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Inhibition of the superantigenic activities of Staphylococcal enterotoxin A by an aptamer antagonist

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ABSTRACT

Staphylococcal enterotoxin A (SEA) is an important component of *Staphylococcus aureus* pathogenesis. SEA induces T lymphocytes activation and proliferation, resulting in the release of a large number of inflammatory cytokines. Blocking the toxic cascade triggered by SEA may be an effective strategy for the treatment of SEA-induced diseases. Through a systematic evolution of ligands by exponential enrichment process, we obtained an aptamer (S3) that could bind SEA with both high affinity and specificity, with a K_d value 36.93 ± 7.29 nM (n = 3). This aptamer antagonist effectively inhibited SEA-mediated human peripheral blood mononuclear cells proliferation and inflammatory cytokines (IFN-γ, TNF-α, IL-2 and IL-6) secretion. Moreover, PEGylated S3 significantly reduced mortality in murine lethal toxic shock models established by lipopolysaccharide-potentiated SEA. Therefore, this novel aptamer antagonist has the potential to become a new strategy for treating *S. aureus* infections and SEA-induced diseases.

1. Introduction

Staphylococcal enterotoxin A (SEA) is one of enterotoxins produced by *Staphylococcus aureus* (*S. aureus*). There are two sites on SEA that can bind to MHC-II. The chief site binds Zn^{2+} with high-affinity, while the other has high similarity to the MHC-II binding site of Staphylococcal enterotoxin B (SEB). SEA recognizes the α chain of one MHC-II molecule and the β chain of another MHC-II molecule, indicating that, SEA can recruit a large number of T cells at relatively low concentrations and can non-specifically stimulate lymphocytes proliferation to enhance superantigen activity and toxicity (Argudin et al., 2010; Ortega et al., 2010; Schad et al., 1995).

As an important component of *S. aureus* pathogenesis, SEA is closely related to the occurrence and development of a variety of diseases, ranging from food poisoning to potentially life-threatening toxic shock (Bette et al., 1993; Kozono et al., 1995). SEA can directly stimulate T lymphocytes activation and proliferation without the need for processing by antigen presenting cells, which leads to the release of large amounts of IFN-γ, TNF-α, IL-2, IL-6 and other cytokines. As a result, as little as 100–200 ng of SEA can produce symptoms (Evenson et al., 1988) and SEA is relatively stable, with strong resistance to protease, high temperatures and extremes in pH. Reports have indicated that SEA retains some biological activity even after incubation at 121 °C for 28 min or at 127 °C for15 min (Balaban and Rasooly, 2000).

Currently, treatment strategies against various SEA-induced diseases center on disrupting the binding of SEA to MHC II and/or T cell receptor (TCR). Formation of the complex between SEA and MHC II and/or TCR can be disrupted by soluble T cell receptor (Sharma et al., 2014), synthetic polypeptides (which inhibit SEA-mediated inflammatory cytokines release) (Maina et al., 2012a), and SEA vaccine or fusion toxins (which stimulate antibody

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production) (Chen et al., 2012; Reddy et al., 2015). Current research has focused on discovering/developing new compounds with greater half-lives, improved SEA binding affinities, and decreased immunogenicity.

Aptamers, which are synthetic oligonucleotide molecules (ssDNA or ssRNA), have recently emerged as novel molecular probes in diagnosis and treatment of diseases (Ellington and Szostak, 1992; Tuerk and Gold, 1990). Due to their unique three-dimensional conformations, high affinity aptamers can bind various target molecules with enhanced specificity, including small molecules, proteins, cells, and whole tissues.

When folded, aptamers form spatial configurations that can bind to specific sites on protein surfaces, forming stable aptamer-protein complexes. The specific sites are usually the active sites or functional regulatory sites of the target protein. After binding with these specific sites, aptamer can inhibit the biological activity of the target protein. As oligonucleotide molecules, aptamers have shown the inherent advantages of low toxicity and immunogenicity. In addition, aptamers composed of modified nucleotides have a long *in vivo* half-life, which can range between hours and days (Santosh and Yadava, 2014; Sun and Zu, 2015; Tsae and DeRosa, 2015). An anti-vascular endothelial growth factor aptamer prodrug (Macugen) has been approved by the FDA for the treatment of age-related macular degeneration (Ng et al., 2006). More aptamer drugs are in clinical trials.

Currently, no aptamer has been designed to selectively inhibit the biological activity of SEA. Therefore, we have screened DNA aptamers that bind SEA *in vitro* and have identified antagonists that were able to inhibit the superantigenic activities of SEA. In summary, study aimed to provide new drug candidates in treating *S. aureus* infections and SEA-induced diseases.

2. Materials and method

2.1. Materials

The ssDNA library consisted of a 40-nucleotide (nt) central random region flanked by 18-nt primer hybridization regions. Primers corresponding to the two ends of the ssDNA library were designed for SELEX (systematic evolution of ligands by exponential enrichment) selection and subsequent analysis. All labeled and unlabeled oligonucleotides were synthesized and purified by high-performance liquid chromatography (HPLC; Invitrogen, Guangzhou, China) and the sequences are listed in Table 1.

For *in vivo* experiments, the aptamer candidate was conjugated at the 5′ end with 40-kDa polyethylene glycol (PEG). The PEGylated aptamer was prepared by treating 40-kDa PEG N-hydroxysuccinimide ester with 5'-amino and 3'-inverted dT modified aptamer candidates. All PEG-aptamer conjugates were purified by anion exchange and then by reverse-phase HPLC (Seebio Biotech, Shanghai, China).

2.2. SELEX process

Firstly, highly purified SEA (Endotoxin Reduced < 1 EU/mg, Toxin Technology, Sarasota, USA) was coupled to M-280 Streptavidin Dynabeads (DYNAL, Oslo, Norway) using the BioMag Plus Carboxyl Protein Coupling Kit (Bangs Laboratories, Indiana USA). Prior to selection, the ssDNA library (dissolved in 500 μL of selection buffer: 50 mM Tris-HCl, pH 7.4,100 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl) was heat-denatured at 95 °C for 5 min and then incubated with SEA-coated beads (100 ng) and 5-fold molar excess of yeast tRNA (Invitrogen, Guangzhou, China) at 37 °C for 1 h.

Unbound ssDNA was washed off with selection buffer (supplemented with 0.2% BSA). To generate SEA-bound ssDNA, 200 μL of ddH_2O was added, followed by incubation at 100 °C, for 5 min. SEA-bound ssDNA was amplified by PCR using the FAM-labeled upstream primer P3 and the biotin-labeled downstream primer P4 (initial denaturation for 5 min at 94 °C, 25–30 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C, and 0.5 min at 72 °C, followed by a final elongation for 5 min at 72 °C). PCR products were purified using a small fragment purification kit (BioTeke Corporation, Beijing, China) to obtain double-stranded DNA fragments (dsDNA) with one end labeled with biotin and the other with the FAM fluorophore. The dsDNA (200 μL) was gently shaken with 100 μL of streptavidin-labeled magnetic beads (5 μg/μl) at 37 °C, for 20 min, followed by three washes with 500 μL of washing buffer (5 mM Tris-HCl, pH 7.5, 1 M NaCl, 500 μM EDTA). Subsequently, 50 μL of 100 mM NaOH was added and then the dsDNA was incubated at 37 °C for 30 min to denature into ssDNA. Afterward, the FAM-labeled ssDNA was isolated from the supernatant and used as the enrichment library for the next round of screening.

In order to obtain high-affinity and specific aptamers, the selective pressure was gradually increased by varying the concentrations of SEA and ssDNA, washing times, amount of KCl and tRNA, and reducing the incubation time (Table 2). In order to reduce non-specific binding, BSA-coupled M-270 beads were used as a target for counter-selection, starting from the third round of selection. To reduce matrix binders, reaction tubes were initially blocked with 1% BSA-PBS.

2.3. Determination of aptamer library binding with SEA

A fluorescently labeled ssDNA binding assay was performed to monitor the enrichment of each SELEX round. The FAM-labeled ssDNA in 200 μL of selection buffer was thermally denatured at 95 °C for 5 min, and then incubated with SEA-coated beads (100 ng) in the dark at 37 °C for 1 h. After incubation, the unbound ssDNA was removed. The ssDNA bound with SEA was incubated with 200 μL of ddH_2O at 100 °C for 5 min and then immediately eluted. A TBS-380 fluorescence quantitative instrument (Turner Biosystems, Sunnyvale, CA) was used to measure the fluorescence intensity of applied, non-bound, and eluted ssDNA to calculate the percentage of ssDNA bound to SEA.

2.4. Cloning and sequencing of aptamers

After SELEX selection, the selected ssDNA pool was PCR amplified using unlabeled primers (P1 and P2) under the same conditions described for screening. Per the manufacturer's instructions, PCR products were ligated into purified pEGM-T vector (Promega, Madison, WI, USA). The ligation product was transformed into *E. coli* XL1-Blue cells and positive colonies were randomly picked and cultured. Plasmid DNA was prepared from bacterial cultures using phenol-chloroform extraction and the inserts were sequenced by Invitrogen. The aptamer sequences were analyzed using ClustalX software and the theoretical secondary structures of aptamers were simulated using mfold based on the principles of minimum free energy (Zuker, 2003).

2.5. Aptamer-SEA binding assay

Enzyme-linked aptamer assay (ELAA) was used for analysis of aptamer binding affinity and specificity with SEA. In brief, 100 μL of SEA (50 μg/mL) was used to coat a microtiter plate. After incubation at 4 °C overnight, the plate was blocked with blocking buffer (1% BSA-PBS, PH 7.4) at 37 °C for 1 h. After washing 4 times with PBS (30 mM NaCl, 140 mM KCl, 2 mM KH_2PO_4, 25 mM Na_2HPO_4, PH 7.4), 100 μL of digoxin-labeled ssDNA (500 nM) was added and incubated at 37 °C for l h. The plate was subsequently washed 4 times