Anti-HIV-1 property of trichosanthin correlates with its ribosome inactivating activity

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Abstract Trichosanthin (TCS) is a type I ribosome inactivating (RI) protein possessing anti-tumor and antiviral activity, including human immunodeficiency virus (HIV). The mechanism of these actions is not entirely clear, but is generally attributed to its RI property. In order to study the relationship between the anti-HIV-1 activity of TCS and its RI activity, three TCS mutants with different RI activities were constructed by using site-directed mutagenesis. The anti-HIV-1 activities of the three mutants were tested in vitro. Results showed that two TCS mutants, namely $TCS_{M(120–123)}$, $TCS_{E160A/E189A}$, with the greatest decrease in RI activity, lost almost all of the anti-HIV activity and cytopathic effect. Another mutant TCS_{R122G}, which exhibited a 160-fold decrease in RI activity, retained some anti-HIV activity. The results from this study suggested that RI activity of TCS may have significant contribution to its anti-HIV-1 property.

Key words: Trichosanthin; Ribosome inactivating protein; human immunodeficiency virus type 1; Antiviral activity

1. Introduction

Trichosanthin (TCS) is a type I ribosome inactivating protein (RIP) isolated from the root tubers of the Chinese medicinal herb *Trichosanthes kirilowii* Maxim (cucurbitaceae). Its crude extracts have been used as an abortifacient agent for centuries in China [1]. TCS possesses a wide spectrum of biological and pharmacological properties, including anti-tumor, antiviral, immunosuppressive and abortifacient activity [2,3]. The finding that TCS preferentially inhibited replication of human immunodeficiency virus type 1 (HIV-1) in both acutely infected T-lymphoblastoid cells and chronically infected macrophages in vitro generated some excitement [4]. Phase I/II clinical trials with TCS alone or in combination with zidovudine or dideoxinosine showed that TCS was able to decrease the serum HIV-1 p24 antigen level and to increase the percentage of $CD4^+$ cells in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex [5]. In addition to TCS, many other RIPs, including MAP30, GAP31, DAP30, DAP32, TAP29, PAP, bryodin, MMC and trichobitacin, have been reported to inhibit HIV-1 replication in vitro [6–12].

The exact mechanism of these RIPs on anti-HIV activity remains to be determined. It is generally believed that the antiviral activity is related to the ribosome inactivating (RI) activity. The argument against it is that not all RIPs have antiviral activity. In this study, the relationship between RI activity of TCS and its anti-HIV-1 activity was investigated. TCS consists of 247 amino acid residues with no disulfide bond. Since the three-dimensional crystallographic structure of TCS complex with its analog substrate has been elucidated, the study of structure–function relationship in RI activity is relatively clear. There are eight α-helices which are relatively concentrated at the center of the molecule and surrounded by 13 β-strands. Two domains in structure of TCS had been found: a larger N-terminal domain with approximately 160 residues and a smaller C-terminal domain possessing about 60 residues. Type I RIPs or the A chain of type II RIPs have some homology in amino acid sequence, and have some conservative amino acid residues that may be involved in the active site, for example in TCS, Tyr14, Arg22, Tyr70, Tyr111, Arg122, Glu160, Ala161, Arg163, Glu189, Trp192 etc., most of which lie at the brink of the cleft formed by the big and the small domain of TCS [1]. In order to study this relationship of the RI activity of TCS and its anti-HIV-1 activity, three TCS mutants namely $TCS_{M(120–123)}$, $TCS_{E160A/E189A}$ and TCS_{R122G} with different RI activities were constructed by using site-directed mutagenesis. The anti-HIV-1 activities were assayed in vitro to test for the relationship.

2. Materials and methods

2.1. Reagents and chemicals

AZT (3′-azido-3′-deoxythymidine) was purchased from Sigma, and horseradish peroxidase (HRP)-labeled goat anti-human IgG was purchased from Sino-America Biotechnology Company. Monoclonal antibody (McAb) to HIV-1 p24 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).

2.2. Natural TCS (nTCS) and TCS mutants

nTCS was purified from the root tuber of *T. kirilowii*. Three TCS mutated genes were constructed by polymerase chain reaction and the mutants were expressed and purified as described previously [13,14].

2.3. Virus and cell lines

C8166 and H9 cells were obtained from Medical Research Council (MRC), AIDS Reagent Project, and maintained in RPMI 1640 sup-

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plemented with 10% heat-inactivated fetal calf serum. 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 the 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.

2.4. Assay for RI activity

The RI activity of nTCS and its 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 [13]. The relative activity compared to nTCS in IC_{50} values was obtained.

2.5. Cytotoxicity assay

The cellular toxicity of compounds on C8166 cells was assessed by MTT colorimetric assay as described previously [15]. Briefly, 100 μl of 3×10^4 cells was seeded on a microtiter plate, 100 μl of various concentrations of compounds was added and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 h. Discard 100 μl supernatant, MTT reagent was added and incubated for 4 h, 100 μl 50% DMF–10% SDS was added. After the formazum was dissolved completely, the plates were read on a Bio-Tek ELx 800 enzyme-linked immunosorbent assay (ELISA) reader at 595 nm/630 nm. The results were shown by absorbance values.

2.6. ELISA for HIV-1 p24 antigen

HIV-1 p24 antigen in a cell-free culture medium was measured using an antigen capture ELISA as described previously [12]. Briefly, 96-well microtiter plates coated with McAb to p24 antigen were blocked by 5% skim milk powder, then detergent-treated cell-free culture medium was added, and incubated for 2 h at 37°C. The plates were then incubated with 1:500 diluted human polyclonal anti-HIV-1 sera, followed by incubation with HRP-labeled goat-anti human IgG and OPD reaction solution was added to the wells. The optical density of the plates was read on a Bio-Tek ELx 800 ELISA reader at 490 nm with reference 630 nm within 30 min of stopping the reaction. 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.

2.7. Anti-HIV activity assay

C8166 cells (3×10^5/ml) were pretreated with nTCS or mutants at various concentrations at 37°C for 1 h, and then were infected with HIV-1_{IIIB} at a multiplicity of infection (M.O.I.) of 0.015. 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 positive control. After 3 days of culture, the number of syncytia (multinucleated giant cells) in each well was counted under an inverted microscope. The percentage inhibition of syncytial cell formation was calculated by the percentage of syncytial cell number in compounds-treated culture to that in infected control culture. Terminated cell culture was centrifuged and harvested, and cell-free supernatant was used to measure the HIV-1 p24 antigen level with antigen capture ELISA assay.

3. Results

3.1. Sequence of nTCS mutants and its comparison with nTCS

A comparison of the amino acid sequence between nTCS and the three mutants is shown as Fig. 1. The Lys120-Ile121-Arg122-Glu123 were replaced by Ser120-Ala121-Gly122-Gly123 in $TCS_{M(120-123)}$. Both Glu160 and Glu189 were substituted by Ala in $TCS_{E160A/E189A}$, and the Arg122 was replaced by Gly122 in TCS_{R122G}.

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                    120             160             189
nTCS        ---AGKIRENIPLG---QSTSEAARYK---LAIISLENSWSA---
TCSM(120-123) ---AGSAGGNIPLG-----------------------------
TCSE160A/E189A ----------------QSTSAAARYK---LAIISLANSWSA---
TCSR122G    ---AGKIGENIPLG-----------------------------
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Fig. 1. Comparison of the amino acid sequence between nTCS and mutants. The changes in amino acids are denoted by bold and italic. '—', indicating the same amino acids compared to nTCS.

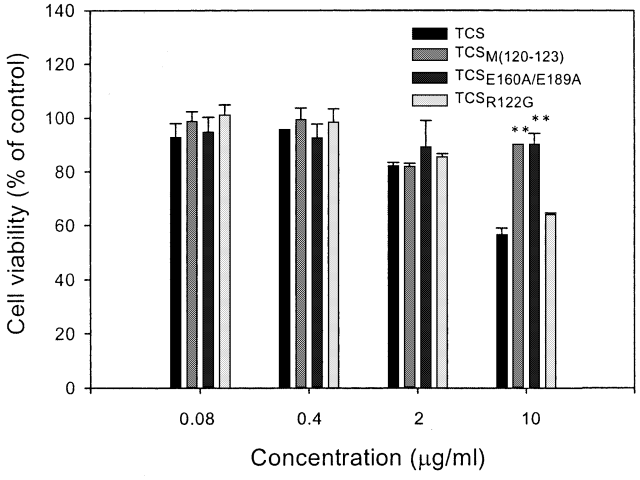

Fig. 2. Cytotoxicity of nTCS and its mutants on C8166 cells. The cell viability was measured by MTT assay. Data are expressed as means ± S.E.M. of triplicate measurements. $^{**}P<0.01$ versus nTCS.

3.2. RI activity of nTCS and its mutants

A rabbit reticulocyte lysate cell-free system was used to measure the RI activity of nTCS and the mutants at different concentrations in vitro. IC_{50} of nTCS active site mutants, $TCS_{M(120-123)}$, $TCS_{E160A/E189A}$ and TCS_{R122G} exhibited a markedly decrease in RI activity. Their RI activities were 4000-, 1800- and 160-fold lower than that of nTCS, respectively. This is consistent with the finding of other investigators [13,14].

3.3. Cytotoxicity of nTCS and its mutants on C8166 cells

The cytotoxicity of nTCS and its mutants on C8166 cells is summarized in Fig. 2. At 10 μg/ml, nTCS and TCS_{R122G} caused about 43.2 ± 2.3% and 36.1 ± 0.9% inhibition of cell growth, respectively. $TCS_{M(120-123)}$ and $TCS_{E160A/E189A}$ exhibited significantly lower cytotoxic effects than that of nTCS. nTCS and mutants slightly affected cell growth and cell viability was over 80% at a concentration below 10 μg/ml.

3.4. Anti-HIV-1 activity of nTCS and mutants

The virus-induced cytopathic effect is quantitated by the syncytium formation. The inhibition of syncytium formation in a dose-dependent manner was observed in the presence of nTCS, an EC_{50} of 0.38 ± 0.09 μg/ml was obtained. TCS_{R122G} exhibited lower effects on inhibition of syncytium formation than that of nTCS. At 10 μg/ml, TCS_{R122G} caused 50.10 ± 0.87% inhibition of syncytium formation (EC_{50} 9.8 ± 2.3 μg/ml). $TCS_{M(120-123)}$ and $TCS_{E160A/E189A}$ lost their effects on inhibition of syncytium formation (Fig. 2A). Simultaneously, inhibition of HIV-1 p24 protein expression on acutely infected cells by nTCS and its mutants was detected to measure HIV-1 replication by an antigen capture ELISA method. Like inhibition of syncytium formation, the inhibition of p24 antigen expression by nTCS was at a dose-dependent manner with an EC_{50} of 0.32 ± 0.02 μg/ml. All the mutants exhibit a decrease in anti-HIV activity at the selected concentration. TCS_{R122G} exhibited 46.4 ± 4.5% inhibition of

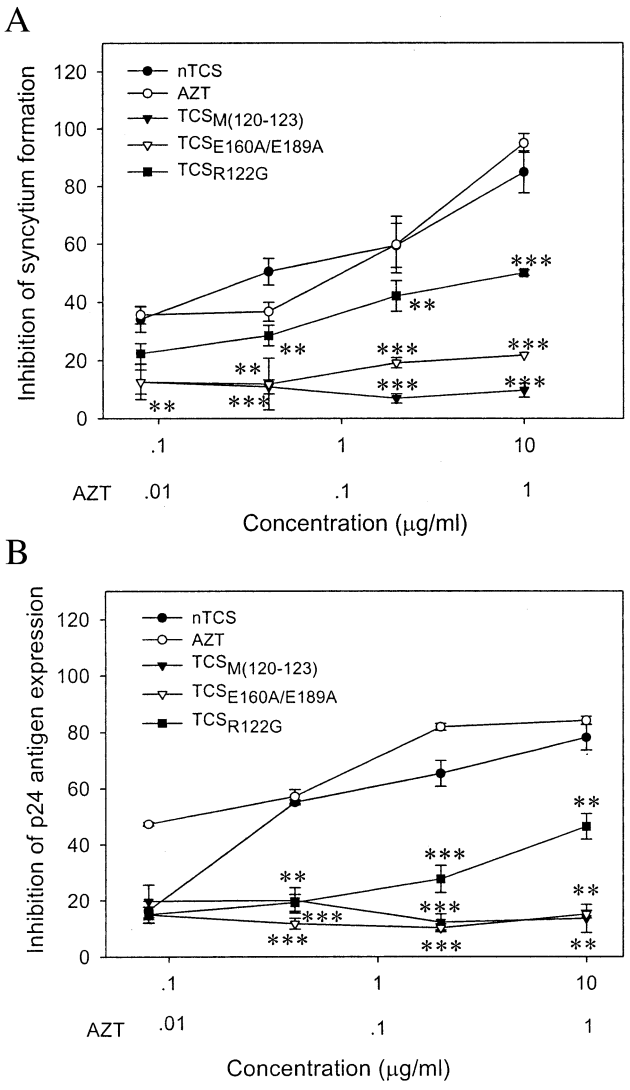

Fig. 3. Anti-HIV activity of nTCS and its mutants. C8166 cells were pretreated and infected with HIV-1_{IIIB} at a M.O.I. of 0.015. Syncytial formation inhibition was quantified under an inverted microscope (A) and p24 antigen expression in supernatants was performed by capture ELISA (B). Data are expressed as means ± S.E.M. of triplicate measurements ($n=9$). $^{**}P<0.01$, $^{***}P<0.001$ versus nTCS.

p24 antigen expression at 10 μg/ml. $TCS_{M(120–123)}$ and $TCS_{E160A/E189A}$ lost the anti-HIV-1 activities, no inhibition of p24 antigen expression was observed at these concentrations (Fig. 3B).

4. Discussion

All RIPs with an anti-HIV property have RI activity, but most of the RIPs with RI and/or abortifacient activities do not possess anti-HIV activity [16]. Therefore, the direct relationship between RI activity and anti-HIV activity of the RIPs remains to be examined. PAP RI activity is distinct from anti-HIV activity [9], while MAP30 and related RIPs inhibit HIV-1 at a concentration that showed little effects on ribosome function [6,17]. The potent antiviral activity of PAP may in part be due to the unique ability of PAP to extensively depurinate viral RNA, including HIV-1 RNA [18].

In order to study the relationship between RI activity of TCS and its anti-HIV activity, three nTCS RI mutants namely $TCS_{M(120–123)}$, $TCS_{E160A/E189A}$ and TCS_{R122G} were constructed.

The 120–123 (Lys-Ile-Arg-Glu) region lying at the entrance of the cleft, is one of the four hydrophilic areas of the nTCS molecule, and has the highest homology of amino acid sequence in several type I RIPs [13]. Crystallographic data demonstrated that this region has strong interaction with other side chains and the protein backbone by forming hydrogen bonding [19]. The substitute mutant $TCS_{M(120–123)}$ was changed from Lys-Ile-Arg-Glu to Ser-Ala-Gly-Gly. This replacement maintained its peptide backbone, but lost most of its interaction with other amino acids due to the change from hydrophilic to hydrophobic properties, resulting in the 4000-fold decrease of in vitro RI activity versus nTCS.

Since Arg122 is the most conservative amino acid, and is related to five hydrogen bonds formation, therefore Arg122 may take on a key role for this region action [1]. The amino acid substitution of Arg122 by Gly leads to a 160-fold decrease in RI activity. TCS_{R122G} is now obtained as a single-sited mutant which possesses the greatest effect on nTCS RI activity.

Both Glu160 and Glu189 lie at the placket area of the nTCS RI activity site. The negative charges of Glu160 may play a role in stabilizing the nTCS transition-state complex which presumably possesses oxocarbonium characteristics. Glu189 can partly substitute for Glu160 by furnishing a carboxylate group for its RI activity when Glu160 is lost [14]. When Glu160, Glu189, the polar residues with long side chains, were replaced by Ala, the non-polar residues with short side chains, the surplus room would be filled with two water molecules, which formed the hydrogen bonding, then substituted partly the carboxylate group effects of Glu160 and Glu189 [20]. $TCS_{E160A/E189A}$, both Glu160 and Glu189 were replaced by Ala, its RI activity was 1800-fold lower than that of nTCS.

Our results showed that along with the decrease in RI activity, the anti-HIV-1 activity of nTCS was declined. When RI activity was 160-fold downward (TCS_{R122G}), the mutant still reserved some anti-HIV activity, but when the RI activity dropped 1800-fold ($TCS_{E160A/E189A}$), the mutant lost almost all of the anti-HIV activity, and so did the mutant $TCS_{M(120–123)}$ with the 4000-fold decrease in RI activity. Suppression of HIV replication was partly due to inhibition of cell proliferation and partly due to direct inhibition of HIV replication by TCS and its mutants. Fig. 3 showed that TCS inhibited cell growth by 43.2% and reduced HIV-1 p24 antigen expression by 78.2% at 10 μg/ml. The difference represented direct inhibition of HIV replication by TCS. At the same concentration, TCS_{R122G} inhibited cell growth by 36.1% and reduced HIV p24 antigen expression by 46.4%. At 2 μg/ml, TCS caused only 18% inhibition of host cells growth, but it possessed significant anti-HIV activity with 65.3% inhibition of p24 production. These results demonstrated that TCS mutant clearly exhibited reduction in anti-HIV activity independent of its effect on cell proliferation. So we can conclude that nTCS anti-HIV-1 activity is related to its RI activity. There is no reason to believe in the mutant active site residues, which located in the active cleft, involved in the membrane binding, endocytosis and translocation process in cytosol.

nTCS cannot inhibit expression of p24 antigen in chronically HIV-1-infected H9 cells. This suggested that disturbing stages of nTCS were not virus maturity, infectivity and release, replication of viral genome. In addition, our data showed that nTCS was unable to block fusion between the chronically HIV-1_{IIIB}-infected H9 cells and uninfected C8166 cells in coculture (data not shown). This result suggested that nTCS did not interfere with the absorption and binding between host cells and viruses. RIPs were reported to possess topological inactivation activity which was also proposed to be important for their antiviral action [21,22], and MAP30, GAP31 and TCS were found to be inhibitors of HIV-1 integrase [23,24].

From this study we can conclude that TCS RI activity contributes to its anti-HIV activity, but the relationship should not be simple and direct, there should be other pathways involved in the effect of TCS anti-HIV.

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