

Extracellular matrix molecules as targets for brown spider venom toxins

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Abstract

Loxoscelism, the term used to describe lesions and clinical manifestations induced by brown spider's venom (*Loxosceles* genus), has attracted much attention over the last years. Brown spider bites have been reported to cause a local and acute inflammatory reaction that may evolve to dermonecrosis (a hallmark of envenomation) and hemorrhage at the bite site, besides systemic manifestations such as thrombocytopenia, disseminated intravascular coagulation, hemolysis, and renal failure. The molecular mechanisms by which *Loxosceles* venoms induce injury are currently under investigation. In this review, we focused on the latest reports describing the biological and physiopathological aspects of loxoscelism, with reference mainly to the proteases recently described as metalloproteases and serine proteases, as well as on the proteolytic effects triggered by *L. intermedia* venom upon extracellular matrix constituents such as fibronectin, fibrinogen, entactin and heparan sulfate proteoglycan, besides the disruptive activity of the venom on Engelbreth-Holm-Swarm basement membranes. Degradation of these extracellular matrix molecules and the observed disruption of basement membranes could be related to deleterious activities of the venom such as loss of vessel and glomerular integrity and spreading of the venom toxins to underlying tissues.

Key words

- Brown spider
- Venom
- Extracellular matrix
- Proteolytic effect

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Introduction

Brown spiders (*Loxosceles* genus) have been reported to cause several clinical manifestations. Envenomation provokes two major kinds of signals, i.e., local lesions at the bite site characterized by edema followed by vasodilatation, blood vessel degeneration, local hemorrhage and a significant cutaneous tissue injury with gravitational spreading, that can exacerbate to necrotic skin ulcers and degeneration (1-4), and systemic

effects that begin as a malaise and can become generalized, with hemolysis, thrombocytopenia, disseminated intravascular coagulation and renal failure. These clinical signs and toxicological effects appear to be phenomena similar for several *Loxosceles* species including the more studied *L. reclusa*, *L. laeta*, *L. intermedia* and *L. gaucho* species (4-8).

Brown spider venoms are highly complex and contain many different proteins (9-11). The exact mechanisms by which the

venoms cause their deleterious effects are currently under investigation, with putative explanations involving an indirect event, as is the case for endothelial cell-dependent neutrophil activation caused by the venoms and seemingly related to the dermonecrotic lesion (4,12-14). The presence of a sphingomyelinase D-like enzyme (32-35 kDa) probably associated with necrotic, hemolytic and thrombocytopenic activities triggered by the venoms has also been identified in different *Loxosceles* species (3,4,6,10,15,16). Other enzymes such as a hyaluronidase have been postulated to be a spreading factor during the lesions (4,17), and protease activities also appear to have some participation in the noxious effects of the venoms (11,18-21). Adult plasma components appear to be required for the deleterious effects of the venoms, since a purified putative dermonecrotic toxin diluted in neonate plasma or synthetic buffer did not induce platelet activation, an event that may be responsible for thrombosis, tissue ischemia and dermonecrosis (8). Indirectly, venoms also seem to cause injury by binding to cell membranes and activating the complement system of plasma (8,22,23) (see Table 1).

The key observations that some physi-

ological events closely dependent on the basement membrane and on connective or plasma extracellular matrix constituents are altered during loxoscelism, as is the case for platelet subendothelial adhesion and aggregation, hemostatic troubles such as disseminated intravascular coagulation, hemorrhage into the dermis, renal failure and even cutaneous tissue injury, point to the presence of molecules potentially deleterious to these structures in the venoms.

This review focuses on the specific degrading effects triggered by *Loxosceles* venoms in extracellular matrix molecules and the biological consequences of these hydrolytic activities related to the clinical signs induced by loxoscelism.

Presence of gelatinolytic enzymes in *L. intermedia* venom

Several extracellular matrix molecules have been described as targets for degradation evoked by proteases present in snake venoms, which cause hemorrhage, necrosis and edema (24-28). Soluble plasma fibrinogen is the major substrate in this family, but it is not the only one since snake venom actions on laminin, entactin, fibronectin and

Table 1. Brown spider venom properties.

Venom effects	Molecules involved	References
Dermonecrotic lesion	32 kDa++, 35 kDa+, +++	3,4,8,10,16,22
Intravascular hemolysis	32 kDa++, 35 kDa+	4,6,16,22
Platelet aggregation and thrombocytopenia	32 kDa++, 35 kDa+	4,8,15
Gravitational spreading	33 kDa++, 63 kDa++	4,17
Fibrinogenolytic activity	20-28 kDa+	19
Fibronectinolytic activity	20-28 kDa+	19
Gelatinolytic activity	32-35 kDa+, 85 kDa+, 95 kDa+	11,19,20
Entactinolytic activity	Unknown+	21,57
Basement membrane-degrading effect	Unknown+	21,57
Complement system activation	32 kDa++, 35 kDa+	4,16,22,23
Heparan sulfate proteoglycan hydrolysis	Unknown+	21

Dermonecrotic lesion, intravascular hemolysis, platelet aggregation and complement system activation are events dependent on the similar brown spider toxins of 32-35 kDa devoid of proteolytic activities.

The 32-35-kDa molecule with gelatinolytic activity is a metalloprotease.

Results obtained by using *Loxosceles intermedia* venom (+), *L. reclusa* venom (++), and *L. gaucho* venom (+++).

gelatin have been described (24,29). With respect to spider venoms and despite some similarities to snake venoms in their activities, little is known about the presence of venom proteases that degrade extracellular matrix molecules. Studying *L. intermedia* venom (the prevalent brown spider in southern Brazil) we were able to detect a 32-35-kDa metalloprotease with gelatinolytic activity. The 35-kDa protease form seems to be a latent pro-enzyme molecule that undergoes cleavage, originating the 32-kDa form (19). The 32-35-kDa proteases are high-mannose glycoproteins (20). We also detected zymogen molecules of proteolytic enzymes in the venom since trypsin activated two gelatinolytic serine proteases of 85 and 95 kDa in the venom. Other proteins such as casein, albumin, hemoglobin and laminin did not suffer any kind of cleavage. The specificity of action of these proteases should be related to spider self-protection. For example, the venom gland of *L. intermedia* is extremely rich in laminin, which separates muscle tissue (involved in venom secretion) from epithelial cells (involved in venom synthesis) (30), and the venom seems to have no lamininolytic activity (19,21). The natural substrate of the 32-35-kDa protease is unknown (since gelatin is denatured collagen and the venom does not display any activity on full length collagen), but, based on gelatinolytic activity, we may assume that this protease has properties like vertebrate gelatinases that appear to cleave connective components (31) and we may propose that this brown spider enzyme is functionally related to the deleterious effects of the venom. Native collagen can suffer an initial effect of collagenase from polymorphonuclear neutrophil leukocytes (which, as described above, are concentrated at the bite site and around it and seem to play a role in dermonecrosis), partially denaturing this molecule, which then can be sequentially degraded by these gelatinase-like venom proteases (see Table 1).

Effect of *Loxosceles* venom proteases on plasma extracellular matrix molecules

Brown spider envenomation, although causing classical skin degeneration, is also responsible for disorders of hemostasis such as hemorrhage into the dermis and disseminated intravascular coagulation. The hemorrhage mechanism has not been fully established at the molecular level. Plasma extracellular matrix molecules are multifunctional proteins involved in blood coagulation on the basis of their ability to bind heparin, as is the case for fibronectin and vitronectin (32-34), to interact with plasminogen, plasminogen activator inhibitors and the thrombin-antithrombin III complex, as is the case for vitronectin (33,35,36), and to promote platelet adhesion and aggregation, as is the case for fibrinogen, fibronectin, von Willebrand factor and thrombospondin (25,28,37-39). They also mediate fibrin formation as fibrinogen (38,39). *L. intermedia* venom degrades both the A and B chains of fibronectin (Figure 1) into a variety of smaller fragments (19,20), and partially digests fibrinogen (Figure 1) (19,20), but, interestingly, has no proteolytic activity on soluble vitronectin (see Figure 1). This fibrinogenolytic effect is closely similar to that of other brown spider venoms (*L. gaucho* and *L. laeta*) (Zanetti VC, unpublished results). The fibronectinolytic and fibrinogenolytic activities of *L. intermedia* venom are produced by a metalloprotease of 20-28 kDa (19). Based on the biological activities of brown spider venoms, we may postulate a relationship of this fibronectinolytic effect of the venom with hemorrhage and difficulties in wound healing. The hemorrhage into the dermis at the bite site, vasodilatation and injury to blood vessel walls could be associated with defective fibronectin surrounding the endothelial cells of capillaries. The hemorrhage could also be related to imperfect platelet adhesion and aggregation, since fibronectin partici-

pates in platelet attachment and aggregation and the proteolytic effect of the venom may disturb this event. Finally, the defective wound healing observed in some cases of envenomation may also be ascribed to the fibronectinolytic activity of the venom, since fibronectin-integrin interactions participate in an essential manner in this phenomenon (32). An identical hypothesis could be raised for the fibrinogenolytic ability of brown spider venom, since it has been well defined that several snake venom proteases with hemorrhagic activities readily degrade fibrinogen-fibrin into nonclotting fragments

(26,27,40). Disseminated intravascular coagulation is a clinical feature detected in a few cases of brown spider envenomation. Venoms from various snakes that partially degrade fibrinogen provoke disseminated intravascular coagulation by fibrin formation (25,28). As previously postulated by Bascur et al. (7) and corroborated by Feitosa et al. (19) and Veiga et al. (20), on the basis of partial fibrinogenolytic activity triggered by brown spider venoms, we may speculate that this ability is involved in disseminated intravascular coagulation, a complication that, together with other hematologic disturbances, has been responsible for most of the deaths due to loxoscelism.

Fibrinogen is a large glycoprotein (340 kDa) formed by dimeric linking of three distinct chains, α , β and γ , linked by disulfide bridges. As described above, fibrinogen α chains are degraded by the venoms. It is interesting to observe that the α chains of fibrinogen have two RGD sequences, a peptide well defined as a major binding site for several integrins (41). The cleaving of fibrinogen RGD sequences by the venoms could reduce the interaction of integrin $\alpha_{IIb} \beta_3$ on the platelet surface with fibrinogen, resulting in the loss of platelet adhesion and functional aggregation and contributing to the hemorrhagic effect of the venom. We may suggest this proteolysis as a conservative event for *Loxosceles* species, indicating some biological function related to the life cycle and envenomation of brown spiders (see Table 1).

Action of brown spider venom on basement membrane constituents

The basement membranes are ubiquitous specialized forms of extracellular matrices produced by several cell types such as endothelial, epithelial, fat, muscle and nervous cells. Molecularly they are characterized by the presence of a considerable variety of biochemically complex components arranged

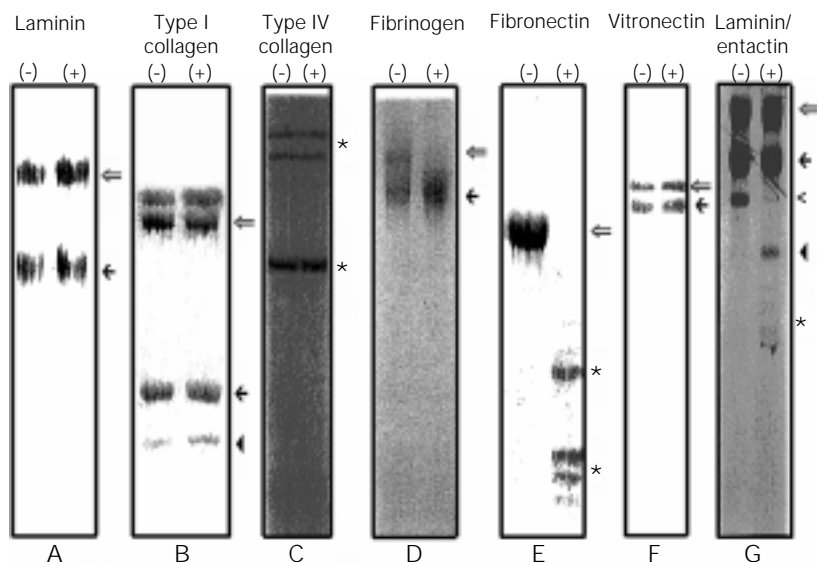


Figure 1. Proteolytic effect of *Loxosceles intermedia* venom on purified extracellular matrix molecules*. EHS-laminin (A), rat tail tendon type I collagen (B), human placental type IV collagen (C), human fibrinogen (D), human fibronectin (E), human vitronectin (F) and EHS-laminin/entactin dimer (G) were incubated with venom (+) or in the absence of venom, as controls for experimental stability (-) under the conditions described in References 19, 20, 21 and 57. The products obtained were analyzed by SDS-PAGE under reducing conditions (except for fibrinogen that was analyzed under nonreducing conditions). In panel A the open arrow indicates the laminin α_1 chain and the filled arrow indicates the β_1 and γ_1 laminin chains that co-migrate. In panel B the open arrow indicates the type I collagen β dimers, the filled arrow depicts α_1 type I collagen chain and the filled arrowhead indicates the α_2 type I collagen chain. In panel C the asterisks represent the major components of trypsin-extracted human placental type IV collagens of 100, 160 and 170 kDa. In panel D the open arrow points to an intact fibrinogen molecule and the filled arrow indicates the fibrinogen fragment. In panel E the open arrow shows the co-migratory fibronectin A and B chains, and asterisks indicate fibronectin fragments. In panel F the open arrow points to the 75-kDa vitronectin molecule and the filled arrow points to the 65-kDa normally processed vitronectin fragment. In panel G the open arrow indicates the laminin α_1 chain, the filled arrow indicates the β_1 and γ_1 laminin chains that co-migrate, the open arrowhead indicates intact entactin, the filled arrowhead indicates the 100-kDa entactin fragment, and the asterisk shows the 50-kDa entactin fragment. *Based on results from References 19, 20, 21 and 57.

as a network and formed by a particular set of proteins such as laminin, entactin, type IV collagen and heparan sulfate proteoglycan. Despite their tissue specificity (different isoforms), these components are present on practically all tissue basement membrane structures. In mammals, basement membranes play several essential roles in angiogenesis, cell differentiation, platelet adhesion, neurogenesis, and blood-urine filtration through the kidney glomeruli, among several other functions (42-46). The effects of venom toxins on basement membranes have been well established for some snake venom hemorrhagic proteases that produce proteolytic degradation of basement membrane constituents isolated from Engelbreth-Holm-Swarm (EHS) tumor (29,40), as well as degradation of purified glomerular basement membrane (47). The pathogenic properties of *Loxosceles* venoms, such as dermonecrotic action, thrombocytopenic activity, local hemorrhage and renal disorders, are events attributable to the presence of proteolytic enzymes that degrade basement membrane molecules. By using the EHS tumor, a transplantable model which produces a characteristic and thick basement membrane as its capsule (48), we detected a disruptive activity of *L. intermedia* venom toward basement membrane structures. The venom apparently has no activity on laminin or type IV collagen (see Figure 1), the major macromolecules of these structures, but has the ability to hydrolyze entactin (19,21), a dumbbell-shaped molecule that links laminin, type IV collagen and heparan sulfate proteoglycan in the basement membrane organization (42,43). The hydrolytic activity of *L. intermedia* venom on entactin resulted in fragments of approximately 100 and 50 kDa when entactin was complexed to laminin and lower fragments that run out the gel when purified entactin was assayed (21). Similar results concerning this differential entactin susceptibility to the venom have been reported for other venoms such as snake

venom toxins (29,40). This difference in cleavage profile for purified entactin and for the laminin-entactin complex treated with *L. intermedia* venom can be explained by the fact that laminin interacting with entactin can hide domains of entactin more susceptible to the degrading action of the venom, as previously described for the entactinolytic effect evoked by atrollysins, metalloproteases from *Crotalus atrox* snake venom (40).

Another essential family of molecules involved in basement membrane assembly and biological activities are the proteoglycans, complex molecules formed by a protein core to which one or more glycosaminoglycan chains are linked (49,50). They have been involved in several biological effects such as extracellular matrix organization (42,43), they are extracellular matrix cell surface receptors (51-53), play a role in cell-cell recognition (54), control cell growth and proliferation (50,55), participate in blood vessel stability and hemostasis (49), and have several other functions (49-51). *L. intermedia* venom degrades the protein core of purified heparan sulfate proteoglycan, but has no activity on heparan sulfate polysaccharide chains (21). On the other hand, the venom degrades chondroitin sulfate polysaccharide chains (Nader HB and Dietrich CB, personal communication). An established endothelial cell line from rabbit aorta (56) submitted to the presence of *L. intermedia* venom increases the liberation of heparan sulfate proteoglycan and sulfated proteins from its extracellular matrix and cell surface into the culture medium (Nader HB and Dietrich CB, personal communication). Based on the fact that *L. intermedia* venom degrades entactin, heparan sulfate protein core, and chondroitin sulfate polysaccharide chains, and releases laminin from basement membranes and that these molecules are extremely important for the structural and functional properties of basement membranes, we may speculate that these effects of the venom are plausible mechanisms for several deleterious conse-

quences of envenomation (see Table 1).

Concluding remarks and future expectations

The molecular mechanisms by which the brown spider venoms cause dermonecrotic injury, local and systemic hemorrhage, thrombocytopenia, hemolysis, disseminated intravascular coagulation and renal failure are currently under investigation, but since the venom is composed of a mixture of several proteins, these mechanisms seem to be molecularly complex and may be dependent on many different toxins.

In the present review, based on previous results obtained by our group (11,19-21,57), we introduce the idea of venom toxins acting as proteases upon molecular constituents of plasma extracellular matrix such as fibronectin and fibrinogen and basement membrane constituents such as entactin and heparan sulfate proteoglycan. Based on these degrading activities on plasma constituents and the disruptive effect on basement membrane structures triggered by venoms, we may speculate that these activities are plausible mechanisms for hemorrhage, delayed wound healing and renal failure and are also related to the spreading of other noxious toxins (for example, dermonecrotic protein), since disruption of the subendothelial basement membrane evokes blood vessel wall instability and increased permeability.

It is clear that more work is needed to elucidate the structure and function of these venom metalloproteases and to fully understand how they interfere with clinical signs of loxoscelism. However, it is interesting that all detected proteolytic activities and

especially the disruptive effect of *L. intermedia* venom on EHS-basement membranes can be inhibited by 1,10-phenanthroline, a metalloprotease inhibitor.

On the other hand, based on biodiversity, venom toxins are excellent tools for investigating molecular mechanisms in the cell biology field, as well as for pharmaceutical applications of newly discovered medicines. In this area, venom toxins could be used as starting materials to design new drugs or directly for therapeutic use as is the case for anacrod, a defibrinogenating enzyme from *Callocelasma rhodostoma* venom, and batroxobin, a defibrinogenating enzyme from *Bothrops atrox* venom, toxins that have been used as defibrinogenating agents for a number of clinical conditions such as deep vein thrombosis, myocardial infarction, pulmonary embolus, acute ischemic stroke, angina pectoris, central retinal vein occlusion and renal transplant rejection (27,58-60). With respect to the pharmaceutical applications of brown spider venom toxins, at least one possibility can emerge from our results. The fibrinogenolytic-fibrinolytic properties of venoms directly indicate that this toxin could be useful for clinical applications in cardiac and thrombotic diseases based on reduced blood fluidity. Other possibilities can directly or indirectly arise from brown spider venom toxins (for example, as a tool in cell biology procedures to study the inflammatory response). A molecular understanding of loxoscelism may greatly enhance our therapeutic approach to this envenoming problem, as well as generate potential toxins as tools for scientific protocols and medicinal applications.

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