

Venoms versus Vasculature and Hemostasis: In Search for Non-Hemorrhagic Anti-Hemostatic Venom Components

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Abstract

Snake venoms target multiple essential organ systems, among them the hemostatic system and the vasculature. Both snake venom serine proteinases (SVSPs) and snake venom metalloproteinases (SVMPs) exist which cleave coagulation factors, platelet receptors and/or extracellular matrix (ECM) components. To treat vascular occlusion and ischemic diseases, cleavage of coagulation factors and platelet receptors is a treatment goal, whereas degradation of the vessel wall ECM results in unwanted and life-threatening hemorrhages. Identification of anticoagulant and antithrombotic snake venom proteinases, which are non-hemorrhagic, provides potential new pharmacological tools. While larger forms of SVMPs belonging to the P-III and P-II- subclasses are hemorrhagic in general, some P-I-SVMPs and SVSPs offer the potential to cleave fibrinogen thereby forming easily dissolvable clots or degrading fibrin, without attacking the vascular ECM. However, it is unclear which features of the molecular structures determine whether a P-I-SVMP belongs to this medically relevant group of fibrin(ogen)olytic, non-hemorrhagic snake venom proteinases.

Keywords: Snake venom metalloproteinase; Fibrinogen; Hemorrhage; Fibrinolysis; Basement membrane; Extracellular matrix; Vasculature

Introduction

A closed and self-sealing circulation system is an evolutionary achievement of vertebrates because it allows the supply of all tissues with nutrients and the disposal of metabolic waste as well as the rapid distribution of signal molecules and hormones in the body. The compartmentalization between intercellular space and blood fluid is essential for the survival. With few exceptions, the two compartments are physically and biochemically separated by a thin but tight vascular wall consisting of vascular cells and a characteristic extracellular matrix (ECM) [1]. For effective homeostasis, this tight vessel wall must be able to self-seal and restore integrity when injured. This is implemented by aggregation of thrombocytes and by conversion of the soluble blood component, fibrinogen, into fibrin. Fibrin is an insoluble and mechanically stable scaffold protein, whose rope-like aggregates stabilize wound-closing platelet aggregates in form of a thrombus. Both vessel integrity and self-sealing function of blood vessels are severely compromised by hemorrhagic toxins from animal venoms, such as from snakes.

Blood Vessel Wall: Cellular Components and Vascular

ECM

Shear forces caused by blood flow and radial forces induced by blood pressure require a mechanically stable vessel wall to avoid rupture and leakage. Moreover, as the blood pressure is regulated by the vessel diameter, blood vessels must not be rigid structures but show plasticity to allow vasoconstriction and vasodilation. Counteracting

blood pressure and transmitting mechanical forces during vasoconstriction require a mechanically stable, yet flexible scaffolding system of blood vessels, which is implemented in a specialized ECM. The vessel wall ECM is in close contact to and interacts with vascular cells, which are arranged in three concentric layers (Figure 1). The inner layer (tunica intima) is made of very thin endothelial cells (ECs), which are tightly connected to each other by various intercellular contacts (marker proteins in parentheses): tight junctions (occludin, ZO-proteins); adherens junctions (VE-cadherin), and gap junctions (connexin) [2-4]. Thus, the endothelial cell lining fulfils various vessel barrier functions, such as selective exchange of nutrients and other low weight molecules between blood and tissue compartments, selective extravasation of blood-borne immune cells into tissue during inflammation, and coverage of ECM components of the subendothelial basement membrane (BM) [5-7]. Uncovering of the latter is a strong stimulus for the vessel-sealing hemostatic system [8,9]. BM-specific proteins, such as collagen types IV, XV, and XVIII, laminins-411 and -511, the heparan-sulfate proteoglycan perlecan, fibronectin and von Willebrand factor (vWF), anchor endothelial cells to the vessel wall [10]. They determine the fate of ECs, such as cellular stiffness and rigidity and propensity to allow leukocyte diapedesis [11-13]. Moreover, the interplay of BM and ECs determines about the quiescent or angiogenically active state of ECs [14,15]. During angiogenesis, ECs produce and deposit a new BM and secrete matrix metalloproteinases (MMPs) for ECM remodeling [16].

Similarly productive in terms of ECM protein synthesis are vascular smooth muscle cells (VSMCs), which form the middle cell layer (tunica media) of blood vessel walls, especially of arterial vessels. VSMCs show phenotypic variation by differentiating into a contractile or ECM-synthesizing/migratory phenotype. The former is abundant in arterial vessels, where individual VSMCs, ensheathed by a BM, align

and allow coordinated constriction of the vessel diameter, thereby increasing the blood pressure.

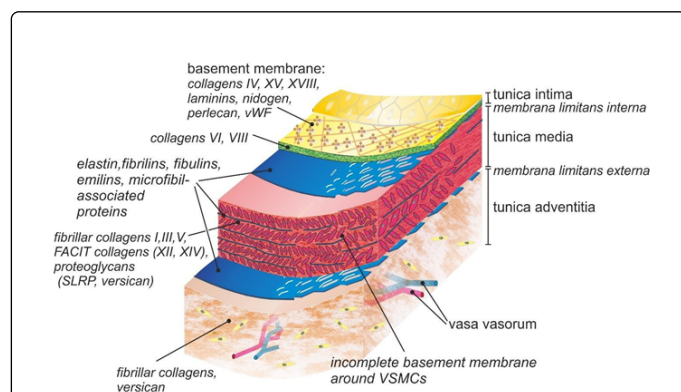


Figure 1: General structure of a blood vessel wall with histological localization of typical vascular ECM components. The vascular wall of an arterial vessel is sketched in Figure 1. However, the common three-layered wall structure of tunica intima, media, and adventitia can be seen with some variation in all arterial, capillary, and venous vessels. The thick layer of smooth muscle cells (tunica media) is much thinner in venous vessels and consists of a single layer of pericytes whose protrusions extend around capillaries. The endothelial cell layer is anchored to the subendothelial BM with its typical components. The BM is fixed to an underlying elastic layer *via* collagens type VI and VIII. The elastic layer is rich in elastin and other elastin-associated proteins. Elastin is also incorporated into the layer of smooth muscle cells, each of which is encased in a BM. The vascular smooth muscle cells are tightly connected *via* the ECM allowing force transmission and vessel contraction. The ECM of the tunica adventitia is similar to the interstitial stromal ECM of the tissue surrounding the vessel. Scheme was adapted from [14].

The contractile forces are transmitted from the VSMC *via* adhesion proteins, such as members of the integrin family and dystroglycans [14,17], to BM-proteins and eventually to ECM fibrils, which contain *inter alia* vessel-typical collagen isoforms, such as collagen-VI and the force-bearing type I/III/V collagen-containing fibrils which are accompanied by the vessel-characteristic proteoglycan, versican [18]. Upon vessel injury of inflammatory processes, such as atherosclerosis, VSMCs differentiate into migratory phenotype, responsible for a fibrotic excess of ECM production and neointima formation [19-21]. Around the endothelial sheath of microvessels, scattered pericytes replace the VSMCs. They wrap long cell protrusions around the EC layer and thereby stabilize the capillaries [22,23].

Collagens play a prominent role within the ECM of the vascular wall. With their triple-helical structure and their ability to form supramolecular fibrils and networks, they are particularly suitable for absorbing mechanical tensile forces necessary to maintain the structure and function of force-loaded blood vessels [18]. The subendothelial BM contains the network forming collagen types IV, XV and XVIII [10,24]. The typical fibrils within the vascular ECM are formed by type I, III, and V collagens. They are the most abundant types of collagen, and their force-bearing fibrils are also found in the interstitial stromal tissue. The microfilaments, which associate with the elastin network, consist of type VI collagen with its typical beaded microfilament structure [25,26]. Meshwork-forming type VIII and the FACIT collagen type XIV and XVI also occur in the vascular ECM,

where they associate with the network-forming collagens of the BM or the interstitial collagen fibrils, respectively [14,18].

A characteristic feature of all collagens is their triple helix with a diameter of 1,5 nm [27]. It consists of three collagen α chains with characteristically repeated GXY tripeptide sequences (G, glycine, X and Y, other amino acid residues, preferentially proline and 4-hydroxyproline in the X and Y position respectively) that form a left-handed poly-L-proline helix. In a right-handed triple-helical quaternary structure, three of these left-handed helices coil around each other in a staggered manner so that the glycine residues which are repeated at every third position of each polypeptide strand alternately line up along the central axis of the triple helix. By lateral association and intermolecular covalent crosslinks, several collagen triple helices associate into higher order supramolecular structures, such as fibrils and networks, which are mechanically stable and bear high tensile forces [5,27]. Together with interlinking ECM-components, such as vascular FACITs, collagens type XIV and XVI, and proteoglycans, these collagenous suprastructures determine the strength and elasticity and thus the resilience of the vessel wall against blood pressure load. The supramolecular aggregation of collagens and the triple helical quaternary structure of each collagen molecule also make collagens highly resistant to most proteinases, and explain their long half-life (approx. 60-70 days for type I collagen).

Elasticity of vessel walls is achieved by their high content of elastin and elastin-associating proteins, such as fibrillins and type VI collagen [28,29]. Elastic proteins are abundant in elastic arterial vessels and especially localized within the elastic membrane flanking a layer of VSMCs towards both the endothelial layer and the adventitial tissue (membrana limitans interna and externa, respectively). The adventitial layer is the outer layer of the vessel wall and contains ECM proteins typical of stromal tissue, such as type I, III, and V collagen as well as the proteoglycans decorin, and biglycan, in addition to versican.

Hemostatic system

Hemostasis seals injured blood vessels, and it provides a provisional ECM to promote tissue regeneration. It relies on a complex and stringently regulated interplay of cellular and humoral components of the blood as well as of the vessel structure. Immediately after injury, the ECM of the vessel wall, among them vWF and collagens, becomes accessible to platelets, which are activated by the ECM components and adhere to the site of injury [8,10,30]. This primary hemostasis is further supported by conversion of soluble fibrinogen into an insoluble meshwork of fibrin during secondary hemostasis [9]. In the course of the coagulation cascade, the fibrinogen-converting enzyme thrombin is activated. It cleaves and releases both fibrinopeptides, A and B, from fibrinogen forming self-aggregating fibrin molecules, $(\alpha\beta\gamma)_2$, which form the thrombus scaffold and seal the injured vessel wall [31]. This secondary hemostasis, together with platelet aggregation, must occur temporally and spatially restricted only at the site of vessel injury. A finely balanced network of interdependent proteolytic activation steps of clotting factors, mostly serine proteinases, as well as several serine proteinase inhibitors (serpins) and clotting factor-degrading enzymes warrant both amplification of hemostasis and its restriction to the site of injury. If hemostatic activity is too little or too excessive, bleeding and thrombotic vessel occlusion, respectively, occur and become life-threatening [32].

Venom components that target blood vessels and the vessel-sealing system

A large proportion of active proteins and peptides found in snake venoms interact with components of the hemostatic system, to promote or inhibit the normal sequence of events leading to thrombus formation. Integrity and tightness of the vasculature are vital, and many snakes use their venoms to target these qualities to immobilize or kill their prey or predators. Two groups of snake venom proteins directly affect the vasculature: proteinases and vascular endothelial growth factor (VEGF)-like venom proteins. The latter form a distinct subgroup of the VEGF family, and these snake venom VEGF-F proteins likely induce vascular permeability *via* endothelial VEGF receptors [33]. Even more drastically, venom proteinases irreversibly attack scaffold proteins in the ECM of the vessel wall and also loosen intercellular contacts of ECs, resulting in vessel leakage. Most snake venom proteinases are either metalloproteinases or serine proteinases. The snake venom metalloproteinases (SVMPs) are modular proteins with a catalytic domain and additional, but not obligatory disintegrin (-like) and cysteine-rich domains [34,35]. The P-I-SVMP subgroup consists of the catalytic metalloproteinase (M) domain only, whereas the P-II- and P-III-SVMPs are C-terminally extended by a disintegrin or disintegrin-like and a cysteine-rich domain, respectively. Based on their susceptibility to partial proteolysis and to post-translational modifications, P-III-SVMPs are divided into subclasses such as those that homodimerize (P-IIIc) or those that are cleaved between M and D domains (P-IIIb). Formerly named P-IV, the heterodimeric class of SVMPs, containing an additional C-type lectin like (snaclec) domain, is now included in the P-III class as subclass (P-IIIId), as no P-IV mRNA has been reported to date [34]. A common feature of all SVMPs is a catalytic domain with a Zn²⁺ ion in its active site followed by a methionine-containing Met-turn. This domain shares high structural homology with the ECM-degrading matrix metalloproteinases (MMPs) [36]. The non-enzymatic domains (D and C) mediate contacts with important proteins in platelet membranes and in the ECM to modulate their functions. After envenomation, SVMPs prevail in the blood of the snakebite victim and are hardly inhibited by any endogenous proteinase inhibitor with the exception of α 2-macroglobulin [34,35,37,38].

The other major class causing snakebite-induced hemorrhage and bleedings are snake venom serine proteinases (SVSP) [39]. Most SVSPs are hardly inhibited by endogenous serine proteinase inhibitors (serpins), whereas serine proteinase inhibitors are substantially involved in the coagulation system [39]. SVSPs structurally resemble clotting factors and, like them, possess a single catalytic domain with the same catalytic triad. Yet, they differ from the substrate-specific coagulation factors by cleaving a plethora of different substrates, including various components of the coagulation cascade and ECM components of the vessel wall. Fibrinogen, the central effector of coagulation, is cleaved by fibrinolytic SVMPs and by SVSPs, albeit in a different manner than by thrombin. Thrombin cleaves and releases both fibrinopeptides, A and B, resulting in fibrin molecules which form very stable aggregates [31]. In contrast, most SVSPs preferentially cleave only one of the two fibrinopeptides. This pseudocoagulant effect results in insoluble fibrin molecules, which, in contrast to the thrombin-cleaved fibrin molecules, aggregate into a less stable and easily degradable thrombus scaffold. Thus, the snake venom proteinase-cleaved fibrin molecules destabilize clot formation and result in dysfunctional hemostasis. Moreover, SVSPs-induced fibrin conversion is not restricted to the site of injury and may cause disseminated intravascular coagulopathy. Other SVSPs cleave fibrin in

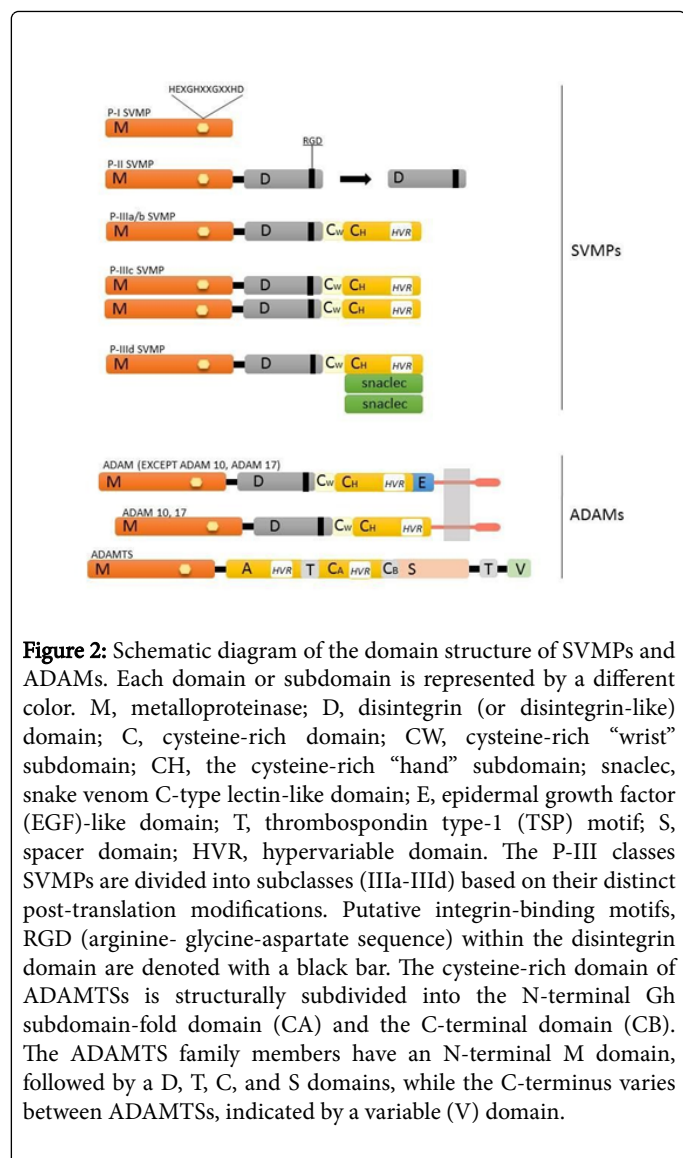
thrombotic aggregates, thereby dissolving already existing wound-closing clots [40-42].

From the two classes of snake venom proteinases, the SVMPs have been reported more frequently for their strong ability to degrade ECM-proteins of the vessel wall, causing disintegration of vessels and severe hemorrhages [43-45]. This leaking of blood into the tissue is exacerbated by the disturbed hemostasis of snakebite victims. The hemostatic system of both coagulation and platelet activation is similarly affected by both SVSPs and SVMPs.

Collagen Degradation by SVMPs and by Endogenous Collagenases of the MMP Family

As collagens are able to bear high mechanical forces, degradation of blood vessel collagens results in hemorrhage. The triple-helical structure bestows proteolytic resistance on collagen. Nevertheless, several hemorrhagic SVMPs are able to cleave triple-helical collagen molecules into fragments, which are unable to withstand the high forces and pressure loads within the vessel wall. Endogenously, triple-helical collagens can be degraded by a particular subclass of MMPs, the collagenases, whose catalytic domain is highly homologous to SVMPs and endogenous membrane-bound A metalloproteinase A Disintegrin (ADAM) proteins (Figure 2). These collagenases include MMP-1, -8, and -13, as well as the membrane-type (MT) MT-MMP1 (MMP14), which is expressed by ECs during angiogenesis [18,36,46-48]. They are involved in developmental processes, tissue remodeling and regeneration [49,50], such as the angiogenic sprouting of new capillaries from preexisting ones, a process which includes the local degradation of BM at the sprouting site and the new formation of BM along the sprout of ECs [16,48]. The structural comparison of hemorrhagic SVMPs with the endogenous collagenolytic MMPs reveals a highly homologous catalytic domain, typical of relatives within the metzincin family of Zn²⁺-dependent endoproteinases, such as ADAMs and ADAM-thrombospondin (ADAMTS) subfamily members (Figure 2) [51].

Despite the homologous catalytic domain, SVMPs and MMP collagenases differ in their exosites, which are auxiliary motifs outside the active site of the catalytic domain. These exosites support the enzymatic activity by tethering the substrate, by unwinding or destabilizing the collagen triple helix, or by directing the catalytic domain to a particular tissue or cell-associated target [52]. Endogenous MMP collagenase, unlike SVMPs, bear a hemopexin domain, which is connected to the catalytic domain *via* a linker sequence [53-55]. The hemopexin domain has a four-bladed β -propeller structure with each blade consisting of a twisted four-stranded β -sheet with a complexed Ca²⁺ ion in the center of the four-bladed propeller domain. The hemopexin domain is essentially required to bind the collagenous triple helix [56-58]. Moreover, collagen binding to the hemopexin domain, along with concomitant substrate binding at the catalytic domain and linker sequence allows a two-point, or most likely three-point binding of the collagen triple helix to the enzyme. This enables the MMP to exert molecular forces onto the triple helix to destabilize it, to bend the triple helix, and to untwist the three collagenous protein chains in a triple helicase action [52,53,59]. This is required for collagen cleavage, as only a single collagen chain with a diameter of 0.5 nm, but not the 1.5 nm thick triple helix sterically fits into the active site of the catalytic domain [59]. Different models of how the triple helix of collagen can be loosened by MMPs have been reviewed by Overall et al. [52].



Despite lacking a hemopexin domain, hemorrhagic SVMPs are highly effective and kinetically rapid in cleaving triple helical collagen [43]. The disintegrin and cysteine-rich domains of P-II- and P-III-SVMPs may offer additional collagen-binding domains, similarly to the hemopexin domain of MMPs. They may act as exosites, which fulfil tethering and helicase activities towards the collagenous triple helix similar to the exosite activities of the hemopexin domain in MMPs. In fact, jararhagin, a P-III-SVMP effectively binds triple helical collagen *via* its non-catalytic domains serving as exosites [43,44,60-62]. However, experimental evidence for a helicase activity of such exosites in P-II- and P-III-SVMPs is lacking to our knowledge.

Fibronectin, elastin and other crosslinked ECM molecules also contribute to the mechanical resilience of the vessel wall. They can be degraded by a whole range of different MMPs [36,63,64]. Laminins, likewise essential components of the BM, are not known as major force-transmitting molecules. Yet, they provide important adhesion sites for ECs and VSMCs to the subjacent and surrounding BM, respectively, regulate differentiation and ensure cell survival. Laminins and their associated nidogens have been reported to be sensitive to

proteolytic attack by snake venom proteinases [65]. Particularly the degradation of the BM proteins, collagen type IV and perlecan, have been considered a criterion to distinguish between BM-degrading hemorrhagic SVMPs and non-hemorrhagic ones [44,62,65].

Hemorrhagic damages caused by snake venoms have been intensively studied by electron microscopy, histochemistry, immunohistochemistry, protein-chemical digestion analyses, including immunoblots, and most recently by proteomic analysis of ECM fragments directly from affected tissues [44,62]. Fragments of nearly all ECM proteins were detected in exudates of mouse tissues envenomed with SVMPs from various viperides. Comparative studies on venom-induced tissue damage also revealed that P-II- and P-III-SVMPs are more hemorrhagic than P-I-SVMPs, and that their repertoire of degradable ECM proteins is wider and includes, *e.g.*, vascular collagen types XV and VI. The different exosites within their additional domains are likely responsible for the stronger tissue-damaging effects of the P-II- and P-III-SVMPs [43]. Noteworthy, exudates from tissues envenomed with P-II- or P-III-SVMP contained more cellular proteins, indicative of more aggressive cell toxicity [45]. Moreover, cell-released endogenous MMPs may further degrade the tissue and hence exacerbate venom-induced tissue damage and hemorrhagic effects [44]. These studies highlighted that BM-components (type IV and XV collagens, perlecan) as well as BM-anchoring and fibril-interconnecting/fibril-organizing collagens (types IX, XII, and XIV) are degraded in hemorrhagic tissue damage. Hence, their degradation constitutes a biochemical marker for hemorrhagic SVMPs [43,44,62,65]. However, it is still difficult to correlate the hemorrhagic activity of an SVMP with its molecular structure. So far, it has become obvious that P-II- and P-III-SVMPs are mostly hemorrhagic, whereas non-hemorrhagic and hemorrhagic proteinases can only be distinguished within the group of P-I-SVMPs.

Non-hemorrhagic Snake Venom Proteinases with Anti-hemostatic Properties

Hemorrhage is an undesirable side effect when considering the use of snake venom proteinases in translational medicine. In contrast, the hemostasis-influencing effect of snake venom proteinases is desired in the treatment of bleeding disorders, thrombosis or thrombotic vascular occlusion. Hence, non-hemorrhagic but fibrinolytic snake venom proteinases may be useful for the development of new medical treatments, especially as they are inhibited differently than endogenous proteinases. Recent research has focused on P-I-SVMPs in this respect, as some of them have been shown to be effective, medically safe, fibrinolytic and directly acting thrombolytic agents that dissolve fibrin clots without requiring an intermediate step of plasminogen activation [35,66]. Yet, whether a specific SVMP is only fibrin(ogen)olytic but not hemorrhagic cannot be predicted from its protein structure and therefore needs to be determined experimentally. Moreover, SVMPs have different abilities to bind relevant target proteins in *in vivo* experiments, such as those of ECM and platelet membrane receptors involved in platelet aggregation. Therefore, it is necessary to study these interactions of SVMPs. Good candidates with the desired activity and selectivity, which are non-hemorrhagic, are likely found within the group of P-I-SVMPs. Recently, several P-I SVMPs with diverse abilities to bind and cleave target proteins and with different hemorrhagic activities have been described [35,67]. Furthermore, a few directly acting fibrinolytic but non-hemorrhagic SVMPs have been identified that also proteolytically inactivate receptors on platelets and thus block for instance vWF- induced

platelet aggregation [35,66,67]. This may provide new opportunities to design new anti-thrombotic agents to treat ischemic diseases.

Comparison of the Catalytic Domains of Hemorrhagic and Non-Hemorrhagic P-I-SVMPs

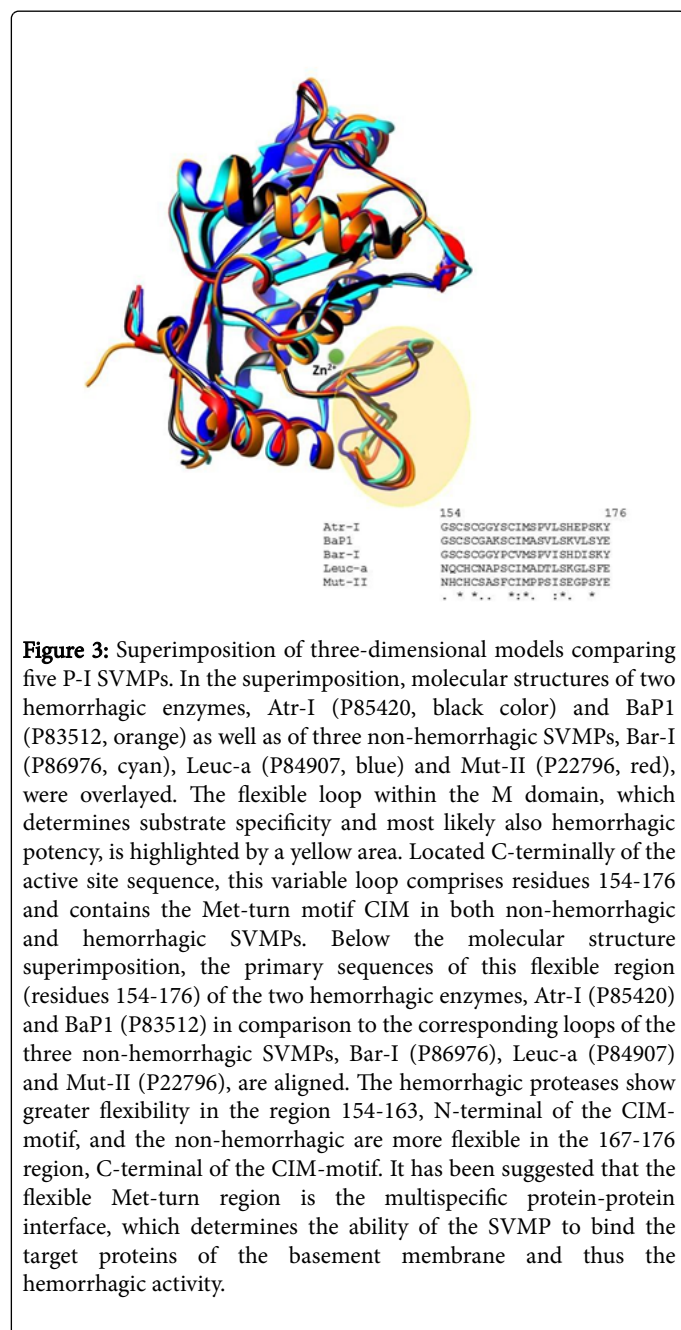


Figure 3: Superimposition of three-dimensional models comparing five P-I SVMPs. In the superimposition, molecular structures of two hemorrhagic enzymes, Atr-I (P85420, black color) and BaP1 (P83512, orange) as well as of three non-hemorrhagic SVMPs, Bar-I (P86976, cyan), Leuc-a (P84907, blue) and Mut-II (P22796, red), were overlaid. The flexible loop within the M domain, which determines substrate specificity and most likely also hemorrhagic potency, is highlighted by a yellow area. Located C-terminally of the active site sequence, this variable loop comprises residues 154-176 and contains the Met-turn motif CIM in both non-hemorrhagic and hemorrhagic SVMPs. Below the molecular structure superimposition, the primary sequences of this flexible region (residues 154-176) of the two hemorrhagic enzymes, Atr-I (P85420) and BaP1 (P83512) in comparison to the corresponding loops of the three non-hemorrhagic SVMPs, Bar-I (P86976), Leuc-a (P84907) and Mut-II (P22796), are aligned. The hemorrhagic proteases show greater flexibility in the region 154-163, N-terminal of the CIM-motif, and the non-hemorrhagic are more flexible in the 167-176 region, C-terminal of the CIM-motif. It has been suggested that the flexible Met-turn region is the multispecific protein-protein interface, which determines the ability of the SVMP to bind the target proteins of the basement membrane and thus the hemorrhagic activity.

Essentially, the P-I-SVMPs consist of an approximately 200 amino acids long metalloproteinase (M) domain. The M domain has two structural features: an active site motif HEXXHXGXHX/D characterized by complexing a Zn^{2+} ion, and a Met-turn motif CIM including a methionine within a conserved turn. To date eleven of these enzymes have been characterized at their structural level as well as their proteolytic activities in correlation with their ability to induce local or systemic bleeding [35]. Despite their high sequence homology,

some P-I SVMPs cause hemorrhage, while others do not cause disintegration of the vessel wall, but still cleave fibrin(ogen). This difference must be related to the structural determinants within the M domain [35,68].

By investigating the protein-protein interfaces of four P-I SVMPs including: hemorrhagic (BaP1 and acutolysin A) and non-hemorrhagic (leucurolysin-a and H2-proteinase) ones. Wallnoefer and co-workers discovered that the backbone flexibility within two loops of the M domain most probably determines their hemorrhagic activity [68]. They concluded that the sequences of the P-I-SVMPs mainly differ in the loop comprising residues 155 to 176. This loop C-terminally flanks the highly conserved active site and contains the so-called Met-turn. It shows an entirely different flexibility for hemorrhagically active and inactive enzymes (Figure 3). Albeit in proximity to the active site, it may be considered as an exosite which is able to recognize, bind, and hydrolyze specific target proteins, such as BM components, and hence determine the hemorrhagic activity of the P-I SVMPs [65].

Outlook

A future task is to identify directly acting fibrinolytic P-I SVMPs devoid of hemorrhagic activity. Their ability to additionally inactivate platelet receptors and to impair platelet functions may be a synergistic effect, which may be harnessed to treat thrombotic disorders. Current thrombolytic intervention using plasminogen activators such as tissue type plasminogen activator (tPA) achieves therapeutic benefits but at a cost of increased risk of bleeding. The directly acting fibrin(ogen)olytic P-I SVMPs offer particular biochemical properties and may possess platelet-directed antithrombotic properties [35,66,67]. Another relevant aspect is the short pharmacological half-life of the fibrin(ogen)olytic P-I SVMPs, likely due to their inactivation by the major plasma proteinase inhibitor α 2-macroglobulin. This is important for clinical application and must be analyzed in *in vivo* hemorrhage models.

Acknowledgements

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