Inhibition of collagen-induced platelet aggregation as the result of cleavage of $\alpha_2\beta_1$ -integrin by the snake venom metalloproteinase jararhagin

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Jararhagin is a high-molecular-mass (52 kDa) haemorrhagic metalloproteinase from *Bothrops jararaca* venom and a member of the metalloproteinase/disintegrin/cysteine-rich protein family. The disintegrin domain of jararhagin has been implicated in the inhibition of platelet responses to collagen by a mechanism that is not entirely known. The present investigation demonstrates that both active and 1,10-phenanthroline-inactivated jararhagin inhibit platelet aggregation by collagen with an IC_{50} of 40 and 140 nM respectively. The apparently higher inhibitory effect of the active enzyme clearly indicates that, in addition to the disintegrin region, the metalloproteinase domain of jararhagin also participates in this inhibition. As collagen interacts with platelets via $\alpha_2\beta_1$ -integrin, we investigated the effects of jararhagin on this integrin using selected function-blocking monoclonal antibodies against both of its subunits. Flow cytometry of

platelets treated with native jararhagin and immunoprecipitation of platelet surface glycoproteins from lysates after jararhagin treatment showed an apparently selective reduction of $\alpha_2\beta_1$ -integrin immunoreactivity with both anti- α_2 and anti- β_1 monoclonal antibodies. The loss of immunoreactivity was not due to integrin internalization, since it also took place in cytochalasin D-treated platelets. Here we show that jararhagin cleaved isolated $\alpha_2\beta_1$ -integrin resulting in the generation of a 115 kDa β_1 fragment. We therefore propose that the inhibition by jararhagin of platelet response to collagen is mediated through the binding of jararhagin to platelet α_2 -subunit via the disintegrin domain, followed by proteolysis of the β_1 -subunit with loss of the integrin structure (conformation) necessary for the binding of macromolecular ligands.

INTRODUCTION

Haemorrhage, local or systemic, is one of the main consequences of viperid and crotalid envenoming. Systemic bleeding can be life-threatening, and cases of fatal intracranial haemorrhage have been reported in envenomed patients [1,2]. A major cause of systemic bleeding tendency is the frequently observed consumption coagulopathy [3]. However, spontaneous systemic haemorrhage has also been observed in the absence of clotting abnormalities, and this led to the proposal that non-coagulant venom haemorrhagic metalloproteinases are also involved in the induction of systemic bleeding [2]. This is supported by animal experiments [4] in which such bleeding was induced by jararhagin, a haemorrhagic metalloproteinase from *Bothrops jararaca* venom [5]. The danger of systemic bleeding is enhanced by the apparent inability of plasma proteinase inhibitors to inactivate large metalloproteinases [6,7].

Although local haemorrhagic effects of venom metalloproteinases clearly depend on their proteolytic activity, the precise mechanism by which these enzymes cause systemic bleeding is not clear. Since platelets play an essential role in haemostasis, it is possible that alterations in platelet function are at least partly responsible for both local and systemic haemorrhage following envenoming. Indeed, *in vitro* inhibition of platelet aggregation by metalloproteinases has been demonstrated [8]. Since these enzymes possess α -fibrinogenase activity, and since fibrinogen is required for platelet aggregation, it was proposed that fibrinogen degradation is responsible for the metalloproteinase-induced abnormalities of platelet function [8]. However, in a recent

investigation of this proposal using jararhagin, we found that the cleavage of fibrinogen A α -chains by this enzyme does not interfere with the binding of fibrinogen to platelet $\alpha_{IIb}\beta_3$ -integrin and ensuing platelet aggregation [9].

Platelet aggregation can be also inhibited by snake venom disintegrins, which are short chain RGD-containing non-enzymic polypeptides found in many viperid and crotalid venoms [10–12]. The finding that a high-molecular-mass venom haemorrhagic metalloproteinase possesses a disintegrin-like domain [13] therefore appeared to be highly relevant, particularly because cDNA cloning of other venom haemorrhagic metalloproteinases confirmed the presence of such disintegrin-type structures in these enzymes [5,14]. However, although in all enzymes this domain shows striking sequence homology to the venom disintegrins, the RGD sequence is generally lacking and replaced by another conserved sequence, most frequently ECD [5]. Thus large venom metalloproteinases show two important domains, an N-terminal metalloproteinase domain containing the Zn^{2+} -binding site and a disintegrin-like domain near the cysteine-rich C-terminus [15]. Although it is well established that the enzyme domain is responsible for the degradation of basement-membrane components and thus causes haemorrhage [16,17], a definite function for the disintegrin-like region has not yet been ascertained.

Our previous observations showed that jararhagin is an inhibitor of collagen-induced aggregation of platelets [18]. In addition, another previous study with jararhagin has shown that this component inhibits platelet adhesion to collagen [19]. It appears that such inhibition is at least partially mediated by the disintegrin domain of jararhagin, since a component isolated

Abbreviations used: gp, glycoprotein; mAb, monoclonal antibody; PRP, platelet-rich plasma.

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from *B. jararaca* venom corresponding to this domain and known as jararhagin C [20] is also a potent inhibitor of platelet aggregation. However, whether or not the metalloproteinase domain of jararhagin is also important for the interference of this enzyme with platelet-collagen interaction has not been clearly addressed. Potentially, proteolysis of either collagen or its platelet receptor can cause such an interference. It is known that jararhagin as well as other venom metalloproteinases do not degrade platelet-aggregating forms of collagen [16,17]. As interaction of platelets with collagen is mediated by the RGD-independent binding to the heterodimeric complex $\alpha_2\beta_1$ -integrin [21], we investigated the effects of jararhagin on the structure and function of this integrin. We demonstrate that the inhibition of collagen-induced platelet aggregation by jararhagin is due to both its binding to and proteolysis of $\alpha_2\beta_1$ -integrin. Thus both domains, disintegrin and metalloproteinase, are relevant in the interference of jararhagin with platelet function.

MATERIALS AND METHODS

Preparation of platelets

Venous blood from healthy donors was anticoagulated with 13 mM sodium citrate and centrifuged for 10 min at 125 *g* at room temperature to obtain platelet-rich plasma (PRP). To prepare washed platelet suspensions, PRP was centrifuged on a discontinuous 25%/34% BSA density gradient, followed by gel filtration [22] on Sepharose 2B (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in Tyrode's/Hepes buffer (140 mM NaCl, 7.9 mM KCl, 1 mM MgCl₂, 3.3 mM NaH₂PO₄, 20 mM Hepes, pH 7.4, 5.5 mM dextrose) containing 0.3% BSA. The platelet count in the final suspensions was adjusted to 3.0×10^8 /ml, and 1 mM CaCl₂ was added.

Platelet aggregation

PRP and platelet suspensions were prewarmed to 37 °C and then treated with jararhagin for 5 min with constant stirring. The platelet incubation with jararhagin was immediately followed by the addition of collagen (Horm Chemie) and aggregation measurements. Both native and inactivated jararhagin were used to investigate the contribution of the metalloproteinase domain of jararhagin. Native jararhagin was purified from *B. jararaca* venom as described by Paine et al. [5]. The inactivation was carried out with 1,10-phenanthroline (Sigma) by dialysis [23] or by direct addition of 5 mM 1,10-phenanthroline to the preparation. The loss of the enzymic activity of jararhagin after these treatments was determined using fibrinogen as substrate [9].

Analysis of platelet $\alpha_2\beta_1$ -integrin by flow cytometry

Platelets in PRP or washed cell suspensions were treated with both active and inactive forms of jararhagin for 10 min and then fixed with 1% paraformaldehyde in PBS. Fixed cells were washed four times in PBS containing 1% BSA and then incubated for 30 min with 4 μ g of anti- α_2 (Gi 9 clone from Immunotech; AK7 clone from Novocastra; CLB150 clone from Central Laboratory of Blood Transfusion, The Netherlands), anti- β_1 (B44 and JB1B clones from Serotec) or anti-glycoprotein (gp)Ib (Dakopatts) monoclonal antibodies (mAbs) at 4 °C. After a wash as before, the cells were incubated with goat anti-mouse IgG-fluorescein isothiocyanate (Becton Dickinson) for a further 30 min at 4 °C. Finally, after repeated washing, platelets were resuspended in FACSFlow solution (Becton Dickinson) and analysed by flow cytometry (Becton Dickinson).

Binding of ¹²⁵I-jararhagin to platelets

Both native jararhagin and jararhagin inactivated by 1,10-phenanthroline were radiolabelled with Na¹²⁵I using Iodobeads[®] (Pierce), followed by gel filtration on Sephadex G-25. Iodinated jararhagin appeared as a single band of 52 kDa on SDS/PAGE [24], confirming the purity of the preparation (not shown). The specific radioactivity of the radiolabelled proteins was 0.007 and 0.015 μ Ci/ μ g for the native and inactivated jararhagin respectively. Binding assay was carried out in triplicate at room temperature by incubating platelets for 10 min in appropriate mixtures of radiolabelled and unlabelled jararhagin. The reaction was terminated by pelleting the platelets by centrifugation through silicone oil as described previously [9]. The radioactivity of pellets was measured in a γ counter and specific binding determined by subtracting the binding in the presence of 10- to 100-fold excess of unlabelled active jararhagin.

Analysis of platelet surface glycoproteins by SDS/PAGE

In order to examine the main proteins on the platelet surface, gpIb and $\alpha_{IIb}\beta_3$ - and $\alpha_2\beta_1$ -integrins, washed platelets were labelled in albumin-free medium with carrier-free Na¹²⁵I (Amersham) by the lactoperoxidase-catalysed reaction [25] and then gel-filtered as above. ¹²⁵I-labelled platelets were then incubated for 10 min at 37 °C with jararhagin, whole *B. jararaca* venom or buffer. The cells were then pelleted by centrifugation. The supernatants were saved for immunoprecipitation; the pellets were washed twice and then either lysed in the SDS-containing sample buffer for SDS/PAGE or resuspended in Tyrode's-Hepes buffer before lysis by mixing with an equal volume of cold 2% Triton X-100 in 100 mM Tris/HCl buffer, pH 7.4, containing 10 mM EGTA, 2 mM PMSF and 100 mM benzamidine. The Triton-soluble lysates were cleared by centrifugation at 16000 *g* for 30 min at 4 °C and used for immunoprecipitation. The immune complexes formed overnight with 5 μ g/ml anti- α_{IIb} (PL2-49; gift from Dr. C. Kaplan, Laboratoire d'Immunologie Plaquettaire, Paris, France), anti- α_2 (Gi 9, AK7 and CLB150 clones) or anti- β_1 (JB1B clone) mAbs were then precipitated with Protein G-Sepharose (Zymed). In some experiments, the protein was first immunoprecipitated from platelet lysates and the immunoprecipitate treated with jararhagin, as above. All samples were then analysed by SDS/PAGE, using 7–12% gels under non-reducing and reducing conditions. In order to solubilize gpIb, in some experiments platelets were resuspended in 10 mM Tris/HCl buffer, pH 7.4, containing 124 mM NaCl, 20 mM EDTA, 2 mM PMSF and 2 mM *N*-ethylmaleimide [26] and lysed with 2% Triton X-100 lysis buffer as before. GpIb in these Triton-soluble lysates was immunoprecipitated with 3.5 μ g/ml anti-gpIb mAb and analysed as described above. Dried gels were autoradiographed using Amersham Hyperfilm. Autoradiographs were analysed by densitometry (Zeineh Video Densitometer, Biomed Instruments).

Investigation of $\alpha_2\beta_1$ -integrin in cytochalasin-D-treated platelets

¹²⁵I-labelled platelets were treated with 10 μ g/ml cytochalasin D (Sigma) dissolved in DMSO for 60 min before jararhagin addition. Platelets were pelleted, washed and lysed as before. Immunoprecipitation was carried out as described above.

Effects of jararhagin on platelet $\alpha_2\beta_1$ -integrin purified by affinity chromatography

The purification of the collagen receptor was carried out using 2–6 units of outdated human platelet concentrates each time, as described [27]. Briefly, platelets were washed twice in

Tyrod's-Hepes buffer, pH 7.4, then labelled with Na^{125}I as described above. After being washed, cell suspensions were lysed with equal volumes of buffer A (50 mM Tris/HCl, pH 7.4, 140 mM NaCl, 2 mM MgCl_2 , 1 mM MnCl_2) containing 100 mM n-octyl β -D-glucopyranoside (Alexis Corp.) and 2 mM PMSF, 100 kallikrein inhibitory units/ml of aprotinin (Bayer), 10 $\mu\text{g}/\text{ml}$ leupeptin and pepstatin (Sigma), for 1 h at 4 °C, followed by centrifugation at 16000 g for 30 min. The lysates were passed three times at 4 °C through a column loaded with 2 ml of CNBr-activated agarose coupled to collagen (Sigma), and the column was then extensively washed with buffer A containing 25 mM n-octyl β -D-glucopyranoside. The platelet collagen receptor was eluted at room temperature with 50 mM Tris/HCl, pH 7.4, containing 20 mM EDTA. In order to eliminate EDTA, the eluates were gel-filtered on a Sephadex G-25 (Pharmacia) column equilibrated with buffer A containing 25 mM n-octyl β -D-glucopyranoside. The untreated purified receptor as well the products of its reaction with jararhagin were then analysed by SDS/PAGE as above. Immunoprecipitation of $\alpha_2\beta_1$ -integrin from the purified preparations was carried out as described above.

RESULTS

Jararhagin inhibits platelet aggregation by collagen

Both native and 1,10-phenanthroline-inactivated jararhagin inhibited platelet responses to collagen with an estimated IC_{50} of 40 and 140 nM respectively (Figure 1). As it is known that, on treatment of metalloproteinase with 1,10-phenanthroline, the protein structure is preserved [23], the higher potency of native jararhagin demonstrates that the proteolytic activity of the enzyme contributes to the inhibition of platelet-collagen interaction.

Figure 1 Inhibition of collagen-induced aggregation of platelets by native and inactivated jararhagin

Human washed platelet suspensions were incubated with various amounts of native or inactivated jararhagin for 5 min at 37 °C. Collagen (2 $\mu\text{g}/\text{ml}$) was added and aggregation was recorded for 4 min. Percentage inhibition was calculated relative to control suspensions incubated with buffer only. ■, Native jararhagin; □, 1,10-phenanthroline-treated jararhagin. Results are means $\pm$ S.D. ($n = 4$).

Figure 2 Flow cytometry of pretreated platelets using anti- α_1 mAbs

Washed platelet suspensions were treated with 200 nM jararhagin. After repeated washings, platelets were allowed to react with mAbs against α_2 -subunit of $\alpha_2\beta_1$ -integrin and examined by flow cytometry. (a) Control platelets; (b) jararhagin-treated platelets; (c) platelets incubated with 1,10-phenanthroline-inactivated jararhagin. Representative results using AK7 and CLB150 mAbs are shown. A similar reduction in total and mean fluorescence of platelets after treatment with jararhagin was observed using Gi 9 (anti- α_2) or B44 (anti- β_1) mAbs (not shown).

Flow cytometry of jararhagin-treated platelets shows a decrease in the immunoreactivity of the subunits of $\alpha_2\beta_1$ -integrin

When platelets were treated with both forms of jararhagin and then incubated with commercially available anti- α_2 mAbs known to block platelet reactivity with collagen [28–30], the flow cytometry showed that both the number of positive cells and mean immunofluorescence were reduced (Figure 2). This demonstrates that jararhagin affected the domain of the α_2 -subunit involved in platelet-collagen binding. Similar results were obtained with B44 anti- β_1 mAb (not shown). In contrast, jararhagin did not cause any change in the platelet reactivity with an anti-gpIb mAb (results not shown). This indicates a relative specificity of jararhagin for $\alpha_2\beta_1$ -integrin. In order to determine the relative contribution of the epitope block by binding competition with mAbs and of epitope loss caused by proteolysis, we next directly measured the platelet binding of active and inactivated jararhagin.

Proteolytic activity has a pronounced effect on jararhagin binding to platelets

Figure 3 shows that equilibrium binding to platelets of 1,10-phenanthroline-treated ^{125}I -jararhagin, but not of active jararhagin, could be demonstrated. These results clearly show that jararhagin possesses a binding site that recognizes platelet $\alpha_2\beta_1$ -integrin and that the binding equilibrium is influenced by the enzymic activity of jararhagin. The latter suggests degradation of the receptor, as already indicated by the aggregation experiments (Figure 1). Thus the binding of inactivated jararhagin points to the significance of the disintegrin domain in the recognition of the platelet collagen receptor, first demonstrated with jararhagin C [20]. In addition, by indicating receptor

Figure 3 Binding of ^{125}I -jararhagin to platelets

Various amounts of radiolabelled native ($\blacktriangle$) and 1,10-phenanthroline-inactivated ($\square$) jararhagin were incubated in triplicate with resting platelets ($1.2 \times 10^8/\text{ml}$) for 10 min at room temperature without stirring. Non-specific binding was estimated by a similar series carried out in the presence of a 10- to 100-fold excess of unlabelled active jararhagin. After centrifugation through silicone oil, radioactivity of the platelet pellets was measured in a γ counter.

degradation by active jararhagin, our results point to the previously unrecognized importance of the metalloproteinase domain in the inhibition of platelet-collagen interaction.

SDS/PAGE of platelet surface glycoproteins further indicates proteolysis of the platelet $\alpha_2\beta_1$ -integrin complex by jararhagin

Analysis of whole lysates from ^{125}I -labelled platelets treated with native jararhagin or *B. jararaca* venom did not show any noticeable changes in the principal radioactive bands (not shown). As the resolving power of this technique may have been insufficient to demonstrate the more subtle changes in platelet surface proteins suggested by flow cytometry, immunoprecipitation of the main individual glycoproteins was carried out. SDS/PAGE patterns of immunoprecipitated $\alpha_{\text{IIb}}\beta_3$ -integrin and gpIb from the Triton-soluble fraction of washed jararhagin-treated platelet pellets (Figure 4, lanes 5 and 7 respectively) did not differ from those of control platelets (lanes 4 and 6 respectively). However, the amount of immunoprecipitated $\alpha_2\beta_1$ complex (160 kDa α_2 -subunit and 130 kDa β_1 -subunit) with Gi 9 mAb was clearly diminished after jararhagin treatment. This was observed under both non-reducing (Figure 4, lane 3) and reducing conditions (not shown). Similar results were obtained using other anti- α_2 mAbs (AK7 and CLB150) (results not shown). These data therefore demonstrate a selective effect of the native jararhagin on the structural integrity of $\alpha_2\beta_1$ -integrin on the platelet surface. As we have demonstrated that the native enzyme is not tightly bound to platelets, proteolysis of the receptor appears to be the most likely cause of the reduced immunoprecipitability. However, it is also possible that this reduction was caused by internalization of the intact integrin on platelet stimulation by jararhagin with or without its subsequent intracellular degradation.

Jararhagin effect is not a result of $\alpha_2\beta_1$ -integrin internalization

Since β_1 -integrin can bind to the cytoskeleton and thus become undetectable in the Triton-soluble fraction of platelet lysate [31],

Figure 4 Effects of jararhagin on the immunoprecipitation of ^{125}I -labelled platelet membrane glycoproteins

Platelets were labelled with Na^{125}I by a lactoperoxidase-catalysed reaction and treated with 200 nM jararhagin for 10 min. Cells were then pelleted, washed, lysed with Triton X-100, and the lysates incubated with anti- α_2 (Gi 9), anti- α_{IIb} or anti-gpIb mAbs overnight, followed by immunoprecipitation on Protein G-Sepharose. Non-reduced immunoprecipitated proteins were analysed by SDS/PAGE in a 7–12% polyacrylamide gel. Dried gels were autoradiographed. Lane 1, whole lysate of untreated ^{125}I -labelled platelets; lane 2, immunoprecipitated $\alpha_2\beta_1$ -integrin, control; lane 3, $\alpha_2\beta_1$ -integrin after platelet treatment with jararhagin; lane 4, $\alpha_{\text{IIb}}\beta_3$ -integrin control; lane 5, $\alpha_{\text{IIb}}\beta_3$ -integrin after platelet treatment with jararhagin; lane 6, gpIb control; lane 7, gpIb after platelet treatment with jararhagin.

Figure 5 Effect of jararhagin on the immunoprecipitation of ^{125}I -labelled $\alpha_2\beta_1$ -integrin from platelets treated with cytochalasin D

Radiolabelled platelets were treated with cytochalasin D for 60 min and then with 200 nM jararhagin for another 10 min. After incubation, cells were pelleted, washed and lysed. The integrin was immunoprecipitated from the lysates with anti- α_2 mAb (Gi 9) and electrophoresed under non-reducing conditions. Lane 1, control untreated platelets; lane 2, jararhagin-treated platelets; lane 3, control platelets with DMSO; lane 4, control cytochalasin D-treated platelets; lane 5, cytochalasin D- and jararhagin-treated platelets; lane 6, platelet lysate. This experiment is representative of three, using platelets from different donors.

we treated platelets with cytochalasin D to inhibit actin polymerization and prevent incorporation of this integrin into the Triton-insoluble platelet residue. The cytochalasin-D treatment completely abolished platelet response to collagen, but did not prevent the reduction by native jararhagin of immunoprecipitable $\alpha_2\beta_1$ -integrin (Figure 5, lane 5). This indicates that platelet $\alpha_2\beta_1$ -integrin was altered by a direct interaction with

Figure 6 SDS/PAGE of collagen-affinity-purified ^{125}I -labelled $\alpha_2\beta_1$ -integrin

The receptor was purified from radiolabelled platelet lysate by collagen-affinity chromatography; the bound receptor was eluted with 20 mM EDTA and then gel-filtered on Sephadex G-25 to remove EDTA. EDTA-free receptor was then treated with 200 nM jararhagin for 30 min at 37 °C. Lane 1, total lysate; lane 2, $\alpha_2\beta_1$ -integrin in EDTA; lane 3, EDTA-free $\alpha_2\beta_1$ -integrin control; lane 4, EDTA-free $\alpha_2\beta_1$ -integrin treated with jararhagin. Lanes 1–4, electrophoresis under non-reducing conditions; lanes 5–8, respective reduced samples shown in lanes 1–4.

jararhagin on the platelet surface. Our next step was to investigate the effects of jararhagin on the isolated integrin.

Jararhagin does not degrade structurally altered affinity-purified platelet $\alpha_2\beta_1$ -integrin

Although the loss of $\alpha_2\beta_1$ -integrin immunoprecipitability with anti- α_2 mAb suggested proteolysis by jararhagin, attempts to detect proteolytic fragments in supernatants of jararhagin-treated platelets and in the platelet lysates by immunoprecipitation with different anti- α_2 mAbs failed. With collagen-affinity-purified receptor, proteolysis by jararhagin could not be demonstrated either. Although when eluted by EDTA from the affinity column, the integrin subunits were distinct and apparently intact (Figure 6, lane 2), in the absence of EDTA the receptor subunits appeared to be associated in the form of high-molecular-mass multimers (lane 3). Since the normal position of the subunits in the gels was restored on 2-mercaptoethanol treatment of the sample (Figure 6, lane 7), it would appear that the integrin chains became mutually disulphide bonded in the absence of EDTA. Treatment of the EDTA-free receptor with jararhagin did not produce any detectable proteolytic fragment (lanes 4 and 8). It thus appears that only structurally fully preserved $\alpha_2\beta_1$ -integrin, as found on the platelet surface, is susceptible to interaction with jararhagin.

Jararhagin degrades the β_1 -subunit of purified structurally preserved $\alpha_2\beta_1$ -integrin

When immunoprecipitation from the Triton-soluble fraction of jararhagin-treated platelets was carried out using an anti- β_1 mAb, a diminution of immunoprecipitated β_1 was seen, under both non-reducing (Figure 7a, lane 2) and reducing conditions (Figure 7a, lane 4). Again no cleavage products could be detected. However, when $\alpha_2\beta_1$ was first immunoprecipitated by the same anti- β_1 mAb and then treated with jararhagin, although no fragment(s) of the integrin could be detected under non-reducing conditions (Figure 7b, lane 2), under reducing conditions, a new radiolabelled product of apparent size 115 kDa became evident,

Figure 7 Proteolysis of ^{125}I -labelled $\alpha_2\beta_1$ -integrin by jararhagin on the platelet surface (a) and after immunoprecipitation (b)

(a) Radiolabelled platelets were treated with 200 nM jararhagin for 10 min at 37 °C. Triton-soluble fractions of the platelet lysates were then immunoprecipitated with anti- β_1 mAb (JB1B) on Protein G–Sepharose and the immunoprecipitates analysed by electrophoresis. Lane 1, untreated platelets; lane 2, jararhagin-treated platelets (both samples run under non-reducing conditions). Lanes 3 and 4 are the respective reduced proteins shown in lanes 1 and 2. (b) Radiolabelled platelets were lysed, and the integrin was immunoprecipitated with anti- β_1 mAb from the Triton-soluble fraction. The immunoprecipitated integrin bound to Protein G–Sepharose was then treated with jararhagin. An SDS-containing sample buffer was added and samples electrophoresed as before. Lane 1, non-reduced untreated immunoprecipitate; lane 2, non-reduced jararhagin-treated immunoprecipitate; lane 3, total platelet lysate; lane 4, reduced untreated immunoprecipitate; lane 5, reduced jararhagin-treated immunoprecipitate. The arrowhead points to the 115 kDa β_1 product generated by jararhagin and seen only under reducing conditions. This experiment is representative of three (including one using anti- α_2 mAb, Gi 9 clone), carried out with platelets from different donors.

together with a diminished amount of β_1 -subunit (Figure 7b, lane 5). Similar results were obtained when $\alpha_2\beta_1$ -integrin was first immunoprecipitated with anti- α_2 mAb (Gi 9) and the immunoprecipitated integrin treated with jararhagin (not shown). Thus jararhagin cleaves platelet $\alpha_2\beta_1$ -integrin in the β_1 -subunit, the immunoreactivity with JB1B anti- β_1 mAb is lost and the resulting product remains disulphide-linked to the whole complex. Apart from the 115 kDa product, no other fragments were detected.

DISCUSSION

Here we demonstrate binding and degradation of platelet $\alpha_2\beta_1$ -integrin by jararhagin and propose that this can fully explain the inhibition of collagen-induced platelet aggregation by this enzyme. This proposal is reinforced by the apparent absence of any effect of jararhagin on the structure of other principal platelet adhesion and aggregation receptors such as gpIb–IX complex and $\alpha_{IIb}\beta_3$ -integrin. Such a selective proteolytic effect of a venom component on $\alpha_2\beta_1$ -integrin has not been described previously.

Recently, it was reported that the inhibition of collagen-induced platelet aggregation by another haemorrhagic metalloproteinase, catrocollastatin, was due to the binding of this venom component to collagen [32]. However, in contrast with jararhagin, catrocollastatin did not directly affect platelets and failed to degrade a number of proteins known to be substrates of jararhagin and other venom metalloproteinases [16,17]. Moreover, jararhagin selectively degrades type-IV collagen [17], which is not the platelet-aggregating component of the preparation used in our experiments. It seems therefore that the effects on the collagen receptor described here, rather than an effect on collagen,

are responsible for the interference of jararhagin with platelet–collagen interaction.

Our results of platelet aggregation inhibition by inactivated jararhagin are quite similar to the reported results obtained with jararhagin C, confirming that the disintegrin-like domain plays a functional role [20]. This domain in jararhagin shows sequence homology to the venom disintegrins [5] which bind to platelet $\alpha_{IIb}\beta_3$ -integrin via their RGD sequence and thereby inhibit fibrinogen-dependent platelet responses [10–12]. In contrast, we now show that jararhagin, which does not contain RGD in its disintegrin domain, recognizes platelet $\alpha_2\beta_1$ -integrin and thus interferes with platelet–collagen interaction. In contrast with the report of De Luca et al. [19], our preparation of jararhagin did not cause platelet agglutination, which is presumed to involve an interaction of the venom component with von Willebrand factor. This discrepancy may be explained by differences in preparation, because the methods used for jararhagin purification were not the same. Also, the presence of different homologous components in *B. jararaca* venom is a well-known fact [5].

Theoretically, the collagen receptor binding of jararhagin alone is sufficient to explain the inhibition of platelet aggregation. In our experiments, however, the differences in the potencies and in the binding of active and inactive enzyme suggest a role for the metalloproteinase domain in the inhibition, in addition to the receptor-blocking role proposed for the disintegrin domain.

During the course of our investigation, we consistently observed a pronounced decrease in the level of functionally and immunologically detectable $\alpha_2\beta_1$ -integrin in jararhagin-treated platelets. This cannot simply be attributed to the binding of jararhagin to the functional domain of $\alpha_2\beta_1$ -integrin, because native jararhagin did not show the required equilibrium binding to platelets. Therefore the decrease in $\alpha_2\beta_1$ -integrin in jararhagin-treated platelets may have been caused by either receptor internalization or proteolytic cleavage. The first hypothesis is based on the fact that the cytoplasmic domain of the β_1 -subunit (47 amino acid residues) interacts with components of the platelet cytoskeleton, namely α -actinin [33] and talin [34]. The function of the short (27 amino acid residues) cytoplasmic tail of the α_2 -subunit has not yet been well characterized, although recent data show an inducible interaction of α_2 and calreticulin which can influence $\alpha_2\beta_1$ -integrin function [35]. The extent of interaction of the $\alpha_2\beta_1$ -integrin with the platelet cytoskeleton and the role of these interactions in collagen-dependent signalling are not clear, however [36].

As we detected a decrease in $\alpha_2\beta_1$ -integrin in the Triton-soluble fraction of jararhagin-treated platelets, we considered that the interaction of $\alpha_2\beta_1$ -integrin with jararhagin caused internalization of the integrin which, as explained above, is known to be under cytoskeletal control. However, as jararhagin in our experiments still diminished immunoprecipitable $\alpha_2\beta_1$ -integrin in cytochalasin D-treated platelets, internalization of the receptor as an alternative to surface receptor proteolysis appears to be unlikely.

Our results, showing that native jararhagin does not bind firmly to platelets and yet decreases $\alpha_2\beta_1$ -integrin, also strongly suggest proteolysis. However, after the treatment of whole ^{125}I -labelled platelets with jararhagin, proteolytic fragments were not detected by a number of anti- α_2 and anti- β_1 mAbs in either platelet supernatants or the Triton-soluble fraction of the platelet pellets. In contrast, when the receptor was first immunoprecipitated and treated with jararhagin, we were able to demonstrate the cleavage of the integrin by jararhagin. The cleavage fragment detected was bound to the rest of the molecule by disulphide bond(s), which explains the lack of detectable product in the supernatants of jararhagin-treated platelets. The new band was only seen in reduced samples (Figure 7b, lane 5) and

appeared to be a remnant of the β_1 -subunit, which was clearly diminished. As the β_1 -chain (798 amino acid residues, 130 kDa) has a conserved extracellular region (residues 466–635) with a large number of internal disulphide bonds [36,37], it is possible that the cleavage by jararhagin occurred near the transmembrane domain within a loop formed by an internal disulphide bond, explaining the absence of fragments in the non-reduced samples. After reduction, the 115 kDa β_1 -subunit remnant was clearly seen, but the expected smaller fragment(s) was absent. This suggests that the cleavage occurred close to the membrane in a non-iodinated region presumably including transmembrane and cytoplasmic parts of the β_1 -chain.

The α_2 -subunit (160 kDa) of $\alpha_2\beta_1$ -integrin exhibits a primary structure consisting of 1152 amino acid residues, with an inserted (I) domain (residues 140–359) that is critically involved in collagen-receptor interactions [36–38] and has sequence homology to the platelet-binding site of von Willebrand factor [37]. It has been proposed that the binding site for jararhagin resides in the I domain of both the α_2 -chain and von Willebrand factor on the basis of the demonstrated interaction of jararhagin and von Willebrand factor and its ability to block platelet adhesion to collagen [19]. Thus it is possible that native jararhagin binds to the I domain of the α_2 -subunit of platelet collagen receptor. We here demonstrate that the binding is followed by a proteolytic attack on the β_1 -chain. Although the demonstrated fragment is retained within the receptor structure via an internal disulphide bond, the ability of the integrin to bind collagen, specific mAbs and jararhagin is grossly altered. This loss of binding ability can be explained by the well-known involvement of both integrin chains in the creation of high-affinity binding sites for macromolecular ligands. Thus mAbs that block ligand binding to $\alpha_2\beta_1$ -integrin recognize regions of either the α_2 -subunit (residues 173–259 within the I domain [30]) or the β_1 -subunit (residues 207–218 [36]).

Affinity-purified $\alpha_2\beta_1$ -integrin, which after dissociation from collagen with EDTA and subsequent removal of this chelator does not maintain its dimeric structure but appears as aggregates, was not affected by jararhagin. Such $\alpha_2\beta_1$ -integrin aggregates were not noticed in the past, presumably because the purified receptor was analysed in the reduced form or in the presence of EDTA. In our experiments, the removal of EDTA during the purification of this Mg^{2+} -dependent integrin was vital, because jararhagin is inactivated by EDTA. After removal of EDTA, the integrin chains clearly fail to reassociate in a way that restores the receptor conformation and instead become disulphide bonded. The lack of an effect of jararhagin on such a ‘scrambled’ receptor further illustrates the strict structural requirements for interaction of $\alpha_2\beta_1$ -integrin and jararhagin.

In conclusion, our studies show that the interaction of jararhagin with platelet $\alpha_2\beta_1$ -integrin is relatively selective, since other major glycoproteins remain unaltered. Jararhagin binding to platelet $\alpha_2\beta_1$ -integrin is followed by cleavage of this collagen receptor and thereby loss of its native conformation which is necessary for high-affinity binding to macromolecular ligands. This binding is a requisite for the proper functioning of blood platelets in haemostasis. For example, it is known that congenital deficiency of platelet α_2 -subunit results in bleeding complications [39]. Such complications are also commonly seen in patients envenomed by *B. jararaca*, in which case the bleeding is thought to be partly related to consumption coagulopathy, also frequently observed in these patients [40]. However, systemic bleeding can also be induced, without any effect on clotting, by haemorrhagic metalloproteinases such as jararhagin [4]. By demonstrating $\alpha_2\beta_1$ -integrin degradation, the present study shows one possible mechanism by which such bleeding can be induced.

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