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Up-regulation of granzyme B and perforin by staphylococcal enterotoxin C2 mutant induces enhanced cytotoxicity in Hepa1–6 cells

Guojun Zhang [a,b], Mingkai Xu [a,*], Huiwen Zhang [a], Yubo Song [a,b], Jian Wang [a], Chenggang Zhang [a]

[a] Institute of Applied Ecology, Chinese Academy of Sciences, No.72 Wenhua Road Shenhe Dis., Shenyang, Liaoning, China
[b] University of Chinese Academy of Sciences, Beijing, China

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ABSTRACT

Staphylococcal enterotoxin C2 (SEC2), a member of bacterial superantigen, is one of the most potent known activators of T lymphocytes. With this property, SEC2 has already been used in clinic as a tumor immunotherapy agent in China. To increase the antitumor activity, a SEC2 mutant named ST-4 (GKVTG102-106WWH) with amino acid substitutions in T cell receptor (TCR)-binding domain was generated by site-directed mutagenesis, and the molecular mechanism of the enhanced antitumor activity was investigated. Results showed that ST-4 could activate much more Vβ 8.2 and 8.3 T cells and NK cells compared with SEC2, and exhibited significantly enhanced immunocyte stimulation and antitumor activity in vitro. The synthetic peptide sequencing the residues of mutant TCR-binding domain could competitively inhibit the immunocyte stimulation activity of ST-4. Most importantly, ST-4 up-regulated granzyme B and perforin at both mRNA and protein levels. We also found that expression of proapoptotic proteins cytochrome c, BAX and activation of caspase-3, 9 was up-regulated, and antiapoptotic protein Bcl-xL was down-regulated in the treatment with either ST-4 or SEC2. When granzyme B inhibitor or perforin inhibitor is presented, tumor cell viability was significantly rescued. Taken together, we demonstrate that increased ST-4-TCR recognition contributed to massive T cells and NK cells activation. These activated cells released up-regulated granzyme B and perforin, which induced the enhanced tumor cells apoptosis by mitochondrial apoptotic pathway, and ultimately led to enhanced tumor cell growth inhibition. ST-4 may be a promising candidate for antitumor clinic usage in future.

1. Introduction

Bacterial superantigen Staphylococcal enterotoxins (SEs) are a class of immunostimulatory proteins secreted by *Staphylococcus aureus* and *Streptococcus aureus* (Bohach et al., 1990). In contrast to conventional antigens, SEs can bind to the region of major histocompatibility complex class II (MHC-II) molecules on antigen-presenting cells (APCs) outside the peptide groove and do not require processing or proteolysis, and then recognize the T cell receptor (TCR) Vβ regions (White et al., 1989). This trimolecular interaction between the SEs, MHC-II and TCR leads to massive proliferation of T cells. Ultimately, activated T cells release massive amounts of inflammatory cytokines such as interleukin-1 (IL-1), IL-2, gamma-interferon (IFN-γ) and tumor necrosis alpha (TNF-α) (Kum et al., 2001), which could activate the CTLs and NK cells to produce granzyme B (GzmB) and perforin (PRF1) (Gorelik and Flavell, 2001; Okada et al., 1997; Fehniger et al., 2007). It is well known that PRF1 and granzymes are released from cytotoxic granules to induce target-cell death (Shi et al., 1992; Shresta et al., 1995). At molecular level, the granule exocytosis pathway partly accounts for cytotoxicity delivered by NK and CTLs (Kägi et al., 1994). Therefore, the characteristics of SEs have been extensively employed in several preclinical studies for cancer therapy (Wang et al., 2010; Wang et al., 2009).

Staphylococcal enterotoxin C2 (SEC2) belongs to SEs family (Hovde et al., 1990). Our previous study found that SEC2 and its mutants exhibit antitumor effect in vitro and in vivo (Liu et al., 2012). Furthermore, SEC2 has been employed in clinic as a promising drug to treat the malignant tumors (Xu et al., 2008). In order to increase the immune-stimulating and tumor-inhibiting activity of SEC2, based on previous reports, we designed and constructed a SEC2 mutant ST-4 (GKVTG102-106WWH) in the present study. We determined whether ST-4 had an enhanced immunocyte stimulation activity as well as the enhanced antitumor effect. Furthermore, the cellular and molecular consequences after ST-4 treatment were investigated to explore the mechanism of this procedure.

Our results indicated that the amino acids substitution ST-4 contributed to the enhanced stimulation activity and antitumor effect. The enhanced antitumor effect was mediated by activated T cells and NK cells via the release of GzmB and PRF1 which induced apoptosis of tumor cells by mitochondrial apoptotic pathway.

* Corresponding author at: Institute of Applied Ecology, Chinese Academy of Sciences, 110016 Shenyang, China.
E-mail address: mkxu@iae.ac.cn (M. Xu).

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2. Materials and methods

2.1. Animals and cell lines

Female wild-type BALB/c mice (6–8 week olds, 19 ± 2 g) were purchased from Chang Sheng Biotechnology Co., Ltd. (Shenyang, China). All mice were maintained under specific pathogen-free conditions on a 12 h light-dark cycle, and with free access to autoclaved food and water. Experiments involving mice were approved by the institutional animal care and use guidelines.

Mouse hepatoma cell line Hepa1–6 was obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO_2 atmosphere.

2.2. Reagents and antibodies

Expression vector pET-28a(+) was purchased from Novagen (USA). Primers and peptides were synthesized from Sangon Biotech (Shanghai, China). The granzyme B inhibitor *Z*-AAD-CMK and perforin inhibitor Concanamycin A were obtained from Calbiochem (San Diego, CA, USA) and ApexBio Technology LLC (Boston, MA, USA), respectively. ELISA kits for granzyme B and perforin were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Monoclonal antibodies CD4-PE and CD8-FITC, CD3-APC and CD49b-FITC were from eBioscience and BioLegend (San Diego, CA, USA), respectively. FITC Annexin V apoptosis Detection Kit with PI was from BioLegend (San Diego, CA). SYBR Premix Ex Taq™ Kit, Primescript RT Master Kit and RNA-extracting reagent RNAiso plus were purchased from Takara Biotechnology Co. (Dalian, China). CFDA SE Cell Proliferation Assay and Tracking Kit, caspase-3 and 9 Activity Assays Kit was from Beyotime (Haimen, Jiangsu, China). Primary antibodies BAX, Bcl-xL, cytochrome c, GAPDH and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from (Proteintech Group Inc., Wuhan, China).

2.3. Construction, expression and purification of SEC2 mutant

The recombinant expression vector pET-28a-SEC2 was used as the template for constructing mutated SEC2 gene (Xu et al., 2008). The residues at position 102–106 of SEC2 were substituted for WWH by overlap PCR. Primers were used for site-directed mutagenesis: ST-4R, 5′-CAAGTTTTACC*ATGCCACCA*TACATTATCTTTGGATG-3′; ST-4F, 5′-CATCCAAAGATAATGTA*TGGTGGCAT*GGTAAAACTTGTATGTATGGAG-3′; SEC2R, 5′-CGGAATTCGAGAGTCAACCAGA-3′; and SEC2F, 5′-TCGCTCGAGTTATCCATTCTTTGTTG-3′. The primers were designed for amino acid substitution. The sequences of mutant residues are indicated in italics. The restriction enzyme sites for *Eco*RI and *Xho*I are underlined. PCR-generated fragments were digested with *Eco*RI and *Xho*I and ligated into plasmid pET-28a(+) digested with the same enzymes. Constructed plasmid was then transformed into *E. coli* BL21(DE3) separately and identified by DNA sequence analysis. Mutant protein ST-4 as well as wide type SEC2 were prepared as described earlier (Xu et al., 2008). Briefly, transformed *E. coli* BL21(DE3) were cultured in LB medium. When OD_{600} reached 0.5–0.8, protein expression was induced with 1.0 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at 30 °C. Cells were harvested and disrupted by sonication on ice. The cell lysate was centrifuged at 12,000 rpm for 30 min. Then the supernatants were collected and loaded onto the Ni-saturated chelating sepharose column. After nonspecifically bound host proteins were washed off with washing buffer (50 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole, pH 7.9), SEC2 or ST-4 bound to the resin specifically was eluted with washing buffer containing 250 mM imidazole and dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Relative protein purity was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R-250 staining.

2.4. CFSE proliferation assay

According to the previous study, the splenocytes of BALB/C mouse had the good sensitivity to SEC2. We selected the BALB/C mouse as model animals. Mice splenocytes were prepared as described previously (Wang et al., 2009). For the proliferation assay, freshly isolated mice splenocytes were labeled with Carboxyfluorescein diacetate succinimidyl ester (CFSE) immediately. Briefly, cells were resuspended in RPMI 1640 with 10% FBS at a final concentration of 5×10^6 cells/mL, and CFSE solution was added for a final working concentration. The cells were incubated at 37 °C for 10 min, and added 10 mL RPMI 1640 with 10% FBS and washed once. Then, staining was fixed by adding 5 mL RPMI 1640 with 10% FBS, and the cells were incubated at 37 °C for 5 min. Cells were then washed once and resuspended in the culture media.

CFSE labeled cells were plated in 96-well plates, stimulated with ST-4 at the concentrations of 100, 1000 and 10,000 ng/mL for 72 h before the proliferation of splenocytes was determined by flow cytometry. RPMI 1640 medium served as negative control. Cells were incubated at 37 °C with 5% CO_2 for 72 h. Splenocytes stained with CFSE were analyzed with BD LSRFortessa, and data were analyzed with FlowJo software (version 7.6.2, TreeStar).

2.5. Proliferation assay and flow cytometry

The cells were maintained in RPMI 1640 medium supplemented with 10% (FBS). Splenocytes were stimulated with series concentrations of 100, 1000 and 10,000 ng/mL SEC2 or ST-4 in 96-well flat-bottomed plates at 1×10^6 cells/well in 0.2 mL culture medium for proliferation assay, or in 24-well flat-bottomed plates at 5×10^6 cells/well in 1 mL culture medium for flow cytometry assay. The plates were incubated for 48 or 72 h at 37 °C in a humidified atmosphere containing 5% CO_2, respectively. After incubation, cell proliferation was determined by MTS assay. Absorbance value was measured with a microplate reader at a test wavelength of 490 nm and a reference wavelength of 620 nm. The proliferation index of splenocyte ($PI_{splenic\ lymphocytes}$) was calculated as follows: Abs value in experimental groups / Abs value in negative control groups.

For flow cytometry assays, surface markers were determined by staining with fluorochrome-conjugated monoclonal antibodies. The panel consisted of PE anti-mouse CD4 for $CD4^+$ T cell, FITC anti-mouse CD8 for $CD8^+$ T cell, and APC anti-mouse CD3 $^+$/FITC anti-mouse CD49b for NK cells. Briefly, the stimulated splenocytes in 24-well plates were centrifuged and washed twice with cold PBS. Then cells (2×10^5 cells/tube) were suspended in 100 μL cold PBS containing appropriately diluted antibodies according to the manufacture's instruction and incubated for 30 min at 37 °C in dark. The flow cytometry were performed using BD LSRFortessa, and data were analyzed with FACSDiva software (BD Biosciences, San Jose, CA, USA). The proliferation index (PI) of splenic $CD4^+$ and $CD8^+$ T cells were calculated with the equation: ($CD4^+$ or $CD8^+$ T cells $\%_{tested}$) / ($CD4^+$ or $CD8^+$ T cells $\%_{control}$) × $PI_{splenic\ lymphocytes}$.

2.6. Competition assay

The M1 and M2 peptides (with amino acid sequences within the TCR binding domain of SEC2: YFSSKDNV*GKVTG*GKT and ST-4: YFSSKDNV*WWH*GKT, respectively) were synthesized by Sangon Biotech (Shanghai, China). The different amino acids of synthetic peptides are indicated in italics. Splenocytes were pretreated with series concentrations of 100, 1000 and 10,000 ng/mL M1 or M2 peptides, and then stimulated with 100 ng/mL ST-4 in 96-well plates at 1×10^6 cells/well in 0.2 mL culture medium for proliferation assay. After incubation for 72 h, proliferation index was calculated according to above-described methods.