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Short communication

Identification of protein components and quantitative immunoassay for SEC2 in staphylococcin injection

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ABSTRACT

In China, staphylococcin injection has been commonly used in combined cancer therapy to enhance the systemic immune response and reduce the toxicities associated with chemotherapy or radiation therapy in the last decade. It is claimed that the main effective component is staphylococcal enterotoxin C2 (SEC2). However, no standard method based on the concentration of SEC2 has been established for quality control of the injection products. In this study, a sensitive and reliable biotin–streptavidin–ELISA (BS–ELISA) method was established for detection and quantification of SEC2. In addition, 1-D SDS-PAGE coupled with nano-LC–MS/MS was performed to identify the protein components in the injection products from one manufacturing company. The results of the BS–ELISA showed that SEC2 only accounted for less than 0.1% of the total protein in the injection products, and the nano-LC–MS/MS results showed that fifty-five proteins of *Staphylococcus aureus* were confidently identified in the injection solution. Seventeen out of these proteins, including SEC2, were well-known virulence factors. In addition, eighteen proteins of other Gram-positive bacteria were also confidently identified. Thus, the results indicated that SEC2 is of very low concentration in the injection products and the process of the injection preparation should be improved for health and safety consideration.

1. Introduction

In China, staphylococcin injection prepared from fermentation broth of *Staphylococcus aureus* (Staphylococcaceae) was widely used as a biological response modifier combined with chemotherapy or radiotherapy in cancer therapy for the last decade. Results of many clinical studies demonstrated that the patients treated with the injection showed significant increases of leucocyte count, CD4/CD8 ratio and IL-2 level, when compared with the control group [1–4]. Short-term efficacy of staphylococcin injection combined with chemotherapy or radiation therapy was confirmed in most reported clinical studies. Also, long-term survival benefit of combined tumour therapy with the staphylococcin injection was reported in several clinical studies. For instance, 63 patients with non-small-cell lung carcinoma (NSCLC) were treated with chemotherapy/radiation therapy alone or combined with the injection. The 1-year, 2-year, 3-year survival rates for patients in the combined group were 70.6%, 35.3%, and 29.4%, respectively, with the median survival of 19.4 months. In the chemotherapy/radiation therapy group, the 1-year, 2-year, 3-year survival rates were 44.8%, 17.2% and 13.8%, respectively, with the median survival of 11.4 months. The results suggested that the injection-treated patients had prolonged survival compared with patients that received chemotherapy/radiation therapy alone [5].

However, adverse events were encountered in approximately 30% of the patients treated with the injection. The most frequent side effect was mild-to-moderate fever, the frequency of which was roughly 10–30% higher than that in the control group [2,6,7]. Local side effects at the injection site such as pain, swelling and redness also commonly occurred in the injection-treated patients. Based on the clinical studies, the adverse events were similar among the patients treated with the injection, which indicates that the staphylococcin injection has a similar toxicological profile in a majority of patients with malignant diseases.

Research and development of the injection are limited by a lack of establishment of a quality standard. It is claimed that the main active component is staphylococcal enterotoxin C2 (SEC2) for its potential to enhance the systemic immune response. However, no standard methods have been developed to monitor the batch-to-batch variation based on the amount of this enterotoxin during the process of the injection preparation.

The research and development of the staphylococcin injection have also met with little success due to the lack of comprehensive identification of the complicated components, including both active

Abbreviations: SE, staphylococcal enterotoxin; MHC, major histocompatibility complex; GST, glutathione *S*-transferase.

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components and impurities. Observations from clinical reports suggested that the anti-tumour effect of the injection was correlated with its immunomodulatory properties. However, whether SEC2 is the most important component for the anti-tumour effect of the injection remains controversial. Consequently, the molecular basis for the efficacy of the injection has not been elucidated clearly, and the application of the injection for cancer therapy is, to a certain extent, limited by the toxicities encountered during the treatment. Thus, component analysis of the injection is highly desirable and the development of the next-generation staphylococcin injection would strongly depend on the assessment of both anti-tumour effect and toxicity of the components in the injection.

In this study, a sensitive biotin–streptavidin–ELISA (BS–ELISA) method for detection and quantification of SEC2 was established. Furthermore, 1-D gel electrophoresis coupled with nano-LC–MS/MS analysis was conducted to identify the protein components in the staphylococcin injection from one manufacturing company.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice and male New Zealand rabbits, weighing 20 ± 2 g and 2.2 ± 0.2 kg, respectively, were purchased from the animal research centre in Academy of Medical Science at Zhejiang province, China. The animals were housed in an air-conditioned room, with temperature $23 \pm 2\,^{\circ}C$, relative humidity 50–60%, controlled illumination of a 12 h light–dark cycle. All procedures described in this study were reviewed and approved by the ethics committee for the use of experimental animals at Zhejiang University, China. Thymine (Bio Basic Inc., Markham, Ontario, Canada), hypoxanthine (Bio Basic Inc., Markham, Ontario, Canada) and aminopterin (Sigma–Aldrich, St. Louis, MO, USA) were used in cell fusion. Biotin conjugated affinity purified Goat anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), biotin conjugated affinity purified Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and streptavidin-labelled horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) were used in the BS–ELISA system. Peroxidase conjugated affinity purified Goat anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and peroxidase conjugated affinity purified Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used in indirect ELISA.

2.2. Drugs and recombinant staphylococcal enterotoxins

Staphylococcin injection A (manufacturing company A, China), staphylococcin injection B (manufacturing company B, China) and staphylococcin injection C (manufacturing company C, China) were purchased from the Second Affiliated Hospital, Zhejiang University College of Medicine, Zhejiang Province, China. Recombinant staphylococcal enterotoxins (rSEA, rSEB, rSEC2, rSEE, rSEG, rSEI, rSEK, rSEM, rSEN, rSEO and rSEQ) obtained from thrombin-digested GST-tagged SEs were purified and preserved in our lab. Recombinant His-tagged SEC2 was purified and preserved in our lab.

2.3. Production of monoclonal and polyclonal antibodies against SEC2

Three male New Zealand rabbits were immunized subcutaneously on the lower back with 200 μg of purified His-tagged SEC2 in complete Freund's adjuvant (Bio Basic Inc., Markham, Ontario, Canada). After the first injection, the rabbits were injected subcutaneously with 200 μg of the immunogen in incomplete Freund's adjuvant (Bio Basic Inc., Markham, Ontario, Canada) at 15-day intervals over a period of 8 weeks. Blood samples were taken 6–8 days after each injection and the titre of the antisera against purified recombinant SEC2 was determined by indirect ELISA.

Six male BALB/c mice were injected subcutaneously with 30 μg of purified recombinant SEC2 emulsified in complete Freund's adjuvant. The mice received a booster injection of 30 μg of the antigen in incomplete Freund's adjuvant every 2 weeks. The immune response was monitored by testing the titre of polyclonal antibody in mouse serum using indirect ELISA and Western blot. Three days before cell fusion, the animals were boosted intraperitoneally with 50 μg of recombinant SEC2 in phosphate buffered saline (PBS, pH 7.4). The splenic lymphocytes obtained from the immunized mice were fused with SP2/0-Ag14 mouse myeloma cells at a ratio of 4:1 using 50% (w/v) PEG 4000 (Sigma–Aldrich, St. Louis, MO, USA). Fused cells were suspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY, USA), supplemented with 10% (v/v) foetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA) and hypoxanthine aminopterin thymidine (HAT), and seeded in 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany). The plates were incubated at 37 °C in a humidified CO_2 incubator (Model 3111, Thermo Forma, USA) for 10–15 days. The hybridoma cells capable of producing antibodies against purified His-tagged SEC2 were screened by indirect ELISA and cloned by limiting dilution. Positive hybridoma cells were further cultured and expanded in DMEM containing 10% (v/v) FBS and injected intraperitoneally to BALB/c mice pretreated with paraffin oil. Ascite fluid was collected from the mice 10–14 days later and the monoclonal antibodies (MAb) against purified His-tagged SEC2 were purified by Protein A chromatography (Amersham Biosciences, Uppsala, Sweden) from the supernatant of ascites on an ÄKTA purifier system (Amersham Biosciences, Uppsala, Sweden). Subtype of the monoclonal antibodies was determined by Mouse Monoclonal Antibody Isotyping Reagents (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

2.4. Establishment of quantitative BS–ELISA system for SEC2

To establish the BS–ELISA system for the detection and quantification of SEC2, two procedures were investigated: (1) use of plate coated with rabbit polyclonal serum and murine MAb as secondary antibody and (2) use of plate coated with murine MAb and rabbit polyclonal serum as secondary antibody. The optimal conditions for BS–ELISA assay were determined by a series of checkerboard titrations with various dilutions of coating antibody, secondary antibody, biotinylated antibody and streptavidin-labelled enzyme. The time of incubation and wash was also optimized. To set up the standard curve, purified His-tagged SEC2 was serially diluted 2-fold from 80 to 0.078 ng/mL in PBS (pH 7.4) with 0.5% (w/v) BSA (Bio Basic Inc., Markham, Ontario, Canada). The LOD was determined by calculating the mean value and the standard deviation of blank samples ($n = 20$): LOD = mean + 3 SD. The LOQ was estimated as the lowest concentration of His-tagged SEC2 that could be measured with acceptable precision (RSD $\leq$ 15%). Intra-day accuracy and precision were assessed by analysing five replicates of each standard sample of His-tagged SEC2 at six concentrations (30, 20, 15, 10, 5, and 2.5 ng/mL) on three separated days. Inter-day accuracy and precision were assessed by analysing ten replicates of standard samples on one day. To estimate the specificity of the BS–ELISA method for SEC2, several types of purified recombinant staphylococcal enterotoxin including rSEA, rSEB, rSEE, rSEG, rSEI, rSEK, rSEM, rSEN, rSEO and rSEQ at a concentration of 1 μg/mL in blocking buffer (0.5% BSA, 0.02% Tween-20 in PBS, pH 7.4) were tested in the established BS–ELISA system.