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Independency of anti-HIV-1 activity from ribosome-inactivating activity of trichosanthin

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Abstract

Trichosanthin (TCS) is a type I ribosome-inactivating (RI) protein possessing multiple biological and pharmacological activities. Its major action is inhibition of human immunodeficiency virus (HIV) replication but the mechanism is still elusive. All evidences showed that this action is related to its RI activity. Previous studies found that TCS mutants with reduced RI activity simultaneously lost some anti-HIV activity. In this study, an exception was demonstrated by two TCS mutants retaining almost all RI activity but were devoid of anti-HIV-1 activity. Five mutants were constructed by using site-directed mutagenesis with either deletion or addition of amino acids to the C-terminal sequence. Results showed that the RI activity of mutants with C-terminal deletion mutants (TCS_{C2}, TCS_{C4}, and TCS_{C14}) decreased by 1.2–3.3-fold with parallel downshifting of its anti-HIV-1 activity (1.4–4.8-fold). Another two mutants, TCS_{C19aa} and TCS_{KDEL} having 19 amino acid extension and a KDEL signal sequence added to the C-terminal sequence, retained all RI activity but subsequently lost most of the anti-HIV-1 activity. These findings suggested that ribosome inactivation alone might not be adequate to explain the anti-HIV action of TCS.

Keywords: Trichosanthin; Ribosome inactivating protein; Human immunodeficiency virus type 1; Antiviral protein

Trichosanthin (TCS) is a single-chain ribosome-inactivating protein (RIP) purified from the root tubers of *Trichosanthes kirilowii* Maxim. It is the active component of the Chinese medicine Tian Hua Fen and is still being used to induce mid-term abortion and to treat choriocarcinoma in China [1]. In addition to the *N*-glycosidase activity that depurinates adenine 4324 of 28S rRNA, TCS exhibits many biological and pharmacological properties, including antitumor, immunosuppressive, and antiviral activities [2,3]. TCS is the first RIP found to inhibit human immunodeficiency virus type 1 (HIV-1) replication in both acutely infected T lymphoblastoid cells and in chronically infected macrophages in vitro [4]. Phase I/II clinical trials with TCS alone or in combination with zidovudine or dideoxyinosine showed that TCS decreased the serum HIV-1 p24 antigen level and increased the $CD4^+$ T cell number in HIV-1 infected patients [5]. Unfortunately, antigenicity of this compound hindered its further development into an anti-AIDS therapeutic agent. Mild to severe anaphylactic side effects were encountered during these trials [5,6].

The exact anti-HIV-1 mechanism of TCS is not entirely clear. It is generally believed that its ribosome inactivating (RI) activity contributes to this action. Our previous studies also found that TCS mutants with reduced RI activity simultaneously lost some anti-HIV activity [7]. However, not all RIPs have antiviral activity [8]. In this study, the effects of C-terminal amino acid sequences on the anti-HIV-1 activity were investigated. It was known that the C-terminal contributed to antigenicity [9]. If the anti-HIV activity can be retained in TCS mutants with a shorter C-terminal, antigenicity can be reduced. Alternatively, if TCS entry into cells can be enhanced, smaller dosage can then produce a greater effect. This can be done by adding some amino acid sequences that were known to lead proteins into cells.

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The TCS mutants used in this study were designed according to these rationales.

The preproprotein of TCS consists of 289 amino acids with a 19 residue C-terminal extension and a 23 residue N-terminal sequence [10]. The role of the two precursor sequences has not been fully elucidated. It was speculated that the 19 amino acid sequences help TCS through the secretary pathway [11]. This 19 amino acid sequence was added to the C-terminal by site-directed mutagenesis in this study.

Some toxins have a C-terminal KDEL or KDEL-like sequence, acting as an endoplasmic reticulum (ER) retrieval signal. It has been proposed that the KDEL system could facilitate the transport of protein to the ER [12,13]. Ricin A-chain mutant containing a C-terminal KDEL sequence was reported to be more toxic to cells than the wild-type [13].

In order to examine the effects of the C-terminal sequences on the anti-HIV-1 activity, deletion and addition mutants were produced by site-directed mutagenesis. The anti-HIV and RI activities were studied.

Materials and methods

Reagents and chemicals. AZT (3′-azido-3′-deoxythymidine) and MTT were purchased from Sigma. The colorimetric reverse transcriptase assay kit was purchased from Roche Molecular Biochemicals and horseradish peroxidase (HRP)-labeled goat anti-human IgG was purchased from SABC (Sino-America Biotechnology Company). Mouse anti-HIV-1 p24 antigen monoclonal antibody (McAb) and human polyclonal anti-HIV-1 serum were kindly donated by Dr. Hiroo Hoshino (Department of Virology and Preventive Medicine, Gunma University School of Medicine, Japan).

Natural TCS and TCS mutants. Natural TCS (nTCS) was purified from the root tuber of the Chinese medicinal plant *T. kirilowii*. TCS mutated genes were constructed by polymerase chain reaction and the mutants were expressed and purified as described previously [9,14,15].

Virus and cell lines. C8166, H9 cells, and HIV-1_{IIIB} strain were kindly donated by Medical Research Council (MRC), AIDS Reagent Project, UK. K562 cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in complete medium and HIV-1_{IIIB} was obtained from the culture supernatant of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by Reed and Muench method. Virus stocks were stored in small aliquots at −70 °C. The titer of virus stock was 9×10^5 $TCID_{50}$ per ml.

Assay for protein-synthesis inhibition activity in vitro. The RI activity of nTCS and various mutants on protein synthesis was measured in a rabbit reticulocyte lysate cell-free system, using an intact rabbit reticulocyte lysate as a source of ribosome, mRNA, and other endogenous factors. Protein synthesis was assessed by [^{3}H]leucine incorporation [14]. Plots of [^{3}H]leucine incorporation versus protein concentration were made for each protein and IC_{50} values were obtained by fitting the curves to third-order regression.

Internalization of TCS_{KDEL}-FITC into cells and confocal imaging analysis. nTCS-FITC and TCS_{KDEL}-FITC conjugate preparation and confocal imaging analysis were processed as previously described [16]. Briefly, nTCS and TCS_{KDEL} were coupled to FITC in 0.1 M bicarbonate buffer (pH 9.0). Then, protein conjugated FITC was separated from free FITC by Sephadex G25. nTCS-FITC or TCS_{KDEL}-FITC was inoculated to K562 cells that were seeded on coverslips which had been treated at 25 μg/ml. At 4 h, cells were washed with ice-cold PBS twice. After mounting a drop of Vectashield mounting medium (Vector Laboratories Inc., CA), the coverslips were ready for microscopy by Bio-Rad MRC 1000/Nikon Diaphot 200 confocal microscope unit. Fluorescence was excited with the 488 nm line of the krypton–argon laser. Emission was collected through a bandpass filter 522/35 nm and stored digitally. All fluorescent images were acquired with a Nikon 40× plan fluor objective (N.A. 1.3).

Inhibition of syncytium formation. This assay is based on the interaction between HIV-1 infected cell and uninfected cell, and quantitates acute cell-free HIV-1 infection [17]. C8166 cells were pretreated with nTCS or mutants at various concentrations at 37 °C for 1 h and then infected with HIV-1_{IIIB} at a MOI of 0.02. They were then cultured in the presence of either nTCS or mutants in a final volume of 200 μl. The plates were incubated in a humidified incubator at 37 °C and 5% CO_2. Each condition was performed in triplicate. AZT was used for drug control. After 3 days of culture, the cytopathic effect was measured by counting the number of syncytia in each well under an inverted microscope. The percent inhibition of syncytial cell formation was calculated by a percentage of the syncytial cell number in compounds treated with culture to that in infected control culture. Terminated cell culture was centrifuged and cell-free supernatant was used to measure the inhibition of HIV-1 p24 antigen expression with antigen capture ELISA assay.

Enzyme-linked immunosorbent assay for HIV-1 p24 antigen. HIV-1 p24 antigen in a cell-free culture medium was measured using an antigen capture ELISA assay as described previously [18]. Briefly, Triton X-100 treated cell-free culture medium was added to 96-well microtiter plates coated with anti-p24 antigen McAb. The plates were then incubated with diluted human polyclonal anti-HIV-1 serums, followed by incubation with HRP-labeled goat-anti human IgG, and OPD substrate was added into the wells. The optical density of the plates was read on Bio-Tek ELx 800 ELISA reader at 490 nm/630 nm. The inhibition (%) of p24 antigen expression was calculated. The concentration of nTCS or mutants reducing p24 antigen expression by 50% (EC_{50}) was determined from the dose–response curve.

HIV-1 reverse transcriptase assay. The quantification of HIV-1 reverse transcriptase (RT) was determined with a commercially available ELISA kit according to the instructions of the manufacturer. Briefly, 4×10^5 C8166 cells were infected HIV-1_{IIIB} at a MOI of 0.02 in the presence of various concentrations of compounds on 24-well plate and the plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 72 h. The virus particles in supernatants were isolated by PEG precipitation and solubilized by lysis buffer. Twenty μl reaction mixture was added and incubated for 15 h at 37 °C. The mixture was transferred to streptavidin-coated microplate modules and incubated for 1 h, and then anti-DIG-POD and ABTS substrate were added. The optical density of the plates was read on ELISA reader at 405 nm/490 nm. The concentrations of HIV-1 RT in the cultured supernatant were determined from a linear calibration curve of HIV-1 RT standards.

Results

C-terminal sequences of mutants and nTCS

A comparison of the carboxyl-terminal amino acid sequence between nTCS and its mutants is shown in Table 1. As many as 2, 4, and 14 amino acid residues were removed from the C-terminal in TCS_{C2}, TCS_{C4}, and TCS_{C14}, respectively. An ER retrieval signal (a C-terminal KDEL sequence) and a 19 amino acid C-terminal

Table 1
The C-terminal sequence of TCS mutants and their RI activity

Proteins	Sequences of C-terminal residues	IC_{50} of RI activity (ng/ml)
	232 247	
nTCS	–VVTSNTALLLNRNNMA	3.8
TCS_{C2}	–VVTSNTALLLNRNN	7.6
TCS_{C4}	–VVTSNTALLLNR	12.6
TCS_{C14}	–VV	9.0
TCS_{KDEL}	–VVTSNTALLLNRNNMA***KDEL***	3.9
TCS_{C19aa}	–VVTSNTALLLNRNNMA***AMDDDVPMTQSFGCGSYAI***	4.4

The addition in amino acids is denoted by bold and italic. The RI activity was measured in a rabbit reticulocyte lysate cell-free system.

propeptide extension (AMDDDVPMTQSFGCGSYAI) were added onto the C-terminal in TCS_{KDEL} and $TCSC_{19aa}$.

Ribosome inactivating activity of nTCS and its mutants

Rabbit reticulocyte lysate cell-free system was used to estimate the RI activity of nTCS and its mutants at different concentrations. The IC_{50} of nTCS and the mutants is shown in Table 1. TCS_{C2}, TCS_{C4}, TCS_{C14}, TCS_{KDEL}, and TCS_{C19aa} exhibited a slightly lowered RI activity than nTCS (3.8 ng/ml). The RI activities of the mutants were 7.6, 12.6, 9.0, 3.9, and 4.4 ng/ml, respectively.

Anti-HIV-1 activities of nTCS and its C-terminal mutants

Virus-induced cytopathic effect is quantified by the syncytium formation. The inhibition of syncytium formation in a dose-dependent manner was exhibited in nTCS, and the EC_{50} value of $0.38 \pm 0.08\,\mu g/ml$ was obtained. TCS_{C2}, TCS_{C4}, TCS_{C14}, and TCS_{KDEL} also showed inhibition of syncytium formation in a dose-dependent manner whose EC_{50} values were 0.53 ± 0.46, 0.83 ± 0.46, 1.84 ± 0.04, and $8.92 \pm 2.02\,\mu g/ml$ which were 1.4-, 2.2-, 4.8-, and 23.5-fold drop compared with nTCS, respectively. TCS_{C19aa} almost lost all of the inhibition effect on syncytium formation, even at 10 μg/ml

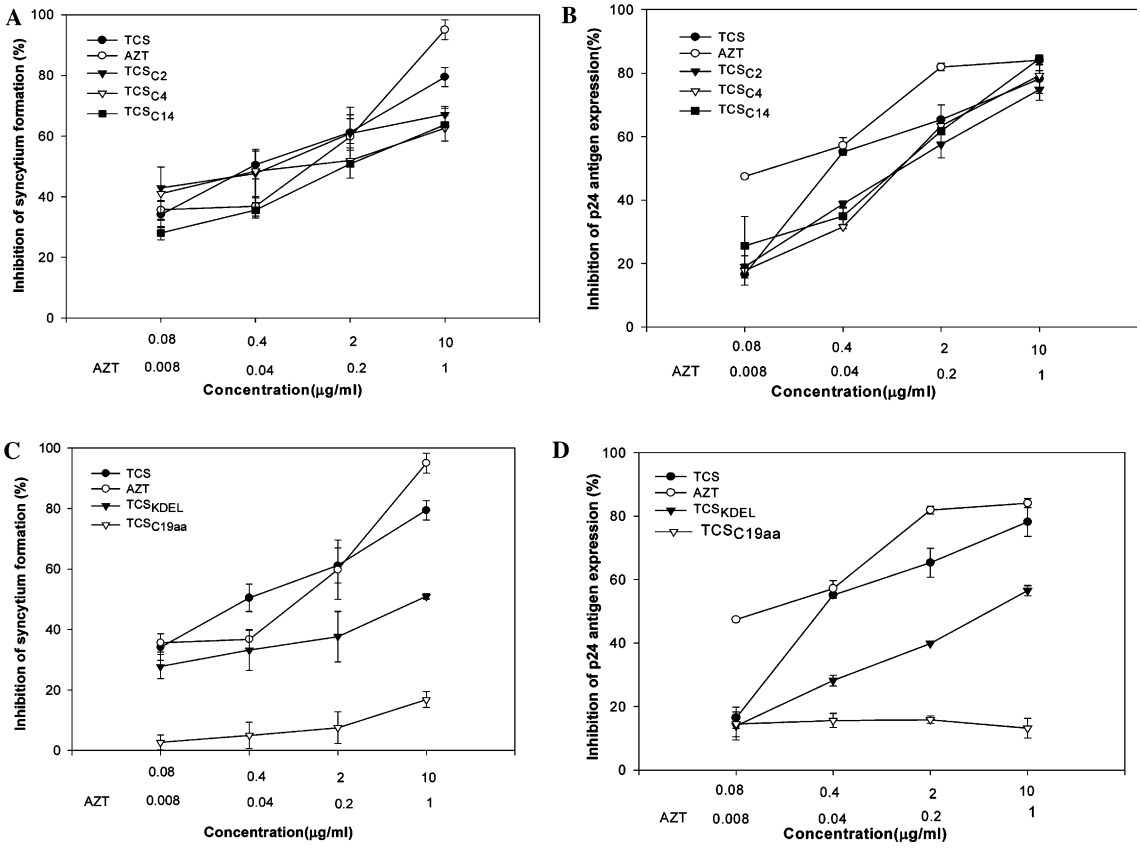

Fig. 1. Anti-HIV-1 activities of nTCS and its mutants. Syncytial formation inhibition was quantified under an inverted microscope (A,C), HIV-1 p24 antigen levels in supernatants were performed by capture ELISA (B,D). Data are expressed as means ± SEM of triplicate measurements.

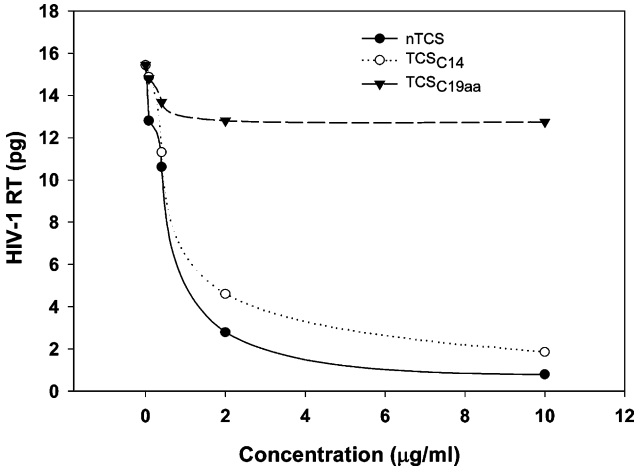

Fig. 2. Effects of nTCS and its mutants on the HIV-1 RT levels. The RT levels in cell-free supernatant were quantified with a commercially available ELISA kit according to the instructions of the manufacturer.

concentration, which showed only $16.8 \pm 2.64\%$ inhibition effect (Figs. 1A and C).

Simultaneously, inhibition of HIV-1 p24 protein expression in acutely infected cells by nTCS and its mutants was assayed to measure HIV-1 replication. Like syncytium formation, the inhibition on p24 protein production by nTCS, TCS_{C2}, TCS_{C4}, TCS_{C14}, and TCS_{KDEL} was exhibited in a dose-dependent manner whose EC_{50} values were 0.32 ± 0.01, 0.97 ± 0.32, 1.02 ± 0.02, 0.99 ± 0.07, and $5.34 \pm 0.53\,\mu g/ml$, respectively. The EC_{50} of TCS C-terminal deletion mutants was about 3-fold decreased, while the TCS_{KDEL} mutant was 16-fold decreased compared with nTCS. However, the TCS_{C19aa} mutant lost almost all of the inhibition effect on p24 antigen production (Figs. 1B and D).

The colorimetric enzyme immunoassay for the quantitative determination of HIV-1 RT activity was used for the corroboration in anti-HIV-1 activities of nTCS, TCS_{C14}, and TCS_{C19aa} mutants (Fig. 2). Like TCS, TCS_{C14} exhibited inhibition on RT production in a dose-dependent manner, but TCS_{C19aa} showed no inhibition property, even at the highest concentration 10 μg/ml.

Internalization of TCS_{KDEL}-FITC and TCS-FITC into K562 cells

Intracellular nTCS-FITC and TCS_{KDEL}-FITC in K562 cells are shown in Fig. 3. The intensity was indistinguishable between TCS-FITC and TCS_{KDEL}-FITC. Similar experiments were also done in several types of cells and the results were almost the same.

Discussion

The original idea of this study was to examine the role of the C-terminal on some TCS actions. It was known that the C-terminal contributed to antigenicity of this compound [9]. Deleting some amino acids from the C-terminal can reduce antigenicity. If the anti-HIV action is not changed substantially, these deletion mutants with reduced antigenicity may become a potential therapeutic agent. Another objective was to add some amino acid sequences that can enhance protein entry into cells [12,13] to the C-terminal of TCS. Higher intracellular TCS concentration hopefully can increase its anti-HIV activity. With all these objectives, the anti-HIV action of the deletion and addition mutants was determined. As expected, sequential deletion of the C-terminal sequence

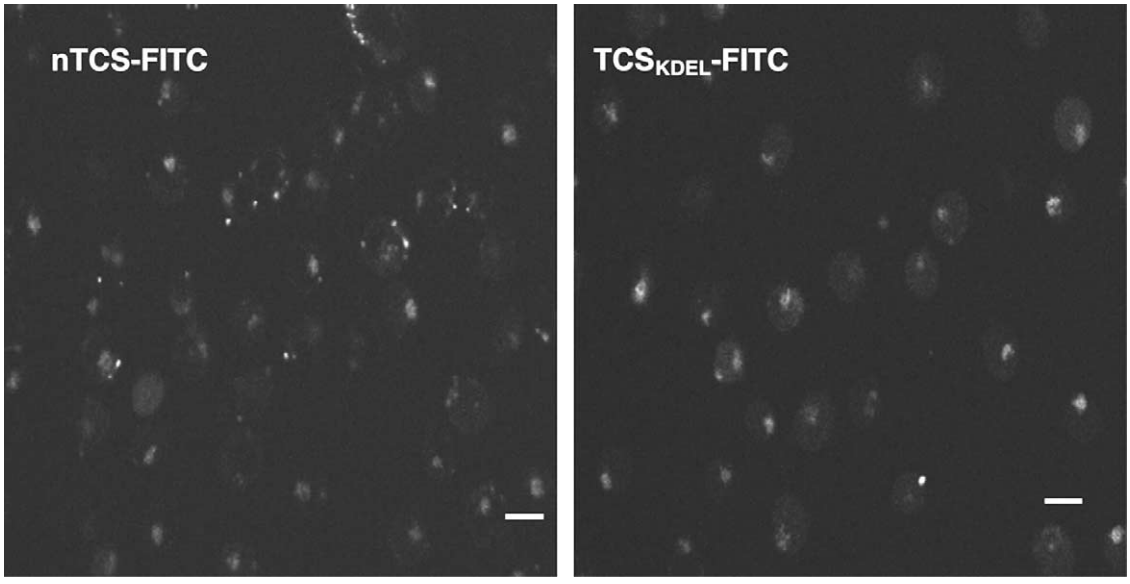

Fig. 3. Confocal images of nTCS-FITC and FITC-TCS_{KDEL}-FITC into K562 cells. Cells were treated with 25 μg/ml nTCS-FITC or TCS_{KDEL}-FITC for 4 h, washed, and processed by confocal microscopy. Scale bar = 5 μm.

resulted in a progressive mild decrease in the anti-HIV activity. Unexpectedly, addition of 19 amino acids and KDEL almost abolished the anti-HIV activity (Figs. 1 and 2). The RI activity of the mutants with added amino acid sequences did not change significantly (Table 1). These results especially the latter led us to examine some aspects of the anti-HIV mechanism of TCS.

TCS has many biological functions. The mechanism for these functions is not entirely clear. It may involve a common mechanism for all its actions or multiple mechanisms for different actions. Since ribosome inactivation is a well-documented action, many investigators believe that other functions of TCS may be totally or in part mediated via this ribosome inactivation. Indeed that in many cases, decrease in RI activity will lead to simultaneous reduction of some functions [7]. The present study demonstrated an exception in two TCS mutants that substantial reduction of its anti-HIV activity was without a concomitant change in RI activity. This will dissociate RI activity from the anti-HIV activity.

Systemic studies of the role of C-terminal on the anti-HIV action of TCS were carried out. Sequential deletion of the C-terminal sequence resulted in progressive decrease of RI activity with a parallel change in anti-HIV activity. Addition of amino acids resulted in loss of anti-HIV activity but not the RI activity. There are basically two plausible explanations to this observation. The first explanation is that the anti-HIV activity is not totally dependent on the RI activity so that the anti-HIV activity can disappear with maximal RI activity. Alternatively, the anti-HIV activity is totally dependent on the RI activity. Loss of anti-HIV activity was due to inability of the TCS_{KDEL} and TCS_{C19aa} mutants to enter cells and therefore were unable to act on the ribosome. This is highly unlikely. The original idea of adding the 19 amino acids and KDEL was to enhance cellular entry of TCS because these amino acid sequence are well known to do this job. In addition confocal study demonstrated that TCS_{KDEL} internalization was similar to nTCS (Fig. 3). Based on the present findings and previous observations, it is likely that the anti-HIV action of TCS is only partially dependent on RI activity.

The MAPK pathway is known to involve in a lot of cellular processes including cell differentiation, growth, and apoptosis [19,20]. It consisted of a number of subfamilies like the ERK, p38 MAPK, and JNK. While the ERK can be activated by receptor binding, the rest is activated by stress signals. Our recent work showed that TCS activated these MAPKs. Inhibitor studies demonstrated that ERK and p38 MAPK worked in opposite direction in TCS induced apoptosis. When these findings are applied to the present study it can be speculated that TCS binds to ERK to produce a signal. At the same time, because of ribosome inactivation a stress signal is also produced. The balance of these signals was ultimately involved in the mechanism of the anti-HIV activity. Addition of amino acids to TCS somehow blocked its binding with receptors and upset the balance of these signals. Anti-HIV activity is therefore reduced or abolished without any change in the RI activity.

In conclusion, the finding from this study showed that shortening the C-terminal will lead to progressive reduction of anti-HIV activity of TCS. Addition of some amino acid sequences expected to enhance TCS internalization almost abolished the anti-HIV activity. These findings also strongly supported that the anti-HIV activity is not entirely dependent on the RI activity of TCS.

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