Platelets from patients with the Quebec platelet disorder contain and secrete abnormal amounts of urokinase-type plasminogen activator

Walter H. A. Kahr, Shilun Zheng, Prameet M. Sheth, Menaka Pai, Alison Cowie, Madeleine Bouchard, Thomas J. Podor, Georges E. Rivard, and Catherine P. M. Hayward

The Quebec platelet disorder (QPD) is an autosomal dominant platelet disorder associated with delayed bleeding and α-granule protein degradation. The degradation of α-granule, but not plasma, fibrinogen in patients with the QPD led to the investigation of their platelets for a protease defect. Unlike normal platelets, QPD platelets contained large amounts of fibrinolytic serine proteases that had properties of plasminogen activators. Western blot analysis, zymography, and immunodepletion experiments indicated this was because QPD platelets contained large amounts of urokinase-type plasminogen activator (u-PA) within a secretory compartment. u-PA antigen was not increased in all QPD plasmas, whereas it was increased more than 100-fold in QPD platelets (P < .00009), which contained increased u-PA messenger RNA. Although QPD platelets contained 2-fold more plasminogen activator inhibitor 1 (PAI-1) (P < .0008) and 100-fold greater u-PA–PAI-1 complexes (P < .0002) than normal platelets, they contained excess u-PA activity, predominantly in the form of two chain (tcu-PA), which required additional PAI-1 for full inhibition. There was associated proteolysis of plasminogen in QPD platelets, to forms that comigrated with plasin. When similar amounts of tcu-PA were incubated with normal platelet secretory proteins, many α-granule proteins were proteolyzed to forms that resembled degraded QPD platelet proteins. These data implicate u-PA in the pathogenesis of α-granule protein degradation in the QPD. Although patients with the QPD have normal to increased u-PA levels in their plasma, without evidence of systemic fibrinogenolysis, their increased platelet u-PA could contribute to bleeding by accelerating fibrinolysis within the hemostatic plug. QPD is the only inherited bleeding disorder in humans known to be associated with increased u-PA. (Blood. 2001;98:257-265) © 2001 by The American Society of Hematology

Introduction

Congenital platelet disorders are usually associated with defective primary hemostasis.1-3 The Quebec platelet disorder (QPD) is an autosomal dominant platelet disorder that has unusual clinical features: it is associated with moderate to severe delayed bleeding, that typically begins 12 to 24 hours after surgery or trauma, and its hemorrhagic manifestations can be controlled with fibrinolytic inhibitors but not with platelet transfusions.1,4-6 This disorder was initially designated as factor V Quebec because of the abnormalities found in platelet factor V of these patients.7 Two families from Quebec have been identified with this condition, which is now known to be associated with other platelet abnormalities that include reduced to low-normal platelet counts, proteolytic degradation of soluble and membrane proteins stored in platelet α-granules, an apparent quantitative deficiency of the α-granule protein multimerin, and defective aggregation with epinephrine.1,3-6,8 Although patients with the QPD have elevated levels of fibrinogen degradation products (FPDs) in their serum (because of platelet fibrinogen degradation), their plasma contains normal amounts of FDPs and D-dimers.9 Complex platelet abnormalities in these patients led us to redesignate their bleeding disorder as the Quebec platelet disorder.3

The cause of the QPD has been uncertain. Affected patients of both families share a characteristic pattern of platelet α-granule protein degradation that is not evident in unaffected family members or in patients with other congenital and acquired platelet disorders1,4-6,9 This degradation affects both plasma-derived and megakaryocyte-synthesized proteins stored in QPD α-granules, but it spares external membrane, dense-granular, and cytosolic platelet proteins.1,4,5 Moreover, some proteins (eg, fibrinogen, von Willebrand factor, and factor V) are degraded in QPD platelets but not in QPD plasma.1,4-6,9 The observation that endogenously synthesized and plasma-derived α-granule proteins were degraded in QPD platelets, despite their normal storage within α-granules,5 suggests some proteolysis occurs late, after megakaryocyte-synthesized and plasma-derived α-granule proteins enter the same compartment. This possibility led us to investigate QPD platelets for the presence of abnormal protease activity. We report that QPD platelets contain abnormal fibrinolytic activity, attributable to their stores of large amounts of urokinase-type plasminogen activator (u-PA). Furthermore, we observed that the consequence of adding similar amounts of exogenous u-PA to normal platelet proteins was the

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proteolysis of α-granule proteins to forms resembling the degraded proteins in QPD platelets.

Patients, materials, and methods

Patients

Blood was collected with informed consent and institutional, ethics board approval from unrelated healthy controls (n = 20) and 5 affected patients with QPD, representing both families with this disorder. Stored samples of washed platelet lysates, pelleted platelet lysates, and plasmas, collected from patients with QPD for previous studies, were included in some analyses.

Materials

The protease inhibitors aprotinin, E-64, AEBSF (4-(2-aminoethyl)-benzenesulfonyl-ethyl), and leupeptin were from Boehringer Mannheim Canada (Laval, QC, Canada). Aldrich (Mississauga, ON, Canada) supplied the other protease inhibitors, PGE1, theophylline, ionophore A23187, adenosine 5′-diphosphate (ADP), and amiloride were from Sigma-Aldrich Canada (Oakville, ON, Canada). Fibrinogen (free of plasminogen and von Willebrand factor), Glu-plasminogen, plasmin, and bovine thrombin were from Enzyme Research (South Bend, IN). Polyclonal and monoclonal antibodies to u-PA were from Monosan (Uden, The Netherlands). Recombinant, active human plasminogen activator inhibitor 1 (PAI-1) was prepared and isolated as described. 11 Polyclonal antibody against PAI-1 was raised in rabbits as previously described. 11 Polyclonal anti–human plasminogen was from Biogenesis (Kingston, ON, Canada). Glu-plasminogen, plasmin, and bovine thrombin were from Enzyme Research (South Bend, IN). Polyclonal and monoclonal antibodies to u-PA were from Biogenesis (Kingston, ON, Canada). Glu-plasminogen, plasmin, and bovine thrombin were from Enzyme Research (South Bend, IN). Polyclonal and monoclonal antibodies to u-PA were from Biogenesis (Kingston, ON, Canada). Glu-plasminogen, plasmin, and bovine thrombin were from Enzyme Research (South Bend, IN). Polyclonal and monoclonal antibodies to u-PA were from Biogenesis (Kingston, ON, Canada). Glu-plasminogen, plasmin, and bovine thrombin were from Enzyme Research (South Bend, IN). Polyclonal and monoclonal antibodies to u-PA were from Biogenesis (Kingston, ON, Canada).

Protein and protease analyses

Zymography was performed using 3% agarose substrate gels (SeaPlaque agarose; BioWhittaker Molecular Applications, Rockland, ME) containing plasminogen-free fibrin or casein (1% wt/vol; Carnation Instant Skim Milk Powder, Nestle Canada, Toronto, ON), with or without added plasminogen (5 µg/mL final), similar to methods previously described. 13, 14 Protease activities were tested by spotting samples directly onto substrate gels, or after proteins were separated by nonreduced SDS-PAGE and renatured with 2% Triton X-100 in PBS, pH 7.4, for 1 hour. Casein gels with or without 1 mM amiloride were used for some determinations. Substrate gels were incubated with samples (37°C, 18 hours) and were photographed wet. Some samples were preincubated with protease inhibitors (same final concentrations as lysates; 20 minutes on ice) or recombinant PAI-1 (0-4000 ng/mL final) after 1:1 dilution in a release pool, prepared with 5 QPD ionophore releases; 1 hour, 22°C before testing their proteolytic activity. Others were tested after immuno- precipitation with rabbit anti–human u-PA or control normal rabbit immunoglobulin G (IgG) bound to protein A Sepharose, similar to methods described. 15 Plasma samples were assayed at 1:5 to 1:100 dilutions in the u-PA ELISA. Platelet lysates were tested at 1:2.5 and larger dilutions. Data for stored, washed, and pelleted platelet lysates were pooled because they contained similar amounts of u-PA at the dilutions tested. Data for new and stored lysates were analyzed separately because the stored samples were prepared with different protease inhibitors. 4, 5 All samples were tested undiluted in the t-PA ELISA, which was modified to include a lower concentration (2 ng/mL) standard. Some normal samples contained less u-PA or t-PA than the lowest standard of the AD ELISA when tested at recommended and lower dilutions. These amounts were reported as “less than” values when ranges for controls were determined, and they were rounded up to the nearest measurable value to calculate means and standard deviations for controls.

Active PAI-1 in platelet ionophore releasate and lysate (without added serine protease inhibitors) was assessed by measuring u-PA–PAI-1 complex generation, similar to methods previously described. 11 Briefly, pooled samples of releasate and lysate, prepared from 5 control and 5 QPD donors, respectively, were incubated (30 minutes, 22°C) with or without added recombinant tcu-PA (200 ng/mL final in 20 µL sample) before measuring u-PA–PAI-1 complexes by ELISA (values expressed as an average of duplicate determinations).

For studies of α-granule protein degradation in vitro, recombinant tcu-PA (0-400 ng u-PA/mL) was incubated overnight (37°C) with releasate from control ionophore-stimulated platelets or with control platelet lysate, prepared without serine protease inhibitors (multimerin digests only) (0.4% Triton X-100, final; all reactions stopped with 4 mM AEBSF). Degraded proteins in these digests were compared to QPD platelet proteins by Western blotting after separation on SDS-PAGE or SDS-multiplier gels. 4, 5 To determine whether u-PA formed high–molecular weight complexes when incubated with secreted platelet proteins, 10 ng recombinant u-PA was incubated with 10 to 60 µL control ionophore release for 1 to 18 hours.

Analyses of platelet mRNA

Total RNA was extracted from platelets and from K562 cells, as previously described. 17 Complementary DNA (cDNA) synthesis was carried out on 1 µg total RNA (20 µL final volume) using oligo dT as a primer and Thermoscript (Life Technologies, Burlington, ON, Canada) reverse transcriptase (RT), as recommended by the manufacturer. Polymerase chain reaction (PCR) was performed on 2 µL cDNA reaction in a final volume of 50 µL using Platinum Taq DNA polymerase (Life Technologies). Primers (synthesized at the Central Facility, McMaster University) were composed of different sizes, and cycle sequences for u-PA and β-actin reverse transcription—PCR were u-PA forward, 5′-GGATGGTCATCTTTCGCG-3′, u-PA reverse, 5′-CTGGCCCTGAAGTCGGTTAG-3′, expected product 1.55 kb, 94°C at 30 seconds, 50°C at 30 seconds, 72°C at 2 minutes for 30 cycles; β-actin forward, 5′-CCTCAGGGTTCCTAGCAAGCC-3′, β-actin reverse, 5′-GGATCCTCATGAGGAGAAGATG-3′, expected product 620 bp, 94°C at 30 seconds, 55°C at 30 seconds, 72°C at 1 minute for 25 cycles. Products were analyzed on 1% agarose gels and visualized with ethidium bromide.

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Results

Because platelet fibrinogen was degraded in patients with the QPD, their platelet releasates and lysates were screened for proteolytic activity using fibrin substrate gels (Figure 1). Fibrinolytic activity was evident in all QPD platelet releasates tested, but it was not detected in the same amounts of control releasates (Figure 1A; data representative of 5 patients and 12 controls). Fibrinolytic activity in QPD platelet releasates was inhibited by the serine protease inhibitor AEBSF (Figure 1A), but it was not blocked by EDTA, leupeptin, the cysteine protease inhibitor E64, the aspartic protease inhibitor pepstatin, or the metalloproteinase inhibitor phenanthroline (not shown). Similar fibrinolytic serine protease activity was present in lysates of QPD resting platelets, whereas it was undetectable in the same volume of control sample (Figure 1A). Fibrinolytic activity released by QPD platelets was not blocked in 1:2 mixtures with normal platelet releasate or lysate (Figure 1B), suggesting the defect was not due to an inhibitor deficiency.

Zymograms indicated there were secretable 50-kd (major band) and 100-kd (minor band) (Mr nonreduced) fibrinolytic enzymes in QPD platelets that were not detectable in similar amounts of normal platelets (Figure 2A-B shows data representative of 5 patients and 12 controls). The activities of these fibrinolytic enzymes were destroyed by reduction (not shown). Comparisons of their activities on fibrin substrate gels, with and without added plasminogen (Figure 2B), indicated the 50- and 100-kd QPD platelet proteases had properties of plasminogen activators. QPD platelets also contained and secreted a 33-kd plasminogen activator that was not detected in the normal samples (Figure 2B).

ELISA and Western blots were used to determine whether the plasminogen activators in QPD platelets were either t-PA or u-PA. Although QPD and control plasmas contained similar amounts of t-PA, neither QPD nor control platelets contained detectable t-PA (Table 1). Both the OS and AD u-PA ELISA indicated there was more than 100-fold more u-PA in QPD platelets than in normal platelets (Table 1). Furthermore, comparisons of platelet u-PA levels in unaffected family members and family members with the QPD indicated that only the affected patients had increased platelet u-PA levels (Table 1; data for stored platelet samples). The OS u-PA ELISA detected approximately 4-fold more u-PA in QPD platelets than the AD u-PA ELISA (Table 1), suggesting these assays differed in their ability to detect some forms of u-PA. The amounts of u-PA in normal plasma, measured by both OS and AD u-PA ELISA (Table 1), were similar to previously reported values.18-21 Each ELISA indicated patients with the QPD had larger increases in u-PA in their platelets than their plasmas because many patients had normal plasma u-PA levels (Table 1). u-PA ELISA confirmed
Table 1. Tissue-type plasminogen activator, urokinase-type plasminogen activator, plasminogen activator inhibitor 1, and u-PA–PAI-1 complexes in patients and controls measured by enzyme-linked immunosorbent assays

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sample</th>
<th>n</th>
<th>ng/mL plasma or ng/10⁹ platelets</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>P</th>
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<tr>
<td>t-PA</td>
<td>Plasma</td>
<td>Q</td>
<td>8</td>
<td>6.3 ± 2.7</td>
<td>3.7-11.2</td>
<td>.87</td>
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<tr>
<td></td>
<td>C</td>
<td>8</td>
<td>6.1 ± 2.0</td>
<td>4.3-9.8</td>
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<td></td>
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<tr>
<td>t-PA</td>
<td>Platelets</td>
<td>Q</td>
<td>5</td>
<td>All values &lt; 2</td>
<td>All values &lt; 2</td>
<td></td>
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<td>C</td>
<td>20</td>
<td>All values &lt; 2</td>
<td>All values &lt; 2</td>
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<td></td>
</tr>
<tr>
<td>u-PA, AD</td>
<td>Plasma</td>
<td>Q</td>
<td>12</td>
<td>2.3 ± 1.2</td>
<td>0.8-4.6</td>
<td>&lt; .0009</td>
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<tr>
<td></td>
<td>C</td>
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<td>0.7 ± 0.3</td>
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<tr>
<td>u-PA, OS</td>
<td>Plasma</td>
<td>Q</td>
<td>12</td>
<td>7.0 ± 6.7</td>
<td>1.9-22.6</td>
<td>&lt; .02</td>
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<tr>
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<td>1.2 ± 0.6</td>
<td>0.6-2.7</td>
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<tr>
<td>u-PA, AD</td>
<td>Platelets</td>
<td>Q</td>
<td>5</td>
<td>123 ± 16</td>
<td>106-143</td>
<td>&lt; .00008</td>
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<tr>
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<td>C</td>
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<td>u-PA, AD</td>
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<td>All values &lt; 5</td>
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<tr>
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<tr>
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<td>816-1161</td>
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<td>20</td>
<td>407 ± 96</td>
<td>284-660</td>
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<tr>
<td>u-PA–PAI-1 complexes</td>
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<td>Q</td>
<td>5</td>
<td>95 ± 14</td>
<td>82-115</td>
<td>&lt; .0002</td>
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<tr>
<td></td>
<td></td>
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<td>20</td>
<td>0.9 ± 0.2</td>
<td>0.6-1.3</td>
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Data for patients and controls were compared using two-tailed Student’s t tests. Results for AD and OD u-PA ELISA are shown separately.

- t-PA indicates tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; ELISA, enzyme-linked immunosorbent assays; AD, American Diagnostica; OS, Oncogene Science; Q, patients; C, controls.
- *Control subjects who were unaffected relatives.
- †Tested at 1:2.5 dilutions.
- ‡Tested at 1:5 dilutions.

Two of 20 control platelet lysates and 5 of 20 control plasmas contained less u-PA than the lowest standard of the AD ELISA. Stored platelet lysates were tested at 1:50 and larger dilutions in the AD u-PA ELISA only because sample volumes were limited.

QPD platelets released significant quantities of u-PA with secretagogue stimulation because their ADP releasates contained approximately 9% of their platelet u-PA, and their ionophore releasates contained approximately 48% of their platelet u-PA (averaged data, AD ELISA; n = 3 patients evaluated).

Western blots (probed with monoclonal and polyclonal u-PA antibodies) confirmed that QPD platelets and platelet releasates contained abnormally large amounts of u-PA (Figure 3A shows data representative of 5 patients). Western blots of stored platelet lysates, from additional affected (n = 9) and unaffected (n = 5) members of both QPD families, confirmed this abnormality was present only in affected patients (not shown).

Western blots were used to determine whether the u-PA in QPD platelets comigrated, nonreduced and reduced, with purified scu-PA, tcu-PA, or LMW u-PA (Figure 3). There was considerable heterogeneity in the forms of u-PA found in QPD platelet lysates, and their releasates contained identical forms (Figure 3). On nonreduced gels (Figure 3A; Figure 3B, left panel), the most abundant form of u-PA in QPD platelets comigrated with scu-PA and tcu-PA, whereas only a small proportion comigrated with LMW u-PA (Figure 3B). After reduction (Figure 3B, right panel), the most abundant form of u-PA in QPD platelets had the mobility of tcu-PA, indicating most u-PA in QPD platelets had been activated. Some of the less abundant forms of u-PA in QPD platelets were proteolyzed and did not comigrate with tcu-PA, scu-PA, or LMW u-PA (Figure 3A-B and longer exposures, not shown). A small proportion of their total u-PA was larger than scu-PA and tcu-PA and resembled high–molecular weight complexes generated by incubating exogenous scu-PA (Figure 3A, right panel) or tcu-PA (not shown) with normal platelet releasate proteins.

Zymograms indicated none of the QPD platelet plasminogen activators comigrated with t-PA or plasmin, and they confirmed the 50- and 33-kd plasminogen activators in QPD platelets comigrated with tcu-PA and LMW u-PA, respectively (Figure 4A). The activities of the 100-, 50-, and 33-kd QPD platelet plasminogen activators were blocked by 1 mM amiloride, which inhibited tcu-PA but not t-PA activity, as previously reported (Figure 4B). All the plasminogen activators in QPD releasates were neutralized when recombinant PAI-1 was added to final concentrations of 3000 ng/mL or more (Figure 4C), which was more than the concentration of PAI-1 in normal and QPD platelet lysates (Table 1). Furthermore, antibodies to u-PA selectively removed all detectable plasminogen activators (Figure 4D) and fibrinolytic proteases (not shown) from QPD releasates. These observations indicated that the fibrinolytic, plasminogen-activating proteases detected in QPD platelets were different forms of the enzyme u-PA.

RT-PCR analyses were performed to determine whether the u-PA abnormalities in the QPD platelets were associated with increased u-PA mRNA levels in platelets. Although platelets from patients and controls contained similar amounts of β-actin mRNA, only QPD platelets contained detectable u-PA mRNA (Figure 5).

Unregulated u-PA activity in QPD platelets was further investigated by measuring platelet PAI-1 antigen and u-PA–PAI-1 complexes using ELISA. QPD platelets contained approximately 2-fold more PAI-1 antigen and more than 100-fold more u-PA–PAI-1 complexes than normal platelets (Table 1). Western blots confirmed some of the PAI-1 in QPD platelets had formed complexes with u-PA, though the proportions of complexed PAI-1 varied slightly between patients (Figure 6A; Pt 3 indicates the patient with the highest concentrations of platelet u-PA–PAI-1 complexes by ELISA). Increased u-PA–PAI-1 complexes were also detected in QPD platelet releasates using ELISA (Figure 6B), but they were difficult to detect by Western blotting (Figure 6A and analyses of larger sample volumes, not shown). The high–molecular weight PAI-1 complexes stored in QPD platelets expressed epitopes recognized by u-PA antibodies (Figure 6A, lane *), and they comigrated with PAI-1 complexes generated in vitro by adding tcu-PA to normal platelet releasate (Figure 6A, right panel). All the QPD platelets tested contained proteolyzed forms of PAI-1 (Figure 6A, arrow) that were not evident in
normal platelets, but only traces of similar proteolyzed forms were detected in control releasates incubated with tcu-PA (Figure 6A and longer exposures, not shown).

Assays of active PAI-1 indicated that although pooled QPD platelet lysates and releasates contained abnormally large amounts of u-PA. The predominant form of u-PA in QPD platelets comigrated, nonreduced, with recombinant scu-PA (scuPA, 10 ng), though larger and smaller forms were also detected. When control platelet releasate was incubated (lanes *) with recombinant scu-PA (lane CR + scuPA*), high-molecular weight u-PA complexes were generated that resembled large forms of u-PA in QPD releasates (arrow indicates the most abundant large form). (B) QPD platelet releasates and lysates (R and L, 5 μL nonreduced, 20 μL reduced) contained forms of u-PA with the characteristic nonreduced/reduced mobility of purified tcu-PA (tcuPA; 4.4 ng nonreduced, 17.6 ng reduced) and LMW u-PA (LMWuPA; 3.2 ng nonreduced, 12.8 ng reduced). Arrows indicate the A chain (A*) of reduced tcu-PA, the B chain (B*) common to reduced tcu-PA and LMW u-PA, and the nonreduced form of LMW u-PA (LMW). A reduced protein with the mobility of the A chain of tcu-PA was seen in prolonged exposures of the QR and QL lanes (not shown).

Western blots were used to determine whether the changes in u-PA in the QPD were associated with plasminogen proteolysis. QPD plasmas contained forms and amounts of plasminogen that were indistinguishable from normal controls (not shown). Although the plasminogen in normal, washed platelets comigrated with purified Glu-plasminogen, in QPD platelets much of the plasminogen was proteolyzed (Figure 7), and there was a form that comigrated with plasmin on reduced (Figure 7) and nonreduced (not shown) gels. When normal platelet releasate was incubated with exogenous tcu-PA, there was loss of detectable intact plasminogen; however, the extent of plasminogen proteolysis was not as complete as in QPD platelets and the tcu-PA digests of purified plasminogen (Figure 7).

Next, we investigated whether exogenous tcu-PA (in concentrations similar to the increased u-PA in QPD platelets) could trigger the proteolysis of other stored platelet proteins to forms that comigrated with degraded proteins in QPD platelets (Figures 8, 9, 10). Adding tcu-PA to normal platelet releasate resulted in the
degradation of α-granule fibrinogen to FDPs that comigrated with QPD platelet FDPs (Figure 8A). α-Granule fibronectin was also degraded when tcu-PA was added to normal platelet releasate (Figure 8B). Although the fibronectin degradation in vitro was not as extensive as in QPD platelets, there were many similarities in the sizes of degradation products (Figure 8B, arrows). When tcu-PA was incubated with normal platelet releasate, thrombospondin-1 was converted to a form that comigrated with the larger thrombospondin-1 degradation product in QPD platelets (Figure 9A, arrow). Osteonectin and von Willebrand factor were degraded when normal platelet releasate was incubated with tcu-PA to forms.
that comigrated with degraded osteonectin and von Willebrand factor in QPD platelets (Figure 9B-C, arrows). Platelet factor V was degraded when normal platelet releasate was incubated with tcu-PA (Figure 10A), resulting in a loss of factor V detectable by Western blotting, as in QPD platelets.4,5 Because the amount of multimerin in platelet releasates was limiting, multimerin proteolysis was investigated by adding tcu-PA to normal platelet lysate, prepared without serine protease inhibitors (Figure 10B). tcu-PA induced a striking loss of multimerin detected by Western blotting in these experiments, as in QPD platelets (Figure 10B shows multimerin multimers; findings in samples analyzed by reduced SDS-PAGE were similar [not shown]).

Discussion

Patients with Quebec platelet disorder have an unusual biochemical defect that causes their α-granule proteins to be degraded. Unlike patients with severe α-granule protein deficiencies, they suffer from bleeding that is paradoxically delayed and cannot be controlled with platelet transfusions.1 The purpose of our current study was to determine whether patients with QPD had a protease abnormality in their circulating platelets. We found that unlike normal platelets, QPD platelets contained large amounts of fibrinolytic, plasminogen-activating proteases. Moreover, we determined this was because QPD platelets contained markedly increased amounts of the enzyme u-PA, within a secretory compartment. These observations suggest u-PA could be involved in the pathogenesis of this unique storage pool disorder and its hemorrhagic complications.

Like normal platelets, QPD platelets store plasma-derived and megakaryocyte-synthesized proteins within their α-granules.5 Some u-PA has been reported to be associated with normal platelets and their external membranes when large quantities of platelet proteins have been analyzed.23-26 We observed that QPD platelets contained more than 100-fold more u-PA than normal platelets, which contained up to 1.3 ng u-PA/10⁹ platelets. Furthermore, unlike normal platelets, QPD platelets released u-PA and high concentrations of u-PA–PAI-1 complexes in response to secretagogue stimulation. QPD platelet lysates, prepared

![Figure 9. α-granule thrombospondin-1, von Willebrand factor, and osteonectin degradation.](image)

![Figure 10. α-granule factor V and multimerin degradation.](image)
with (Table 1) or without (Figure 6B) high concentrations of serine protease inhibitors, also contained high concentrations of u-PA–PAI-1 complexes, suggesting QPD platelets costore u-PA with PAI-1 in \( \alpha \)-granules.\(^{27} \) We suspect the increased u-PA in QPD platelets is synthesized by their megakaryocytes because only some patients had increased u-PA in plasma, and, unlike normal platelets, QPD platelets contained u-PA mRNA.

u-PA has a number of different forms, and its tcu-PA form has much greater plasminogen-activating activity than uncleaved scu-PA.\(^{28-31} \) These forms can be distinguished from each other and from LMW u-PA using nonreduced and reduced SDS-PAGE.\(^{28-31} \) Whereas normal platelets have been reported to contain mostly scu-PA,\(^{23,24} \) we found QPD platelets contained predominantly active tcu-PA, minimal scu-PA, some LMW u-PA, and a small amount of u-PA in high–molecular weight complexes. Moreover, unlike normal platelets, QPD platelets contained plasminogen that was proteolized and that comigrated with plasmin. The high–molecular weight u-PA complexes in QPD platelets resembled the complexes generated by incubating exogenous u-PA with normal releasate, and they included forms recognized by PAI-1 antibodies. These data suggest the very large forms of u-PA in QPD platelets have been reported to contain mostly scu-PA,\(^{23,24} \) we found QPD platelets contained more than 100-fold more u-PA–PAI-1 complexes, we were unable to generate any further u-PA–PAI-1 complexes, suggesting QPD platelets costore u-PA with PAI-1 in \( \alpha \)-granules.\(^{27,35,38-40} \) We observed that some of the u-PA in QPD platelets had been neutralized by PAI-1. However, the amount of exogenous, recombinant PAI-1 required to completely neutralize the u-PA secreted by QPD platelets exceeded the amount of PAI-1 in normal and QPD platelets. Because we found QPD platelets contained more than 100-fold more u-PA–PAI-1 complexes and 2-fold more total PAI-1 antigen than normal platelets, our data exclude a functional or quantitative PAI-1 deficiency as the cause of the their unregulated u-PA activity. Although only a minority of the total PAI-1 in QPD platelets was contained in high–molecular weight complexes, we were unable to generate any further u-PA–PAI-1 complexes by adding u-PA to QPD platelet lysates or releasates in vitro. This suggests the increased u-PA in QPD platelets depletes their stores of active PAI-1. The relative proportions of total PAI-1, u-PA, and u-PA–PAI-1 complexes detected in QPD platelets by ELISA (954 ng PAI-1, 505 ng u-PA [OS assay], and 95 ng u-PA–PAI-1 complexes/10\(^9\) platelets) and the similar molecular masses of PAI-1 and u-PA\(^{41} \) infer that only a limited amount (approximately 5%) of the total PAI-1 in QPD platelets was active in neutralizing u-PA in vivo. Interestingly, this estimate corroborates previous reports that normal platelets contain less than 10% active PAI-1.\(^{27,35,38,39} \) The large amounts of u-PA in QPD platelets, and the relatively limited supply of u-PA inhibitors in platelets, could be part of the reason patients with QPD experience delayed bleeding that cannot be controlled with platelet transfusions.

The diversity of proteins degraded in QPD platelets has suggested that fairly broad-specificity protease(s) are involved. The FDPs secreted by QPD platelets are not recognized by a monoclonal antibody specific for plasmin-degraded fibrinogen.\(^{5} \) Using sensitive Western blots, we observed that QPD platelets contained proteolyzed forms of plasminogen with the mobility of plasmin, but we were unable to detect plasmin activity in QPD platelet releasates by zymography, even after u-PA was immunodepleted. tcu-PA and LMW u-PA are known to proteolyze fibrinogen in addition to plasminogen,\(^{42} \) but where they cleave fibrinogen has not been determined. Moreover, it is not yet known whether tcu-PA and LMW u-PA can cleave other potential substrates within platelets. We observed that the net effects of adding exogenous tcu-PA to normal platelet secretory proteins (in concentrations similar to the increased u-PA in QPD platelets) were a loss of intact plasminogen and the proteolysis of many \( \alpha \)-granule proteins. There were phenotypic similarities in the sizes of the fibrinogen, fibronectin, von Willebrand factor, thrombospondin-1, and osteonectin degradation products generated to degraded proteins in QPD platelets. Furthermore, platelet multimerin and factor V were proteolyzed after adding tcu-PA, resulting in a loss of the forms detectable by Western blotting, as in QPD platelets. These observations provide indirect evidence that the changes to \( \alpha \)-granule proteins, including multimerin, in the QPD likely reflect a complex process of proteolysis that may be initiated by decreased platelet u-PA. The less extensive proteolysis of plasminogen—and some of the other \( \alpha \)-granule proteins—in the in vitro digests compared to QPD platelets could reflect differences in the duration of substrate protein exposure to u-PA, or it could reflect contributions of factors, such as the environment within platelets, that enhance \( \alpha \)-granule protein proteolysis in vivo. Although platelets have receptors on their external membranes for scu-PA and tcu-PA,\(^{45,44} \) that could modulate some aspects of u-PA proteolysis in the QPD, we observed that membrane-tethered proteins were not required to degrade \( \alpha \)-granule proteins to forms found in QPD platelets.

u-PA is normally expressed in many different tissues,\(^{45} \) and it is thought to play a role in diverse physiological and pathological processes.\(^{31} \) In mice, u-PA deficiency causes problems with excess fibrin deposition, whereas its overexpression in the liver results in bleeding, marked hypofibrinogenemia, and systemic fibrinogenolysis.\(^{46,47} \) The QPD has biochemical abnormalities distinct from other platelet storage pool disorders,\(^{2} \) and from congenital bleeding disorders associated with increased t-PA levels or t-PA–related proteins in plasma.\(^{48,49} \) Like \( \alpha \)-antiplasmin deficiency,\(^{50} \) the QPD is not associated with systemic fibrinogenolysis.\(^{6} \) This may be because plasma u-PA inhibitors effectively regulate the normal to increased u-PA levels in the plasma of patients with QPD. Our observations indicate patients with the QPD have an inherited, autosomal dominant defect that increases u-PA expression and storage in their megakaryocytes and platelets. Fibrinolytic inhibitors (such as tranexamic acid and epsilon amino caproic acid) rapidly and effectively control bleeding in patients with the QPD, yet they do not measurably improve QPD \( \alpha \)-granule protein degradation, even when they are given for several weeks of therapy.\(^{6} \) This observation suggests that the moderate to severe bleeding in patients with the QPD could result from accelerated fibrinolysis within the hemostatic plug, where the concentrations of released u-PA may overwhelm protease inhibitors.

The QPD is the only inherited bleeding disorder in humans associated with increased levels of u-PA in blood. Unraveling its genetic cause is likely to provide further insights into this unusual and sometimes fatal bleeding disorder.

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References

Platelets from patients with the Quebec platelet disorder contain and secrete abnormal amounts of urokinase-type plasminogen activator

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