T-cell proliferation and antitumour activities of a truncated mutant of staphylococcal enterotoxin C2 with decreased cytokine secretion

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As a superantigen, staphylococcal enterotoxin C2 (SEC2) has commonly been used as an antitumour immunotherapy agent in China. However, the clinical application of SEC2 has been hampered by its pyrogenic toxicity and the presence of neutralizing antibody in patients. Thus, an improvement in its superantigen-based immunotherapy is highly needed. In this study, a truncated SEC2 mutant, SEC(14–128), was constructed without the N-terminal 13 and C-terminal 111 aa. This mutant retained T-cell proliferation and antitumour activities in vitro experiments. However, it induced a significantly decreased release of the main inflammatory cytokines interleukin-2 and gamma interferon. Moreover, SEC(14–128) exhibited reduced toxicity and affinity to anti-SEC2 IgG compared with native SEC2. Based on the considerable antitumour activity and low toxicity, it is proposed that the mutant SEC(14–128) could be a potential candidate for cancer treatment.

INTRODUCTION

Staphylococcal enterotoxins (SEs) are a class of disease-causing and immunostimulatory heat-stable proteins secreted by Staphylococcus aureus and Streptococcus aureus (Llewelyn & Cohen, 2002). The SE family has various serological types, from SEA to SEE and from SEG to SEU (Munson et al., 1998; Letertre et al., 2003; Ono et al., 2008). Based on minor differences in the amino acid sequence at several residues, SEC has been further classified into three subtypes, SEC1–SEC3 (Hovde et al., 1990). These three subtypes have been speculated to share some important biological properties because of their high degree of homology (Dinges et al., 2000).

Compared with conventional antigens, SEs are superantigens that can bind to major histocompatibility complex class II (MHC II) molecules outside the antigenic groove without being processed by antigen-presenting cells. They can also stimulate the vigorous proliferation of T-cells bearing certain T-cell receptor β-chain variable regions (Dinges et al., 2000; Müller-Alouf et al., 2001). As a result, superantigens induce strong cell-mediated cytotoxicity, preferentially against MHC II-positive target cells, and release large amounts of inflammatory cytokines (Dohlsten et al., 1994; Mondal et al., 2002). Therefore, SEs have been studied extensively as an antitumour immunotherapy (Dohlsten et al., 1994; Shaw et al., 2007; Xu et al., 2011). SEC2 has also been used in clinics as a supplementary therapeutic agent for malignant tumour treatment in China (Chen, 2005). However, the pyrogenic toxicity and high molecular mass of SEs, as well as the presence of highly neutralizing antibodies in patients, affect the delivery efficiency of SEs and their therapeutic effects. Therefore, SE molecules have to be reconstructed to improve immunotherapy.

For clinical applications, low-molecular-mass proteins are highly superior compared with their high-molecular-mass counterparts. Moreover, molecular truncation is a feasible method for medical protein reconstruction. Thus, elucidation of the structure–activity relationship of SEs is the premise and basis of their truncation reconstruction.

The molecular architecture of the SE family has been studied extensively to explore the relationship between their structure and function (Schad et al., 1997; Papageorgiou et al., 2004). In most cases, an SE molecule consists of two closely spaced N- and C-terminal domains. Its main secondary structures are α-helices and β-sheets (Dinges et al., 2000). For the three SEC subtypes, Hoffmann et al. (1994) found that the deletion of aa 1–13 did not affect the stimulatory activity of SEC1 on T-cell proliferation, whereas further deletion of the protein inhibited its T-cell proliferative activity. Moreover, our previous study indicated that deletion of the C-terminal 77 or N-terminal 11 aa had little effect on the T-cell proliferation and pyrogenic activities of SEC2; however, deletion of the C-terminal 127 or N-terminal 18 aa significantly depressed its activities (Wang et al., 2009).
To date, it is still uncertain whether the C-terminal 77 or N-terminal 11 residues are the borders of the shortest critical length for SEC2 activity and whether a key residue governing the activities in the C-terminal aa 77–127 or N-terminal aa 11–18 exists. Aiming to obtain rational answers to these questions and to overcome the limitations of SEC2 in its clinical application, we constructed a deletion mutant, SEC(14–128), without the N-terminal aa 1–13 and C-terminal aa 129–239 of native SEC2 and determined its biological activity using in vitro and in vivo experiments. SEC(14–128) exhibited considerable antitumour activity and low toxicity; thus, it could be a potential candidate for cancer treatment.

**METHODS**

**Bacterial strain, plasmids and cell line.** *Escherichia coli* BL21(DE3) was grown on Luria–Bertani (LB) medium for plasmid transformation and protein expression. The expression vector pET-28a(+) was purchased from Novagen. A recombined plasmid, pET-28a-sec2, which contained the entire SEC2 cDNA (GenBank accession no. AY450554), was constructed previously in our laboratory (Xu & Zhang, 2006). The murine Hepa1-6 cell line was purchased from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FBS.

**Animals.** Six- to 8-week-old female BALB/c mice with body weights ranging from 20 to 25 g and adult New Zealand white rabbits with body weights from 2.0 to 2.5 kg were provided by the Experimental Animal Center (China Medical University, Shenyang, PR China). The animals were raised under strict pathogen-free conditions in 12:12 h light:dark cycles. All in vivo and in vitro experiments were conducted following the regulations for the administration of affairs concerning experimental animals approved by the State Scientific and Technological Commission of China.

**Chemicals and enzymes.** IPTG and methyhiotiazol tetrazolium (MTT) were purchased from Sigma Chemical Co. Restriction enzymes and *Pfu* DNA polymerase were purchased from TaKaRa Biotechnology. The Ni-NTA agarose was from Qiagen. The DNA Gel Extract kit and Mini Preparation of Plasmid kit were from BioDev-Tech Co. The rabbit anti-SEC2 IgG ELISA kit was from the National Institute for the Control of Pharmaceutical and Biological Products, and the mouse cytokine ELISA kit was from KeyGEN Biotechnology Co.

**Site-directed mutagenesis.** pET-28a-sec2 was used as template to amplify the truncated SEC(14–128) using a sense primer (5'-GGTTGGAATCTCAAGTGAAGTTGTTATGGG-3') and an antisense primer (5'-CGGCCCTCGATTTAGTTCAATTTCAATCGAG-3'). The PCR fragments were digested using EcoRI and *XhoI* and ligated into plasmid pET-28a(+), which was digested using the same enzymes. The constructed plasmid was then transformed into *E. coli* BL21(DE3) and verified by DNA sequencing.

**Protein expression and purification.** The transformed BL21(DE3) cells were inoculated into LB medium supplemented with 50 μg kanamycin ml⁻¹, grown overnight and then subcultured at a 1:100 dilution into fresh medium at 37 °C. Protein expression of SEC(14–128) was induced using 1 mM IPTG for 4 h at 30 °C. The cells were harvested by centrifugation at 4 °C and the pellet was resuspended in buffer A (50 mM NaH₂PO₄, 500 mM NaCl, 10 mM imidazole, pH 7.9) and sonicated on ice. The solution was clarified by centrifugation at 12 000 g for 30 min. The supernatant was collected and loaded onto a Ni-saturated chelating Sepharose column pre-equilibrated with buffer A. After non-specifically bound proteins had been washed off with buffer A containing 30 mM imidazole, the target protein specifically bound to the resin was eluted with buffer A containing 250 mM imidazole. The eluate was dialysed in PBS (pH 7.4) and the concentration of the mutant protein measured using a Bradford assay with BSA as the standard. The purity of the mutant protein was determined by SDS-PAGE and Coomassie Brilliant Blue R-250 staining.

**Murine T-cell proliferation assay.** Splenocytes isolated from 6–8-week-old female BALB/c mice were seeded into 96-well microtitre plates at a concentration of 8 x 10^5 cells per well in RPMI 1640 with 10% FBS. The cells were treated with SEC2 or SEC(14–128) that was serially diluted tenfold from 10 to 1000 ng ml⁻¹. RPMI 1640 with 10% FBS was used as a negative control. Splenocyte proliferation was determined by an MTT assay (Mosmann, 1983). The splenic lymphocytes were cultured at 37 °C with 5% CO₂ for 72 h. The incubation was continued for 4 h after the addition of 30 μl MTT (5 mg ml⁻¹ in PBS) to each well. The cells were collected by centrifugation at 1000 g for 10 min. The pellet was resuspended in 120 μl DMSO at room temperature for 10 min, and the absorbance (A) was measured using a microplate reader at 570 nm, with a reference wavelength of 630 nm. The difference between the two readings was taken as the final absorbance. The proliferation effect was reported as a proliferation index (PI) calculated as: PI = Avalue of experimental well/A value of negative-control well.

**In vitro growth inhibition assay.** Tumour cell death induced by T-cell cytokotoxicity (superantigen-dependent cellular cytotoxicity) was evaluated by an MTT assay (Wang et al., 2009). Briefly, murine splenocytes were used as effector cells and the murine hepatoma cell line Hepa1-6 was used as the target cells. The diluted SEC2 and SEC(14–128) were placed separately into 96-well plates at concentrations ranging from 10 to 1000 ng ml⁻¹. The target cells (2.5 x 10⁶ cells per well) were mixed with splenocytes at an effector:target ratio of 20:1 in each well and incubated at 37 °C for 72 h in a humidified atmosphere with 5% CO₂. The blank wells (RPMI 1640 only), unsettled-cell control wells (Hepa1-6 cells only) and lymphocyte-releasing wells (lymphocytes and proteins) were used as controls. BSA was used as a negative control. The plates were treated as described above and the absorbance value was measured.

The rate of tumour cell growth inhibition (%) was calculated as 100–[(A value in wells with protein-treated cells–A value in wells with lymphocyte-releasing cells)/(A value in control wells with unssettled cells–A value in blank control wells)] x 100.

**In vivo pyrogenic toxicity assay.** A rabbit model was used to evaluate the febrile response induced by SEC2 and SEC(14–128). The rectal temperature of each rabbit was stable for at least 90 min before the proteins were injected. Only rabbits with body temperatures of 38.6–39.5 °C were used. Fever was characterized by a mean body temperature increasing by >0.5 °C over a 4 h period (US Pharmacopeia, 2002). For the treatment group, three rabbits were given an initial intravenous injection of SEC(14–128) [10 μg (kg body weight)⁻¹] dissolved in PBS. The positive- and negative-control groups were injected with SEC2 and PBS, respectively. The rectal temperatures of the rabbits were measured using an indwelling rectal thermometer for 3 h after injection. The rectal temperature changes (ΔT) were calculated by subtracting the temperature immediately before injection from the temperature at each designated time point after injection.

**Affinity to anti-SEC2 IgG.** ELISA was performed according to the manufacturer’s instructions. ELISA polystyrene plates were coated overnight with 100 μl 5 μg rabbit anti-SEC2 IgG ml⁻¹ diluted in
carbonate buffer. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 1% gelatine in PBS at 37 °C for 30 min. Thereafter, the native SEC2 and SEC(14–128), each dissolved in PBST to final concentrations of 25, 50 and 100 ng ml⁻¹, were placed into separate wells and incubated at 37 °C for 2 h. After three washings with PBST, a rabbit anti-SEC2 IgG–horseradish peroxidase conjugate was added to all wells and incubated at 37 °C for 1.5 h. After three more washings with PBST, 100 µl substrate (0.04% o-phenylenediamine) was added to the wells and incubated at 37 °C for 30 min. The reactions were terminated by adding 50 µl 2 M H₂SO₄ to each well, and the absorbance was measured at 492 nm.

**Detection of cytokine production in vitro.** Splenocytes from 6–8-week-old BALB/c female mice were seeded into the wells of 96-well microtitre plates at a concentration of 8 × 10⁵ cells per well in RPMI 1640 with 10% FBS. Native SEC2 and SEC(14–128) were serially diluted tenfold in RPMI 1640 and added to individual wells at concentrations from 10 to 1000 ng ml⁻¹. BSA was used as a negative control. The splenic lymphocytes were cultured with 5% CO₂ at 37 °C. After 48 h, the culture supernatant was collected, and the levels of interleukin-2 (IL-2), gamma interferon (IFN-γ) and tumour necrosis factor α (TNF-α) were measured separately using the corresponding mouse cytokine ELISA kits according to the manufacturer’s instructions.

**Statistical analysis.** Statistical analysis was performed using Student’s t-test. A value of P<0.05 was considered statistically significant.

**RESULTS**

**Construction and expression of SEC(14–128)**

The expression vector of the mutant protein SEC(14–128) was constructed and confirmed by DNA sequencing. SEC(14–128) expression was induced by IPTG at 30 °C for 4 h and its soluble expression was detected by SDS-PAGE. After purification with Ni-saturated chelating Sepharose, the purified SEC(14–128) was analysed by SDS-PAGE and visualized using Coomassie Brilliant Blue staining.

**Murine T-cell proliferation**

The diluted native SEC2 and SEC(14–128) were tested for their ability to stimulate murine T-cell proliferation using an MTT assay. Although half of the native SEC2 molecule was truncated in the protein SEC(14–128), it retained potential T-cell proliferation activity compared with native SEC2 (P>0.05; Fig. 1), suggesting that the amino acid residues at positions 1–13 and 129–239 are not necessary for the T-cell proliferation activity of SEC2.

**In vitro growth inhibition assay**

We also performed MTT assays to evaluate the antitumour activity of the SEC(14–128) mutant. Thus, we determined the growth inhibition of Hepa1-6 cells induced by SEC(14–128) in the presence of murine lymphocytes. SEC(14–128) was shown to exhibit an antitumour activity similar to that of native SEC2 (P>0.05). This activity was dose dependent from 10 to 1000 ng ml⁻¹ (P<0.01; Fig. 2). These results indicated that amino acid residues from both aa 1–13 and aa 129–239 of SEC2 are not important in its antitumour activity.

**In vivo pyrogenic toxicity**

A rabbit model was used to compare the pyrogenic toxicity of SEC2 and SEC(14–128). Native SEC2, but not SEC(14–128), caused severe fever from 1 h after the start of the...
experiment until the end (Fig. 3). In contrast, SEC(14–128) induced only a modest increase in temperature during the procedure. The maximum increase in temperature occurred at 1.5 h but was still less than 0.5 °C for SEC(14–128). Thereafter, the temperatures of all three rabbits generally decreased until the end of the experiment (Fig. 3). This result implied that the truncated amino acid sequences are critical for the pyrogenic toxicity of SEC2.

**Epitope change of SEC(14–128)**

The epitope change of SEC(14–128) was determined by ELISA. SEC(14–128) was shown to have no significant binding affinity to a SEC2-specific IgG compared with the native SEC2 ($P < 0.01$; Fig. 4), suggesting that SEC2 truncation led to a remarkable epitope change and that SEC(14–128) might be neutralized by the anti-SEC2 IgG much less effectively than the native SEC2 when used in vivo.

**In vitro cytokine level**

We performed an ELISA to determine the cytokine levels secreted by splenocytes after treatment with SEC2 and the mutant protein. Native SEC2 induced significantly higher levels of IL-2 and IFN-γ than SEC(14–128) ($P < 0.01$; Fig. 5). However, SEC(14–128) and native SEC2 exhibited a similar activity in terms of the induction of TNF-α ($P > 0.05$; Fig. 5). As fever can be caused by the release of cytokines, this result indicated a possible reason for the decreased pyrogenic toxicity of SEC(14–128).

**DISCUSSION**

Many studies have proven the therapeutic potential of SEs against malignancy (Dohlsten et al., 1994; Shaw et al., 2007). In China, SEC2 has been used as a supplementary medicine for tumour treatment, and positive results have been obtained (Chen, 2005). In Sweden, a mutant SEA with low affinity to anti-native SEA IgG has been constructed for treating solid tumours in phase II trials (Shaw et al., 2007). However, as a protein macromolecule, the complete SE molecule had high immunogenicity in the human immune system, which led to it being neutralized by patient anti-SE antibodies and attenuation of its therapeutic effects (Shaw et al., 2007). The pyrogenic toxicity of SE is another barrier for its clinical application (Dinges et al., 2000; Shaw et al., 2007). In addition, the high molecular mass of SE, usually around 2.8–3.0 kDa, hinders the successful permeation of the biological barrier in vivo. Thus, constructing low-molecular-mass SEs is a feasible way to solve these problems.

Our previous study found that the first 11 N-terminal residues of SEC2 could be deleted without affecting its mitogenicity and pyrogenicity, whereas deleting the first 18 N-terminal residues of SEC2 led to a substantial loss of its superantigen activity (Wang et al., 2009). Hoffmann et al. (1994) found that the deletion of aa 1–13 of SEC1 did not affect its T-cell proliferation activity. Aa 1–13 of both SEC1 and SEC2 are highly conserved. Thus, we assume that residues 11–18, particularly aa 13–18, are significant for SEC2 activity. Crystallographic studies have shown that aa 13–18 of SEC2 comprise a helix (α2) that could stabilize the conformation of the region participating in both MHC II and T-cell receptor binding (Schad et al., 1997; Papageorgiou et al., 2004). In this study, we deleted aa 1–13 of SEC2 and found that the deletion did not affect the T-cell proliferation activity, thereby confirming our assumption.

In our previous study, aa 163–239 were not required for the T-cell proliferation activity of SEC2 (Wang et al., 2009). The region of SEC2 from aa 129 to 163 is composed of...
three β-sheets (β6, β7 and β8; Schad et al., 1997), among which only β8 is a possible binding site for the MHC molecule. Thus, we assumed that aa 129–163 were also not required for the T-cell proliferation activity of SEC2. Our results confirmed that the absence of aa 129–163 and 163–239 did not severely impair the T-cell proliferation activity of the native SEC2.

Surprisingly, compared with the native SEC2, SEC(14–128) minimally induced T-cells to release the cytokines IL-2 and IFN-γ, even with a relatively high concentration of 1000 ng ml⁻¹, at different time points (data not shown). We assume that the considerable T-cell proliferation activity of SEC(14–128) might be performed through a mechanism independent of IL-2 and IFN-γ secretion, and that other cytokines not tested in this study might be involved. Moreover, SEs can also activate CD8⁺ T-lymphocytes and induce the addition of CD8⁺ cytotoxic T-lymphocytes (Sundstedt et al., 1998). Cytotoxic T-lymphocytes could effectively lyse the target cells bound to SEs, which may be a possible explanation for the antitumour effect of SEC(14–128) in vitro. Further studies are needed to explain this phenomenon. Residues 1–13 and 129–239 appeared to be unimportant in the T-cell proliferation activity but significantly important in the induction of IL-2 and IFN-γ cytokines by SEC2.

SEC(14–128) did not induce a fever response in the rabbit model. Our previous study showed that aa 163–239 were not required for the T-cell proliferation and pyrogenic activities, whilst aa 113–162 were important for both activities (Wang et al., 2009). The present study further showed that aa 129–162 are not required for the T-cell proliferation activity but are important for the pyrogenic activity of SEC2. Furthermore, these two activities were not necessarily correlated.

The massive release of inflammatory cytokines is one of the reasons for immune-mediated fever by SEs (Roggiani et al., 1997). Our results indicated that SEC(14–128) minimally induced mouse T-cells to secrete IL-2 and IFN-γ in vitro. We speculate that SEC(14–128) did not induce the rabbit T-cells to secrete any inflammatory cytokines because of the lack of anti-rabbit cytokine antibodies. Thus, a lower pyrogenic toxicity was observed in the rabbit model. Moreover, compared with the native SEC2, SEC(14–128), with a lower molecular mass, had lower immunogenicity. Consequently, this low immunogenicity may have decreased the immune response in vivo, making it a possible reason for the lower toxicity of SEC(14–128). The deletion of amino acid residues changed the original antigenic determinants of the native SEC2. Our study showed that SEC(14–128) was barely recognized by anti-SEC2 IgG, allowing it to avoid being neutralized by the existing anti-SEC2 antibodies in patients.

In summary, we obtained a truncated mutant consisting of aa 14–128 of the native SEC2 protein. The immunostimulation and antitumour activities of the mutant were not affected by the decreased molecular mass. In addition, the pyrogenic toxicity of SEC(14–128) and its affinity to the anti-native SEC2 IgG were severely reduced, and the secretion of IL-2 and IFN-γ by stimulated T-cells was decreased. Overall, SEC(14–128) has significant potential as a safe and effective superantigen agent for cancer treatment.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Science & Technology Major Specific Projects of China for ‘Significant Creation
REFERENCES


