Thrombosis and Hemostasis

PLATELETS AND PLATELET-DERIVED FACTOR VA CONFER HEMOSTATIC COMPETENCE IN COMPLETE FACTOR V DEFICIENCY

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Running title: Platelet-derived factor Va imparts hemostasis
KEY POINTS

● Administration of plasma to a factor V deficient individual yields a stable platelet factor V/Va pool derived from megakaryocyte endocytosis

● Platelets and platelet-derived factor V/Va promote and extend hemostasis well after depletion of the plasma-derived factor V pool
ABSTRACT

Whole genome sequencing of an individual completely devoid of plasma- and platelet-derived factor V (FV) identified 167 variants in his F5 gene including previously identified and damaging missense mutations at rs6027 and Leu90Ser. Since the administration of fresh-frozen plasma (FFP) prevents gastrointestinal bleeding in this individual, its effects on his plasma- and platelet-derived FV concentrations were assessed. The patient’s plasma FV levels peaked by two hours following FFP administration and were undetectable 96 hours later. In contrast, increased platelet-derived FV/Va concentrations were observed within six hours, peaked at 24 hours, decreased slowly over two weeks, and originated from megakaryocyte endocytosis and intracellular processing of plasma FV. Ten days after transfusion, no thrombin was generated in a tissue factor-initiated whole blood clotting assay unless exogenous FV was added, consistent with the complete absence of plasma FV. In marked contrast, release of the patient’s platelet-derived FV/Va (7% of normal) following platelet activation, resulted in robust thrombin generation, similar to that in an individual with normal plasma-and platelet-derived FV concentrations. Thus, total FV deficiency can be corrected by plasma administration, which partially repletes and sustains the platelet cofactor pool, thereby highlighting the critical role of platelet-derived FV/Va in ensuring hemostatic competence.
INTRODUCTION

Congenital factor (FV) deficiency is a rare autosomal recessive bleeding disorder (prevalence ~1:1,000,000). Individuals heterozygous for this disorder are usually asymptomatic. However, the bleeding phenotype in individuals with undetectable levels of FV antigen and activity (<1%) in their plasma varies dramatically. While the majority of the total FV pool circulates in plasma, ~20-25% is stored in platelet α-granules (4,600-14,000 molecules/platelet). This platelet-derived FV pool originates solely from megakaryocyte endocytosis of the plasma procofactor through a process which results in the formation of a partially proteolytically-activated cofactor (FV/Va), and phenotypically alters it to a more procoagulant phenotype.

The most common treatment for individuals with symptomatic FV deficiency is administration of fresh frozen plasma (FFP) to temporarily maintain plasma FV at minimally hemostatic levels (20-30%). Its effect on platelet-derived FV/Va concentrations is unknown. In the current investigation, an individual with undetectable levels of both plasma- and platelet-derived FV/Va, who receives FFP transfusions to control gastrointestinal (GI) bleeding, was studied.

MATERIALS & METHODS

Patient history: A 67-year old male with congenital FV deficiency (<1% plasma- and platelet-derived FV antigen and activity) was recruited and consented according to a protocol approved by the University of Vermont (UVM) Committee on Human Research. The patient experienced recurrent epistaxis and prolonged bleeding after dental surgery and underwent left hip hemiarthroplasty and right knee total arthroplasty for end-stage arthropathy caused by recurrent
hemarthroses. Both surgeries required subsequent revision. When studied initially (February 2005), the patient was receiving two units of FFP/week to prevent GI bleeding. At follow-up (August 2008 and October 2012), he only required two units of FFP every two weeks.

**Whole genome sequencing:** Whole genome sequencing and subsequent analyses were performed by the UVM Advanced Genome Technologies Core Facility. DNA, isolated from peripheral blood, was sequenced on an Illumina HiSeq 1000 sequencer (average of 25±5 reads). The data were analyzed using the Genome Analysis Toolkit. Variants were evaluated for biological relevance with Polymorphism Phenotyping v2 and Scale-invariant feature transform algorithms.

**Assessment of plasma-derived factor V antigen and activity:** FV antigen was determined by a competitive radioimmunoassay. Plasma-derived FV levels between 0-2 hrs of FFP administration were extrapolated based upon the FV turnover rate in a nonhuman primate model assuming a starting plasma volume of 3,200mL (hematocrit=36%) and a constant transfusion rate (3.75mL plasma/min).

**Western blotting analyses of platelet- and plasma-derived factor V:** Plasma and platelet-derived FV/Va were visualized by western blotting as detailed previously. For quantitative western blotting analyses, platelet lysates were treated with thrombin (2 U/mL, 10 min, 37°C) to convert all platelet-derived FV/Va to FVa. The density of the platelet-derived FVa heavy and light chains were compared to a standard curve prepared from an unaffected control presumed to have ~10,000 molecules FV/platelet.
**Whole blood coagulation:** Tissue factor (TF)-initiated whole blood clotting assays and quantification of serum thrombin-antithrombin complex (TAT) formation were performed as described.\textsuperscript{13} Whole blood clotting reactions contained: 1) TF (5pM) and corn trypsin inhibitor (CTI) (100µg/ml); 2) TF, CTI and FV (2nM); 3) TF, CTI, PARS 1 (100µM) and 4 (500µM); and 4) CTI alone.

**Measurement of tissue factor pathway inhibitor (TFPI):** Plasma TFPI was quantified using Quantikine® Human TFPI Immunoassay (R&D Systems, Minneapolis MN, USA).

**RESULTS & DISCUSSION**

Whole genome sequencing identified 167 variants in the patient’s F5 gene. Two variants, rs6027 (A6755G mutation causing an Asp2194Gly substitution in the FV C2 domain) and L90S [an A to G mutation at chr1:169541563 causing a Leu90Ser (Leu62Ser) substitution in the FV A1 domain], were classified as damaging, having been shown previously to be associated with FV deficiency.\textsuperscript{14,15} The patient is heterozygous at both loci, which may explain his complete absence of FV; however, other variants may play a role.

Following FFP administration, the patient’s plasma FV concentration increased from undetectable (t=0 hrs) to 1.3µg/mL (2 hrs) (Figure 1A, circles), declined rapidly and was undetectable by 96 hours. These data were confirmed by western blotting (Figure 1B). In contrast, quantifiable levels of platelet-derived FV/Va were observed prior to FFP administration (~124 molecules/platelet) [Figure 1A (triangles) and Figure 1C], which presumably represented platelet-derived FV/Va remaining from the previous transfusion. Platelet-derived FV/Va nearly doubled by 6 hrs, peaked at 24 hrs post FFP administration (609 molecules/platelet) (Figure 1A,
triangles), with a substantial amount remaining (434 molecules/platelet) 96 hrs post transfusion (Figure 1A, triangles). A follow-up study confirmed that the rapid acquisition of FV by the patient’s platelets was the result of megakaryocyte and not platelet endocytosis of the plasma molecule (Supplemental Figure 1), and that following endocytosis, the patient’s platelet-derived FV/Va was proteolytically processed normally (Supplemental Figure 2).

The ability of the patient’s platelets and platelet-derived FV/Va to support thrombin generation ten days after FFP administration was assessed in a TF-dependent, contact pathway-suppressed, whole blood clotting assay following platelet activation with PAR1 and PAR4 peptides (Supplemental Figure 3). Simultaneous addition of TF and the agonist peptides had little effect on whole blood clotting and thrombin formation in an unaffected individual (Supplemental Figure 4). While no thrombin was generated in the absence of added FV (Figure 2, closed squares), the simultaneous addition of PAR1 and PAR4 to the patient’s blood resulted in platelet clumping by 3.8 min and clot formation by 7.8 min. Thrombin generation was robust (37.5nM thrombin/min) with a maximum level of thrombin equal to 369.4nM (Figure 2, closed circles). In comparison, FV addition (2nM) to the patient’s blood shortened the clot time (~2.8 min) but effected thrombin generation at a nearly identical rate (40.6nM thrombin/min) and amplitude (360.9nM thrombin) (Figure 2, open circles). When the effects of the agonist peptides on the whole blood clotting profiles of the patient (Figure 2, closed circles) and two unaffected individuals (Figure 2, triangles) were compared, only the durations of the initiation phases were substantially different (~7.8 min vs. ~2.8±0.35 min).

Following its endocytosis by megakaryocytes, FV is re-tailored to form a physically distinct molecule that exhibits an increased procoagulant potential (4-10). Due to its localized release from the platelets’ α-granules at vascular injury sites, platelet-derived FV/Va is the predominant
cofactor in thrombin generation at the platelet surface. Thus, these combined observations suggest that despite a complete absence of a plasma-derived FV and the presence of ~7% normal levels of platelet-derived FV/Va, the persistence of the highly procoagulant cofactor in the patient’s platelets confers hemostatic competence. The importance of platelets and platelet-derived FV/Va in sustaining normal hemostasis is supported by several studies. A patient with a neutralizing inhibitor to plasma- but not platelet-derived FV showed no bleeding tendency following extensive surgical challenge. In contrast, individuals with platelet-derived FV/Va inhibitors exhibit severe GI bleeding. Other reports describe the success of platelet transfusions in the cessation of severe bleeding resulting from FV deficiency or FV inhibitors. In a recent study, Duckers et al. described three individuals with severe plasma-derived FV/Va deficiency (<1% activity) but expression of detectable platelet-derived FV/Va antigen and activity (1.7–6.4%) who exhibited only a mild bleeding diathesis. The authors speculate that this residual platelet-derived FV/Va coupled with the decreased TFPI levels observed in these individuals allows for sufficient thrombin generation to prevent fatal bleeding. Indeed, our patient’s plasma TFPIα level (5.9±0.65 ng/mL) was dramatically lower than that observed in a normal plasma pool (13.0±0.95 ng/mL) consistent with previous observations made in FV-deficient individuals.
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AUTHORSHIP

B.A.B. performed research, analyzed data and prepared the manuscript. J.C., K.E.B.Z. and P.D. performed research, analyzed data, and critically reviewed the manuscript. N.S.K. oversaw the participation and clinical management of the patient and critically reviewed the manuscript. P.B.T. conceived of the research, analyzed data, and critically reviewed the manuscript. The authors disclose no competing conflicts of interest.
**LITERATURE CITED**


FIGURE LEGENDS

Figure 1. Quantification of plasma- and platelet-derived FV levels in a FV deficient patient prior to and subsequent to administration of FFP. (A) Plasma-derived FV antigen and activity was measured prior to (time = 0 hrs) and subsequent to FFP administration, at the times indicated, using a double antibody competitive radioimmunoassay (circles). The FV concentrations between 0 and 2 hrs (circles, dashed line) were extrapolated based upon the human FV turnover rate in a nonhuman primate model as described in Materials & Methods. Platelet-derived FV (triangles) was measured using a quantitative western blot described in Materials & Methods using washed platelets lysed with triton X-100 in the presence of leupeptin. Platelet lysates were treated with thrombin (2 U/mL, 10 min, 37 °C) to convert all platelet-derived FV and its partial activation products to FVa. Following SDS-PAGE, immunoblotting was performed using a mixture of an anti-FV heavy chain and an anti-FV light chain monoclonal antibody. (B) FV in plasma was immunoblotted prior to to (0 hrs) and subsequent to FFP administration (6, 24, 96, and 168 hrs) as described above. The position of single chain FV (SC) is indicated. C = plasma from an unaffected individual. (C) Platelet-derived FV was immunoblotted in whole platelet lysates following its conversion to FVa prior to (0 hrs) and subsequent to FFP administration (6, 24, and 96 hrs), as described above. The positions of the FVa heavy chain (HC) and light chain (LC) are indicated.
Figure 2. The patient’s platelet-derived FV pool remaining 2 weeks after plasma administration supports thrombin generation in a TF-initiated whole blood clotting model. Whole blood from the patient was incubated (at 37°C with rocking) with TF (5pM) alone (closed squares), TF (5 pM) plus PARS 1 (100 μM) and 4 (500 μM) agonist peptides (closed circles), or TF (5 pM) plus FV (2nM) (open circles). TAT formation was measured by ELISA as described in Materials & Methods. For comparison, TAT formation in the presence of PAR agonist peptides was also assessed in two unaffected individuals assayed in duplicate (mean ± SD) (open triangles).
Figure 1

A

Graph showing the plasma factor V (μg/mL) over time (hrs) and the number of molecules per platelet. The graph includes data points at various time intervals from 0 to 1000 hours.

B

Table showing hours following FFP administration:

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C

Images showing Western blot analysis with time points at 0, 6, 24, and 96 hours following FFP administration, indicating SC, HC, and LC bands.
Platelets and platelet-derived factor Va confer hemostatic competence in complete factor V deficiency

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