Snake venom metalloproteinases and disintegrins: interactions with cells

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Abstract

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Metalloproteinases and disintegrins are important components of most viperid and crotalid venoms. Large metalloproteinases referred to as MDC enzymes are composed of an N-terminal Metalloproteinase domain, a Disintegrin-like domain and a Cys-rich C-terminus. In contrast, disintegrins are small non-enzymatic RGD-containing cysteine-rich polypeptides. However, the disintegrin region of MDC enzymes bears a high degree of structural homology to that of the disintegrins, although it lacks the RGD motif. Despite these differences, both components share the property of being able to recognize integrin cell surface receptors and thereby to inhibit integrin-dependent cell reactions. Recently, several membrane-bound MDC enzymes, closely related to soluble venom MDC enzymes, have been described in mammalian cells. This group of membrane-anchored mammalian enzymes is also called the ADAM family of proteins due to the structure revealing A Disintegrin And Metalloproteinase domains. ADAMs are involved in the shedding of molecules from the cell surface, a property which is also shared by some venom MDC enzymes.

Introduction

Venoms of viperid and crotalid snakes are rich sources of components that can affect hemostasis by causing changes in blood coagulation and platelet function. Venom components affecting the clotting system include thrombin-like enzymes (TLE) which convert fibrinogen to fibrin, and activators of prothrombin, factor V and factor X. Platelet function can be affected by venom components such as hemorrhagic metalloproteinases, phospholipases and RGD-containing disintegrins. As a result, it is common to find consumption of clotting factors and blood incoagulability accompanied by hemorrhage in victims of snakebite. Various reviews on this subject have been published (1-4).

Several venom enzymes lacking coagulant activity but possessing the ability to cause local hemorrhage have been characterized, as recently reviewed by Bjarnason and Fox (5). They are zinc-containing metalloproteinases varying in size from 20 to 100 kDa, capable of inducing rapid local bleeding. Metalloproteinases of different sizes are often present in the same venom. The high molecular weight enzymes are classified as

metalloproteinase/disintegrin-like/cysteine-rich (MDC) proteins, according to a structure containing an N-terminal catalytic site, a disintegrin-like domain followed by a cysteine-rich C-terminus. These enzymes have a potent proteolytic effect on the extracellular matrix proteins. Their effects on the blood vessel wall components have been extensively studied (5,6). The arrest of bleeding from the damaged blood vessels, however, depends on normal function of blood platelets and clotting factors, which are the principal blood components involved in hemostasis. The extent to which these latter components are directly affected by venom hemorrhagic metalloproteinases has not been explored in detail.

Another group of snake venom components that affect platelet function is comprised of small non-enzyme polypeptides (5-10 kDa) called disintegrins. Disintegrins have also been recently investigated in many other cells besides platelets. Since disintegrin domains of venom MDC enzymes and disintegrins show pronounced structural homology, they will be reviewed together here, referring particularly to their effects on cell-cell interactions using platelets as a model.

Mechanism of hemostasis

Blood fluidity in the circulation is maintained by the non-thrombogenic properties of the intact blood vessel walls. Damage to blood vessels triggers a prompt response of hemostatic reactions to prevent hemorrhage (7). Very briefly, these reactions include contraction of the vessel wall itself due to the action of released vasoactive agents, adhesion and aggregation of circulating platelets to form a hemostatic plug and activation of clotting factors leading to the formation of fibrin clots. In order to allow full tissue healing, the clots are subsequently removed by the fibrinolytic enzyme, plasmin. In situations where any component of these mechanisms is altered, hemostasis is compromised

and the result could be either thrombosis or hemorrhage (bleeding due to platelet and/or clotting factor deficiencies).

In small blood vessels, platelets alone can arrest bleeding. In their inactive form, platelets are discoid but once activated they become round, extend numerous pseudopods and then aggregate. This occurs when platelets are exposed to ADP, thrombin, adrenaline, collagen, and other agonists. Each agonist stimulates platelets via a specific receptor, whereby the receptor for collagen belongs to the superfamily of $\alpha\beta$ dimeric proteins or integrins (8). Indeed, the first reaction of platelets to vessel damage is their adhesion to the adhesive proteins, von Willebrand factor (vWF) and collagen on the exposed subendothelium. The respective platelet receptors for these proteins are the glycoprotein (gp) Ib/IX complex (9,10) and $\alpha_2\beta_1$ integrin also known as the gpIa/IIa complex (11). Engagement of these receptors stimulates platelets to secrete their granular contents and in particular ADP, which promotes activation of platelet $\alpha_{IIb}\beta_3$ integrin or gpIIb/IIIa. This receptor then binds the RGD-containing ligands (fibrinogen and von Willebrand factor) and thereby promotes platelet aggregation, resulting in the formation of a platelet plug which stops bleeding. Thus, the mechanism by which platelets act clearly depends on their surface receptors for vessel wall and plasma proteins and on normal content and release of their granular ADP stores.

Venom metalloproteinase/disintegrin-like/cysteine-rich enzymes

Mechanism of hemorrhage by venom MDC enzymes

As mentioned above, the presence of clotting factor activators in many venoms frequently causes consumption coagulopathy in envenomed victims and this is often associated with systemic bleeding. In some

envenomed patients, systemic bleeding can be fatal (12,13) and, interestingly in one case, fatal intracranial hemorrhage has been described apparently in the absence of co-agulopathy (14). Moreover, there have been other reports describing spontaneous systemic bleeding following snake bite in which no coagulation defects have been recorded (12,15,16). Systemic bleeding without coagulopathy has also been reported in a case of envenoming by the snake *Philodryas olfersii* (17), which led to the discovery of one hemorrhagic metalloproteinase in the venom of this species (18). Experimental studies have shown that venoms of various snakes can cause pronounced local hemorrhage in the absence of coagulant activity (15,19). In one such study using a purified non-coagulant hemorrhagic toxin, systemic bleeding was observed (20), whereas in another, using a different hemorrhagic toxin, this effect could not be demonstrated (21). Therefore, different metalloproteinases appear to differ in their ability to cause systemic effects; this clearly requires further in-depth studies of the effects of these enzymes on the hemostatic mechanism.

Various studies have demonstrated that purified venom metalloproteinases alone cause local hemorrhage. Hemorrhagic metalloproteinases from crotalid and viperid venoms produce local bleeding by causing lesions in the walls of small blood vessels (22,23). It is believed that this is caused by proteolysis of components of the basal lamina of the microvasculature (24-26). In animal experiments, such disruption of microvessels becomes evident within minutes of an intradermal injection of venom metalloproteinases. These enzymes degrade all major proteins of the extracellular matrix (ECM) (6,27,28) and in this respect they resemble the cell-secreted soluble matrix metalloproteinases (MMP). A positive correlation exists between the proteolytic activity of venom metalloproteinases and their hemorrhagic potencies. Larger enzymes are more potent than the smaller ones in degrading the extracellular matrix (6,26). However, the proteolytic attack of these enzymes on ECM proteins is slow, while the *in vivo* hemorrhagic effect of the venom occurs within minutes of the bite or experimental injection. This indicates that the mechanism of action of these enzymes may be considerably more complex (23,29). It has been well documented that the venom hemorrhagic metalloproteinases, because of their broad substrate specificity, cause digestion of the extracellular matrix proteins and damage the integrity of blood vessels. Although this can explain the phenomenon of local bleeding, past studies have shed little light on the possibility that metalloproteinases can have effects on other components of the hemostatic mechanism and thereby also contribute to systemic bleeding.

We have investigated the effects of envenoming by *Bothrops jararaca*, because this species constitutes the major problem in southeastern Brazil, and because local and systemic hemorrhage is one of the most prominent results of envenoming by this species (30-32). The main venom component responsible for this effect is thought to be jararhagin (33), an MDC enzyme which causes intense local hemorrhage, inhibits platelet aggregation *in vitro* and, as shown in animal experiments, also contributes to systemic bleeding (34). The reason why jararhagin contributes significantly to the systemic bleeding is because this enzyme is not effectively inhibited by plasma proteinase inhibitors (34). When radiolabelled jararhagin was incubated with whole plasma and its binding to plasma proteins examined by immunoelectrophoresis, only α_2-macroglobulin (α_2-M) was capable of binding jararhagin. However, jararhagin retained considerable proteolytic activity towards high molecular weight substrates such as fibrinogen and hide powder azure in the presence of a large molar excess of α_2-M (34). Others have also shown that some venom metallo-

proteinases are not completely inhibited by equimolar amounts of α_2-M (28,35), but the precise reason why the inhibitor-enzyme reaction failed to proceed to completion was not clarified.

Of all venom components known to interfere with hemostasis, the hemorrhagic metalloproteinases have been the least investigated. Little attention has been paid to the effects of these enzymes on platelets which, as mentioned before, play a central role in the hemostatic mechanism.

Venom MDC enzymes and platelet function

Earlier studies of venom metalloproteinases showed that they can inhibit platelet aggregation *in vitro* (36). As the majority of these enzymes are also known to hydrolyze the α-chain of fibrinogen (α-fibrinogenases; 37), and since fibrinogen is an important cofactor in platelet aggregation (38,39), it has been proposed that the inhibition of platelet aggregation caused by these enzymes is due to degradation of fibrinogen (2,40). However, we have shown that the proteolysis by jararhagin of the C-terminal fibrinogen α-chain, which contains an RGD sequence, does not affect fibrinogen-dependent platelet aggregation (41). This is mainly because jararhagin and other venom α-fibrinogenases do not attack the fibrinogen γ-chain, which contains the more important platelet binding site.

The venom MDC enzymes, such as jararhagin, have a disintegrin-like domain whose function is not precisely known. However, we have reported that this domain may play an important role in the substrate specificity of the enzyme (42,43). Jararhagin has a very selective effect on platelets in platelet-rich plasma *in vitro* with inhibition of both collagen- and ristocetin-induced cell aggregation only. This suggested either the proteolysis by jararhagin of collagen and vWF, or an interference with their respective platelet receptors, $\alpha_2\beta_1$ integrin and gpIb. The

fibrillar type I collagen currently used in platelet aggregation studies is not degraded by venom MDC enzymes, the preferential substrate structure being type IV collagen (5).

In jararhagin-treated platelets, no alterations of gpIb could be detected either by flow cytometry or by immunoprecipitation (44). This indicated that jararhagin inhibition of platelet responses to the ristocetin/vWF complex may have been due to some effect on the multimeric plasma protein, vWF. The 225-kDa vWF subunit contains the Val[449]-Lys[728] N-terminal domain responsible for ristocetin-induced binding to platelet gpIb, and one binding site for platelet $\alpha_{IIb}\beta_3$ integrin containing the C-terminal RGDS[1744-1747] sequence (45). We have shown that the treatment of vWF with jararhagin resulted in the proteolysis of vWF subunits in the N-terminal half of the molecule which contains the platelet gpIb binding site (44). Thus, jararhagin inhibited platelet responses to ristocetin by cleaving the ligand vWF, with loss of the gpIb binding site.

There are several proposed platelet collagen receptors including $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins, gpIV and gpVI. It is proposed that collagen first binds to the I domain of the $\alpha_2\beta_1$ integrin; this binding generates signals for the activation and expression of a binding site on $\alpha_{IIb}\beta_3$ integrin (46) resulting in collagen binding, presumably by the collagen RGD sequence. These reactions result in a full platelet aggregation response. The $\alpha_{IIb}\beta_3$ integrin has also been found to be both structurally and functionally unaltered in jararhagin-treated platelets (41). This was expected from the preserved fibrinogen-dependent platelet aggregation which is mediated by the $\alpha_{IIb}\beta_3$ integrin. GpIV is also functional in jararhagin-treated platelets (Kamiguti AS and Zuzel M, unpublished data). In contrast, the expression of the $\alpha_2\beta_1$ integrin following platelet treatment by jararhagin is markedly reduced and we found that this was due to the proteolysis of the β_1 subunit of this integrin (42). There is evi-

dence that the proteolysis of the platelet $\alpha_2\beta_1$ integrin by jararhagin is a two-step process in which jararhagin first recognizes the α_2 I domain (47) and subsequently cleaves the β_1 subunit (42). Jararhagin, as an intact enzyme, does not remain bound to platelets. This can be explained by the alteration of the integrin structure following proteolysis which results in the loss of both collagen and jararhagin binding (42). An equilibrium binding could be demonstrated when the enzyme was inactivated by treatment with 1,10-phenanthroline (42). Therefore, it is most likely that the recognition of the platelet $\alpha_2\beta_1$ integrin by jararhagin occurs via the disintegrin-like domain including the Cys-rich region. Furthermore, by using synthetic ECD-containing peptides based on the disintegrin-like domain of jararhagin, we showed that these peptides effectively inhibited platelet responses to collagen, thus confirming the importance of this domain in cell recognition (43).

Platelet interaction with collagen results in increased protein tyrosine phosphorylation, one of the earliest signals being the phosphorylation of the non-receptor 72-kDa tyrosine kinase Syk or pp72^{syk} (48,49). The activation of pp72^{syk} depends on both $\alpha_2\beta_1$ integrin and gpVI because the absence of either one of these receptors results in decreased Syk phosphorylation and a poor platelet response to fibrillar collagen (50-53). We found that the proteolysis of the $\alpha_2\beta_1$ integrin by jararhagin inhibited protein tyrosine phosphorylation in collagen-stimulated platelets, with greatly reduced tyrosine phosphorylation of pp72^{syk} (54). These findings not only explain the altered responses of jararhagin-treated platelets to collagen, but also support the hypothesis that $\alpha_2\beta_1$ integrin plays a vital role in the platelet/collagen interaction (55, 56). Moreover, since congenital deficiency of this receptor leads to bleeding manifestations (52), it is reasonable to expect that jararhagin-treated platelets would not be hemostatically fully competent.

Venom disintegrins

Disintegrins are cysteine-rich low molecular weight polypeptides, which contain an RGD sequence recognized by integrins. Trigramin, from the venom of *Trimeresurus gramineus*, was the first venom disintegrin characterized as a competitive inhibitor of fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin of platelets activated by ADP (57). Following this, a number of other disintegrins isolated from different snake venoms have been reported and reviewed (58). It is interesting that, unlike other RGD-containing ligands (i.e. fibrinogen), venom disintegrins bind to $\alpha_{IIb}\beta_3$ integrin without requiring prior activation of this integrin. This is because disintegrins have a unique RGD-containing loop which can express the ligand-induced binding site (LIBS) on the β_3 subunit (59).

Recently, a relatively large disintegrin (28 kDa) containing an ECD sequence instead of RGD, isolated from the venom of *B. jararaca* has been reported. This disintegrin has exactly the same primary structure as the disintegrin-like domain of jararhagin and was therefore named jararhagin C (60). Unlike jararhagin, which in our studies showed no interference with platelet response to ADP, jararhagin C inhibited both the ADP- and collagen-induced responses of platelets. Nevertheless, the finding that jararhagin C inhibits collagen-induced platelet aggregation confirms our conclusion that the disintegrin-like domain of the enzyme jararhagin recognizes the platelet collagen receptor, $\alpha_2\beta_1$ integrin.

Effects of venom disintegrin on cell function

As mentioned above, disintegrins have the ability to inhibit the binding of RGD-containing ligands to platelet $\alpha_{IIb}\beta_3$ integrin and, because of this, they impair platelet aggregation responses dependent on this receptor. Thus, platelets treated with disintegrins do not aggregate in response to collagen stimulation. However, these platelets

show normal phosphorylation of the tyrosine kinase pp72[syk] (54,61). As mentioned above, collagen first binds to the $\alpha_2\beta_1$ integrin, signaling for pp72[syk] phosphorylation and an inside-out activation of $\alpha_{IIb}\beta_3$ integrin. In the disintegrin-treated platelets, the $\alpha_{IIb}\beta_3$ integrin is already occupied by the venom ligand so that the aggregation response is lost. However, pp72[syk] phosphorylation is preserved, confirming that free $\alpha_2\beta_1$ can bind collagen and signals in these platelets. In this respect, we found results using both jararhagin and contortrostatin particularly revealing. Contortrostatin (62) is a dimeric RGD-containing disintegrin from *Agkistrodon contortrix contortrix* venom. Suboptimal ADP stimulation of jararhagin-treated platelets resulted in a full aggregation response to collagen and this could be inhibited by either RGDS peptide or contortrostatin (54). Our explanation for this is that ADP caused activation of the $\alpha_{IIb}\beta_3$ integrin in these platelets, which then bound collagen. Inhibition of this binding by contortrostatin emphasizes the importance of $\alpha_{IIb}\beta_3$ in addition to $\alpha_2\beta_1$ integrin in the platelet/collagen interaction.

Recently, disintegrins have been also shown to interfere with other integrin-mediated cell functions; for example, inhibition of tumor cell-extracellular matrix adhesion (63-65) and metastasis (62,66), of adhesion of human umbilical vein endothelial cells to matrix proteins (59), and also of egg fertilization through inhibition of sperm-oolemmal adhesion (67).

Venom MDC enzymes and disintegrins: structure/function relationships

The elucidation of the primary structure of different members of the venom hemorrhagic metalloproteinase-disintegrin family (33,68-70) has generated new interest in the function of these enzymes. Comparison of amino acid sequences have indicated that, despite differences in their molecular sizes,

all these enzymes may be related through a common ancestral gene encoding a prodomain, metalloproteinase, disintegrin and cysteine-rich domains (33,69,71). The prodomain contains a conserved sequence PRCGVPD, called a cysteine-switch, which is responsible for the enzyme activation and is lacking in the mature protein. A multidomain structure characterizes the large hemorrhagic metalloproteinases; the N-terminal region possessing a zinc-containing metalloproteinase domain is followed by a disintegrin-like and a C-terminal cysteine-rich domain. Moreover, disintegrin-like regions of MDC enzymes possess a high sequence homology to the venom disintegrins. Disintegrins contain an RGD sequence, but a KGD sequence has also been reported (72). In contrast, the disintegrin-like regions of most venom MDC enzymes have a conserved ECD sequence overlapping the region where an RGD sequence occurs in the homologous disintegrins. However, this is not a constant motif found in all disintegrin-like domains because some venom MDC enzymes have a DCD sequence instead of ECD (73,74).

Since the large hemorrhagic metalloproteinases possess a disintegrin-like domain (MDC enzymes) it is thought that some disintegrins may originate from autoproteolysis of these enzymes (75). The discovery that jararhagin C is identical to the disintegrin-like plus Cys-rich regions of jararhagin in *B. jararaca* venom (60) supports this proposal.

Membrane-anchored cell MDC enzymes (ADAM family proteins)

A number of membrane-anchored MDC enzymes have been reported. They belong to the ADAM family proteins, with <u>A</u> <u>D</u>isintegrin <u>A</u>nd <u>M</u>etalloproteinase domain (76). They are unique cell surface proteins possessing both a potential adhesion domain as well as a potential protease domain, not related to other surface adhesion molecules and proteases, but rather to domains found in

the snake venom MDC enzymes. Sequence homology to the disintegrin-like domain of venom hemorrhagic metalloproteinases has also been found in a sperm protein, fertilin ß, which mediates egg binding in sperm-egg fusion (77). Recently, it has been shown that the disintegrin-like region of this sperm protein recognizes the $\alpha_6\beta_1$ integrin on the egg membrane (78). In addition, six other distinct ADAMs have been identified in spermatogenic cells; all are considered to be potential ligands for integrins on the cell surface or receptors for extracellular matrix and are, therefore, expected to play important roles in cell-cell and cell-matrix interactions (79).

The presence of ADAM proteins is not confined to reproductive tissue only. In 1989, a metalloproteinase isolated from bovine brain myelin membrane preparations appeared to be distinct from the other MMP described at that time (80). The determination of the primary structure of this metalloproteinase by cDNA cloning revealed that significant similarity exists between the deduced sequence of the bovine protein and jararhagin, the greatest homology being found in the disintegrin domain (81). Similar findings involve another ADAM protein, meltrin-α, which participates in myoblast fusion (82). More recently, a widely expressed cellular ADAM or MDC enzyme, designated as MDC9, has been described to have a cytoplasmic tail that can bind the SH3 domain of signal molecules of the Src family proteins, thus implicating MDC9 in interactions with the cytoskeleton or with intracellular signaling molecules (83).

The cytokine tumor-necrosis factor-α (TNF-α) has physiological importance in inflammation. The release of soluble TNF-α from its membrane-bound precursor involves an ADAM enzyme called TNF-α-converting enzyme (TACE), recently purified and cloned from monocytes (84). TACE has been shown to be present in monocytes, peripheral blood T cells, neutrophils, endothelial and smooth muscle cells. TACE has also been purified and cloned from porcine spleen and the recombinant protein shown to correctly process precursor TNF-α to the mature form (85). Thus, it appears that the membrane-anchored ADAM proteins play an important function in shedding cell surface proteins (84). It is interesting that our investigations demonstrated that also the soluble venom MDC enzyme jararhagin can release TNF-α in a similar fashion to TACE (86). Thus, there is an indication that jararhagin may play a role in the inflammatory response evoked by *B. jararaca* venom, although this has not yet been fully investigated.

Conclusions

Venom disintegrins and MDC enzymes are excellent tools for investigating integrin cell receptor function. Interference with integrins and their ligands is the major mechanism by which these proteins contribute to the toxicity of snake venoms. Moreover, the understanding of the precise mechanism of action of these venom components and the finding of the minimum structure required for their action will certainly extend their application in studies of cell physiology. Thus, the studies of venom disintegrins and MDC enzymes have already greatly aided the advance which has been recently achieved in understanding the function of similar mammalian proteins.

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