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Expression and bioactivity analysis of *Staphylococcal enterotoxin* M and N

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Abstract

Staphylococcal enterotoxins (SEs) are powerful superantigens that stimulate non-specific T-cell proliferation produced by *Staphylococcus aureus* and draw considerable attention as ideal drugs for cancer therapy. The filtrate of *S. aureus* culture has been used as ampul named *Staphylococcal enterotoxin C injection* in clinic for 10 years in China and proved to be effective. The superantigen SEC claimed to be the only active component without certifiable evidences. For further investigations of the active components of this injection and establishment of foundations for the development of novel anti-cancer drugs, in this research we extracted total DNA from *S. aureus* (FRI 1230), cloned, expressed and purified recombinant proteins of *Staphylococcal enterotoxin* M and N (rSEM and rSEN). The MTT assay of the purified rSEM and rSEN demonstrated that their abilities of stimulating T cells and inhibiting the proliferation of K562–ADM cells and B16 cells were equivalent to that of purified SEC2 *in vitro*. These findings suggested that SEC was not the only active component of *Staphylococcal enterotoxin C injection* and the effective procedure of expression and purification may be useful for mass productions of these therapeutically important proteins.
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Keywords: Enterotoxins; Superantigen; *Staphylococcal enterotoxin C injection*; Expression; MTT

Staphylococcal enterotoxins (SEs)[1] are a family of 13 major serological types of heat stable enterotoxins (SEA through SEE, SEG through SEQ, TTST-1, TTST-2 and SEC including subtypes of SEC1, SEC2 and SEC3) produced by *Staphylococcus aureus* [1–3] and share a number of genetic and biochemical characteristics, with similar functions [3]. SEs are powerful superantigens that stimulate non-specific T-cell proliferation based on their V_β receptor usage. Their ability to stimulate lymphocyte proliferation and lymphokine production at concentrations as low as 10^{-13}–10^{-16} M makes them among the most potent T-cell activators known and their ability of inducing apoptotic death of tumor cells is of particular interest [3–5]. The filtrate of *S. aureus* culture has been used as ampul named *Staphylococcal enterotoxin C Injection* for cancer therapy in clinic for 10 years in China and proved to be effective [6], but its active components have not been confirmed yet.

For further investigations of the active components of the injection and establishment of foundations for development of novel anti-cancer drugs, in this research we will extract total DNA from *S. aureus* (FRI 1230), clone the genes of *Staphylococcal enterotoxin* M and N, obtain recombinant proteins (rSEM and rSEN), and compare their abilities of stimulating T cells and inhibiting the proliferation of K562–ADM cells and B16 cells with SEC2 by MTT assay.

Materials and methods

Cell lines and cultures

Anti-adriamycin (ADM) human chronic myelogenous leukemia cell line: K562–ADM, K562 gradually induced

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[1] *Abbreviations used:* SEs, *Staphylococcal enterotoxins;* ADM, anti-adriamycin; FBS, fetal bovine serum; IPTG, isopropyl thiogalactoside.

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with 1 μg/ml ADM. Murine B16 melanoma cell line: B16. These cell lines were provided and conserved by our lab and cultivated with RMPI1640 (Gibco, Eggenstein, Germany) supplemented with 10% FBS (fetal bovine serum) at 37 °C in an atmosphere with 5% (v/v) CO_2, 0.01% ampicillin and streptomycin.

Animals

ICR mouse, outbred stock, produced in 1947 by Hauschka at the Institute of Cancer Research, derived from Swiss mice of the Rockefeller Institute and now widely distributed [7]. Weighting 20 ± 2 g, provided by animal research center, Academy of Medical Science, Zhejiang province, China.

Bacterial strains, plasmids, enzymes, reagents and media

Staphylococcus aureus (FRI 1230) was used for the extraction of the total DNA. *Escherichia coli* (*E. coli*) DH5α was used for the transformation of the vectors. *E. coli* strain BL21 (DE3) was applied as host strain for expression. The plasmid pGEM-T (Promega, USA) was used to sequence the genes of interest. The plasmid pGEX-4T-1 (Promega, USA) was used for the expression of the GST-SEM and GST-SEN genes in *E. coli*. The restriction endonucleases BamHI and XhoI, *Taq* polymerase, T4 DNA ligase were purchased from NEB (USA). IPTG and low melting-point agarose were purchased from BBI (USA). DNA sequencings were completed by Shanghai Association-gene (China); glutathione Sepharose 4B was from Amersham Phamacia Biotech (Sweden); thrombin was from Changzhou Qianhong Co. (China); BCA kit was from Beyotime (China); other biochemical reagent MTT, ConA and DNA gel extraction kit were from Shanghai Sangon (China). *S. aureus* C2 (SEC2) was prepared and conserved by our lab [8]. The culture media LB (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) and 2× YT (tryptone 16 g/L, yeast extract 10 g/L and NaCl 5 g/L) were prepared as described.

DNA extraction and PCR amplification

Total DNA was extracted from *S. aureus* (FRI 1230) with protein K and SDS–Tris solution and purified with saturate phenol by centrifugation extracting to collect the aqueous phase. Mixed the collected aqueous phase with NaAc (pH 5.5) and ice-cold dehydrated alcohol at −20 °C for 1 h. Then purified DNA was separated by centrifugation, dissolved with ion-free water and conserved at −20 °C.

PCR amplification [9] was performed on a Mastercycler gradient (Effendorf, Germany). Two microliters plasmid template was subjected to amplification in 100 μl mixture containing 1 U of *Taq* polymerase, 10 μl 10× PCR buffer, 6 μl 25 mM $MgCl_2$, 4 μl 10 mM each of dATP, dCTP, dGTP and dTTP, 4 μl 20 μM primer P1 and P2 or P3 and P4 (see below) and fill water to 100 μl, and then divided the 100 μl mixture to 4×25 μl for use. PCR conditions were initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min.

The mature peptide of SEM coding region was amplified with following primer pair according to the literature [10] and the estimation of SignalP 3.0 Server: forward primer P1: 5′-CAG GAT CCT TTT GCT ATT CGC AAA ATC ATA TCG CA-3′ (the scribed part is an introduced BamHI recognition sequence); and reverse primer P2: 5′-GCC TCG AGT CAA CTT TCG TCC TTA TAA GAT ATT TCT AC (the scribed part is an introduced XhoI recognition sequence).

The mature peptide of SEN coding region was amplified with following primer pair according to the estimation of SignalP 3.0 Server: forward primer P3: 5′-GGA TCC GAA GTA GAC AAA AAA GA-3′ (the scribed part is an introduced BamHI recognition sequence); and reverse primer P4: 5′-CTC GAG ATA ATC ATC AAT CAC TTA (the scribed part is an introduced XhoI recognition sequence). All primers used in the paper were synthesized in Shanghai Sangon (China).

Construction and sequencing of pGEM-SEM and pGEM-SEN

After recovery of the amplified PCR products, the products were cloned into pGEM-T vectors with T4 DNA ligase and the resultant plasmids were transformed to DH5α competent cells. The transformants were cultured in LB plate with IPTG/X-gal and ampicillin, and white clones were selected. The recombinant plasmids with SEM or SEN genes were identified with restriction endonucleases cleavage and confirmed by sequencing in Shanghai Sangon (China). The sequences of the inserts were compared to DNA sequences of the estimations of SignalP 3.0 Server and confirmed to be right.

Construction and verification of GST-SEM and GST-SEN expression vectors

The recombinant plasmids pGEM-SEM and pGEX-4T-1 were cleavaged with restriction endonucleases BamHI and XhoI. The cleavaged products of mature peptide of SEM with sites of restriction endonucleases and the products of the fragment of plasmid pGEX-4T-1 were recovered and connected to construct the expression vector of GST-SEM, named pGEX-4T-SEM. The resultant expression vector plasmids were transformed to DH5α competent cells and the amplification products were identified with restriction endonucleases BamHI and XhoI.

In addition, the expression vector of GST-SEN, named pGEX-4T-SEN was constructed and verificated in the same way.

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