International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-009, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

The functional roles of the residue tyrosine at position 26 in staphylococcal enterotoxin C2

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Accepted 11 January, 2019

Bacterial superantigen *Staphylococcus aureus* enterotoxin C2 (SEC2) is a very potent activator of T cells. Previous crystal studies on SEC2 showed that residue Tyr26 which is located near the T cell receptor (TCR) binding sites could potentially participate in T cell activating. In SEC1, a superantigen highly homologous to SEC2, the residue Val26 played a critical role in stimulating certain V expansion. Moreover, residues 20, 22 and 26 in SEC1 determined its serologic cross-reaction. In order to investigate the potential roles of Tyr26 in SEC2, two SEC2 mutants SEC2 (Y26V) and SEC2(Y26A) were constructed by site-directed mutagenesis. The activities and toxicities of the two mutants were determined both *in vivo* and *in vitro*. Results showed that both SEC2 (Y26V) and SEC2 (Y26A) remained potent immune stimulating activities compared with native SEC2. SEC2 (Y26V) had a decreased pyrogenic effect on rabbit model. Both of the two mutants had significantly decreased binding affinities to anti-SEC2 IgG. Our result indicated that Tyr26 is a critical site for the serological characters of SEC2 rather than for its superantigen activity. This may also provide an insight in constructing a novel mutant to substitute native SEC2 in cancer immunotherapy avoiding being neutralized by anti-SEC2 IgG *in vivo*.

Key words: Staphylococcal enterotoxin C2, Site-directed mutagenesis, superantigen, serological, property.

INTRODUCTION

Microbial superantigens are a family of enterotoxins that share the ability to trigger excessive and aberrant activation of T cells (Llewelyn and Cohen, 2002). Unlike conventional antigens, SAgs bind to invariant regions of MHC class molecules outside the antigen-binding groove and are presented as unprocessed proteins to T lymphocytes expressing appropriate motifs on the variable domain of the chain(V) of the T cell receptor (TCR) (Müller-Alouf et al., 2001). As a consequence, SAgs can activate an unusually high percentage of T lymphocytes and initiate massive releasing of cytokines which plays a pivotal role in T cell-mediated immune responses. The best characterized SAgs are Staphylococcal enterotoxins (SEs) and Streptococcal pyrogenic exotoxins (Spes) to date. Staphylococcal enterotoxins (SEs), a series of serological types of heat stable enterotoxins, produced by *S. aureus*, are the leading causes of gastroenteritis resulting from consumption of contaminated food (Balaban and Rasooly, 2000). These toxins share common phylogenetic relationship, structure, function, and sequence homology. SECs are a group of highly conserved proteins with significant immunological cross-reactivity (Marr et al., 1993). According to minor epitope differences, SECs were further classified into three subtypes (C1-C3), which differ in only several amino acid residues near the N terminus (Hovde et al., 1990).

Staphylococcal enterotoxin C2 (SEC2) can effectively activate the immune system and result in tremendous releasing of cytokines such as tumor necrosis factors- (TNF-), interleukin-2 (IL-2), and interferon- (INF-). This attribute makes SEC2 an effective ingredient for cancer immunotherapy in clinic in China (Chen, 2007).

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But as an enterotoxin produced by *S. aureus*, SEC2 can cause toxic shock syndrome (TSS) at certain concentration, which limits its clinical application. Moreover, anti-SEC2 antibodies in patients' serum could neutralize injected SEC2 and impair its effects. In Sweden, a mutant SEA has been fused to the Fab-fragment of tumor-targeted monoclonal antibody for treatment of solid tumors in phase II trial. But toxicity and anti-SEA antibody were also the limiting factors for therapy (Shaw et al., 2007). For construction of novel superantigen variants with decreased toxicity and low reactivity to human anti-SEC2 antibodies, it is necessary to give insight into the key residues in SEC2.

In SEC1, valine in position 26 (Val26) was likely to interact with TCR directly and cause certain V expan-sion (Deringer et al., 1996). Previous researches on antigenic uniqueness indicated that residues 20, 22 and 26 were critical for forming SECl- and SEC2-specific epitopes (Turner et al., 1992). While in SEC2, the crystallographical data showed that tyrosine in position 26 (Tyr26) might locate in putative TCR binding site (Schad et al., 1997; Fields et al., 1996), and it was likely to expose on the molecular surface and influence molecular interaction between SEC2 and TCR (Turner et al., 1992; Papageorgiou et al., 1995).

SEC2 and SEC1 are different in only seven residues near the N terminus (Bohach and Schlievert, 1989), while their superantigen activities exhibited some differences (Hovde et al., 1994; Wang et al., 2009a). Based on the high homology between SEC1 and SEC2, we hypothesize that the residue of Tyr26 might be important to the superantigen activities and serological characters of SEC2.

In order to investigate the potential roles of Tyr26 in SEC2, in this study, two SEC2 mutants SEC2(Y26V) and SEC2(Y26A) have been constructed and their superantigen activities and toxicities were determined both *in vivo* and *in vitro*. Our results showed that the Tyr26 was not function- related with the immune stimulating activities of SEC2, but important for the serological characters of SEC2. The mutant SEC2(Y26V) with potent immune stimulating activities showed decreased toxicity and significantly reduced binding by SEC2-specific IgG, which suggested that the newly constructed mutant protein could be used as a potentially powerful candidate for cancer immunotherapy in the future.

MATERIALS AND METHODS

Plasmids, bacterial strains and cell line

Expression vector pET28a(+) from Novagen (Darmstadt, Germany) was used to transform SEC2 mutant gene into the host. *E. coli* BL21(DE3) from Novagen was cultured in Luria–Bertani (LB) medium for expressing SEC2 mutant protein. Murine hepatoma Hepal-6 cell line used in this study was purchased from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium with 10% (v/v) fetal bovine serum (Gibco, USA). Recombined plasmid pET-28a-SEC2 which contains the entire SEC2 cDNA (Accession number AY450554) was constructed and conserved in our lab (Xu and Zhang, 2006).

Chemicals and enzymes

All chemicals are analytical grade. Pfu DNA polymerase and restriction enzymes were purchased from Takara Biotechnology Co (Dalian, China); isopropyl-D-thiogalactopyranoside (IPTG) and methylthiazol tetrazolium (MTT) were from Sigma Chemical Co (St Louis, MO, USA); Ni-NTA His-Bind resin was from Qiagen (Germany); the DNA Gel Extract kit and Mini-preparation of plasmid kit were from BioDev-Tech Co (Beijing, China).

Experimental animals

Female BALB/c mice (6-8 weeks old, 20-25 g) and adult New Zealand white rabbits weighing 2.0-2.5 kg were purchased from the Experimental Animal Center, China Medical University (Shenyang, China).

All experimental animals were maintained under specific-pathogen-free conditions on a 12 h light/dark cycle. All experiments were carried out under the guiding principles for the care and use of laboratory animals approved by Animal Care Committee of China Medical University.

Site-directed mutagenesis

We constructed two mutant SEC2, SEC2(Y26V) and SEC2(Y26A), by converting the Tyr at position 26 to Val and Ala respectively. The conceptual ideas behind this substitution design were as follows: we introduce Val into position 26 of SEC2 because valine is the residue in position 26 in SEC1, furthermore we introduce Ala into position 26 of SEC2 to diminish the effect of Tyr in this position.

The SEC2 mutant genes were constructed by sequence overlap extension. All the primers related with over -lap PCR were listed in Table 1. The PCR-generated fragments containing substituted oligonucleotides were digested by *Eco*R I and *Xho* I, and ligated into plasmid pET28a(+) digested with the same enzymes. The recombined expression plasmid was transformed into *E.* coli BL21(DE3) and positive clone was identified by DNA sequencing.

Expression and purification of mutant protein

For expressing SEC2 mutant protein, the positive clone of transformed *E*. coli BL21(DE3) was incubated in LB for 10 h. The culture was then inoculated at a ratio of 1:100 (v/v) into LB medium supplemented with 50 g kanamycin ml^{-1} and incubated at 37°C with vigorous shaking until the OD600 reached 0.7. Then 1.0mM IPTG was added into the culture and the incubation was continued for 4 h more with vigorous shaking at 30°C. The cells were harvested by centrifugation at 5,000 rpm and resuspended with ice-cold loading buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 7.9). The cells were disrupted by sonication on ice followed by centrifugation (10,000 rpm, 30 min). The supernatants were collected and loaded onto a Ni-saturated chelating Sepharose column pre-equilibrated with loading buffer. After removing non-specifically binding protein with washing bufferl (50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 7.9), SEC2 mutant protein was released from the column by washing buffer (50 mM NaH_2PO_4 , 500 mM NaCl, 250 mM imidazole, pH 7.9) and dialysed against PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) at 4°C for 48 h to remove the high concentration of

Table 1. Primers for amino acid substitution by over-lap PCR.

Primers	Amino acid substitution	Primer sequence [a]
P-1		5 -CGGAATTCGAGAGTCAACCAGA-3
P-2	26Y V	5 -CATCATATAATACTTTCATATTACCCATCG-3
P-3		5 -GGGTAATATGAAAGTATTATATGATGATC-3
P-4	26Y A	5 -CATCATATAATGCTTTCGTATTACCCATCG-3
P-5		5 -GGGTAATACGAAAGCATTATATGATGATC-3
P-6		5 -TCGCTCGAGTTATCCATTCTTTGTTG-3

[a] The primers were designed for special amino acid substitution. The sequences of mutant residues are stressed in boldface. The recognition sites of *Eco*R I and *Xho*I are underlined.

imidazole. The purity of SEC2 mutant protein was estimated by SDS-PAGE and the concentration of mutant protein was determined by Bradford assay using BSA as the standard.

Stimulating murine T-cell proliferation assay

To investigate the activity of stimulating T cell proliferation by mutant SEC2, splenocytes from 6- to 8-week-old BALB/c female mice were collected as experimental target. The mutant SEC2 and the native SEC2 dissolved in 1640 medium were filled into the wells of 96- well microtitre plates in four different concentrations (10, 100, 1000, and 10,000 ng/ml). There were three repeated wells at each concentration. RPMI 1640 medium with 10% FBS was served as negative control. Murine T cells were seeded into the experimental and negative control wells at a density of 8×10^5 cells per well.

The proliferation index (PI) was determined by MTT assay. The splenocytes were incubated at 37°C with 5% CO_2 for 72 h. The incubation was continued for 4 h more after 25 l MTT (5 mg/ml, dissolved in PBS) had been added to each well. Then the T cells were collected by centrifugation at 1,000 rpm for 10 min. 120 l DMSO was added into each well to redissolve the pellet on the bottom. To fully dissolve the formazan produced by T cells, the plate was vigorously shaking for 20 min. The absorbance was measured with a microplate reader at 570 nm, using a reference wavelength of 630 nm. The final absorbance is the difference between these two readings. The proliferation index (PI) was calculated with the following equation:

PI=Abs value of the experimental well / Abs value of the negative control well.

Antitumor activity

To further investigate the superantigen activity of mutant SEC2, the inhibitory effects on tumor cell was determined by MTT assay. Splenocytes from 6- to 8-week-old BALB/c female mice were served as effector cells and Hepal-6 cell as target. Native SEC2 and the mutants diluted with RMPI 1640 containing 10% FBS were separately filled into the wells of 96-well microtitre plate at 10ng/ml, 100 and 1,000 ng/ml in triplicate. In experimental wells, effector cells (5×10^5 cells/well) and target cells (2.5×10^4 cells/well) were mixed in the present of native SEC2 or the mutants in different concentrations. Wells contained protein and lymphocytes only were served as lymphocytes releasing control. Wells contained Hepal-6 cells alone were served as unsettled tumor cell control. The blank wells were RMPI 1640 with 10% FBS only, and BSA was served as negative control.

After incubation for 72 h at 37°C with 5% CO_2, 25 l MTT was added into each well, and the cells were incubated for 4 h more. The cells were collected by centrifugation and redissolve with DMSO. The absorbance was measured with a microplate reader at 570 nm, using a reference wavelength of 630 nm. Tumor growth inhibition (%) was calculated with the equation: 100 - [(Abs value in protein-treated cells well - Abs value in lymphocytes-releasing well) / (Abs value in unsettled tumor cells control wells - Abs value in blank control wells)] ×100.

Rabbit model assay

Rabbit model was used to compare the febrile response of SEC2 and the mutants *in vivo*. Each of the experimental animals was fixed and the rectal temperature was measured for at least 90 min before injection. Qualified rabbits used in this assay must have stable body temperatures ranging from 38.6 to 39.5°C. Native SEC2 and the mutants dissolved in PBS were injected into rabbits at the does of 10 g/kg (body weight). Three animals were injected with each protein. PBS was served as negative. Rectal tempera-tures of rabbits were measured with indwelling rectal thermometers right after pyrogen administration continually for 3 h. The rectal temperature change (T) of rabbit was accepted as an index of pyrogen effect *in vivo*, and it was calculated by subtracting the temperature immediately before injection from the temperature at each time point after the pyrogen injection.

Proteolysis susceptibility assays

To investigate whether the trypsin-resistance of SEC2 mutants were affected by amino acid substitution, purified mutanted proteins and native SEC2 (100 g/ml) were respectively incubated at 37°C with trypsin at a trypsin/protein ratio of 1:25 (w/w). After desired periods of time, the enzymolysis was terminated by boiling in SDS-PAGE sample buffer for 5 min and analyzed by SDS-PAGE.

Detection of mutant SEC2 by ELISA

ELISA assay was carried out in accordance with the instruction of the kit (National institute for the control of pharmaceutical and biological products, Beijing, China). Briefly, the polystyrene plates were coated with 100 l of 5 g/ml rabbit anti -SEC2 IgG diluted in carbonate buffer overnight. The excessive IgG which did not bind to the wells was washed off by PBS containing 0.05% Tween 20 (PBS-T20), then unabsorbed sites were blocked with 1% gelatin in PBS for 30 min at 37°C. Mutant SEC2 and the native dissolved in PBS-T20 buffer were added into the wells separately. The plate was incubated for 2 h at 37°C. Then the wells were emptied and washed by PBS-T20 buffer. Rabbit anti-SEC2 IgG-HRP conjugate was added, and the plate was incubated for 1.5 h at 3 7°C. The

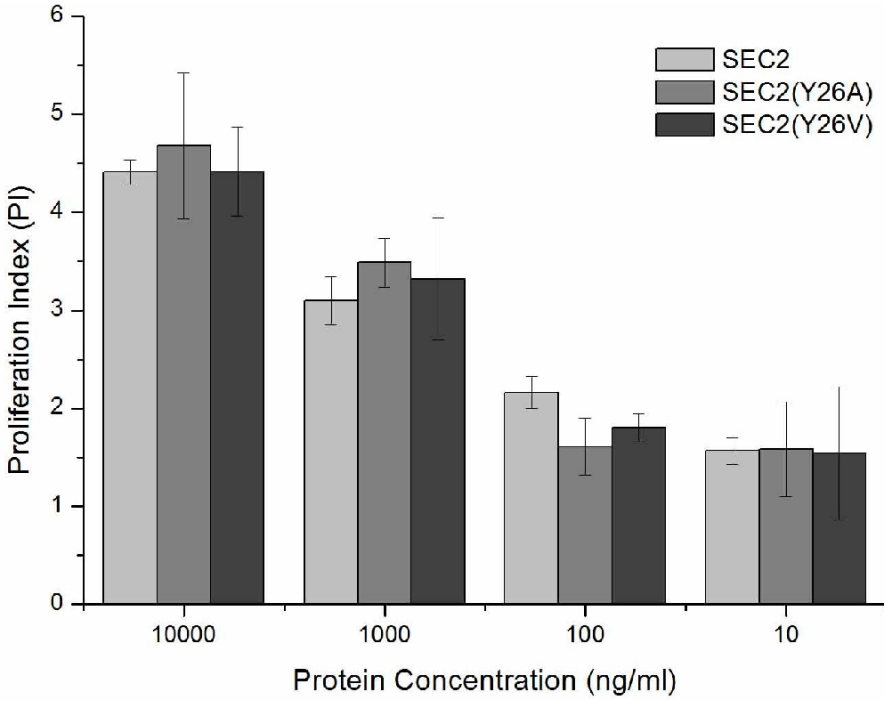

Figure 1. Results of murine T-cell proliferation by SEC2 mutants and native SEC2. Purified proteins of SEC2 mutants or the native were incubated with murine splenocytes for 72 h before the proliferation effects were determined by MTT assay. Value on the *y*-axis represents the average proliferation index (PI)± SD of triplicate values. Each purified protein was tested in at least three separate assays.

uncombined enzyme- linked antibody was washed off by PBS-T20, and 100 l substrate (0.04% *ortho*-phenylene-diamine) was added into the wells and incubated for 30 min at 37°C. Reactions were terminated by addition of 50 l 2 mol/L H_2SO_4, and the absorbance was measured at 450 nm.

Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using Student's *t* test. A P-value < 0.05 was considered statistically significant.

RESULTS

Construction and expression of the mutant SEC2

To construct SEC2 mutant protein, over-lap PCR was employed. The mutant SEC2 genes were successfully constructed and cloned into the expression vector pET-28a (+). The recombinant expression vectors were transformed into *E. coli* BL21 (DE3) respectively for expressing mutant proteins. The positive clones were identified by DNA sequencing.

For expressing SEC2 mutant proteins, the positive clones with the right mutagenesis introduced were respectively cultured in LB medium containing 50 mg kanamycin ml^{-1} till the OD 600 reached 0.7. The expressions of mutant proteins were performed by IPTG inducing at 30°C for 4 h. After purification with Ni-NTA His Bind Resin, both of the two mutant proteins shown as a single band on SDS-PAGE were of purity of more than 95%.

Murine T-cell proliferation activity

The activities of stimulating T cell proliferation by SEC2 and the mutants were investigated. Series of ten-fold dilutions of native SEC2 and the mutants dissolved in PBS were filled into the wells of a 96-well microtitre plate with murine T cells. After 72 h incubation, the proliferation index of murine T cell was determined by MTT assay. As the experimental data reflected, both native SEC2 and the mutants could induce murine T cells proliferation in a dose-dependent manner. The ability to induce T cell proliferation of SEC2 mutants were similar with that of the native, without significant differences at any concentration (P>0.05; Figure 1) . This result indicated that the amino acid substitution at position 26 with Ala or Val had not significantly affected the stimulating activity of SEC2.

Examination the antitumor activity

To further investigate the superantigen activity of mutant SEC2, MTT assay was employed to compare the

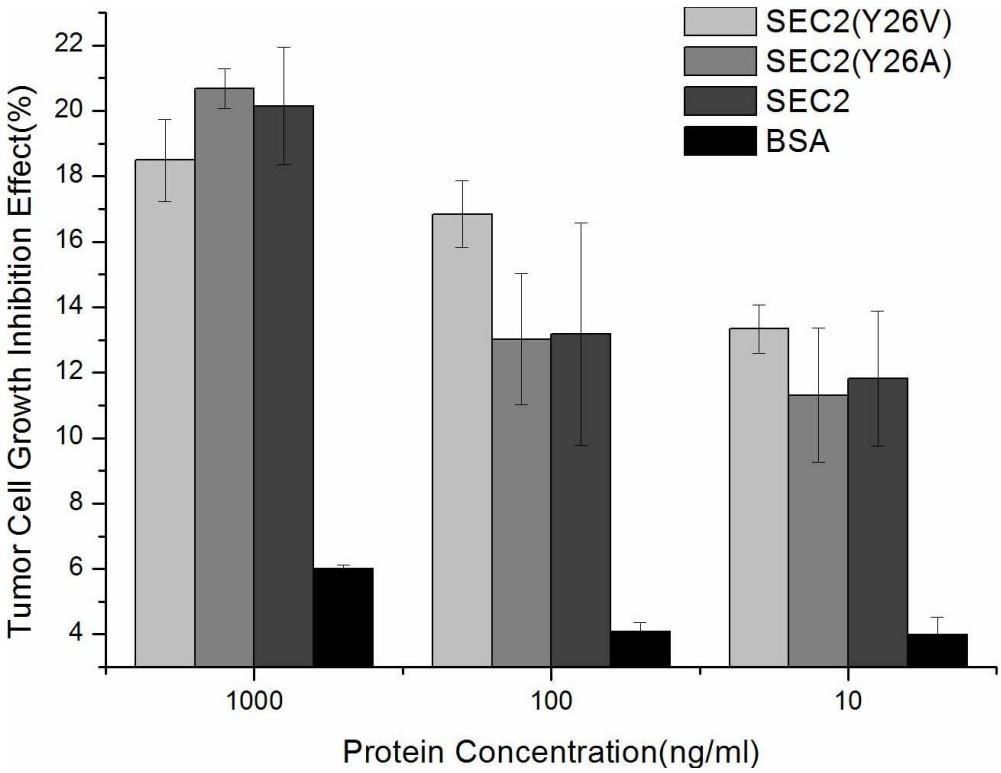

Figure 2. MTT assay was employed to compare the tumor cell growth inhibition effects between SEC2 and the mutants. Tumor cells and the splenocytes were mixed and seeded into the wells at the density of 2.5×10^4 cells/well and 5×10^5 cells/well respectively. Different concentrations of SEC2 or the mutants were incubated with the cells at 37°C for 72 h before the absorbance was measured. BSA was used as negative control. Data shown are representative of at least three separate experiments, and the values represent the mean ± standard error of the mean.

anti-tumor activity of SEC2 and the mutants. Hepal-6 cells (target cells) and splenocytes (effector cells) were incubated in the present of proteins in different concentrations for 72 h before the absorbance was measured.

All the proteins tested in this study exhibited predominant anti-tumor abilities compared with negative control ($P<0.05$, Figure 2). The abilities of the two mutants to inhibit tumor cell proliferation was comparable with that of the native SEC2 ($P>0.05$). This indicated that the amino acid substitution in position 26 had little influence on the superantigen activity of SEC2.

Toxicity

The Rabbit model assay was carried out to investigate the pyrogenic effect of mutant SEC2. The rectal temperature of each experimental animal was continually measured for 3 h after pyrogen injection. The results showed that both SEC2 and the mutants could induce the enhancement of temperature in experimental animal significantly compared with negative control ($P<0.05$, Figure 3). SEC2(Y26A) exhibited comparable pyrogenic effects with native SEC2 in the whole experimental process ($P>0.05$). Whereas SEC2(Y26V) exhibited a decreased ability to induce fever at 2h, 2.5h, and 3h compared with SEC2. Furthermore, the enhancement of temperature in experimental animal injected with SEC2(Y26V) reached its top at 1.5 h after pyrogen injection. But it is noteworthy that the body temperature of animal injected with SEC2 or SEC2(Y26A) rose continually although the experiment.

Susceptibility to trypsin

In order to determine whether the minor decreased pyrogenic effect of SEC2(Y26V) in the later stage of pyrogen experiment was resulted from its degradation, trypsin digestion assay was performed to evaluate the stability of the two mutants. The result was determined by 15% SDS-PAGE and showed that there was no significant difference in the trypsin susceptibility between native SEC2 and the mutants (Figure 4). This result suggested that the amino acid substitution at position 26 did not affect the instability of SEC2, and the decreased pyrogenic activity of SEC2(Y26V) was not resulted from its degradation.

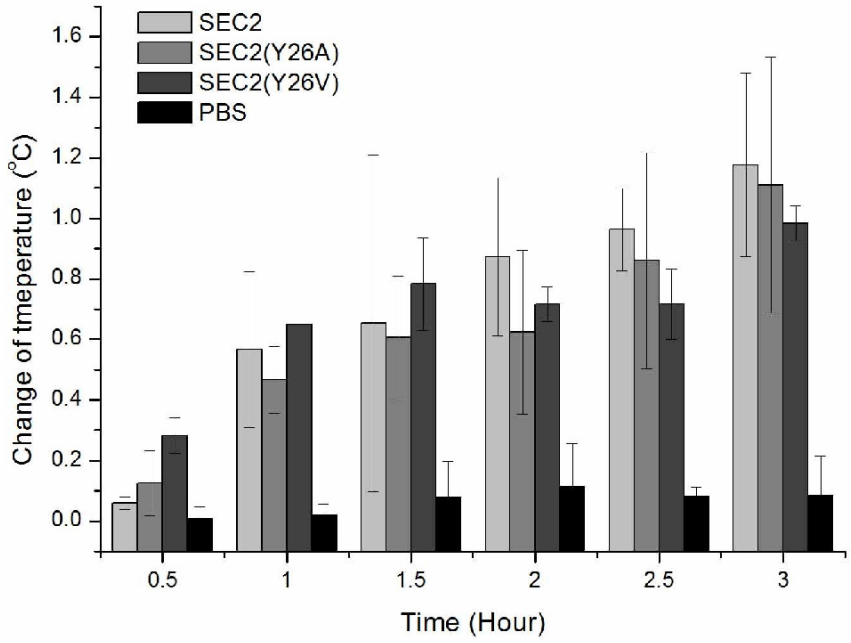

Figure 3. The pyrogenicities of SEC2 and the mutants in rabbit model. The body temperature of experimental rabbit was continually measured for 3h right after protein injection. PBS solution was served as negative control. The value of y-axis represents the rise of temperature.

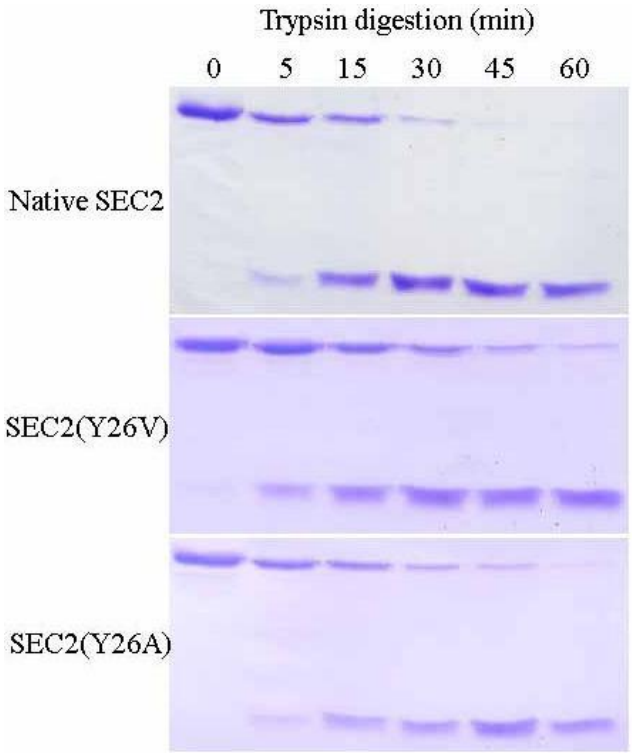

Figure 4. Susceptibilities of SEC2(Y26V), SEC2(Y26A), and native SEC2 to trypsin were compared. Each protein was mixed with trypsin at a trypsin/protein ratio of 1:25 (w/w). The digestive reaction was performed at 37°C for time gradient and terminated by boiling for 5 min at 100°C. The result was analyzed by 15% SDS-PAGE.

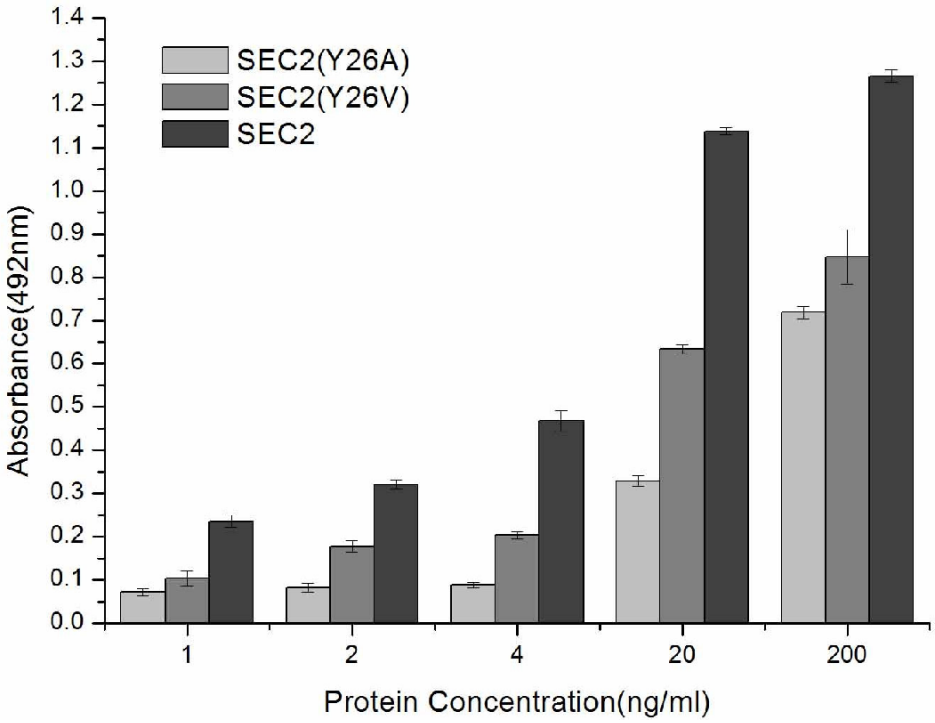

Figure 5. Comparison the anti-SEC2 IgG binding affinity of native SEC2 and the mutants through ELISA. The absorbance was measured and calculated as the mean readings of triplicate samples ± SD.

Evaluating epitope change of mutant SEC2 by ELISA

ELISA assay was employed to investigate whether the Tyr at position 26 determines the epitope of SEC2. Both of the two mutants showed significant decreased binding affinities to SEC2-specific IgG compared with native SEC2 ($P<0.05$; Figure 5) in all concentrations. This result indicated that Tyr26 was a critical site for the serological characters of SEC2. Substitution at position 26 in SEC2 results in remarkable change of epitope and further influence on the immunogenic activity of native SEC2. Furthermore, the binding affinity of SEC2(Y26A) was significantly lower than that of SEC2(Y26V) ($P<0.05$) especially in concentration from 2 to 20 ng/ml, which suggested that the substitution with Ala results in more alteration of antigenic determinants of native SEC2 than that with Val.

DISCUSSION

Recently, much attention has been focused on the tumor-therapeutic potential of SEs because the ability of SEs to induce strong T cell-mediated immune responses and elicited systemic antitumor effect (Wang et al., 2009b; Sundstedt et al., 2009). In China SCE2 has been used as a supplementary- drug in tumor treatment for many years and encouraging effect has been obtained (Chen, 2007). Recently, a tumor-targeted superantigen (TTS) consisting of a mutant SEA fused with a Fab-fragment of anti-tumor monoclonal antibody had been studied in phase II trial for tumor treatment (Shaw et al., 2007).

But as serial toxins produced by *Staphylococcus aureus*, SEs play roles in several disorders, especially infectious and autoimmune diseases as well as toxic shock syndrome in general (Pastacaldi et al., 2010; Strandberg et al., 2010). High does SEC2 injection can result in toxin shock syndrome in humans (Chen, 2005). Furthermore, the main side effects of SEA TTS treatment were fever and being neutralized by patients' anti-SEA antibody, which limits the wide clinical application (Shaw et al 2007; Sundstedt et al., 2009). So improved superantigen reagents with approximate antitumor activity and depressed toxicity but lower serological activity were required (Shaw et al., 2007). This promotes us to investigate the relationship between crucial amino acids and bioactivity of SEs.

In order to investigate the potential roles of Tyr26 in SEC2, in this study, two SEC2 mutants SEC2(Y26V) and SEC2(Y26A) have been constructed and their superantigen activities and toxicities were determined both *in vivo* and *in vitro*.

The experimental data showed that both of the two mutants exhibited comparable abilities to stimulate murine T cell proliferation and inhibit the growth of Hepal-6 cells. This is unexpected to us because the functional

side chain of Try26 was completely removed by Ala substitution in SEC2(Y26A). A reasonable explanation for this phenomenon is that Try26 was not the key residue for the immune stimulating activity of SEC2.

Previous crystal structure study on SEC2 conveyed that residues 20, 22 and 26 potentially interacted with TCR (Papageorgiou et al., 1995). Although there was no direct evidence that Tyr26 in SEC2 participated in the interaction of SEC2 with TCR, some researchers reported that Val26 in SEC1 recognized the specificity of TCR V of SEC1. Mutant study in SEC1 showed that SEC1(V26 Y)with a single amino acid substitution at position 26 could significantly enhance the expansion of V 13.1 but decreased the expansion of V 3 (Deringer et al., 1996).

So, on the basis of the high homology between SEC1 and SEC2, we constructed SEC2(Y26V) and SEC2(Y26A) to respectively change and diminish the TCR V recognization ability of SEC2. It was widely accepted that the affinity of SAgs for TCR can significantly affect their mitogenic potency (Andersen et al., 2001) . The weak affinity of the TCR-Sag interaction results in poor T cell response. But in this study, both of the two mutants exhibited comparable stimulating ability with SEC2, which suggested that substitution in position 26 could not alter the TCR affinity of SEC2.

In rabbit model assay, SEC2(Y26V) exhibited a decreased ability to induce fever at 2h, 2.5h, and 3h compared with SEC2. This promoted us to investigate whether the decreased toxicity *in vivo* was due to its instability resulted from amino acid substitution. Our result from proteolysis susceptibility assays has exhibited that both of the two mutants maintained comparable resistance to trypsin with SEC2, which indicates that the amino acid substitution in position 26 did not result in the exposure of additional tryptic cleavage sites. So it is suggested that the decreased toxicity of SEC2(Y26V) does not due to its degradation. SEC2(Y26V) has potent immune stimulating activities compared with SEC2 in all concentration, which is inconsistent with the previous theory that the febrile activity of SE is a direct consequence of T cell stimulation. On the other hand, macrophages and monocytes stimulated by superantigen could secrete some endogenous pyretogenic cytokines such as TNF- and IL-1, which was the major cause of immune-mediated diseases such as fever (Roggiani et al., 1997). Further investigation was needed to interpret it.

Previous researches on antigenic uniqueness indicated that residues 20, 22 and 26 were critical for forming SECl- and SEC2-specific epitopes. SEC1 mutants with substitutions at all three positions reacted only with an SEC2-specific antibody (Turner et al., 1992). On the basis of the homology between SEC1 and SEC2, we hypothesized that Tyr26 was important for the serological characters in SEC2. The result from ELISA confirmed this hypothesis that SEC2(Y26V) and SEC2(Y26A) showed significantly decreased binding affinities to SEC2-specific IgG. The residue of Val26 in SEC2(Y26V) was homolo-gous to Val26 in native SEC1, so Val26 could mediate the cross-reactivity of SEC2(Y26V) with rabbit anti-SEC2 polyclonal IgG used in this study. It could be a possible explanation for that SEC2(Y26V) showed higher binding affinity to SEC2-specific IgG than SEC2(Y26A) did. For the lack of SEC1-specific IgG, we could not determine whether SEC2(Y26V) and SEC2(Y26A) were able to induce cross-reactivity with SEC1-specific IgG. However it was quite sure that the mutation introduced into position 26 influenced the recognition of SEC2 by SEC2-specific antibodies, which suggested that Tyr 26 was critical for the antigenic uniqueness of SEC2.

Concluded from our result, the roles of residue at position 26 in SEC2 are different from those of in SEC1, which may result from continuous evolution. This slight difference can display different serological specificity to avoid the cross-reactivity with antibodies from the host.

In summary, we have investigated the potential roles of Tyr26 in SEC2 by site-directed mutagenesis. Our results indicated that Tyr26 is not important for the immune stimulating activities, but a critical site for the serological characters of SEC2. A low toxic mutant SEC2(Y26V) constructed in this study with potent superantigen activities and significantly decreased binding affinities to anti-SEC2 IgG has the potentiality to be a novel mutant to substitute native SEC2 in cancer immunotherapy.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Science and Technology Major Specific Projects of China for "Significant Creation of New Drugs" (2009ZX09103-692).

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