Endogenous factor V synthesis in megakaryocytes contributes negligibly to the platelet factor V pool

M. Christella L.G.D. Thomassen, Elisabetta Castoldi, Guido Tans, Elke J.P. Magdeleyns, Christian Delaunoit, Louisette Debusscher, Kris J.A.J. Van Assche, Jan Rosing

Background and Objectives. Coagulation factor V (FV) is distributed between two pools: 80% circulates in plasma and 20% is stored in platelets. The aim of the study was to determine the origin of platelet FV.

Design and Methods. We investigated a FV Leiden heterozygous patient who had received an allogeneic bone marrow transplant from a normal donor. The patient had been referred to our laboratory for his marked activated protein C (APC) resistance in the apparent absence of FV Leiden. Analysis of the DNA from a buccal swab showed that the patient was indeed a heterozygous carrier of FV Leiden. The difference in FV genotype between the hepatocytes (heterozygous FV Leiden) and the blood cells (homozygous normal) of the patient provided a good model to investigate the origin of platelet FV. Platelets were isolated from the patient and the bone marrow donor and activated with thrombin and ionomycin to release and activate FV. APC was then added and the inactivation of platelet FVa was followed over time with a highly sensitive prothrombinase-based assay.

Results. While the donor's platelet FVa showed a normal inactivation time course, the patient's platelet FVa was considerably resistant to APC. The kinetic pattern of APC-catalyzed inactivation of the patient's platelet FVa was indistinguishable from that of plasma FVa from a FV Leiden heterozygote.

Interpretation and Conclusions. These data indicate that platelet FV is derived from plasma and that endogenous FV synthesis by megakaryocytes contributes negligibly to the platelet FV pool.

Key words: platelet factor V, factor V Leiden, bone marrow transplantation, megakaryocyte.

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From the Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands (MCLGDT, EC, GT, EJPM, IR) The Centre Hospitalier Universitaire de Tivoli, La Louvière, Belgium (CD, LD), Instrumentation Laboratory, Breda, The Netherlands (KJAJVA).

Correspondence: Dr. Guido Tans, Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands. E-mail: g.tans@bioch.unimaas.nl

Coagulation factor V (FV)[1] is a large multi-domain glycoprotein synthesized in the liver and released into the bloodstream as an inactive pro-cofactor. After limited proteolysis by factor Xa (FXa) or thrombin, FV is converted to the active co-factor FVa, which greatly enhances FXa-catalyzed prothrombin activation. Under physiologic conditions FVa activity is down-regulated by the anticoagulant protein, activated protein C (APC), which cleaves FVa at positions 306, 506 and 679. A natural FV mutant lacking the APC-cleavage site at Arg^{506} (R506Q, also known as FV Leiden)[2] is relatively common in Caucasians, having a prevalence of 3-15% in the European population. *In vitro* studies have demonstrated that FVa Leiden is less efficiently inactivated by APC.[3,4] This results in a plasma phenotype known as APC resistance, which is characterized by a relative insensitivity of plasma to the anticoagulant action of APC.[5] Since APC resistance represents a major risk factor for venous thrombosis,[6-8] several functional assays have been developed for its diagnosis.

Plasma FV accounts for ~80% of total FV present in blood, the remaining ~20% resides in platelets.[9] Platelet FV is stored in α-granules[10] in a non-covalent complex with multimerin.[11] It is released upon platelet activation and is believed to play a crucial role in the early phase of the coagulation process. Accumulation of FV starts already in bone marrow megakaryocytes,[12,13] but the origin of platelet FV is still a matter of debate. Although studies on isolated and cultured megakaryocytes have provided evidence for both endogenous FV synthesis[13-16] and secondary endocytosis of plasma FV,[13] it is not clear in what proportions these processes contribute to the platelet FV pool. Western blot analysis of the APC-mediated inactivation of platelet FVa in two FV Leiden heterozygous patients, one who had received a liver transplant and the other who had received bone marrow from wild-type donors, has lent support to the plasma origin of most of the platelet FV pool.[17] However, since Western blot does not lend itself well to quantitative analysis, it was argued that the contribution of endogenous FV synthesis might have been underestimated.[18]

In the present investigation we have used the same model (allogeneic bone marrow transplantation) but a complementary and more quantitative approach to the functional characterization of FV present in platelets, i.e. detailed kinetic analysis of APC-catalyzed inactivation of platelet FVa.

Design and Methods

The chromogenic substrates D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were from Chromogenix, Mölndal, Sweden (supplied by Nodia, Amsterdam, The Netherlands). Tissue factor (Dade Innovin) was purchased from Behring, Germany. Phospholipids were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. Small, unilamellar phospholipid vesicles, composed of a mixture of 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) (10/90 M/M), were prepared as described elsewhere.[19] Activated protein C (Enzyme Research Laboratories) and human FXa were purchased from Kordia Laboratory Supplies, Leiden, The Netherlands. The APC concentration was determined according to Sala.[20] Human prothrombin was purified as described by Di Scipio *et al.*[21] All proteins used in the APC resistance test were diluted in buffer I (25 mM Hepes pH 7.5, 175 mM NaCl and 5 mg/mL bovine serum albumin (BSA). Stopping buffer (buffer II) contained 50 mM Tris HCl pH 7.9, 175 mM NaCl, 0.5 mg/mL ovalbumin and 20 mM EDTA. Platelets were washed and resuspended in platelet buffer containing 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.2% glucose and 2 mg/mL BSA, pH 6.6 during isolation and pH 7.5 in the final re-suspension step. Acid-citrate dextrose (ACD), the anticoagulant used for platelet experiments, contained 80 mM trisodium citrate, 52 mM citric acid and 183 mM glucose; 1 mM H-Arg-Gly-Asp-Ser-OH (RGDS, Bachem AG, Bubendorf, Switzerland) was added to prevent platelet aggregation. To activate the platelets, 7 nM human thrombin was added, in combination (when appropriate) with 2.6 μM ionomycin added from a stock solution dissolved in dimethyl sulfoxide (DMSO, Calbiochem Hoechst, San Diego, CA, USA).

Case history

The patient, who is presently a 30-year-old man, was admitted to Tivoli Hospital in La Louvière (Belgium) in September 1988, at the age of fifteen years, because of aggressive acute lymphoblastic leukemia. In 1989, after chemotherapy, he received a bone marrow transplantation from an HLA-compatible sister. In October 2000 he experienced two brief neurological episodes consisting of visual disturbance, speech difficulties and hand weakness. The clinical and radiological evaluation was entirely negative. Laboratory coagulation tests were all normal, except for the presence of APC resistance (APC sensitivity ratio 1.65, normal range 1.90-2.74) determined with the Coatest® APC™ Resistance V kit (Chromogenix, Mölndal, Sweden). However, the FV Leiden mutation could not be detected by DNA analysis. Since the patient's plasma showed no other coagulation abnormalities that could account for the observed APC resistance, a sample of the patient's blood was sent to our center for further investigations. At this stage we were not informed that the patient had undergone an allogeneic bone marrow transplantation.

Blood collection and plasma preparation

Blood was collected from the patient and his sister (the bone marrow donor), after informed consent, at Tivoli Hospital in La Louvière by venipuncture with an open system. For experiments in plasma and for DNA analysis 9 mL blood were drawn in 1 mL 0.13 M trisodium citrate (pH 7.8), while for platelet experiments 45 mL blood were drawn in 7.5 mL ACD. Platelet-poor plasma preparation and platelet isolation were started approximately 2.5 hours after blood collection. Platelet-poor plasma (PPP) was prepared by double centrifugation of the citrated blood for 15 minutes at 3000 g at room temperature and frozen at –80 °C.

In addition to blood, mouth mucosal cells were collected with a swab from both the patient and his sister. The patient's buccal swab was used as a source of his own genomic DNA.

Prothrombinase-based test for the detection of FV Leiden

The prothrombinase-based test for the diagnosis of carriership of the FV Leiden mutation[22,23] relies on the different patterns of APC-mediated inactivation of normal FVa and FVa Leiden in highly diluted plasma samples. Briefly, PPP was diluted 1000-fold in buffer I with 3 mM $CaCl_2$, and FV was activated with 5 nM thrombin in the presence of 20 mM phospholipid vesicles (DOPS/DOPC 10/90 M/M) during 12 minutes at 37°C. After complete activation of FV, 0.2 nM APC was added to the reaction mixture and FVa inactivation was followed over time. In order to do this, aliquots were drawn from the FVa inactivation mixture at regular time points and transferred to a prothrombinase mixture containing 1 μM prothrombin and 0.3 nM human FXa (final concentrations). The prothrombinase reaction was stopped after 1 minute by the addition of cold buffer II containing 20 mM EDTA. The amount of thrombin formed was measured with the chromogenic substrate S2238. The FVa concentration in the plasma sample was calculated from the amount of thrombin generated using a calibration curve made with known amounts of purified FVa. Residual FVa activity was expressed as a percentage of the FVa activity present before the addition of APC.

Platelet isolation

ACD blood was centrifuged for 15 minutes at 250 g. Platelet-rich plasma (PRP) was collected and centrifuged for 5 minutes at 160 g to remove residual

erythrocytes. The PRP was then transferred to a new tube and, after the addition of 1 mL ACD, centrifuged again for 15 minutes at 500 g. The platelet pellet was resuspended in 20 mL platelet buffer pH 6.6 and 1 mL ACD was added. This platelet suspension was centrifuged again for 15 minutes at 500 g and the platelet pellet was resuspended in 15 mL platelet buffer pH 6.6 with 750 μL ACD added. The final centrifugation step was 15 minutes at 500 g, after which the platelet pellet was resuspended in 3.5 mL platelet buffer pH 7.5 and the platelet concentration was measured on a Microdiff 18 Coulter counter (Beckman Coulter Nederland BV, Mijdrecht, The Netherlands). RGDS was added to a final concentration of 1 mM to prevent platelet aggregation. All steps were performed at room temperature.

Platelet activation

Washed platelets (2×10^8 platelets/mL) were activated for 15 minutes at 37 °C in the presence of 7 nM thrombin, 2.5 mM $CaCl_2$ and 2.6 μM ionomycin under continuous stirring. After complete platelet activation, part of this platelet mixture was diluted 50 times in buffer I (with 3 mM $CaCl_2$ added) and further incubated at 37°C for 12 minutes with 5 nM thrombin and 20 μM phospholipids (DOPS/DOPC 10/90 M/M) to ensure full activation of platelet FV. Then 0.2 nM APC was added and FVa inactivation was followed over time as described above.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Buccal swabs were soaked in 200 μL PBS and stirred vigorously to release the cells. The cell suspension was digested with proteinase K for 1.5 hours and genomic DNA was subsequently isolated by a standard procedure. Carriership of the FV Leiden mutation was checked by amplification of FV exon 10 (32 cycles) followed by both direct sequencing and Mnl I-restriction analysis. Moreover, all FV gene exons and splicing junctions were PCR-amplified and sequenced. The list of primers and amplification conditions used for this purpose is freely available on request (*e.castoldi@bioch.unimaas.nl*).

Results

Functional studies in plasma

The plasma of the patient had an APC sensitivity ratio of 1.65 in the aPTT-based APC resistance test performed after 1:5 dilution of the plasma in FV-deficient plasma. This result indicated an APC resistance comparable to that of FV Leiden heterozygotes and attributable to a functional abnormality of FV. In order to find out whether this abnormality was indeed the FV Leiden mutation, we subjected the patient's plasma to a functional test for carriership of the FV Leiden mutation.[22] As shown in Figure 1, this assay can readily discriminate normal, FV Leiden heterozygous and FV Leiden homozygous individuals on the basis of the APC-mediated inactivation pattern of their respective FVa. When the patient's plasma FV was analyzed, the time-course of APC-mediated FVa inactivation was indistinguishable from that of a FV Leiden heterozygous control (Figure 1). In contrast, the FV present in the plasma of the patient's sister yielded a FVa inactivation time-course identical to that of a normal control (Figure 1).

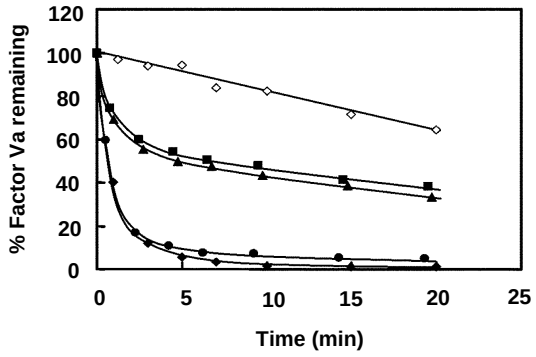

Figure 1. APC-catalyzed inactivation of plasma FVa on synthetic phopholipids. Plasma, 1000-fold, diluted was incubated with 5 nM thrombin, 3 mM calcium and 20 μM phospholipid vescicles (DOPS/DOPC 10/90 M/M) in order to activate FV completely. After 12 min incubation at 37°C, 0.2 nM APC was added and the loss of FVa activity was followed over time. FVa activity was quantified with a prothrombinase-based assay performed with 1 μM prothrombin and a low amount of FXa (0.3 nM) as described in the Methods section. The residual FVa activity was expressed as a percentage of the FVa activity present before APC addition. (■) patient; (●) patient's sister (bone marrow donor); (◆) normal control; (▲) FV Leiden heterozygous control; (◆) FV Leiden homozygous control.

Genetic studies

Before being referred to us, the patient had already been tested and found negative for the FV Leiden mutation at DNA level. However, we repeated the DNA analysis to exclude the possibility of a sample swap. After extraction of genomic DNA from peripheral blood leukocytes, the patient's FV exon 10 was amplified, along with those of a normal and a FV Leiden heterozygous control. Both direct sequencing (Figure 2A) and Mnl I-restriction analysis (Figure 2B, lane 3) of the PCR product confirmed the absence of FV Leiden in the patient. Since the functional assays pointed to the patient's FV as the cause of the APC resistance, all FV gene exons and splicing junctions were amplified

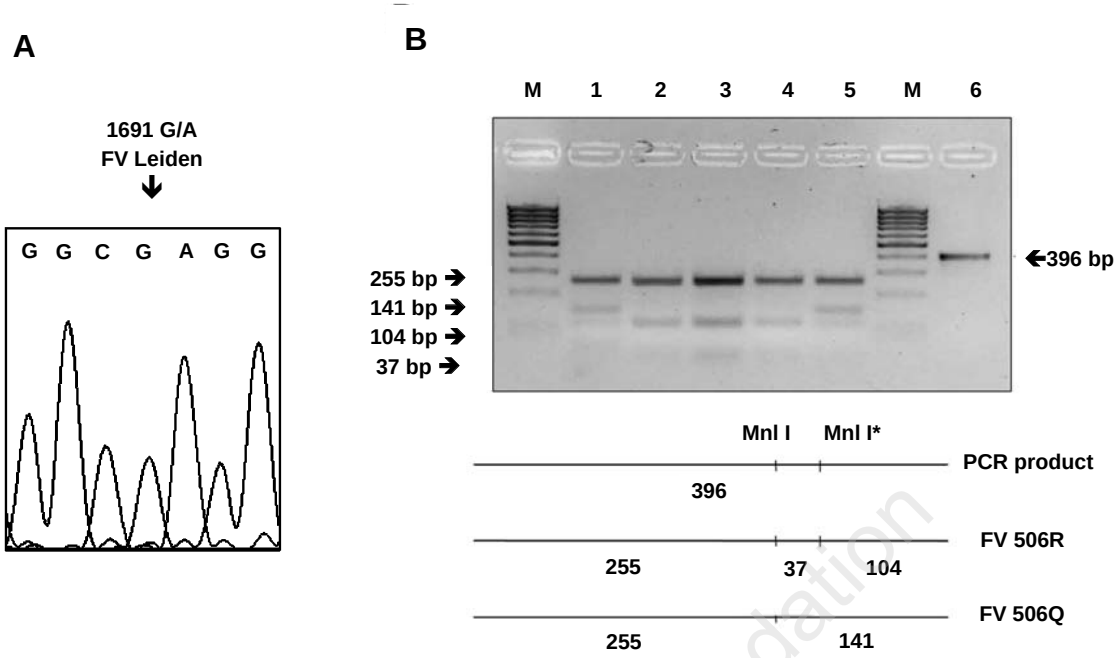

Figure 2. DNA analysis. A) Sequencing plot of exon 10 of the FV gene amplified from the DNA extracted from the patient's peripheral blood leukocytes. Only the G (normal) allele is visible at position 1691. B) Mnl I-restriction analysis of exon 10 of the FV gene amplified from the DNA extracted from: the patient's mouth mucosa (lane 1), the bone marrow donor's mouth mucosa (lane 2), the patient's leukocytes (lane 3), a normal control (lane 4) and a FV Leiden heterozygous control (lane 5). M, molecular weight marker; lane 6, undigested PCR product. The numbers in the restriction map below the gel indicate the size of the DNA fragments in base pairs.

and sequenced to identify the mutation responsible. Several neutral (non-functional) polymorphisms were detected, but no new mutation was found.

When we were informed that the patient had undergone an allogeneic bone marrow transplantation, we realized that the leukocyte-derived DNA that we had analyzed was not the patient's own genetic material but actually that of the donor. This could explain the genotype/phenotype discrepancy, provided that the patient was a carrier of FV Leiden and his donor sister was not. To verify this, mouth swabs were taken from both the patient and his sister and used as a source of genomic DNA for PCR amplification of FV exon 10. Subsequent digestion of the PCR products with Mnl I showed that the patient indeed carried the mutation while his sister did not (Figure 2B, lanes 1-2).

Functional studies of platelet FV

The difference in FV genotype between the patient's hepatocytes (heterozygous FV Leiden) and hematopoietic cells (homozygous normal) offered us the opportunity to investigate the origin of platelet FV by studying the kinetic pattern of APC-mediated inactivation of platelet FVa. If platelet FVa showed a normal susceptibility to APC, it would be synthesized endogenously by megakaryocytes, whereas resistance to APC-mediated inactivation would be indicative of secondary internalization from the plasma pool. An intermediate phenotype would indicate a contribution of both mechanisms. The patient's sister who had donated the bone marrow served as a normal control for this analysis.

Platelets were isolated from blood from the patient and from his sister and resuspended in platelet buffer at a concentration of 2×10^8 platelets/mL. Following incubation with a mixture of thrombin, calcium and the synthetic calcium agonist ionomycin, 2.0 and 2.7 nM FVa were released from the platelets of the patient and his sister, respectively. Subsequently we determined the time-courses of APC-catalyzed inactivation of platelet FVa from both the patient and his sister. In order to minimize interference by other proteins (e.g. protein S present in α-granules)[24] and platelet debris, activated platelets were diluted 50-fold in buffer I with 3 mM $CaCl_2$. To ensure full activation of platelet FV, the diluted platelet suspension was incubated with thrombin and phospholipid vesicles for 12 more minutes, after which APC was added and FVa inactivation was followed over time as described in the Methods section. Figure 3 shows that the sister's platelet FVa lost most of its

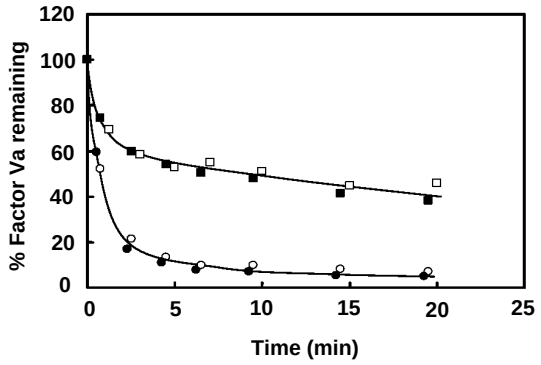

Figure 3. Comparison of plasma and platelet FVa inactivation by APC on synthetic phospholipid vesicles. Platelets were activated in the presence of 7 nM thrombin, 2.5 mM calcium and 2.6 mM ionomycin for 15 minutes at 37°C, to release and activate FV. Activated platelets were then diluted 50 times in buffer I (3 mM $CaCl_2$) and 5 nM thrombin and 20 mM phospholipids were added to activate FV completely. After the addition of 0.2 nM APC, loss of FVa activity was followed over time using a prothrombinase-based assay performed with 1 μM prothrombin and a low amount of FXa (0.3 nM). The residual FVa activity was expressed as a percentage of the FVa activity present before APC addition. (□) patient, (○) patient's sister (bone marrow donor). Closed symbols represent the data obtained for plasma FVa as described and shown in Figure 1.

co-factor activity within 5 minutes, whereas the patient's FVa was inactivated considerably more slowly and still retained approximately 40% of its co-factor activity after 20 minutes. In the absence of APC, platelet FVa activity was stable throughout the time period of the experiment. The same results were obtained when the experiment was repeated at lower (1×10^8 platelets/mL) or higher (4×10^8 platelets/mL) platelet concentrations, and when platelets were activated with thrombin and $CaCl_2$ in the absence of ionomycin (data not shown). Moreover, since the time courses of inactivation of platelet FVa inactivation were virtually indistinguishable from those obtained with the corresponding plasma FVa, both for the patient and for the sister (Figure 3), it could be excluded that megakaryocyte FV synthesis contributed to the platelet FV pool.

Discussion

Comparison of genetic and functional tests, which are routinely used to diagnose carriership of the FV Leiden mutation and APC resistance, occasionally leads to the identification of genotype/phenotype discrepancies. An APC resistance phenotype not associated with FV Leiden may be attributable to genetic and acquired conditions that cause APC resistance, such as other FV gene mutations, altered levels of plasma proteins, lupus erythematosus, pregnancy or the use of female hormones.[25] Homozygosity for FV Leiden without APC resistance, due to co-segregation of a FV null mutation preventing the expression of both FV Leiden alleles, has also been reported.[26] Moreover, several cases of false positivity or false negativity for FV Leiden following allogeneic liver[17,27-32] or bone marrow/stem cell transplantation[17,32-34] have been described over the past few years. Since genotyping for the FV Leiden mutation is normally performed on blood cells (produced by bone marrow progenitors), while the plasma phenotype depends on circulating FV (synthesized in the liver), bone marrow or liver transplantation between individuals with different FV Leiden genotypes will result in a genotype/phenotype discrepancy. Therefore, as transplantation practices become more widespread, additional care is warranted in the evaluation of genetic and functional tests for APC resistance.

Patients who have received a bone marrow or a liver transplant from a donor with a different FV Leiden genotype provide an excellent model for studying the origin of platelet FV. Although it has been convincingly shown that human megakaryocytes contain FV mRNA[15,16] and actually synthesize FV,[13,16] it is not clear to what extent endogenous biosynthesis contributes to the platelet FV pool *in vivo*. Camire *et al.*[17] addressed this issue by analyzing two FV Leiden heterozygous patients, one of whom had received a bone marrow transplant and the other a liver transplant from donors with a normal genotype. In both patients, APC-mediated inactivation of platelet FVa post-transplantation, as monitored by Western blotting, showed a pattern consistent with the respective liver cell genotype, providing strong evidence for the plasma origin of most platelet FV. Although it was claimed that the Western blot technique used was sufficiently quantitative to warrant this conclusion,[35] concerns were raised that the contribution of endogenous FV synthesis might have been underestimated.[18] In the present study, we investigated a FV Leiden heterozygous patient who had received a bone marrow transplant from a normal sister. Bone marrow chimerism was excluded by PCR (32 cycles), which showed that only the donor's normal genotype was represented in the peripheral leukocytes of the patient (Figure 2). APC-mediated inactivation of platelet FVa was followed by means of a prothrombinase-based assay that yields clearly distinct patterns for normal, FV Leiden heterozygous and FV Leiden homozygous individuals and that can detect FV concentrations as low as 0.2 pM.[22] The high-resolution power and sensitivity of this test make it ideal for detecting contributions of the endogenously synthesized normal FV to the typically heterozygous inactivation pattern of the patient's platelet FVa.

Since the patient's plasma and platelet FVa showed superimposable time-courses of APC-mediated FVa inactivation (Figure 3), our experiment demonstrates that FV synthesis in megakaryocytes contributes negligibly to the (functional) platelet FV pool. In accordance with Camire *et al.*[17] and a recent congress abstract,[36] we therefore conclude that platelet FV derives from secondary internalization of plasma FV by bone marrow megakaryocytes.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MCLGDT performed the functional characterization of the plasma and platelet FV and wrote the paper; EC carried out the DNA analyses and helped to write the paper; GT was responsible for the conceptual design of the work andinterpretation of the data; EJPM participated in the functional studies; CD and LD recruited the patient and collected the clinical history; KJAJVA provided precious technical advice; JR supervised the whole work and gave final approval of the manuscript. All authors contributed critically to the content of the article. Author taking primary responsibility for the paper: GT; primary responsibility for Figures 1 and 3: MCLGDT; primary responsibility for Figure 2: EC. EC is the recipient of a grant from the European Molecular Biology Organisation (EMBO).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Valerio de Stefano, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor De Stefano and the Editors. Manuscript received July 30, 2003; accepted August 8, 2003.

In the following paragraphs, Professor De Stefano summarizes the peer-review process and its outcomes.

What is already known on this topic

Factor V is synthesized by the liver. The origin of the factor V stored in platelets is debated, since the contribution of endogenous synthesis to the platelet factor V pool is not clear. Allogenic bone marrow transplantation to an individual heterozygous for factor V Leiden from a donor with a normal factor V genotype provided a good and simple model to investigate this issue.

What this study adds

An accurate functional investigation of the factor V of the recipient showed that all the platelet factor V was APC-resistant like the factor V obtained from plasma. Thus the platelet factor V pool was proven to derive from plasma, with no significant contribution from endogenous synthesis by the megakaryocytes originating from the normal donor.