NECROSIS AND REGENERATION OF THE SKELETAL MUSCLE WITHOUT A MARKED HEMORRHAGE INDUCED IN THE RAT BY ENVENOMATION OF *TRIMERESURUS FLAVOVIRIDIS* VENOM AND ITS COMPONENTS, PHOSPHOLIPASE A_2 ISOZYMES

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Abstract

A histopathological study of the lesions caused by sublethal doses of *Trimeresurus flavoviridis* (*Tf*) venom injected into the anterior thigh muscle (quadriceps femoris muscle; QFM) of rats was done. A dose of 50 μg of *Tf* venom (crude venom, and three kinds of the phospholipase A_2 isozymes of *Tf* venom; [Asp^{49}]PLA_2, BPI and BPII) produced a large area of necrosis in QFM. Myonecrosis was evident 30 minutes after the injection, and over the following 72 hours the necrotic muscles remained acellular and devoid of inflammatory reaction except at the very edge where liquefaction necrosis associated with inflammatory infiltrate was marked from the beginning. Blood vessels in and around the necrotic foci were often involved in inflammatory changes and thrombosis, to some degree. However, marked hemorrhage was never noted. Phagocytosis of debris proceeded from the periphery, and after 24 hours the muscle was replaced by granulaion tissue containing many regenerating myoblastic cells. Interestingly, the myonecrogenic *Tf* venom itself may cause marked and extensive activation of muscle fiber regeneration, and the venom seems to be a great myotoxin but not a hemorrhagenic toxin for the rat.

Key words: *Trimeresurus flavoviridis*(*Tf*), Envenomation, Myotoxin, Skeletal muscle, Quadriceps femoris muscle (QFM), Myonecrosis, Regeneration, Myoblast, PhospholipasesA_2 isozymes, Hemorrhage

Introduction

Envenomation of *Trimeresurus flavoviridis* (*Tf*) snake venom causes two main symptoms, one local and the other systemic in humans. The local symptoms are severe pain, swelling, hemorrhage and necrosis in compliance with the bitten extremity, result in permanent disability or amputation (HOMMA and TU, 1971). In the Amami islands of Kagoshima Prefecture, this accident accounts for about 90% of snake bites. Subsequently, experimental pathological studies with the venoms of the snake have centered on local muscle necrosis and on local hemorrhage (HOMMA and TU, 1971).

We have examined the short term effects of the various components of *Tf* venom on rat skeletal muscle, quadriceps femoris muscle (QFM), with closer attention to impairment, necrosis and regeneration of muscle fibers, the latter occurring even at the earlier stages. Moreover, the rats have shown a strong resistance to hemorrhage by envenomation of *Tf* venom. Renewed interest in this venom has stemmed from our recent studies of its action on muscle and vascular wall of the rat and from its fractionation of the venom, which may shed light on the pathogenesis of local

lesions caused by the snake bites.

This article is written based upon an oral presentation to Symposium I 'Habu (*Trimeresurus flavoviridis*) in Amami Islands' at the 26th Kyushu Regional Meeting of the Japanese Society of Tropical Medicine, held on January 26, 2002, in Kagoshima. Interested readers may refer to the original publication for detailed data (KITANO et al., 2001).

Materials and Methods

Venom and its phospholipase A_2 isozymes

Tf venom was collected in Amami-Oshima island and lyophilized. Its phospholipase A_2 (PLA_2) isozymes, [Asp^{49}]PLA_2, BPI and BPII, the latter two being [Lys^{49}]PLA_2s, were separated by conventional chromatographies at Sojo University (LIU et al., 1990; KIHARA et al., 1992). The crude venom and PLA_2 isozymes were weighed and dissolved in sterile physiological saline just before use.

Experimental design

Young adult female and male rats of F344, Dark-Agouti, Wistar/Furth strains were divided into two to five groups, each group consisting of 6 rats (total; 90) (Table 1).

The rats were anesthetized with pentobarbital sodium (Nembutal). The crude venom and three PLA_2 isozymes (50μg each) in 100μl physiological saline were injected into each rat of four groups at the upper two-thirds of the right QFM. The fifth group, the control rats, were similarly injected with 100μl of only physiological saline.

The animals were allowed to survive for the periods ranging from 30 minutes to 72 hours and were sacrificed under ethyl ether anesthesia. Both the right and left hindlimbs, the visceral organs and 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 stained with hematoxylin and eosin for histopathological examination.

Histopathological Findings

The muscle tissue injected with only physiologic saline had a typical histology of skeletal muscle with no abnormality in muscle fibers, nerves or blood vesseles.

In the rats inoculated with toxins, no significant differences were detected among the four kinds of toxins, among three strains of rats and among female and male rats, so we preferred to describe here all together.

There was a more or less marked sign of liquefaction necrosis with a depressive or atrophic features of the muscle masses of QFM in the venom-injected right hind leg. A close examination of the injected QFM revealed an absence of marked hemorrhage except for a few rats (Fig. 1).

As early as 30 minutes after the inoculation of *Tf*-venom, there were groups of affected cells accompanied by the decrease of fine cytoplasmic structures to some degree, which were located in the periphery of the muscle. At this time edema without inflammatory cells were observed, and many of the skeletal muscle cells presented edematous swelling.

Six hours after injection, the necrosis of muscle fibers in QFM was indicated by nuclear pyknosis and fragmentation of the myofibrils into homogenous eosinophilic masses separated by empty-looking segments. The earliest alterations seem to take place in the periphery of the necrotic focus where a very mild inflammatory infiltrate was observed at this time.

After the elapse of 24～48 hours there was widespread myonecrosis (Fig. 2); the myofibrillar

Table 1. Experimental rats with snake venom injection into the QFM.

Venom of snake (*Tf*)	Durat-ion	N of rat*	Skeletal muscle fibers and endomysium					Perimysium, epimysium and surrounding connective layers				
			Necrosis	Inflam-matory infiltr-ation	Hemo-rrhage	Edema	Muscular re-generat-ion	Inflam-matory infiltr-ation	Changes in the vascular walls**	Hemo-rrhage	Edema	Others#
Crude venom	30m	6	-	-	-~+	+	-	-	-	-	+	-
	6h	6	-~+	+	-~+	+	-	-~+	-	-	+	-
	24h	6	++~+++	+++	-~+	++	+	++	-~+ (Th)	-~+	++	-
	48h	6	++~+++	+++	-~+	++	++	++	-~+ (Th)	-~+	++	+
	72h	6	++~+++	++	-~+	++	++	++	-~+ (Th)	-~+	++	+
	Total	30										
[Asp49] PLA_2	24h	6	++	++	-~+	++	+	++	-~+ (Th)	-	++	-
	48h	6	++	++	-~+	++	+	++	-	-	++	-
	Total	12										
BPI	24h	6	++	+~++	-~+	++	+	+	-~+ (Th)	-	++	-
	48h	6	++	+~++	-~+	+	++	+	-~+	-	+	-
	Total	12										
BPII	24h	6	++	+~++	-~+	+	++	+	-~+	-	++	-
	48h	6	++	+~++	-~+	+	+++	+	+	-	++	-
	72h	6	+	+	-~+	+	+++	+	-	-	++	-
	Total	18										
Control (physio-logical saline)	30m	6	-	-	-	-	-	-	-	-	-	-
	24h	6	-	-	-	-	-	-	-	-	-	-
	48h	6	-	-	-	-	-	-	-	-	-	-
	Total	18										
Rat, total N		90										

*One group consists of male and female rats of three strains (F344, Dark-Agouti, Wistar/Furth). However, there is neither strain nor sex differences in the essential histopathological changes among nenom kinds and duration.

**Inflammatory changes are noted in the medium-sized vascular walls. Th means 'thrombosis'.

Necrotic changes are noted in the fatty tissue around the fascia.

material in necrotic cells was more amorphous and its distribution within the cellular space was more homogeneous, instead of being dense and clumped masses (Fig. 3). A marked and dense inflammatory infiltrate was present outside the necrotic cell nests. There was little hemorrhage at this or later times, although edema was considerably marked. Simultaneously, regenerative proliferation of myoblasts. The regenerating cells were spindle in shape with scanty basophilic cytoplasm and a central nucleus, some revealing mitotic activities at 48 hours (Fig. 4). Muscle regeneration was observed in the peripheral fibers of necrotic muscles, but the inner parts of those muscles appeared still necrotic. The regenerating cells gradually became hypertrophic with an acidophilic light cytoplasm at 72 hours. The muscle tissue examined histologically 8 weeks after the onset of muscle necrosis was characterized by the presence of abundant regenerated muscle cells with centrally-located nuclei and a diameter similar to that of normal muscle cells, indicating that regeneration took place successfully (data, not shown).

There was little hemorrhage through the whole duration. 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 veins and arteries, occasionally forming occlusive masses. There were several peripheral nerves showing marked edema of the endoneurium, but no apparent structural disintegration was found.

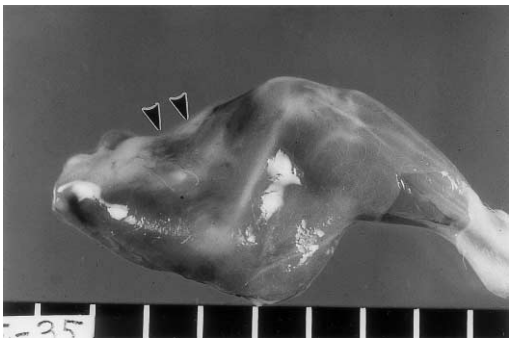

Fig.1. A large necrotic lesion in the QFM of a F344 male rat 48 hours after the inoculation of the crude venom of *Tf*. Note the severe and extensive atrophy of the skeletal muscles of the anterior thigh due to liquefaction necrosis (arrow heads). Slight focal hemorrhage is noted in the necrotic area.

Fig.2. Massive necrosis of the QFM of a F344 male rat 48 hours after the inoculation of the crude venom of *Tf*. Marked reactive changes (Re) are seen in the peripheral areas of the necrotic layer, most of which is composed of exudated inflammatory cells and regenerated myoblastic cells. There is no detectable hemorrhage in this case.

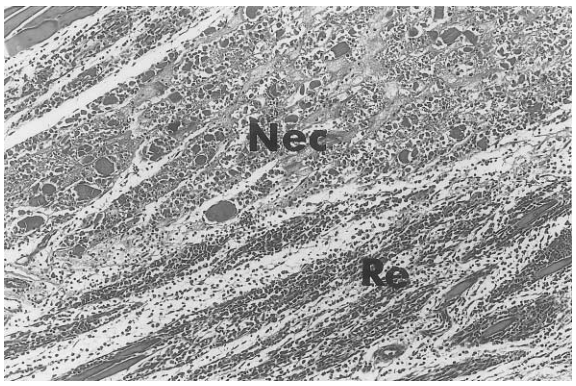

Fig.3. Massive and extensive liquefaction necrosis of the skeletal musle fibers of QFM of a F344 male rat 48 hours after the inoculation of the crude venom of *Tf*.

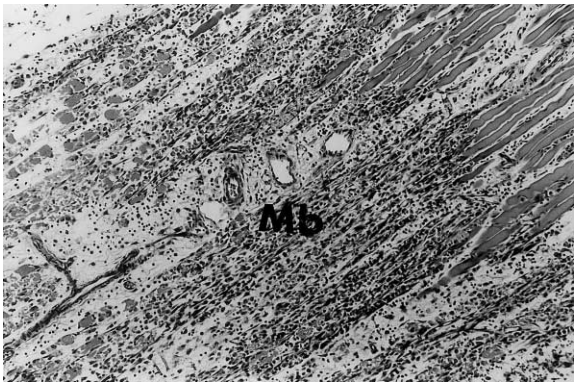

Fig.4. A reactive tissue in the periphery of a necrotic focus in a female F344 rat 48 hours after inoculation of the crude venom. Many myoblastic cells (Mb) are sprouted from the viable skeletal muscle fibers. Mitotic figures are noted in the regenerating cells, some of which have two nucleoli.

Discussion

Thirty minutes after the injection of *Tf* venom, initial changes of muscle fiber necrosis was widespread in QFM, where most blood vessels looked normal and contained no thrombi. These findings show that necrosis is an early event, which probably results from a direct action of the venom on the muscle fibers.

On the other hand, muscle necrosis by the local inoculation of various kinds of snake venoms is usually followed by the rapid phagocytosis of debris at the periphery of the necrotic area. The clearing of necrotic material 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. The success of regeneration after myonecrosis induced by *Tf* myotoxin might be due to the fact that neither nerves nor blood vessels are markedly affected by this toxin, since adequate blood supply and innervaton are essentially requirements for the muscle regeneration (QUEIROZ et al., 1984; GUITIERREZ et al., 1989).

The most striking effects produced by the injection of many snake venoms, including venom

of *Tf*, are local, consisting of marked hemorrhage, myonecrosis, and edema in animals (OHSAKA et al., 1960; OKONOGI et al., 1960; LOMONTE et al., 1994). It is well known that the pathological signs characteristic of *Tf* bite in humans, rabbits and mice are marked by extensive hemorrhage and necrosis (OKONOGI et al., 1960). Our experimented rats, however, failed to demonstrate that hemorrhage was a predominant sign of snake venom injection. We used three strains of rats and four kinds of preparations of *Tf* venom for our experiments. In spite of the difference of strains of rats and kinds of venom-preparations, we could not note marked hemorrhage in and around the injection area, although a few rats showed focal slight hemorrhage in the necrotic areas.

It is of great interest whether or not the same principle is responsible for necrosis and hemorrhage. Many researchers stated that hemorrhage is due, at least in part, to the action of myonecrotic enzymes, such as phospholipase A_2 (LOMONTE et al., 1994). Concerning local hemorrhage, our results did not agree with the general view that snake venoms produce bleeding at the site of injection. Pathological observations indicated that the easily discernible local changes produced in the humans, rabbits and mice by *Tf* bite injury were hemorrhage. However, in the present experiment, *Tf* venom did not give rise to any prominent bleeding locally, but produced myolysis with edema, in the rats.

Some investigators have suggested that proteolytic enzymes are factors in hemorrhage and necrosis (OHSAKA et al., 1960; OKONOGI et al., 1960). Our observations concerning the degree of hemorrhage and necrosis were not always in parallel with proteolytic activities. Furthermore, there is a report that a crystalline trypsin produces local hemorrhage and almost no myonecrosis (HOMMA and TU, 1971). This seems to indicate that both changes depend not only on proteolytic enzymes, but also on other enzymes or factors.

A few reports have described the occurrence of vascular lesions in a poisonous snake bite (MANDELBAUM et al., 1989; MATSUI et al., 2000; ESTEVAO-COSTA et al., 2000). It was confirmed that the venoms containing both myonecrogenetic and hemorrhagic activities, *crotalinae* and *viperinae* venoms, inflicted damage on the arteries and that the venoms devoid of hemorrhagic activity, *elapidae* venoms, fail to cause significant changes in the arterial walls. The important changes responsible for developing arterial lesions appear to be the injury of the endothelium and the disintegration of the media. It has been reported that *Tf* venom attacks the endothelium and smooth muscles of the media when the venom is injected close to the arteries. This suggests that both hemorrhagic and myonecrogenetic factors play an important role in the involvement of local blood vessels (MATSUI et al., 2000).

In conclusion, the *Tf* venom seems to be a great myotoxin but not a hemorrhagenic toxin for the rat. Simultaneously the myonecrogenic *Tf* venom itself may cause marked and extensive activation of muscle fiber regeneration.

Acknowledgements

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

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