Skeletal muscle necrosis in rat induced by *Trimeresurus flavoviridis* venom can be prevented by its serum proteins

Motoo KITANO[1]*, Masato HIRANO[1], Takashi ISHIHARA[1],
Aichi YOSHIDA[2], Shosaku HATTORI[3],
Naoko UEDA[4], Takahito CHIJIWA[4], Motonori OHNO[4]

Abstract

Trimeresurus flavoviridis (Tf) serum proteins were fractionated by ammonium sulfate precipitation to five portions depending on the differences of its saturation percentages, that is, 0-20%, 20-30%, 30-40%, 40-50%, and 50-70%. The effects of these proteins on *Tf* venom-induced rat skeletal muscle damage were investigated with closer attention to histopathological features of impairment, necrosis, and regeneration of muscle fibers. The knowledges which portion of *Tf* serum proteins is effective for prevention of local lesions caused by *Tf* venom should shed light on the effective medical treatment after bitten by *Tf* snake. In consequence, it was found that the necrotic change of the rats inoculated with *Tf* crude venom together with the serum protein fraction of ammonium sulfate saturation percentage 40-50% was the smallest compared to those of the rats tested with other *Tf* serum protein fractions.

Key words: *Trimeresurus flavoviridis* (*Tf*), envenomation, skeletal muscle necrosis (myonecrosis), regeneration, myoblast, *Tf* serum proteins, ammonium sulfate

Introduction

In conformity with recent improvement of the medical treatment of *Trimeresurus flavoviridis* (*Tf*: habu snake) snake bites, the majority of recent victims has been saved from severe generalized symptoms and death. However, local tissue damage is inevitable, and in some severe cases this is responsible for sequelae such as a serious slough in the major extremity and eventually leads to amputation (HOMMA and TU, 1971). In the Amami islands of Kagoshima Prefecture and the several islands of Okinawa Prefecture, this accident accounts for about 90% of *Tf* snake bites. Therefore, it is an important task to prevent the severe and extensive damage of skeletal muscle tissues caused by myotoxins contained in *Tf* venom.

Five phospholipase A_2 (PLA_2) isozymes have been isolated from *Tf* venom (TANAKA *et al.*, 1986; LIU *et al.*, 1990; YOSHIZUMI *et al.*, 1990; KIHARA *et al.*, 1992): [Asp^{49}]PLA_2 called PLA2 (pI 7.9, highly lipolytic to egg-yolk emulsion and myolytic), more basic [Asp^{49}]PLA_2 called PLA-B (pI 8.6, edema-inducing), most basic [Asp^{49}]PLA_2 called PLA-N (pI 10.3, neurotoxic), and two [Lys^{49}]PLA_2s called BPI and BPII (pIs, 10.1 and 10.2, respectively, extremely low lipolytic to egg-yolk emulsion but strongly

[1]Department of Oral Pathology, Kagoshima University Dental School, [2]Department of Biopathology, Kagoshima University School of Health Sciences, Faculty of Medicine, [3]Amami Laboratory of Injurious Animals, Institute of Medical Science, University of Tokyo and [4]Applied Life Science, Faculty of Engineering, Sojo University
*Corresponding Author

myolytic)(LIU *et al.*,1990;KIHARA *et al.*,1992;SHIMOHIGAGASHI *et al.*,1995). BPI and BPII can cleave arachidonate at *sn*-2 position of phospholipids in micellar state and in bilayer membranes with similar activity as PLA2. Thus, most of PLA_2 isozymes can be considered to be myotoxins. The myotoxic action of PLA_2s is not necessarily associated with their catalytic activity toward ordinary micellar substrates such as egg-yolk emulsion. They are able to disrupt the integrity of skeletal muscle plasma membranes and possibly cause an increased permeability to Ca^{2+} and other ions.

Recently, NOBUHISA *et al.* (1997) reported that the proteins with binding affinity to *Tf* venom PLA_2s were fractionated from its serum on four colums, each conjugated with one of four PLA_2 isozymes, PLA2, PLA-B, BPI, and BPII. Five PLA_2 inhibitory proteins, termed PLA_2 inhibitors, PLI-I~PLI-V, were obtained. PLI-IV and PLI-V is mostly bound to PLA2, whereas PLI-I has affinity toward basic PLA_2s, PLA-B, BPI, and BPII.

Purification of PLA_2 inhibitors from *Tf* serum by affinity chromatography is quite laborous and this method is difficult to obtain the inhibitors in a large quantity. For the reasons, *Tf* serum proteins were fractionated by ammonium sulfate precipitation to five portions depending on the differences of its saturation percentages, 0-20%, 20-30%, 30-40%, 40-50%, and 50-70%. The effects of these proteins on *Tf* venom-induced rat skeletal muscle damage were investigated with closer attention to histopathological features of impairment, necrosis, and regeneration of muscle fibers. Such studies have stemmed from our recent detailed studies of the injurious action of *Tf* venom and its components, some PLA_2s, on the rat skeletal muscle fibers (KITANO *et al.*, 2001). The knowlegde which portion of *Tf* serum proteins is effective for prevention of local lesions caused by *Tf* venom should contribute to advancement of the effective medical treatment after bitten by *Tf* snake.

Materials and Methods

Rats

Young adult female and male rats (80~100 g in body weight) of Wistar/Furth strain maintained in Department of Oral Pathology, Kagoshima University Dental School by sister-brother mating (KITANO *et al.*, 1992; KITANO *et al.*, 2001) were divided into seven groups, each group consisting of 6 rats (total; 42) (Table,1).

Crude Tf Venom

Tf venom was collected in Amami Laboratory of Injurious Animals, Institute of Medical Science, University of Tokyo and lyophilized after dilution with a small volume of water. The crude venom were weighed and dissolved in sterile physiological saline immediately prior to use.

Preparation and Fractionation of Tf Serum Proteins

Blood of *Tf* snake was collected at Amami Laboratory of Injurious Animals. Its serum was prepared by removing precipitated erythrocytes and fibrous proteins after keeping the blood at 4℃ overnight and stocked at –80℃. At Faculty of Engineering, Sojo University, ammonium sulfate was added to the serum portionwise at 0℃. At 20% saturation of ammonium sulfate the precipitate was collected by centrifugation. The

supernatant was then brought to 30% ammonium sulfate saturation and the precipitate was collected. The same procedure was repeated. The proteins collected were dissolved in water (or 0.05 M Tris-HCl, pH 8.0), dialyzed against water, and lyophilized. The proteins obtained from 50 ml of *Tf* serum were 0.15g, 0.36g, 0.35g, 1.33g, and 0.55g for 0-20%, 20-30%, 30-40%, 40-50%, and 50-70% ammonium sulfate saturation, respectively. Each serum protein fraction was weighed and dissolved in sterile physiological saline just before use.

Experimental Procedures

The animal experiments were conducted at the Animal Center of Kagoshima University Dental School following "Guidline of the Animal Experiment" of this School. All the rats of seven groups were anesthetized with pentobarbital sodium (Nembutal: Abbot Lab., U.S.A.). A mixture of *Tf* crude venom (50 μg each) and each serum protein fraction (50 μg each) in 100 μl physiological saline were injected into the anterior aspect of the quadriceps femoris muscle (QFM) of each rat of first five groups (Groups 1-5) in the order of increasing ammonium sulfate saturation percentage, 0~20%, 20~30%, 30~40%, 40~50%, and 50~70% (Table 1). The crude venom (50 μg) in 100 μl physiological saline was injected into the rats of the sixth group (Group 6). Sterile physiological saline alone was injected into the rats of the seventh group (Group 7) as a control. The animals were allowed to survive for a period of 48 hours and sacrificed under ethyl ether anesthesia. Both the right and left hindlimbs and visceral organs including the brain were immersed for several days in 10% buffered formalin (pH 7.4), and processed for paraffinic embedding. The sections of 5~6 μm thickness were cut and stained with hematoxylin and eosin.

Pathological findings

The muscle tissue of the rats of Group 7 injected only with physiological saline had a typical histology of skeletal muscle with no abnormality in muscle fibers, nerves, blood vessels, and fibro-adipose layers.

In the rats inoculated with *Tf* crude venom (Group 6), and with a mixture of the crude venom and a serum protein fraction of ammonium sulfate saturation percentages (Groups 1~5), no histopathologically significant differences were detected among the female and male rats of each group, so we preferred to describe here all together (Table 1).

In the rats inoculated with only *Tf* crude venom (Group 6) and with a mixture of the crude venom and the serum protein fraction of ammonium sulfate saturation percentage 0~20% (Group 1), there was a widespread muscle necrosis. The myofibrillar materials in necrotic cells were more amorphous and their distribution within the cellular space was dense. Clamped masses showed liquefaction necrosis (Fig. 1) instead of being more homogeneous looking coagulative necrosis. An abundant inflammatory infiltrate was present outside the necrotic cell areas, mainly at the surrounding tissue layers of the necrotic muscle fibers. Simultaneously, regenerative proliferation of myoblasts, which were characterized by the presence of scanty basophilic cytoplasm, was observed in the periphery of the necrotic cell areas. The regenerating cells were spindle in shape with a central nucleus, some revealing mitotic activities. There was little hemorrhage, although

edema was considerably marked. Fibrosis was not so conspicuous.

The sizes of the necrotic areas became smaller and smaller in accord with the phenomenon that proliferation of small regenerating muscle fibers seemed more and more marked in the Groups 2, 3, 4 in the order of increasing ammonium sulfate saturation percentage, 20~30%, 30~40%, and 40~50% (Figs. 2-4). However, the rats of Group 5 of ammonium sulfate saturation percentage 50~70% showed relatively greater necrotic changes than those of Groups 3 or 4.

Table 1. Histopathologic findings in QFM of the rats when treated with the mixtures of TF venom and its serum protein fractions indicated.

Tf venom + Fraction* of *Tf* serum protein (Group of rats)**	Necrosis of skeketal muscle tissue	Inflamm atory Infiltration	Regeneration of skeletal muscle fibers	Hemorrhage	Edema	Others
0~20%* (Group 1)	++~+++	+~++++	++	-~+	+++	
20~30% (Group 2)	++	+~++	++	-~+	+~++	
30~40% (Group 3)	-~++	+~++	++	-~+	-~++	
40~50% (Group 4)	-~++	+~++	++	-~+	-~+	
50~70% (Group 5)	+~++	+~++	++	-~+	-~++	
Crude venom (Group 6)	+++	++	++	-~+	+++	thrombosis -~+
Saline only (Group 7)	-	-	-	-	-	

* The values of % represent the ammonium sulfate saturation ranges for fractional precipitation of the serum proteins.

** Each group consists of 6 rats (Wistar/Furth strain; F + M).

\# The necrotic changes are composed mainly of liquefaction necrosis but coagulative necrosis are also present.

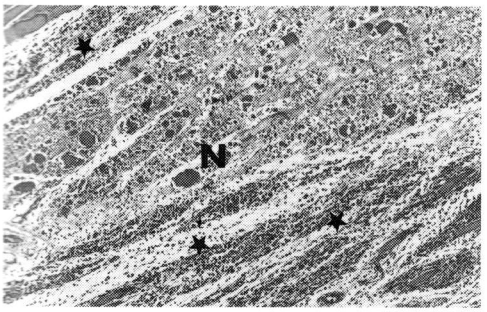

Fig. 1

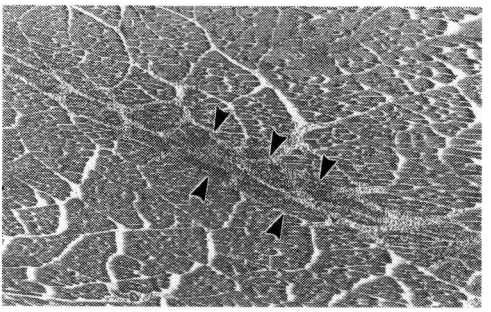

Fig. 2

Fig. 1(left): A photomicrograph of a peripheral area of a large focus of liquefaction necrosis (N) observed in the QFM of a rat of Group 6. The necrosis was induced by *Tf* crude venom, inoculated 48 hours before. The focus is surrounded by a granulation tissue (Asterisks) containing numerous inflammatory cells and regenerating myoblastic spindle cells (HE).

Fig. 2(right): A photomicrograph of a very small necrotic focus (Arrow heads) in the QFM of a rat of Group 4 (HE).

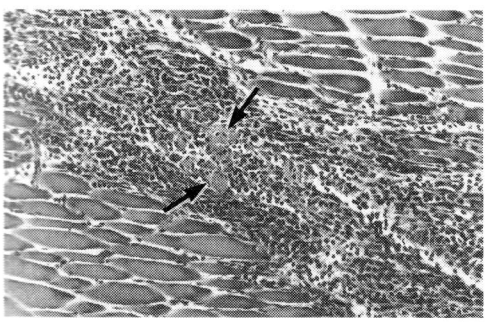

Fig. 3

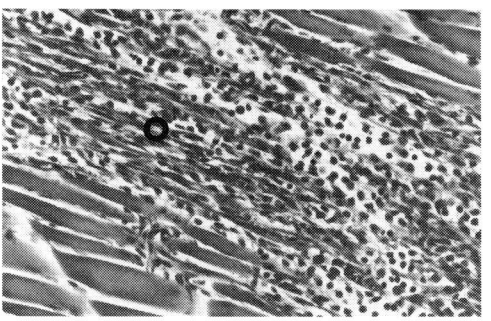

Fig. 4

Fig. 3(left): A larger magnification of Fig. 2. The focus is composed mainly of a numerous inflammatory cells and regenerating myoblastic spindle cells. A couple of necrotic muscle fibers (Arrows) are intermingled with them at the central region of the focus.

Fig. 4(right): A peripheral area of the granulation tissue of Fig. 2. Note the characteristic myoblastic spindle cells (Open circle).

Discussion

Many venomous snakes are resistant to their own venoms. Their natural resistance to their toxins is due to neutralizing factors in their sera. These factors must protect against toxicity due to accidental bites by the snake itself or by other snakes of the same species. Inhibitors of snake venom PLA_2s have been isolated from the sera of various snakes and their primary structures determined. Two kinds of PLA_2 inhibitors against *Tf* venom PLA2, named PLI-A and PLI-B, were purified from its serum (INOUE *et al.*, 1991), and an inhibitor that neutralizes *Agkistrodon blomhoffii siniticus* (mamushi snake) PLA_2 was isoslated from its serum (OHKURA *et al.*, 1993). *Crotalus* PLA_2-neutralizing factor was isolated from *Crotalus durissus terrificus* serum (FORTES-DIAS *et al.*, 1994; PERALES *et al.*, 1995).

NOBUHISA *et al.* (1997) reported the isolation of five PLA2 inhibitory proteins (PLI-I~V) from *Tf* serum in which PLI-IV and PLI-V are the same as PLI-A and PLI-B, respectively. However, the contents of the inhibitors in *Tf* serum seem to be to a minor extent, so that their separation from *Tf* serum is very difficult. Thus, the establishment of the effective method for preparation of the serum fractions containing the inhibitors was required. Our present study has started to fractionate *Tf* serum proteins to several portions by precipitation with increasing concentration of ammonium sulfate.

It is well known that the pathological signs characteristic of *Tf* bites in humans, rabbits, and mice are marked and extensive hemorrhage and muscle necrosis (OKONOGI *et al.*, 1960). Previously we reported that our experimental rats, however, failed to demonstrate the hemorrhage as a predominant sign when *Tf* venom was injected. *Tf* venom did not give rise to any prominent local bleeding in the rats, but produced a massive necrosis in the skeletal muscles accompanied by marked edema (KITANO *et al.*, 2001). Additionally we reported that in 48 hours after the injection of *Tf* venom into the QFM of the rats, a widespread myolytic muscle necrosis was found, which probably resulted from a direct venom action on the muscle fibers. Muscle necrosis by the local inoculation of *Tf* venom is usually followed by rapid phagocytosis of debris at the periphery of the necrotic area. Then the clearing of necrotic materials by phagocytes was followed by a rapid regeneration process. Numerous myoblasts were observed. The excess and marked regeneration activity of myoblasts after muscle necrosis induced by *Tf* myotoxins might be ascribed to the conditions that neither nerves nor blood vessels are markedly damaged by these toxins, since adequate blood supply and innervaton are the essential requirements for muscle regeneration (QUEIROZ *et al.*, 1984; GUITIERREZ *et al.*, 1989). Therefore, for analysis of the effective functions of *Tf* serum inhibitors against the myolytic injuries by *Tf* venom, the rat is considered to be one of the most appropriate and useful experimental animals.

In the present study, the rats of Groups 3 and 4 inoculated by *Tf* venom together with the serum protein fractions of ammonium sulfate saturation percentages 30-40% and 40-50% demonstrated the most dominant changes in the foci of muscle necrosis. The necrotic foci were limited in a narrow area and were surrounded mainly by granulation tissues containing numerous macrophages and regenerative myoblastic cells. Edema was not so marked, either. These facts suggested that the effective myotoxin inhibitory proteins were contained in the serum fractions precipitated in the particular concentration ranges of ammonium sulfate.

Conclusively, the results obtained in the present study suggest that the myotoxin inhibitors in *Tf* serum are effective in healing the myotoxic injuries induced by *Tf* crude venom. Since application of excessive doses and/or repetitive application of anti-*Tf* venom antisera are known to bring about serious medical problems, we certainly expect that application of *Tf* serum inhibitors in appropriate ways could provide a useful therapy for *Tf* snake bites in near future.

Acknowledgements

This work was supported, in parts, by a Fund for Promotion and Development of Amami Islands sponsored by Kagoshima Prefecture and Ministry of Land, Infrastructure and Transport of Japan and by a Kagoshima University Fund for Improvement of Education and Research Activities.

References

FORTES-DIAS, C. L., LIN, Y., EWELL, J., DINIZ, C. R. and LIU, T.-Y. 1994. A phopholipase A2 inhibitor from the plasma of the South American rattlesnake (*Crotalus durissus terrificus*). J. Biol. Chem. 269:15646-15651.

GUTIERREZ, J. M., CHAVES, F., GENE, J. A., LOMONTE, B., CAMACHO, Z. and SCHOSINSKY, K. 1989. Myonecrosis induced in mice by a basic myotoxin isolated from the venom of the snake *Bothorops nummifer* (jumping viper) from Costa Rica. Toxicon, 27: 735-745.

HOMMA, M. and TU, A.T. 1971. Morphology of local tissue damage in experimental snake envenomation. Br. J. Exp. Pathol., 52:538-542.

INOUE, S., KOGAKI, H., IKEDA, K., SAMEJIMA, Y. and OMORI-SATOH, T. 1991. Amino acid sequences of the two subunits of a phopholipase A2 inhibitor from the blood plasma of *Trimeresurus flavoviridis*. J. Biol. Chem., 266:1001-1007.

KIHAREA, H., UCHIKAWA, R., HATTORI, S. and OHNO, M. 1992. Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipases A2. Biochem. Int., 28:895-903.

KITANO, M.,HATANO, H., and SHISA,H.1992. Strain difference of susceptibility to 4-nitroquinoline 1-oxide-induced tongue carcinoma in rats. Jpn. J.Cancer Res.,83:843-850.

KITANO, M., HIRANO, M., YAHATA, M., UMEMURA, E., YOSHIDA, A., HATTORI, S., UEDA, N., TSURU, D. and OHNO, M. 2001. Muscle necrosis and regeneration with lack of marked hemorrhage induced in the rat after envenomation of *Trimeresurus flavoviridis* venom and its components, phopholipase A2 isozymes. South Pacific

Study, 22:31-39.

LIU, S. Y., YOSHIZUMI, K., ODA, N., OHNO, M., TOKUNAGA, F., IWANAGA, S. and KIHARA, H. 1990. Purification and amino acid sequence of basic protein II, a lysine-49-phospholipase A2 with low activity, from *Trimeresurus flavoviridis* venom. J. Biochem. (Tokyo), 107:400-408.

NOBUHISA, I., INAMASU, S., NAKAI, M., TATSUI, A., MINORI, T., OGAWA, T., SHIMOHIGASHI, Y., FUKUMAKI, Y., HATTORI, S. and OHNO, M. 1997. Characterization and evolution of a gene encoding a *Trimeresurus flavoviridis* serum protein that inhibits basic phospholipose A2 isozymes in the snake's venom. Eur. J. Biochem. 249:838-845.

OHKURA, N., INOUE, S., IKEDA, K. and HAYASHI, K. 1993. Isolation and amino acid sequence of a phopholipase A2 inhibitor from the blood plasma of *Agkistrodon blomhoffii siniticus*. J. Biochem. (Tokyo), 113:413-419.

OKONOGI,T., HOSHI, S., HOMMA, M., MITSUHASHI, S., MAENO, H. and SAWAI, Y. 1960. Sudies on the *Habu* snake venom. 3-2. A comparative study of histopathological changes caused by crude venom, purified *Habu*-proteinase and other proteinases. Jpn. J. Microbiol., 4:189-192.

PERALES, J., VILLELA, C., DOMONT, G.B., CHOMET, V., SALIOU, B., MOUSSATCHE, H., BON, C. and FAURE, G. 1995. Molecular structure and mechanism of action of the cotoxin inhibitor from *Crotalus durissus terrificus* serum. Eur. J. Biochem., 227:19-26.

QUEIROZ, L.S., SANTO-NEO, H., RODRIGUES-SIMIONI, L. and PRADO-FRACESCHI, J. 1984, Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom.Toxicon, 22:339-346.

SHIMOHIGASHI, Y., TANI, A., MATSUMOTO, H., NAKASHIMA, K., YAMAGUCHI, Y., ODA, N., TAKANO, Y., KAMIYAMA, H., KISHINO, J., ARITA, H. and OHNO, M. 1995. Lysine-49-phospholipase A2 from *Trimeresurus flavoviridis* venom are membrane-acting enzymes. J.Biochem., 118:1037-1044.

TANAKA, S., MOHRI, N., KIHARA, H., and OHNO, M. 1896. Amino acid sequence of *Trimeresurus flavoviridis* phospholipase A2. J. Biochem. (Tokyo), 99:281-289.

YOSHIZUMI, K., LIU, S-Y., MIYATA, T., SAITA, S., OHNO, M., IWANAGA, S. and KIHARA, H. 1990. Purification and amino acid sequence of basic protein I, a lysine-49-phospholipase A2 with low activity, from the venom of *Trimeresurus flavoviridis* (*habu* snake). Toxicon, 28:43-54.