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Increments in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake *Bothrops asper*

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Envenomations by the snake *Bothrops asper* are characterized by prominent local tissue damage (i.e. myonecrosis), blistering, hemorrhage and edema. Various phospholipases A_2 and metalloproteinases that induce local pathological alterations have been purified from this venom. Since these toxins induce a conspicuous inflammatory response, it has been hypothesized that inflammatory mediators may contribute to the local pathological alterations described. This study evaluated the local production of cytokines and matrix metalloproteinases (MMPs) as a consequence of intramuscular injections of an Asp-49 myotoxic phospholipase A_2 (myotoxin III (MT-III)) and a P-I type hemorrhagic metalloproteinase (BaP1) isolated from *B. asper* venom. Both enzymes induced prominent tissue alterations and conspicuous increments in interleukin (IL)-1β, IL-6 and a number of MMPs, especially gelatinase MMP-9, rapidly after injection. In contrast, no increments in tumor necrosis factor-α (TNF-α) and interferon-γ were detected. In agreement, MT-III and BaP1 did not induce the synthesis of TNF-α by resident peritoneal macrophages *in vitro*. Despite the conspicuous expression of latent forms of MMPs in muscle, evidenced by zymography, there were no increments in activated MMP-2 and only a small increase in activated MMP-9, as detected by a functional enzymatic assay. This suggests that MMP activity was regulated by a highly controlled activation of latent forms and, probably, by a concomitant synthesis of MMP inhibitors. Since no hemorrhage nor dermonecrosis were observed after injection of MT-III, despite a prominent increase in MMP expression, and since inflammatory exudate did not enhance hemorrhage induced by BaP1, it is suggested that endogenous MMPs released in the tissue are not responsible for the dermonecrosis and hemorrhage characteristic of *B. asper* envenomation. Moreover, pretreatment of mice with the peptidomimetic MMP inhibitor batimastat did not reduce myotoxic nor edema-forming activities of MT-III, suggesting that MMPs do not play a prominent role in the pathogenesis of these effects in this experimental model. It is concluded that MT-III and BaP1 induce a local inflammatory response associated with the synthesis of IL-1β, IL-6 and MMPs. MMPs do not seem to play a prominent role in the acute local pathological alterations induced by these toxins in this experimental model.

Key words: Snake venom, *Bothrops asper*, Matrix metalloproteinases, Cytokines, Snake venom metalloproteinases, Phospholipases A_2

Introduction

Envenomations induced by the bites of snakes from the family *Viperidae* are characterized by drastic local tissue damage involving necrosis of muscle, blistering, hemorrhage and a prominent inflammatory response, associated with edema, pain and a leukocyte infiltrate.[1–3] The pathogenesis of these alterations is rather complex, involving both the direct action of tissue-damaging toxins present in the venom and the participation of endogenous mediators that might contribute to such alterations. However, the precise role of each of these elements in venom-induced local effects has not been fully elucidated.

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Bothrops asper is the most important poisonous snake in Central America, where it inflicts more than 50% of snakebites.[4] Its venom induces prominent local tissue damage.[1,5] Acute muscle damage is caused by a group of highly basic phospholipases A_2 and phospholipase A_2 homologs that bind to and disrupt skeletal muscle cell plasma membrane, promoting a calcium influx that culminates in irreversible cell damage.[5,6] Hemorrhage is induced by the action of zinc-dependent metalloproteinases that hydrolyze proteins of the basement membrane of capillaries. As a consequence, endothelial cells are affected, with disruption of the capillary wall and extravasation.[7–10] Metalloproteinases are also responsible for blistering, probably by degrading basement membrane components at the dermal–epidermal junction.[11]

Besides the direct action of venom phospholipases A_2 and metalloproteinases, *B. asper* envenomations are also characterized by the synthesis and release of a number of endogenous mediators that promote edema and pain.[12–14] However, little is known on the local synthesis of cytokines and matrix metalloproteinases (MMPs) in *B. asper* envenomations. Previous investigations have documented elevated serum levels of some cytokines after experimental injections of various *Bothrops* sp. venoms,[15–17] and increments in MMPs in skin and exudates after intramuscular injection of *B. asper* P-I type hemorrhagic metalloproteinase BaP1 were described.[11] Furthermore, it was observed that venom metalloproteinases are able to release tumor necrosis factor-α (TNF-α) from its recombinant precursor *in vitro*.[18] Cytokines and MMPs are important mediators of tissue damage and inflammation in a variety of pathologies,[19,20] and they may play a role in venom-induced local alterations, enhancing the tissue damage initiated by venom components, a hypothesis that needs to be addressed.

The aim of the present work was to determine whether cytokine and MMP levels increase in muscle tissue injected with two tissue-damaging toxins from *B. asper* venom, myotoxic phospholipase A_2 myotoxin III (MT-III) and hemorrhagic metalloproteinase BaP1. Furthermore, the possible role of MMPs in the pathogenesis of acute local tissue damage was investigated in this experimental model.

Materials and methods

Toxins

MT-III and metalloproteinase BaP1 were isolated from a venom pool obtained from more than 40 adult specimens of *B. asper* collected in Costa Rica and kept at the Serpentarium of Instituto Clodomiro Picado. MT-III was isolated by ion-exchange chromatography on CM-Sephadex C-25,[21,22] whereas BaP1 was purified by ion-exchange chromatography on CM-Sephadex C-25, gel filtration on Sephacryl S-200 and affinity chromatography on Affi-gel Blue.[11,23] Homogeneity of the enzymes was demonstrated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions.[24]

Determination of increments in MMPs

Groups of four Swiss mice (18–20 g) were injected intramuscularly (i.m.), in the right gastrocnemius, with 60 μg of either MT-III or BaP1, dissolved in 60 μl of sterile, endotoxin-free saline solution (0.15 M NaCl). All animal experiments in this study were approved by the Animal Ethical Committee of the University of Costa Rica. Control mice received 60 μl of sterile saline solution alone. At various time intervals, mice were killed by CO_2 inhalation, and the injected gastrocnemius were dissected out and homogenized in 2.0 ml of endotoxin-free saline solution using a PT 10/35 homogenizer (Brinkmann Instruments Co., New York, USA). Muscle homogenates were centrifuged at 6000 × *g* for 5 min and the supernatants collected, stored at –70°C and used within the following week. The protein concentration of all samples was assessed according to Spector[25] in order to load the same amount of protein in the gels. Metalloproteinase activity was visualized by zymography, according to the procedure of Herron *et al.*,[26] with the modifications described by Rucavado *et al.*[11] Briefly, electrophoreses were performed at 100 V in a Mini-Protean cell (Bio-Rad, CA, USA), using a 5–20% gradient SDS-polyacrylamide gel containing Type A gelatin (Sigma Chemical Co, St Louis, MO, USA). Molecular weight markers were included. After a washing step with 2.5% Triton X-100 with shaking for 30 min, gels were incubated at 37°C for 18 h in 50 mM Tris–HCl (pH 8.0) buffer, containing 5 mM $CaCl_2$ and 20 mg/dl of sodium azide. Gels were then stained with 0.5% Coomassie Blue R-250 in acetic acid:isopropyl alcohol:water (1:3:6) and destained with distilled water. Gels run under identical conditions were incubated in the presence of 20 mM ethylenediamine tetraacetic acid (EDTA) to inhibit metalloproteinases.

Since the zymography assay described detects both latent and active MMPs, as well as those forming complexes with TIMPs, additional experiments were performed to estimate the concentration of latent and active MMPs in muscle homogenates. Active MMP-2 and MMP-9 were quantitated by the functional assay system Biotrak (codes 2630 and 2631; Amersham Pharmacia Biotech, UK). In conditions where *p*-aminophenylmercuric acetate (APMA) is not added to the enzyme, this assay detects only active MMPs. Addition of APMA allows detection of both latent and active forms. Homogenate samples, standardized by protein concentration,[25] were added to microplate wells in which either anti-MMP-2 or anti-MMP-9

antibodies had been adsorbed. After an overnight incubation at 4°C and a washing step, the pro-enzyme form of a detection enzyme was added, together with its substrate, and the plates were incubated at 37°C. Active MMPs cleave this pro-enzyme, activating it to hydrolyze its substrate with the formation of a colored product. Absorbances at 405 nm were recorded in a Multiskan microplate reader and enzymatic activitiy was determined on the basis of a standard curve prepared with purified MMP-2 and MMP-9.

Quantification of cytokine levels

For quantification of cytokine levels in muscle, the same homogenization protocol already described was followed. TNF-α, interleukin (IL)-1β, interferon-γ (IFN-γ) and IL-6 were quantitated by enzyme-immunoassays (Quantikine™ M; R & D Systems, MN, USA) following the manufacturer's instructions. TNF-α was also quantitated by a cytotoxic assay using the fibroblast cell line L-929,[27] with the modifications described by Petricevich *et al.*[17] Briefly, monolayers of L-929 cells were seeded in microtiter plates and incubated in a humidified air atmosphere with 5% CO_2 at 37°C for 18 h. Then, samples of muscle homogenates in RPMI-1640, containing 1.0 μg/ml of actinomycin D, were added. After incubation for 18 h at 37°C, supernatants were removed and viable cells were assessed after fixation and staining with crystal violet (0.2% in 20% methanol). Negative controls included cells incubated with RPMI medium. A standard curve was prepared with known concentrations of recombinant TNF-α.

Effects of BaP1 on macrophages *in vitro*

Resident, non-elicited macrophages were collected from anesthetized Swiss mice by lavage of the peritoneal cavity with sterile 0.15 M NaCl solution. Cells were counted in a hemocytometer, centrifuged and pelleted. Macrophages were resuspended in RPMI medium and applied to plates (1.5×10^5 cells/well) and incubated with MT-III or BaP1 (6.25, 12.5 and 25.0 μg/ml) for 60 min at 37°C under a 95% air, 5% CO_2 atmosphere. In some experiments, supernatants of macrophage cultures were assessed for the presence of TNF-α by the cytotoxic assay on L929 cells already described. An additional step was introduced in some experiments to increase the sensitivity of the assay. After incubation with toxins, macrophages were harvested and 2×10^4 cells, suspended in RPMI medium, were directly applied on monolayers of L929 cells. After 24 h of incubation at 37°C, the viability of L929 cells was assessed as already described. Recombinant TNF-α and anti-TNF-α antibodies were used in some controls to assure that the cytotoxic effect was indeed caused by this cytokine.

Histological studies

To determine whether treatment with MT-III or BaP1 induces skin lesions, mice were injected with 60 μg of these toxins i.m. in the gastrocnemius, as described, and were killed at 1 or 3 h. The skin overlying the injected area was dissected out, cut into small pieces and fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer; pH 7.2). Postfixation was performed with 1% osmium tetroxide. Afterwards, samples were dehydrated in ethanol and embedded in Spurr resin.[7] One micrometer sections were cut and stained with toluidine blue for histological observation.

Inhibition by the peptidomimetic hydroxamate batimastat

To determine whether MMPs synthesized after MT-III injection contribute to muscle damage and edema, mice were pretreated with an intraperitoneal (i.p.) injection of the peptidomimetic inhibitor batimastat (BB-94; British Biotech Pharmaceuticals Ltd., Oxford, UK). Batimastat suspension was prepared by sonication in phosphate-buffered saline solution (PBS) (pH 7.2), containing 0.01% Tween 80 and injected intraperitoneally at a dose of 30 mg/kg. This dose was selected since it has been used in previous studies dealing with the effect of this inhibitor in experimental cancer models,[28] in which a systemic concentration above the IC_{50} values for MMPs was reached. Three hours after batimastat administration, mice were injected with MT-III either i.m. (50 μg), in the gastrocnemius, or subcutaneously (20 μg), in the footpad, for the assessment of myonecrosis and footpad edema, respectively.[11,29] Myotoxicity was evaluated 3 h after MT-III injection by determining the plasma creatine kinase (CK) activity.[30] Edema was evaluated by measuring the increase in footpad thickness at various time intervals after toxin injection with the aid of a low-pressure spring caliper.[15,29]

Potentiation of BaP1-induced hemorrhage and dermonecrosis by inflammatory exudate

Inflammatory exudate was collected from mice 3 h after i.m. injection of 60 μg of MT-III in the right gastrocnemius. Animals were killed and a small incision was made in the skin overlying the injected muscle. Exudate was collected into heparinized capillary tubes and centrifuged at $2000 \times g$ for 5 min. For studying the possible effect of MMPs present in inflammatory exudate on the hemorrhagic and dermonecrotic activities of BaP1, groups of five mice were injected intradermally, in the abdominal region, with either BaP1 plus PBS, BaP1 plus exudate, or exudate plus PBS. Animals were killed after either 2 or 72 h for quantification of hemorrhagic and necrotizing lesions, respectively.[31,32] Hemorrhagic and necrotic areas

were measured in the inner side of the skin. Then, hemorrhagic areas were cut and placed into 2 ml of distilled water overnight for elution of hemoglobin. Tubes were centrifuged and the absorbances of supernatants recorded at 540 nm as a quantitative assessment of hemoglobin concentration.

Statistical analysis

The significance of the differences between the mean values of two experimental groups was determined by the Student's *t*-test, using $p < 0.05$ to establish significance.

Results

Studies on MMPs and cytokines in muscle

Samples of muscle homogenates obtained from mice injected with saline solution showed two main proteolytic bands of 100 and 60 kDa when analyzed by zymography (Fig. 1). A conspicuous increment in the intensity of the 100 kDa band was observed in samples obtained 1 and 6 h after MT-III and BaP1 injections (Fig. 1). In addition, bands of 270, 230, 135, 115, 74, 65 and 57 kDa were also detected. When incubations were carried out in the presence of EDTA, proteolytic activity was abrogated. There were no qualitative differences in the zymographic pattern between samples obtained from mice injected with MT-III and BaP1.

Enzymatic activity of MMP-2 and MMP-9 was quantified in muscle homogenates by a functional assay that includes an antibody-binding step, followed by a quantitative enzymatic determination. When compared with homogenates from mice injected with saline solution, no increments in either latent or activated forms of MMP-2 were detected. In contrast, both toxins induced increments in MMP-9 activity. When only the active form of the enzyme was detected, activity was: BaP1, 1 h, 17 ± 2 ng/g tissue; BaP1, 6 h, 8 ± 3 ng/g; MT-III, 1 h, 17 ± 2 ng/g; MT-III, 6 h, activity below the detection limit of the assay. When both latent and active forms were quantified (i.e. when APMA was included in the assay), activity increased significantly: BaP1, 1 h, 180 ± 5 ng/g tissue; BaP1, 6 h, 78 ± 28 ng/g; MT-III, 1 h, 789 ± 181 ng/g; MT-III, 6 h, 65 ± 0.2 ng/g. Thus, most of the expressed MMP-9 was in the latent form at these time intervals.

A prominent increment in muscle levels of IL-1β and IL-6 was observed early after injection of MT-III and BaP1, higher levels being induced by myotoxin administration (Fig. 2). Maximum levels were attained between 3 and 6 h, decreasing afterwards. In contrast, no evidence of TNF-α nor IFN-γ increments were detected in muscle homogenates by the enzyme

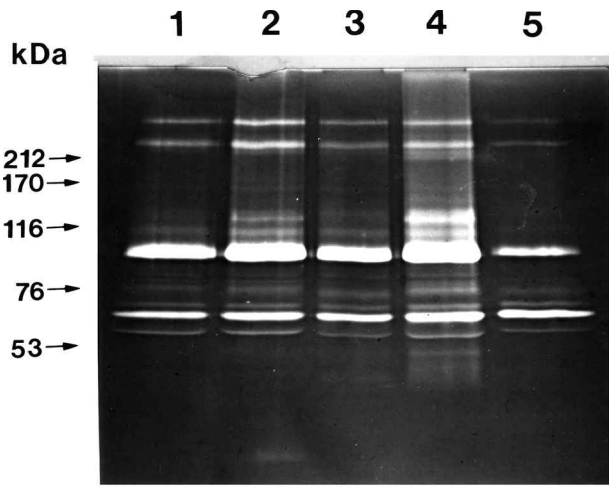

FIG. 1. Zymograms of muscle homogenates from mice injected i.m. with 60 μg of MT-III or BaP1. Gelatinase activity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5–20% acrylamide gradient) containing 1% type A gelatin. Muscle homogenates after injection of: (1) BaP1, 1 h; (2) BaP1, 6 h; (3) MT-III, 1 h; (4) MT-III, 6 h; (5) saline. Molecular weight markers are depicted to the left.

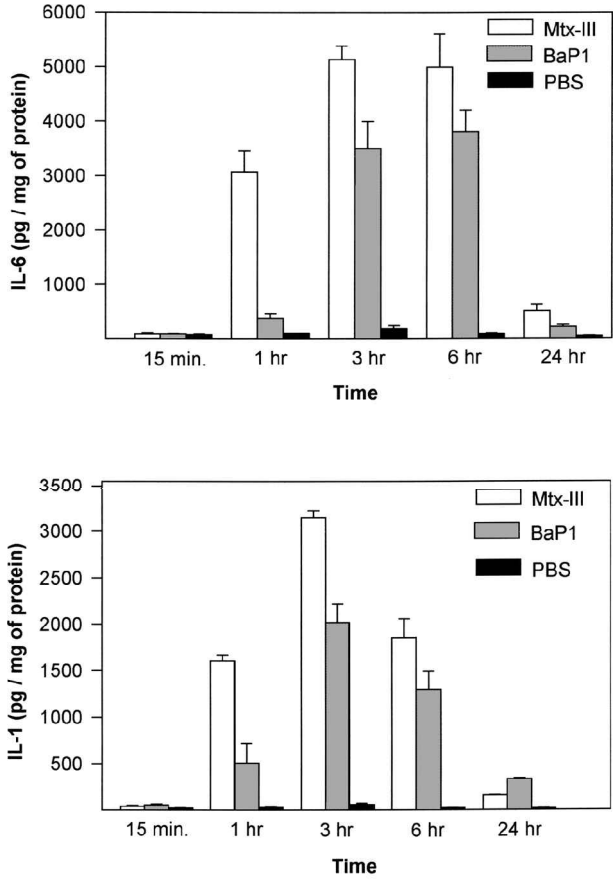

FIG. 2. Increments of IL-1β and IL-6 in muscle homogenates after i.m. injection of 60 μg of MT-III or BaP1, dissolved in 60 μl of saline solution. Control mice received saline solution alone. Mice were injected in the right gastrocnemius and, at various time intervals, they were killed and the injected muscle was excised, weighed and homogenized in 2.0 ml of saline solution. After centrifugation, cytokine levels were quantitated by enzyme-linked immunosorbent assay (see Materials and methods). Results are presented as mean ± standard deviation ($n = 4$).

immunoassay utilized. In these cases, cytokine concentration did not differ significantly from those of saline-injected muscle and were below the detection limit of the assay. In agreement with this, TNF-α was not detected in muscle homogenates by the cytotoxic assay on fibroblasts L929.

Effect of BaP1 on TNF-α production by macrophages *in vitro*

Neither MT-III nor BaP1 induced the synthesis of TNF-α by resident non-elicited peritoneal macrophages on incubation *in vitro* for 60 min, as evidenced by lack of cytotoxicity of supernatants on L929 cells (results not shown). To increase the sensitivity of the assay, macrophages that had been incubated with toxins were harvested and directly applied to cultures of L929 cells. Again, negative results were obtained at the doses tested.

Histological studies

Pathological alterations induced by *B. asper* myotoxins and BaP1 in muscle have been previously described in detail.[6,10] In the present study, we investigated the alterations induced by these toxins in the skin. Control mice injected with saline solution showed a normal morphology, with the characteristic structure of epidermis, dermis and hypodermis. In contrast, samples from mice injected with BaP1 showed prominent hemorrhage and inflammation in the hypodermis and dermis, together with the formation of blisters, characterized by the separation of epidermis from the underlying dermis, in agreement with previous observations[11] (Fig. 3A). Skin samples from mice injected with MT-III showed only a mild inflammatory reaction in the dermis. No evidence of blisters were observed (Fig. 3B), although there was necrosis of the panniculus carnosus muscle (not shown), indicating that the toxin reached the skin.

Inhibition by batimastat and effect of exudate on BaP1-induced hemorrhage

Administration of batimastat by the i.p. route 3 h before MT-III injection did not decrease the extent of edema induced by this myotoxin (Table 1). Regarding myotoxicity, plasma CK levels of mice injected with MT-III alone were 4051 ± 268 U/l, CK levels of MT-III-injected mice that had been pretreated with batimastat were 3880 ± 297 U/l, and CK levels of MT-III-injected mice pretreated with batimastat and receiving an additional local injection of batimastat were 3524 ± 399 U/l. None of these treatments promoted a significant reduction of myotoxicity ($p > 0.05$). Inflammatory exudate collected 3 h after MT-III injection, having a protein concentration of 4.00 ± 0.24 g/dl, did not increase nor reduce the extent of hemorrhage induced by intradermal injections of BaP1 (Table 2). Inflammatory exudate itself lacked hemorrhagic activity in these assays. Moreover, mice receiving an intradermal injection of BaP1 alone presented a necrotic skin lesion of 3.73 ± 0.15 mm ($n = 3$) 3 days after injection. When BaP1 was co-injected with exudate, the diameter of the necrotic lesion was 3.57 ± 0.15 mm ($p > 0.05$ when compared with BaP1 alone), exudate alone being devoid of necrotizing effect.

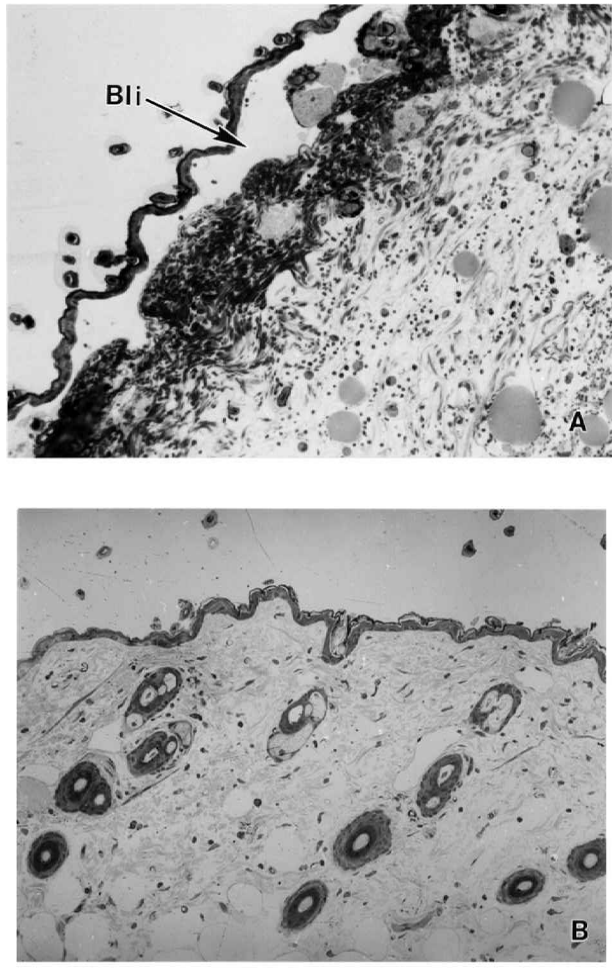

FIG. 3. Light micrographs of sections of skin from mice injected i.m. in the gastrocnemius with 60 μg of either BaP1 (A) or MT-III (B). Tissue samples were obtained 3 h after injection, processed and embedded in Spurr resin. Thick (1 μm) sections were stained with toluidine blue. Notice the presence of hemorrhage in the dermis and the formation of a blister (Bli) in mice injected with BaP1, whereas no evident pathological alterations in dermis and epidermis were observed after injection of MT-III.

Discussion

A conspicuous increase in IL-1β and IL-6, as well as a prominent increment in the expression of MMP-9, were observed in gastrocnemius muscle injected with MT-III and metalloproteinase BaP1. Local tissue pathology induced by *B. asper* venom, and probably by other crotaline snake venoms as well, is mainly

Table 1. Lack of inhibition of edema-forming activity of MT-III by pretreatment with the MMP inhibitor batimastat

Time (min)	Treatment*	
	Control	Batimastat
0†	1.72 ± 0.15‡	1.69 ± 0.09
15	3.18 ± 0.08	3.15 ± 0.09
60	2.94 ± 0.09	2.99 ± 0.13
180	2.69 ± 0.07	2.76 ± 0.12

* Mice received an i.p. injection of batimastat (30 mg/kg in PBS-0.01 % Tween), 3 h afterwards they were injected subcutaneously (s.c.) in the right footpad with 20 μg of MT-III in 50 μl of PBS. Control mice received an i.p. injection of PBS–Tween followed by a s.c. injection of MT-III as described. Edema is expressed as footpad thickness (mm).
† 0 represents footpad thickness immediately before injection of MT-III.
‡ Mean ± standard deviation (n = 4). No significant differences were observed at any time interval ($p > 0.05$).

characterized by a direct muscle damage induced by phospholipases A_2, and by blistering and microvessel disruption and bleeding induced by metalloproteinases.[5,8,11,33] Interestingly, despite the different targets and mode of action of these toxins, MT-III and BaP1 induced a qualitatively similar pattern of cytokine and MMP expression in injected muscle. Thus, it is likely that muscle tissue responds in a rather stereotyped fashion to locally acting agents of different nature present in snake venoms. Cytokine and MMP increments are likely to be related since a variety of cytokines, including TNF-α and IL-1β, induce MMP expression.[20,34]

Prominent increments of IL-1β and IL-6 occurred, whereas no elevations of TNF-α and IFN-γ were detected. In the case of TNF-α, this observation was corroborated using both an enzyme immunoassay and a cytotoxicity assay. This lack of correlation between IL-1β and TNF-α increments might be due to the fact that the former is produced by a number of different cells types, such as macrophages, endothelial cells and other cell types,[35,36] whereas TNF-α is mainly produced by macrophages. Resident macrophages are present in skeletal muscle,[37] but they are not elicited under normal conditions. Thus, when MT-III and BaP1 are injected, these resident macrophages do not seem to synthesize levels of TNF-α that could be detected by the assays performed. In agreement with this contention, TNF-α was not detected in cultures of non-elicited resident peritoneal macrophages incubated with these toxins. In contrast with our observations, Clissa *et al.*[38] described the production of TNF-α by peritoneal macrophages incubated with jararhagin, a P-III hemorrhagic metalloproteinase from *B. jararaca* venom. This apparent discrepancy may be related to the type of macrophage used, since Clissa *et al.*[38] worked with thioglycollate-elicited macrophages, whereas our experiments were performed with resident, non-elicited cells. It would be relevant to compare the effect of tissue-damaging toxins on cytokine production by resident, elicited and activated macrophages.

Table 2. Effect of the inflammatory exudate, obtained from mice injected with MT-III, on the hemorrhagic activity of metalloproteinase BaP1*

Treatment	Diameter of hemorrhagic lesions	Absorbance at 540 nm
PBS	0	0.008 ± 0.002†
PBS + exudate	0	0.009 ± 0.002
BaP1 + PBS	14.5 ± 2.3 mm	0.327 ± 0.165
BaP1 + exudate	13.8 ± 1.9 mm	0.358 ± 0.117

* Groups of mice were injected i.m. with 60 μg of MT-III and inflammatory exudate was collected from underneath the skin 3 h after injection. Then, groups of four mice were injected intradermally with either PBS (90 μl), PBS (50 μl) + exudate (40 μl), BaP1 (40 μg/50 μl) + PBS (40 μl), or BaP1 (40 μg/50 μl) + exudate (40 μl). Hemorrhagic activity was assessed 2 h after injection by measuring the diameter of the hemorrhagic spots. Then, skin samples containing the hemorrhagic areas were dissected out and placed into 2 ml of distilled water overnight for elution of hemoglobin. Tubes were centrifuged and the absorbances of supernatants recorded at 540 nm as a quantitative assessment of hemoglobin concentration.
† Mean ± standard deviation (n = 4). The differences between the mean values of mice receiving BaP1 + PBS and those receiving BaP1 + exudate were not significant ($p > 0.05$).

Increments in serum levels of IL-6 have been observed in mice injected with *B. asper* and *B. atrox* venoms.[15–17] IL-6 is a major mediator of inflammation,[39] inducing the synthesis of acute-phase proteins in the liver.[40] In addition, IL-6 induces myoblast proliferation in culture,[41] suggesting that the observed increments in this cytokine may influence skeletal muscle regeneration after snake venom-induced myonecrosis. IL-1β plays a number of roles in inflammation, inducing the expression of adhesion molecules,[42] and the release of leukocyte chemotactic factors,[43] besides altering the functional properties of endothelial cells.[35] IL-1β promotes inflammation after skeletal muscle injury.[44] In addition, these cytokines induce MMP expression[34] and probably contribute to the synthesis of MMPs observed in our experimental models, since early increments in these cytokines correlated with the expression of MMPs. Increments in the serum levels of a number of cytokines have been described in patients after snakebite envenomations.[45,46] Thus, cytokines may play a relevant role in the pathophysiology of these envenomations in humans.

MMPs constitute a group of more than 20 enzymes, with the common ability to degrade extracellular matrix components, that play essential roles in embryonic development, reproduction, tissue remodeling and inflammation.[19,20] These enzymes are usually not constitutively present in cells, being synthesized as prepro-enzymes and secreted as inactive pro-enzymes by a variety of cell types in inflammatory processes.[19,47] However, a constitutive expression of MMP-2 has been described for mouse skeletal muscle,[48] in agreement with our observations in control muscle. Zymography results indicate that a

number of MMPs, especially MMP-9, are expressed in muscle injected with MT-III and BaP1. Bands of 60 and 100 kDa observed in zymography probably correspond to the latent forms of MMP-2 and MMP-9, respectively, as these molecular masses have been described for these enzymes in mice.[48] High activity was detected in muscle homogenates when both latent and active MMP-9 were quantitated, whereas activity was greatly reduced when only the active form was quantitated by a specific enzymatic assay. This strengthens the conclusion that most of the increment in MMP-9 corresponds to the latent form of the enzyme. MMP-2 and MMP-9 have been reported to increase in a variety of pathologies, such as myonecrosis,[48] chronic inflammation,[49] meningitis,[50] intracerebral hemorrhage and ischemia,[51,52] and acute lung injury,[53] among others. In addition to these two MMPs, other gelatinolytic bands were observed in muscle homogenates and exudates in our experimental model, which probably correspond to other members of the MMP family. We also detected two bands of 270 and 230 kDa, which might represent MMP aggregates.[54] Although the cellular source of MMPs was not addressed in this study, it is suggested that resident macrophages, fibroblasts and endothelial cells constitute the main sources of MMPs during the first hours after injection of these toxins.[34,53,55,56]

In the physiological processes in which MMPs participate, the balance between MMP expression, activation and inhibition by TIMPs and other inhibitors is finely regulated.[20] On the contrary, exaggerated or unregulated expression of MMPs may lead to uncontrolled extracellular matrix degradation and tissue damage, as occurs in chronic wounds.[49] Thus, the question was raised in this study as to whether the observed increment in MMP expression contributes to the acute local tissue damage characteristic of *B. asper* envenomation. Several lines of evidence strongly suggest that MMPs do not contribute significantly to the acute tissue damage induced by MT-III and BaP1 during the first 6 h after injection of the toxins. Pretreatment with batimastat, a potent MMP inhibitor having broad specificity, did not reduce the extent of edema and myonecrosis induced by MT-III. Moreover, MT-III injection did not induce local hemorrhage nor blistering, despite the conspicuous increase in MMP-9 expression. Thus, in the case of BaP1, it is suggested that hemorrhage and blister formation are mainly due to the direct action of this venom metalloproteinase at the basement membrane of capillary vessels and the epidermis. This conclusion is strengthened by the observation that concomitant injection of BaP1 and inflammatory exudate containing MMPs induced similar hemorrhagic and dermonecrotic lesions when compared with mice injected with BaP1 alone. In addition, local injection of inflammatory exudates obtained from MT-III-injected mice did not induce dermonecrosis nor hemorrhage.

As evidenced by zymography and by a specific enzymatic assay, MMPs in muscle homogenates corresponded predominantly to the latent forms, therefore lacking activity under non-denaturing conditions, such as those operating *in vivo*. Thus, the apparent lack of pathological effects of MMPs in this model might be associated with the very low concentration of the active forms of these enzymes in the tissue. It is likely that MMP activity is finely controlled in our experimental conditions by keeping most of the enzymes in their latent form and, probably, by a parallel expression of TIMPs. As a consequence, free metalloproteinase activity is maintained under control. It has been observed that the acute wound microenvironment, such as that of our model, is characterized by a controlled balance of MMPs and their inhibitors, whereas in chronic wounds this balance is disrupted, with the concomitant increment in MMP free activity associated with prominent extracellular matrix degradation and pathology.[49,57] It is therefore likely that MMP expression in our experimental model is a regulated event that does not seem to play a prominent role in the acute local tissue damage induced by these toxins within the first hours after injection. Instead, MMPs probably participate in the inflammatory response, by remodeling extracellular matrix and promoting events associated with inflammatory cell influx, release of matrix-embedded growth factors and onset of tissue regeneration. The role of MMPs in the inflammatory and reparative processes taking place after the onset of acute local tissue damage induced by snake venom toxins remains to be investigated.

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