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Research article

Protective efficacy of a peptide derived from a potential adhesin of *Pseudomonas aeruginosa* against corneal infection

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ABSTRACT

Dissecting the interactions between *Pseudomonas aeruginosa* and corneal cells is important to identify a novel target for prevention and treatment of *Pseudomonas* keratitis. The current study began with a peptide identified by phage display, and was to investigate the protective efficacy against *P. aeruginosa* infection in cornea. The original peptide Pc-E, with high homology to a hypothetical membrane protein (HmpA) in *P. aeruginosa,* and the derived peptide Pc-EP, with the same sequence as a region in HmpA, were synthesized. Peptide Pc-EP could directly bind to HCEC, stronger than Pc-E, and specifically activate toll-like receptor 5, and thereby significantly induce the production of pro-inflammatory factors, such as IL-1β, IL-6, IFN-γ and IL-17. Moreover, Pc-EP could act as an antagonist to inhibit the adhesion of wild-type *P. aeruginosa* to HCEC and mouse corneas. No inhibitory effect was observed on the adhesion of the strain loss of HmpA. When compared to the wild-type strain, the adhesion of the *hmpA* mutant to corneal cells was significantly decreased. Treatment of infected mouse corneas with Pc-EP before infection significantly decreased the bacterial load in the cornea and attenuated the corneal pathology. These results indicate that Pc-EP can be a useful prophylactic agent for *P. aeruginosa* keratitis.

1. Introduction

Pseudomonas aeruginosa is the most common pathogen involved in bacterial keratitis caused by trauma or contact lens wear (Robertson et al., 2007; Ti et al., 2007). *Pseudomonas* keratitis is a rapidly progressive disease that can affect the entire cornea within 48 h and can quickly lead to corneal perforation and blindness (Fleiszig et al., 1994). Current antibiotic therapies for *P. aeruginosa* infections often fail to control this excessive tissue damage (O'Brien et al., 1995). Bacterial adhesion to the corneal cell surface is the first step in the establishment of an infection (Beachey, 1981), and this adhesion is often mediated by the binding of pathogen ligands to host receptors. Theoretically, the interruption of this adhesion could be an effective method to inhibit further bacterial invasion and, thus, serves as an optimal target for the treatment of infections, particularly at an early stage.

The adhesion between the host and the pathogens includes both specific and non-specific binding. The Pathogen-Associated Molecular Patterns (PAMP) of bacteria and the pattern recognition receptors (PRRs) on the surface of host cells are an example of specific binding. Previous reports indicate that extracellular matrix (ECM) proteins such as laminin, fibronectin and collagen are utilized by pathogens to adhere to host cells (Singh et al., 2012; Vercellotti et al., 1985), whereas, in recent years, the toll-like receptors (TLR) have been more defined as the PRRs on the cell surface (Akira and Hemmi, 2003). On the bacterial side, the pili of

Abbreviations: HmpA, hypothetical membrane protein; PRRs, pattern recognition receptors; HCEC, human corneal epithelial cells; CFTR, cystic fibrosis transmembrane conductance regulator; ARVO, Association for Research in Vision and Ophthalmology.

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Pseudomonas have been confirmed to be a primary adhesin during infection (Zolfaghar et al., 2003). Flagellin was also reported to be adhesin on the *P. aeruginosa* surface that bind to mucin on host epithelial cells (Lillehoj et al., 2002). Alginate in *Pseudomonas* biofilm and several membrane proteins have also been confirmed to be adhesins on *P. aeruginosa* (Borlee et al., 2010; Orgad et al., 2011). In addition, lipopolysaccharide (LPS) is a well-known ligand of the majority of bacteria and induces an inflammatory response (Ernst et al., 2003). Moreover, the primary *P. aeruginosa* adhesin identified in corneal cells is the outer core of LPS, which binds to the cystic fibrosis transmembrane conductance regulator (CFTR) (Zaidi et al., 1996, 1999). Nevertheless, studies of additional ligands on *P. aeruginosa* surfaces are relatively lacking, making it necessary to expand our insights into how these ligands contribute to the onset and progression of infection.

Phage display has been used as a powerful tool for studying protein–protein or protein-tissue interactions (Pande et al., 2011). In the area of host–pathogen interactions, phage display has been successfully used to discover new pathogen ligands that bind to host receptors during the adhesion stage (Antonara et al., 2007). Previously, we used phage display to screen for peptides that bind to human corneal epithelial cells (HCEC) and discovered two peptides with a high homology to *Aspergillus* proteins that significantly inhibit *Aspergillus* adhesion to HCEC (Zhao et al., 2012). Among the fourteen screened peptides in that study, one peptide designated Pc-E, with the amino acid sequence of YALRPGMPQWLE, shares high homology with a hypothetical membrane protein (NP_253222.1) of *P. aeruginosa* PAO1 at amino acids 172-177 (MPPWLE). Therefore, in the current study, the role of this hypothetical membrane protein, designated as HmpA, during *P. aeruginosa* invasion in the cornea was investigated. A 12-mer peptide designated as Pc-EP, with the same sequences of HmpA at 167-178 (N-DGLFPMPPWLED-C), was also synthesized. The amino acid sequence of HmpA and Pc-EP were highly conserved between various *P. aeruginosa* strains, with an identity of >99% and 100%, respectively, when analyzed using BLAST. The segment of Pc-EP is relatively unconstrained in the homology modeling of the HmpA structure as predicted with the CPH models 3.2 server (Nielsen et al., 2010). Thus, its role during the interaction between *P. aeruginosa* and corneal epithelial cells was analyzed along with the host responses to Pc-EP and the cytotoxicity of the peptide.

2. Materials and methods

2.1. Strains, cells, and culture conditions

P. aeruginosa PAO1 was from our laboratory stocks; clinical isolates 6294 (ExoS-producing invasive strain of serogroup O6) and 6077 (ExoU-producing cytotoxic strains of serogroup O11) (Fleiszig et al., 1996) were kindly provided by Gerald Pier (Harvard Medical School). All bacterial strains were cultured at 37 °C in nutrient broth, and the bacterial concentration was adjusted according to measured OD_{600nm} value. The SV40-immortalized HCEC (ATCC CRL-11135) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. ELISA assay of synthesized peptides binding to cells

Synthetic peptide Pc-EP was biotinylated at the N-terminus and aminated at the C-terminus (Bootech Company, Shanghai, China) with a purity of 98%. After the cultured HCEC reached 80–90% confluence in a 96-well plate, cells were starved in serum-free DMEM/F12 for 2 h, and then synthesized peptides were added to 3 wells for each concentration and incubated at 37 °C for 1 additional hour. Unbound peptides were removed by 6 washes with PBST. Then, horse radish peroxidase-conjugated streptavidin (BD Biosciences, San Jose, CA, USA) was added for 1 h, followed by 6 washes. Tetramethyl benzidine (BD Biosciences) was added at room temperature, followed by incubation in the dark for 30 min; finally, 1N H_3PO_4 was added to stop the reaction. The absorbance was read at 405 nm using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Pc-E, Pc-ER (a peptide with residues identical to Pc-EP but in random order, N-WPGLPMFDDEPL-C) and pBSA (a BSA derived peptide, Zhao et al., 2012) were used for comparison.

2.3. Effect of synthetic peptides on HCEC gene transcription

To measure the effect of peptide Pc-EP on the transcription of genes of interest, 80%–90% confluent HCEC were stimulated by 100 μM of Pc-EP for 2 h in triplicate. For antibody blocking, 200 ng of antibody to TLR5 were added to the cells along with the peptide during the incubation. At the end of the culture period, cells were harvested and total RNA extracted using a NucleoSpin® RNA II Kit (MACHEREY-NAGEL, Düren, Germany). Reverse transcription was performed using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) with 500 ng total RNA, followed by Taqman real-time PCR in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA). The sequences of primers and probes of the target genes are listed in Table S1. Amplification was set for 10 s at 95 °C followed by 45 two-step cycles (15 s at 95 °C and 1 min at 60 °C). Data were analyzed with the SDS System Software (Applied Biosystems) using hB2-M as a reference gene.

2.4. Construction of the PAO1(ΔhmpA) mutant

For construction of PAO1 mutant with the altered *hmpA* gene, the previously described *sacB*-based strategy was employed (Schweizer and Hoang, 1995). The up-stream and down-stream regions of *hmpA*, *hmpA*F and *hmpA*R, were obtained by PCR using the primer pairs *hmpA*Fup/*hmpA*Fdown (5-GCTGAATTCGTGGTTACCTCCAGCACCT-3, 5-ATGGATCCTAGATGGCCCAGCCCAGTT-3) and hmpRup/hmpRdown (5-ATAGGATCCGGAAGGACGAAGAGCGCAAG-3, 5-GTCAAGCTTACGACGTGCTGGTCAACGAC-3), respectively. The purified fragments were cloned into the vector pEX18Tc (Tc^r) using EcoRI/BamHI and BamHI/HindIII (TaKaRa, Dalian, China) for digestion. The final construction contained the DNA fragment spanning the *hmpA* gene with the middle portion (687 bp) omitted. This *hmpA* construct, including the corresponding position of Pc-EP, were confirmed by sequencing. The suicide plasmid pEX18Tc-*hmpA*F-*Ω*-*hmpA*R for gene replacement was constructed by inserting the *Ω* (Sm^r) sequence digested by *BamHI* from pHP45Ω (Schweizer and Hoang, 1995) (Amp^r and Sm^r). The pEX18Tc-*hmpA*F-*Ω*-*hmpA*R plasmids were transferred to PAO1 using a triparental mating procedure including the helper vector pRK2013. The colonies that were able to grow on *Pseudomonas* isolation agar (PIA) containing Sm and sucrose but unable to grow on PIA containing Tc were collected and deletion of *hmpA* verified by PCR using the primers *hmpA*Fup and *hmpA*Rdown. The correct knockout mutant clones selected were those with which no PCR product could be amplified. These clones were further confirmed by sequencing the PCR products using the primer *hmpA*check1 (5-CTTCTGCTCCGACCGCTGCAAGCTGATC-3) and *hmpA*check2 (5-TCCACCCTGGAGCCCTGGACGTTGCAG-3).

2.5. Growth and antibiotic sensitivity of the hmpA mutant

Overnight cultures of the *hmpA* mutant and wild type PAO1 were separately inoculated into the wells of a 96-well plate in a