Biological Activities and Assays of the Snake Venom Metalloproteinases (SVMPs)

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Contents

Abstract

Snake venom metalloproteases (SVMP) are a key group of enzymes abundant in Viperidae venoms. Structurally, secreted SVMPs are typically organized into three main groups based on the presence or absence of domains: PI – which contains only a metalloproteinase domain; PII – includes also a disintegrin domain; and PIII – in addition to the first two domains, possesses a cysteine-rich domain. Diverse functions have been described to this group of proteases including their well-known hemorrhagic activity. Fibrin(ogen)olysis, prothrombin activation, interaction and lysis of von Willebrand factor, cytotoxicity, obstruction of angiogenesis, interference with platelet aggregation, myotoxicity, and proinflammatory

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are some of the other activities described by SVMPs. The SVMPs possess a broad range of biological activities, many with pathological consequences, that a brief description of the more common assays for biological activity associated with these proteins is of value to those entering the field. This chapter discusses the numerous activities attributed to the SVMPs and outlines the major assays utilized in systematic investigations of SVMPs.

Introduction

Snake venoms are complex mixtures of biologically active proteins and peptides that induce a wide variety of biological effects, many of which give rise to pathological outcomes (Tu 1996). In snake venoms, there are many different toxins organized into a relatively smaller number of toxin families associated with molecular targets that impact a variety of physiological systems. The key systems affected by Viperidae and Elapidae families of snake venoms include the hemostatic system, neurological system, renal system, as well as tissue integrity (Warrell 2010). It is the somewhat unique combination of the toxins in each venom and subsequently their molecular targets that give rise to the varied pathologies observed in patients envenomated by snakes from different genera and species.

Snake venom metalloproteinases (SVMPs) are an important group of enzymes with molecular masses ranging from approximately 20–100 kDa (Jia et al. 1996). They have been characterized according to both their function and structure. Structurally, the SVMPs have been organized into three main groups primarily based on the presence or absence of nonproteinase domains in context with the metalloproteinase domain. The three major classes are: PI – containing only a metalloproteinase domain; PII – including the metalloproteinase domain and a disintegrin domain; and PIII – which possesses metalloproteinase, disintegrin-like and cysteine-rich domains and in some cases additional lectin-like domains linked posttranslationally to the canonical PIII cysteine-rich domain structure. This classification can be further divided into 11 subclasses (PIa, PIIa, PIIb, PIIc, PIId, PIIe, D-I, PIIIa, PIIIb, PIIIc, and PIIId) according to the proteolytic processing the structures undergo and/or formation of dimeric structures (Fig. 1) (Fox and Serrano 2008).

Over the past 10 years, numerous new reports of SVMPs structures, primarily at the cDNA level, have been reported of which 35 have been isolated and assayed for biological activities (Table 1). While it is generally recognized that the *in vitro* activities associated with toxins in general and SVMPs in particular may only partially recapitulate the function of the toxin in the context of the whole venom in the course of envenomation, such assays are of critical value in terms of assisting with the isolation of the toxins as well as providing some level of understanding as to what role the toxin may be playing in actual envenomations (Gallagher et al. 2005). With this in mind, we have assembled some of the key assays associated with SVMPs' function (*in vitro* and *in vivo*) with the aim of providing the field with the technological landscape available when characterizing SVMPs.

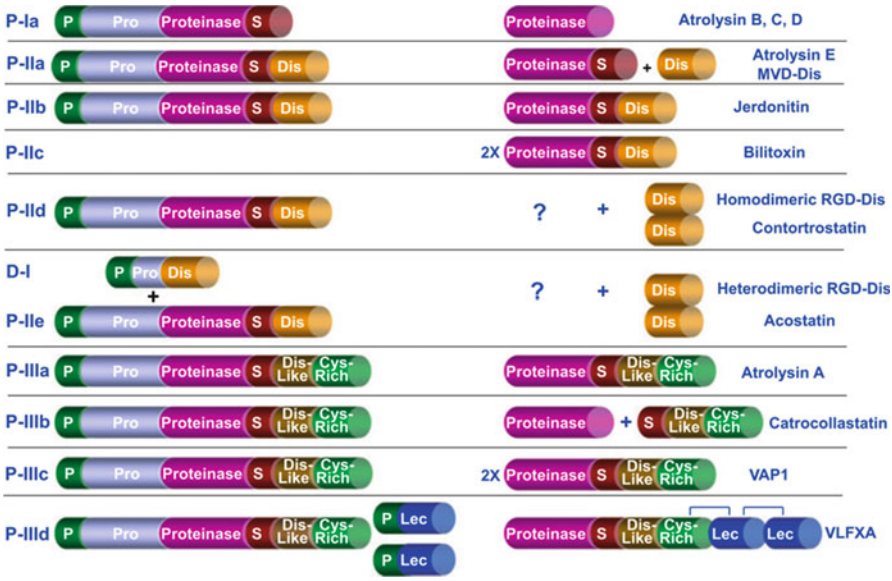

Fig. 1 Schematic of SVMP classes. Question marks (?) in the figure indicate that the processed product has not been identified in the venom (Fox and Serrano 2008)

SVMP: Proteolysis Assays *In Vitro*

Of the pathologies associated with envenomation by snake venoms, the SVMPs have been established as primarily contributing to the hemostatic disorders including hemorrhagic activity and the proteolytic and nonproteolytic disruption of key elements of the coagulation pathway. The hemorrhagic activity is strictly attributed to the proteolytic degradation of components of basement membrane by hemorrhagic SVMPs, since zinc chelation by EDTA, *o*-phenanthroline, or synthetic peptidomimetic hydroxamates completely abrogates this effect (Bjarnason and Fox 1994; Escalante et al. 2000). As mentioned, SVMPs may also disrupt hemostasis by perturbation of the coagulation pathways with a pro- or anticoagulation outcome which in general is accomplished by degrading plasma proteins such as fibrinogen and von Willebrand factor as well as other key proteins involved in these pathways (Baramova et al. 1989; Serrano et al. 2007).

Caseinolytic Activity – One of the first methods published for measuring nonspecific proteolytic activity was developed by Kunitz (1947) using the milk protein casein as the protein substrate. Using this assay, the SVMP is incubated with casein and after a set reaction time the mixture is subjected to acid precipitation with trichloroacetic acid and the resultant soluble digestion fragments are then spectrophotometrically quantitated at 280 nm, and the value used as a measure of the proteolytic action of the enzyme on the substrate.

Table 1 List of SVMPs fully sequenced in the last 10 years and their respective biological activities

Name	Accession #	*Species*	Activities	MHD	References
PI class					
Atroxlysin-I	P85420.2	*B. atrox*	Fibrinolytic and fibrinogenolytic (6.53 U/mg and 16.8 U/lg), digests fibronectin, binds to integrins α7β1 and α1β1 but not α2β1 and α3β1. Also inhibits platelet aggregation	19.9 μg	(Sanchez et al. 2010)
Batx-1	P0DJE1.1	*B. atrox*	Proteolytic activity (minimal and maximal doses: 4,800 U/mg and 1,670 U/mg), Fibrinogenolytic activity, induces a mild myotoxicity, lacks coagulant activity on human plasma or bovin fibrinogen and defibrinating activity	17 μg	(Patiño et al. 2010)
BjussuMP-II	Q7T1T4.1	*B. jararacussu*	Did not induce hemorrhage, myotoxicity or lethality, but dis- played proteolytic activity on fibrinogen, collagen, fibrin, casein and gelatin and does not show any clotting or anticoagulant activity, in contrast to its inhibitory effects on platelet aggregation	N.H.	(Marcussi et al. 2007)
BleucMP	P0DJJ6.1	*B. leucurus*	Acts on fibrinogen and fibrin, provoking blood incoagulability, however, devoided of hemorrhagic activity and did not induce relevant biochemical, hematological and histopathological alterations in mice	N.H.	(Gomes et al. 2011)
BmHF-1	P86802.1	*B. marajoensis*	Fibrinogenolytic, caseinolytic, induces dose-dependent edema and is weakly (or non)-hemorrhagic	41.14 μg	(Torres-Huaco et al. 2010)
BmooMPalpha-I	P85314.2	*B. moojeni*	Proteolytic activity towards azocasein, fibrinogen and fibrin, provoking blood incoagulability, but devoided of hemorrhagic and thrombin-like activities	N.H.	(Bernardes et al. 2008)
BnP1	P0C6S0.1	*B. pauloensis*	It is able to hydrolyze fibrinogen and fibrin, but devoided of significant myotoxic and hemorrhagic activities. Induces cell detachment, a decrease in the number of viable endothelial cells and apoptosis	>50 μg	(Baldo et al. 2008)

BnP2	P0C6S1.1	*B. pauloensis*	It is able to hydrolyze fibrinogen and fibrin, but devoid of significant myotoxic and hemorrhagic activities	N.H.	(Baldo et al. 2008)
Bothrojaractivase	P0C7A9.1	*B. jararaca*	Is a cofactors-independent prothrombin activator. Also has fibrinolytic and fibrinogenolytic activity. A dose-dependent procoagulant activity is shown in human plasma	N.D.	(Berger et al. 2008)
BpirMP	P0DL29.1	*B. pirajai*	Proteolytic activity on azocasein, and weak (or no) hemorrhagic activity. Degrade fibrin, fibrinogen and blood clots *in vitro*	50 μg	(Bernardes et al. 2013)
Leucurolysin-A	P84907.2	*B. leucurus*	Nonhemorrhagic, proteolytic activity on dimethylcasein, fibrin, fibrinogen, and plasma fibronectin, but not laminin. Inhibits ADP-induced platelet aggregation and is thrombolytic. Moreover, it induces changes in cell morphology and detachment followed by cell death, increases microvessel permeability and edema in mouse paw	N.H.	(Bello et al. 2006; Gremski et al. 2007)
[a]VMP1	B7U492.1	*A. piscivorus leucostoma*	*Recombinant protein* show proteolytic activity on fibrinogen but is devoid of hemorrhagic activity and had no capacity to inhibit platelet aggregation	N.H.	(Jia et al. 2009)
PII class					
BlatH1	U5PZ28.1	*Bothriechis lateralis*	Potent local and systemic hemorrhagic activity in mice, hydrolyzed azocasein, gelatin and fibrinogen, and inhibits human platelet aggregation	0.23 μg	(Camacho et al. 2014)
Stejnitin	P0DM87.1	*Trimeresurus stejnegeri*	It is fibrinogenolytic, inhibits platelet aggregation in human platelet-rich plasma (IC50 = 175 nM). It is also able to inhibit proliferatin of ECV304 cells by inducing apoptosis	N.D.	(Han et al. 2007)
[a]CamVMP-II	J9Z332.1	*C. adamanteus*	Expressed disintegrin domain (r-Cam-dis) and was able to inhibit all processes of platelet thrombus formation including platelet adhesion with an estimated IC50 of 1 nM, collagen- and ADP-induced platelet aggregation with the estimated IC50s of 18 and 6 nM, respectively, and platelet function on clot retraction	N.D.	(Suntravat et al. 2013)

(*continued*)

Table 1 (continued)

Name	Accession #	*Species*	Activities	MHD	References
PIII class					
AAV1	P0DJH3.1	*Deinagkistrodon acutus*	Induced platelet aggregation and the tyrosine phosphorylation of intracellular signaling proteins (LAT, SYK, p76SLP76, PIK3C, and PLCG2) that follows GP6 activation. Interestingly, blocks platelet aggregation but does not affect shape change	N.D.	(Wang 2007)
Ammodytagin	P0DJE2.3	*V. ammodytes ammodytes*	Fibrinogenolytic and hemorrhagic	10.86 μg (rat) 0.65 μg (mice)	(Kurtović et al. 2011)
Atragin	D3TTC2.1	*N. atra*	Exhibits an inhibitory activity toward cell migration of both NIH3T3 and CHOK1	N.D.	(Wei et al. 2006; Guan et al. 2010)
Atrahagin	P0DJJ1.1	*N. atra*	Failed to induce skin hemorrhage at doses of 5 or 50 μg per injection point. Causes mast cell degranulation and histamine release. Selectively degrades the alpha-chain of human fibrinogen (FGA)	N.H.	(Leonardi et al. 2008)
Atrase-A	D5LMJ3.1	*N. atra*	Fibrinogenolytic, edematogenic and bactericidal activity against *Staphylococcus aureus*. Did not show cytotoxicity on A549 and K562 cells, but detached adherent A549 cells. Also did not show significant inhibition of platelet aggregation, proteolytic activities towards fibrin, azocasein and *N*-alpha-benzoyl-L-arginine ethyl ester (BAEE), nor hemorrhagic activity. On the other hand, it significantly inhibited the growth and caused detachment of adherent HMEC, increased releasing of IL-8, ICAM-1 and MCP-1 and expression of caspase-3/7 and caspase-8	N.H.	(Qianyun et al.; Ye et al. 2009; Sun and Bao 2010)

Atrase-B	D6PXE8.1	*N. atra*	Non hemorrhagic	N.H.	(Sun and Bao 2010)
BjussuMP-1	Q1PHZ4.1	*B. jararacussu*	Hemorrhagic, inhibits platelet aggregation induced by collagen and ADP. Has moderate edema activity, but no myotoxic activity. It is unable to clot plasma. It also shows bactericidal activity against *E. coli* and *S. aureus*	4.0 μg	(Mazzi et al. 2004)
Daborhagin-K	B8K1W0.1	*Daboia russellii russellii*	High hemorrhagic activity when subcutaneously injected into mice. May also potently degrade alpha chain of fibrinogen (FGA)	0.82 μg	(Chen et al. 2008)
Daborhagin-M	P0DJH5.1	*D. siamensis*	High hemorrhagic activity when subcutaneously injected into mice. Has potent fibrinogenolytic activity on alpha-chain of fibrinogen (FGA). Hydrolyzes model substrate (beta-chain of insulin) at Ala(14)-Leu(15) and Tyr(16)-Leu(17) followed by His(10)-Leu(11) and Phe(24)-Phe (25)	0.86 μg	(Chen et al. 2008)
EoVMP2	Q2UXQ5.1	*Echis ocellatus*	Hemorrhagic, inhibits collagen-induced platelet aggregation and activates prothrombin (F2)	10 μg	(Howes et al. 2005)
Jerdohagin	P0DM88.1	*Protobothrops jerdonii*	High hemorrhagic activity. It may also inhibit platelet aggregation	0.04 μg	(Chen et al. 2004)
Kaouthiagin-like	D3TTC1.1	*N. atra*	Exhibits an enzymatic specificity toward pro-TNF-alpha with low inhibition of cell migration	N.D.	(Guan et al. 2010)
Leucurolysin-B	P86092.1	*B. leucurus*	Hemorrhagic	30 ng (rabbit)	(Sanchez et al. 2007)
Moojenin	P0DKR0.1	*B. moojeni*	Fibrinogenolytic and coagulant. Nonhemorrhagic	N.H.	(de Morais et al. 2012)
Ohanin	A3R0T9.1	*Ophiophagus hannah*	Has hemorrhagic activity. Inhibits ADP-, TMVA- and stejnulxin-induced platelet aggregation in a dose-dependent manner (on washed platelet, but not on platelet rich plasm)	N.D.	(Guo et al. 2007)

(*continued*)

Table 1 (continued)

Name	Accession #	*Species*	Activities	MHD	References
Oxiagin	P0DJJ4.1	*N. oxiana*	Inhibits the classical complement pathway dose-dependently. Also induces cation-independent hemagglutination that can be prevented by D-galactose pretreatment	N.D.	(Shoibonov et al. 2005)
TSV-DM	Q2LD49.1	*T. stejnegeri*	Non hemorrhagic, inhibits cell proliferation and induces cell morphologic changes transiently on human umbilical vein endothelial cells	N.H.	(Wan et al. 2006)
VAFXA-I	P0C8I7.1	*V. ammodytes ammodytes*	Activates coagulation factor X (F10) in a calcium-dependent manner	N.H.	(Wei et al. 2006)
VLAIP-A	Q4VM08.1	*M. lebetina*	Induces apoptosis in vascular endothelial cells and inhibits endothelial cell adhesion to extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, collagen I, and collagen IV. Also hydrolyzes azocasein, and oxidized insulin B-chain	N.D.	(Trummal et al. 2005)
VLAIP-B	Q4VM07.1	*M. lebetina*	Inhibits endothelial cell adhesion to extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, and collagen. Induces apoptosis in vascular endothelial cells	N.D.	(Trummal et al. 2005)

NH non hemorrhagic
ND non determined
[a]Sequence obtained from cDNA library

A colorimetric version of the caseinolytic activity utilizes azocasein which upon digestion releases the soluble azo dye which is measured colorimetrically. The reaction is stopped by the addition of perchloric acid and the solution is then filtered and the absorbance measured at 390 nm.

Fibrinogenolytic Assay – Fibrinogen or coagulation factor I is a 340 kDa glycoprotein synthesized in the liver and found with a plasma concentration of 1 to 5–4.0 g/L. Fibrinogen is a potent hemostatic agent playing an essential role in controlling bleeding by bridging activated platelets and being the key substrate for thrombin in establishing a fibrin network (Sorensen et al. 2012). The fibrinogenolytic activity of SVMPs can be classified according to their specificity of hydrolysis of fibrinogen chains. Proteases that are responsible for cleaving Aα chains are α-fibrinogenases and those responsible for cleaving Bβ chains are termed β-fibrinogenases (Markland 1998b). It should be noted that this activity is not directly related with hemorrhage since some SVMP may contain a high fibrinogenolytic activity yet are devoid of hemorrhagic activity (Girón et al. 2013).

Fibrinogenolytic activity is typically evaluated by SDS-PAGE on the basis of proteolytic activity on fibrinogen (Edgar and Prentice 1973) with slight modifications. Fibrinogen and SVMP are mixed and incubated in buffer with different pH values for different time intervals. Substrate to SVMP ratios should be adjusted for optimal results. The reaction is stopped by the addition of a denaturing buffer containing 2 % sodium dodecyl sulfate (SDS) and 10 % β-mercaptoethanol. Reaction products are then analyzed by SDS-PAGE. Heat stability can also be tested dissolving the SVMP in 50 mM Tris-HCl buffer, pH 8.0, then incubating for 15 min at 0–70 °C. The remaining fibrinogenolytic activity is followed as described above. Similarly, different inhibitors of fibrinogenolytic activity can be determined by incubating the enzyme in buffer containing inhibitors such as: EDTA, aprotinin, benzamidine, and β-mercaptoetanol for 15 min. In the Fig. 2a, the SVMP named BmooMPalpha-I, isolated from the venom of *Bothrops moojeni*, was evaluated by this method and showed the cleavage of the Aα-chain of fibrinogen first, followed by the Bβ-chain, and showed no effects on the γ-chain. Studies with inhibitors demonstrated that BmooMPa-I is a metalloproteinase (Fig. 2b) and revealed the importance of the disulfide bonds in the stabilization of the native structure and retention of activity. Like many venom SVMPs, BmooMPalpha-I was observed to be stable at pH values between 4 and 10 and exhibit its maximum fibrinogenolytic activity between 20 °C and 50 °C (Fig. 2c) (Bernardes et al. 2008).

Fibrinolyic Assay – The action of the protease thrombin on fibrinogen generates fibrin (Factor Ia), a fibrous protein that participates in blood coagulation. As it polymerizes, it traps platelets and forms a clot that functions as a hemostatic plug at the wound site (Mosesson 2005). Many of the venom fibrinolytic enzymes are metalloproteinases, and similarly most of the SVMP are fibrinolytic (Markland 1998a). The fibrinolytic activity of an SVMP may be evaluated by the same principle as the fibrinogenolytic assay described above by observing the fibrin degradation pattern in SDS-polyacrylamide electrophoresis (Fig. 3) (Bernardes et al. 2008). It can also be assayed based on the fibrin plate method described by Marsh and Arocha-Piñango (1972). The assay consists of measuring the diameter of

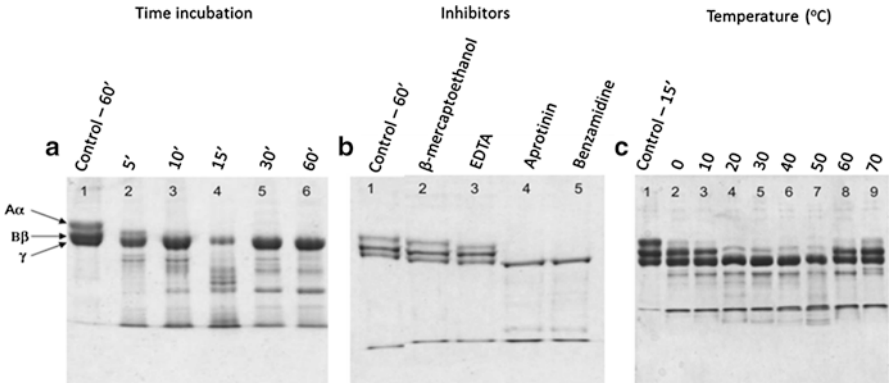

Fig. 2 Fibrinogenolytic activity evaluated by the fibrinogen degradation pattern on SDS-polyacrylamide electrophoresis. (**a**) Proteolysis of bovine fibrinogen by the BmooMPa-I in different times and (**b**) effect of inhibitors on BmooMPa-I. Lanes *1* – control fibrinogen incubated without enzyme for 60 min; (**c**) Effect of temperature on BmooMPa-I. Lanes *1* – control fibrinogen incubated without enzyme for 15 min; Lanes *2–9* – control fibrinogen incubated with enzyme for 15 min at 0, 10, 20, 30, 40, 50, 60 and 70 °C, respectively (Adapted from Bernardes et al. 2008)

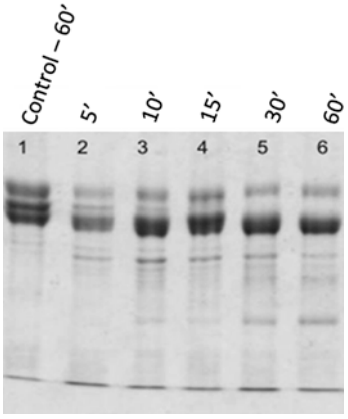

Fig. 3 Fibrinolytic action by BmooMPa-I SVMP. Lanes: *1* – Control incubated without enzyme for 60 min; *2–6* – control incubated with enzyme for 5, 10, 15, 30 and 60 min, respectively (Adapted from Bernardes et al. 2008)

the halos of lysis produced after 24 h of incubation with aliquots of the SVMP solution in different concentrations (Table 2). A solution of purified fibrinogen (containing 10 % added plasminogen) is coagulated by adding bovine thrombin at room temperature for 30 min. Human plasmin, single-chain t-PA (sct-PA), and two-chain u-PA (tcu-PA) may be used as controls. Fibrinolytic activity may also be evaluated in the presence of inhibitors such as, serine protease (SBTI, PMSF, benzamidine/HCl, and aprotinin), metalloprotease (EDTA, EGTA, and 1,10-phenanthroline), DTT, and cysteine protease (iodoacetic acid) (Marsh and Arocha-Piñango 1972; Da Silva et al. 2009; Girón et al. 2013).

Table 2 Purified fibrinolytic metalloproteinases activity yields from *Bothrops colombiensis* purification steps Purification step (Adapted from Girón et al. 2013)

	Protein (mg)	Active fractions	Fibrinolytic activity ($mm^2/\mu g$)	Hemorrhagic activity
Crude venom	127.00		30.6	+
Mono S 10/10	12.11	F1		
		F2	96.1	+
		F3		
Phenyl Sepharose	3.05	F2-A	40.8	–
	0.39	F2-B	162.2	–
Superose F 2-A	2.44	Colombienase-1	94.6	–
Bio-Select SEC F 2-B	0.33	Colombienase-2	211.57	–

Extracellular Matrix Protein Degradation – Many biologically relevant substrates for proteolytic assays with SVMPs have been described. Some of these substrates include type IV collagen, fibronectin, laminin, plasminogen, and protein mixtures such as matrigel. Detection of proteolysis is generally accomplished by SDS-polyacrylamide electrophoresis as described above (Cintra et al. 2012). The obvious caveat with these assays is that they are *in vitro* and not in the context of other proteins which may be interacting with them and altering their proteolysis susceptibility. An excellent example would be the extracellular matrix proteins which are know to be involved in numerous protein–protein interactions which contribute to their structure and function (Hynes and Naba 2012). One approach to attenuate those concerns is the use of matrigel, which represents an extract of proteins found in the specialized extracellular matrix and basement membranes (Kleinman and Martin 2005). While this mixture may better mimic *in vivo* substrate organization, it lacks authentic basement structure composition in that most of the collagen IV is lacking. Nevertheless, matrigel as a complex substrate is better than individual components. Matrigel proteolysis by SVMPs has demonstrated that different P classes of SVMPs generate different patterns of limited proteolysis and that the component basement membrane proteins show a somewhat different digestion pattern when in complex compared with their digestion as a lone substrate.

Thrombolytic Activity – The ability of some SVMPs to target different factors (fibrin or fibrinogen) gives them the ability to degrade fibrin-rich clots and prevent progression of clot formation (Markland 1998a). The thrombolytic activity of SVMPs assayed *in vitro* by clot formation can be evaluated by incubation in 24-well plates of SVMPs with whole rabbit blood (Gremski et al. 2007) or human blood (Cintra et al. 2012). After incubation, samples can be the quantitatively evaluated based on the clot density. Figure 4 illustrates the thrombolytic activity of Batroxase in a 24-well plate assay showing thrombus clot reduction.

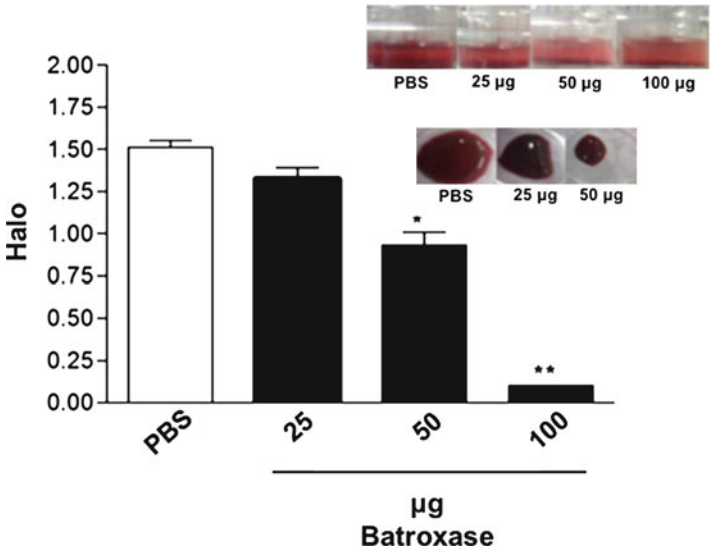

Fig. 4 Thrombolytic activity of Batroxase (25, 50 and 100 µg). The results are expressed as the mean thrombus diameter (cm) ± SD ($n = 3$). The Tukey test was used for statistical analysis. *$p < 0.05$ and **$p < 0.001$ compared with the control (PBS). (*B insert*) Side and upper views of a 24-well plate showing thrombus clot reduction. *Upper panel*, side view; *Lower panel*, upper view after supernatant removal for thrombus measurement (Cintra et al. 2012)

Protein Interaction/Proteolysis Assays

von Willebrand factor (vWF) Cleavage – von Willebrand factor is a multimeric glycoprotein involved in endothelial cell–matrix interactions (Cheresh 1987; Whittaker and Hynes 2002) that plays an essential role in hemostasis and thrombosis (Wagner 1990; Zhou et al. 2012) and is also involved in the angiogenesis regulation (Starke et al. 2011). The binding of vWF to platelets occurs through binding with platelet glycoprotein Ib (gpIb), one of the major platelet receptors for ligands such as vWF and thrombin. This binding is known as the first event in the formation of hemostatic plugs (Kroll et al. 1991).

To examine whether SVMPs may affect vWF–glycoprotein Ib interaction and subsequent platelet aggregation, vWF is pretreated with the SVMP for different time intervals, and then ristocetin is added to trigger platelet aggregation of washed platelets from human blood. The platelet aggregation levels are continuously monitored by turbidimetry. Also, proteolytic degradation of vWF may be evaluated and analyzed utilizing SDS-PAGE. The reactions are carried out with different substrate:enzyme ratios and time intervals and then stopped by adding reducing or nonreducing buffer and boiling at 95 °C for 5 min. The products of the reactions are then subjected to 12 % SDS-PAGE (Wang et al. 2004).

Cell-Based Assays

In addition to the ability to degrade extracellular matrix proteins (Souza et al. 2000; Baldo et al. 2010), some of the PII and PIII class members can also interact with cell surface integrins (Isabelle Tanjoni et al. 2010). Thus, it is likely that some of the pathologies associated with SVMPs may involve interference or disruption of cellular activities (Moura-da-Silva et al. 2007). Accordingly, assays designed to assess the ability of SVMPs to interfere in diverse biological processes, such as platelet aggregation, cell viability, migration, adhesion, and angiogenesis have been developed.

Platelet aggregation – Platelet aggregation is a well-studied process by which platelets adhere to each other at sites of vascular injury and has been recognized as critical for plug formation and thrombosis (Osler 1886). As fibrinogen is required for platelet aggregation and metalloproteinases possess α-fibrinogenase activity, it was initially proposed that fibrinogen degradation is responsible for the abnormalities of platelet function (Teng and Huang 1991). However, a study using jararhagin, a hemorrhagic PIII SVMP, showed that the cleavage of fibrinogen Aα-chain by this enzyme does not interfere with the binding of fibrinogen to the platelet integrin and therefore, jararhagin-induced inhibition of platelet function is not caused by proteolysis of fibrinogen (Kamiguti et al. 1994).

Subsequently, it was proposed that the inhibition of platelet response by jararhagin is mediated by its disintegrin-like domain by binding to integrin α2-subunit of platelets followed by cleavage of the β1-subunit, causing loss of the integrin native conformation, necessary for binding of macromolecular ligands (Kamiguti et al. 1996). Since the disintegrin-like domains in SVMP are similar to disintegrins (Jia et al. 1997), it was suggested that this domain would be most likely responsible for the inhibition of platelet aggregation. However, the recombinant cysteine-rich domain of atrolysin A was demonstrated to inhibit collagen-stimulated platelet aggregation, showing that this domain by itself is able to bind to α2β1 receptors on platelets and inhibit platelet aggregation (Jia et al. 2000). Moreover, another study demonstrated two complete peptide sequences from the homologous cysteine-rich domains of atrolysin A and jararhagin interact with α2β1 integrin and interfere with platelets aggregation response as well as the interaction of other cells to collagen, thus clarifying the mechanism of action of SVMPs in relation to platelet aggregation (Kamiguti et al. 2003).

Inhibition of platelet aggregation by SVMPs may be tested using the protocol described by Howes and colleagues (Howes et al. 2005). Platelet-rich plasma (PRP) is initially separated by centrifugation of fresh human blood mixed with sodium citrate. The platelet pellet is resuspended in Tyrode's HEPES buffer and platelets are then isolated by gel filtration on a Sepharose column. To evaluate the action of the SVMP on platelets, the washed platelet suspension is stirred in a dual-channel aggregometer with increasing amounts of SVMP added and incubated for 5 min. Platelet aggregation is stimulated by introduction of an agonist (such as thrombin, epinephrine, platelet-activating factor, collagen, or ADP to specific platelet membrane receptors) and the aggregation monitored as a change in optical density. Figure 5 provides an

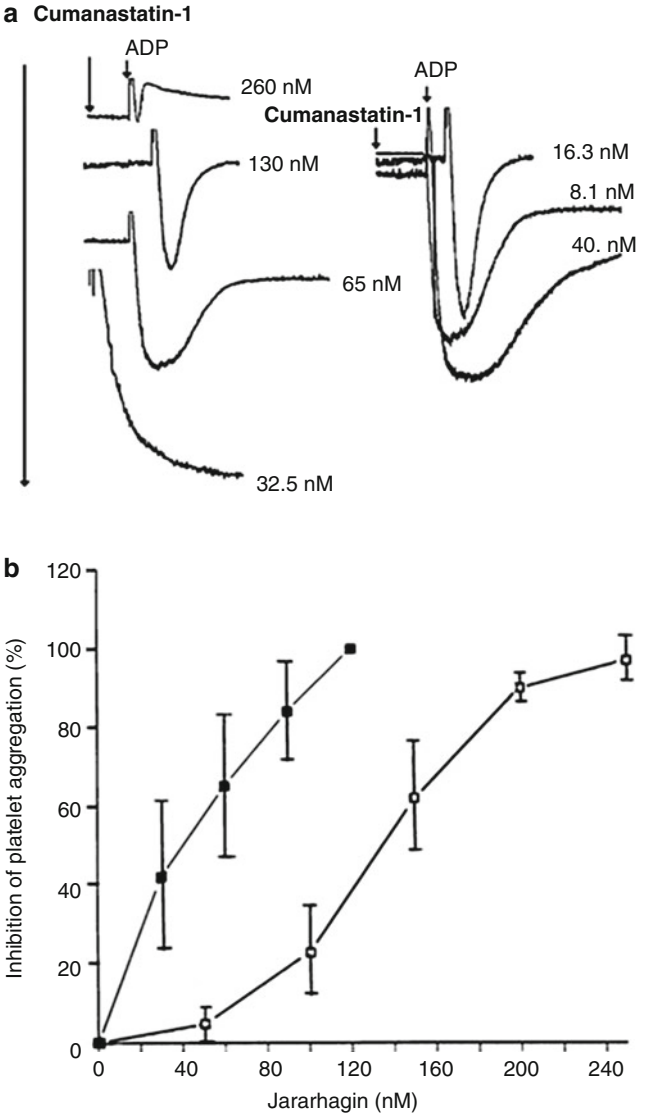

Fig. 5 Inhibition of platelet aggregation induced by two different mechanisms. (**a**) Inhibition of ADP-induced platelet aggregation by cumanastatin-1. The percentage of inhibition was calculated by comparing light transmittance obtained in presence of venom against the control sample. The IC50 value determined from the curve is 158 nM (Da Silva et al. 2009). (**b**) Inhibition of collagen-induced platelet aggregation by native and inactivated jararhagin. Percentage inhibition was calculated relative to control suspensions incubated with buffer only., ■ Native jararhagin; □ 1,10-phenanthroline-treated jararhagin (Kamiguti et al. 1996)

example of the inhibition of platelet aggregation induced by two different mechanisms, collagen-induced, which is inhibited by native and inactivated jararhagin (Kamiguti et al. 1996) and ADP-induced which is inhibited by cumanastatin-1 (Da Silva et al. 2009).

Cell viability – The viability of cultured cells (which implies in the ability of growing and dividing) is frequently evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method, a colorimetric technique based on the ability of mitochondrial reductases in viable cells to convert MTT into purple formazan crystals (Mosmann 1983). In this assay, the cells are seeded on a 96-well plate. Following adhesion, the medium is changed to media containing different concentrations of the SVMP. After 24 h, cells are incubated with MTT for 3 h and then formazan crystals are solubilized using DMSO and the intensity measured by optical density. Another method is an adaptation of the neutral red uptake method (Chaim et al. 2006), adapted by Gremski and collaborators (2007). In this protocol, the neutral red solution in PBS is added after the cell incubation with SVMP, and allowed to sit for 3 h. Then, the plate is washed with PBS followed by the addition of a fixing solution. The incorporated dye is finally released from cells with 1 % acetic acid in 50 % ethanol and the absorbance measured at 540 nm.

Cytotoxicity activity – Cytotoxicity of SVMPs *in vitro* with cultured cells may also be evaluated by using dyes and flow cytometry, in order to check cell membrane integrity and DNA fragmentation. Incubation of treated cells with the nucleic acid dye Sytox Green (Molecular Probes), with subsequent cell counting by flow cytometer, allows the measurement of cell death (Kim et al. 2006). Alternatively the use of annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) allows the measurement of apoptotic and necrotic cells respectively (Teklemariam et al. 2011).

The disruption of cell adhesion and subsequently initiation of apoptosis seems to be dependent on the metalloproteinase domain of the SVMP. Treatment with jararhagin, a SVMP from *Bothrops jararaca*, induces changes in cell shape and causes anoiksis following loss of cell adhesion. However, when the catalytic activity of jarahagin was inhibited, these effects were abolished, suggesting the importance of the catalytic domain (Tanjoni et al. 2005).

Angiogenesis – The influence of SVMPs on endothelial cell tube formation may be evaluated *in vitro* by matrigel tube formation assays. In the protocol described by Yeh and collaborators (2001), matrigel is diluted with or without basic fibroblast growth factor (bFGF) as a chemoattractant and added to 24-well plates to form a gel layer. HUVECs are then incubated with or without the toxin and then stained with toluidine blue and analyzed microscopically. Fields are chosen randomly and the total tube length is quantified using an image analysis software (Fig. 6).

Cell migration – All nucleated cell types during at least one point in their development undergo cell migration. In most cases, this process occurs during morphogenesis, stops at the terminal differentiation, and may be restarted only during tissue regeneration or neoplastic processes (Friedl and Wolf 2010).

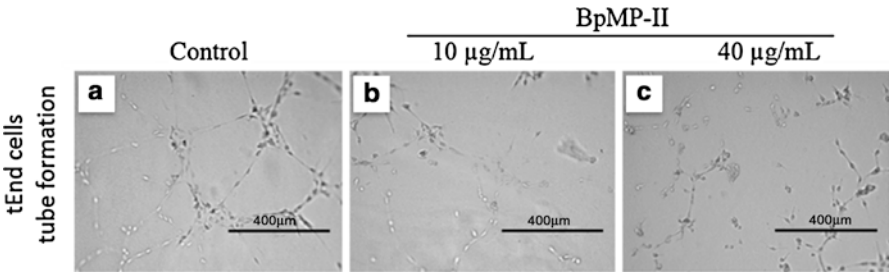

Fig. 6 Effect of BpMP-II on tube formation of endothelial cells inmatrigel. (**a**) tEnd cells preincubated with medium (Control) arranged capillary-like form. Samples containing 10 mg/mL (**b**) or 40 mg/mL (**c**) of BpMP-II were preincubated with tEnd cells for 30 min at room temperature (Achê et al. 2014)

The migration of tumor cells *in vitro* is evaluated by measuring the migration of cells on the bottom of the well after scratching a line through them. The experiment is performed in a 24-well plate. Cells are plated and after 24 h of incubation, the medium is discarded and the confluent monolayer is scratched at the center of the well. The detached cells are then washed with medium and 900 µL of new medium containing the SVMP is added. A disintegrin that blocks migration of the cells (such as the disintegrin echistatin) must be used as a positive control and PBS may be used as a negative control. After incubation, microscopic images at different times (0, 3, 6, 12, and 24 h) are taken and the percent of closure of the scratch is calculated.

In Vivo Assays

Considering the multitude of physiological mechanisms involved in the maintenance of hemostasis, it is clear that tissue, physical and biological factors play an important role in this process. Thus, performing *in vivo* assays is extremely important to better understand the action of these enzymes in envenomation. Despite the knowledge about the role of SVMPs on hemostasis *in vitro*, a few studies have evaluated its effects *in vivo*.

Defibrinating Activity – Many snake envenomations are characterized by a defibrination syndrome which is a systemic event (Fig. 7) and may be clinically utilized to monitor the time course of envenomation and success of treatment (Barrantes et al. 1985).

In vivo, this activity may be tested by the method based on Gené and collaborators (1989) with slight modifications. The minimum defibrinating dose (MDD) is defined as the amount of venom capable of making the plasma incoagulable. In this method, mice are injected i.p. with 200 µL of saline and increasing doses of the SVMP. After 1 h, animals are anesthetized and whole blood is kept at 25–30 °C until clotting occurs (Bernardes et al. 2008).

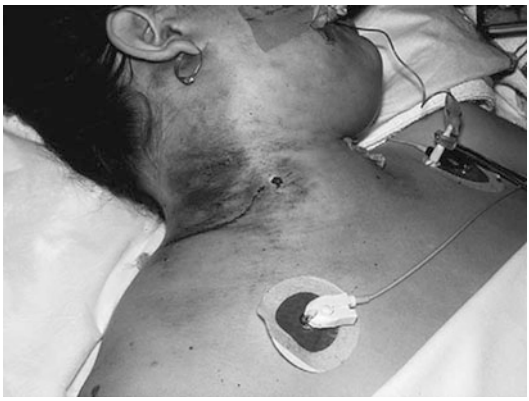

Fig. 7 A case of severe defibrination coagulopathy after a taipan bite causing extensive bleeding following failed IV insertion into the right jugular vein. The resultant hemorrhage resulted in a halving of hemoglobin levels in 3 h (Photo copyright q Dr. Julian White) (White 2005)

Fibrinogenolytic activity – The fibrinogenolysis provoked by proteinases in *B. jararaca* venom was also studied *in vivo* by Yamashita and colleagues (Yamashita et al. 2014). The venom was preincubated with Na_2-EDTA or 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), which are inhibitors of SVMPs and serine proteases, respectively, and injected subcutaneously or intravenously into rats. Samples of blood, lung, and skin were analyzed at 3 and 6 h. Degradation levels of fibrin(ogen) in plasma were estimated by ELISA assay using three different antibodies (rabbit antirat fibrinogen IgG for coating, rat fibrinogen as standard, and biotinylated rabbit antirat fibrinogen IgG). The preincubation with the chelating agent markedly reduced plasma fibrinogen consumption. SVSP, on the other hand, did not seem to be important *in vivo*. Another effect was the elimination of the marked elevation in tissue factor (TF – a cellular receptor essential for initiating blood coagulation *in vivo*) levels in plasma by both administration routes.

Myotoxicity – Studies have recognized that SVMPs are also able to induce acute muscle damage (Rucavado et al. 1999). Myotoxic activity can be assayed by intramuscular injection of the SVMPs in the gastrocnemius muscle of mice and after 3 h, the animals are anesthetized, bled by cardiac puncture, and their sera is assayed for creatine-kinase activity with a commercial kit (Baldo et al. 2008). The skeletal muscle alterations induced by SVMPs may also be assayed using transmission electron microscopy. In this case, mice are injected intramuscularly in the gastrocnemius and at different times postinjection, the animals were sacrificed and the gastrocnemius muscle dissected out and processed for microscopy, thus allowing a histological examination of the damage (Baldo et al. 2010). However, one must take care in the interpretation of these experiments as it is not clear if this effect is owing to direct cytotoxic action on muscle cells or weather other indirect mechanisms related to ischemia due to microvasculature damage and hence tissue death (Gutiérrez and Rucavado 2000).

Hemorrhagic activity – Binding to and subsequent hydrolysis of the ECM components by SVMPs are central to their hemorrhagic action, since these constituents ensure the mechanical stability of microvessels (Escalante et al. 2011). Experimental evidence indicates a two-step mechanism of SVMP-induced hemorrhage

(Gutiérrez et al. 2005). The first comprising of proteolysis of the major components of basal membrane (BM), laminin, type IV collagen, nidogen, and perlecan (Escalante et al. 2006) and of proteins that play a key role in the mechanical stability of BM, such as nonfibrillar collagens weakens the capillary wall (Escalante et al. 2009).

The second step involves the transmural pressure acting on the weakened capillary wall causing distention and eventually disruption and as consequence; extravasation of blood occurs (Gutiérrez et al. 2005). In addition, the hemorrhagic effect of SVMPs is further potentiated by their ability to inhibit platelet aggregation and to hydrolyze plasma proteins involved in the process of blood clotting, such as different coagulation factors and von Willebrand factor (vWF) (Sajevic et al. 2011), which have been described previously.

Determination of the hemorrhagic activity was initially described by Kondo and colleagues (1960) in which solutions of toxin or saline are subcutaneously injected into the dorsal skin of mice. Hemorrhagic spots are measured on the inside surface of the skin. The minimum hemorrhagic dose (MHD) is defined as the least amount of protein that causes a hemorrhagic spot 5 mm in diameter 6 h after injection (Bjarnason and Tu 1978). Modifications of this protocol were proposed by Gutiérrez and collaborators (1985) in which the animals are sacrificed after 2 h and the minimal hemorrhagic dose as the amount of toxin that causes a 10 mm hemorrhagic spot (Fig. 8).

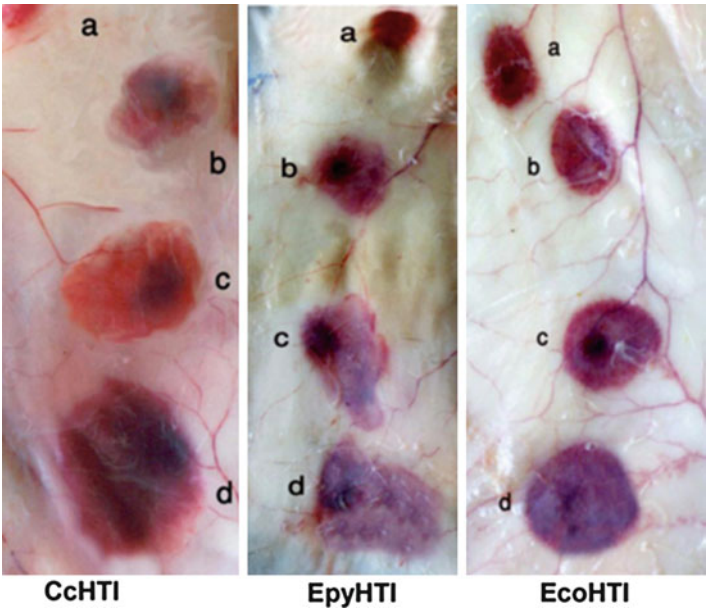

Fig. 8 Determination of the SVMPs hemorrhagic activity. Calculation of the hemorrhagic dose using 1 mg (*a*), 2 mg (*b*), 4 mg (*c*), and 8 mg (*d*) of three viper P-III hemorrhagic SVMPs: EpyHTI, EcoHTI, and CcHTI of the vipers *Echis pyramidum*, *Echis coloratus*, and *Cerastes cerastes*, respectively (Wahby et al. 2012)

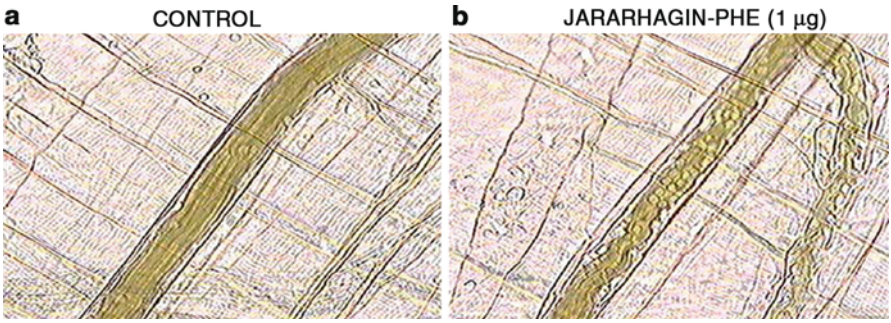

Fig. 9 Intravital micrograph of cremaster muscle after topical application of inactivated jararhagin: (**a**) control; (**b**) inactivated jararhagin. The presence of PMN cells rolling in the vascular well after topical application of inactivated jararhagin can be observed (Clissa et al. 2006)

The hemorrhagic activity may also be evaluated *in vivo* by intravital microscopy (Fig. 9). Mice are anesthetized and placed on a water-heated bed. The *cremaster* muscle is exposed and the SVMP placed onto the muscle and immediately covered with Mylar® to prevent dehydration. The alterations are then observed for a period of 30 min (Lomonte et al. 1994).

Miscellaneous Assays

Inflammation – Inflammation can be initially defined as a beneficial response to injury that gives rise to a combination of clinical signs and symptoms. The four well-known signs of inflammation are redness, swelling, heat, and pain, which may end up in loss of function, characterized by loss of mobility in a joint due to the edema and pain or to the replacement of functional cells with scar tissue (Punchard et al. 2004; Scott 2004).

However, this clinical view of inflammation includes many cellular and molecular implications, which makes possible the analysis of inflammation by different approaches, such as counting of inflammatory cells, measurement of proinflammatory transcripts such as interleukins, chemokines, and tumor necrosis factors, in addition to quantifying edema.

The formation of blisters and infiltration of leukocytes into dermis may be accessed through macroscopic and histological observations following intramuscular injection of the toxin in the gastrocnemius of mice (Rucavado et al. 1998). Also, the increase of blood neutrophil numbers and their accumulation in the peritoneal cavity in the early phase followed by an increase in mononuclear cells in the late period is another way to assess the inflammatory effect of SVMPs. For this approach, mice are injected with the SVMP or sterile saline (control) into the mouse peritoneal cavity. Total leukocyte, polymorphonuclear, and mononuclear cells are then determined in peritoneal washes harvested at different times after injections. For determination of the number of leukocytes in the blood different

times after SVMP injection, blood aliquots are collected from the tail vein and the leukocyte number is determined with a Neubauer chamber (Fernandes et al. 2006).

The induction of TNF-α, interferon-γ (IFN-γ), and interleukins may be quantitated by enzyme immunoassays. Basically, mice are injected in the gastrocnemius and at different time intervals; the injected muscle is then excised, weighed, and homogenized in saline solution. After centrifugation, cytokine levels are quantitated by enzyme-linked immunosorbent assay (Rucavado et al. 2002).

Inflammation caused by SVMPs was described for all of the different classes of SVMPs. The first studies showed that inflammation induced by BaP1, a P-I class SVMP from *Bothrops asper* venom (Gutiérrez et al. 1995) is able to induce formation of blisters and infiltration of leukocytes into the dermis and peritoneal cavity as well as increasing their number in blood circulation (Rucavado et al. 1998; Fernandes et al. 2006). Also, BaP1 induces a prominent increase of IL-1b and IL-6, but no elevation of TNF-α and IFN-γ were detected (Rucavado et al. 2002).

Edema induced by SVMPs treatment is assayed by injecting the toxin subcutaneously into the subplantar area of a mouse paw along with the same volume of vehicle in the contra paw as a control. The paw edema is then determined by measuring paw thickness with a digital caliper or a digital water plethysmometer at different times after injection, and the results calculated as the difference in thickness between the right and left paws (Sharma et al. 2004).

The PIII class SVMPs are also important proinflammatory molecules. Jararhagin, for example, in addition to directly stimulating TNF-α, IL-1β, and IL-6 expression (Clissa et al. 2001), has been shown to induce influx of leukocytes into mouse air pouch and its activity seems to be dependent upon proteolytic activity and the presence of macrophages (Costa et al. 2002). Gene expression profiles of human fibroblasts *in vitro* and mouse tissue *in vivo* also revealed significant upregulation of proinflammatory transcripts such as IL-1β, IL-6, CXCL1, CXCL2, IL-8, and apoptosis and inflammation-related transcripts such as TNF-α induced protein 6 (Gallagher et al. 2005).

SVMPs belonging to PI-class trigger inflammatory signals *in vivo* and *in vitro* models and therefore provides evidence that proteolytic domain is able to cause inflammation, while the disintegrin-like domain in turn, seems to stimulate leukocyte functions through integrin-mediated pathways (Teixeira et al. 2005). In order to evaluate the importance of disintegrin-like/cysteine-rich domains, a study using either inactivated jararhagin and jararhagin-C, which lacks the catalytic domain, showed that both are able to increase the number of rolling leukocytes without interfering with microvasculature (Fig. 9), thus suggesting that the disintegrin-like/cysteine-rich domains are sufficient to locally activate the early events of an acute inflammatory response (Clissa et al. 2006).

The cysteine-rich domain via its hypervariable region (HVR) were shown to play a role in inflammation by analyzing the effect of recombinant proteins and peptides of HF3, a hemorrhagic P-III SVMP, containing the cysteine-rich but not the disintegrin-like domain. These proteins were able to significantly increase the leukocyte rolling in the microcirculation. The proinflammatory effect of HF3 and its disintegrin-like/cysteine-rich domains expressed together or individually were

also evaluated by analyzing the alterations on the microcirculatory network through intravital microscopy and the results corroborate the role of the cysteine-rich domain in SVMPs targeting *in vivo* as well as confirming the role of integrin $\alpha_M\beta2$ in the proinflammatory effects of HF3. Taken together, these results show for the first time that the cysteine-rich domain and its HVR play a role in triggering inflammation which is mediated by integrins (Menezes et al. 2008).

Conclusion

Understanding the structure and the mode(s) of action of the SVMPs has long been the focus of venom studies due to the important role of these enzymes in the pathophysiology of envenomation. In spite of the decades of research conducted in the characterization of SVMP structure and function, there are still numerous unanswered questions, some of which we know and certainly some as yet to be asked. As new technologies become available in conjunction with the rich databases being generated on these toxins as well as others, we are optimistic that a more complete understanding of SVMP activities *in vitro* and more importantly *in vivo* will be gained. Furthermore, novel approaches for understanding SVMP activity in conjunction with other bioactive toxins in the venoms must be developed in order to have a complete system-based understanding of envenomation which will in turn hopefully lead to novel modes of therapeutic intervention.

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