



## Experimental pathology of local tissue damage induced by *Bothrops asper* snake venom

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### ARTICLE INFO

#### Article history:

Received 6 November 2008

Received in revised form 22 January 2009

Accepted 23 January 2009

Available online 18 March 2009

#### Keywords:

*Bothrops asper*

Local effects

Hemorrhage

Myonecrosis

Edema

Blistering

Muscle regeneration

Metalloproteinases

Phospholipases A<sub>2</sub>

### ABSTRACT

Envenomations by *Bothrops asper* are often associated with complex and severe local pathological manifestations, including edema, blistering, dermonecrosis, myonecrosis and hemorrhage. The pathogenesis of these alterations has been investigated at the experimental level. These effects are mostly the consequence of the direct action of zinc-dependent metalloproteinases (SVMPs) and myotoxic phospholipases A<sub>2</sub> (PLA<sub>2</sub>s). SVMPs induce hemorrhage, blistering, dermonecrosis and general extracellular matrix degradation, whereas PLA<sub>2</sub>s induce myonecrosis and also affect lymphatic vessels. In addition, the prominent vascular alterations leading to hemorrhage and edema may contribute to ischemia and further tissue necrosis. The mechanisms of action of SVMPs and PLA<sub>2</sub>s are discussed in detail in this review. Venom-induced tissue damage plays also a role in promoting bacterial infection. A prominent inflammatory reaction develops as a consequence of these local pathological alterations, with the synthesis and release of abundant mediators, resulting in edema and pain. However, whether inflammatory cells and mediators contribute to further tissue damage is not clear at present. Muscle tissue regeneration after venom-induced pathological effects is often impaired, thus resulting in permanent tissue loss and dysfunction. SVMP-induced microvessel damage is likely to be responsible of this poor regenerative outcome. Antivenoms are only partially effective in the neutralization of *B. asper*-induced local effects, and the search for novel toxin inhibitors represents a potential avenue for improving the treatment of this serious aspect of snakebite envenomation.

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### 1. Envenomations by *Bothrops asper* are characterized by drastic local pathological effects

*B. asper* is responsible for the vast majority of snakebite envenomations in Central America and in some regions of northern South America (Bolaños, 1982; Gutiérrez, 1995; Otero et al., 2006). This is due to a number of factors, i.e. the widespread distribution of this species in low-land humid areas devoted to agricultural activities, the high capacity of this species to adapt to altered environments, such as agricultural fields and pastures (Savage, 2002; Solórzano, 2004), and the consequent close contact between *B. asper*

and humans in rural areas. In addition, *B. asper* is able to deliver a relatively large volume of venom (Bolaños, 1972), thus being capable of provoking severe envenomations. The relevance of *B. asper* goes beyond human medicine, since it is also responsible for a great number of envenomations in domestic animals, particularly cattle, dogs and horses (Villalobos, 2008).

Envenomations by *B. asper* are characterized by a prominent and complex series of local pathological alterations, which appear rapidly after the bite at the anatomical site where venom is injected. Such effects include edema and pain, hemorrhage, myonecrosis, blistering, and dermonecrosis (Picado, 1931; Bolaños, 1982; Gutiérrez, 1995; Otero et al., 2002; Warrell, 2004). Furthermore, local infection by bacteria present in the mouth of the snake or in the skin of

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patients often complicates these cases (Otero et al., 2002), as described in other *Bothrops* sp. envenomations as well (Jorge et al., 1994). The pathogenesis of local pathology induced by *B. asper* venom has been the subject of many experimental studies that have clarified the mechanisms through which toxins present in this venom inflict such a drastic and complex pathological picture. The present work reviews this information and highlights some unsolved issues that demand further investigation.

## 2. Edema and pain

### 2.1. The synthesis and release of endogenous mediators play a central role in edema, hyperalgesia and allodynia

Local edema and pain appear very rapidly after injection of *B. asper* venom in experimental animals (Gutiérrez and Lomonte, 2003; Chaves et al., 1995; Chacur et al., 2001). The time-course of edema in rodent footpad assays greatly depends on the dose of venom injected. At low doses, edema peaks within the first hour and drops at later time intervals (Lomonte et al., 1993). However, when higher doses are administered, edema persists for more prolonged time intervals (Gutiérrez et al., 1980a; Lomonte et al., 1993; Chaves et al., 1995). This probably reflects different mechanisms in the genesis of fluid imbalance in the tissues which leads to edema. The pathophysiology of edema formation induced by *B. asper* venom is multifactorial. There is a direct damage on microvessels, i.e. capillaries and venules, by the action of hemorrhagic toxins (see Section 3), with the consequent extravasation. In addition, edema is mediated by the action of inflammatory mediators, released or synthesized in the course of envenomation, which induce increments in the permeability of microvessels (Gutiérrez and Lomonte, 2003; Teixeira et al., 2003a).

Pharmacological studies in mice and rats have identified a number of mediators involved in *B. asper* venom-induced footpad edema, such as prostaglandins, nitric oxide and others (Chaves et al., 1995, 2006; Olivo et al., 2007). This agrees with observations performed with other *Bothrops* sp. venoms (Bonta et al., 1979; Trebien and Calixto, 1989). Similarly, the pharmacological basis of hyperalgesia and allodynia induced by this venom has been investigated, and the involvement of various mediators, such as bradykinin and leucotrienes, evidences a multifactorial mechanism in the onset of pain (Chacur et al., 2001). Myotoxic phospholipases A<sub>2</sub> play a key role in *B. asper* venom-induced hyperalgesia (Chacur et al., 2003). The pharmacological mediation of edema and pain is covered in detail in another contribution in this volume (see Teixeira et al., this issue).

### 2.2. Pathological effects on lymphatic vessels and their possible role in the pathogenesis of edema

The observation that high doses of *B. asper* venom provoke a long-standing edema suggests that pathological, in addition to pharmacological, mechanisms may be at work in these conditions, i.e. that pathological alterations in the tissues and the vasculature are responsible for a prolonged interstitial fluid imbalance. It is possible that pathological damage to microvessels may be responsible

for this phenomenon, since venom drastically affects the integrity of capillaries, venules and small arteries (Arce et al., 1991; Moreira et al., 1992). Recent findings suggest that a direct effect of the venom on collecting lymphatic vessels may be also involved. When *B. asper* venom is directly applied onto exposed mouse mesentery preparations and observed by intravital microscopy, there is a rapid and marked reduction in the lumen of collecting lymphatics, an effect associated with a halting in the flow of lymph in these vessels (Mora et al., 2008).

This effect on lymphatics was not reproduced when a hemorrhagic metalloproteinase or a coagulant serine proteinase was applied, thus evidencing that it is not the consequence of clotting of fibrinogen or extracellular matrix degradation. Instead, the effect is reproduced when a myotoxic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) homologue, myotoxin II, is applied (Mora et al., 2008). In agreement, the effect induced by crude *B. asper* venom was abrogated by fucoidan, a polysaccharide inhibitor of myotoxins, but not by the metalloproteinase inhibitor batimastat (Mora et al., 2008). Myotoxin II was shown to lyse smooth muscle cells in culture and it was suggested that the reduction in lymphatic vessel lumen is the result of smooth muscle cell membrane perturbation by myotoxins, with the consequent calcium influx and contraction of muscle cells of the collecting lymphatic wall (Mora et al., 2008). The functional consequences of this phenomenon are obvious, since an adequate lymph flow depends on the periodic contraction of the lymphangions associated with the opening and closure of the lymphatic valves (Schmid-Schönbein, 2006). Thus, if the vessel is permanently contracted, due to the action of venom myotoxic PLA<sub>2</sub>s, the lymph flow is halted. Furthermore, if lymphatic smooth muscle cells are irreversibly damaged by the venom, as occurs in cell culture conditions, then the pumping of lymph would be permanently impaired. In agreement with these observations, fucoidan was highly effective in the inhibition of *B. asper* venom-induced mouse footpad edema (Mora et al., 2008).

Venom-induced vascular disturbances leading to edema may contribute to the overall pathophysiology of envenomation in two additional ways: (a) the fluid imbalance resultant from these alterations represents a significant displacement of fluid from the vascular compartment to the interstitial compartment, thus contributing to the hypovolemia and consequent hemodynamic alterations typical of severe envenomations by viperid snakes (Warrell, 1996, 2004). (b) The pronounced increment in interstitial fluid in some muscle compartments, such as the anterior tibial compartment, may result in an increment in intracompartamental pressure. When this pressure increases over 40 mm Hg, a compartmental syndrome may develop (Warrell, 1999), with the consequent effect in the perfusion to distal regions and the onset of ischemic damage (see Section 6.5).

## 3. Local hemorrhage

### 3.1. Zinc-dependent metalloproteinases are responsible for local hemorrhagic effects

*B. asper* venom, and the majority of viperid venoms, are rich sources of zinc-dependent proteinases, a group of

enzymes that belong to the M12 reprotolysin family of metalloproteinases, comprised by the snake venom metalloproteinases (SVMPs) and the ADAMs (A Disintegrin And Metalloproteinase enzymes) (Fox and Serrano, 2005). All hemorrhagic components isolated from viperid snake venoms are SVMPs, although not all SVMPs are able to induce hemorrhage (Fox and Serrano, 2005). SVMPs are classified within four different groups, depending on their domain composition. Group P-I is comprised by enzymes presenting only the metalloproteinase domain, where the characteristic zinc-binding signature (HEXXHXXGXXH followed by a Met-turn) is present (Bode et al., 1993; Fox and Serrano, 2005). Group P-II includes enzymes containing the metalloproteinase domain and a disintegrin domain. SVMPs belonging to group P-III comprise metalloproteinase, disintegrin-like (Dis-like) and cysteine-rich (Cys-rich) domains in a single polypeptide chain, many of them being glycosylated. Finally, P-IV SVMPs are heterodimers, with one subunit corresponding to a P-III chain and another being a C-type lectin-like domain. Some SVMPs have been classified in a number of subgroups within this general structural frame (Fox and Serrano, 2005).

In general, the most potent hemorrhagic toxins belong to the P-III SVMPs, and there is strong evidence suggesting that the additional Dis-like and Cys-rich domains contribute to the high hemorrhagic activity by targeting the enzymes to relevant locations in the microvasculature and, perhaps, by affecting coagulation as well (see below). SVMPs of the groups P-I and P-III have been isolated from the venom of *B. asper* (Aragón-Ortiz and Gubensek, 1987; Borkow et al., 1993; Gutiérrez et al., 1995a; Franceschi et al., 2000; Loría et al., 2003). The concentration of P-I SVMPs is higher than that of P-III SVMPs in the venom of adult specimens of *B. asper*, whereas venoms of neonates have a predominance of P-III SVMPs (Alape-Girón et al., 2008).

### 3.2. Pathogenesis of hemorrhage induced by *B. asper* venom and isolated SVMPs

Injection of *B. asper* venom or isolated SVMPs in experimental animals results in rapid and prominent hemorrhage. The Minimum Hemorrhagic Dose of *B. asper* venom, i.e. the amount of venom that induces a hemorrhagic halo of 10 mm diameter in the skin of mice 2 h after injection, is 1.5 µg for the venom of specimens collected in the Pacific versant of Costa Rica, and 2.5 µg for venom from specimen of the Caribbean region of the country (Gutiérrez et al., 1985). Similar values have been described for venoms from Honduras and Guatemala (Rojas et al., 1987; Saravia et al., 2001). Hemorrhagic activity of *B. asper* venom and isolated SVMPs is completely abrogated upon incubation of venom with metalloproteinase inhibitors, indicating that SVMPs are the only hemorrhagic components in this venom (Borkow et al., 1997; Escalante et al., 2000; Rucavado et al., 2000). These findings also evidence that metalloproteinase activity is an absolute requirement for exerting hemorrhagic effect.

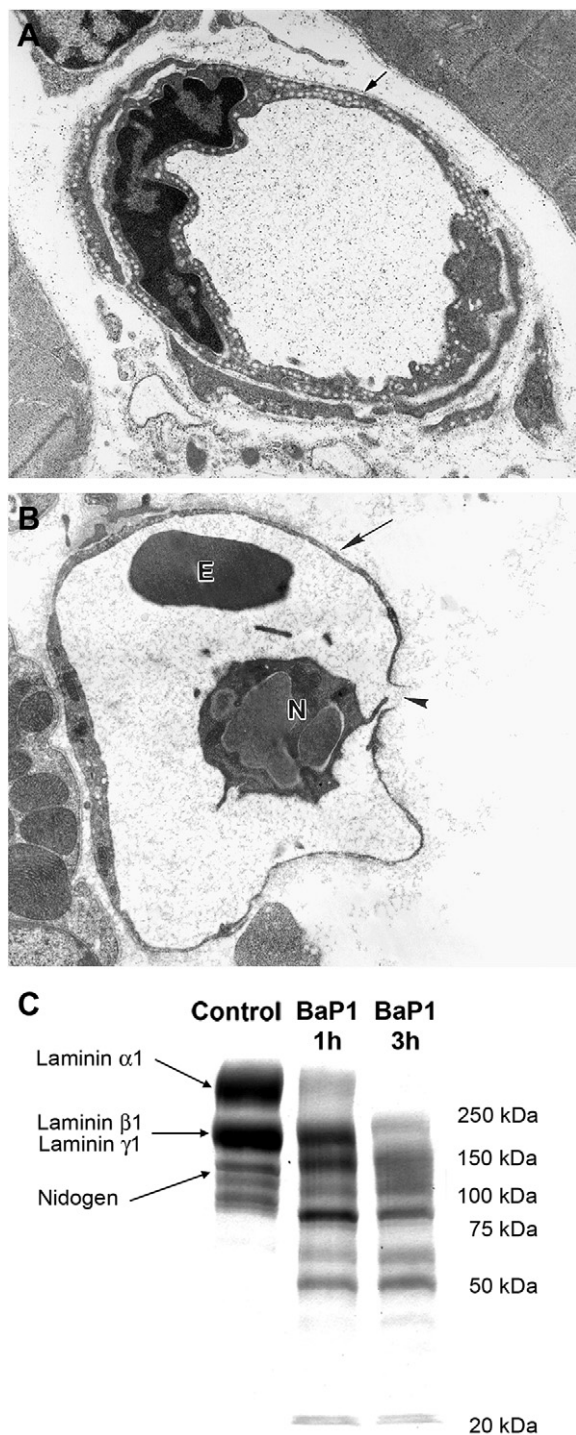
Intramuscular injection of crude *B. asper* venom or purified hemorrhagic SVMPs BaH1 (a P-III enzyme) and BaP1 (a P-I enzyme) results in rapid damage to microvessel structure, especially capillary vessels (Moreira et al., 1992,

1994; Gutiérrez et al., 2006). Capillary endothelial cells show the following structural alterations: reduction in cell thickness and in the number of pinocytotic vesicles, appearance of small blebs protruding from the cell to the vascular lumen, mitochondrial swelling and loss of endothelial cell integrity (Moreira et al., 1992, 1994; Gutiérrez et al., 2006). In addition, the basement membrane (BM) that surrounds the capillaries loses its continuity and is absent in some locations (Moreira et al., 1992, 1994). Distention of endothelial cells is associated with the disruption in cell integrity leading to extravasation through focal lesions (Fig. 1).

These observations are highly similar to those performed with other hemorrhagic SVMPs from viperid venoms (Ownby et al., 1978; Ownby and Geren, 1987; Anderson and Ownby, 1997; Escalante et al., 2003). Similar ultrastructural alterations were also observed in intravital microscopy when *B. asper* venom was applied to mouse cremaster muscle (Lomonte et al., 1994a). Using this methodology, which allows the analysis of the dynamics of pathological events in vivo, it was evident that hemorrhage occurred in discrete locations in the microvasculature, with 'explosive' hemorrhagic events occurring at various sites in the capillary network, characterized by a rapid bursting of blood out of the microvessels forming localized hemorrhagic foci in the tissue (Lomonte et al., 1994a; Rucavado et al., 1995).

SVMPs, including enzymes from *B. asper* venom, degrade extracellular matrix proteins in vitro (Ohsaka et al., 1973; Ohsaka, 1979; Baramova et al., 1989, 1991; Rucavado et al., 1995; Franceschi et al., 2000; Escalante et al., 2006) (Fig. 1). Since SVMPs hydrolyze components of the BM, such as laminin, nidogen/entactin, and type IV collagen, it was proposed that such degradation of BM proteins plays a central role in the pathogenesis of hemorrhage (Ohsaka et al., 1973; Ohsaka, 1979; Bjarnason and Fox, 1994). However, one puzzling observation in these in vitro studies was that BM protein hydrolysis occurred at relatively late time intervals, whereas in vivo hemorrhage develops within minutes of injection.

On the other hand, the action of SVMPs on endothelial cells has been investigated in cell culture conditions. In contrast to the rapid disruption of endothelial cells in vivo after injection of hemorrhagic SVMPs, incubation of these enzymes with endothelial cells in culture did not result in rapid cytotoxicity. Instead, the most notorious effect upon incubation with *B. asper* SVMPs BaH1 and BaP1 was the detachment of cells from their substratum, without rapid loss in viability (Lomonte et al., 1994b; Borkow et al., 1995; Rucavado et al., 1995; Díaz et al., 2005). Similar observations were performed with the P-III SVMP jararhagin, from the venom of the South American species *Bothrops jararaca* (Tanjoni et al., 2005). At later time intervals of incubation with SVMPs, endothelial cells may undergo apoptosis. However, even though it was previously suggested that the mechanism could be anoikis, resultant from the detachment of cells and the interruption in viability signals that depend on the contact between the cells and the extracellular matrix (Díaz et al., 2005; Tanjoni et al., 2005), new results indicate that this might not be the case (O. Brenes et al., unpublished results). These new data, obtained using



bovine aortic endothelial cells (BAEC), show that BaP1 concentrations that induce a significant decrease in cell viability are not able to promote cell detachment from the plates, indicating that no strict correlation exists between both events and suggesting that other mechanisms of cytotoxicity are probably being triggered. However, regardless of such apoptotic outcome, observations with crude *B. asper* venom and isolated hemorrhagic SVMPs in cell culture conditions clearly show that the rapid and drastic endothelial cell pathology occurring in vivo is not reproduced in cell culture.

This apparently puzzling situation could be explained by a hypothesis concerning the mechanism of action of hemorrhagic SVMPs, which incorporates both biochemical and biophysical events in the pathogenesis of microvessel damage and extravasation. According to this hypothesis, capillary damage leading to hemorrhage occurs by a two-step mechanism. In the first step, hemorrhagic SVMPs hydrolyze proteins of the capillary BM, as has been documented for *B. asper* SVMPs in vitro (Rucavado et al., 1995; Franceschi et al., 2000; Escalante et al., 2006) and, more recently, in vivo (Escalante et al., 2006). Such hydrolysis is likely to result in the mechanical weakening of BM structure. BM plays the role of a mechanical scaffold of capillary structure, being largely responsible for the Young's elastic module of capillaries (Milnor, 1980), thus resisting the hemodynamic forces that tend to distend the capillary wall. As a consequence of this proteolysis-mediated weakening, in the second step of this pathological event the biophysical forces normally operating in the microvasculature, mainly hydrostatic pressure and tangential shear stress, induce the distention of the capillary wall, eventually resulting in the disruption of endothelial cell integrity and the consequent extravasation (Gutiérrez et al., 2005).

The ultrastructural observation of a drop in the number of pinocytotic vesicles can be interpreted in the light of this hypothesis as an adaptive response of endothelial cells to distention, thus mobilizing membrane from the vesicles to the plasma membrane (Lee and Schmid-Schönbein, 1995). Eventually, however, the distention overcomes this adaptive mechanism and endothelial cells become disrupted. In this scenario, endothelial cell pathology corresponds to a mechanically-induced necrosis. Clearly, this phenomenon is not reproduced in cell culture conditions because the

**Fig. 1.** Effect of the P-I SVMP BaP1 on capillary vessel integrity and on basement membrane. (A) Electron micrograph of a capillary vessel from the gastrocnemius muscle of a mouse injected with saline solution. Notice the typical ultrastructure of endothelial cell and the presence of basement membrane (arrow), 17,000 $\times$ . (B) Electron micrograph of a capillary vessel of mouse gastrocnemius muscle 5 min after injection of BaP1. The endothelial cell is distended and has a reduced thickness, being devoid of pinocytotic vesicles. There is a rupture in the integrity of endothelial cell (arrow head) and loss of structure of basement membrane (arrow). A neutrophil (N) and an erythrocyte (E) are present in the capillary lumen. 10,000 $\times$ . (C) Hydrolysis of Matrigel proteins by BaP1. Matrigel was incubated with the enzyme at a 1:50 (w:w) enzyme:substrate ratio for 1 h and 3 h at 37 °C. A control of Matrigel incubated with saline solution was included (Control). Mixtures were separated by SDS-PAGE run under reducing conditions in 4–15% polyacrylamide gradient gel. Laminin chains and nidogen are shown in the Matrigel control. Molecular mass references are indicated at the right. (A) and (B) reprinted from *Microvascular Research*, Vol. 71, Gutiérrez et al., copyright 2008, with permission from Elsevier.

biophysical forces operating *in vivo* are not present in cell culture experiments. Experimental support for this two-step hypothesis came from experiments in which blood flow in muscle tissue was interrupted. In these circumstances, the injection of hemorrhagic SVMP BaP1 did not induce endothelial cell damage, thus evidencing that biophysical forces associated with blood flow play a key role in endothelial cell damage and the ensuing hemorrhage (Gutiérrez et al., 2006).

A pending issue is the analysis of the cleavage sites of hemorrhagic SVMPs in key substrates in BM and the consequences of the various cleavage patterns on BM stability. The observation that non-hemorrhagic SVMPs are also able to hydrolyze BM proteins *in vitro* (Rucavado et al., 1999) suggests that it is not the cleavage of BM *per se*, but the particular pattern of such cleavage, what is relevant for the pathogenesis of hemorrhage. Different cleavage pattern by jararhagin and BaP1 was described for nidogen, with potentially different effects on the mechanical integration of nidogen and other BM components (Escalante et al., 2006). A detailed account on the *in vitro* and *in vivo* BM degradation patterns induced by SVMPs having different hemorrhagic potencies is required for a more in-depth understanding of the pathogenesis of hemorrhage. Furthermore, the role played by the Dis-like and Cys-rich domains in the action of P-III SVMPs is another important issue. Clearly, these additional domains contribute to the higher hemorrhagic potency of this group of enzymes, as compared to P-I SVMPs (Fox and Serrano, 2005).

The most likely mechanism to explain this role is the ability of sequences found in these domains to direct the enzyme to specific targets in extracellular matrix or endothelial cells, in such a way that the enzyme is positioned in a location where the proteinase activity is exerted in the most effective way to affect capillary integrity. Although these studies have not been carried out with *B. asper* venom SVMPs, observations performed with jararhagin clearly suggest that sequences in these domains allow the binding to relevant substrates, such as proteins having von Willebrand factor A domains like collagens XII and XIV, matrilins 1, 3 and 4 and von Willebrand factor (Serrano et al., 2005, 2006, 2007), and to collagens I and IV (Moura-da-Silva et al., 2008). Moreover, P-III SVMPs are poorly inhibited by  $\alpha$ 2-macroglobulin, whereas P-I SVMPs are readily inhibited by this plasma macromolecule (Baramova et al., 1990; Kamiguti et al., 1994; Escalante et al., 2003, 2004; Loría et al., 2003). Finally, the ability of P-III SVMPs, but not P-I SVMPs, to inhibit collagen-induced platelet aggregation (Kamiguti et al., 1996; Jia et al., 1997; Moura-da-Silva et al., 1999; Estevao-Costa et al., 2000), may also contribute to their higher hemorrhagic activity.

### 3.3. Does apoptosis play a role in SVMP-induced endothelial cell damage *in vivo*?

As described above, incubation of endothelial cells with SVMPs in culture results in apoptosis (Wu et al., 2001; You et al., 2003; Díaz et al., 2005; Tanjoni et al., 2005). In the case of *B. asper* SVMP BaP1, human capillary endothelial cell apoptosis was independent of two Bcl-2 family members, Bcl-xL and Bax, whereas procaspase-8 disappeared,

probably reflecting its activation by a death receptor. Thus, BaP1 induces apoptosis by a mechanism dependent on caspase 8 activation (Díaz et al., 2005). On the basis of these observations in cell culture, it has been assumed that endothelial cell apoptosis also occurs *in vivo* as a consequence of the action of SVMPs. However, recent observations performed with *B. asper* hemorrhagic SVMP BaP1 question this assumption. No apoptosis was observed in endothelial cells in the dermis of mouse ear skin after injection of BaP1, as determined by the lack of TUNEL-positive cells in capillaries (Jiménez et al., 2008). It is suggested that capillary endothelial cells are mostly affected by mechanically-induced necrosis, as discussed above. The role of *in vivo* apoptosis in capillary endothelial and other cells in tissues demands further attention, but caution should be taken when extrapolating conclusions from *in vitro* observations to the more complex *in vivo* scenario where these toxins exert their action.

### 3.4. Are arteries and arterioles also affected?

A number of experimental pathological observations with crude *B. asper* venom indicate that larger vessels, especially arterioles and arteries, are affected. Angionecrosis and thrombosis were described, together with loss of immunostaining for endothelial cell markers in these vessels (Tu and Homma, 1970; Arce et al., 1991). The occurrence of angionecrosis in these experimental models is a relevant finding that deserves further consideration, owing to the implications that such pathology would have in the overall context of local tissue damage. The toxins involved and the mechanisms behind these effects have not been investigated, although it is tempting to suggest that SVMPs and myotoxic PLA<sub>2</sub>s are likely candidates. The ability of a *B. asper* myotoxic PLA<sub>2</sub> homologue to induce cytotoxicity on arterial smooth muscle cells in culture has been demonstrated (Mora et al., 2008). In addition, the presence of thrombi in arterioles and arteries (Tu and Homma, 1969; Homma and Tu, 1971; Arce et al., 1991) may also contribute to vascular alterations leading to a deficient perfusion to distal muscle tissue.

## 4. Skin pathology induced by *B. asper* venom: blistering and dermonecrosis

Intramuscular or subcutaneous injection of the venom of *B. asper*, and other viperid and some elapid venoms, in human victims results in acute damage to the skin, provoking hemorrhage, dermonecrosis and blistering (Gutiérrez, 1995; Otero et al., 2002; Warrell, 2004). Such effects have been reproduced in experimental models, which have allowed the study of the mechanisms involved and the toxins responsible. A laboratory test was developed by Theakston and Reid (1983) in which rodents receive an intradermal injection of a venom or toxin, and the area of dermonecrosis is measured in the inner side of the skin 72 h after injection. The activity of venoms is expressed as the Minimum Necrotizing Dose, defined as the dose of venom that induces a necrotic area of 5 mm diameter (Theakston and Reid, 1983). This assay assesses the ability of venoms to induce dermonecrosis. Using this test, it was

shown that dermonecrosis induced by the venom of *B. asper* was completely abrogated by SVMP inhibitors, thus evidencing that in this venom SVMPs are the main components responsible for the effect (Rucavado et al., 2000).

Two investigations have addressed the mechanism of skin damage by *B. asper* SVMP BaP1 using intramuscular and intradermal routes of injection (Rucavado et al., 1998; Jiménez et al., 2008). In the dermis, BaP1 induced a hemorrhagic effect of rapid onset, associated with loss of immunostaining for capillary endothelial cell markers and BM components. Simultaneously, a marked edema developed in the dermis. Blisters also formed within the first hours of injection, with a clear separation of dermis and epidermis and with the accumulation of a proteinaceous exudate (Fig. 2). No apparent histological damage was observed in epithelial cells of epidermis, although TUNEL staining revealed widespread apoptosis in these cells at 6 h (Jiménez et al., 2008). Immunostaining of BM components revealed that laminin and type IV collagen mostly remained at the base of the blister, with little or no staining in the roof of the blister (Jiménez et al., 2008). This observation strongly suggests that blisters are formed predominantly by the proteolytic cleavage of components of the

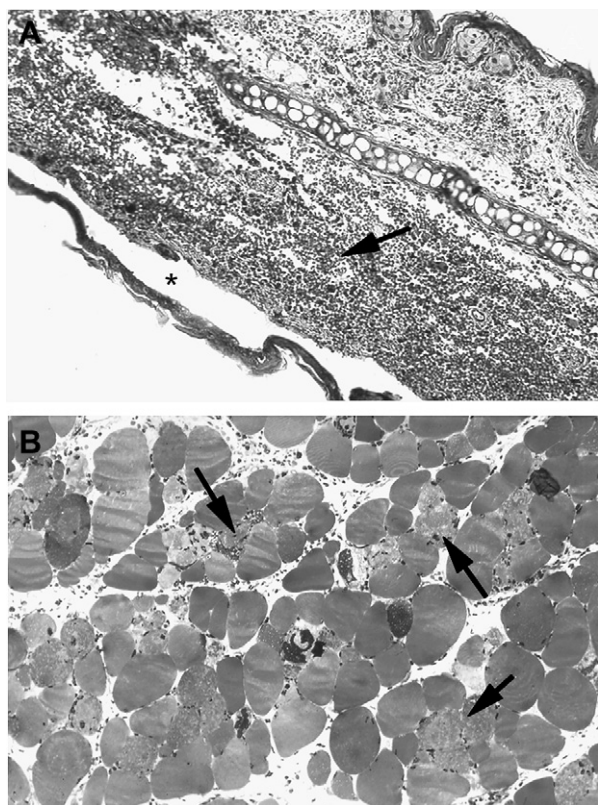
dermal–epidermal interface at the region of lamina lucida, with the consequent separation of epidermis from dermis and with components of the lamina densa and the anchoring fibrils remaining attached to the dermis. In agreement, extravasated erythrocytes are not released from the dermis to the blister fluid.

This proteolytic cleavage may be due to the direct action of the SVMP or, alternatively, to the action of endogenous proteinases synthesized and released in the tissue as a consequence of the ensuing inflammatory reaction. An increment in the expression of matrix metalloproteinase-9 (MMP-9) after injection of an SVMP was shown by zymography on gelatin gels (Rucavado et al., 1998). However, the large majority of MMP-9 and MMP-2 in the tissue corresponded to the latent forms of these enzymes, with very little activation, suggesting that MMPs are regulated and remain mostly inactive by the time blisters are developing, i.e. within the first hours. In muscle tissue injected with *B. asper* venom, activated variants of MMP-9 are observed at later time intervals (Saravia-Otten et al., 2004) and, therefore, these are not likely to be involved in the first stages of acute damage. Neutrophil and other endogenous proteinases are associated with the pathogenesis of some blistering disorders (Ray et al., 2002; Greenberg et al., 2006). However, in the case of BaP1-induced skin pathology, elimination of neutrophils, by pretreatment with an anti-granulocyte monoclonal antibody, did not affect the main pathological outcomes after toxin injection, i.e. hemorrhage and blistering (Jiménez et al., 2008). Taken together, these observations strongly suggest that skin hemorrhage and blistering induced by BaP1 depend on the direct proteolytic cleavage of capillary BM and dermal–epidermal junction components.

Blistering is followed, in many regions of the affected skin, by loss of epidermis and ulceration, with the formation of an eschar (Rucavado et al., 1998; Jiménez et al., 2008). A process of re-epithelialization ensues afterwards, with replication of epithelial cells at the borders of the eschar, and migration over the denuded skin. In this experimental model, after a period of two to four weeks, the epidermis shows a complete regeneration, with the presence of skin appendages and the absence of scar tissue and fibrosis (Rucavado et al., 1998; Jiménez et al., 2008). In parallel, the drastic reduction in the microvascular density in the dermis, as a result of the direct damage induced by BaP1 in capillary vessels, is followed by rapid angiogenesis and revascularization, with microvascular density reaching values approaching those of control tissue preparations (Jiménez et al., 2008).

## 5. Extracellular matrix degradation

Extracellular matrix constitutes a highly complex and multifunctional structure that plays a relevant role in many aspects of tissue homeostasis, such as establishment of tissue architecture and cellular spatial organization, cell migration in tissue differentiation, participation in orderly cell replacement after tissue repair or regeneration, provision of storage sites for a number of growth factors and other intercellular mediators, presence of cryptic molecular regions that release a plethora of molecules for the



**Fig. 2.** Hemorrhage, blistering and myonecrosis induced by *B. asper* SVMP BaP1. (A) Light micrograph of a section of mouse ear injected intradermally with BaP1. Abundant hemorrhage is observed in the dermis (arrow). A blister is observed (\*) with a clear separation of epidermis from the dermis. 200 $\times$ . (B) Light micrograph of a section of gastrocnemius muscle 6 h after injection of BaP1. Scattered necrotic muscle fibers are observed (arrows), together with an inflammatory infiltrate. 200 $\times$ .

regulation of many cellular processes, and regulation of extracellular volume and composition, among other roles (Kalluri, 2003). Therefore, the injection of venom hydrolases in the tissues after snakebite, with the consequent hydrolysis of extracellular matrix components, constitutes a highly relevant aspect of venom-induced local pathology. The effects of SVMPs on a specialized type of extracellular matrix, i.e. the BM, were discussed above in relation to the pathogenesis of hemorrhage and blistering. However, a more general degradation of extracellular matrix is likely to occur in these envenomations as well. In the case of *B. asper* venom, SVMPs, serine proteinases and hyaluronidases, among other components, are candidates for playing a relevant role in extracellular matrix damage. Unfortunately, with the exception of the discussed effects of hemorrhagic and non-hemorrhagic SVMPs on various BM components and fibronectin, mostly by in vitro studies, this aspect of local pathology has received little attention from an in vivo experimental standpoint.

The hydrolysis of hyaluronic acid by hyaluronidase, which has been shown to occur in vitro by *B. asper* venom (Gené et al., 1985), is likely to play a role as a spreading factor, as demonstrated by the increment in local hemorrhagic activity of a SVMP when injected with a purified hyaluronidase (Tu and Hendon, 1983). Accordingly, it has been proposed that the inhibition of hyaluronidase, by synthetic inhibitors such as cromoglycate and aurothiomalate, reduces the extent of local tissue damage induced by several snake venoms (Yingprasertchai et al., 2003). In addition, extracellular matrix degradation by SVMPs may contribute to the local and systemic diffusion of venom components (Anai et al., 2002). On the other hand, in addition to the direct action of venom hydrolases on extracellular matrix components, the inflammatory response of tissues to venom injection involves the expression of endogenous proteinases, especially MMPs (Rucavado et al., 1998; Saravia-Otten et al., 2004). However, the increments in MMP-9 correspond to the latent form of this enzyme during the acute inflammatory reaction after injection of the SVMP BaP1 (Rucavado et al., 1998). The active form of MMP-9 is observed only at later time intervals after injection of crude *B. asper* venom (Saravia-Otten et al., 2004). Experiments performed with *B. asper* venom and fibroblast cell cultures also evidenced that MMPs synthesized by these cells, upon incubation with *B. asper* venom, are activated by the action of a serine proteinase (Saravia-Otten et al., 2004). Nevertheless, injection of a purified serine proteinase from the venom of *B. asper* did not induce any evidence of local pathology in mice, nor did it activate proMMP-2 or -9 (Pérez et al., 2007), thus suggesting that venom serine proteinases probably play a very minor role in the pathogenesis of local tissue damage in these envenomations. It is therefore likely that SVMPs, and perhaps hyaluronidase, are the components that play the predominant role in the degradation of extracellular matrix in envenomations by *B. asper*. Consequently, the in situ inhibition of SVMPs, by the local injection of metalloproteinase inhibitors, represents a potentially useful therapeutic approach to prevent various aspects of venom-induced local tissue damage, such as hemorrhage, myonecrosis secondary to microvessel damage, blistering,

dermonecrosis, and extracellular matrix degradation (Escalante et al., 2000; Rucavado et al., 2000; Gutiérrez et al., 2007).

## 6. Local myonecrosis

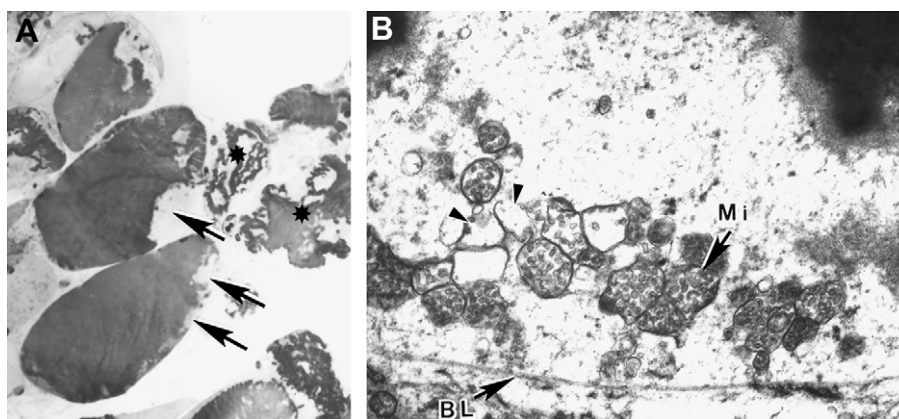
### 6.1. PLA<sub>2</sub>s and PLA<sub>2</sub> homologues are the main myotoxic components in *B. asper* venom

The venom of *B. asper* induces prominent local myonecrosis in experimental animals (Gutiérrez et al., 1980a, 1984b; Arce et al., 1991), in agreement with clinical observations (Otero et al., 2002; Warrell, 2004). This local myonecrosis is predominantly caused by a group of basic components which include PLA<sub>2</sub>s and PLA<sub>2</sub> homologues (Gutiérrez and Lomonte, 1995, 1997). They are myotoxins I and III, which are catalytically-active Asp49 PLA<sub>2</sub>s (Gutiérrez et al., 1984a; Kaiser et al., 1990), and myotoxins II and IV, which are catalytically-inactive PLA<sub>2</sub> homologues that present critical substitutions at position 49 and at residues forming the calcium-binding loop (Lomonte and Gutiérrez, 1989; Francis et al., 1991; Díaz et al., 1995; Lizano et al., 2001). The complete amino acid sequence of several of these myotoxic components has been determined (Kaiser et al., 1990; Francis et al., 1991; Lizano et al., 2001) and the crystal structure of myotoxin II, a Lys49 homologue, has been described (Arni et al., 1995). The lack of enzymatic activity in the Lys49 PLA<sub>2</sub> homologues derives from the inability of these variants to bind the calcium ion required for catalysis, owing to the substitutions occurring at residue 49 and at various residues of the calcium-binding loop (Francis et al., 1991; Arni et al., 1995; Lomonte et al., 2003a). Overall, this group of basic myotoxic components comprises ~15–35% of the total proteins in *B. asper* venom (Gutiérrez and Lomonte, 1995; Alape-Girón et al., 2008).

### 6.2. Basic myotoxic components affect the integrity of the plasma membrane of skeletal muscle fibers

Intramuscular injection of crude *B. asper* venom or these basic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues results in rapid myonecrosis, revealed by histological alterations and by an increment in the plasma activity of muscle-derived enzymes, such as creatine kinase (CK) (Gutiérrez et al., 1980a, 1984a; Lomonte and Gutiérrez, 1989). A cytotoxic activity also occurs in myoblasts and especially in myotubes in culture (Lomonte et al., 1999; Angulo and Lomonte, 2005) and in isolated muscle preparations in vitro (Gutiérrez et al., 1986a; Bultrón et al., 1993a), in agreement with observations carried out with other *Bothrops* sp. venoms and myotoxins (Melo and Suárez-Kurtz, 1988; Rodrigues-Simioni et al., 1995).

The following experimental observations strongly indicate that the initial attack of *B. asper* myotoxins on muscle fibers occurs at the plasma membrane, with a rapid and drastic perturbation in the integrity of sarcolemma: (a) At the ultrastructural level, there are abundant interruptions in the integrity of plasma membrane (Gutiérrez et al., 1984b) (Fig. 3). (b) The earliest histopathological alteration in vivo corresponds to focal lesions at the periphery of muscle fibers, known as 'delta lesions'



**Fig. 3.** Acute pathological effects induced by *B. asper* myotoxic Asp49 phospholipase A<sub>2</sub> (myotoxin I) in mouse skeletal muscle. (A) Light micrograph of muscle tissue 30 min after toxin injection. Focal lesions ('delta lesions') are observed at the periphery of muscle fibers (arrows). 350 $\times$ . (B) Electron micrograph of a peripheral portion of a muscle fiber 30 min after injection of myotoxin. The integrity of plasma membrane has been lost, and only the basal lamina remains (BL). There is a group of degenerated mitochondria (Mi) with disruption of their membranes (arrowheads). A portion of hypercontracted myofilaments is observed. 24,000 $\times$ . Reprinted from *Experimental and Molecular Pathology*, Vol. 40, Gutiérrez et al., copyright 1984, with permission from Elsevier.

(Gutiérrez et al., 1984a, 1984b) (Fig. 3). These lesions correspond to peripheral areas of cell degeneration that develop secondarily to plasma membrane damage (Mokri and Engel, 1975). (c) A rapid increment in cytosolic calcium occurs in myotubes in culture (Villalobos et al., 2007) and in muscle cells in vivo (Gutiérrez et al., 1984a), probably as a consequence of plasma membrane disruption, and loss in the control of calcium permeability. The ability of these myotoxins to affect the integrity of cell membranes has also been described in a variety of cell types in culture, as evidenced by a rapid release of cytosolic markers, such as lactic dehydrogenase (Lomonte et al., 1994c, 1999), and the incorporation of extracellular markers (Bultrón et al., 1993b; Villalobos et al., 2007; Mora et al., 2008). Moreover, *B. asper* myotoxins are capable of disrupting the integrity of liposomes by catalytically-dependent and independent mechanisms (Díaz et al., 1991, 2001; Rufini et al., 1992).

The mechanisms by which *B. asper* myotoxins disrupt the integrity of muscle cell plasma membrane remain unknown in their details. However, it is clear that catalytically-dependent and independent events are involved. Regarding enzymatically-inactive Lys49 PLA<sub>2</sub> homologues, it is evident that membrane disruption occurs without phospholipid hydrolysis. In the case of *B. asper* myotoxin II, a stretch of cationic and hydrophobic residues, located at the C-terminus of the molecule, plays a key role in membrane perturbation (Lomonte et al., 1994d, 2003a; Calderón and Lomonte, 1998). A similar mechanism has been advocated for a number of additional Lys49 PLA<sub>2</sub>s from other *Bothrops* sp. venoms (Lomonte et al., 2003a,b; Chioato et al., 2002; Chioato and Ward, 2003). Interestingly, this region also plays a determinant role in other biological activities displayed by myotoxin II, such as hyperalgesia (Chacur et al., 2003) and bactericidal activity (Páramo et al., 1998).

A structural feature characteristic of local myotoxic PLA<sub>2</sub>s is a cationic face which is likely to interact with negatively-charged phospholipid bilayers (Falconi et al., 2000; Murakami and Arni, 2003; Gutiérrez et al., 2008).

This initial electrostatic interaction is probably followed by the penetration and disorganization of the membrane by the C-terminal region (Lomonte et al., 2003a). Additional structural features might also contribute to the membrane-perturbing activity (Díaz et al., 1994). The dimeric character of many of these myotoxins would enable a shift in quaternary structure that facilitates membrane penetration (da Silva-Giotto et al., 1998). More recently, the observation of a fatty acid moiety within the structure of Lys49 PLA<sub>2</sub> homologues (de Azevedo et al., 1999; Ambrosio et al., 2005) has been proposed to induce the exposure of a knuckle constituted by hydrophobic residues, contributing to the ability of these proteins to disturb the bilayer (Ambrosio et al., 2005).

In the case of *B. asper* myotoxins I and III, which are Asp49 catalytically-active PLA<sub>2</sub>s, phospholipid hydrolysis plays a critical role in muscle fiber plasma membrane destabilization, as revealed by a prominent drop in myotoxicity in conditions that inhibit phospholipase A<sub>2</sub> activity (Bultrón et al., 1993b; Díaz-Oreiro and Gutiérrez, 1997). The role played by enzymatic phospholipid hydrolysis may be associated with the perturbation of membrane integrity, secondary to hydrolysis, or to the effect exerted by the products of phospholipid degradation, i.e. lysophospholipids and fatty acids (Montecucco et al., 2008). Lysolecithin has been shown to induce sarcolemmal disruption and myonecrosis (Pestronk et al., 1982) and to affect the function of membranes in a number of ways (Montecucco et al., 2008). In addition, *B. asper* myotoxic Asp49 PLA<sub>2</sub>s are still capable of inducing myotoxicity even in conditions that abrogate phospholipase activity (Gutiérrez et al., 1986a; Bultrón et al., 1993b), thus suggesting that molecular regions distinct from the catalytic site are able to interact and disrupt the integrity of muscle fiber plasma membrane. The identity of these molecular regions remains largely unknown, although it is likely that they may comprise hydrophobic-cationic stretches similar to the ones present in the C-terminus of Lys49 PLA<sub>2</sub> homologues. Observations with myotoxic Asp49 PLA<sub>2</sub>s agree with the general



hypothesis proposed by Kini and Evans (1989) to explain the pharmacological profile of venom PLA<sub>2</sub>s, i.e. that these enzymes have molecular regions that determine their toxicity and tissue specificity in addition to the catalytic activity.

### 6.3. Are *B. asper* myotoxins 'specific' for muscle cells?

When the first myotoxic PLA<sub>2</sub> from the venom of *B. asper* was isolated (Gutiérrez et al., 1984a) it was named '*B. asper* myotoxin', implicitly assuming that the main toxic activity of this enzyme is cytotoxicity on muscle fibers. However, further studies evidenced that this, and other basic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues present in this venom, exerted a wider cytotoxic profile, affecting many different cell types (Bultrón et al., 1993b; Lomonte et al., 1994c), although mature muscle cells and myotubes showed the highest susceptibility to these toxins (Lomonte et al., 1999; Angulo and Lomonte, 2005). The widespread cytotoxic profile of these molecules has been ascribed to their ability to interact with low-affinity sites, probably phospholipids, in the plasma membrane of a variety of cell types. This property is likely to depend on the presence of a cationic face in these toxins which enables them to interact with negatively-charged phospholipids in membranes (Falconi et al., 2000; Murakami and Arni, 2003; Gutiérrez et al., 2008). The role of negatively-charged phospholipids as low-affinity 'acceptors' of *B. asper* myotoxins is supported by the higher susceptibility to these toxins of liposomes made of negatively-charged phospholipids (Díaz et al., 1991, 2001; Rufini et al., 1992), and by the observation that erythrocytes are rendered susceptible to the lytic activity of these toxins by the addition of phosphatidylserine to their membrane, thus increasing the density of negatively-charged phospholipids (Díaz et al., 2001).

On the other hand, the high susceptibility of muscle fibers to these toxins may be a consequence of the presence of 'acceptors' of higher affinity, perhaps of protein nature, in the muscle fiber membrane. The identity of these 'acceptors' remains largely unknown. The M-type PLA<sub>2</sub> receptor identified in rabbit skeletal muscle (Lambeau et al., 1990) does not seem to play a relevant role in myotoxicity induced by *B. asper* myotoxin II (G. Lambeau, personal communication). Recently, a high affinity binding has been described between Lys49 PLA<sub>2</sub> homologues, including *B. asper* myotoxin II, and vascular endothelial growth factor receptor-2 (VEGFR-2) (Fujisawa et al., 2008), although the functional implications of such finding are not clear at present.

Myotoxic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues present in *B. asper* and other *Bothrops* sp. venoms are mostly 'locally-acting myotoxins', in the sense that their intramuscular injection results in necrosis in muscles located in the vicinity of the injection site, with little action on distant ones (Gutiérrez and Ownby, 2003; Gutiérrez et al., 2008). In addition, intravenous injection of these myotoxins results in very little increment in plasma CK activity (Moreno and Gutiérrez, 1988; Gutiérrez et al., 2008). A toxicokinetic analysis confirmed the local concentration of *B. asper* myotoxin I at the injected muscle (Moreno and Gutiérrez, 1988). This situation contrasts with what has

been described for other myotoxic PLA<sub>2</sub>s, such as crotoxin, from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Kouyoumdjian et al., 1986; Salvini et al., 2001), and a number of class I myotoxic PLA<sub>2</sub>s isolated from elapid venoms (Fohlman and Eaker, 1977; Ponraj and Gopalakrishnakone, 1996). These PLA<sub>2</sub>s induce both local and systemic myotoxicity, owing to the higher selectivity of these toxins for muscle fibers and to their ability to act on muscles distant from the site of injection. These 'systemically-acting myotoxins' provoke rhabdomyolysis and prominent increments in plasma CK activity and in myoglobin, resulting in myoglobinuria (Ponraj and Gopalakrishnakone, 1996). This dichotomy between local and systemic myotoxicity has a well-defined clinical correlate, since envenomations by *B. asper*, and other *Bothrops* species, results in prominent local myonecrosis, but no systemic myotoxicity (Otero et al., 2002; Gutiérrez, 1995; Warrell, 2004). In contrast, envenomations by *C. d. terrificus* and several elapid snakes result in rhabdomyolysis and acute renal failure (Azevedo-Marques et al., 1987; Warrell, 1996). Such drastically different toxicologic and clinical profiles seem to depend on how specific these types of myotoxic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues are for muscle fiber membranes.

### 6.4. Plasma membrane perturbation is followed by a prominent calcium influx and a stereotyped sequence of cellular pathological effects

The rapid and drastic plasma membrane perturbation induced by *B. asper* myotoxic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues promotes an influx of calcium from the extracellular fluid to the cytosol, rapidly elevating the cytosolic concentration of this cation (Gutiérrez et al., 1984a; Villalobos et al., 2007; Montecucco et al., 2008). Such calcium influx triggers a number of deleterious cellular processes that rapidly bring the cell beyond the 'point of no return'. The most important consequences of such calcium increment are: (a) hypercontraction of myofilaments, resulting in the formation of amorphous masses of myofibrils and cytoplasmic spaces devoid of myofilaments (Gutiérrez et al., 1984b). In addition to impairing the mechanical integration of the contractile apparatus, such drastic hypercontraction induces further mechanical disruption of the integrity of the plasma membrane, thus promoting a vicious circle that results in more calcium entering the cell. (b) Mitochondria begin to transport excess calcium, through the action of the uniporter and using the proton gradient that exists across the inner mitochondrial membrane. This affects the synthesis of ATP and results in the accumulation of large amounts of calcium in the mitochondrial matrix, with the formation of hydroxyapatite crystals (Gopalakrishnakone et al., 1984). As a consequence, mitochondria become swollen and flocculent densities appear in their matrix; furthermore, mitochondrial membranes appear interrupted. It is likely that these alterations also result in the formation of the 'permeability transition pore', with the release of 'death factors' from mitochondria (Dong et al., 2006; Montecucco et al., 2008). In the end, mitochondria become irreversibly damaged and ATP synthesis is severely hampered. (c) Activation of calcium-dependent proteinases

and phospholipases A<sub>2</sub>. Calpains, which are calcium-dependent cysteine proteinases, become activated and hydrolyze a series of cellular substrates, particularly cytoskeletal components responsible for the mechanical integration of myofibrillar proteins (Dargelos et al., 2008). Early loss of immunostaining of desmin and  $\alpha$ -actinin was described in muscle injected with a myotoxic PLA<sub>2</sub> from *B. asper* venom (Gutiérrez et al., 1990). On the other hand, increments in cytosolic calcium may result in the activation of calcium-dependent cytosolic PLA<sub>2</sub>s, which would further degrade phospholipids from plasma membrane and the membranes of intracellular organelles, such as mitochondria, sarcoplasmic reticulum and T tubules (Montecucco et al., 2008).

#### 6.5. What is the role of ischemia in myonecrosis induced by *B. asper* venom?

Incubation of *B. asper* venom with polyclonal antibodies against a myotoxic PLA<sub>2</sub> reduces the myotoxic activity of this venom by approximately 70%, confirming the antagonistic role played by these molecules in myonecrosis (Lomonte et al., 1987). However, the fact that there is still 30% of muscle damage in these conditions reveals that other venom components also contribute to acute muscle damage. Experimental observations demonstrate that hemorrhagic SVMPs, such as *B. asper* enzymes BaH1 and BaP1, induce myonecrosis as well (Gutiérrez et al., 1995b; Rucavado et al., 1995) (Fig. 2). The mechanism by which hemorrhagic SVMPs cause myonecrosis has not been firmly established, although a very plausible hypothesis is that they promote muscle damage secondary to the ischemia resultant from the action of these SVMPs on the microvasculature (Gutiérrez et al., 1995b).

The increment demonstrated in the levels of lactic acid in muscle injected with BaH1, and the lack of in vitro myotoxicity of this enzyme in muscle preparations in conditions where oxygen is supplied support this hypothesis (Gutiérrez et al., 1995b). Thus, the interruption of blood flow and perfusion in a tissue in which the microvasculature has been severely hampered by the action of SVMPs is a mechanism that contributes to local myonecrosis. In addition, other vascular alterations, such as angioneurosis and thrombosis, may further impair tissue perfusion. Finally, the increment in muscle intracompartmental pressure, as a consequence of the prominent extravasation associated with edema, inflammation and bleeding, might be another contributing factor to ischemia. There have been no experimental studies on the role of increments in intracompartmental pressures in myonecrosis in the case of *B. asper* envenomations. However, a study performed in pigs with the venom of *Crotalus atrox* demonstrated that surgical decompression reduces the increment in intracompartmental pressure, but increases the extent of myonecrosis (Tanen et al., 2004), thus suggesting that, at least in this model, muscle necrosis is more the result of the direct toxic activity of venom components than the indirect effect of compression-induced ischemia. Owing to its evident clinical implications, this is a subject that requires further experimental analysis.

## 7. Ontogenetic variations in venom and implications in local tissue damage

Pronounced differences have been described between the venoms of newborn/neonate specimens and those of adult specimens of *B. asper* from Costa Rica and Colombia (Gutiérrez et al., 1980b; Chaves et al., 1992; Saldarriaga et al., 2003; Alape-Girón et al., 2008). Regarding local effects, the venoms of newborn specimens induce stronger hemorrhagic and edema-forming activities than those of adults, whereas the latter induce stronger myonecrosis (Gutiérrez et al., 1980b; Saldarriaga et al., 2003). Such different pathophysiological profiles can be understood in the light of the proteomes of these venoms, since those from newborns have a large content of P-III SVMPs and a very low content of myotoxic basic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues. In contrast, venoms from adults present a relatively low content of P-III SVMPs, and a high amount of myotoxic PLA<sub>2</sub>s (Lomonte and Carmona, 1992; Alape-Girón et al., 2008). Thus, venoms from newborn *B. asper* have a predominantly 'vasculotoxic' profile, whereas those from adults present a predominantly 'myotoxic' profile. The clinical implications of these findings, however, have to be analyzed with caution, since the volume of venom delivered by newborn and adult specimens is very different.

## 8. Does inflammation contribute to local pathology?

Injection of *B. asper* venom, and viperid venoms in general, results in a complex and multifactorial inflammatory reaction (Farsky et al., 2005). The description and mechanisms of inflammatory events induced by *B. asper* venom are covered in detail in another contribution of this issue (see Teixeira et al., this issue) and, therefore, are beyond the scope of this review. However, the possible pathological consequences of such inflammatory reaction need to be considered herein. Owing to the extent of such reaction, and to the well-known pathological role that some inflammatory pathways and mediators play in tissue damage in many diseases (Gallin and Snyderman, 1999), it has been often assumed that local inflammation contributes to the local pathological effects in viperid snakebite envenomation. A number of studies have addressed this issue in the case of *B. asper* venom by an experimental approach in which specific inflammatory cells and mediators have been depleted or blocked, and the effects of these interventions in the extent of local pathology have been assessed.

### 8.1. Role of neutrophils

As part of a typical inflammatory reaction after injection of *B. asper* venom or myotoxins, neutrophils accumulate in the affected tissues within the first hours of venom injection, with a peak at 24 h, in the case of acute muscle damage (Gutiérrez et al., 1986b, 1990; Teixeira et al., 2003b). Elimination of neutrophils in a mouse model, by administration of a monoclonal rat antibody against mouse granulocytes, has been used to assess the role of these cells in the local pathology induced by *B. asper* venom and a myotoxin (Teixeira et al., 2003b). Results clearly show that

the extent of local edema, hemorrhage and myonecrosis in neutrophil-depleted animals did not differ from the extent of damage in mice having neutrophils, strongly suggesting that these cells do not participate in the pathogenesis of tissue damage, at least in this model. The only difference observed between these groups of mice occurred in skeletal muscle regeneration, since neutropenic mice presented a deficient and delayed regenerative response, as compared to control mice (Teixeira et al., 2003b). This evidences that neutrophils play a relevant role in muscle regeneration, probably by removing necrotic debris and providing mediators that stimulate macrophage recruitment and other aspects of the regenerative response.

### 8.2. Role of cytokines

Injection of *B. asper* venom, myotoxins or hemorrhagic toxins results in the increments of several cytokines in the affected tissue and in blood (Lomonte et al., 1993; Rucavado et al., 2002; Petricevich et al., 2000; Chaves et al., 2005). The highest increments in muscle tissue were described for IL-6 and IL-1 $\beta$ , whereas TNF- $\alpha$  did not show elevations (Rucavado et al., 2002). When a dose of *B. asper* venom corresponding to one LD<sub>50</sub> was injected by the intraperitoneal route, there were increments in the serum levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and IFN- $\gamma$  (Petricevich et al., 2000). Studies with the SVMP jararhagin suggest that TNF- $\alpha$  and IL-6, but not IL-1 $\beta$ , play a role in the local dermonecrotic effect induced by this toxin (Moura-da-Silva et al., 1996; Laing et al., 2003). However, pretreatment of mice with pentoxifylline, an inhibitor of the transcription of TNF- $\alpha$ , or with antibodies against TNF- $\alpha$ , IL-1 $\beta$  or IL-6 did not modify the extent of local edema, hemorrhage, dermonecrosis or myonecrosis induced by *B. asper* venom in mice (Chaves et al., 2005), thus not supporting a role for these cytokines in the pathogenesis of the acute local tissue damage induced by this venom. However, it is necessary to assess the role of cytokines in other aspects of local pathology in *B. asper* envenomation.

### 8.3. Role of nitric oxide

Local and systemic increments in nitric oxide (NO) have been documented after injection of *B. asper* venom in mice (Petricevich et al., 2000; Chaves et al., 2006). Inhibition of NO synthase by pretreatment of mice with L-NAME and aminoguanidine did not reduce the extent of local hemorrhage and myonecrosis induced by *B. asper* venom, but reduced the extent of local edema, albeit to a limited extent (Chaves et al., 2006). Thus, this mediator does not participate in the local pathological effects, but contributes to the pharmacological processes that lead to edema, together with other inflammatory mediators.

Taken together, the experimental assessment of the role played by neutrophils, cytokines and NO in the local tissue damage induced by *B. asper* venom strongly suggests that these inflammatory cells and mediators do not contribute to a significant extent in the pathogenesis of acute local hemorrhage and myonecrosis in our animal models. The scenario that emerges from these observations is one in which the local pathology is mostly caused by the direct

action of venom PLA<sub>2</sub>s and SVMPs in the tissue. The prominent inflammatory reaction that ensues is likely to participate in the processes of tissue repair and regeneration. Therefore, the manipulation of the local inflammatory response has to be analyzed in the light of this hypothesis, in order to promote its favorable aspects that contribute to tissue regeneration (Gutiérrez et al., 2007). Clearly, more research is required in other aspects of local inflammation and in the development of additional experimental models, in order to obtain a more complete view of the role of inflammation in *B. asper* venom-induced local pathology. For instance, the assessment of the role played by macrophages and mast cells is pending, as well as the possible involvement of complement in these alterations.

## 9. The interplay between local pathology and local infection in *B. asper* envenomation

Local infection occurs in a percentage of patients envenomated by *B. asper* and other viperid species (Otero et al., 1992, 2002; Jorge et al., 1994; Avila-Agüero et al., 2001), and local abscess formation is a risk factor for amputation in human snakebite victims (Saborío et al., 1998; Jorge et al., 1999). Bacteria isolated from local abscesses in these patients include species present in the mouth and venom of the snakes (Arroyo and Bolaños, 1980; Jorge et al., 1994), as well as in the skin of the victims, such as *Staphylococcus aureus* (Avila-Agüero et al., 2001; Otero et al., 2002). Such local infection, which leads to cellulitis, local abscesses and, in some cases, sepsis, demands the use of antibiotics as a frequent therapeutic intervention in the management of these envenomations (França and Málague, 2003; Gutiérrez, 1995). The role of venom-induced local pathology in promoting local infection by *S. aureus* was investigated in a mouse model. Results clearly showed that local tissue damage induced by either *B. asper* venom, a myotoxin or a hemorrhagic SVMP strongly favors staphylococcal infection (Saravia-Otten et al., 2007). It was proposed that the binding of bacteria to soluble tissue factors released as a result of the action of venom components may hamper the recognition of bacteria by the immune mechanisms. Moreover, the degradation of innate immune host factors by venom proteinases may also contribute to the infection (Saravia-Otten et al., 2007). Thus, there is an evident interplay between local pathological alterations induced by venom toxins and local infection by bacteria: tissue damage clearly favors local infection and, in turn, local infection further enhances local pathology in these envenomations.

## 10. Towards an integrative scenario: multiple interacting pathological events and multiple tissue responses mediate a complex outcome

The injection of a viperid snake venom, such as that of *B. asper*, in muscle tissue results in a multiplicity of cellular and tissue reactions, some of which are the consequence of the direct action of venom components whereas others depend on the tissue responses that follow venom-induced damage. An additional observation is that venom PLA<sub>2</sub>s and SVMPs, which promote cell and tissue damages, may also

induce cell activation in some circumstances. For instance, a Lys49 PLA<sub>2</sub> homologue from *B. asper* venom induces cellular responses as varied as necrosis, apoptosis and proliferation in a lymphoblastoid cell line in culture, depending on the concentration of the toxin (Mora et al., 2005), and these diverse effects are associated with variable levels of cytosolic calcium increments (Mora et al., 2006). The ability of *B. asper* myotoxins to activate macrophages, when tested at sub-cytotoxic concentrations, has been also documented (Zuliani et al., 2005), as well as the increment in the expression of a number of genes in fibroblasts incubated with sub-cytotoxic concentrations of *B. asper* SVMP BaP1 (unpublished results), similarly to what has been described for the action of jararhagin and *B. jararaca* venom in fibroblasts and endothelial cells (Gallagher et al., 2003, 2005). Moreover, P-III SVMPs from other *Bothrops* sp. venoms activate endothelial cells (Schattner et al., 2005). These observations suggest that different cell types may have different thresholds for different cellular responses when incubated with a particular toxin. Therefore, depending on the concentration reached by a particular toxin in a particular tissue location, and also depending on the susceptibility/reactivity of a specific cell type to a given toxin, cellular responses after injection of venom may vary from overt necrosis, to apoptosis, to activation leading to proliferation or synthesis of mediators of various sorts.

The simplistic view that injection of a viperid venom into a tissue only results in widespread direct damage to cellular and extracellular matrix components has to be substituted by a more integrative scenario in which different cells respond in different ways to different toxins. In the tissue regions that may be considered 'ground zero', i.e. where the maximum concentration of venom components is attained, it is likely that cytotoxic and tissue-destructive effects greatly predominate. However, in more peripheral areas of the envenomated tissue, where the concentration of venom components may be lower, a mixed response may occur. Thus, some cells may reach thresholds of cytotoxicity, by either necrosis or apoptosis, whereas other cell types may not be directly damaged and, instead, may respond by activation or proliferation. Likewise, degradation of extracellular matrix in heavily damaged regions might be widespread, whereas in other regions it might give rise to fragments of proteins that may induce diverse cellular responses. Such integrative scenario may help to understand the responses associated with direct and acute tissue damage and those associated with the onset of processes responsible for tissue repair, remodeling and regeneration. Consequently, a temporal and spatial perspective in the tissue response to envenomation has to be considered for an integrated analysis of local alterations in these pathological models. The study of venom-induced tissue alterations from this broader perspective demands novel hypotheses and renewed research efforts.

## 11. Beyond tissue damage: reparative and regenerative events

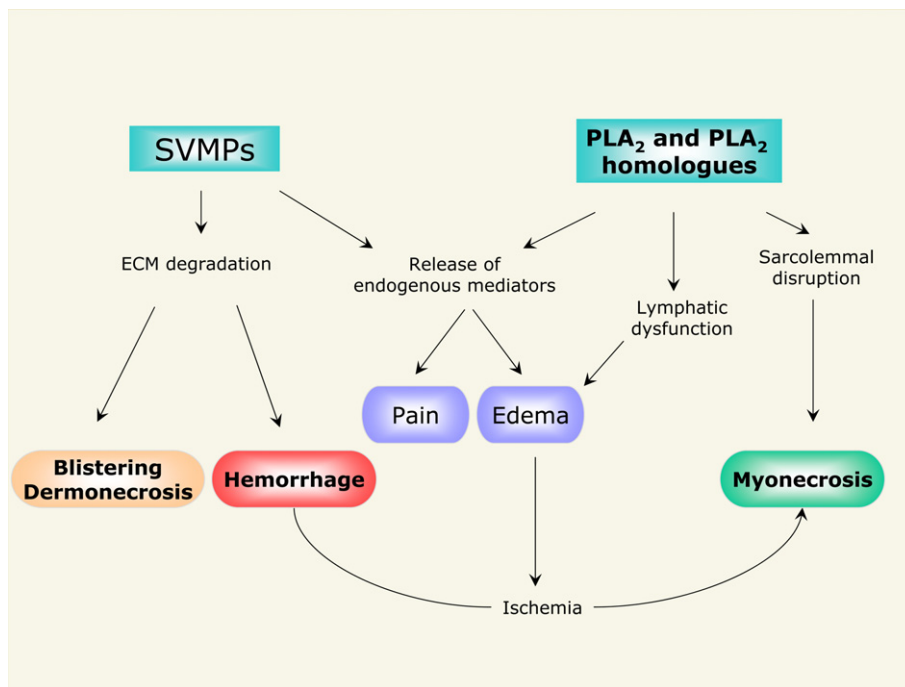
Acute local pathology after injection of *B. asper* venom is characterized, as described above, by prominent

myonecrosis, dermonecrosis, hemorrhage, and damage of larger vessels, lymphatic vessels and nerves. Such acute local events are followed by a complex inflammatory reaction, characterized by the infiltration of inflammatory cells, mostly neutrophils and macrophages. As a consequence, necrotic tissue is removed and this is followed by reparative and regenerative processes. The final outcome of local pathology after injection of *B. asper* venom, and other viperid snake venoms, may vary from a largely complete restitution of form and function, in mild and some moderate cases, to a deficient reparative/regenerative response that ends up in permanent tissue loss and deficient functional recovery, a common consequence of severe envenomations (Otero et al., 2002; Gutiérrez and Lomonte, 2003; Warrell, 2004). Therefore, the analysis of the factors that mediate tissue repair and regeneration is highly relevant for the design of interventions aimed at promoting a more successful regenerative response.

### 11.1. Muscle regeneration is adequate after myotoxin-induced necrosis but is impaired after venom-induced tissue damage

When mice are injected with purified myotoxic PLA<sub>2</sub>s or PLA<sub>2</sub> homologues isolated from *B. asper* and other *Bothrops* sp. venoms, acute muscle necrosis is followed by successful muscle regeneration (Gutiérrez et al., 1984c, 1989, 1991). After necrosis, neutrophils and macrophages infiltrate the tissue and remove the necrotic debris. Concomitantly, muscle satellite cells, which are myogenic cells present at the periphery of muscle fibers, within the BM but outside the myocyte plasma membrane, are activated and become myoblasts. In addition to satellite cells, myoblasts can also arise from other non-myogenic cells, such as mesenchymal stem cells and bone marrow cells (Grounds, 1999). Myoblasts proliferate, by the action of a variety of mediators released in the affected tissue, and eventually they start a process of cell fusion that ends up with the formation of multinucleated myotubes (Grounds, 1999). These proliferative and fusion processes occur within the space demarcated by the BM of the necrotic muscle fibers, thus guaranteeing the maintenance of tissue architecture. Upon the formation of myotubes, these cells mature to become regenerated adult muscle fibers, although they maintain the nuclei located in a central position for a prolonged time span. All these processes are coupled to the regeneration of neuromuscular junctions (see Harris, 2003 for a discussion of muscle regeneration after venom PLA<sub>2</sub>-induced necrosis). The good regenerative response observed after injection of myotoxic PLA<sub>2</sub>s can be explained on the grounds that the factors required for a successful muscle regeneration, i.e. adequate blood supply, innervation, removal of necrotic debris, and permanence of BM, are fulfilled in these conditions (Gutiérrez et al., 1984c, 1991; Harris, 2003).

In contrast, when mice are injected with crude *B. asper* venom, or purified hemorrhagic SVMPs, the process of muscle regeneration is partially impaired (Gutiérrez et al., 1984c, 1995b; Arce et al., 1991; Rucavado et al., 1995), a phenomenon also described for other *Bothrops* sp. venoms (Queiroz et al., 1984; Salvini et al., 2001; Santo-Neto and Marques, 2005). In these conditions, areas of



**Fig. 4.** Diagrammatic summary of the experimental pathogenesis of local tissue damage induced by *B. asper* venom. The main types of toxins responsible for these effects are metalloproteinases (SVMPs) and phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) and PLA<sub>2</sub> homologues. SVMPs degrade extracellular matrix components and induce hemorrhage and skin damage (blistering and dermonecrosis). PLA<sub>2</sub>s and PLA<sub>2</sub> homologues, in turn, induce direct damage to the plasma membrane of skeletal muscle fibers, causing acute myonecrosis, and also damage the walls of collecting lymphatic vessels. Myonecrosis is also provoked by ischemic alterations that develop secondary to local hemorrhage and to the increment in intracompartamental pressures as a result of extensive extravasation. Both SVMPs and PLA<sub>2</sub>s promote the synthesis and/or release of a wide variety of inflammatory mediators (kinins, eicosanoids, nitric oxide, cytokines, complement anaphylatoxins, matrix metalloproteinases, etc.) which induce a strong local inflammatory reaction associated with edema and pain (hyperalgesia and allodynia). The effect of myotoxic PLA<sub>2</sub>s on collecting lymphatic vessels also contributes to edema.

necrotic tissue remain for several days after venom injection, revealing an evident delay in the removal of necrotic debris by phagocytes. In addition, crude venom and isolated hemorrhagic SVMPs drastically affect the microvasculature, as revealed by a decrease in the microvascular density (Gutiérrez et al., 1984c; Escalante et al., 2006). A similar scenario has been described after local myonecrosis induced by *B. jararacussu* venom (Santo-Neto and Marques, 2005). Moreover, *B. asper* venom affects intramuscular nerves (Arce et al., 1991), an observation also performed with the venom of *B. jararacussu* (Queiroz et al., 2002). As a consequence, areas of damaged muscle are replaced by fibrotic tissue, and even in areas where regenerative muscle fibers are observed, they have a small diameter, evidencing an incomplete regeneration and atrophy (Gutiérrez et al., 1984c; Queiroz et al., 1984, 2002; Arce et al., 1991). Interestingly, microscopic observations indicate that some small regenerative fibers present evidences of degeneration (Arce et al., 1991), suggesting that these cells may be halted in their differentiation, probably as a consequence of the lack of adequate microenvironmental conditions required for appropriate regeneration.

It has been postulated that such poor regeneration observed after *B. asper* venom injection is mostly the consequence of the drastic effect of venom components in the microvasculature and intramuscular nerves (Gutiérrez et al., 1984c; Arce et al., 1991). This hypothesis is supported by the observation that inhibition of hemorrhagic activity

of *B. asper* venom significantly improves the regenerative outcome (Gutiérrez et al., 1986c). In addition, the injection of a combination of a myotoxic PLA<sub>2</sub> and a hemorrhagic SVMP reproduces the poor regenerative outcome observed when crude venom is injected (Gutiérrez et al., 1995b). Whether this effect induced by hemorrhagic SVMPs depends on the reduction of blood supply to regenerating cells, or whether it depends on other actions induced by SVMPs in the tissue, remains to be investigated. Moreover, the components that affect intramuscular nerves have yet to be identified, together with the analysis of the role of additional venom components, i.e. hyaluronidases and other proteinases that affect the extracellular matrix, in the regenerative process. The understanding of the factors affecting regeneration in venom-induced myonecrosis may contribute to the manipulation of such process by the addition, or inhibition, of tissue mediators, with the aim of improving the regenerative outcome in patients envenomed by *B. asper* and other viperid species. Owing to the effect of SVMPs in the impairment of muscle regeneration, it is likely that the administration of factors that promote angiogenesis and revascularization might contribute to a better regenerative response.

## 12. Concluding remarks

The complexity of the local pathological changes induced by the injection of *B. asper* venom has been

partially unraveled by the studies reviewed in this work (Fig. 4). Some basic ideas emerge from this analysis: (a) Local tissue damage is mainly provoked by the direct action of venom components in the tissues, especially by the action of SVMPs in microvessels and extracellular matrix and of myotoxic PLA<sub>2</sub>s and PLA<sub>2</sub> homologues in skeletal muscle fibers and lymphatic vessels. The extremely rapid onset of these effects precludes their neutralization by antivenoms, especially when there is a delay in antivenom administration (Gutiérrez et al., 1998). (b) The tissue response to the venom goes beyond the direct pathological effects, as it includes a prominent inflammatory response and the activation of resident and infiltrating cells which synthesize and release a plethora of mediators that influence the outcome of tissue derangement. Further studies are required to fully understand the pathogenesis of local pathology induced by *B. asper* venom and the role played by endogenous inflammatory and reparative processes in these alterations.

Such investigations may pave the way for the development and application of novel inhibitors that could be injected at the site of venom injection in field conditions, and which may halt the progression of local tissue damage by inhibiting SVMPs and PLA<sub>2</sub>s in situ, rapidly after the bite (Gutiérrez et al., 2007). Furthermore, these studies will identify endogenous inflammatory and regenerative events that can be modulated in order to prevent deleterious effects of local tissue responses and, at the same time, promote cellular and tissue processes that result in improved regeneration (Gutiérrez et al., 2007). A better understanding of the local pathology induced by *B. asper* venom will be of value for understanding viperid snakebite envenomation in general, owing to the common processes involved in local tissue damage by many viperid species. In addition, an in-depth understanding of this pathological model may also contribute to illuminate the processes of tissue damage from a broader perspective, highlighting mechanisms involved in tissue pathology and repair in other diseases.

## Acknowledgements

The authors thank their colleagues and students at Instituto Clodomiro Picado and other laboratories who have contributed to the many of the studies reviewed in this communication. Our investigations on local pathology induced by snake venoms have been supported by Vice-rectoría de Investigación (Universidad de Costa Rica), NeTropica, International Foundation for Science (IFS), AID, and the Wellcome Trust.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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