Inflammatory effects of snake venom metalloproteinases

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Metalloproteinases are abundant enzymes in crotaline and viperine snake venoms. They are relevant in the pathophysiology of envenomation, being responsible for local and systemic hemorrhage frequently observed in the victims. Snake venom metalloproteinases (SVMP) are zinc-dependent enzymes of varying molecular weights having multidomain organization. Some SVMP comprise only the proteinase domain, whereas others also contain a disintegrin-like domain, cysteine-rich, and lectin domains. They have strong structural similarities with both mammalian matrix metalloproteinases (MMP) and members of ADAMs (a disintegrin and metalloproteinase) group. Besides hemorrhage, snake venom metalloproteinase induce local myonecrosis, skin damage, and inflammatory reaction in experimental models. Local inflammation is an important characteristic of snakebite envenomations inflicted by viperine and crotaline snake species. Thus, in the recent years there is a growing effort to understand the mechanisms responsible for SVMP-induced inflammatory reaction and the structural determinants of this effect. This short review focuses the inflammatory effects evoked by SVMP.

Key words: metalloproteinase - snake venom - inflammation

Envenomation by snakes of the family Viperidae is characterized by prominent local effects, including necrosis, hemorrhage, edema, and pain, which develop rapidly after the accident and often result in permanent sequelae (Gutiérrez & Lomonte 1989, 1997, Dart et al. 1992, Warrell 1995). In addition, systemic alterations such as hemorrhage, coagulopathy, shock, and acute renal failure may occur. Both local and systemic effects of these snake venoms have been associated with the action of a variety of venom components, which include metalloproteinases (Ownby 1990, Gutiérrez & Lomonte 1997).

Snake venom metalloproteinases (SVMP) comprise a subfamily of zinc-dependent enzymes of varying molecular mass, which are responsible for the hemorrhagic effect characteristic of viperine and crotaline snake envenomations (Bjarnason & Fox 1994, Kamiguti et al. 1998, Hati et al. 1999). In addition, more recent investigations have evidenced that these enzymes are also involved in the pathogenesis of local myonecrosis (Gutierrez et al. 1995, Rucavado et al. 1999), skin damage (Rucavado et al. 1998), and inflammatory reaction (Gutiérrez et al. 1995, Moura da Silva et al. 1996). Although anti-venom is the recognized therapy for systemic envenoming, it is unable to neutralize the toxins responsible for the rapid onset of local tissue damage by snake venoms. Therefore, in the recent years there is a growing effort to understand the mechanisms responsible for SVMP-induced inflammatory reaction.

The SVMP are members of the Reprolysin subfamily of zinc metalloproteinases, which also includes a group of mammalian homologous proteins, a disintegrin and metalloproteinase (ADAM) (Fox & Long 1998). In turn, SVMP are part of the "metzincin" family of zinc-dependent metalloproteinases, together with matrix metalloproteinases (MMP), astacins, and serralysins, all of them exhibiting an identical zinc-dependent motif, with the sequence HEXXHXXGXXH and the presence of a methionine turn (Bode et al. 1993). SVMP can be divided into four classes depending on their domain organization (Hite et al. 1994): P-I, comprising only the metalloproteinase domain; P-II, having a metalloproteinase domain followed by a disintegrin-like domain; P-III, comprising metalloproteinase, disintegrin-like and cysteine-rich domains, and P-IV, containing additionally a lectin-like domain linked by disulfide bonds. The disintegrin-like domain shows high sequence identity with venom disintegrins (Markland 1998), which are proteolytically released from P-II precursors. However, the disintegrin-like domain does not contain the typical RGD sequence found in disintegrins, showing different sequences in this region (Paine et al. 1992, Bjarnason & Fox 1994, Hite et al. 1994). All these metalloproteinases are synthesized as zymogens, and are proteolytically processed to yield the active enzyme (Bjarnason & Fox 1994).

Studies on the crystal structures of venom P-I class metalloproteinases reveal similar structures, being ellipsoidal molecules with a shallow active site clef that separates a lower subdomain from the main domain composed of five stranded β-sheets and four α-helices (Gomis-Rüth et al. 1993, Zhang et al. 1994, Kumasaka et al. 1996, Gong et al. 1998). The catalytic Zn^{2+} ion in the active-site clef is surrounded by three His residues (142, 146, and 145) and a water molecule is anchored to Glu143 in a tetrahedral manner.

Hemorrhagic activity has been associated with enzymatic proteolytic activity, since chelation of the zinc atom abolishes both proteolytic and hemorrhagic effects (Bjarnason & Tu 1978, Bjarnason & Fox 1994). The role of the others domains in the toxicity of high molecular weight

Financial support: Fapesp (grant 02/13863-2), CNPq
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Received 8 November 2004
Accepted 30 DEcmber 2004

enzymes is not clear, although it has been shown that large hemorrhagic metalloproteinases having disintegrin-like and high-cysteine domains, are more active at inducing hemorrhage than enzymes comprising only the metalloproteinase domain (Bjarnason & Fox 1994, Hite et al. 1994). Interestingly, there are metalloproteinases in snake venoms which are devoid of hemorrhagic activity (Willis & Tu 1988, Markland 1998). The structural basis of this observation is not clear, although some comparative studies have identified residues which may be required to exert this activity (Takeya et al. 1990, Hite et al. 1992, Ramos & Selistre-de-Araújo 2004).

P-III class SVMP have strong similarities in sequence and domain organization with ADAM (Fox & Long 1998). Members of the ADAM group have been implicated in the control of several physiological events including cytokine shedding and cell migration. (Blobel 1997, Peschon et al. 1998). In addition, increased expression of some mammalian MMP has been found in a variety of inflammatory conditions such as rheumatoid arthritis and periodontal diseases (Cawston 1996, Cawston & Billington 1996). Therefore, mechanisms involved in the proinflammatory action of mammalian metalloproteinases and SVMP are under active investigation. This short review deals with the proinflammatory effects evoked by SVMP and their mechanisms of action.

Inflammatory effects

Besides inducing hemorrhage and myonecrosis, venom metalloproteinases play a relevant role in the complex and multifactorial inflammatory response characteristic of viperine envenomation.

The first experimental evidence that SVMP cause inflammation was provided by Gutierrez et al. (1995), who showed that a P-I class SVMP isolated from *Bothrops asper* venom (BaP1) induced paw edema in mice. Upon intramuscular injection, this weakly hemorrhagic SVMP induced formation of blisters and infiltration of leukocytes into dermis. This was associated with degranulation of mast cells and enlarged macrophages (Rucavado et al. 1998, 1999). Release of IL-1 and IL-6 induced by BaP1 in the muscle tissue was further described, suggesting that besides mast cell-stored mediators, cytokines could mediate the local inflammatory events induced by BaP1 (Rucavado et al. 2002). In agreement with this hypothesis, increased levels of IL-1 have also been detected in peritoneal exudates collected after BaP1 injection into peritoneal cavity of mice, and this event was followed by an increased expression of leukocyte adhesion molecules (our unpublished results).

The ability of BaP1 to induce leukocyte migration has also been investigated in an in vitro model using Boyden's chamber. In this study, the authors have demonstrated absence of a direct stimulatory effect of BaP1 on neutrophil chemotaxis (Fasky et al. 2000). However, this SVMP was able to activate events of the classic and alternative complement system, generating the chemotactic C5a factor. Accordingly, BaP1 treated serum was chemo-attractant to neutrophils in vitro.

Hemorrhagic SVMPs are also able to induce inflammatory events. Jararhagin, a P-III class SVMP isolated from *Bothrops jararaca* venom has been shown to induce influx of leukocytes into mouse air pouch (Costa et al. 2002). This effect was dependent on the presence of macrophages as well as on the proteolytic activity of this SVMP. Further investigations have also shown that jararhagin can directly stimulate the expression of mRNA encoding for TNF, IL-1 and IL-6 by elicited macrophages (Clissa et al. 2001). This suggests that macrophages are important targets for SVMP, although the cell binding site has not yet been characterized. Inactivation of the catalytic activity of jararhagin had no effect on jararhagin-induced stimulatory effect on macrophages, pointing to a role for the disintegrin-like/cysteine domain in this effect (Clissa et al. 2001). Investigations using knockout mice deficient in TNF receptors and IL-6 have demonstrated that both are relevant for development of jararhagin-induced necrosis but not edema nor hemorrhage (Laing et al. 2003).

More recently, it was reported that HF3 a P-III class SVMP isolated from *B. jararaca* venom induced phagocytosis of opsonized zymosan particles by elicited macrophages in vitro. This effect was inhibited by antibodies anti-α_m or anti-β_2 or a combination of both antibodies, thereby indicating that the integrin $\alpha_m\beta_2$ is involved in the HF3-induced phagocytosis by macrophages (Silva et al. 2004).

Not only native P-I or P-III class hemorrhagic metalloproteinases have been shown to induce inflammatory reaction. The disintegrin and disintegrin-like domains have also been shown to evoke such phenomena in some experimental models. Most disintegrins contain a RGD/KGD sequence within an amino acid hairpin loop maintained by disulfide bridges. The RGD motif confers disintegrins the ability to selectively bind to integrins in different cell systems (Mariano-Oliveira et al. 2003). Initially considered merely as antagonists of integrins, the SVMP-derived disintegrins have been shown to interact with and activate integrin-signalling pathways in inflammatory cells. Integrin-signaling pathways mediate important functions in leukocytes, including migration, spreading, activation of the respiratory burst, binding of complement, cell adhesion, cytokine gene expression and apoptosis (Williams & Solonkin 1999, Lowell & Berton 1999, Larsson et al. 2000). Thus, ocellastusin, an RGD-containing short monomeric disintegrin, isolated from venom of *Echis ocellatus*, has been shown to induce chemotaxis of human neutrophils in vitro (Smith et al. 2002). Similarly, jarastatin, an RGD-disintegrin isolated from *B. jararaca* venom showed a potent chemotactic activity on neutrophils and the ability to increase the levels of mRNA encoding for IL-8. Stimulation of leukocyte chemotaxis by jarastatin has been associated to its ability to trigger intracellular signaling pathways mediated by integrin activation (Coelho et al. 2004). Further studies have shown that this disintegrin interacts with $\alpha_m\beta_2$ integrin on neutrophil surface, thereby triggering integrin-mediated signaling, activating PI3K and MAPK pathways and interfering with neutrophil chemotaxis and production of chemokines. In the same study, it was described that EC3, a heterodimeric MLD-disintegrin, isolated from *E. carinatus suchoreki* venom, is also able to induce chemotaxis of human neutrophils in vitro, through its interaction

with $\alpha_9\beta_1$ integrin (Coelho et al. 2004). In addition, it was reported that alternagin-C, an ECD-disintegrin-like/cysteine-rich protein purified from *B. alternatus* venom, has a chemotactic activity on neutrophils and this effect involves actin cytoskeleton rearrangement, and FAK, PI3-kinase and Erk-2 activation,which are characteristic of integrin-activated pathways (Mariano-Oliveira et al. 2003). The interaction of disintegrins with macrophages has also been investigated. DC-HF3, a recombinant disintegrin-like cisteine rich protein derived from HF3 has been shown to be able to stimulate $\alpha_m\beta_2$-mediated phagocytosis of opsonized zymosan particles by macrophages (Silva et al. 2004). However, in vivo inflammatory effects of SVMP-disintegrins have not yet been described, a study which must be addressed.

Taken together, these findings indicate that the proteolytic domain of SVMPs *per se* is able to trigger inflammatory events in both in vivo and in vitro experimental models. The SVMP disintegrin-like domains can stimulate leukocyte functions in vitro through integrin-mediated pathways. Activation of distinct signaling pathways by these domains appears to be dependent on the structure of each domain and on the type of cell surface receptors.

Concluding remarks

The recent advance on venom metalloproteinases-induced inflammatory reaction are briefly reviewed in this article. Exogenous administration of SVMP into experimental animals triggers a cascade of inflammatory events, characterized by edema formation, leukocyte recruitment into tissues, and release of inflammatory mediators, which mimic a number of systemic and local inflammatory disorders in humans. Recent studies have shown that both disintegrin and disintegrin-like/cysteine-rich domains of SVMP are able to trigger leukocyte functions and cell signaling inflammatory pathways in vitro, and that proteolytic domain *per se* can induce inflammatory events in vivo. However, further studies are necessary to identify the domain structural features which are required for these inflammatory effects. Moreover, studies on characterization of protein-ligand interactions have evidenced that integrins present in membrane surface of neutrophils and macrophages are important acceptors for SVMP and their domains. Despite these significant advances, the bases for ligand recognition as well as identification of the different membrane high-affinity ligands involved in SVMP-induced leukocyte inflammatory activities have only begun to be investigated. More detailed analysis of the effects induced by these unique venom proteinases, and investigations on the structure/function as well as on their putative endogenous ligands need to be carried out to better understanding their mechanisms of inflammatory action. Given the structural similarities of the SVMP with mammalian MMP, these studies may help in clarifying the role of these proteins in diverse inflammatory processes. In addition, the knowledge of the mechanisms of action of SVMP may provide important clues for understanding snakebite envenomation and, in the future, to pave the way for novel, more effective therapeutic strategies to treat the local inflammatory reactions characteristics of snakebites.

ACKNOWLEDGMENTS

To Dr Solange MT Serrano for valuable suggestions and for critical reviewing the manunscript.

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