Technology CO.LTD (Shanghai, China). *Escherichia coli* (*E. coli*, ATCC25922), *Staphylococcus aureus* (*S. aureus*, ATCC25923) and *Yersinia enterocolitica* (*Y. enterocolitica*, ACTT23715) were obtained from the American Type Culture Collection. *Listeria monocytogenes* (*L. monocytogenes*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Bacillus subtilis* (*B. subtilis*) were provided by Nanjing Normal University, China. The linearized plasmid pBR322 vector from *E. coli* was obtained from New England BioLabs. The live/dead® Baclight™ bacterial viability kit (Thermo Fisher, USA) was used for the fluorescent dye method. The fluorescence microscope was purchased from Zeiss, Imager.A2, Germany. The other materials, such as those used for cell culture, were all purchased from Sigma (St. Louis, MO, USA).

2.2. Inhibitory concentration curves

All the strains (*S. aureus, Y. enterocolitica*, *L. monocytogenes*, *K. pneumoniae*, *E. coli* and *B. subtilis*) were incubated in liquid growth media at 37 °C for 24 h to enhance the activity of the bacteria. Then, the bacteria were diluted to 1×10^6 colony-forming units (CFU)/ml. The bacteria were added to each well of a sterile 96-well plate containing PDC213 in serial 2-fold dilutions (final concentration between 50 and 0.390 μg ml^{-1}). The plates were incubated at 37 °C for 12 h with agitation, and the bacterial number was determined by absorbance measurements at 600 nm using a microplate autoreader. All experiments were repeated three times independently.

2.3. Disk diffusion test

Agar plates were coated with *S. aureus* and *Y. enterocolitica* and incubated for 24 h at 37 °C. Disks soaked with PDC213 or sterile water were placed on the inoculated agar plates. The disks with sterile water were added as a negative control. The plates were incubated overnight at 37 °C. The baseline antimicrobial activity was assessed after 16–24 h by measuring the zones of inhibition in millimeters from each center.

2.4. Fluorescence microscopy assay

Both *S. aureus* and *Y. enterocolitica* were grown to the late log phase at 37 °C; then, we followed the manufacturer instructions of the live/dead® Baclight™ bacterial viability kit to wash, centrifuge, incubate and resuspend the cells. The excitation/emission maxima for these dyes are approximately 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide (PI). All experiments were repeated three times independently, and at least three different fields were observed for each culture.

2.5. Scanning electron microscopy (SEM)

SEM was used to gain insight on the direct effects of PDC213 on the morphology of bacterial cells. In brief, *S. aureus* and *Y. enterocolitica* were seeded into Luria-Bertani (LB) and incubated for 20–24 h at 37 °C with constant agitation (220 rpm) under aerobic conditions. Then, the bacteria were harvested and centrifuged at 500 rcf for 10 min and washed 3 times with sterilized deionized water. The bacterial pellets were resuspended in sterilized water and incubated at 37 °C for up to 1 h in the presence of PDC213 solvent. The same amount of bacteria without PDC213 was used as a negative control. We used 2.5% glutaric dialdehyde to fix the cells. Then, the samples were dehydrated with ethanol concentrations of 50%, 70%, 85%, 95% and 100%. After air-drying and gold coating, the samples were sent to the Nanjing Medical University for further treatment and analysis on a SEM.

2.6. Transmission electron microscopy (TEM)

Samples containing *S. aureus* and *Y. enterocolitica* were inoculated into LB media, and they were incubated at 37 °C overnight with constant agitation at 220 rpm under aerobic conditions. We centrifuged the samples at 500 rcf for 10 min and then washed the samples in sterilized deionized water for 3 times. Bacteria containing PDC213 solvent were incubated at room temperature for 1 h. Bacteria with sterile water were used as the negative control. The bacteria were centrifuged, washed and fixed in 2.5% of glutaric dialdehyde for at least 4 h at 4 °C. After fixation, the bacteria were examined and photographed before they were sent to Nanjing Medical University.

2.7. DNA gel retardation assay

The gel-retardation experiment was performed with a fixed amount (100 ng) of the linearized plasmid pBR322 vector from *E. coli*; the linearized plasmid was incubated with increasing amounts of PDC213 (0–500 μg/ml) for 30 min. The complexes were maintained at 20 μl by adding 5% glycerol, 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 20 mM KCl and 50 mg/ml BSA at pH 7.4. After the addition of loading dye, the samples were placed into a 1.0% agarose gel in TBE (Tris-borate-EDTA) buffer and run at 120 V for 90 min.

2.8. Data analysis

All biological experiments were repeated three times. The data from the experiments were analyzed by IBM SPSS 20, and Student's t tests were performed. All date are presented as the mean ± standard deviation (SD).

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