Influence of Factor V HR2 on Thrombin Generation and Clinical Manifestation in Rare Bleeding Disorders

Rüdiger F. Strey^a Annelie Siegemund^b Thomas Siegemund^b
Christine Schubert^c Gudrun Schuster^a Karin Wulff^a Falko H. Herrmann^a

^aDepartment of Human Genetics, EMA University of Greifswald, Greifswald, ^bDepartment of Clinical Biochemistry and Pathobiochemistry, University of Leipzig, Leipzig, and ^cKlinikum Erfurt GmbH, Erfurt, Germany

Key Words

Factor V HR2 haplotype · Endogenous thrombin potential · Thrombosis

Abstract

In this study we investigated the influence of the presence of the factor V HR2 haplotype, defined by the factor V gene mutation H1299R (FV_{HR2}), on thrombin generation. Measurements were performed in platelet-poor plasma of individuals with factor V_{HR2} or factor V_{Leiden} in comparison to a control group carrying none of these mutations. Coagulation was triggered by low concentrations of recombinant tissue factor in the presence of activated protein C. Thrombin generation was monitored by a fluorogenic substrate. The endogenous thrombin potential was calculated from the obtained curves. As a result we observed an increased thrombin generation both for individuals heterozygous and homozygous for FV_{HR2}. The level of endogenous thrombin potential is in the same range as in samples of patients heterozygous or homozygous for FV_{Leiden}. The results indicate that FV_{HR2} plays a role as a risk factor for venous thrombosis in homozygous patients through an increased thrombin

generation. The association between different clinical manifestations in individuals with FVII deficiency and endogenous thrombin potential and the presence of FV_{HR2} was studied.

Copyright © 2005 S. Karger AG, Basel

Introduction

Activated protein C (APC) resistance caused by the point mutation FV_{R506Q} (FV_{Leiden}) is the most important genetic cause of venous thrombosis [1–8]. Several other mutations of the factor V gene (M385T, H1299R, M1736V and D2194G) define the factor V HR2 haplotype (FV_{HR2}) which is associated with a mild APC resistance, but an association with an increased risk of thrombosis is not definitely clarified [9].

An additional clinical effect of APC resistance of FV_{Leiden} consists of a moderation in the clinical expression in individuals with severe bleeding disorders. However, this effect has been studied by several groups with inconsistent results [10].

The clinical effect of FV_{HR2} may be explained by two different mechanisms. The first consists in an impaired

APC cofactor activity of factor V resulting in a reduced degradation of factor VIIIa [11]. An alternative mechanism is based on a changed ratio of the isoforms FV₁ and FV₂ in plasma.

In this article we present indications for an association between increased endogenous thrombin potential (ETP) values and the expression of FV_{Leiden} as well as FV_{HR2}. The association is studied between different clinical manifestations in individuals with FVII deficiency on the one hand and ETP and the presence of FV_{HR2} on the other hand.

Patients and Methods

Citrated plasma samples from 8 control subjects without FV_{Leiden} or FV_{HR2} (factor V wild-type; FV_{wt}) were used as a reference. We used the mutation H1299R as marker for FV_{HR2} [13, 14]. The FV_{Leiden} mutation has been determined according to Bertina et al. [5].

The samples with FV_{Leiden} or FV_{HR2} were collected from 7 patients with thrombosis and 7 persons without thrombotic events. The FV mutations of subjects without thrombosis were identified according to corresponding family history. Plasma concentrations of fibrinogen, antithrombin III, homocysteine, protein C, protein S and coagulation factor XII were within the normal range and lupus anticoagulant testing was negative in all study subjects. Blood samples from patients were collected more than 6 months after the thrombotic event, and in the case of patients on oral anticoagulation (phenprocoumon), the measurements were repeated 4 and 6 weeks after the treatment had been stopped.

Plasma samples of 4 FVII-deficient patients of the Greifswald Factor VII Deficiency Study [15], who are homozygous for the same FVII gene mutation A294V [16], were also included in the present study. Two of these patients had spontaneous bleeding symptoms, such as epistaxis, gum bleeding, hemarthrosis or menorrhagia, and the other 2 patients had never had spontaneous bleeding symptoms in relation to the FVII deficiency (further referred to as 'asymptomatic' subjects). No correlation was found between FVII activity and clinical manifestation in homozygous patients for the identical mutation A294V. There was no significant difference ($p = 0.47$) between FVII:C values of asymptomatic patients (13 ± 7) and symptomatic patients (10 ± 9).

All samples, including the pooled normal plasma, were centrifuged for 10 min at 4,000 *g* and the obtained platelet-poor plasma was stored at -70°C until analysis.

Informed consent was obtained from all patients and donors of reference samples.

Measurement of Thrombin Generation in Platelet-Poor Plasma

The measurements were performed in a final volume of 75 μl containing 50 μl sample, 0.6 ng/ml recombinant tissue factor (2.5 μl $0.1 \times$ Innovin[®], Dade Behring, Marburg, Germany), 2.7 $\mu\text{g/ml}$ activated protein C (1 μl $0.1 \times$ Xigris[®], Lilly & Co., Giesen, Germany), 0.8 ng/ml phospholipids, isotonic saline, CaCl₂, Tris buffer and fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Heidelberg, Germany) [17, 18]. Calibration was per-

formed without recombinant tissue factor but with fondaparinux (Arixtra[®], Sanofi-Synthelabo, France) as a coagulation inhibitor and a dilution series of up to 20 nM thrombin. The initial conversion rate of the calibration measurements is linearly correlated to the initial thrombin concentration.

Fluorescence intensity was detected using fluorimetric detection (Fluoroscan Ascent, Labsystem Helsinki, Finland) at wavelengths of 340 nm (excitation) and 440 nm (emission). The measurements lasted 240 min with a cycle time of 1 min. All measurements were repeated three times.

Mathematical Computation

In plasma, α_2 -macroglobulin ($\alpha_2\text{M}$) builds a complex with free thrombin (IIa) that converts the fluorogenic substrate, but has no known clotting activity. Thus the influence of the complex $\alpha_2\text{M} \cdot \text{IIa}$ to the signal has to be subtracted by mathematical processing to obtain the thrombin curve. Assuming the formation of $\alpha_2\text{M} \cdot \text{IIa}$ to be a pseudo 1st-order process [19] leads to:

$$\frac{\partial}{\partial t} [\alpha_2 \text{M} \cdot \text{IIa}] = k_n \cdot [\text{IIa}]$$

With a 1st-order approximation of the conversion of the substrate with the same rate constant for IIa and $\alpha_2\text{M} \cdot \text{IIa}$ [19] and a linear correlation between the concentration of the converted fluorogenic substrate and the obtained signal (which was given within the measurement range used), the signal f is characterized by the differential time equation:

$$\frac{\partial}{\partial t} f = k_s \cdot ([\text{FIIa}] + k_n \int [\text{FIIa}] dt + A_0) \cdot (f_{\infty} - f)$$

The maximum signal f_{∞} (for complete conversion of the fluorogenic substrate), the rate constant k_s for the conversion of the substrate by IIa or $\alpha_2\text{M} \cdot \text{IIa}$ as well as the background activity A_0 (mainly caused by APC) were determined by calibration. With the starting values $f_0 = 0$, $[\alpha_2\text{M} \cdot \text{IIa}]_0 = 0$ and $[\text{IIa}]_0 = 0$ as well as the end condition $[\text{IIa}]_{t \rightarrow \infty} = 0$, the rate constant k_n may be obtained by a suitable nonlinear optimization method. We used a modified Marquardt-Levenberg algorithm.

In this article the average ETP values of the 8 references without any risk factors for thrombosis were set to 100%. Thus ETP values of <100% represent reduced thrombin generation and ETP values of >100% represent increased thrombin generation compared to the references in our measurements.

Statistical analysis was performed by paired *t* test.

Results

ETP in Samples of FV_{Leiden} or FV_{HR2} Subjects

In our measurements without addition of APC, the ETP values of samples with FV_{wt}, FV_{HR2} or FV_{Leiden} did not show any significant differences by detection. This is in accordance with results reported by other authors [11, 20]. In the presence of APC, a reduction in thrombin generation by an average of 46% was observed for reference plasma (wild-type for both FV mutations) compared with measurements without addition of APC. In our assay

Fig. 1. Average ETP values in the presence of high APC concentrations for references (8 samples), heterozygous FV_{HR2} (n = 4), homozygous FV_{HR2} (n = 3), heterozygous FV_{Leiden} (n = 4) and homozygous FV_{Leiden} (n = 3) individuals.

Fig. 2. Effect of oral anticoagulation on ETP in the presence of APC in patients homozygous for FV_{HR2}. Average ETP values of samples from 2 patients collected during and after oral anticoagulation (phenprocumon) compared to the average reference values obtained from 8 persons with FV wild-type for both FV mutations (fig. 1).

with APC, obvious differences appear ($p < 0.05$) between reference samples and samples from subjects with FV_{Leiden} or FV_{HR2} mutations (fig. 1).

In samples with the FV_{Leiden} and FV_{HR2} mutations, thrombin generation is less reduced for both homozygous and heterozygous entities by addition of APC compared to the references. This means that in the presence of APC, FV_{Leiden} as well as FV_{HR2} lead to increased ETP values compared to the references.

These data indicate a dysfunction in the regulation of the coagulation system which results from the presence of FV_{Leiden} or FV_{HR2}. The diminished regulation causes a higher thrombin generation compared to the references without these mutations.

No influence of a history of thrombosis on ETP was observed.

In the patients homozygous for FV_{HR2} the thrombin generation in the corresponding plasma samples was below the detection limit during oral anticoagulation (phenprocumon). Four weeks after termination of medication, the ETP values remained below the reference value. On repeat testing 4 and 6 weeks after oral anticoagulation had been stopped, ETP markedly increased above the reference value (fig. 2).

The difference between the ETP values 4 and 6 weeks after treatment had been stopped indicates the sensitivity of this method to low concentrations of vitamin K antagonists.

Fig. 3. Average ETP values of samples from patients homozygous for FVII_{A294V} with bleeding symptoms (2 samples) and without bleeding symptoms (2 samples) compared to the average reference values obtained from 8 persons with FV wild-type for both FV mutations (fig. 1).

ETP in FVII-Deficient Patients Homozygous for Mutation FVII_{A294V}

For the samples of patients homozygous for the mutation FVII_{A294V} the ETP measurements in the presence of APC resulted in heterogeneous data despite the same FVII genotype. However, a very good correlation was observed between ETP on the one hand and the presence of FV_{HR2} and bleeding symptoms on the other (fig. 3). ETP

in patients with bleeding symptoms (epistaxis, gum bleeding, menorrhagia or hemarthrosis) was markedly below the reference values. None of these patients carried FV_{HR2} or FV_{Leiden}. The ETP values of the asymptomatic cases were increased compared to the reference values. These asymptomatic persons were heterozygous or homozygous for FV_{HR2} and did not carry FV_{Leiden}.

Discussion

FV_{HR2} Increases ETP with APC-Like FV_{Leiden}

The endogenous thrombin potential is a sensitive indicator of hyper- and hypocoagulability [21, 22]. APC resistance as demonstrated by ETP is associated with an increased risk for venous thromboembolism [23]. An association between the APC resistance of FV_{Leiden} and an increased risk of venous thrombosis has already been published by other authors [1–3].

The present study shows an increased thrombin generation in the presence of APC both for FV_{Leiden} and for FV_{HR2} compared to the references. The ETP values are correlated to the presence of FV_{HR2} and likewise to the presence of FV_{Leiden}. Our measurements show an increased ETP in 3 subjects homozygous for FV_{HR2} and for the first time in 4 heterozygous individuals. This increase is comparable to that in subjects with FV_{Leiden} ($p > 0.3$).

Recently a similar result was reported by Castoldi et al. [11] for a single individual homozygous for FV_{HR2}.

These high ETP values are caused by an APC resistance, which is an indication for an increased risk of venous thrombosis [23]. Thus, our results provide evidence for the role of FV_{HR2} as a risk factor for venous thrombosis in homozygous patients through an increased thrombin generation. The increased ETP values in the presence of APC also suggest an increased risk of thrombosis for heterozygous FV_{HR2}. However, this is not conclusive due to the wide data range. Thus, further investigation with a large number of subjects is necessary to confirm this statement.

Clinical Manifestation in Rare Bleeding Disorders and Thrombin Generation

Several cases of patients with severe deficiency of factors VIII (hemophilia A) [24] or factor IX (hemophilia B) [25] have been reported which show only mild bleeding symptoms because of the simultaneous occurrence of FV_{Leiden}. The mild clinical phenotype can be explained by an increased thrombin generation as a consequence of the APC resistance of FV_{Leiden} [26]. A moderating effect

of FV_{Leiden} on bleeding symptoms has also been described recently in patients homozygous for the mutation FVII_{9726 + 5G>A} (FVII_{Lazio}) with 1 patient carrying heterozygous FV_{Leiden} [20]. However other groups have not confirmed this moderating effect of FV_{Leiden} in severe hemophilia A patients [27, 28]. Thus, mild clinical expression of severe hemophilia cannot be exclusively attributed to the coexistent presence of FV_{Leiden} [29].

In this study we have shown that the presence of FV_{Leiden} and of the FV_{HR2} alleles (heterozygous or homozygous) is correlated with an increase of the thrombin generation in the corresponding plasma samples. Our measurements show that homozygous FV_{HR2} is correlated with an increased thrombin generation comparable to FV_{Leiden}. On this basis the effect of FV_{HR2} on thrombin generation was studied in homozygous FVII-deficient patients (caused by the same mutations) of variable severity.

In the Greifswald FVII Deficiency Study we described a great variability in the clinical manifestation of inherited FVII deficiency [15]. The most frequent F 7 gene lesion among FVII-deficient patients in Germany is the missense mutation A294V [16].

Among 4 homozygous patients with the identical mutation A294V, 2 had bleeding symptoms and 2 never had spontaneous bleeding symptoms related to the FVII:C deficiency. ETP measurements in the plasma samples of these patients carrying the homozygous mutation FVII_{A294V} showed the reported correlation of severity and thrombin generation. The asymptomatic homozygous A294V patients have a significantly higher thrombin generation than symptomatic homozygous patients for the same mutation.

On the other hand, the symptomatic FVII-deficient homozygous patients are characterized by the FV wild-type and the asymptomatic patients by the FV_{HR2} genotype (homozygous or heterozygous). An association is shown between severity and ETP (measured with addition of APC) as well as the presence of FV_{HR2}.

In analogy to FV_{Leiden} our findings may allow the conclusion that the APC resistance of FV_{HR2} may moderate the severity of bleeding symptoms in patients with inherited bleeding disorders by an increased thrombin generation.

These studies indicate that the presence of the thrombotic risk factors, FV_{Leiden} or FV_{HR2}, may modulate the degree of bleeding symptoms in patients with FVII deficiency, similar to that observed in patients with hemophilia A [24]. Further investigation on homozygous patients with identical mutations is necessary to verify these findings.

Acknowledgements

The authors thank Angelika Batorova (National Haemophilia Centre, Institute of Haematology and Blood Transfusion, University Hospital, Bratislava, Slovakia), Volker Aumann (Universitäts-

Kinderklinik, Kiel, Germany), Harald Lenk (Universitäts-Kinderklinik, Leipzig, Germany) and Sirak Petros (Clinical Haemostaseology, Medical Clinic I, University of Leipzig, Leipzig, Germany) for providing the plasma samples of FVII_{A294V} patients.

References

- Dahlbäck B, Carlsson M, Svensson P: Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993;90:1004–1008.
- Rosendaal FR, Koster JP, Vandenbroucke JP, Reitsma PH: High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood* 1995;85:1504–1508.
- Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP: Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. *N Engl J Med* 1995;332:912–917.
- Koster T, Rosendaal FR, de Ronde H, Briët E, Vandenbroucke JP, Bertina RM: Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet* 1993;342:1503–1506.
- Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PJ: Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994;369:64–67.
- Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B: Activated protein C resistance caused by Arg506→Gln mutation in factor Va. *Lancet* 1994;343:1361–1362.
- Voorberg J, Roelse J, Koopman R, Büller H, Berends F, ten Cate JW, Mertens K, van Mourik JA: Association of idiopathic venous thromboembolism with single point mutation at Arg506 of factor V. *Lancet* 1994;343:1535–1536.
- Zöller B, Dahlbäck B: Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet* 1994;343:1536–1538.
- Castaman G, Faioni EM, Tassetto A, Bernardi F: The factor V HR2 haplotype and the risk of venous thrombosis: a meta-analysis. *Haematologica* 2003;88:1182–1189.
- Dargaud Y, Meunier S, Negrier C: Haemophilia and thrombophilia: an unexpected association! *Haemophilia* 2004;10:319–326.
- Castoldi E, Brugge JM, Nicolaes DAF, Girelli D, Tans G, Rosing J: Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood* 2004;103:4173–4179.
- Castoldi E, Rosing J, Girelli D, Hoekema L, Lunghi B, Migozzi F, Ferraresi P, Friso S, Corrocher R, Tans G, Bernardi F: Mutations in the R2 FV gene affect the ratio between the two FV isoforms in plasma. *Thromb Haemost* 2000;83:362–365.
- Bernardi F, Faioni EM, Castoldi E, Lunghi B, Castaman G, Sacchi E, Mannucci PM: A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood* 1997;90:1552–1557.
- Herrmann FH, Salazar-Sanchez L, Jimenez-Arce G, Grimm R, Schroder W: High prevalence of FVHR2 polymorphism in Costa Rican Indians who have no FVL. *Thromb Haemost* 2001;85:1020–1021.
- Herrmann FH, Wulff K, Auberger K, Aumann V, Bergmann F, Bratanoff E, Franke D, Grundeis M, Kreuz W, Lenk H, Losonszy H, Maak B, Marx G, Mauz-Körholz C, Pollmann H, Serban M, Sutor A, Syrbe G, Vogel G, Weinstock N, Wenzel E, Wulff K: Molecular biology and clinical manifestation of hereditary factor VII deficiency. *Semin Thromb Hemost* 2000;26:393–400.
- Wulff K, Herrmann FH: Twenty-two novel mutations of the factor VII gene in factor VII deficiency. *Hum Mutat* 2000;15:489–496.
- Siegemund A, Petros S, Siegemund T, Scholz U, Seyfarth H-J, Engelmann L: The endogenous thrombin potential and high levels of coagulation factor VIII, factor IX and factor XI. *Blood Coagul Fibrinolysis* 2004;15:241–244.
- Siegemund T, Petros S, Siegemund A, Scholz U, Engelmann L: Thrombin generation in severe haemophilia A and B: the endogenous thrombin potential in platelet-rich plasma. *Thromb Haemost* 2003;90:781–786.
- Hemker HC, Béguin S: Thrombin generation in plasma: its assessment via the endogenous thrombin potential. *Thromb Haemost* 1995;74:134–138.
- Castoldi E, Govers-Riemsag JWP, Pinotti M, Biondini D, Tans G, Berrettini M, Mazzucconi MG, Bernardi F, Rosing J: Coinheritance of factor V (FV) Leiden enhances thrombin formation and is associated with a mild bleeding phenotype in patients homozygous for the FVII 9726 + 5G>A (FVII Lazio) mutation. *Blood* 2003;102:4014–4020.
- Zotz RB, Gerhardt A, Kluff C, Scharf RE: Venous thromboembolism during pregnancy is not associated with persistent elevated activated protein C (APC) sensitivity ratio based on the endogenous thrombin potential. *Thromb Haemost* 2005;93:306–310.
- Wielders S, Mukherjee M, Michiels J, Rijkers DTS, Cambus JP, Knebel RWC, Kakkar V, Hemker HC: The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability. *Thromb Haemost* 1997;77:629–636.
- Tans G, Rosendaal FR, Curvers J, Thomassen MCL, Bertina RM, Rosing J: APC resistance determined with the endogenous thrombin generation potential is associated with venous thrombosis: a blinded clinical evaluation (abstract 640). 17th Congr Int Soc Thromb Haemost, Washington, 1999. *Thromb Haemost* 1999;82(suppl):202.
- Nichols WC, Amano K, Cacheris PM, Figueiredo MS, Michaelides K, Schwaab R, Hoyer L, Kaufman RJ, Ginsburg D: Moderation of hemophilia A phenotype by the factor V R506Q mutation. *Blood* 1996;88:1183–1187.
- Vianello F, Belvini D, Dal Bello F, Tagariello G, Zanon E, Lombardi A-M, Zerbinati P, Girolami A: Mild bleeding diathesis in a boy with combined severe haemophilia B (C10400→T) and heterozygous factor V Leiden. *Haemophilia* 2001;7:511–514.
- van't Veer C, Golden NJ, Kalafatis M, Simioni P, Bertina RM, Mann KG: An in vitro analysis of the combination of hemophilia A and factor V (LEIDEN). *Blood* 1997;90:3067–3072.
- Arbini AA, Mannucci PM, Bauer KA: Low prevalence of the factor V Leiden Mutation among 'severe' haemophiliacs with a 'milder' bleeding diathesis. *Thromb Haemost* 1995;74:1255–1258.
- Arruda VR, Annichio-Bizzachi JM, Antunes SV, Costa FF: Association of severe haemophilia A and factor V Leiden: report of three cases. *Haemophilia* 1996;2:51–53.
- Chan J, Weinmann AF, Thompson AR: Factor V Arg/Gln 506 has no dominant influence on the severity of haemophilia when inherited concurrently. *Thromb Haemost* 1995;73:1368.