Intracellular activation of the fibrinolytic cascade in the Quebec Platelet Disorder

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Summary
The Quebec Platelet Disorder (QPD) is an unusual bleeding disorder associated with increased platelet stores of urokinase-type plasminogen activator (u-PA) and proteolysis of platelet α-granule proteins. The increased u-PA and proteolysis of plasminogen in QPD platelets led us to investigate possible contributions of intracellular plasmin generation to QPD α-granule proteolysis. ELISA indicated there were normal amounts of plasminogen and plasmin-α2-antiplasmin (PAP) complexes in QPD plasmas. Like normal platelets, QPD platelets contained only a small proportion of the blood plasminogen, however, they contained an increased amount of PAP complexes compared to normal platelets (P < 0.005). The quantities of plasminogen stored in platelets were important to induce QPD-like proteolysis of normal α-granule proteins by two chain u-PA (tcu-PA) in vitro. Moreover, adding supplemental plasminogen to QPD, but not to control, platelet lysates, triggered further α-granule protein proteolysis to forms that comigrated with plasmin degraded proteins. These data suggest the generation of increased but limiting amounts of plasmin within platelets is involved in producing the unique phenotypic changes to α-granule proteins in QPD platelets. The QPD is the only known bleeding disorder associated with chronic, intracellular activation of the fibrinolytic cascade.

Keywords
Inherited platelet disorders, fibrinolysis, α-granule proteins, urokinase-type plasminogen activator, plasmin

Introduction
Activation of the fibrinolytic cascade in vivo is commonly associated with increased plasma plasmin-α2-antiplasmin (PAP) complexes, fibrin(ogen) degradation products (FDPs) and D-dimers (1-6). Inherited disorders with increased plasminogen activators are rare and only one has been described with increased urokinase-type plasminogen activator (u-PA) and delayed bleeding, a condition formerly known as factor V Quebec and more recently designated as the Quebec Platelet Disorder (QPD) (7-11). In the QPD, the increased expression and storage of u-PA in megakaryocytes/platelets, and normal to increased plasma u-PA, are associated with α-granule protein degradation (not evident in other storage pool disorders), without increased plasma FDPs or D-dimers, and with impaired platelet aggregation with epinephrine (6-8, 10-14).

At the present time, the mechanism of intraplatelet proteolysis in the QPD is not fully understood. Although QPD platelets contain large amounts of unregulated u-PA without detectable plasmin activity (8), it has not been established if there is sufficient plasminogen in platelets to measurably increase plasmin generation [detectable by markers such as PAP complexes (15,
and mediate α-granule protein degradation by u-PA. In vitro, plasmin cleaves many α-granule proteins degraded in QPD platelets, including thrombospordin-1, fibrinogen, fibronectin, von Willebrand factor and factor V (17-20). Less is known about the substrate specificity of u-PA which can degrade plasminogen-free fibrinogen (21) and trigger QPD-like proteolysis of normal platelet proteins in vitro (8, 21). The observation that QPD serum contains platelet FDPs that are not recognized by an assay for plasmin degraded fibrinogen has raised doubts about plasmin contributions to the proteolysis in QPD platelets (6, 22).

Unanswered questions about the mechanism of platelet protein degradation in the QPD led us to investigate markers of plasmin generation in the QPD and the contributions of platelet plasminogen to α-granule protein degradation by tcu-PA. We report that the QPD is associated with chronic intraplatelet activation of the fibrinolytic cascade and provide evidence that platelet plasminogen contributes to the unique changes to QPD α-granule proteins.

Materials and methods

Materials

Purified fibrinogen (plasminogen-free), Glu-plasminogen, and plasmin were from Enzyme Research (South Bend, IN). Purified fibrinogen fragment D was from Haematologic Technologies (Essex Junction, VN), tcu-PA was from Dr Jack Henkens (Abbott Laboratories, IL). Aprotinin, E-64, AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride) and leupeptin were from Boehringer Mannheim Canada (Laval, PQ, Canada). All other protease inhibitors, bovine serum albumin (BSA), PGE$_1$, and theophylline (used to prepare resting platelets, as described [7, 8]) and Ca$^{2+}$ ionophore A23187 were from Sigma-Aldrich Canada (Oakville, ON, Canada). PAP complex ELISA was from American Diagnostica of Canada LP (Montreal, PQ, Canada). The FDP Plasma assay, which detects D-dimer and fragment D, but not E, epitopes in plasmin degraded fibrinogen (22), was from Diagnostica Stago (Asnières, France). Goat anti-plasminogen IgG and Plasminogen ELISA were from Affinity Biologicals, (Hamilton, ON, Canada). Normal pooled plasma (NPP, from George King Biomedical Inc, Overland Park, KS) was used as the standard for the plasminogen ELISA, which recognized plasminogen and plasmin and detected 12 fold more antigen after purified Glu-plasminogen (1.86 mg/mL) was converted to plasmin (18 hour digest with 500 ng/mL tcu-PA).

Supplies, antibodies and procedures for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were as previously described (7, 8, 10). BSA and goat anti-plasminogen were coupled to cyanogen bromide-activated Sepharose beads from Amersham Pharmacia Biotech AB (Uppsala, Sweden) as recommended by the manufacturer.

Sample preparation

Blood was collected with informed consent and institutional ethics board approval from unrelated healthy controls (n = 20, ages: 23–55; 15 males, 5 females) and 5 individuals with the QPD (ages: 20–50; all males).

Methods previously described (7, 8) were used to prepare washed platelets, releasates from ionophore stimulated platelets, and platelet lysates (containing inhibitors: 2.8 µM E64, 10 mM EDTA, 5 mM N-ethyl-maleimide, 1 µM pepstatin, 100 µM 1,10-phenanthroline monohydrate, with or without the serine protease inhibitors 4 µM AEBSF, 0.3 mM aprotinin, 100 µg/mL soybean trypsin inhibitor and 1 µM leupeptin) except releasates were prepared using 1x10$^{10}$ platelets/ml. Lysates without serine protease inhibitors were used exclusively for digests. Plasma samples were double centrifuged as described (7, 10), using blood collected into buffered 3.2% sodium citrate anticoagulant (vol/vol: 9:1) with added protease inhibitors (final: 20 mM benzamidine and 2000 KUI aprotinin). Samples were stored in aliquots at ~70°C before analysis. Platelet lysates were centrifuged (5000g, 10 min) to remove insoluble materials before quantitative analysis. Immuno-depleted platelet lysates (1 x 10$^9$ platelets/ml) for digests were prepared using methods similar to described (8) except sham (BSA-coated) and anti-plasminogen beads were used to prepare samples that respectively contained approximately 400 and <6 ng /ml plasminogen.

Protein analyses

PAP complexes and plasminogen antigen were measured using samples processed with serine protease inhibitors. A two-tailed, student t-test was used to compare patient and control values. Plasmin degraded fibrinogen was quantitated in plasma, serum, platelet releasates, plasmin-treated fibrinogen, and purified fragment D using the Plasma FDP assay.

The proteolysis of α-granule proteins in response to tcu-PA was evaluated by Western blotting as described (8). Briefly, α-granule proteins in untreated and immunodepleted normal platelet lysates without serine protease inhibitors were analyzed after incubation (37°C, 24 h) without or with (final concentrations): 500 ng/ml recombinant tcu-PA, 5 or 200 µg/ml Glu-plasminogen, or 200 µg/ml plasmin. Digests were terminated with 4 mM final AEBSF. For some analyses, degradation products were compared to plasmin degraded fibrinogen, prepared by incubating 140 µg/ml fibrinogen with 200 µg/ml plasmin (in phosphate buffered saline, 37°C, 24 h; complete conversion of fibrinogen to fragments D and E confirmed by Western blotting). The respective gels, types of antibodies (sources as described (8), and volumes of lysates used to evaluate platelet proteins by Western blotting were: fibrinogen – nonreduced 8% SDS-PAGE, polyclonal, 5 µl; factor V - reduced 8% SDS-PAGE, polyclonal, 15 µl; von Willebrand factor – reduced 10% SDS-PAGE, polyclonal, 20 µl; thrombo-
 gambles – reduced 12% SDS-PAGE, monoclonal, 10 µl; multimerin – nonreduced 1.25% agarose/1.5% acrylamide multimer gels, pooled monoclonal and polyclonal, 20 µl; P-selectin - reduced 12% SDS-PAGE, polyclonal, 20 µl.

**Table 1:** Plasminogen and PAP (plasmin-α2-antiplasmin) complexes in patient (Q) and control (C) platelet lysates and plasma measured by ELISA. P values compare patient and control values, evaluated by a two-tailed, student t-test.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sample</th>
<th>n</th>
<th>Mean ± S.D.</th>
<th>Range</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen</td>
<td>platelet lysate (ng/10⁹ platelets)</td>
<td>C</td>
<td>462 ± 90</td>
<td>316–589</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>536 ± 197</td>
<td>244–730</td>
<td></td>
</tr>
<tr>
<td>plasma (µg/ml)</td>
<td></td>
<td>C</td>
<td>236 ± 44</td>
<td>170–330</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>219 ± 53</td>
<td>159–280</td>
<td></td>
</tr>
<tr>
<td>PAP complexes</td>
<td>platelet lysate (ng/10⁹ platelets)</td>
<td>C</td>
<td>27 ± 3</td>
<td>23–33</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>113 ± 23</td>
<td>93–152</td>
<td></td>
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<tr>
<td>plasma (ng/ml)</td>
<td></td>
<td>C</td>
<td>73 ± 36</td>
<td>32–125</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>53 ± 12</td>
<td>39–70</td>
<td></td>
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</table>

**Results**

Like control platelets, QPD platelets contained very little plasminogen compared to plasma (Table 1). QPD and control

![Figure 1](https://example.com/figure1.png)

**Figure 1:** The impact of stored platelet plasminogen on tcu-PA mediated degradation of platelet α-granule proteins. Western blots compare fibrinogen (A), factor V (B), von Willebrand factor (panel C), thrombospondin-1 (D), multimerin (E) and P-selectin (F) in control (lanes C) and QPD (Q) platelet lysates with digests of sham and plasminogen (Pg) depleted control platelet lysates, incubated with (+) or without (-) tcu-PA (NR and R indicate nonreduced and reduced gels). Like QPD platelets, tcu-PA digests of sham-depleted, normal platelet lysates contained degraded α-granule proteins. Minimal to no proteolysis of α-granule proteins occurred in tcu-PA digests of plasminogen-depleted normal platelet lysates.
plasmas contained similar amounts of PAP complexes (Table 1, \( P = 0.7 \)), whereas QPD platelet lysates contained about 4-fold more PAP complexes than control platelet lysates (Table 1, \( P < 0.005 \)). When representative platelet lysates without added serine protease inhibitors were incubated with or without exogenous plasmin (200 \( \mu g/mL \), final), the measured PAP complexes increased 8 fold for the control, and 2 fold for the QPD sample (data not shown). These data suggested there was increased plasmin generation in QPD platelets, with partial consumption of platelet \( \alpha_2 \)-antiplasmin.

The increased PAP complexes in QPD platelets led us to investigate if QPD platelets contained detectable plasmin degraded fibrinogen, using the monoclonal antibody Plasma FDP assay. Although this assay required >100 \( \mu g/ml \) fragment D for FDP detection and it could not detect FDPs in a plasmin digest of 140 \( \mu g/ml \) fibrinogen, it detected plasmin-degraded fibrinogen in QPD platelet releasates (\( \mu g \) FDPs released from \( 10^9 \) platelets: QPD, \( n = 4 \): all 0.25 \( \mu g \); controls, \( n = 7 \): all <0.25 \( \mu g \) ) but not in QPD serum and plasma, as previously reported (6). These data indicated QPD platelets contained some plasmin degraded fibrinogen but in amounts that were too low to be detected in serum using the Plasma FDP assay.

Because platelets contained only small amounts of plasminogen, we investigated if platelet plasminogen was important for the proteolysis of \( \alpha \)-granule proteins by u-PA. There was minimal to no proteolysis of platelet fibrinogen, factor V, von Willebrand factor, thrombospondin-1, multimerin, P-selectin (Fig. 1A-F), fibronectin and osteonectin (not shown) in tcu-PA digests of plasminogen depleted normal platelet lysates. However, like QPD platelets, tcu-PA digests of sham depleted normal platelet lysate contained degraded \( \alpha \)-granule fibrinogen, factor V, von Willebrand factor, thrombospondin-1, multimerin, P-selectin (Fig. 1A-F), fibronectin and osteonectin (not shown) as previously reported (8). These data indicated that platelet plasminogen was important for \( \alpha \)-granule protein degradation by tcu-PA.

Further studies were done to determine if the limited plasminogen in platelets influenced \( \alpha \)-granule protein degradation by tcu-PA. When plasma concentrations of Glu-plasminogen (200 \( \mu g/ml \) final) were incubated overnight with normal platelet lysates, without added tcu-PA, \( \alpha \)-granule protein degradation did not occur (Fig. 2A-D, lanes C+Pg – tcu-PA). In contrast, the same amount of Glu-plasminogen triggered further proteolysis of QPD, \( \alpha \)-granule fibrinogen, von Willebrand factor, and P-selectin (Fig. 2A-D, lanes Q+Pg – tcu-PA), thrombospondin-1, factor V, and multimerin (data not shown) and converted QPD platelet fibrinogen (Fig. 2A, lane Q+Pg – tcu-PA) to forms that comigrated with fragments D and E in plasmin digested fibrinogen.
fibrinogen (Fig. 2A, lane C+Pn). It also converted QPD α-granule fibrinogen, fibronectin, von Willebrand factor, P-selectin (Fig. 2B-D, lanes Q+Pg), osteonectin and thrombospondin-1 (data not shown) to forms that co-migrated with plasmin degraded normal platelet proteins, generated without tcu-PA (Fig. 2B-D, lanes C+Pn – tcu-PA). When small amounts of Glu-plasminogen (5 µg/ml final) were added to normal platelet lysate along with tcu-PA, there was increased (data not shown) to complete proteolysis of α-granule fibrinogen, fibronectin, von Willebrand factor, P-selectin (Fig. 2A-D, lanes C+Pg* + tcu-PA), factor-V, osteonectin and thrombospondin-1 (data not shown) to forms that co-migrated with plasmin degraded platelet proteins, prepared using high concentrations of plasmin (200 µg/ml final) without tcu-PA (Fig. 2A-D, lanes C+Pn – tcu-PA). The proteolysis of multimerin in these digests, in plasmin digests, and in QPD platelet lysates incubated with additional Glu-plasminogen, was manifested by a loss of detectable multimerin in Western blots (data not shown). These data indicated that the amount of plasminogen in platelets influenced the phenotype of α-granule protein degradation by tcu-PA and limited the changes to α-granule proteins in QPD platelets.

Discussion

A number of pathological states have been described in which activation of the fibrinolytic cascade in the extracellular environment leads to proteolysis of fibrin and other proteins (1, 3, 23, 24). The marked increase in u-PA in QPD platelets, more modest increases in u-PA in QPD plasma, and the unique proteolytic changes to QPD stored platelet proteins including plasminogen (8) led us to investigate if there was increased plasmin generation in the QPD. We found PAP complexes were increased in QPD platelets, but not in QPD plasma, indicating that there is chronic intracellular, rather than extracellular, activation of the fibrinolytic cascade in the QPD. These data provide evidence that a normal balance between plasminogen and plasmin in biological samples have not been developed and the ELISA that we used measured more antigen after plasminogen was converted to plasmin. Nonetheless, based on measured protein concentrations and respective molecular weights, there appear to be similar molar amounts of u-PA and plasminogen (and possibly 1.5 fold more u-PA (8)) in QPD platelets. Locally increased enzyme/substrate ratios, from the compartmentalization of u-PA, plasminogen and α-granule proteins within QPD platelets probably influences and enhances intracellular plasmin generation and QPD α-granule protein degradation in vivo. In vitro, platelet plasminogen was important for tcu-PA to induce QPD-like proteolysis of α-granule fibrinogen, thrombospondin-1, fibronectin, von Willebrand factor, factor V, multimerin, P-selectin and osteonectin. The limited platelet stores of plasminogen influence the phenotype of QPD platelet protein degradation as additional plasminogen triggered more extensive degradation of QPD platelet proteins ex vivo. With additional plasminogen, QPD FDPs with the mobility of fragments D and E (1) (Fig. 2A) were generated, without producing alternatively degraded forms to implicate other proteases. These data, the ratios of plasminogen to u-PA in QPD platelets, and the plasmin degraded fibrinogen in QPD platelets suggest plasmin generation contributes to the proteolysis of QPD platelet proteins, including the degradation of multimerin, P-selectin and osteonectin which are not known plasmin substrates. However, we cannot entirely exclude the possibility that some QPD α-granule proteins are degraded directly by u-PA in vivo where intraplatelet enzyme and substrate concentrations are higher.

The changes to proteins in the QPD suggest that triggering proteolysis within cells requires changes to the normal balance of zymogens, activators and inactivators, similar to the mechanisms that activate fibrinolysis in the extracellular environment. Normal platelets store fibrinolytic inhibitors, including α2-antiplasmin (25), PAI-1 (26) and α2-macroglobulin (27) that may help to limit QPD intracellular protein degradation by neutralizing some of the plasmin and u-PA (8) in QPD platelets. However, the sequestration of u-PA within QPD platelets may be a more important mechanism that limits extracellular plasmin generation in the QPD. The origins of some fibrinolytic components in platelets are unclear although the small quantities of plasminogen in platelets suggest it comes from plasma (28). When and where plasminogen encounters u-PA in QPD megakaryocytes and/or platelets has implications for the timing, restriction, and phenotype of protein degradation in the QPD. If like fibrinogen (29, 30), plasminogen continues to traffic into circulating QPD platelets, this could affect the phenotype of QPD α-granule protein degradation.

Therapeutically, there has been interest in developing thrombolytic agents that do not trigger systemic fibrinolysis (24). The relatively infrequent spontaneous bleeding episodes in the QPD (G.E.R., unpublished observations) and normal plasma levels of PAP complexes in QPD plasma indicate that there is an advantage to sequestering profibrinolytic activity. Exposure to surgery or trauma often triggers delayed bleeding in the QPD (7, 10), perhaps because these stimuli
induce u-PA release and increase extracellular plasmin generation. Fibrinolytic inhibitors are the only known effective therapy for bleeding in the QPD yet these drugs do not measurably reduce QPD α-granule protein degradation (6). This suggests hemostatic mechanisms can compensate for the proteolysis of α-granule proteins but not for the dysregulation of fibrinolysis induced by hemostatic challenges in the QPD.

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References


