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Insights of local tissue damage and regeneration induced by BnSP-7, a myotoxin isolated from *Bothrops (neuwiedi) pauloensis* snake venom

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ABSTRACT

Envenomations caused by *Bothrops* snake venoms are characterized by prominent local tissue damage due to myonecrosis, hemorrhage, edema and acute muscle damage which is widely correlated with phospholipases A_2 (PLA_2). In the present study, the progression of local tissue damage and inflammation induced by BnSP-7, a myotoxin isolated from *Bothrops (neuwiedi) pauloensis* snake venom, was evaluated. Local tissue damages characterized by edema, necrosis and inflammation were evaluated until 24 h after inoculation of BnSP-7. The regeneration of myofibers, analyzed by light microscopy, was observed from 72 h to 2 weeks post-inoculation of toxin. MMP-2 was expressed in gastrocnemius muscle at all time points tested, while the expression of MMP-9 increased expressively at the same time interval of regenerating muscle, suggesting the involvement of MMP-9 in the regeneration process. The production of pro-inflammatory cytokines was also increased, whereas IL-1β showed the highest level. Modification of BnSP-7 with BPB decreased the release of IL-8, IL-6 and IL-1β when compared to native BnSP-7. These data suggest that BnSP-7 acts as pro-inflammatory incentives (mediators), inducing MMP and cytokine production from the inflammatory and satellite cells, and thus it may play an important role in inflammatory process and, consequently, in the evolution of local tissue damage and regeneration.

1. Introduction

Local tissue damage induced by the envenoming by *Bothrops* snakes is characterized by hemorrhage, edema, inflammation and myonecrosis (Nishioka and Silvera, 1992). Muscle necrosis can occur due to direct action of myotoxic phospholipases A_2 on plasma membranes of muscle cells. Beyond those effects, regeneration was also verified after treatment of mice muscles with myotoxins (Harris, 2003; Vignaud et al., 2005).

The inflammatory response that settles down soon after envenoming is also of relevance for progress of tissue damage, considering that besides snake venom toxins on site of bite, there is also the participation of endogenous mediators which can contribute with these alterations. Activated leukocytes release a broad spectrum of cytokines such as IL-1, IL-6 and IL-8, which amplify the inflammatory response contributing to necrosis process (Voronov et al., 1999).

The matrix metalloproteinases (MMPs) are also important mediators of tissue damage and inflammation of

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a variety of pathologies (Shapiro, 1998; Nagase and Woessner, 1999), thus they could have an important role in local alterations induced by the venom (Kherif et al., 1999; Rucavado et al., 2002).

Several studies showed that MMP-2 and MMP-9 are increased in a variety of pathologies, as myonecrosis (Kherif et al., 1999), chronic inflammation (Trengove et al., 1999), meningitis (Leib et al., 2000), hemorrhage and cerebral ischemia (Rosenberg and Navratil, 1997; Gasche et al., 1999), among others. Rucavado et al. (2002) reported an increase of MMP-9 in gastrocnemius muscle induced by the toxins BaP1 and MT-III, isolated from *Bothrops asper* venom, suggesting a role not in induction of injury but in inflammatory response and regeneration.

BnSP-7 PLA_2 (Rodrigues et al., 1998; Soares et al., 2000) is a catalytically inactive protein with molecular mass around 13.5–14 kDa and its structure is essentially identical to the structure of other myotoxic Lys49 PLA_2s. It presents an amino acid content composed by many basic and hydrophobic residues, where a serine is the N-terminal amino acid and Gly30, Gly33, His48, Lys49, Asp99 residues and the 115–129 regions are conserved. This toxin has an isoeletric point of 8.8 and can induce necrosis of mice muscle fibers (Rodrigues et al., 1998; Soares et al., 2000; Magro et al., 2003).

BnSP-7 was first isolated and characterized from *Bothrops (neuwiedi) pauloensis* by Soares et al. (2000). This snake was described by Amaral (1925) as one out of 12 subspecies of *B. neuwiedi* complex. Following the systematic revision of this complex by Silva (2004), the 12 subspecies resulted in seven distinct species, where *B. (neuwiedi) pauloensis* has become *Bothrops pauloensis*, as accepted by the Brazilian Society of Herpethology (2005).

In the present study, the progression of local tissue damage and inflammation induced by BnSP-7 was evaluated. We further investigated the MMPs expression (MMP-2 and MMP-9) during the process of degeneration and regeneration of the gastrocnemius skeletal muscle. Finally we aimed to investigate the myotoxic activity of BnSP-7 in the release of cytokines.

2. Material and methods

2.1. Venom and toxins

Myotoxin BnSP-7 was purified from the crude venom of *B. (neuwiedi) pauloensis* snake, as previously described (Rodrigues et al., 1998). The myotoxic activity of BnSP-7 was inactivated by the alkylation of His48 using 4-bromophenacyl bromide (BPB). This process resulted in BnSP-7–BPB complex (Soares et al., 2000). Both myotoxin BnSP-7 and BnSP-7–BPB were kindly provided from Andreimar Martins Soares, PhD, from Faculty of Pharmaceutical Sciences, University of Sao Paulo. The protein concentration was estimated by the method of Bradford (1976).

2.2. Animals

BALB/c male mice were maintained under standard conditions (temperature 22 ± 1 °C, relative humidity 60 ± 5%, 12 h light/dark cycle) with diet and water *ad libitum*. The experimental protocol was approved by the Committee of Ethics for the Use of Animals of Butantan Institute (411/07).

2.3. Edema inducing-activity

Groups of four male BALB/c mice (18–22 g) were injected in the sub-plantar region of foot paw with 10 μg/10 μl of the myotoxin BnSP-7 or crude venom. Control animals received an injection of PBS under identical conditions. After 0.5, 1, 3, 6 and 24 h the paw edema was measured using a low pressure pachymeter (CALIPER). The thickness obtained before the injection (zero time point) was subtracted from all the values. The results were expressed as the media of percentage of induced edema ± SD.

2.4. Myotoxic activity

The assay of creatine kinase (CK) was carried out using the CK-NAC kinetic kit (*Bioclin*). 50 μg/25 μl of the myotoxin BnSP-7 or crude venom were injected intra-muscularly in groups of four BALB/c male mice (18–22 g). Control animals received an injection of 25 μl of PBS under identical conditions. At intervals of 1, 3, 6 and 24 h after injection, blood was collected from cardiac by cardiac puncture and the plasma CK activity was determined. Activity was expressed as U/ml, where one unit is defined as the phosphorylation of 1 μmol of creatine/min at 25 °C.

2.5. Histological analysis

Myotoxic activity was also evaluated on the basis of morphological alterations induced by i.m. injection of 50 μg/25 μl of the myotoxin BnSP-7 or crude venom in the left gastrocnemius skeletal muscle of BALB/c male mice (18–22 g). Control animals received an injection of 25 μl of PBS under identical conditions. After time intervals of 1, 3, 6, 24 and 72 h, 1 and 2 weeks of injection the animals were sacrificed in CO_2 chamber and a small section of the central region of the muscle was excised. The material was then soaked in fixing solution (10% formaldehyde in PBS, v/v) and dehydrated by increasing concentrations of ethanol and processed for inclusion in paraffin. The resulting blocks were sliced in 6.0 μm thick sections, stained with 0.25% (w/v) Hematoxilin and Eosin (HE) or Masson and examined under a light microscope.

2.6. RNA extraction

Total RNA was extracted from gastrocnemius muscle by Tri-Reagent (*Sigma*) method following the manufacture's instructions, after i.m. injection of 50 μg/25 μl of BnSP-7 or 25 μl of PBS at different time intervals (1, 3, 6, 24, 72 h and 1 and 2 weeks). RNA extraction was carried out in an RNAse-free environment. RNA was quantified by reading the absorbance at 260 nm according to the method described by Sambrook et al. (1989).

2.7. Semi-quantitative RT-PCR analysis

The reverse transcription of 1 μg RNA was carried out using M-MLV RT (2 U/μl) (Invitrogen), oligo $(dT)_{15}$, primers