Mapping the antigenic determinants and reducing the immunogenicity of trichosanthin by site-directed mutagenesis

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Summary

Trichosanthin (TCS) is a type I ribosome-inactivating protein (RIP) possessing multiple pharmacological properties. One of its interesting properties is to inhibit human immunodeficiency virus (HIV) replication but its strong immunogenicity has limited the repeated clinical administration. To map the antigenic determinants and reduce the immunogenicity of TCS, two potential antigenic sites (YFF81–83 and KR173–174) were identified by computer modeling, and then three TCS mutants namely TCS_{YFF81–83ACS}, TCS_{KR173–174CG}, and TCS_{YFF-KR} were constructed by site-directed mutagenesis. The RI activity and DNase-like activity of the three constructed TCS mutants were similar to natural TCS but with much lower immunogenicity. Results suggested that the two selected sites are all located at or near the antigenic determinants of TCS. In toxicity studies, the LD₅₀ of the three TCS mutants was not different from natural TCS. These findings would be useful in designing a better therapeutic agent for AIDS.

Introduction

Trichosanthin (TCS) is a 27 kDa active plant protein isolated from the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii* Maximowicz. It is processed from a larger precursor protein, containing a 23 amino acid amino (N)-terminal sequence (pre sequence) and a 19 amino acid carboxy (C)-terminal extension (pro sequence). TCS is classified as a type I ribosome-inactivating protein (RIP) that cleaves specifically the N-glycosidic bond of A-4324 of 28S rRNA and

inhibits protein synthesis [1–3]. TCS also has DNase-like activity and cleaves the supercoiled double-stranded DNA to produce nicked circular and linear DNA [4]. Recently, TCS has been found to stimulate the production of reactive oxygen species (ROS) and induce apoptosis [5–8], enhance the action of chemokines and inhibit human immunodeficiency virus type 1 (HIV-1) integrase [9–11].

TCS has multiple pharmacological properties including anti-tumor, immunomodulatory and antiviral activity [12–15]. It is traditionally used to induce mid-term abortion in china. In the late 1980s, TCS was the first RIP found to possess anti-HIV activity. It preferentially inhibited the replication of HIV-1 in both acutely infected

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T-lymphoblastoid cells and chronically infected macrophages *in vitro* [16]. Therefore, it is also a potential therapeutic agent for AIDS. Clinical trials demonstrated that HIV infected patients failing treatment with antiretroviral agent such as zidovudine showed a significant decrease of the serum HIV-1 p24 antigen level and an increase of the percentage of CD4⁺ T-lymphocytes when TCS is added to the antiretroviral agent regimen [17–19]. However, strong immunogenicity of this compound hindered its further development into an anti-HIV therapeutic agent. As a protein foreign to a host, TCS elicits specific antibodies such as IgG and IgE [19, 20]. Repeated uses will result in severe anaphylactic reaction leading to death.

Since the TCS crystal structure [21, 22] is available, we can use three-dimensional computer modeling to analyze the potential antigenic sites. The following major parameters were considered: hydrophilicity, exposure on the surface, chain flexibility, and formation of loops or turns. Two sites, YFF81–83 and KR173–174 with higher antigenic index and remote from the active cleft were selected. The objective of this study was to construct the TCS mutants and examine its immunogenicity and biological activities.

Materials and methods

Reagents and bacterial strains

Natural TCS (nTCS) was purified from the root tuber of *Trichosanthes kirilowii* and obtained from Jinshan Pharmaceutical Limited Company (Shanghai, China). Anti-TCS rabbit antiserum was prepared in our laboratory using purified nTCS as immunogen. The restriction endonucleases, T4 DNA ligase and Taq DNA polymerase were purchased from Promega. The Ni-NTA purification system, expression vector pRSET-A, *E. coli* DH5 α and *E. coli* BL21 (DE3) came from Invitrogen. All other chemicals were of analytical grade.

Site-directed mutagenesis

By computer modeling based mainly on hydrophilicity, exposure on the surface, chain flexibility,

and formation of loops or turns, two sites namely, YFF81–83 and KR173–174 were identified as potential antigenic sites of TCS. By site-directed mutagenesis, the two sites, YFF81–83 and KR173–174, were replaced by ACS81–83 and CG173–174 respectively, three TCS mutants namely TCS_{YFF81–83ACS}, TCS_{KR173–174CG} and TCS_{YFF81–83ACS-KR173–174CG} (TCS_{YFF-KR}) were constructed by polymerase chain reaction using the overlapping primer method [23]. The PCR-amplified DNA fragments were confirmed by dideoxy DNA sequencing to ensure that no secondary mutation had occurred.

Protein expression and purification

By site-directed mutagenesis, the wild-type TCS (wTCS) and mutant TCS (mTCS) genes were digested with *Bam*HI and *Eco*RI, cloned into pRSET-A (This vector contains a 6 $\times$ histidine affinity tag for ease of purification, Invitrogen), and then transformed to *E. coli* BL21 (DE3). The transformed *E. coli* strains were cultured at 37 °C in LB medium with ampicillin (100 μ g/ml) until the cell density reached OD₆₀₀ = 0.6. Then, the IPTG was added to a final concentration of 0.1 mM and the cells were cultured at 37 °C for 4~5 h. Collect the bacteria and Resuspend all pellets in lysis buffer using the Ni-NTA purification system for native purification according to the instructions (Invitrogen).

Characterization of the expressed proteins

The purity and molecular weight of the expressed proteins were determined by 12% SDS-PAGE. Immunological analysis was performed by western blot analysis using anti-TCS rabbit antiserum as the first antibody.

DNase activity assay

The assay for DNase-like activity of TCS was carried out according to a previously described method [4]. In brief, 1 μ g supercoiled DNA (pUC19) was incubated with 2 μ g of various forms of TCS in a total volume of 20 μ l of 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM CaCl₂, 100 mM NaCl, pH 8.0 at room temperature for 1 h. After finished the incubation, the electrophoresis was carried out under non-denaturing conditions in

standard TAE buffer in 1.2% agarose gel at 50 V until the tracking dyes moved to the appropriate positions. The gel was stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$), and then photographed on a UV transilluminator.

Ribosome inactivating activity assay

The inhibitory activity of nTCS and its derivatives on protein synthesis *in vitro* was determined by measuring the incorporation of [^3H]-leucine into protein in a rabbit reticulocyte lysate cell-free system (Promega). The assay was based on that of Pelham et al. [24] using an untreated rabbit reticulocyte lysate as a source of ribosome, mRNA, and other endogenous factors. Briefly, various amounts of nTCS or its derivatives were mixed with 11.5 μl of rabbit reticulocyte lysate in 20 mM Tris-HCl (pH 7.8) containing 4 mCi/l [^3H]-leucine, 1.5 mM MgCl_2 , 5 mM dithiothreitol, and 50 mM KCl, followed by incubation at 30 $^{\circ}\text{C}$ for 90 min. The reactions were precipitated with 25% trichloroacetic acid and collected on glass microfiber filters by filtration with Whatman GF/C, and the radioactivities were determined with a liquid scintillation counter. Each reported inhibition point is calculated as the mean of triplicate individual tests.

Assay for immunogenicity: IgG and IgE responses

BALB/c mice (6~7-weeks-old, about 20 g) were used for the experiment. Mice in groups of five were immunized subcutaneously at the back with 8 μg of various forms of TCS. Booster injections were given 2 weeks after the first immunization. Then blood samples were collected from each mouse 1 week after the immunization, and sera were stored at -20°C for later assay. Specific IgG and IgE antibodies against TCS were detected by ELISA. ELISA plates were coated with antigen (10 $\mu\text{g}/\text{ml}$ nTCS) overnight at 4 $^{\circ}\text{C}$ with coating buffer. After washing several times with PBST, serum samples were added to the ELISA plates at 100 μl per well. Then biotin-conjugated rat anti-mouse IgE, streptavidin-HRP, or HRP-conjugated goat anti-mouse IgG antibodies were added after incubation and washing. Finally, 50 μl substrate OPD were added and incubated at 37 $^{\circ}$ for 20 min. Optical density was measured by ELISA reader at 490 nm.

Acute toxicity assay

Female and male BALB/c mice (6~7-weeks-old, about 20 g) were divided into five groups of 7 each. Each group of animals was injected i.v. with various forms of TCS in a dose of 31.50, 22.05, 15.44, 10.80 and 7.56 mg/kg body weight. Mortality of these animals was monitored up to 10 days after administration. The LD_{50} is the calculated dose of various forms of TCS that kills 50% of the animals.

Results

Mutation, expression, and purification of TCS

In this study, computer prediction of antigenic determinants was adopted to analyze the potential antigenic sites based on hydrophilicity, exposure on the surface, chain flexibility, and formation of loops or turns. Two sites, YFF81–83 and KR173–174, were selected (Fig. 1). YFF81–83 are all aromatic amino acid with larger side chain and are located at the surface of the TCS molecule. KR173–174 have long and positively charged side-chains, which can form a hydrophilic surface with higher polarity than other residues. In addition, the computer prediction showed that the antigenic indexes of YFF81–83 and KR173–174 are much higher and may involve in the formation of TCS antigenic determinants.

By site-directed mutagenesis, the two selected sites were mutated as follows: Tyr81-Phe82-Phe83 to Ala81-Cys82-Ser83, and Lys173-Arg174 to

Figure 1. The three-dimensional structure of trichosanthin indicating the location of YFF81–83 and KR173–174.

Cys173-Gly174, respectively. Three TCS mutants namely TCS_{YFF81-83ACS}, TCS_{KR173-174CG} and TCS_{YFF81-83ACS-KR173-174CG} (TCS_{YFF-KR}) were constructed by PCR using appropriate mutagenic primers. As for the choice of residues that were used to substitute these identified antigenic sites, we usually select the amino acid with smaller molecule and shorter side chain that is not possibly involved in the construction of antigenic determinants, such as Gly, Ala and Ser etc. according to our experiences. TCS is a polypeptide without cysteine residues. In this study, a cysteine residue has been introduced by site-directed mutagenesis to TCS at Phe82 and Lys173 respectively, and then site-directed PEGylation of TCS can be possible.

To express the various forms of TCS, the plasmid pRSET containing the wild-type TCS (wTCS) or mutant TCS (mTCS) gene was expressed at high level in *E. coli* strain BL21 (DE3). All the TCS protein were expressed in soluble form and purified with the Ni-NTA native purification system. After expression and purification, SDS-PAGE and Western blotting were used to detect the recombinant wTCS and mTCS protein (Fig. 2).

DNase activity

Under normal enzymatic digestion conditions, TCS cleaves the supercoiled double-stranded DNA to produce nicked circular and linear DNA. To demonstrate the DNase activity of the expressed TCS proteins, supercoiled DNA (pUC19) was incubated with various forms of TCS. As shown in Fig. 3, all of them exhibited the DNase-like activity, suggesting that the expressed wTCS and mTCS had a similar functional activity to nTCS.

Ribosome inactivating activity

Rabbit reticulocyte lysate cell-free system was used to estimate the ribosome inactivating (RI) activity of various forms of TCS. As shown in Table 1, the ability of expressed wTCS and mTCS to inhibit protein synthesis *in vitro* is comparable with that of nTCS, the IC₅₀ values of nTCS, wTCS, TCS_{YFF81-83ACS}, TCS_{KR173-174CG}, and TCS_{YFF-KR} were 4.69 ± 0.30 , 5.06 ± 0.29 , 4.95 ± 0.22 , 5.34 ± 0.32 , and 5.30 ± 0.25 ng/ml, respectively.

Figure 2. (A) Analysis of expressed TCS and its mutants by 12% SDS-PAGE. The gel was stained using Coomassie brilliant blue. Lane 1, molecular weight marker; lane 2, 3, 4 & 5, proteins extracted from *E. coli* strain BL21 (DE3) transformed with recombinant plasmid pRSET-wTCS, pRSET-TCS_{YFF81-83ACS}, pRSET-TCS_{KR173-174CG}, and pRSET-TCS_{YFF-KR}, respectively; lane 6, proteins extracted from *E. coli* strain BL21 (DE3) transformed with empty plasmid pRSET were served as negative control. (B) Purification of expressed TCS and its mutant proteins using Ni-NTA native purification system. Lane 1, molecular weight marker; lane 2, wTCS; lane 3, TCS_{YFF81-83ACS}; lane 4, TCS_{KR173-174CG}; lane 5, TCS_{YFF-KR}. (C) The expressed TCS and its mutant proteins were confirmed by western blot analysis using anti-TCS rabbit antiserum as the first antibody. Lane 1, wTCS; lane 2, TCS_{YFF81-83ACS}; lane 3, TCS_{KR173-174CG}; lane 4, TCS_{YFF-KR}.

Immunogenicity: IgG and IgE responses

Mice were immunized with various forms of TCS, and the sera IgG and IgE levels determined by ELISA are shown in Fig. 4. It appeared that all the mTCS produced a lower

Figure 3. The DNase-like activity of TCS and its mutants on supercoiled pUC19 DNA. Supercoiled pUC19 DNA was incubated with various forms of TCS under normal enzymatic digestion conditions for 1 h. Then, the electrophoresis was carried out in 1.2% agarose gel and the gel was stained with ethidium bromide. Lane 1, Supercoiled pUC19 DNA was served as negative control; lane 2, 3, 4, 5 & 6, Supercoiled pUC19 DNA was incubated with nTCS, wTCS, TCS_{YFF81-83ACS}, TCS_{KR173-174CG}, and TCS_{YFF-KR} respectively. Nc, nicked circular DNA; L, linear DNA; S, supercoiled DNA.

Table 1. The summary of RI activity and acute toxicity of TCS and its mutants.

Protein species	RI activity (IC ₅₀ , ng/ml)	Acute toxicity (LD ₅₀ , mg/kg)
nTCS	4.69 ± 0.30	13.64
wTCS	5.06 ± 0.29	13.71
TCS _{YFF81-83ACS}	4.95 ± 0.22	14.35
TCS _{KR173-174CG}	5.34 ± 0.32	13.02
TCS _{YFF-KR}	5.30 ± 0.25	13.71

The RI activity of TCS and its mutants was assessed by inhibition [³H]-leucine incorporation, and acute toxicity is represented by dose to kill 50% of the mice (LD₅₀).

IgE response than wTCS and nTCS, while the IgG response of mTCS was not much different from wTCS and nTCS.

Acute toxicity

For toxicity studies, several different doses of each type of TCS were injected intravenously into BALB/c mice and mortality was monitored up to 10 days after administration. The LD₅₀ of TCS and its mutants is summarized in Table 1. Acute toxicity of expressed wTCS and mTCS was not different from nTCS, and their LD₅₀ values were about 14.0 mg/kg.

Figure 4. Mice in groups of five were immunized with various forms of TCS, and blood samples were collected from each mouse after immunization. Specific IgG and IgE antibodies against TCS were detected by ELISA. mTCS1, TCS_{YFF81-83ACS}; mTCS2, TCS_{KR173-174CG}; mTCS3, TCS_{YFF-KR}. Values are means ± SD (*n* = 6).

Discussion

Although TCS has multiple biological and pharmacological activities, its strong immunogenicity hindered the further development into a therapeutic agent. In this study, to map the antigenic determinants and reduce the immunogenicity of TCS, computer modeling was adopted to select the potential antigenic sites. Two sites were selected: YFF81-83 and KR173-174. By site-directed mutagenesis, three TCS mutants namely TCS_{YFF81-83ACS}, TCS_{KR173-174CG} and TCS_{YFF-KR} were constructed successfully, and expressed at high level in soluble form in *E. coli* BL21 (DE3) and purified easily to homogeneity by using Ni-NTA purification system.

TCS is classified as a type I RIP that inhibits protein synthesis [1-3]. It is generally believed that the anti-HIV activity of TCS is related to its RI activity. TCS also has DNase-like activity and cleaves the supercoiled double-stranded DNA to produce nicked circular and linear DNA [4]. In this study, the RI activity and DNase-like activity of TCS and its mutants were tested. There had no effect on its RI activity and DNase-like activity when mutation of TCS at YFF81-83 and KR173-174. This indicated that the overall structure of the three mTCS was probably maintained.

Immunogenicity and acute toxicity of TCS and its mutants were also examined in this study. The results indicated that there was a marked decrease in immunogenicity. This suggests that the two selected sites are all located at or near the antigenic determinants of TCS. In toxicity studies, the LD₅₀ of the three mTCS was not different from nTCS.

This indicates that mutation of TCS does not bring about significant changes in acute toxicity.

Covalent modification of proteins with polyethylene glycol (PEG) has been widely used to increase plasma half-life and reduce immunogenicity. This method has been proved to be effective with many other molecules [25–30]. TCS is a polypeptide without cysteine residues. In this study, a cysteine residue has been introduced by site-directed mutagenesis to TCS at Phe82 and Lys173, respectively, and then site-directed PEGylation of TCS can be possible. In the next step, the three promising mTCS will be coupled to PEG via the newly created cysteine residue, and their activities, immunogenicity and pharmacokinetics will be explored further.

In conclusion, this study described a method of combining computer modeling and site-directed mutagenesis to map the antigenic determinants and reduce the immunogenicity of TCS. The *in vitro* biological activities of the three constructed mTCS were similar to nTCS but with much lower immunogenicity. Results suggested that the two selected sites, YFF81-83 and KR173-174, are all located at or near the antigenic determinants of TCS. These findings would be useful in designing a better therapeutic agent for AIDS.

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