Prevention of Muscle Necrosis Induced in Rats after Envenomation of *Trimeresurus flavoviridis* (Amami-Oshima Island) Venom and Its Components, Phospholipase A_2 Isozymes

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Abstract

The lesions caused by sublethal doses of *Trimeresurus flavoviridis* (*Tf*) venom injected into the anterior thigh muscle (quadriceps femoris muscle; QFM) of rats were studied using paraffin sections. A dose of 50 μg of *Tf* venom produced a large area of necrosis in the QFM together with the neighboring muscles. Phagocytosis of necrotic remnants was followed by marked regeneration of muscle fibers. Myonecrosis was microscopically evident 30 minutes after injection, and over the following 72 hours the necrotic muscles remained acellular and were devoid of inflammatory reaction, except at the very edge where liquefaction necrosis associated with inflammatory infiltrate was marked from the beginning. Blood vessels within and outside the necrotic foci were occasionally involved in collapse and/or in inflammatory changes with thrombosis, to some degree. However, marked hemorrhage was never noted in and around the foci. Phagocytosis of debris proceeded from the periphery, and after 24 hours the muscle was replaced by granulation tissue containing many regenerating myoblastic cells. Abscesses developed in the vicinity of the injection site in several rats receiving *Tf* venom but never after injection of its phospholipase A_2 isozymes, PLA2, BPI, or BPII. Muscle necrosis after envenomation of *Tf* venom seems to be due primarily to direct action of the venom, although vascular thrombosis and ischemia may also contribute. Interestingly, in rats, myonecrotic *Tf* venom itself may cause marked and extensive activation of muscle fiber regeneration. The venom seems to be a strong myotoxin but not a hemorrhagenic toxin.

Tf serum proteins were fractionated by ammonium sulfate precipitation to six portions depending on its degree of saturability, 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 results indicated that the rats injected with *Tf* crude venom together with the serum protein fraction of ammonium sulfate saturation 40–50% showed necrotic changes were smallest of all in the rats treated together with the other serum protein fractions. The knowledge that *Tf* serum proteins are effective in preventing local lesions caused by *Tf* venom should shed light on effective medical treatment following *Tf* snake bites.

Keywords:

Trimeresurus flavoviridis(*Tf*), Snake venom, Phospholipase A_2 (PLA_2) isozymes, Envenomation, Myotoxin, Myonecrosis, Skeletal muscle, Quadriceps femoris muscle (QFM), Regeneration of muscle fibers, Myoblasts, Hemorrhage, Serum proteins, Ammonium sulfate

Introduction

Envenomation of *Trimeresurus flavoviridis* (*Tf*) snake venom causes two main symptoms in humans, one local and the other systemic. The local symptoms are severe pain, swelling, hemorrhage, and necrosis in the bitten extremity and often result in permanent disability or amputation.[1] In the Amami islands of Kagoshima Prefecture, this accident accounts for more than 90% of snake bites.[2] Consequently, experimental pathological studies of venom of *Tf* of Amami-Oshima island, one of the Amami islands, have been centered on local muscle necrosis and on local hemorrhage.

We have examined the short-term effects of the various components of *Tf* venom on the rat quadriceps femoris muscle (QFM), with closer attention to histopathological changes in the skeletal muscles. Impairment, necrosis, and regeneration of muscle fibers have occurred even several hours after the exposure to the *Tf* venom.[3] However, the rats were strongly resistant to hemorrhage by envenomation of *Tf* venom. Renewed interest in this venom has stemmed from our recent studies[3,4] of its action on the muscle and the vascular wall of the rat and of its fractionated components which may intimately relate to the pathogenesis of local lesions caused by snake bite.

In our recent studies,[3,4] *Tf* serum proteins were fractionated by ammonium sulfate precipitation to five portions depending on its degree of saturability — 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 histopathologically. A knowledge of which portion of *Tf* serum proteins are most effective for prevention of local lesions caused by *Tf* venom should provide a clue for the future medical treatment of those having been bitten by a *Tf* snake. In all the rats treated with the mixtures of *Tf* venom and its serum protein fractions, those that received a mixture of *Tf* venom and its serum protein fraction of ammonium sulfate at 40-50% saturation had the smallest necrotic changes.[4]

Phospholipase A_2 isozymes of *Tf* venom

The main components of *Tf* venom contributing to myonecrosis are phospholipase A_2 (PLA_2) isozymes. Five PLA_2 isozymes have been isolated from *Tf* venom[5,6,7,8]: [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 weakly lipolytic to egg-yolk emulsion but strongly myolytic). BPI and BPII can cleave arachidonate at the *sn*-2 position of phospholipids in the micellar state and in bilayer membranes with activity similar to PLA2.[9] Thus, most 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.

Rats

For histopathological analysis of the susceptibility to tissue injury by *Tf* venom, myonecrosis was induced by envenomation of *Tf* venom in young adult female and male rats (80-100 g) of F344 (Charles River Japan, Inc.,), Dark-Agouti (Shizuoka Laboratory Animal Center), or Wistar/Furth (maintained in our laboratory[10] by sister-brother mating) strains.

Envenomation and muscle preparation for pathological examination

Tf crude venom and its PLA_2 isozymes were weighed and dissolved in sterile physiological saline immediately prior to use. The experimental rats were anesthetized with pentobarbital sodium (Nembutal: Abbot Lab, U.S.A.). The crude venom or one of the three PLA_2 isozymes, [Asp^{49}]PLA_2, BPI, and BPII (50 μg each), in 100 μl physiological saline were injected into the upper two thirds of the right QFM of the rats, which had been divided into four groups. The control rats were similarly injected with 100 μl sterile physiological saline.

The animals were allowed to survive for periods ranging from 30 minutes to 72 hours and were 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). All tissue was embedded in paraffin, sectioned at 5-6 μm and routinely stained for histopathological examination.

Pathological observations in skeletal muscle after envenomation (Table 1)

Macroscopically, there were more or less marked changes of liquefaction necrosis (Fig. 1) with mild to marked edematous swelling of the muscle mass in the venom-injected right hind leg. A close examination of the injected QFM revealed an absence of marked hemorrhage.

Histologically, there were groups of affected cells accompanied by some decrease in fine cytoplasmic structures, which were located in the periphery of the muscle as early as 30 minutes after inoculation. At this time edema without inflammatory cells was observed and many of the skeletal muscle cells showed edema-

Table 1: Histopatholotical data* following *Tf* venom injection into the QFM of rats

Tf venoms	Time following envenomation duration**	Necrosis of skeletal muscle	Inflammatory infiltration	Regeneration of skeletal muscle fibers	Hemorrhage	Edema	Others#
Crude venom	30m	−	−	−	−～+	+	
	6h	−～+	+	−	−～+	+	
	24h	++～+++	+++	+	−～+	++	Thrombosis (+; focal)
	48h	++～+++	+++	++	−～+	++	Thrombosis (+; focal)
	72h	++～+++	++	++	−～+	++	Thrombosis (+; focal)
[Asp[49]] PLA_2	24h	++	++	+	−～+	++	Thrombosis (+; focal)
	48h	++	++	+	−～+	++	
BP I	24h	++	+～++	+	−～+	++	Thrombosis (+; focal)
	28h	++	+～++	++	−～+	+	
BP II	24h	++	+～++	++	−～+	+	
	48h	++	+～++	+++	−～+	+	
	72h	+	+	+++	−～+	+	
Control (physiological saline)	30m	−	−	−	−	−	
	24h	−	−	−	−	−	
	48h	−	−	−	−	−	
Total No of rats***		90 (45 males, 45 females)					

*There were no strain differences in the fundamental histopathological changes between the various types of venom or time post-envenomation. There is no sex difference in the histopathological changes among the various kinds of venom and duration.
**Each group consists of 6 rats (2 Fischer 344 rat, 2 DA rats, and 2 WF rats, one of each sex).
***The total number of experimental rats was 90 (45 males and 45 females).
#There are occasional findings of angiitis with thrombi.

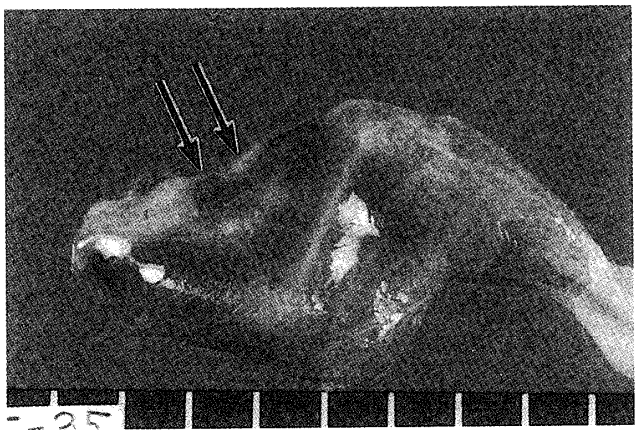

Fig. 1: A large necrotic lesion in the QFM of a F344 male rat 48 hours after the inoculation of the crude venom of *Tf*. Note the severe and extensive atrophy of the skeletal muscles of the anterior thigh (arrows) due to changes of liquefaction necrosis without marked hemorrhage.

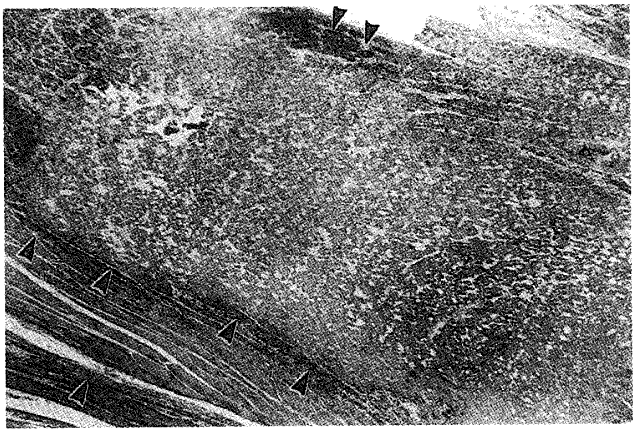

Fig. 2: Massive liquefaction necrosis of the QFM of a F344 male rat 48 hours after the inoculation of the crude venom of *Tf*. Marked inflammatory infiltration is seen in the peripheral layer (arrow heads). Hemorrhage is not noted.

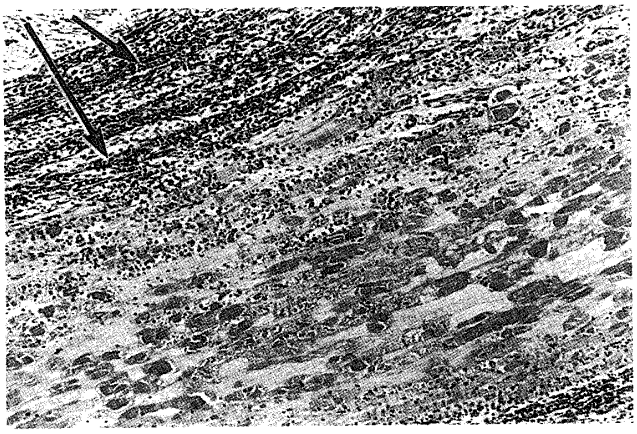

Fig. 3: Necrotic mass is surrounded by a granulation tissue (arrows) with marked inflammatory infiltration. Note numerous spindle-shaped myoblastic cells in the granulation tissue. The QFM of a F344 female rat 48 hours after the inoculation of the crude venom of *Tf*.

tous swelling. Six hours after injection, necrosis of muscle fibers in the QFM was indicated by nuclear pyknosis and fragmentation of myofibrils into homogenous eosinophilic masses separated by empty-looking segments. There were many areas where necrotic cells were predominant. Some of them appeared in the initial stage of cell degeneration, i.e. some muscle cells had only edematous swelling, whereas other cells were in the more advanced stage of damage, with formation of dense clumps of myofibrils alternating with cellular spaces apparently devoid of myofibrillar material. The findings support the view that the earliest alterations seem to take place in the periphery of the necrotic focus. A very mild inflammatory infiltrate was observed at this time.

After an elapse of 24 hours there was widespread myonecrosis; the myofibrillar material in necrotic cells was more amorphous and its distribution within the cellular space was more homogeneous, instead of forming dense and clumped masses charactristic to the features of liquefaction necrosis (Fig. 2). A marked and dense inflammatory infiltrate was present outside the necrotic cell nests.

Forty-eight hours after toxin inoculation, there was an abundant inflammatory infiltrate mainly in the surrounding layers of necrosis (Fig. 3). Even after 72 hours, the QFM still showed an extensive area of coagulative necrosis, but in some rats the central mass of necrotic fibers was surrounded by active-looking granulation tissue with neutrophils, macrophages, and fibroblasts. Very little fibrosis was observed.

Simultaneously, regenerative proliferation of myoblasts, which are characterized by the presence of myofibrils and rows of centrally-located nuclei, was observed in the periphery of necrotic cells. Proliferation of small regenerating muscle fibers was most marked in the rats treated with BPII and was seen to a lesser amount in those treated with BPI, the crude venom, and PLA2. Twenty-four hours after toxin inoculation, regenerating cells were spindle shaped with scant

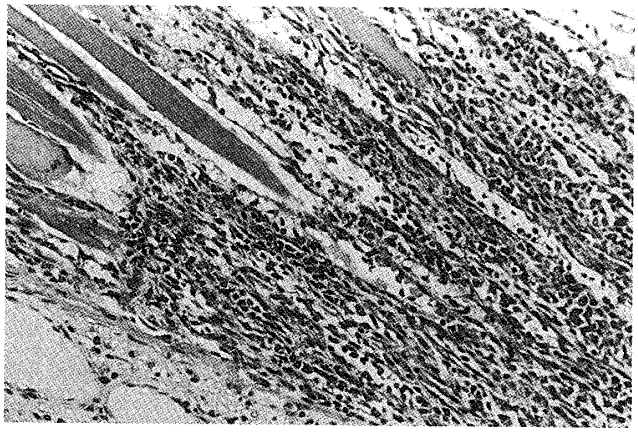

Fig. 4: A granulation tissue in the periphery of a necrotic focus in a DA male rat 48 hours after inoculation of the BPI. Many spindle-shaped myoblastic cells have sprouted from the viable skeletal muscle fibers around a necrotic mass.

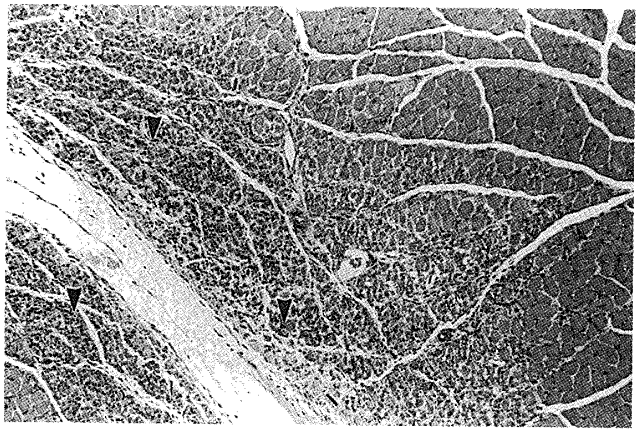

Fig. 5: Numerous myoblastic cells around the necrotic focus in a F344 male rat 72 hours after inoculation of the BPII. Myoblastic cells are hypertrophied and have a round nucleus in their central cytoplasm (arrow heads). Inflammatory infiltration becomes mild.

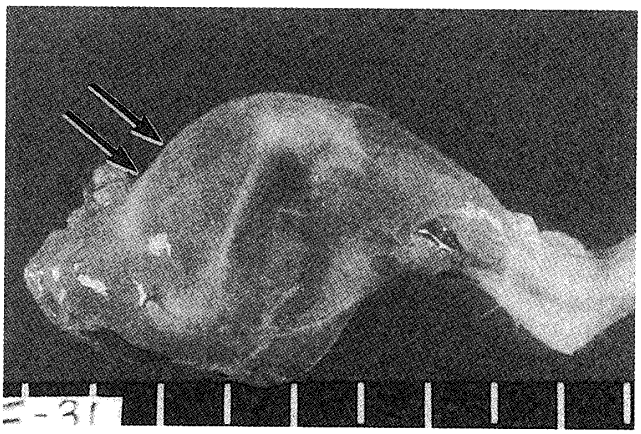

Fig. 6: Macroscopical findings of the QFM of a WF male rat 48 hours after the inoculation of a mixture of the crude venom of *Tf.* and its serum protein of ammonium sulfate saturation percentage, 40-50%. Slight edematous swelling of the anterior thigh (arrows) without a hemorrhage and there is neither marked necrotic change nor atrophy of the skeletal thigh muscles.

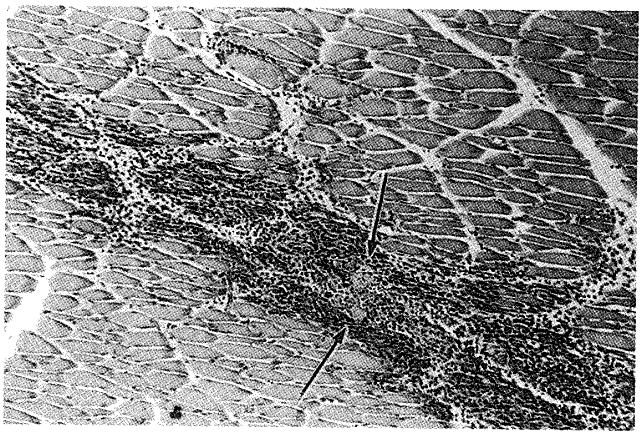

Fig. 7: A photomicrograph of a very small necrotic focus (arrows) in the QFM of the same rat as shown in Fig. 6.

basophilic cytoplasm and a central nucleus. Some of these cells revealed mitotic activity after 48 hours (Fig 4). Muscle regeneration was observed in the peripheral fibers of necrotic muscles, while the inner parts of those muscles were still necrotic. The regenerating cells gradually became hypertrophic with a light acidophilic cytoplasm after 72 hours (Fig. 5).

Histological examination of muscle tissue 8 weeks after the onset of muscle necrosis revealed the presence of abundant regenerated muscle cells with centrally-located nuclei and a diameter similar to that of normal muscle cells, indicating that regeneration had taken place successfully (data, not shown).

There was little hemorrhage through the whole duration of 8 weeks. Most blood vessels looked normal, but in some animals inflammatory infiltrate was noted in the vascular walls and platelet thrombi were found in some medium-sized blood vessels. There were several peripheral nerves showing marked edema in the endoneurium, but without apparent structural disintegration.

Muscle necrosis followed by muscle regeneration

Thirty minutes after injection of *Tf* venom the

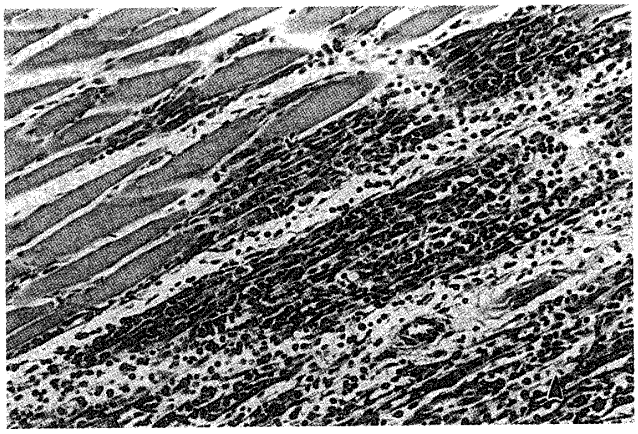

Fig. 8: A higher magnification of Fig.7. The field is composed mainly of numerous inflammatory cells and regenerating myoblastic spindle cells.

initial change of muscle fiber necrosis was widespread in the QFM, where most blood vessels looked normal and contained no thrombi. These findings suggest that necrosis is an early event, which probably results from a direct venom action on muscle fibers. However, 24 hours later the venom caused collapse of blood vessels with or without vasculitis and thrombosis. The resulting ischemia may have contributed to the genesis of the myonecrosis, as seen in animals receiving the various types of snake toxins, i.e., the crude venom, BPI, BPII, and PLA2.[6] The absence of inflammatory cells and unphagocytosed necrotic muscle fibers suggest that there was no blood flow through the central necrotic area. A delay in phagocytosis was also observed after inoculation of the venoms from other snakes. The collapse of blood vessels was also considered important in the pathogenicity.

On the other hand, muscle necrosis induced by local inoculation of the various kinds of snake venoms is usually followed by rapid phagocytosis of debris at the periphery of the necrotic area. The clearing of necrotic materials by phagocytes was followed by a rapid regeneration process. Myoblasts were observed 24 hours after the onset of myonecrosis, and by 8 weeks regeneration was complete (data not shown). It was common to find many regenerative cells of a size similar to that of adult muscle fibers but with centrally-located nuclei. The success of regeneration after myonecrosis induced by *Tf* myotoxins might be due to the fact that neither nerves nor blood vessels are markedly affected by these toxins, since adequate blood supply and innervation are essential requirements for muscle regeneration.[11, 12]

Tf venom and hemorrhage

The most striking effects produced by parenteral injection into animals of many snake venoms including *Tf* venom are local and consist of marked hemorrhage, myonecrosis, and edema.[13, 14, 15] It is well known that the pathological signs characteristic of *Tf* bite in humans, rabbits, and mice are extensive hemorrhage and necrosis.[14] Our experimental rats, however, failed to demonstrate hemorrhage as a predominant sign of snake venom injection. We used three strains of rats and four kinds of preparations derived from *Tf* venom for our experiments. In spite of the different strains of rats and the kinds of venom preparations, we could not observe hemorrhage in and around the injection area.

It is of great interest whether or not the same factor of this venom is responsible for necrosis and hemorrhage. However, little is known about this point.[3] Many researchers stated that hemorrhage is due, at least in part, to the action of myonecrotic enzymes such as PL A_2s.[15] However, in terms of local hemorrhage, our results did not agree with the general view that snake venoms produce bleeding at the injection site. Pathological observations indicated that the easily discernible local changes produced in humans, rabbits, and mice by *Tf* bite injury were related to hemorrhage. In the present experiment, *Tf* venom did not give rise to any prominent bleeding in rats even locally, but produced myolysis accompanied by edema.

Some investigators have suggested that proteolytic enzymes are the factors in hemorrhage and necrosis.[13,14] We observed, however, that the degrees of hemorrhage and necrosis were not always in parallel with proteolytic activities. Furthermore, there is a report that crystalline trypsin produces local hemorrhage but almost no myonecrosis.[1] This seems to indicate that both changes depend not only on proteolytic enzymes but also on other certain enzymes or factors.

A few reports have described the occurrence of vascular lesions in a poisonous snake bite.[16,17,18] It was confirmed that *crotalinae* and *viperinae* venoms containing both myonecrogenic and hemorrhagenic

activities inflicted damage on the arteries and *elapidae* venoms devoid of hemorrhagic activity fail to cause significant changes inarterial walls. The important changes responsible for developing arterial lesions appear to be injury of the endothelium and disintegration of the media. It has been reported that *Tf* venom attacks endothelium and smooth muscle of the media when the venom is injected close to the arteries. This suggests that both hemorrhagenic and myonecrogenic factors play an important role in the involvement of local blood vessels.[17] Thrombi were observed in local arteries at the sites injected with several kinds of venoms. In our experiments the frequency of thrombus formation was not always in parallel with the severity of arterial lesions and of local hemorrhage and swellings. Important factors causing thrombosis are slowing or eddying of the blood stream, injury to the lining of a blood vessel, and alterations of the blood. If a longer period of time had been allowed to elapse between injection and autopsy, there would probably have been more thrombus formation, as *viperinae* and *crotalinae* venoms produce the conditions bringing about this kind of lesion.[1]

Serum proteins acting as inhibitors against *Tf* venom

In conformity with improvement of the medical treatment of *Tf* snake bite, the majority of recent victims have been saved from severe generalized symptoms and death. However, local tissue damage is inevitable, and in some severe cases this is responsible for subsequent serious slough in the major extremity and eventually leads to amputation. Therefore, it is still an important task to prevent the severe and extensive damage of skeletal muscle tissues caused by myotoxins in *Tf* venom.

Recently, the proteins with binding affinity to *Tf* venom PLA_2s were fractionated from the serum of *Tf* itself on four columns, each conjugated with one of four PLA_2 isozymes, PLA2, PLA-B, BPI, and BPII.[19] 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 mostly toward basic PLA_2s such as PLA-B, BPI, and BPII.

Purification of PLA_2 inhibitors from *Tf* serum by affinity chromatography is quite laborious and it is difficult to obtain the inhibitors in large quantities using this method. For these reasons, *Tf* serum proteins were fractionated into six portions, using ammonium sulfate precipitation. The fractions were based upon degree of saturability — 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 skeletal muscle fibers.[3]

Fractionation of *Tf* serum proteins

Blood of *Tf* was collected and its serum was prepared by removing precipitated erythrocyte and fibrous proteins after keeping the blood at 4℃ overnight and stored at -80℃. Ammonium sulfate was added to the serum portion 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 0.05M 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.[19] Each serum protein fraction was weighed and dissolved in sterile physiological saline just before use.

Injection of *Tf* venom together with its serum proteins into rat skeletal muscle

The rats were anesthetized with pentobarbital sodium (Nembutal: Abbot Lab, U.S.A.). A mixture of *Tf* crude venom (50 ㎍ each) and each serum protein fraction (50 ㎍ each) in 100 ㎕ physiological saline was injected into the anterior aspect of the QFM of each rat of the first five groups (Group 1-5) in the order of increasing degrees of ammonium sulfate saturation — 0～20%, 20～30%, 30～40%, 40～50%, and 50～70% (Table 2). The crude venom (50 ㎍) alone in 100 ㎕ physiological saline was injected into the rats of the

Table 2 Histopathological findings in QFM of rats treated with mixtures of *Tf* venom and its serum protein fractions

Tf venom + Fraction* of *Tf* serum protein (Group of rats)**	Necrosis# of skeletal muscle tissue	Inflammatory 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 percentage values represent the ammonium sulfate saturation ranges for fractional precipitation of the serum proteins.
**Each group consits of 6 rats (Wistar/Furth strain; 3 females + 3 males).
#The necrotic changes are composed mainly of liquefaction necrosis. Coagulative necrosis is focally noted.

sixth group (Group 6). Sterile physiological saline 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. Hindlimb muscles for pathological observations were prepared as described in Table 2.

Effects of *Tf* serum proteins on rat skeletal muscle necrosis induced by *Tf* venom

In the rats inoculated with *Tf* venom (Group 6) and with a mixture of *Tf* venom and its serum protein fraction of ammonium sulfate saturation percentage 0～20% (Group 1), there was a widespread myonecrosis. The myofibrillar material in necrotic cells was more amorphous and distributed within the cellular space as dense and clumped masses which show liquefaction necrosis instead of being more homogeneous looking coagulative necrosis. An abundant inflammatory infiltrate was seen outside the necrotic cell areas, mainly at the surrounding tissue layers of the necrotic muscle cells. Simultaneously, regenerative proliferation of myoblasts, which are characterized by the presence of scanty basophilic cytoplasm, was observed in the periphery of the necrotic cell areas. The regenerating cells were spindle-shaped with a central nucleus, with some revealing mitotic activity. There was little hemorrhaging, although edema was considerably marked. Fibrosis was not conspicuous.

The size of the necrotic areas became smaller and smaller in Groups 2, 3, and 4 in the order of increasing ammonium sulfate saturation — 20～30%, 30～40%, and 40～50% — which agreed well with the fact that proliferation of small regenerating muscle fibers seemed more and more marked (Fig. 4). However, the rats of Group 5 (ammonium sulfate saturation 50～70%) showed relatively greater necrotic changes than those of Groups 3 or 4.

Significance of serum proteins for treatment of snake bite

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 a venomous snake itself or by fellow snakes. Inhibitors against snake venom PLA_2s have been isolated from the sera of various snakes and their primary structures determined. A PLA_2 inhibitor (PLI) that neutralizes *Agkistrodon blomhoffii siniticus* (mamushi snake) PLA_2 was isolated from its serum.[21] *Crotalus* PLA_2-neutralizing factor was isolated from *Crotalus durissus terrificus* serum.[22,23] As mentioned above, five PLIs (PLI-I-V) were isolated from *Tf* serum.[19] PLI-IV and PLI-V are identical to PLI-A and PLI-B purified by Inoue et al.[20] However, purification of these inhibitors from snake sera is laborious and the yields are low. Thus, an effective method for the preparation of a massive volume of inhibitors is necessary. The ammonium sulfate precipitation procedure was thus employed in the present study to obtain the effective fraction in a large quantity.

The present study demonstrated that the rats of Groups 3 and 4 inoculated with the mixtures of *Tf* venom and its serum protein fractions of ammonium sulfate saturation 30-40% and 40-50% had much smaller necrotic foci in muscle as compared with the rats which were treated with *Tf* venom alone and with the mixtures of *Tf* venom and its serum protein fractions of ammonium sulfate saturation 0-20 and 20-30%. The foci for Groups 3 and 4 were surrounded mainly by granulation tissue containing numerous macrophages and regenerative myoblastic cells. Edema was also not so marked. These facts suggested that inhibitory proteins against myotoxins were contained at high concentration in the *Tf* serum protein fractions precipitated by ammonium sulfate saturation 30-40% and 40-50%.[4]

The results obtained in the present work suggest that the inhibitors in *Tf* serum against its venom myotoxins were effective in improving the myotoxic injury caused by *Tf* bite. Since application of excessive doses and/or repetitive application of anti-*Tf* venom antisera induce serious medical problems, it is expected that application of *Tf* serum inhibitors in an appropriate way could provide a useful method of therapy for snake bite in the near future.

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