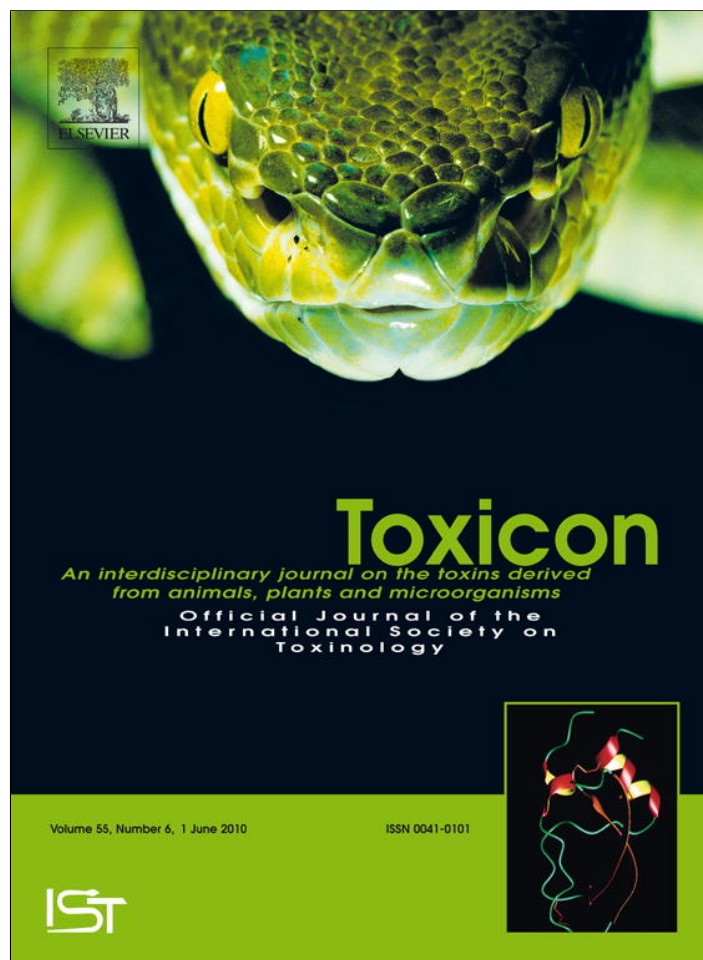


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Different regions of the class P-III snake venom metalloproteinase jararhagin are involved in binding to $\alpha_2\beta_1$ integrin and collagen

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ABSTRACT

SVMPs are multi-domain proteolytic enzymes in which disintegrin-like and cysteine-rich domains bind to cell receptors, plasma or ECM proteins. We have recently reported that jararhagin, a P-III class SVMP, binds to collagen with high affinity through an epitope located within the Da-disintegrin sub-domain. In this study, we evaluated the binding of jararhagin to $\alpha_2\beta_1$ integrin (collagen receptor) using monoclonal antibodies and recombinant jararhagin fragments. In solid phase assays, binding of jararhagin to $\alpha_2\beta_1$ integrin was detectable from concentrations of 20 nM. Using recombinant fragments of jararhagin, only fragment JC76 (residues 344–421), showed a significant binding to recombinant $\alpha_2\beta_1$ integrin. The anti-jararhagin monoclonal antibody MAJar 3 efficiently neutralised binding of jararhagin to collagen, but not to recombinant $\alpha_2\beta_1$ integrin nor to cell-surface-exposed $\alpha_2\beta_1$ integrin (α_2 -K562 transfected cells and platelets). The same antibody neutralised collagen-induced platelet aggregation. Our data suggest that jararhagin binding to collagen and $\alpha_2\beta_1$ integrin occurs by two independent motifs, which are located on disintegrin-like and cysteine-rich domains, respectively. Moreover, toxin binding to collagen appears to be sufficient to inhibit collagen-induced platelet aggregation.

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1. Introduction

During the evolution of snakes, venom proteins were selected with the aims of inducing the rapid immobilisation of prey and to serve as a defence against predators. However, in rural areas and forests, humans are at great risk to accidentally come into contact with venomous snakes. In cases of snakebites, a severe pathology takes place with symptoms dependent on several factors, for which the type of snake venom is a determinant. Their venom contains a large proportion of biologically active proteins or peptides that interact with components of the haemostatic system, including the coagulation cascade,

endothelial cells, basement membrane and platelets. As result, bites by viper snakes cause haemorrhage and coagulation disorders.

The most important snake venom proteins that interfere with platelet functions are C-type lectins, disintegrins and metalloproteinases. Their targets on platelet surfaces include the receptors GPIb-IX-V, GPIIb-IIIa ($\alpha_2\beta_3$ integrin), GPIa-IIa ($\alpha_2\beta_1$ integrin) and GPVI and also their ligands, von Willebrand factor, fibrinogen, and collagen (Andrews et al., 2001). Snake venom metalloproteinases (SVMPs) P-III class have a particular effect on collagen-induced platelet aggregation. SVMPs are multi-domain enzymes composed of a catalytic domain typical of metzincin metalloproteinases, a disintegrin-like domain devoid of the RGD sequence, and a cysteine-rich domain, also present in ADAMs (Fox and Serrano, 2008). The inhibition of collagen-induced platelet aggregation by P-III SVMPs has been

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primarily related to interference of the interaction between $\alpha_2\beta_1$ integrin receptors and collagen.

Jararhagin, a P-III SVMP isolated from *Bothrops jararaca* venom (Paine et al., 1992), inhibits collagen-induced platelet aggregation (Kamiguti et al., 1996). It has been shown that jararhagin cleaves the β_1 subunit of the integrin receptor, which then loses its function for aggregation by modifications at the functional I-domain of the α_2 subunit, which is responsible for the recognition of native collagen (Kamiguti et al., 1996). However, inhibition of collagen-induced platelet aggregation also occurs in the absence of proteolytic activity. Isolated disintegrin-like/cysteine-rich domains of native jararhagin or recombinant jararhagin-C (Usami et al., 1994; Moura-da-Silva et al., 1999) also inhibit platelet aggregation by interfering with the binding between $\alpha_2\beta_1$ integrin and collagen. In this regard, it has been shown that P-III SVMPs or their fragments bind to the integrin receptor. Synthetic peptides containing the sequence RKKH of the jararhagin catalytic domain have been shown to bind to the I-domain of the α_2 subunit (Ivaska et al., 1999). Peptides based on the disintegrin-like region (De Luca et al., 1995; Kamiguti et al., 1997; Jia et al., 1997) or cysteine-rich domains (Kamiguti et al., 2003) have been shown to inhibit collagen-induced platelet aggregation. Moreover, SVMPs may also block the interaction between platelets and collagen by binding to collagen fibres (Zhou et al., 1995; Liu and Huang, 1997) resulting in the inhibition of collagen-induced platelet functions. Taken together, these observations indicate that jararhagin, as other SVMPs, displays multiple mechanisms probably related to different structural motifs to reach its effect on platelet inhibition.

We have recently studied the interaction of jararhagin and collagen as a mechanism involved in SVMP-induced haemorrhagic activity (Moura-da-Silva et al., 2008). Jararhagin strongly binds to collagen I and IV generic triple-helix structure and the binding was completely inhibited by a monoclonal antibody, MAJar 3, which recognises a conformational epitope located on the Da sub-domain of the jararhagin disintegrin-like domain. In the present study, we assessed jararhagin binding to $\alpha_2\beta_1$ integrin and used MAJar 3 to show that jararhagin displays different epitopes to bind to $\alpha_2\beta_1$ integrin and collagen, highlighting the role of binding to collagen for class P-III SVMP platelet aggregation inhibition and spatial features that could enhance the toxicity of SVMPs.

2. Material and methods

2.1. Jararhagin and recombinant fragments

Jararhagin, a haemorrhagic metalloproteinase from *B. jararaca* venom, was obtained as previously described (Moura-da-Silva et al., 2003). Recombinant fragments corresponding to segments of jararhagin disintegrin-like and cysteine-rich domains were produced in an *Escherichia coli* expression system (Tanjoni et al., 2003). Briefly, the cDNA sequences comprising the disintegrin-like domain (residues 210–298 – **JD89**), the Da-disintegrin sub-domain (residues 250–298 – **JD49**), the cysteine-rich domain (residues 304–421 – **JC116**) and the cysteine-rich domain

hyper-variable region (residues 344–421 – **JC76**) were amplified by polymerase chain reaction (PCR) from jararhagin BJD4 clone (Paine et al., 1992) and cloned into pGEX4T-1 vector (Amersham Biosciences). The expression was achieved in the protease-free strain *E. coli* BL21 induced with 0.1 mM IPTG. The GST fusion peptides were affinity-purified with Glutathione Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions.

2.2. Antibodies

Anti-jararhagin polyclonal antibodies were raised in rabbits by immunisation with the purified protein (Moura-da-Silva et al., 1999). Monoclonal antibodies were produced from hybridomas previously produced and maintained in our laboratory (Tanjoni et al., 2003). MAJar 3 and MAJar 2 antibody-secreting hybridomas were cultured in RPMI 1640 medium supplemented with 10% FCS (GibcoBRL) at 37 °C and 5% CO₂. Immunoglobulins were purified from culture supernatants by affinity chromatography on protein A Sepharose equilibrated in borate-buffered saline, pH 8.5, and eluted in citrate buffer 0.15 M, pH 5.0. MAJar 2 and MAJar 3 antibodies are IgG1 isotypes and recognise conformational epitopes located on jararhagin Ds- and Da-disintegrin sub-domains, respectively. MAJar 3 neutralises jararhagin binding to collagen and thus its haemorrhagic activity. MAJar 2 was used as a control for binding to a non-related epitope, since it does not neutralise jararhagin-induced haemorrhaging and only partially neutralises the toxin binding to collagen. A monoclonal antibody raised against a non-related antigen (*Taenia crassiceps*-secreted antigen) was used as control of IgG1 isotype.

2.3. Cell lines

K562 cells transfected with cDNA coding for α_2 integrin subunit were a gift from Prof. S. Niewiarowski (Temple University, Philadelphia, USA). Cells were cultured under standard conditions in RPMI or DMEM media containing 10% FBS, L-glutamine, penicillin, streptomycin and geneticin for the transfected cells. The non-transfected k562 were used as a control. Each cell line was evaluated for its surface integrin contents by flow cytometry using anti-integrin monoclonal antibodies.

2.4. Binding of jararhagin to $\alpha_2\beta_1$ integrin

The binding of jararhagin to $\alpha_2\beta_1$ integrin was analysed by solid phase assay using the recombinant extracellular domains of human $\alpha_2\beta_1$ integrin (Eble et al., 1998) and by dot blot experiments using washed platelets and K562 cells transfected with α_2 subunit (α_2 -k562). For experiments with soluble integrins, microtitre plates were coated with 5 µg/mL recombinant $\alpha_2\beta_1$ integrin diluted in Tris-buffered saline containing 3 mM MgCl₂. After blocking with 1% BSA in Tris-buffered saline, plates were incubated with samples containing only jararhagin or jararhagin pre-incubated (2 h at 37 °C) with saturating amounts of monoclonal or polyclonal antibodies. Then, bound jararhagin was detected using anti-jararhagin serum raised in rabbits (1:100)

followed by incubation with goat IgG anti-rabbit IgG labelled with horseradish peroxidase (1:2000) and with ortho-phenylenediamine/H₂O₂ as enzyme substrates. The products were detected at 492 nm.

In dot blot experiments, 2 μ L samples containing 4×10^4 cells (α_2 -k562 and non-transfected k562) or 10^5 washed platelets were dotted on nitrocellulose membranes. After blocking with 5% defatted milk in Tris-buffered saline, membranes were incubated with different concentrations of jararhagin or jararhagin pre-incubated (1 h at 37 °C) with saturating amounts of monoclonal or polyclonal antibodies. Membranes were washed and incubated with anti-jararhagin serum raised in rabbits (1:2000) followed by incubation with goat IgG anti-rabbit IgG labelled with horseradish peroxidase (1:2000). The dots were developed by addition of 0.05% 4-chloro-1-naphthol, 0.03% H₂O₂ in 15% methanol.

2.5. Human platelet aggregation

Blood was collected from healthy human adult volunteers who had not taken any medication two weeks prior to collection, and 3.8% sodium citrate (9:1, vol/vol) was added as an anticoagulant. Citrated blood was immediately centrifuged for 10 min at 120 g at 25 °C, and the supernatant (platelet-rich plasma, PRP) was obtained. PRP was diluted in 0.1% EDTA and centrifuged for 30 min at 850 g. The pellets containing the platelets were washed two times with Tyrode's solution and platelet concentration was adjusted to 3×10^8 platelets/mL for aggregation assays using a Chrono-log lumi-aggregometer (Chrono-log Corporation, USA). Samples of 400 μ L washed platelets were incubated for 5 min with increasing concentrations of jararhagin or jararhagin pre-incubated (1 h at 37 °C) with saturating amounts of monoclonal or polyclonal antibodies and challenged with 2 μ g/mL collagen. The extent of aggregation was calculated as the increase in light transmission for 3 min after the addition of collagen.

3. Results

We have previously shown that jararhagin binds with high affinity to collagen I and IV and the binding is blocked by incubation of jararhagin with MAJar 3 monoclonal antibody (Moura-da-Silva et al., 2008). In the present study, we evaluated jararhagin binding to $\alpha_2\beta_1$ integrin and its structural regions related to the binding, correlating to the role of integrin or collagen binding for the inhibition of platelet aggregation induced by jararhagin. As shown in Fig. 1A, binding of jararhagin to $\alpha_2\beta_1$ integrin was detectable at concentrations of 20 nM when tested by solid phase assays with the soluble recombinant integrin molecule coated onto microtitre plates. The binding was dose-dependent, showing a significant increase in OD 492 nm when 200 nM concentrations were used. Jararhagin bound at similar concentrations to $\alpha_2\beta_1$ integrin displayed in the membrane of K562 cells transfected with the α_2 subunit cDNA (α_2 -K562). The specificity of the reaction was confirmed since incubation of jararhagin with non-transfected K562 cells, devoid of the α_2 subunit, resulted in lower signals close to the background values (Fig. 1B).

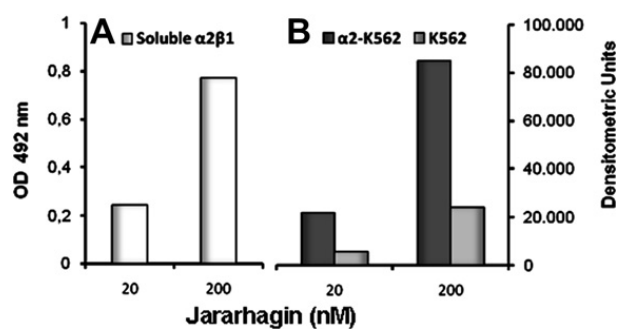


Fig. 1. Jararhagin binding to $\alpha_2\beta_1$ integrin: (A) Soluble recombinant extracellular domains of human $\alpha_2\beta_1$ integrin (5 μ g/mL) were coated onto microtitre plates in Tris-buffered saline containing 3 mM MgCl₂. After blocking, plates were incubated with jararhagin and the bound toxin was detected using anti-jararhagin serum raised in rabbits followed by incubation with goat IgG anti-rabbit IgG labelled with horseradish peroxidase (HRP) and with ortho-phenylenediamine/H₂O₂ as enzyme substrates. The products were read at 492 nm (B) 2 μ L samples containing 4×10^4 cells (α_2 -k562 and non-transfected k562 as control) were dotted on nitrocellulose membranes. After blocking, membranes were incubated with jararhagin. Membranes were washed and incubated with polyclonal anti-jararhagin serum raised in rabbit followed by incubation with goat IgG anti-rabbit IgG labelled with HRP. The dots were developed by addition of 0.05% 4-chloro-1-naphthol, 0.03% H₂O₂ in 15% methanol and the densitometric units of each dot estimated using the ImageJ software from NIH (<http://rsbweb.nih.gov/ij/>).

Attempting to identify the region of the jararhagin molecule involved in its binding to $\alpha_2\beta_1$ integrin, competition assays with anti-jararhagin monoclonal antibodies were carried out. The same monoclonal antibodies used to map the collagen-binding epitope, located at the D₂ disintegrin sub-domain, were used and jararhagin binding to either collagen or $\alpha_2\beta_1$ integrin was accessed after pre-incubation of the toxin with antibodies under saturating conditions. As shown in Fig. 2, pre-incubation of jararhagin with MAJar 3 resulted in a drastic reduction in the binding of the toxin to collagen (83%), as previously reported (Moura-da-Silva et al., 2008). In opposition, only a mild reduction of jararhagin binding to recombinant $\alpha_2\beta_1$

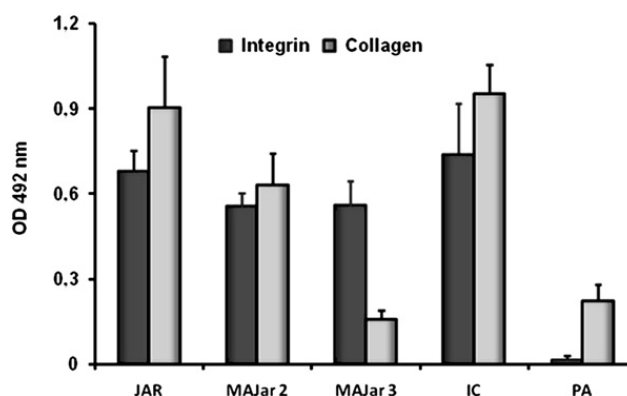


Fig. 2. Neutralisation of jararhagin binding to $\alpha_2\beta_1$ integrin and to collagen by monoclonal antibodies: Soluble recombinant extracellular domains of human $\alpha_2\beta_1$ integrin (dark grey columns) or collagen I (light grey columns) were coated onto microtitre plates and incubated with jararhagin (JAR) or jararhagin pre-incubated (1 h at 37 °C) with saturating amounts of monoclonal antibodies MAJar 2, MAJar 3, the isotype control (IC) or anti-jararhagin polyclonal antibodies (PA). Bound toxin was detected using anti-jararhagin serum raised in rabbits followed by incubation with goat IgG anti-rabbit IgG labelled with HRP and with ortho-phenylenediamine/H₂O₂ as enzyme substrates. The products were read at 492 nm.

integrin (18%) was observed after incubation with MAJar 3, suggesting that jararhagin binds to collagen and $\alpha_2\beta_1$ integrin by distinct motifs. Pre-incubation of jararhagin with MAJar 2, an antibody that recognises an epitope at the Ds-disintegrin sub-domain, resulted in a small decrease in the binding of the toxin to both collagen and $\alpha_2\beta_1$ integrin (19% and 30%, respectively), suggesting that this sub-domain is apparently not involved in the binding to either collagen or $\alpha_2\beta_1$ integrin. The values of jararhagin binding to collagen or $\alpha_2\beta_1$ integrin were similar when the toxin was incubated with the non-related antibody (isotype control). Only the serum containing polyclonal anti-jararhagin antibodies, raised by rabbit immunisation, was able to neutralise the binding of jararhagin to both collagen and $\alpha_2\beta_1$ integrin (Fig. 2), indicating that the interaction between jararhagin and $\alpha_2\beta_1$ integrin can also be neutralised by antibodies although by interaction with distinct epitopes. Similar results were observed when antibody neutralisation activity was tested using jararhagin and $\alpha_2\beta_1$ integrin displayed at cell surfaces (Fig. 3). Specific binding of jararhagin to washed platelets or α_2 -K562 cells, accessed by dot blots, was inhibited only by polyclonal anti-jararhagin antibodies. MAJar 3 and MAJar 2 were unable to block jararhagin binding to $\alpha_2\beta_1$ integrin on α_2 -K562 cells and platelet surfaces (Fig. 3), similarly to the isotype control. These data confirm that the epitope recognised by MAJar 3 is involved only in jararhagin binding to collagen and suggest that Ds- and Da- disintegrin sub-domains are not involved in jararhagin binding to $\alpha_2\beta_1$ integrin.

Recombinant fragments were then used in an attempt to identify the jararhagin region responsible for the binding to $\alpha_2\beta_1$ integrin. Fragments JD89, JD49, JC116 and JC76 were obtained from expression in *E. coli* and correspond to the entire disintegrin-like domain, the Da-disintegrin sub-domain, the entire cysteine-rich domain and the C-terminal segment of the cysteine-rich domain comprising the hyper-variable region, respectively. As previously reported

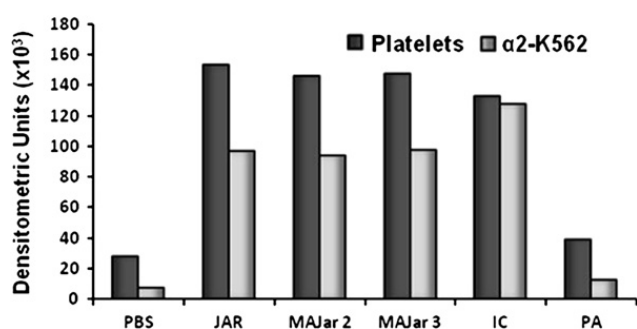


Fig. 3. Neutralisation of jararhagin binding to cell-surface $\alpha_2\beta_1$ integrin by monoclonal antibodies: 2 μ L samples containing 4×10^4 washed platelets (dark grey columns) or α_2 -k562 cells (light grey columns) were dotted on nitrocellulose membranes. After blocking, membranes were incubated without jararhagin (PBS) as a negative control or with jararhagin (JAR) or jararhagin pre-incubated (1 h at 37 °C) with saturating amounts of monoclonal antibodies MAJar 2, MAJar 3, the isotype control (IC) or anti-jararhagin polyclonal antibodies (PA). Membranes were washed and incubated with polyclonal anti-jararhagin serum raised in rabbit followed by incubation with goat IgG anti-rabbit IgG labelled with HRP. The dots were developed by addition of 0.05% 4-chloro-1-naphthol, 0.03% H₂O₂ in 15% methanol and the densitometric units of each dot estimated using the ImageJ software from NIH (<http://rsbweb.nih.gov/ij/>).

(Tanjoni et al., 2003), MAJar 3 recognises JD49 and JD89 fragments and MAJar 2 recognises only the JD89 fragment. Polyclonal, but not monoclonal antibodies, recognised the fragments contained in the cysteine-rich domain (JC76 and JC116). Binding of each fragment to $\alpha_2\beta_1$ integrin-coated plates was accessed as described. Fig. 4 shows the values obtained for each fragment after decreasing the values of non-specific binding resulting from the GST incubation. A significant increase of binding was observed only after incubation with JC76 fragment, suggesting that the $\alpha_2\beta_1$ integrin-binding motif is located close to or within the hyper-variable region of the cysteine-rich domain.

Our next step was to check the involvement of collagen binding of a MAJar 3-related epitope in the inhibition of collagen-induced platelet aggregation by jararhagin. Fig. 5 shows the platelet aggregation profiles observed after incubation with monoclonal antibodies in a representative experiment. A short while after challenge with collagen, washed platelets enlarged in size and aggregated (Fig. 5 A). This effect was completely inhibited by the addition of 20 nM jararhagin (Fig. 5B). However, when the toxin was pre-incubated with MAJar 3, the inhibition of platelet aggregation was no longer observed (Fig. 5C), indicating that neutralisation of jararhagin binding to collagen also impairs its inhibitory effect on platelet aggregation. Inhibition of platelet aggregation was similar when platelets were pre-incubated with jararhagin only or jararhagin pre-incubated with MAJar 2 (Fig. 5B and D, respectively) or the isotype control (not shown). Table 1 summarises the results described above pointing out that jararhagin binding to collagen and $\alpha_2\beta_1$ integrin occurs by two independent motifs, located on disintegrin-like and cysteine-rich domains, respectively. Moreover, toxin binding to collagen is sufficient to inhibit platelet aggregation.

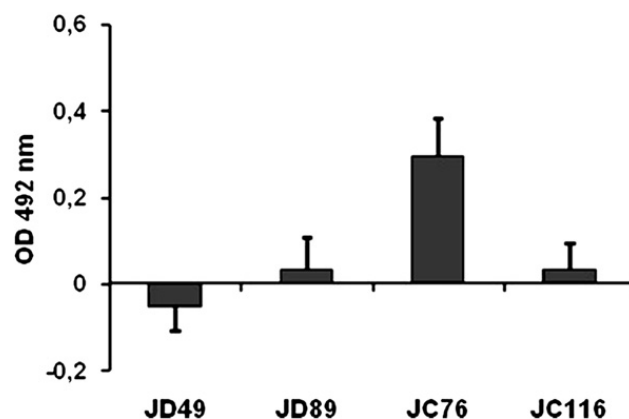


Fig. 4. Binding of fragments of disintegrin-like and cysteine-rich jararhagin domains to $\alpha_2\beta_1$ integrin: Soluble recombinant extracellular domains of human $\alpha_2\beta_1$ integrin (5 μ g/mL) were coated onto microtitre plates. After blocking, plates were incubated with jararhagin fragments comprising the disintegrin-like domain (residues 210–298 – JD89), the Da-disintegrin sub-domain (residues 250–298 – JD49), the cysteine-rich domain (residues 304–421 – JC116) and the hyper-variable region (residues 344–421 – JC76). Binding was detected using anti-GST polyclonal antibodies raised in rabbits followed by incubation with goat IgG anti-rabbit IgG labelled with HRP and with ortho-phenylenediamine/H₂O₂ as enzyme substrates. The products were read at 492 nm and absorbance values were normalised by subtracting the OD obtained in wells incubated with GST only, used as a negative control.

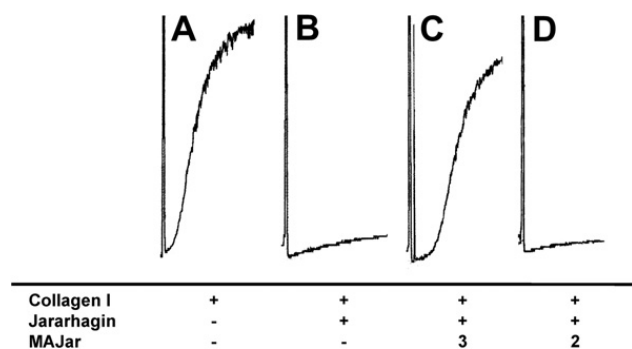


Fig. 5. Inhibition of platelet aggregation: Washed platelets were isolated from blood of healthy human adult volunteers, and adjusted to a concentration of 3×10^8 platelets/mL in Tyrode's solution. For aggregation assays, samples of 400 μ L washed platelets were challenged with 2 μ g/mL collagen (A) or incubated for 5 min with 20 nM jararhagin (B) or jararhagin preincubated (1 h at 37 °C) with saturating amounts of MAJar 3 (C) or MAJar 2 (D) before collagen challenge. The extent of aggregation was calculated as the increase in light transmission for 3 min after the addition of collagen using a Chrono-log lumi-aggregometer (Chrono-log Corporation, USA).

4. Discussion

Platelet aggregation involves a complex series of steps that begins with the binding of agonists to receptors on the plasma membrane. Subendothelial collagen fibres, exposed by injury to blood vessels or disease, are a relevant thrombogenic stimulus. Exposed collagen fibres support adhesion of platelets to the damaged subendothelium by platelet receptor GPIa-IIa ($\alpha_2\beta_1$ integrin). In contrast, GPVI, a non-integrin receptor of the immunoglobulin family, is an activator receptor (Raynal et al., 2006) that in response to collagen activates signalling pathways that upregulate the $\alpha_2\beta_1$ integrin and $\alpha_{IIb}\beta_3$ integrin fibrinogen receptor. $\alpha_{IIb}\beta_3$ integrin binds the RGD-containing ligands, such as fibrinogen and von Willebrand factor, allowing platelet aggregation (Andrews and Berndt, 2004).

Snake venom metalloproteinases interfere with platelet receptors, impairing platelet aggregation. SVMPs shed collagen receptors from platelet surfaces. Kistomin binds to and cleaves GPVI (Hsu et al., 2008), and jararhagin cleaves the β_1 subunit of GPIa-IIa ($\alpha_2\beta_1$ integrin), inhibiting the aggregation induced by collagen (Kamiguti et al., 1996). However, the effects of class P-III SVMPs on $\alpha_2\beta_1$ integrin are not solely due to proteolytic shedding of the integrin chain from the platelet surface. Several references in the literature report that the binding of these toxins to the integrin receptors or their ECM ligands elicits an antagonist

response, blocking their function, or even eliciting an agonist response and inducing integrin-mediated signal transduction (Moura-da-Silva et al., 2007). These evidences indicate that mechanisms involved in interactions of SVMPs with distinct targets may include different structural motifs of the toxins.

Although the structure/function relationships are essential to enlighten the molecular mechanisms resulting in pathological lesions, the 3D structure of P-III SVMPs may be a limiting factor. The presence of 28 cysteines in the non-catalytic domains leads to a great problem in folding correctly the disulfide bridges in recombinant proteins or fragments, explaining apparently discrepant results. In this study, the whole cysteine-rich domain (JC116 fragment) failed to bind to $\alpha_2\beta_1$ integrin while its JC76 fragment, comprising only the HVR was active probably because the surface exposure of the binding motif was correct only in the later. Even with these concerns, important observations on SVMPs structure/function relationships arose from studies using recombinant fragments, despite their lower biological activity than native SVMPs (Menezes et al., 2008). The importance of conformation-dependent motifs was confirmed when the first cristal-structure of P-III SVMPs was published. For example, the motif of catrocollastatin responsible for its binding to collagen was first described as the region homologous to the RGD loop of classical disintegrins, including the sequence CRASM-SECDPAEHC, present also on jararhagin (Zhou et al., 1996). However, in the 3D structure, many of these residues are buried inside the tertiary structure or they coordinate calcium ions (Takeda et al., 2006). In order to approach conformation-dependent motifs, our group has been using neutralising monoclonal antibodies. However, one concern inherent to the use of monoclonal antibodies for structure/function studies is the possibility of steric hindrance. Against this possibility it can be argued that MAJar 3 did not inhibit neither jararhagin catalytic activity (Tanjoni et al., 2003) nor its binding to I-domain-containing proteins as $\alpha_2\beta_1$ integrin, assuring that in this study the inhibition of the binding to collagen and neutralisation of jararhagin action towards platelets were not due to steric hindrance.

The binding of jararhagin to $\alpha_2\beta_1$ integrin in platelets usually results in the inhibition of collagen-induced aggregation (Kamiguti et al., 1996), and may also inhibit the binding of cells to collagen mediated by $\alpha_2\beta_1$ integrin, as occurs in endothelial cells and α_2 -K562 transfected cells (Moura-da-Silva et al., 2001). In this paper, we characterised the structural regions of jararhagin involved in its binding to $\alpha_2\beta_1$ integrin. A region located at the C-terminal region of the cysteine-rich domain was related to $\alpha_2\beta_1$ integrin binding. This region is spatially distinct from the collagen-binding region, since the antibodies that block jararhagin binding to collagen did not affect the binding of the toxin to the integrin. It has recently been described that interaction of P-III SVMPs with von Willebrand factor is mediated by the toxin's cysteine-rich domain targeting the von Willebrand factor A domain (Serrano et al., 2007). Similarly, interactions of the cysteine-rich domain of P-III SVMPs were observed with extracellular matrix proteins such as collagen XII and XIV (FACITs), and the matrilins 1, 3 and 4, all of which contain von Willebrand factor A

Table 1
Neutralising ability of MAJar 3.

Antibody	Platelet aggregation (% aggregation)	$\alpha_2\beta_1$ Integrin (inhibition of binding)		Collagen I (inhibition of binding)
		Soluble integrin	α_2 -K562 cells	
Isotype control	0%	0%	0%	0%
MAJar 2	0%	19%	4%	30%
MAJar 3	91%	18%	0%	83%
Polyclonal serum	-	98%	87%	75%

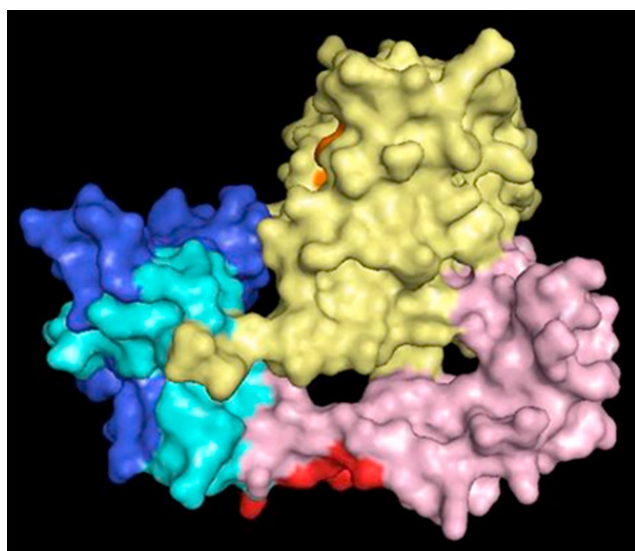


Fig. 6. Schematic representation of jararhagin collagen and integrin-binding regions. Jararhagin sequence (accession number CAA48323.1) was modelled as previously described (Moura-da-Silva et al., 2008). The metalloproteinase domain is shown in pale yellow, disintegrin-like domain in pale red and cysteine-rich domain in pale blue. The region involved in zinc binding is highlighted in orange, the collagen-binding epitope in red and the JC76 fragment in blue.

domains, resulting in specific proteolysis of those substrates (Serrano et al., 2006). Recently, the determination of the crystal structure of VAP1 and catrocollastatin/VAP2B (Takeda et al., 2006; Igarashi et al., 2007) highlighted a Hyper-variable region, located at the C-terminal part of the cysteine-rich domain. This region was described as a novel substrate interaction site that allows the binding of extracellular matrix proteins for their hydrolysis by the SVMP catalytic domain located spatially very close to the hyper-variable region in the crystal structure. The $\alpha_2\beta_1$ integrin also has an I-domain similar to the von Willebrand factor A domain. Thus, the integrin I-domain could be the target of the jararhagin motif involved in the integrin-binding, which was shown in this paper to be within or very close to the hyper-variable region within the cysteine-rich domain.

As occurs with extracellular matrix proteins containing A domains (Serrano et al., 2006), the consequences of integrin-binding as a jararhagin mechanism of action would be the approximation of the integrin to jararhagin catalytic domain, allowing hydrolysis and explaining $\alpha_2\beta_1$ shedding from platelet surfaces. In opposition, binding of proteolytic-inactivated toxin or the non-catalytic domains of jararhagin to $\alpha_2\beta_1$ integrin would trigger signal transduction in platelets (Kamiguti et al., 1997, 2000), which are not sufficient for inducing aggregation, the apparent result being inhibition. Signal transduction also occurs in other cell types, such as fibroblast cultures, where jararhagin activates similar $\alpha_2\beta_1$ integrin pathways to the ones activated by collagen (Zigrino et al., 2002).

The evidence that class P-III SVMPs bind to collagen and integrin by different motifs brings new insight regarding the action of these complex molecules. The first evidence highlighted is that the binding of jararhagin to collagen is sufficient to inhibit platelet aggregation. After incubation

with MAJar 3, the toxin was as able to bind the $\alpha_2\beta_1$ integrin to the same extent as the toxin incubated with the isotype control antibody. Despite that, MAJar 3 completely inhibited the ability of jararhagin to bind to collagen and to inhibit platelet aggregation. The second implication is the location of the integrin-binding motif opposite to the collagen-binding epitope (Fig. 6). This indicates a multi-domain function in $\alpha_2\beta_1$ integrin/collagen interaction by these enzymes and the possibility of assembling multi-structural complexes with the toxin interposing the contacts between endothelial cells and extracellular matrix, displacing the focal adhesion contacts. This hypothesis would explain endothelial cell apoptosis by anoikis induced by jararhagin (Tanjoni et al., 2005) and also the tissue localisation of jararhagin around blood vessels after injection into mice tissues, which is essential for the expression of jararhagin-induced haemorrhagic activity.

5. Conclusions

Our data suggest that jararhagin binding to collagen and $\alpha_2\beta_1$ integrin occurs by two independent motifs, which are located on disintegrin-like and cysteine-rich domains, respectively. Moreover, toxin binding to collagen appears to be sufficient to inhibit collagen-induced platelet aggregation. Considering that the interaction of $\alpha_2\beta_1$ integrin with collagen is essential for endothelial cell and platelet function, the existence of two independent motifs in jararhagin that are able to interfere with integrin–matrix interaction would improve the efficiency of the toxin to inhibit platelet function, to detach endothelial cells leading to apoptosis and also to concentrate within basement membrane and endothelial cells, enhancing its haemorrhagic activity.

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Conflict of interest

The authors included in this work or the author's institutions have no financial or other relationship with people or organizations that may inappropriately influence the work.

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