RAPID COMMUNICATION

Factor V Quebec Revisited

By Claude M. Janeway, Georges E. Rivard, Paula B. Tracy, and Kenneth G. Mann

Factor V Quebec has been described as a bleeding disorder that exhibits an autosomal dominant inheritance pattern and presents severe bleeding after trauma. Two members of a fourth-generation (IV.13 and IV.15) Canadian family have been studied in detail and are the subject of this report. Their clinical presentations and histories were previously described (Tracy et al. J Clin Invest 74:1221, 1984). Persistent abnormalities include mild thrombocytopenia and defective platelet factor V. Plasma factor V is present at near normal concentration and is fully functional. Thus, the bleeding diathesis appears to reflect the absence of platelet factor V activity. The recent report (Hayward et al: Blood 84:110a, 1994 [suppl, abstr]) of multimerin deficiency in these individuals led us to reevaluate these patients. Western blot analysis of platelet lysates developed with a variety of monoclonal antibodies show that the α-granule proteins, fibrinogen, von Willebrand factor, factor V and osteonectin are decreased in concentration and significantly degraded in the platelets of these patients. Thrombospondin, while not degraded, is substantially decreased. In contrast, platelet factor 4 and β-thromboglobulin do not appear to be affected. These observations suggest that the α-granules are correctly assembled but the contents are subsequently subjected to proteolytic degradation. The results indicate that factor V Quebec disorder is probably associated with a generalized defect that leads to degradation of most proteins of the α-granules.

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Electrode's solution containing PGE, 5 pmol/L (Sigma, St Louis, MO) and heparin, 5 U/mL (Organon, Inc, West Orange, NJ) in the initial wash. After the third and final wash, the platelets were resuspended in Tyrode’s medium wash, 5 mMol/L HEPES, without albumin, pH 7.4. The platelets were counted with a Technicon H1 counter (Technicon, Puerto Rico) and adjusted to a final concentration of 1 × 10^9/mL. The Tyrode’s HEPES solution was removed by centrifugation, and the platelets were lysed by replacing the Tyrode’s HEPES solution with an identical volume containing 0.5% Triton X-100 20 mMol/L HBS, 100 mMol/L NaCl, pH 7.4 and the following protease inhibitors: 10 mMol/L EDTA, 0.02 mg/mL soybean trypsin inhibitor, 0.1 mMol/L leupeptin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The platelet lysates, 1 × 10^8 platelets/mL, were frozen on dry ice. All experiments were carried out in parallel on the normal and the two patients.

Proteins, antibodies, and assays. Human factor V was prepared from fresh frozen human plasma using immunoaffinity chromatography as described previously. Factor Va was prepared by the incubation of factor V with catalytic amounts of thrombin (1.5 U/mL). Fibrinogen and platelet factor 4 were gifts from Haematologic Technologies, Inc (Essex Junction, VT). Human platelet osteonectin was prepared in the laboratory as described by Stenner et al. An MoAb against the heavy chain of human factor VNa, a-HFV-17, an MoAb that recognizes the light chain of factor Va a-HFV-9, and an MoAb recognizing the α-chain of human fibrinogen ω-Fng-3A, were obtained from Dr W.R. Church (Department of Biochemistry, University of Vermont College of Medicine). An anti-osteonectin MoAb, IIIA-A1, was prepared in the laboratory as described by Stenner et al. An MoAb to thrombomodulin, P10 α-TSP, was obtained from AMAC Inc (Westbrook, ME). Burro antihuman platelet factor 4 (PF4) IgG was prepared in our laboratory by Dr M. Rand as described and an MoAb against vWF, α-vWF-95, was a gift from Dr D. Meyer. Bioassays of platelet factor V were previously reported. Radioimmunoassay of factor V were performed as described. Densitometry was accomplished with a Microscan 1000 scanning densitometer (Tri Inc, Nashville, TN). Radioimmunoassays of β-thromboglobulin were performed in the Laboratory for Clinical Biochemistry Research, University of Vermont.

Gel electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using 4% to 12% and 8% to 20% gradient gels according to the method of Laemmli. The proteins were transferred into nitrocellulose membranes (Trans-Blot nitrocellulose; Bio-Rad, Hercules, CA) using a previously described method. The nonspecific sites of the membranes were blocked by the addition of 5% nonfat milk (Carnation Co, Los Angeles, CA) (wt/vol) in TBS/Tween for 1 hour at room temperature followed by incubation with usually a purified MoAb. The final concentration of the antibodies varied from 1 to 5 μg/mL, depending on the antibody used. After another wash in TBS/Tween, the nitrocellulose membrane was incubated with conjugated goat-antimouse horseradish peroxidase IgG (Southern Biotechnologies Associates, Inc, Birmingham, AL) at 1:5,000 dilution. The peptide fragments were detected using the chemiluminescent Lumilum Developer (ECL Western blotting detection system; Amersham Corp, Arlington Heights, IL).

RESULTS

Factor V/Va. The platelet factor V was analyzed by Western blots using two MoAbs, one that recognizes the heavy chain as shown in Fig 1A and the other the light chain, Fig 1B. Comparable amounts of platelets of a normal individual and two patients were placed on the 4% to 12% SDS-PAGE gels. In all samples from patient IV.13 (lanes 9 through 12), and patient IV.15 (lanes 13 and 14), factor V is much decreased when compared with the normal (lanes 5 through 8). It is apparent that platelet factor V from the patients (lanes 9 through 14) is degraded and results in many fragments different from those observed in the normal (lanes 5 through 8). Normal activation of platelet factor V with thrombin results in fragments M, = 150,000 and M, = 74,000 associated with the light chain, and M, = 105,000 and M, = 71,000 associated with the heavy chain. Fractions of the platelet lysates were treated with thrombin (+lIl), 5 U/mL, 5 minutes 37°C, a high concentration to overcome the protease inhibitors. In both immunoblots, lanes 7 and 8 represent the normal with thrombin added, and lanes 11, 12, and 14 represent patients IV.13 and IV.15 with thrombin. In both immunoblots, we see little, if any, change of factor V/Va in the lanes of the patients after thrombin addition. The components expected after thrombin activation, especially the M, = 150,000 light chain precursor and the M, = 105,000 heavy chain are for all practical purpose missing in the patients. We also observe that the platelet factor V of the patients is proteolyzed to discrete fragments (ie, M, = 45,000 to 35,000), and shows a pattern of proteolysis different from the normal. The results of these Western blots show that factor V is decreased and degraded in the platelet lysates of these patients when compared with the normal.

The factor V in the plasma of the patients and the normal controls were also analyzed using the two MoAbs to the factor Va heavy and light chains. The results (data not shown) indicate that no difference in factor V or factor Va was found quantitatively or qualitatively in the platelet-poor plasma of the patients as compared with the normal and upon activation by thrombin the expected fragments were obtained. These results confirm our previous observation that indicated that plasma factor V in the patients is normal and functional.

vWF. vWF is normally present in both plasma and platelets and is composed of a series of high-molecular-weight multimers that range between M, 0.5 to 10 × 10^9. When the disulfide bonds are reduced with β-mercaptoethanol, a single predominant band is seen on SDS-PAGE with an apparent molecular weight of M, = 270,000. The platelet lysates were analyzed on a 3% to 15% SDS-PAGE gel under
Fig 1. Immunoblots of factor V and factor Va. The samples were run on 3% to 15% SDS-PAGE gels under reducing conditions. (A) Immunoblots were developed with an MoAb against the heavy chain, α-HFV-17. Lanes 1 and 2 contain 50 and 100 ng of purified human factor V, lanes 3 and 4 contain the same amount of purified human factor Va. Lanes 5 through 8 contain platelet lysates from a normal individual; lanes 7 and 8, the normal platelet lysate treated with 5 U thrombin/mL/5 min. Lanes 9 through 12 contain platelet lysates from patient IV.13; lanes 11 and 12, these lysates treated with thrombin as above. Lanes 13 and 14 contain platelet lysates from patient IV.15; lane 14, this lysate was treated with thrombin. Samples in lanes 5, 7, 9, and 11 are derived from 2 x 10^7 platelets; samples in lanes 6, 8, 10, 12, 13, and 14 are derived from 5 x 10^7 platelets. (B) The membrane of (A) was stripped with 0.1 mol/L glycine, 0.5 mol/L NaCl, pH 2.8 for 16 hours, treated with milk, and washed as described in Materials and Methods. The membrane was developed with the light-chain MoAb α-HFV-9. The samples in (B) are identical to those in (A).

reducing conditions. The immunoblots were developed with a primary MoAb against vWF as described in Materials and Methods. In Fig 2A, comparable amounts of platelets from the normal, lanes 4 and 5, and the two patients, lanes 6 and 7, patient IV.13, and lane 8, patient IV.15, were run in parallel on the gel. The results indicate that the total vWF factor antigen levels (intact plus fragments) are not substantially decreased but the molecule is degraded in both patients when compared with the normal and the fragments are similar in both patients.

Osteonectin. Osteonectin is a noncollagenous bone protein that is also contained and secreted from human platelets on activation with thrombin or collagen. Purified platelet osteonectin is a single-chain molecule that exhibits a slightly larger apparent molecular weight (39,000) than that of osteonectin from bone. Osteonectin immunoblot analyses using MoAb IIIA3A are shown in Fig 2B. The platelets lysates were analyzed on a 3% to 15% SDS-PAGE gel under reducing conditions. The samples in lanes 4 through 8 are identical to those in Fig 2A. The immunoblots show that in the patients’ platelet lysates, osteonectin is degraded when compared with the normal and shows a distinctive pattern of degradation in both patients.

Thrombospondin. Thrombospondin is a glycoprotein released from the α-granules of stimulated platelets, which appears to play a role in platelet aggregation by stabilizing the platelet:fibrinogen interaction. The apparent molecular weight of the protein, M_r = 540,000, is decreased under reducing conditions to M_r = 170,000. The platelet lysates were run on a 4% to 12% SDS-PAGE gel under reducing conditions. The immunoblot was developed with the MoAb P10 α-TSP and is shown in Fig 3A. Comparable amounts...
of platelets of a normal individual, lanes 3 and 4, and the two patients lanes 5 and 6, patient IV.13, and lane 7, patient IV.15, were run in parallel. Thrombospondin is substantially decreased in the platelet lysates of both patients when compared with the normal. It is not known if thrombospondin is degraded, but the MoAb did not reveal any fragments.

**Fibrinogen.** Fibrinogen is present in the α-granules of platelets, where it normally constitutes 4% to 10% of the total platelet protein concentrations. The apparent molecular weight of the protein is $M_r = 269,000$; under reducing conditions it separates into three chains, $\alpha$: $M_r = 68,000$, $\beta$: $M_r = 54,000$, and $\gamma$: $M_r = 49,000$. Fibrinogen was assayed on 4% to 12% SDS-PAGE gels in the presence and in the absence of β-mercaptoethanol. Detection was accomplished by immunoblotting with an MoAb against the α-chain. The results are shown in Fig 4A under nonreducing conditions, and in Fig 4B under reducing conditions.

![Fig 2. Immunoblots of vWF (A) and of osteonectin (B). The samples were run on a 3% to 15% SDS-PAGE gel run under reducing conditions. (A) Lanes 1, 2, and 3 contain 0.25, 0.50, and 0.75 μg of human vWF purified from plasma. Lanes 4 and 5 contain platelet lysates from a normal; lanes 6 and 7 contain platelet lysates from patient IV.13; lane 8 contains the platelet lysate from patient IV.15. The samples in lanes 4 and 6 are derived from $2 \times 10^7$ platelets and in lanes 5, 7, and 8 from $5 \times 10^7$ platelets. (B) Lanes 1, 2, and 3 contain 0.17, 0.35, and 0.70 μg of purified human platelet osteonectin, respectively; the samples in lanes 4 through 8 are identical to those in (A).](image)

![Fig 3. Immunoblots of thrombospondin (A) and of PF (B). (A) Samples were run on a 4% to 12% SDS-PAGE under reducing conditions. Lanes 1 and 2 contain 0.6 and 1.2 μg of purified human thrombospondin, respectively; lanes 3 and 4 contain the platelet lysates from the normal; lanes 5 and 6 contain the platelet lysate from patient IV.13; lane 7 contains the platelet lysate of patient IV.15. The samples in lanes 3 and 5 are derived from $2 \times 10^7$ platelets and in lanes 4, 6, and 7 from $5 \times 10^7$ platelets. (B) Samples were run on an 8% to 20% SDS-PAGE under reducing conditions. Lanes 1 through 4 contain 0.16, 0.325, 0.65, and 1.3 μg of purified human PF, respectively; lanes 5 and 6 contain the platelet lysates of the normal, lanes 7 and 8 contain the platelet lysates of patient IV.13, and lane 9 contains the platelet lysate of patient IV.15. The samples in lanes 5 and 7 are derived from $2 \times 10^7$ platelets, and in lanes 6, 8, and 9 from $5 \times 10^7$ platelets.](image)
Fig 4. Immunoblots of fibrinogen. The samples were run on 4% to 12% SDS-PAGE gels under nonreducing conditions in (A) and reducing conditions in (B). (A) Lanes 1, 2, and 3 contain 50, 100, and 200 ngm of purified human fibrinogen, respectively; lanes 4 and 5 and lanes 6 and 7 contain platelet lysates from two normals; lanes 8 and 9 contain platelet lysates from patient IV.13; lanes 10 and 11 contain platelet lysates from patient IV.15; the samples in lanes 4, 6, 8, and 10 are derived from 2 x 10^6 platelets while in lanes 5, 7, 9, and 11 from 5 x 10^6 platelets. (B) The gel was developed under reducing conditions and the samples are identical to those in (A), with the following exceptions: lanes 9 and 10 contain platelet lysates from patient IV.13, and lanes 12 and 13 contain platelet lysates from patient IV.15. The samples in lanes 9 and 12 are derived from 2 x 10^6 platelets and in lanes 12 and 13 from 5 x 10^6 platelets.

The results indicate that fibrinogen is present at decreased concentration and is degraded in the two patients (lanes 8 through 11 in Fig 4A and lanes 8 through 13 in Fig 4B), when compared with two normals (lanes 4 and 5, and 6 and 7, Fig 4A and B).

PF₄. PF₄ is a low-molecular-weight heparin-binding protein that is secreted from agonist-activated platelets and is localized within the α-granules where its concentration normally ranges from 11.2 to 12.4 μg/l × 10^9 platelets. On a molar basis, PF₄ is one of the most abundant proteins in the platelets. The platelet factor 4 monomer has a molecular weight of Mᵣ = 7,800. In its native state, PF₄ is a homotetramer that complexes with a high-molecular-weight proteoglycan carrier protein. The results of the Western blots analyses of PF₄ are shown in Fig 3B. Comparable amounts of the platelets were run on an 8% to 20% SDS-PAGE gel, followed by immunoblotting with a polyclonal antibody to PF₄. The platelets of the normal are in lanes 5 and 6, those of patient IV.13 in lane 7 and 8, and patient IV.15 in lane 9. By densitometry and comparison with purified human PF₄, the normal platelet lysate was calculated to contain 15.2 μg/l × 10^9 platelets PF₄, whereas those of the patients were, respectively, 122% and 113% of this value. In this case we attempted to calculate a concentration for the PF₄ because the protein is not degraded into fragments. β-Thromboglobulin was assayed by radioimmunoassay and found to be at normal levels in both patients (data not shown).

We speculate that either there is a gross packaging and/or storage defect of proteins in the α-granules or that these proteins are degraded by a specific protease in the platelets. These results indicate that factor V Quebec disorder is probably associated with a generalized defect which leads to degradation of most proteins of the α-granules. These findings likely have a direct bearing on the severity of the symptoms in these patients.
Table 1. Determination of Platelet Factor V by RIA and of Multimerin by ELISA

<table>
<thead>
<tr>
<th>Factor V</th>
<th>Multimerin</th>
<th>Symptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>µg/mL*</td>
<td>U/mL*</td>
</tr>
<tr>
<td>Normal</td>
<td>100</td>
<td>2.7 (± 0.2) (0.5-2.0)</td>
</tr>
<tr>
<td>Patient IV.13</td>
<td>62</td>
<td>1.7</td>
</tr>
<tr>
<td>Patient IV.15</td>
<td>29</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The RIA was developed in our laboratory; the determinations were done in our laboratory on platelet lysates previously prepared by Dr C Hayward.

*Platelet lysates contain 1 x 10^9 platelets/mL.
†These values were obtained from Hayward et al.
‡Average value for patients IV.13 and IV.15.

**Average value for patients IV.13 and IV.15.

DISCUSSION

The index family exhibits a serious bleeding disorder previously attributed to the presence of inactive platelet factor V/Va. In that study a gross discrepancy was observed between the amount of platelet associated factor V determined by either bioassay or radioimmunoassay (RIA) for both patients. In the case of patient IV.13, the RIA indicated near-normal level of antigen; however, only 2% of the molecules expressed FVa activity after thrombin activation. In the case of patient IV.15, the platelet factor V antigen was reduced and only 4% of the factor V present expressed cofactor activity. The only functional abnormality observed was inactive platelet factor V. Thus, a more general defect in the ability of the platelets to support coagulation was not suspected.

Subsequently, Hayward et al investigated the same individuals using a multimerin assay. They had identified multimerin as a complex multimeric protein that is stored within platelet α-granules and endothelial cell Weibel-Palade bodies. Because it also appears to be a factor V binding protein, Hayward et al have suggested that multimerin plays a role in the storage of factor V within platelet α-granules. Using an enzyme-linked immunosorbent assay (ELISA), platelets from three afflicted members of the index family, including patients IV.13 and IV.15, were found to be deficient in platelet multimerin, with values ranging from 0.09 to 0.16 U/1 x 10^9 platelets (normal range, 0.5 to 2.0 U/1 x 10^9 platelets). Multimer analyses identified only the smallest multimers of multimerin in the platelet lysates of the affected family members. Two unaffected family members had normal multimerin levels. These investigators also reported that the level of several other α-granular proteins, including β-thromboglobulin and vWF were normal in both affected and nonaffected family members. Recently, the same investigators reported a multimerin deficiency in a second Quebec family with an autosomal bleeding disorder, with no relations common to the factor V Quebec pedigree. Platelet factor V antigen levels, evaluated using the ELISA assay and polyclonal antisera, were also reduced (20%; normal range, 48% to 148%). These observations led us to reinvestigate the factor V levels in the affected siblings and extend our analyses to other α-granule proteins. In the present study we have obtained evidence that factor V and multimerin are not the only altered proteins present in the platelet α-granules of these patients. With the exception of PF4 and β-thromboglobulin, all of the α-granule proteins analyzed, factor V, fibrinogen, vWF, thrombospondin, and osteonectin, were either degraded or were present at decreased levels. Thrombospondin is very much decreased in the patients, although we do not have any evidence that is degraded. However, there is always the possibility that the MoAb would not recognize the fragments. For the same reason we have not attempted to calculate total protein content from the immunoblots when the protein is degraded. Fibrinogen also show a large decrease in the α-granules of the Quebec patients.

Table 1 compares the results of factor V radioimmunoassays performed recently in our laboratory on platelet lysates obtained from patients IV.13 and IV.15 and from a pool of normal individuals and the multimerin results reported by

![Composite graph showing the quantities of intact proteins of the platelets. The films developed from the immunoblots were subjected to scanning densitometry. Density is expressed relative to the maximum density observed for the patients in comparison with the normals. The intact proteins measured by densitometry are vWF, osteonectin, thrombospondin, and PF4. The graph represents the relative quantity of intact proteins present without taking peptide fragments into account. Factor V and multimerin were measured by radioimmunoassay and ELISA, respectively (see Table 1). Thus, the amount of these two proteins represents the concentration of the total protein as total antigen in the platelet lysate. Multimerin is represented as the average of the range given in units/1 x 10^9 platelets as reported.](image-url)
Hayward et al\textsuperscript{26} on the same patients. The results of the radioimmunoassays indicate that factor V antigen is present at a decreased level in the platelet lysates of the two patients compared with the normal. Multimerin, given as a range, is also decreased in the affected individuals. These assays represent total protein concentrations. We observe from Table 1 that the decrease in multimerin does not correlate well with factor V. As observed in Table 1 for the patients, the decrease in multimerin varies between 4.5\% and 32\% whereas that of factor V varies between 30\% and 65\%. This could indicate that multimerin deficiency is not necessarily associated with factor V as has been suggested,\textsuperscript{26} but may be just another macromolecule that is altered in these patients.

A composite display of the intact proteins present in the affected individuals is shown in Fig 5. The immunoblots were subjected to densitometry and only the intact protein is represented here as explained in the legend of Fig 5. Thus, the decreases seen in Fig 5 represent the loss of the intact protein only. In contrast, the RIA and ELISA results for factor V and multimerin represent the quantities of the total antigen concentrations of these proteins.

Immunoassay quantitation of plasma and platelet proteins yields a result that reflect total protein and peptide content without necessarily distinguishing between intact and degraded proteins. However, immunoblotting analysis has the advantage of allowing for the visualization of degradation products and thus is a more complete and accurate analysis of the status of the proteins. In view of our results, which show that more than one protein of the \( \alpha \)-granules may be altered, a reassessment of the disorder in these patients is necessary. We have shown in a previous paper that human platelet factor V is released as a partially proteolyzed species possessing cofactor activity in the prothrombinase complex.\textsuperscript{4}

All attempts so far to isolate human platelet factor V as a single-chain procofactor in platelet lysates have failed,\textsuperscript{3} and thus it is probable that factor V is stored in platelets as a partially proteolyzed cofactor.\textsuperscript{4} Our present results, visible on the immunoblots in Fig 1 lanes 5 and 6, for the factor V of the normal individual attest to these findings. In this report we speculate that another protease is responsible for the degradation observed of factor V and of the other macromolecules in the \( \alpha \)-granules of the patients. Calpain, a calcium-dependent protease present in platelets is a likely candidate. Bradford et al\textsuperscript{25} reported that purified platelet calpain cleaves human factor V and the cleavage pattern reported by Viskup et al\textsuperscript{24} from our laboratory may have similarities to that of calpain cleaved factor V. Calpain has also been reported to cleave vWF, fibrinogen, and cytoskeletal proteins.\textsuperscript{35,36} An increase in the concentration or the activity of this enzyme or the absence of its suppressor could be responsible for the degradation observed on the \( \alpha \)-granule proteins in the Quebec patients.

A second possibility is that there is a defect in the storage and packaging mechanism of the \( \alpha \)-granules of these patients that would affect all these proteins. Inherited abnormalities of platelet \( \alpha \)-granules have been described.\textsuperscript{37} Studies of patients with the rare condition Gray platelet syndrome have shed light on the chemical content localized within the different types of \( \alpha \)-granules. In the Gray platelet syndrome, platelets are virtually devoid of \( \alpha \)-granules and are deficient in products confined to these organelles, including PF\(_4\) and \( \beta \)-thromboglobulin.\textsuperscript{38} The presence of normal levels of intact PF\(_4\) and BTG suggest that the \( \alpha \)-granule is assembled normally in factor V Quebec.

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