Biochemical and Biophysical Research Communications 460 (2015) 170–176

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Single chain antibody fragment with serine protease inhibitory property capable of neutralizing toxicity of *Trimeresurus mucrosquamatus* venom

Yu-Ching Lee [a, b, 1], Wang-Chuan Chen [c, d, 1], Meng-Huei Liang [b], Chi-Hsin Lee [e], Keng-Chang Tsai [f], Jen-Ron Chiang [g], Liao-Chun Chiang [g], Chi-Ching Chen [h], Chang-Yu Chang [i], Ching-Hsiao Lee [i], Sy-Jye Leu [e, j], Yi-Yuan Yang [b, k, l, *]

[a] *The Center of Translational Medicine, Taipei Medical University, Taipei, Taiwan*
[b] *Antibody and Hybridoma Core Facility, Taipei Medical University, Taipei, Taiwan*
[c] *The School of Chinese Medicine for Post Baccalaureate, I-Shou University, Kaohsiung, Taiwan*
[d] *Department of Chinese Medicine, E-Da Hospital, Kaohsiung, Taiwan*
[e] *Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[f] *National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei, Taiwan*
[g] *Center for Research, Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare, Taiwan*
[h] *Department of Pathology and Laboratory Medicine, Landseed Hospital, Taoyuan, Taiwan*
[i] *Department of Medical Technology, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan*
[j] *Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[k] *School of Medical Laboratory Sciences and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan*
[l] *Department of Laboratory Medicine, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan*

ARTICLE INFO

Article history:
Received 16 February 2015
Available online 10 March 2015

Keywords:
Trimeresurus mucrosquamatus
Phage display technology
Single chain variable fragment
Venom serine protease
Molecular modeling

ABSTRACT

Trimeresurus mucrosquamatus (TM) is one of majorities of snake envenomation with necrotic and hemorrhagic toxin in Taiwan. In this study, chickens were used as an alternative animal model for immunization with TM venom. Using phage display technology to process four rounds of panning, selected single chain variable fragments (scFv) could specifically recognize TM venom proteins, which were later identified as a group of homogeneous venom serine protease. The specific scFv antibodies showed various inhibitory effects on sheep RBC lysis induced by TM venom using an indirect hemolytic assay *in vitro*. In addition, the survival times of mice were extended to certain degrees when treated with these scFv antibodies individually or in a combination. To elucidate the inhibitory mechanism, we used molecular modeling to build up the serine protease structure to simulate the possible interactions with scFv antibodies. The results suggested that the CDR-loop of the scFv antibodies (3S10 or 4S1) might bind at the 99-loop of venom serine protease so as to affect substrate access due to the partial collapse of the subsite S2 and the partial movement of the subsite S4. It is hoped these chicken-derived antibodies could be applied to develop diagnostic and therapeutic agents against snakebites.

1. Introduction

In developing parts of the world, venomous snakebites are still important medical problems to be dealt with. *Trimeresurus mucrosquamatus* (TM) is one of majorities of snakebite envenomation with necrotic and hemorrhagic toxicity in Taiwan. Systemic edema with some pathological complications, such as tissue necrosis and myonecrosis are the major pathological symptoms after snakebite envenomation by TM [1]. TM venom with hemorrhagic activity is complicated and mainly affects blood coagulation and platelet function. These proteins in venom include snake venom metalloproteinases (SVMPs), thrombin-like serine proteinases, disintegrins, C type lectin-like proteins, phospholipases A2 (PLA2) and other anticoagulants [2,3].

* Corresponding author. School of Medical Laboratory Sciences and Biotechnology, College of Medical Science and Technology, Taipei Medical University, #250 Wu-Hsing Street, Taipei 110, Taiwan. Fax: +886 2 27324510.
E-mail address: yangyuan@tmu.edu.tw (Y.-Y. Yang).
[1] These authors contribute equally in this work.

http://dx.doi.org/10.1016/j.bbrc.2015.02.169
0006-291X/

In general, snake venom serine proteinases (SVSPs) were determined to be about 10% of the venom toxins among the venom components [4]. They are involved in proteolysis process directly by affecting corresponding substrates, resulting in disturbances on hemostasis and thrombosis after envenomation [5]. Although molecular weight of the naive SVSPs are around 35–40 kDa, sequence variations and various glycosylation levels of SVSPs usually result in distinct forms with slightly different molecular weights and pIs under electrophoresis [6]. These serine proteinases share a common characteristic in that the endogenous Cys residues are all disulfide-linked, and their pairing is identical to that of mammalian trypsin [7].

Up to date, the major and effective treatment against snakebite is to administrate the species-specific anti-venom antibodies by immunizing horses and later purify polyclonal antibodies from the crude serum. Alternatively, hens provide a more hygienic, cost-effective and convenient choice, as compared to that of obtaining antibodies from mammalian serum [8]. The yolks of egg laid by immunized chickens have been recognized as an excellent source of polyclonal antibodies for over a decade [9]. Currently, considerable number articles related to clinical and experimental treatments using IgY for passive therapy have been reported [9]. Exemplified by the study of developing anti-venom therapies, this way has been proved to be applicable in providing significant protective effect [10,11].

Since the first recombinant antibody library was established by Huse et al., in 1989, phage display technology not only opened up an opportunity for monoclonal antibody preparation but also in the current development of target therapy [12]. Noticeably, many studies confirmed the benefits exploited by this novel technology to generate anti-venom antibodies against snakebites [13,14]. However, due to the complexity of snake venom, the potential targets recognized by these specific monoclonal antibodies have not been fully unveiled. In this study, to produce monoclonal antibodies capable of neutralizing the toxicity of venom proteins for better understanding their poisoning mechanisms, a panel of specific monoclonal antibodies in the configuration of single chain variable fragments (scFvs) was isolated by phage display technology after immunizing chickens with TM venom. The isolated antibodies not only inhibited the venomous hemolysis but also protected the mice from lethal injection of venom when used individually or in the combination. Moreover, we analyzed the possible interactions of these neutralizing antibodies with target venom serine protease to delineate the possible inhibitory mechanisms by molecular modeling approach. Hopefully, these antibodies could provide a basis in the development of diagnostic and therapeutic agents for snakebites.

2. Materials and methods

2.1. Animals

All animal experimental protocols in this study had been approved by the Institutional Animal Care and Use Committee of the Taipei Medical University before study initiation. Female white leghorn (*Gallus domesticus*) chickens and 4 week old ICR mice were purchased from the National Laboratory Animal Center, Taiwan, and maintained in the animal facility of the Taipei Medical University.

2.2. Chicken immunization, antibody library construction and panning

Female white leghorn chickens were immunized intramuscularly with TM venom to induce humoral response. After immunization, libraries containing single chain variable fragment (scFv) were generated according to the published protocols with minor modifications [15]. Panning procedures were carried out according to the previously published papers [16,17]. The panning cycle was repeated for four times. After final panning, total library DNAs were purified and transformed into *Escherichia coli* Top10 F' strain for next single colony analysis.

2.3. Western blotting

To validate binding ability of antibodies, TM venom proteins were transferred onto nitrocellulose membranes after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with polyclonal IgY, horse-derived polyclonal IgG or purified scFv antibodies, respectively. After blocking with 5% skim milk and washing with PBST (PBS containing 0.05% Tween 20), goat anti-chicken light chain antibodies (Bethy, USA) or rabbit anti-horse antibodies (Sigma, USA) were added and incubated for additional 1 h at room temperature. Thereafter, the blots were washed and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA). Finally, the recombinant scFvs were visualized by the addition of diaminobenzidine (DAB).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Snake venom protein was coated on microtiter plate wells at 4 °C overnight. After blocked with 5% skim milk, the expressed scFv molecules were added to wells in duplicate and incubated for 1 h at room temperature. Goat anti-chicken light chain antibodies were added and incubated for additional 1 h at room temperature. Wells were washed with PBST after samples were removed. Subsequently, HRP-conjugated donkey anti-goat IgG antibodies were incubated for 1 h at room temperature. After washing as above, 3,5,5-Tetramethubezidine dihydrochloride (TMB) was added for color development. Reaction was stopped by addition of 1 N HCl and absorbance was taken at 450 nm.

2.5. Hemolytic inhibition assay

Hemolytic activity of TM venom was evaluated by an indirect hemolytic assay *in vitro*. In brief, 2 μl of venom proteins diluted from 10 mg/ml to near 80 μg/ml in 2-fold serial condition was spotted on blood agar plate (BAP, Trypticase Soy Agar plates containing 5% sheep blood (TSA II) (BBL, BD, USA)). After incubation at 37 °C for 24 h, the diameter of each hemolytic area was measured to determine hemolytic activity.

To test the inhibitory effect on hemolysis, 2 μl of purified scFv antibodies (1 μg or 0.5 μg) were individually mixed with equal volume of TM venom (5 μg) and incubated for 1 h at 37 °C. Two μl of each mixture was spotted on TSA II plates, followed by determination of hemolytic activity as described as above.

2.6. Lethality neutralization assay

To test the neutralizing activity, the ICR mice (four-week-old) were injected intra-peritoneally (i.p.) using various scFv antibodies mixed with 1× minimal lethal dose (MLD) of TM venom. MLD was defined as the total amount of venom leading to the death of mice within 1 h after administration. In brief, 1× MLD of TM venom was incubated with 1 mg of polyclonal IgY or 0.5 μg of each purified scFv individually or with a mixture of four different scFv (each 0.125 μg) for 1 h at 37 °C. Three ICR mice were included in each group. The control group of mice received 1× MLD of TM venom alone or 1× MLD of venom with 1 mg of horse-derived polyclonal IgG. The survival of mice was recorded at a half hour interval for 24 h after different treatments as described above.

2.7. Molecular modeling and docking

To further look into the mechanisms of VSP antigen and the scFv antibody interaction, we chose 3S10 antibody, which was modeled as a scFv scaffold, and a venom serine protease type 2 (VSP2) using the homology modeling program. Full details are provided in the supplementary data.

3. Results

3.1. Humoral response in chickens, antibody library construction and biopanning

TM venom powders was dissolved in 1× PBS for chicken immunization. Their identity was analyzed on SDS-PAGE using Coomassie blue staining. The protein pattern showed a complex of components containing one major band with molecular weight of 30 kD (lane TM venom, Fig. 1A). By Western blotting, the polyclonal anti-TM IgY antibodies recognized a panel of proteins (lane Y) which were also identified by polyclonal anti-TM IgG antibodies purified from horse sera (lane H) as shown in Fig. 1A. The results indicated that immunization with TM venom elicited similar humoral antibody responses in chickens and horses. Interestingly, a protein which consists of 50% of the total components in the venom was not recognized by both polyclonal antibodies, suggesting that this particular protein is not immunogenic to the animals. Thus, its identity and biological functions remain to be investigated.

The complexity of two antibody libraries was estimated to be 1×10^7 and 4×10^7, respectively. After four rounds of panning cycle, the TM venom-binding phage variants were enriched for more than one order of magnitude (Table 1). These results suggested that the majority of nonspecific phage particles displaying scFv antibodies were removed throughout the panning process and the clones with specific binding affinity were enriched.

3.2. ScFv expression and characterization

The ELISA analysis of isolated scFv after panning showed that 3S10, 4S1, 4S9, 4S15 and 3L6 scFv antibodies exhibited significant binding activity to TM venom but not BSA as compared to an irrelevant scFv (Fig. 1B). For further characterization, these specific scFv were probed with TM venom proteins separated on SDS-PAGE and immobilized on nitrocellulose membrane. Similar binding patterns were visualized on the blots. The results suggested that purified scFv antibodies of 3S10, 4S1, 4S9, 4S15 but not 3L6 can recognize the same target proteins in the venom (Fig. 1C). However, the recognized pattern with several major proteins was not expected. We speculated all the recognized proteins may possess similar linear amino acid sequences, leading to the formation of antigenic epitope. The problem could be solved using mass spectrometry analysis to confirm their identity.

In addition, these scFv antibodies were assessed for their cross-reactivity with crude venoms of *Deinagkistrodon acutus*, *Bungarus multicinctus*, *Trimeresurus stejnegeri*, *T. mucrosquamatus, Naja naja atra* and *Daboia russellii* by ELISA. Given the complexity and similarity of venom components in the same genus, the results unexpectedly showed that all 4 scFv antibodies tested had significant binding specificity only to TM snake venom (Fig. 1D).

3.3. Analysis of heavy (H) and light (L) variable (V) regions of antibody genes

To scrutinize the gene usage of the isolated scFv clones with significant binding signals against TM venom, VL and VH genes were sequenced. The alignment of putative amino acid sequences to the germline genes of chicken immunoglobulin revealed that these clones contained 6–12% and 31–62% variations in framework (FR) and CDR regions, respectively, leading to 13–25% mutation rates in the entire V region (Fig. S1). Intriguingly, it was observed that the identical VL gene was used by clones 3S10, 4S09 and 4S15 to pair with different VH genes for scFv antibody expression.

3.4. Inhibition of scFv antibodies on hemolytic activity

These scFv antibodies were evaluated for their inhibitory effect on RBC lysis by an indirect hemolytic assay on BAP. In Fig. 2A, TM snake venom diluted serially (2^0–2^{-7}) produced various hemolytic halos in diameter. The 2^{-3} dilution containing 2.5 μg of venom resulting in a hemolytic halo of 8 mm in diameter was selected for the inhibitory assay. As shown in Fig. 2B, RBC hemolysis was suppressed effectively by 1 and 0.5 μg of 3S10 or 4S1 scFv antibodies in a dose-dependent manner. Similarly, scFv 4S9 and 4S15 also showed a partial inhibitory effect. By contrast, 3L6 scFv antibody exhibited no neutralization activity which was also seen when an irrelevant scFv (panel NC) or PBS alone was used.

3.5. In vivo animal assay

ICR mice were used to evaluate the antibody-mediated neutralization against TM venom challenge *in vivo*. As shown in Fig. 3A, bivalent anti-TM/TS (*T. stejnegeri*) IgG from horse serum provided complete protection against TM venom to rescue all mice survived. The treatment using bivalent anti-TM/TS polyclonal IgY prevented 2/3 of the mice from dying of envenomation. In addition, the anti-TM polyclonal IgY antibodies offered partial protective effect, delaying the mice death. However, all the mice received TM venom alone died within 1 h after intraperitoneal injection. Similar protective effect was observed when monoclonal scFv 3S10, 4S1, 4S9 or 4S15 were used individually or as a mixture of all scFv for the test. As compared to the result of venom injection solely, all four individual scFv were able to protect mice from lethality and prolonged the survival time of mice (Fig. 3B). It is noticeable that 3S10 scFv antibody exhibited the most effective activity against envenomation. Moreover, the results showed that the monoclonal antibody mixture including 3S10, 4S1, 4S9 and 4S15 in equal amount not only prolonged the survival time of mice but also kept 1/3 of the treated mice alive from the venom challenge.

3.6. Target recognition by antibody

In order to identify the compounds in TM venom mixture recognized by the scFv antibodies on Western blots, four protein bands (p1, p2, p3 and p4 as arrowed) on Coomassie blue-stained acrylamide gel were cut out for mass spectrometry analysis (Fig. S2A). The alignment analysis using protein data bank showed that the potential targets were different isoforms of TM venom serine protease (VSP). Of which, amino acid sequences of proteins p1, p2, p3 and p4 showed 24%, 24%, 42% and 25% homology to those of VSP5, VSP5, VSP2 and VSP1, respectively (Fig. S2B). Additionally, the alignment of seven serine proteases of TM venom (VSP1-7) showed a high similarity in amino acid sequences, containing a conserved catalytic site consisting of His57, Asp102 and Ser195 residues (Fig. S3).

3.7. Molecular modeling and docking

Homology model of 3S10 was generated by MODELER v.9.4 program in Discovery Studio v.4.0 (Accelrys Software Inc., San Diego, CA, USA) using the crystallographic structure of the anti-ptau antibody (PDB id: 4GLR) derived from the same species,

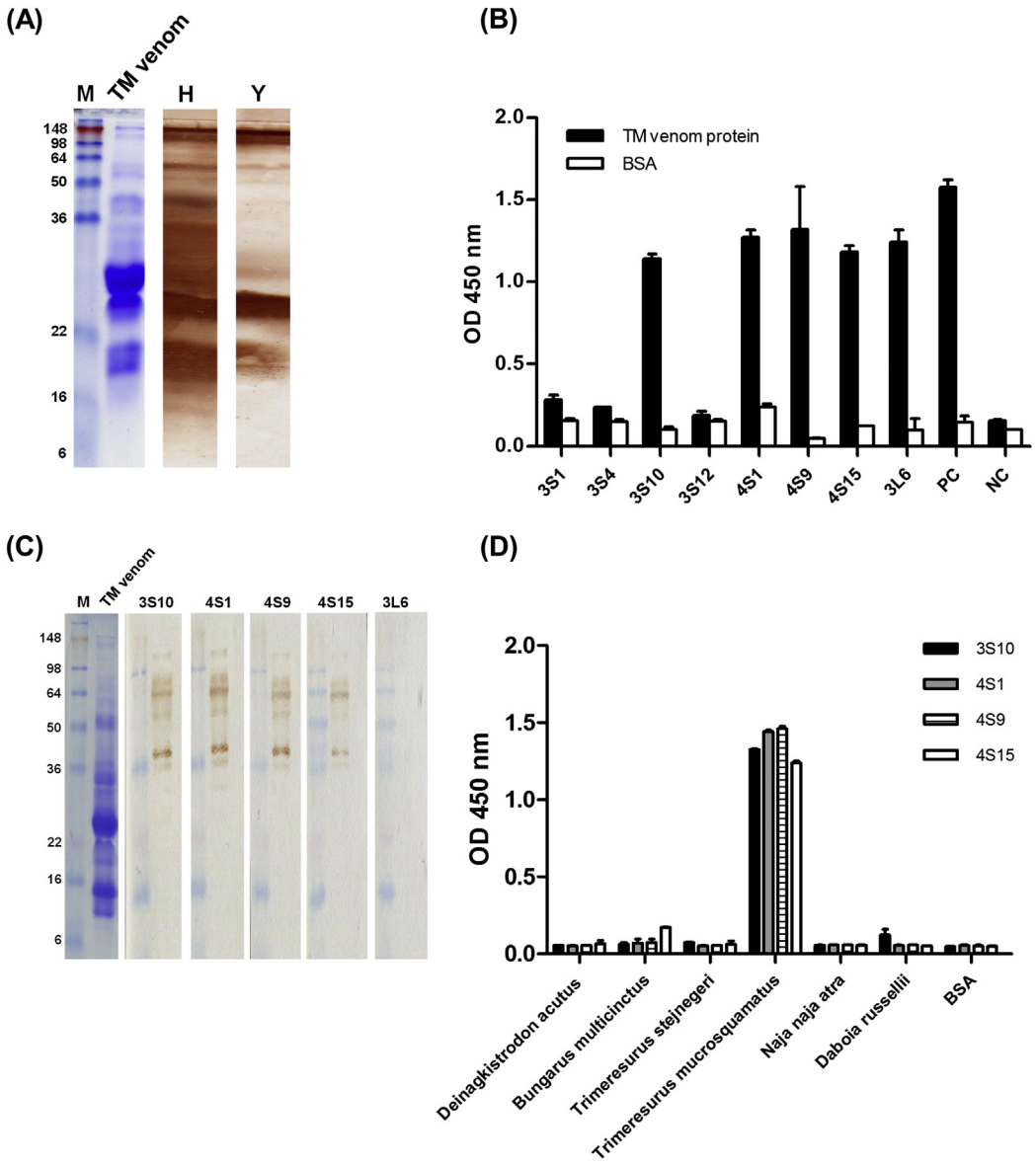

Fig. 1. Characterization of anti-TM polyclonal IgY and monoclonal scFv antibodies. (A) TM venom proteins were visualized by Coomassie blue staining or transferred onto nitrocellulose paper, which was subsequently probed with horse-derived polyclonal IgG (lane H) or with polyclonal IgY antibodies from 5th-immunized chicken (lane Y). (B) Binding activity of isolated scFv was examined using ELISA. Purified polyclonal IgY from 5th-immunized chicken was included as a positive control (PC). An irrelevant scFv was used as a negative control (NC). (C) TM venom proteins were detected by five purified scFv using Western blotting individually. (D) Binding specificity of scFv was examined against a panel of six different venoms coated on ELISA plate wells.

G. domesticus, as a structural template (Fig. S4). Similarly, homology model of VSP2 antigen was built by the same program using another antigen (PDB id: 2AIP) as a structural template (Fig. S3). The refined 3S10 and VSP2 models were validated with Profiles-3D and Ramachandran Plot. The strategy of antibody-antigen docking details is provided in the Supplementary data.

Table 1
Evolution of panning process.

Library	Linker[a] length	Complexity	Eluted phage numbers after each time of panning cycle			
			1st	2nd	3rd	4th
csc-TMS	7 aa	4×10^7	3.7×10^4	8.1×10^3	2.1×10^4	7.4×10^5
csc-TML	18 aa	1×10^7	6.8×10^4	8×10^3	1.2×10^4	9.8×10^5

[a] Linker length of 7 aa and 18 aa are GGSSRSS and GGSSRSSGGGG.

4. Discussion

Currently, the horse-derived anti-venom IgG antibodies are the most reliable and effective agent for deadly snakebite. However, the cost for the production of such therapeutics is high and alternative choice for cost-effective production is needed. As comparable to the horses, chickens have been demonstrated suitable for eliciting strong humoral response in our previous studies [17,18]. In this study, our data indicated that bivalent anti-TM/TS IgY antibodies were used for neutralization test, they provided an efficient protection comparable to that offered by bivalent anti-TM/TS IgG antibodies (Fig. 3A). Thus, it is evident that chickens are excellent alternative choice as the source of antibody production. Due to the presence of isoforms with similar structure in the various components in snake venom (such as VSP, Fig. S3), it is reasoned that the bivalent antibodies elicited by immunizing with a mixture of two

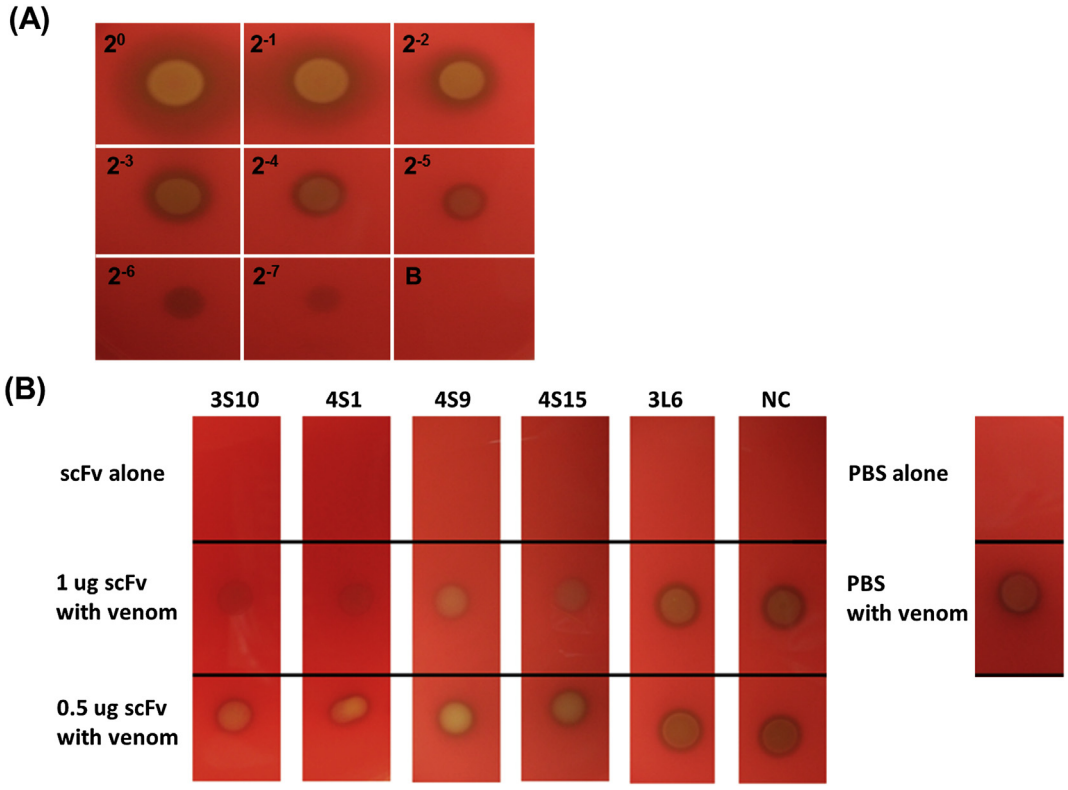

Fig. 2. Inhibitory effect of scFv antibodies on hemolytic activity of venom. (A) TM venom was serially diluted (2^0–2^{-7}) to examine its hemolytic activity. B denoted treatment with PBS only. (B) TM venom at 2^{-3} dilution was mixed with 1 or 0.5 μg of purified scFv and spotted on the sheep RBC plates. The inhibition of hemolytic effect of venom on RBCs was clearly demonstrated by 3S10, 4S1 and 4S15 scFv antibodies. In accordance, less inhibitory effect was observed in the groups of venom mixed with lower dosage of scFv antibodies. NC denoted the treatment of an irrelevant scFv antibody.

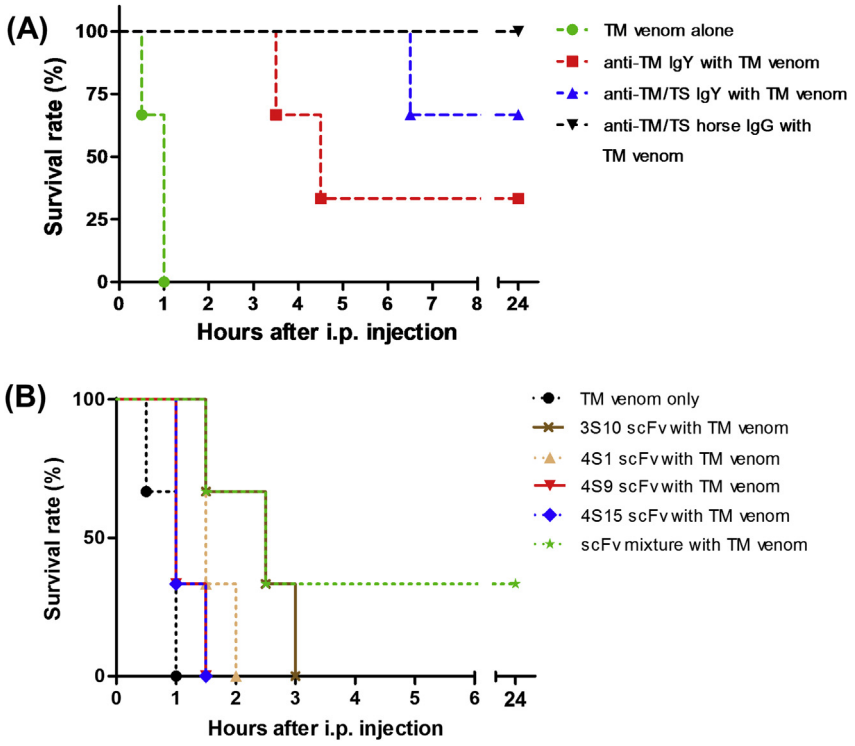

Fig. 3. Protective effect of anti-TM IgY and scFv antibodies on lethal challenge *in vivo*. (A) Groups of mice were individually injected with 1× MLD of TM venom (circle), a mixture of venom and anti-TM IgY (square) or a mixture of venom and anti-TM/TS IgY (triangle). Horse-derived polyclonal IgG containing divalent anti-TM/TS IgG antibodies was also included as a protective control (inverted triangle). (B) Similarly, mice were treated with TM venom together with individual scFv antibody or with a mixture of 3S10, 4S1, 4S9 and 4S15 scFvs.

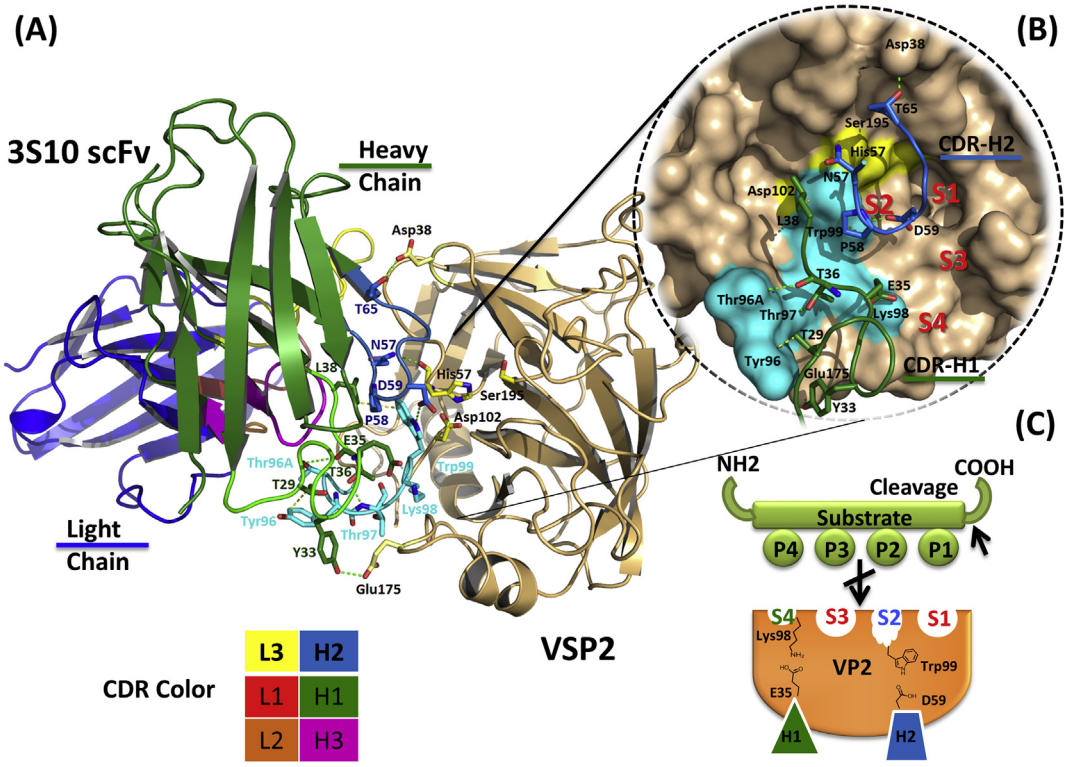

Fig. 4. (A) Secondary structure for 3S10 scFv antibody-VSP2 antigen model (light chain in blue and heavy chain in green). VSP2 antigen and 99-loop are colored in light orange and cyan, respectively. The format of residues of VSP2 antigen and H1/H2 loops are abbreviated as three words and residues codes for distinguishing between antigen and antibody, respectively. The green, cyan and yellow dotted lines in the two panel represent H-bonding, electrostatic and hydrophobic interactions of 3S10 with VSP2, respectively. (B) The interface structure of the 3S10-VSP2 was focused on the CDR-H1 (colored in green) and CDR-H2 (colored in blue) loops. The substrate binding subsites S1–S4 are indicated. The format of residues of VSP2 antigen and CDR-H loops are abbreviated as the same codes as in Fig. S5. (C) A cartoon model illustrating the binding mechanism of inhibition. The side chain of Glu35 on the H1 loop and Asp59 side chain on the H2 loop interact with the Lys98 side chain and Trp99 side chain on 99-loop via ion interaction and H-bonding, respectively, so as to affect substrate access due to the partial collapse of the subsite S2 and the partial movement of the subsite S4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

different venoms would have a better chance to provide more efficient neutralizing reactivity. This observation was further supported by the use of a mixture of 4 monoclonal scFv antibodies to protect mice from lethal challenge (Fig. 3B).

As compared to those of the chicken germline, the amino acid sequences of isolated scFv antibodies showed a greater mutation rates in the CDRs than in the framework regions. The hyper mutation in CDRs implied antibodies with high affinity could be generated through an antigen-driven humoral response after the immunization [19]. Similar patterns of somatic mutation were also observed in our previous studies with genetic analysis of scFv antibodies [17,18]. Notably, the light chain used by clones 3S10, 4S9 and 4S15 are identical, suggesting that this particular light chain may play an important role on structure stability and the protective effect of these scFv antibodies against RBC hemolysis and lethal *in vivo* venom challenge. However, it's interesting to find that a few changes of amino acid residues in the frameworks and CDR2 of scFv 3S10, 4S9 and 4S15 make significant difference in their neutralizing and protective activity (Fig. 3).

Currently, antibodies in the PDB that bind to serine protease can be categorized into two classes according to the binding mechanism. The first class includes the active site-directed antibody [20], implying these antibodies might sterically block substrate access by their CDR loops to occupy the important Ser-His-Asp catalytic triad of serine protease (Fig. S5A). The second class antibody binds to the 99-loop or 220-loop [21], indirectly causing structural change at the active site and thereby hindering the substrate from entering the active site, which is known as the allosteric mechanism (Fig. S5B). In this study, the protein docking analysis of antibody-antigen interaction indicated that scFv 3S10 might bind to 99-loop of VSP2 and get close to the active site. Meanwhile, the H1 and H2 loops of 3S10 that dominate the interaction with VSP2 are shown in Fig. 4A and B. Based on the interaction between CDR-H1 loop and subsite S4 of VSP2, we found that a key Glu35 side chain on the CDR-H1 loop acted on Lys98 side chain on the subsite S4 of VSP2, which involved an electrostatic interaction. Such an interaction might lead to the partial movement of the subsite S4, so that the substrate couldn't enter the active site. In addition, these interactions of the Leu38 of H1 loop with the Pro58 and Asp59 side chains on the H2 loop surrounding the Trp99 on the 99-loop involved in the hydrophobic and H-bonding forces might lead to a partial collapse of the subsite S2. As a result, the movement of Trp99 on the S2 subsite and the Lys98 on the S4 subsite perturbed the interactions with substrate residues P2 and P4, respectively (Fig. 4C). Viewed as a whole, our study showed that anti-VSP scFv antibodies have an excellent binding ability and the neutralization of toxicity with computational structural investigation is in line with the experiments.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgments

We thank the National Science Council (NSC102-2320-B-038-037 and NSC102-2622-B-038-001), Ministry of Health and Welfare (MOHW103-TD-B-111-01 from Health and welfare surcharge of

tobacco products) and Centers for Disease Control (DOH100-DC-1017) for financial supports. We are also grateful to the National Center for High-performance Computing in Taiwan for Discovery Studio software and facilities.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.02.169.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.169.

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