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ORIGINAL PAPER

Staphylococcus aureus enterotoxin C2 mutants: biological activity assay in vitro

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Abstract Staphylococcal enterotoxin C2 (SEC2) is one member of bacterial superantigens produced by *Staphylococcus aureus*. It can be attributed to its superantigenic activity to cross-link major histocompatibility complex class II molecules with T-cell receptors and activate a large number of resting T cells resulting in release of massive cytokines, which will produce significant tumor inhibition in vivo and in vitro. However, it could be not broadly applied to cure malignant tumors in clinic because of emetic activity of SEC2. The aim of this study was to inactivate emetic activity of SEC2 through site-directed mutagenesis. Cys93, Cys110 and His118 were selected as substitutional sites based on the functional sites responsible for emesis. The mutated proteins were used to determine Peripheral blood mononuclear cell proliferation activity and anti-tumor activity in vitro. Results showed that these mutated proteins efficiently stimulated T cell and exhibited the same tumor-inhibition effect as SEC2. It is possible to inactivate emetic activity of SEC2 through site-directed mutagenesis and provide satisfying agents for tumor treatment in clinic.

Keywords Staphylococcal enterotoxin · Site-directed mutagenesis · T-cell stimulation · Anti-tumor activity

Jing Hui and Yan Cao have equal contribution.

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Introduction

The nature of the activity of staphylococcal enterotoxins (SEs) on cells of the immune system puts them in a class of molecules designated as superantigen (SAg). Unlike conventional antigens, they bind to major histocompatibility complex (MHC) class II molecules outside the antigen-binding groove and are presented as unprocessed proteins to certain T cells expressing specific T-cell receptors (TCRs) Vβ genes [1, 14]. Very low concentrations of SAg are able to activate a number of resting T cells, thereby inducing massive cytokine release, including interleukin-2, interferon-γ and tumor necrosis factor, which produce significant tumor inhibition in vivo and in vitro [18]. In China, SEC2 has been used in clinics as an effective therapeutic agent for tumor treatment since 1996, and some encouraging results have been reported [3, 4]. However, the clinical application of SEC2 and cure of malignant tumors is seriously restricted because the question of emetic activity of SEC2 is not still solved [2, 12].

Although the mechanisms of SEs-inducing emesis have not been completely understood, the functional domains of SEC2 associating with emetic activity have been addressed [5–11, 17, 21, 22]. Loop structure, forming through disulfide bond, is necessary for emetic activity of SEs. Disulfide bond has a role in stabilizing a structure involving in emesis. SEC1 differ from SEC2 by a few amino acids. The SEC1 mutants with alanine substitutions were not emetic, but those with serine substitutions retained this activity [8]. In addition, His118, is thought to correlate with emetic activity of SEs [15, 23], and also was not essential for T-cell stimulation [5]. His-to-tyr substitution of SEB does not cause the confirmation change and the mutant proteins retain the MHC-binding ability [11]. In this paper, for the first time, we report that the mutated SEC2, obtained

through site-directed mutagenesis, exhibit nearly the same superantigenic activities and anti-tumor activities in vitro as SEC2, but no longer induce emetic response.

Materials and methods

Cell line, bacterial strains, vectors and culture conditions

Human colorectal cancer cells (Cx-1) purchased from China Medical University was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (TBD). *Staphylococcus aureus* o165Z-1 (Shenyang Xiehe Pharmaceutical Group Company, China) and *Escherichia coli* strains (JM109, BL21) (TaKaRa, China) were cultivated at 37 °C on Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl). Expression vector pET-28a was purchased from Novagen. Kanamycin (40 μg/ml), ampicillin (100 μg/ml) and/or Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mmol/l) was added to the medium when needed. In all cases, the cultures were incubated in conical flask at 200 rpm (NBS, Series 25D, New Brunswick, USA).

DNA manipulation and site-directed mutagenesis

All molecular manipulations were performed according to standard procedures [20] or those recommended by the manufacturers. All restriction endonucleases were purchased from Biolab (New England Biolabs, USA), *EX Taq* DNA polymerase from TaKaRa (Dalian, China), and T4 DNA ligase from Promega (USA).

The DNA sequence of SEC2 (GenBank Accession number AY450554) was amplified through PCR. PCR products amplified from genomic DNA of *S. aureus* o165Z-1 using primers S1 and S2 (Table 1) were purified and ligated into the *Eco*RI/*Xho*I sites of expression vector pET28a, resultant in plasmid pET1 [26]. Site-directed mutagenesis was performed using MutanBEST kit (TaKaRa, China) according to the methods recommended by the manufacturers. PCR primers (Table 1) were designed in inverted tail-to-tail directions to amplify pET1 together with the target sequence for amino acid substitution. DNA was sequenced by TaKaRa (Dalian, China). All primers were synthesized by TaKaRa (Dalian, China).

Preparation of proteins

Expression of the proteins was induced with 0.2 mmol/l IPTG after growth 4 h at 30 °C. Purification of the proteins was done through the Ni^{+}-NTA His•Bind Superflow column (Qiagen). The purity of the protein was determined by SDS-PAGE [24].

His-tag fused to the amino terminus was removed using Novagen recombinant enterokinase kits. Protein without His-tag was purified with the Ni^{+}-NTA His•Bind Superflow column (Qiagen), and then desalted, concentrated and determined by SDS-PAGE [24].

Proteolytic lability assay

To compare the general stability of SEC2 and its mutant derivatives, purified preparations of each were incubated at

Table 1 SEC2 mutant derivatives produced through site-directed mutagenesis

Plasmids	Site-directed mutations[a]	Amino acid substitution[b]	Protein designation	Primers[c]
pET1	–	–	SEC2	S1: 5′-<u>GAATTC</u>GAGAGTCAACCAGACCCTA-3′
				S2: 5′-<u>CTCGAG</u>TTATCCA TTCTTTGTTGTA-3′
pET2	T277 → A	C93 → S	C93S	P1: 5′-TCAAATTACTATGTAAACAGCTATTT-3′
				P2: 5′-TCCATACACATCAACTACTTCATC-3′
pET3	T328 → G; G329 → C	C110 → A	C110A	P3: 5′-GTGGTAAAACTGCTATGTATGGAGG-3′
				P4: 5′-CTGTAACTTTACCTACATTATCTTTGG-3′
pET4	T328 → A	C110 → S	C110S	P5: 5′-GTGGTAAAACTAGTATGTATGGAGG-3′
				P4
pET5	T277 → A; T328 → A	C93 → S; C110 → S	C93S, C110S	P1, P2, P5, P6
pET11	C352 → T	H118 → Y	H118Y	P7: 5′-ATAACAAAATATGAAGGAAA-3′
				P8: 5′-TCCTCCATACATACAAGTT-3′

[a] Specific point mutation produced in particular amino acid codon. Numbers in parentheses refer to the positions of nucleotide bases within the *sec2* gene

[b] Specific amino acid substitution. Numbers in parentheses refer to the positions within the primary sequence of mature SEC2

[c] Restriction enzyme sequences are underlined

25 °C with trypsin (TaKaRa, China) at various toxins: enzyme (w/w) ratio ranging from 20:1 to 400:1. After incubation, the digestions were terminated by boiling 5 min in SDS-PAGE sample buffer. The extent of proteolysis was assessed by SDS-PAGE as described above.

Biological activity assay in vitro

The biological activities of SEC2 and its mutant derivatives were determined by methyl thiazol tetrazolium (MTT) assay in vitro. Peripheral blood mononuclear cell (PBMC) proliferation assay was done according to the method described previously [26]. PBMC from the blood of healthy donors was isolated by lymphocytes separation medium (TBD) and aliquoted to 1×10^5 cells/well in 96-well plate in RPMI 1640 (Hyclone) supplemented with 10% characterized FBS (TBD). A series of concentration of SEC2 and its mutant derivatives were added to triplicate wells. Bovine serum albumin (BSA) and phytohemagglutinin-P (PHA-P) were used as negative and positive controls, respectively. After 96 h of incubation at 37 °C in 5% CO_2, 50 µl 5 mg/ml MTT in PBS was added to each well, and the incubation was continued for another 4 h. The cells were collected by centrifugation for 10 min at room temperature and 3,000 rpm. The pellet was re-dissolved in 120 µl DMSO for 37 °C 20 min, and the absorbance was measured at a wavelength of 570 nm in a microplate reader (Thermo Labsystems).

Tumor cells Cx-1 was seeded in 96-well plate at a density of 1×10^4 cells/well in DMEM supplemented with 10% FBS. SEC2 and its mutant derivatives were added separately to triplicate wells, and the cells were added along with 2×10^5 PBMC cells. The blank wells (DMEM only), unsettled tumor cells control wells (tumor cells only), and PBMC releasing wells (PBMC and toxin protein) were used as control. The medium was removed from each well after incubation at 37 °C for 36 h, and 50 µl MTT (5 mg/ml) in PBS were added into each well. Incubation was continued for another 4 h. Cells were then collected by centrifugation for 10 min at room temperature and 3,000 rpm. The pellet was re-dissolved in 120 µl DMSO for 37 °C 20 min, and the absorbance was measured at a wavelength of 570 nm in a microplate reader (Thermo Labsystems). Tumor growth inhibition (%) = 100 − [(the OD_{570} of protein-treated cells well − the OD_{570} of PBMC-releasing wells)/(the OD_{570} of unsettled tumor cells control wells − the OD_{570} of blank control wells)] × 100. These data were presented as mean values of SD from three independent experiments and statistically analyzed by Student's *t*-test.

The ferret feeding assay for SEs [25] was used for comparing the emetic capability of SEC2 and its mutant derivatives. Experiments were performed with adult female ferret (ShenYang Pharmaceutical University, China) with a mean body weight of 700 g.

Statistical analysis

Data were presented as means ± standard errors of the mean. All data are averaged from triplicate tests. Statistical comparisons were performed using the one-way ANOVA and Student's *t*-test with SPSS software (SPSS, Germany), *P*-values <0.01 were considered statistically significant.

Results

Site-directed mutagenesis of *sec2* gene

Cys93, Cys110 and His118 of SEC2 were selected as substitution sites. Site-directed mutagenesis of *sec2* gene was performed using MutanBEST kit (TaKaRa, China). Amino acid-substitution mutants (Table 1) were generated, in which Cys93, Cys110, or His118 was replaced by other amino acids, respectively. The mutated *sec2* genes were individually expressed in *E. coli* BL21, and production of each recombinant protein was checked through SDS-PAGE analysis by using whole-cell extracts. Similar levels of recombinant proteins production were observed in all mutants.

Proteolytic lability assay

Despite having 31 lysine and three arginine residues, all potential sites of SEC2 for trypsin cleavage, only the bonds at positions 59 and 103 are highly susceptible to cleavage by the enzyme in native toxin [8]. Therefore, a significant change in the overall conformation associated with loss of the disulfide bond could be assessed through comparing tryptic liability of the mutants with that of SEC2.

All mutants were degraded more rapidly than native SEC2 when treated with trypsin, indicating that their trypsin-sensitive sites were more accessible (Fig. 1). Although the rates of degradation varied, the three mutants with either alanine or

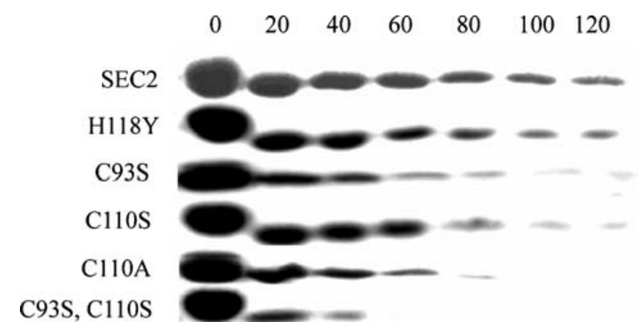

Fig. 1 Trypsin digestion rate of SEC2 and its mutant derivatives. Purified proteins were incubated with trypsin at a trypsin: toxin ratio of 1: 200 (w/w). After digestion for various times (0-120 mins), samples were analyzed by SDS-PAGE

serine substitution at position 110 degraded more rapidly than mutants with an identical substitution at position 93. For example, the rate of degradation for H118Y and C93S were similar to each other and only higher than native SEC2. Regardless of position, mutants with a single serine substitution were more resistant to trypsin than toxins with single alanine substitutions at the same location, proving that hydrogen bond produced by the substituted serine could stabilize the toxin in the absence of the disulfide bond [8].

Biological activity assay in vitro

The ability of SEC2 mutants to induce an emetic response was assessed in a Ferret model. The minimal emetic dose of SEC2 for ferret was 1 mg. In initial experiments, the emetic ability of C110A was assessed and administered at 10 mg to ensure an excess over the minimal emetic dose. C110A was not emetic (Table 2) even if this dose represented a tenfold excess of the amount of SEC2. However, others mutants were emetic, indicating that His118 did not correlate with emetic activity of SEs. Indeed, the gold standard to assess emetic activity of SEs is oral administration to primates. Therefore, further experiment using primate model is needed to confirm loss of emetic activity of the mutated SEC2.

Peripheral blood mononuclear cell proliferation was quantified following stimulation of enriched T cell cultures with SEC2 or each of the five mutants. BSA and PHA-P were used as negative and positive controls, respectively. Although all of the mutants possessed superantigenic activity, the SEC2 mutants had reduced potency in PBMC assays. Only the mutant C110A which had an alanine substitution at position 110 displayed T cell proliferation similar to that of SEC2 (Fig. 2).

Substitution of residue 93 depressed T cell stimulation to a greater extent than analogous alterations at position 110. In PBMC assays, this effect was best demonstrated by comparing the two alanine mutants since each had considerably different effects on proliferation induction.

To confirm whether the mutated SEC2 possess the anti-tumor activity similar to that of SEC2, tumor cells Cx-1 was used to assess anti-tumor activity assay. BSA and SEC2 were used as controls. Compared with SEC2, the mutated SEC2 exhibited almost the same anti-tumor activity as SEC2 (Fig. 3).

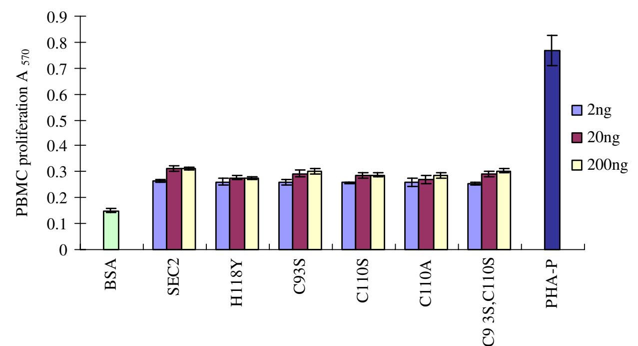

Fig. 2 PBMC proliferation assay of SEC2 and its mutant derivatives by MTT assay. All values are averaged from triplicate tests, $n = 3$, $x \pm s$. Compared with negative control group: $P < 0.01$

Discussion

Superantigen SEs stimulate T cells at a high frequency, thereby giving rise to potent cell-mediated immunological responses and producing a large variety of cytokines inducing the final result of apoptotic death of tumor cells [13]. However, the application of SEC2 is seriously restricted

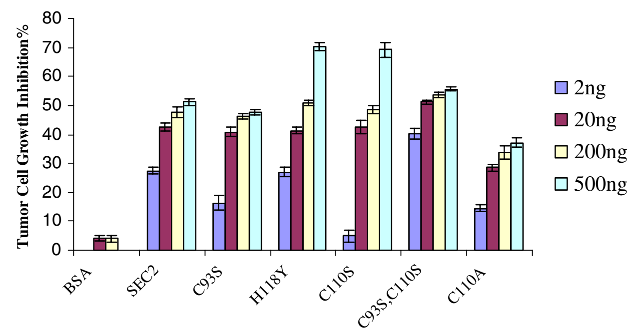

Fig. 3 Anti-tumor activity of SEC2 and its mutant derivatives by activating PBMC in vitro. All values are averaged from triplicate tests, $n = 3$, $x \pm s$. Compared with negative control group: $P < 0.01$

Table 2 Emetic response induced by SEC2 mutants

Dose (mg)	No. of animals[a] exhibiting emesis/total no. of animals following administration					
	SEC2	C93S	C110A	C110S	C93S, C110S	H118Y
1	3/3	ND	ND	3/3	ND	3/3
10	ND	3/3	0/3	ND	3/3	ND

ND not determined at that dose

[a] Prior to dosing with samples, animals were deprived of food for 24 h but were allowed free access to water. Following dosing with protein sample, the incidence of vomiting was monitored over a period of 3 h by trained observers

due to the emesis question [2, 12]. SAgs are able to cross-link MHC class II molecules and TCR in a variety of subtly different ways through the use of various structural regions within each toxin [1, 6, 23]. The crucial functional sites responsible for binding MHC class II and TCR have been confirmed according to the crystal structure of SEs [15, 16, 19, 21].

The cystine loop was composed of Cys93 and Cys110. The local confirmation adjacent to each cysteine residues is substantially different [8]. Residue 93 is situated at one end of a loop just beyond the end of the fourth β-strand that completes the same domain. Despite the different structural environments surrounding both positions, there is no obvious reason for the decreased stability of positions-110 mutants compared with those with substitutions of residue 93. Perhaps substituting cysteine 110 with a residue that cannot form the intra-molecular disulfide linkage is more likely to increase loop flexibility and exposes additional trypsin-sensitive cleavage sites.

The events leading to SAg-induced T cell blastogenesis are multi-factorial, requiring contribution from several molecular regions. Therefore, this biological activity is sensitive to change of protein confirmation. SEC2 mutants, lacking of the disulfide bond, had the least impairment of T cell proliferation even under different concentrations, proving that substitution of cysteine (93 and/or 110) did not affect T cell blastogenesis. It was consistent with the opinion which the disulfide linkage is not critical for superantigenic activity by SEC1 [8]. Among these mutants, C93S mutant exhibited the best activity of T cell proliferation. It may result from position 93 that does not participate in the interaction with both MHC class II and TCR. T cell proliferation activity was not affected after Cys93 was replaced with Ser.

The disulfide bond may be more important with regard to the structure of SEA than to that of SEC1 [8, 12]. Although all SE contain a single disulfide bond, the loop regions internal to the two cysteine residues are not highly conserved. The 16-residues disulfide loop of SEC2 is longer than that of SEA which only contains nine residues. Site-directed mutagenesis of the shorter loop in SEA could predictably have a greater influence over conformation than the same changes in toxins with longer loops. SEC2 cysteine mutants retained more critical native structure. Although all mutants degraded more rapidly than native SEC2 when treated with trypsin, the rates of degradation varied. The three mutants with either alanine or serine substitution at position 110 degraded more rapidly than mutants with an identical substitution at position 93. The rate of degradation for H118Y and C93S were similar to each other and only higher than native SEC2. Regardless of position, mutants with a single serine substitution were more resistant to trypsin than toxins with single alanine substitutions at the same location, indicating that hydrogen bonding by the substituted serine could stabilize the toxin in the absence of a disulfide, which was consistent with the published results [8].

Either the loop residues or residues adjacent to the loop are important for emesis. Therefore, it is feasible to inactivate emetic activity of SEC2 through site-directed mutagenesis and obtain satisfying agents for tumor treatment.

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