Construction of Novel Bifunctional Chimeric Proteins Possessing Antitumor and Thrombolytic Activities

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Based on their respective antitumor and thrombolytic activities, the superantigen staphylococcal enterotoxin C2 (SEC2) and staphylokinase (Sak) were chosen for the construction of the novel chimeric proteins Sak-linker-SEC2 and SEC2-linker-Sak using a linker composed of nine Ala residues. Both chimeric proteins possessed nearly the same PBMC proliferation stimulating activity and antitumor activity as SEC2 and thrombolytic activity as Sak. Neither the SEC2 or Sak component of each chimeric protein affected the activity of the other component. The results presented in this study provide a possible strategy to prevent and cure tumor thrombus.

Keywords: Chimeric protein, antitumor, thrombolytic, staphylokinase, staphylococcal enterotoxin C2

Thromboembolic diseases have often appeared in patients with cancer, the first complicated case of malignant tumor thrombus having been seen in 1865 [19]. Such thromboembolic diseases as deep vein thrombosis, pulmonary embolism, disseminated intravascular coagulation, portal vein thrombosis, arterial thromboembolism, and migratory superficial thrombophlebitis often result in disability and mortality of cancer patients [20]. In addition, thrombosis in tumor patients has usually been induced by procoagulant factors, including anticancer therapy, the release of procoagulant by tumor cells, cancer-causing genes, etc. [3]. Thrombosis has remarkably affected tumor angiogenesis and metastasis of patients, indicating that the occurrences of tumors and thrombosis are related. However, the interaction between tumors and blood coagulation is complex. Tumor-derived procoagulant factors, such as tissue factor, are associated with systemic activation of blood coagulation. The host response to tumors, particularly activation of monocytes, may also be associated with tissue-factor-dependent activation of blood coagulation. Other elements of the hemostatic system are also activated in patients with malignant disease. These include platelets and endothelium disease [18]. Until now, there have been no efficient methods or appropriate drugs to prevent and cure tumor thrombus, clinically named Trousseau syndrome [3]. This syndrome has attracted increasing attention from the scientific and clinical communities.

Staphylokinase (Sak) produced by Staphylococcus aureus is a promising thrombolytic agent [6, 33]. It is not a proteolytic enzyme [3]. Rather, it forms a 1:1 stoichiometric complex with plasmin(ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix. Compared with other thrombolytic agents, Sak has many merits and characteristics including strong thrombolytic activity, high thrombolytic specificity, and low immunogenicity and incidence of allergic reactions. As soon as the thrombus is dissolved, plasminogen activation of Sak is inhibited by α2-antiplasmin in vivo, which remarkably prevents bleeding. Therefore, Sak is a type of safe and credible drug for use against cardiovascular disease at present [5, 10, 22, 32].

Staphylococcal enterotoxins (SEs) are a class of bacterial superantigens (SAg) produced by S. aureus [17]. Their superantigenic activity can be attributed to cross-linking of major histocompatibility complex class II molecules with the variable region of the β chain (Vβ) of T-cell receptors (TCR) and activation of a large number of resting T cells at a very low concentration, causing the release of massive amounts of cytokines including interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF); the result is a very strong immune response and significant tumor inhibition in vivo and in vitro [11, 12, 15, 26, 30]. Therefore, SE has been extensively employed for studies of antitumor immunotherapy [1, 2, 13, 14, 24, 25], particularly SEC2,
which has been used to cure malignant tumors including hepatoma, rectal cancer, lung cancer, ovarian cancer, etc. [8] in clinics in China since 1996.

To take advantage of the thrombolytic activity of Sak and the antitumor activity of SEC2, the chimeric proteins Sak-linker-SEC2 and SEC2-linker-Sak were constructed using a linker composed of nine Ala residues. The chimeric proteins not only efficiently inhibited the growth of tumor cells in vitro, but also exhibited nearly the same thrombolytic activity as Sak alone. Therefore, these proteins are valuable for further research and investigation in vivo so as to provide novel bifunctional agents to patients suffering from tumors and thrombosis in the clinic.

MATERIALS AND METHODS

Cell Lines, Bacterial Strains, Vectors, and Culture Conditions
Human carcinoma of the cervix cells (Hela) from China Medical University were cultured in RPMI 1640 (Hyclone) supplemented with 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml).

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. SEC2-producing S. aureus (o65Z-1) was provided by Shenyang Xiehe Pharmaceutical Group Company (China). S. aureus 26112 was provided by the Liaoning Entry-Exit Inspection and Quarantine Bureau. The other wild-type S. aureus strains were from CGMCC. S. aureus and Escherichia coli strains were cultivated at 37°C on Luria–Bertani (LB) medium. Kanamycin (40 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 flasks at 200 rpm (Series 25D; NBS, New Brunswick, NJ, USA).

Construction of Expression Plasmids
All molecular manipulations were performed according to standard procedures [29] or those recommended by the manufacturers. All restriction endonucleases and Ex Taq DNA polymerase were purchased from TaKaRa (Dalian, China) and T4 DNA ligase was from Promega (USA).

Construction of expression plasmids is shown in Fig. 1. The full-length gene encoding Sak was amplified from the genomic DNA of S. aureus strains through PCR using primers SAK3 and SAK4 (Table 1). Based on the DNA sequences of SEC2 and Sak, the primers SAK5, SAK6, SEC2a, SEC2b, SEC2c, and SEC2d, respectively, were designed (Table 1). PCR products obtained with SAK3/SAK5 and SEC2a/SEC2b were used as templates, using primers SAK3/SEC2b, for gene splicing by overlap extension PCR.

Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Important features</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>S. aureus o165Z-1</td>
<td>Wild type, Source of sec2</td>
<td>Shenyang Xiehe Company</td>
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<tr>
<td>S. aureus Jinp</td>
<td>Wild type, Source of sak_jinp</td>
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<td>SEC2c</td>
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<td>5'-AGCAGCAGACAGACAGACAGACAGACAGCTTTTTTGTGAA-3'</td>
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Restriction enzyme sequences are underlined.
Expression and Purification of Chimeric Proteins

Expression of chimeric proteins was induced with 0.2 mM IPTG after growth for 4 h at 30°C. Cells were harvested by centrifugation for 10 min at 4°C and 8,000 rpm, and the cell pellets were resuspended in ice-cold buffer A (20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl). Cells were disrupted by sonication at 0°C and centrifuged for 30 min at 4°C and 15,000 rpm. The supernatants were collected and loaded onto a Ni²⁺-NTA HisBind Superflow column (Qiagen, Germany) equilibrated with buffer A. After nonspecifically bound proteins were washed off with buffer A containing imidazole, specifically bound proteins were eluted with buffer A containing imidazole from 50 to 250 mM [16]. The purity of the eluted protein was determined by SDS-PAGE and staining with Coomassie brilliant blue [34].

Thrombolytic Activity Assay of Chimeric Proteins

The thrombolytic activity of the chimeric proteins in vitro, based on the proteolytic activity of human plasmin towards casein [28], was determined on a 5% agar plate containing 1 NIHU/ml of human thrombin (Sigma), 10 µg/ml of human plasminogen (Roche), and 0.8 mg/ml of fibrinogen (Chinese Medical Academy) at pH 7.5. Ten-µl samples with serial dilutions of chimeric proteins and Sak were added to the precast wells. The plates were incubated at 37°C for 4 h and the fibrinolytic activity was expressed as the diameter of the clear zone around the wells. Sak and urokinase standards were purchased from the Chinese National Institutes for Food and Drug Control.

PBMC Proliferation Assay of Chimeric Proteins

The peripheral blood mononuclear cell (PBMC) proliferation stimulating activity of the chimeric proteins was determined by methyl thiazol tetrazolium (MTT) assay in vitro according to a previously described method [16]. PBMCs from the blood of...
Antitumor Activity Assay of Chimeric Proteins In Vitro

The antitumor activity of the chimeric proteins in vitro was analyzed according to a previously described method [16]. SEC2 and chimeric proteins were added separately to triplicate wells, and 1×10^5 tumor cells were added along with 2×10^5 PBMC cells/well. The blank wells (DMEM only), unsettled tumor cell control wells (tumor cells only), and PBMC-releasing wells (PBMC and toxin protein) were used as controls. The medium was removed from each well after incubation at 37°C for 36 h, and 50 µl MTT (5 mg/ml) in PBS was added to each well. Incubation was continued for another 4 h. Cells were then collected by centrifugation at 3,000 rpm for 10 min at room temperature. The pellet was redissolved in 120 µl of DMSO at 37°C for 20 min, and the absorbance was measured at a wavelength of 570 nm in a microplate reader (Thermo Labsystems).

Tumor growth inhibition (%) = 100 – [(OD sample of protein-treated cell well – OD blank of PBMC-releasing wells)/(OD blank of unsettled tumor cell control wells – OD blank of blank control wells)] × 100.

Sequence Analysis
DNA sequences were analyzed using Vector NTI suite 8.0 software (Informax; Invitrogen, USA).

Statistical Analysis
Data are presented as means ± standard error of the mean (SEM). Statistical comparisons were performed using the one-way ANOVA and Student’s t-test with SPSS software (SPSS, Germany), and P values <0.01 were considered statistically significant.

RESULTS

Cloning of Novel sak Genes
The full-length gene encoding Sak was obtained by PCR of the genomic DNA of S. aureus strains using primers SAK3 and SAK4 (Table 1). According to the sequence alignment result, two novel genes, sak5SD and sakJinpu, were found. The other sequences, sakJinpu1 and sak26112, can already be found in GenBank. Compared with the published sak sequence, the homology of sakJinpu is more than 95%. The novel sequences have been deposited in GenBank under accession numbers GU966685 and EU146839. The expression vectors pET28a-sak5SD, pET28a-sak26112, pET28a-sakJinpu1, and pET28a-sakJinpu were constructed and transformed into E. coli BL21 (DE3) for protein expression under IPTG induction. Expression of the soluble products was high. High-purity Saks were obtained by passage through the Ni²⁺-NTA His-Bind Superflow column. The thrombolytic activities of Sak5SD, Sak26112, and SakJinpu1 were the same as that of commercial Sak, but that of SakJinpu was higher than that of standard Sak (Fig. 2). Therefore, SakJinpu and SEC2 were selected for construction of the bifunctional chimeric protein.

Plasmid Construction, Expression, and Purification of Chimeric Proteins
The PCR product sak-linker was 442 bp using primers SAK3/SAK5. The PCR product linker-sec2 was 757 bp using primers SEC2a/SEC2b. When sak-linker and linker-sec2 were used as templates for SOE-PCR with primers SAK3/SEC2b, the sak-linker-sec2 product was 1,172 bp (Fig. 1). The PCR product was purified and ligated into the EcoRI/XhoI sites of the expression vector pET28a, resulting in plasmid pET28a-sak-linker-sec2 (Table 1). The PCR product linker-sak was 452 bp using primers SAK4/SAK6. The PCR product sec2-linker was 757 bp using primers SEC2c/SEC2d. When sec2-linker and linker-sak were used as templates for SOE-PCR with primers SAK4/SEC2c, the sec2-linker-sak product was 1,172 bp (Fig. 1). The PCR product was purified and ligated into the EcoRI/XhoI sites of the expression vector pET28a, resulting in plasmid pET28a-sec2-linker-sak (Table 1). The plasmids were verified by restriction endonuclease mapping and DNA sequencing.

The chimeric proteins produced in recombinant E. coli BL 21 harboring pET28a-sak-linker-sec2 or pET28a-sec2-linker-sak under IPTG induction were purified through the Ni²⁺-NTA His-Bind Superflow column and termed Sak-linker-SEC2 and SEC2-linker-Sak, respectively. SDS-PAGE showed a single band for each, indicating that the purity of the proteins was more than 95% (Fig. 3).
Bioactivity Assay of Chimeric Proteins In Vitro

Native SEC2 and the chimeric proteins were tested for their ability to stimulate PBMC proliferation and inhibit the growth of tumor cells. The results showed that the chimeric proteins very efficiently stimulated PBMC proliferation even at a concentration of 20 ng/ml, a result equivalent to that of the SEC2 standard (Fig. 4). Compared with the negative control, the P value was less than 0.01. Both chimeric proteins, Sak-linker-SEC2 and SEC2-linker-Sak, exhibited nearly the same PBMC-stimulating activity, indicating that the Sak component did not affect the PBMC-proliferation stimulating activity of the SEC2 component.

To confirm whether the chimeric proteins still possess antitumor activity similar to SEC2, Hela cells were used to assess their antitumor activity in vitro. The results showed that they significantly inhibited the growth of Hela cells at a concentration between 20 and 500 ng/well. Both chimeric proteins exhibited almost the same antitumor activity as SEC2 (Fig. 5). Compared with the negative control, the P value was less than 0.01. The results also showed that the chimeric proteins maintained good inhibiting activity against tumor cells as that of the standard SEC2.

Based on the proteolytic activity of human plasmin towards casein, the thrombolytic activity of the chimeric proteins was also evaluated. The results showed that they exhibited nearly the same thrombolytic activity as Sak when...
the concentration of the proteins was between 25 and 100 µg/ml (Fig. 6). At 50 µg/ml, the thrombolytic activity of the chimeric proteins was higher than that of urokinase (500 U/ml). Moreover, the thrombolytic activity of both chimeric proteins did not show obvious differences, indicating that their SEC2 component did not affect the thrombolytic activity of the Sak component.

**DISCUSSION**

Clinical cases of thromboembolic disease have been increasingly reported since 1865. In a large series of over 230000 cases with five different cancer types, the rate of symptomatic thromboembolism within 700 days of diagnosis ranged from under 1% for breast cancer to approximately 5% for pancreatic cancer, for those patients with early-stage disease. The rate increased for those with advanced disease, with a near doubling for those with breast cancer and a rate nearly three times as high for patients with pancreatic cancer who presented with metastatic disease [9]. Thromboembolic disease, which may be fatal, is seen more commonly in patients with cancer. Moreover, disability and mortality of cancer patients caused by Trousseau syndrome in clinics is prevalent and commonplace [19, 27]. It is necessary to take effective measures or find appropriate drugs in order to prevent and cure tumor thrombus. Usually, tumor-thrombus patients are treated through surgical intervention and randomized to receive either low-dose unfractionated heparin, low-molecular-weight heparin, or urokinase [18]. Although such therapy may help to prolong survival, many questions and risks have yet to be solved and avoided according to clinical trials.

SAg SEs deserve growing attention as ideal drugs for cancer therapy owing to their ability to efficiently stimulate T-cell proliferation, thereby giving rise to potent cell-mediated immunological responses and producing a large variety of cytokines that induce the final result of apoptotic death of tumor cells [31]. The SEC2 drug, termed Gaojusheng, has been used in clinics in China since 1994 [7, 8]. At the same time, as a promising thrombolytic agent, Sak is a type of safe and credible drug against cardiovascular disease at present [5, 10, 22, 32]. Considering the antitumor activity of SEC2 and the thrombolytic activity of Sak, it seemed feasible to construct novel bifunctional chimeric proteins possessing both activities. Such proteins could help to prevent and cure tumor thrombus. For this reason, sak genes (GU966685 and EU146839) were cloned. Compared with a Sak standard, SakJinpu exhibited very good thrombolytic activity and was selected for construction of the bifunctional chimeric protein along with SEC2. To construct a functional chimeric protein, the selection of a linker peptide is critical [23]. Amino acids such as Ala, Gly, and Ser have usually been chosen to compose the linker peptide [21, 35]. In this study, a linker composed of nine Ala residues was selected to link SEC2 and Sak because Ala is small and stable, characteristics that could diminish spatial interruption and maintain the native conformations of the two proteins. Using this linker, plasmids encoding the chimeric proteins Sak-linker-SEC2 and SEC2-linker-Sak were successfully constructed by SOE-PCR. However, the T-cell proliferation efficiency of SEC2 is directly correlated with affinity for MHC class II [4], which determines the antitumor effect of SEC2 [4, 26].

The thrombolytic activity of Sak also depends on whether it can effectively bind plasminogen. Thus, the key question herein was whether the chimeric proteins maintained their antitumor effect and thrombolytic activity. Both chimeric proteins stimulated PBMC proliferation and significantly inhibited the growth of Hela cells, with activity equivalent to that of native SEC2, indicating that these proteins preserve the distinctive features of SEC2. Moreover, the chimeric proteins did not exhibit remarkable differences from each other in this regard. The position of SEC2 or Sak in the chimeric proteins did not affect the PBMC proliferation activity or antitumor effect. The chimeric proteins possessed nearly the same thrombolytic activity as Sak and did not exhibit remarkable differences in this regard either, indicating that the position of SEC2 or Sak in the chimeric proteins did not affect the thrombolytic activity of Sak. The results of the bioactivity assay also showed that the linker was efficient at maintaining the functions of both proteins.

**Fig. 6.** Thrombolytic activity assay of chimeric proteins. 1: Sak (100 µg/ml); 2: Sak (50 µg/ml); 3: Sak (25 µg/ml); 4: physiological saline; 5: SEC2-linker-Sak (100 µg/ml); 6: SEC2-linker-Sak (50 µg/ml); 7: SEC2-linker-Sak (25 µg/ml); 8: urokinase (500 U/ml); 9: Sak-linker-SEC2 (100 µg/ml); 10: Sak-linker-SEC2 (50 µg/ml); 11: Sak-linker-SEC2 (25 µg/ml); 12: BSA (1,000 µg/ml).
The results of this study prove the validity of our design. From this finding, chimera genesis has been demonstrated to be a useful method for the construction of novel bifunctional chimeric proteins possessing antitumor and thrombolytic activity. The results presented in this study provide a possible strategy for the prevention and cure of tumor thrombus. In conclusion, desirable chimeric proteins were obtained. They are valuable for further research and investigation in vivo.

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