Thrombosis Research (2006) **118**, 269–273

THROMBOSIS RESEARCH

intl.elsevierhealth.com/journals/thre

REGULAR ARTICLE

Endogenous or exogenous coagulation factor level and the response to activated protein C

Laura C. Gennari *, Alicia N. Blanco, Maria P. Domínguez, Silvia H. Grosso, Maria A. Lazzari

División Hemostasia, Departamento de Hemostasia y Trombosis, Instituto de Investigaciones Hematológicas "Mariano R. Castex", Academia Nacional de Medicina, Buenos Aires, Argentina

Received 30 December 2004; received in revised form 13 April 2005; accepted 22 July 2005
Available online 6 September 2005

KEYWORDS
Activated protein C resistance;
Coagulation factors;
Thrombosis

Abstract

Background: The abnormal response to activated protein C could be the mechanism to explain the prothrombotic role of elevated coagulation factor levels.
Objective: We evaluated the effect of factor VIII, II, or X (FVIII, FII, or FX) levels on activated protein C resistance technique and its association with the resistant phenotype.
Materials and methods: The correlation between APCR and FVIII was assessed in 36 samples, after Desmopressin infusion and the correlation between FII or FX and APCR in 15 patients with plasma levels between 100–125 U/dl. Also, the effect of the addition of purified human factors (FII, FX) to a normal plasma pool (final concentration: 100, 120, 140, 180, 220 U/dl) was estimated on the APCR technique.
Results: APCR values correlated with FVIII increase ($r_{Spearman} = 0.839$; $p < 0.001$); APCR was abnormal (<2.4) in 9 / 36 samples, showing higher FVIII values in the abnormal group ($VIII_{abnormalAPCR} = 176.7 \pm 14.2$; $VIII_{normalAPCR} = 103.5 \pm 8.0$). APCR did not correlate with endogenous FII ($r_{Spearman} = 0.423$) or FX ($r_{Spearman} = -0.169$). However, the addition of human FII or FX to the normal plasma pool caused a decrease in APCR ($r_{SpearmanFII} = -0.843$; $r_{SpearmanFX} = -0.958$) without reaching abnormal (<2.4) results. FVIII levels may be associated with a resistant phenotype at values >153.0 U/dl, according to the linear regression analysis. Exogenous FII or FX levels greater than 120 U/dl would affect the APCR, without obtaining abnormal results.
Conclusions: The data do not allow the direct association of the FII or FX increase with a defect in the protein C system in the current conditions.

Abbreviations: APC, activated protein C; APCR, activated protein C resistance; APTT, activated partial thromboplastin time; DDAVP, Desmopressin; FII, Factor II; FVIII, Factor VIII; FX, Factor X; FVL, Factor V Leiden; SEM, standard error of the mean.

* Corresponding author. Pacheco de Melo 3081, CP:1425, Buenos Aires, Argentina. Fax: +54 11 4805 0712.
E-mail address: laugennari@hematologia.anm.edu.ar (L.C. Gennari).

0049-3848/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2005.07.022

Introduction

An abnormal activated protein C (APC) response is considered one of the main causes of venous thrombosis [1—3], and it is defined as a poor response of plasma to APC addition. About 80% of the resistant phenotypes are associated with the FVR506Q mutation, known as factor V Leiden (FVL) [2,3]. There are also other congenital or acquired conditions that may affect the protein C system and lead to an abnormal response, such as the presence of antiphospholipid antibodies [4] and the increase in coagulation factors [5—7]. In both cases, the abnormal response to APC would be an independent risk factor for thrombosis [8], hence the significance of its detection [9]. Plasma levels of factor VIII (FVIII) rise during pregnancy and the intake of oral contraceptives, and have also been considered a cause for the development of the resistant phenotype in these situations [5]. The relationship between FVIII increase and the poor response to APC has been validated by in vitro assays [10]. High levels of factor II (FII) in plasma have also been described as a determinant of the resistant phenotype [7], although probably through a different mechanism. FVIII would act through an increase in the procoagulant activity (determined by the activated partial thromboplastin time [APTT]) and FII by direct inhibition of APC [11].

Elevated levels of FVIII [12], FII [13], FIX [14] or FXI [15] have been described as independent thrombotic risk factors [16]. However, the thrombotic risk associated with high levels of FX disappears after the adjustment of the concentration of the other vitamin K factors [17].

A negative response to APC may contribute to the thrombotic risk associated with the increase of coagulation factors. This contribution has been described for FVIII based on in vivo and in vitro studies, and based on in vivo studies for FII.

There are few studies of FX effect on APCR, and the results are contradictory [18,19]. Because of this, we proposed to evaluate the effect of FX in vivo and in vitro parallel with the evaluation of FII in the same conditions and FVIII in vivo.

We analyzed samples from patients with plasma levels of FII or FX between 100 and 125 U/dl and a pool of normal plasmas with increasing amounts of purified human factors. In the latter the only variable would be the exogenous addition of FII or FX.

We also analyzed plasma samples obtained after the administration of Desmopressin (DDAVP). Among changes induced by DDAVP [20], the FVIII increase would be the only variable at least directly involved in the protein C system.

Materials and methods

Samples

Patient and normal samples were collected into tubes containing 0.11 M sodium citrate (9:1) and platelet poor plasma was obtained by double centrifugation at 3500 g for 15 min. FII, FVIII and FX activity was measured on fresh plasma samples, while the APCR technique was carried out on samples stored at $-80\ ^{\circ}C$.

Techniques

Coagulant activity of FII, FVIII and FX was determined by one-stage method, using deficient plasmas (IL Test™, Factor II deficient plasma; FVIII deficient plasma, Grifols; IL Test™, Factor X deficient plasma) and either reagent for APTT (IL Test™ APTT-SP) or prothrombin time (Thromboplastin-S, Biopool).

APC resistance (APCR) was determined using the original technique [1] (Coatest APC Resistance, Chromogenix), without prediluting target plasma in factor V deficient plasma. APCR values below 2.4 were considered abnormal according to previous results in normal controls [21].

Endogenous FVIII

Samples ($n=36$) from patients taken before and 1 and 2 h after the infusion of DDAVP (Desmopressin®, Ferring; 0.3 μg/kg) were analyzed; FVIII concentration and APCR were determined in each sample. Patients have neither FVL nor history of thrombotic events, and FII or FX plasma levels $\leq$100 U/dl.

Endogenous FII and FX

We selected plasma samples from 15 patients with previous venous thrombotic events and FII or FX levels between 100 and 125 U/dl, without FVL or prothrombin G20210A mutation; APCR was measured in each sample.

Exogenous FII and FX

Increasing amounts of human purified FII (50 U/ml, factor II, F-5132, Sigma) or FX (55 PEU/ml, Purified factor X, Diagnostica Stago) were added to a normal plasma pool ($n=20$). For each factor added, five plasma fractions were obtained with final concentrations of 100, 120, 140, 180 and 220 U/dl, respectively. The coagulant activity of the added

factor was verified and the APCR assay was performed fourfold in each aliquot.

Statistical analysis

Results were collected in a database (Microsoft Excel, version 2000) and analyzed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 9.0 for Windows). The following non-parametric tests were applied: the Kruskal–Wallis one-way analysis for comparison between groups and the Mann–Whitney rank sum test to compare values between two groups; correlations between APCR and the coagulation factor activities were determined with the Spearman's rank correlation test. Statistical significance was taken at 5% level.

Results

Endogenous FVIII

APCR values inversely correlated with FVIII values ($r_{Spearman} = -0.839$; $p < 0.001$) (Fig. 1). Mean ± standard error of the mean (SEM) for APCR was 2.9 ± 0.1 (range: 1.9–4.5) and for FVIII 121.8 ± 8.7 U/dl (range: 45–250 U/dl). Abnormal APCR values (<2.4) were obtained in 9 samples (median: 2.3; range: 1.9–2.4), with FVIII values between 120 and 250 U/dl (median: 190 U/dl). Significant differences ($p_{MW} < 0.001$) were observed when comparing FVIII plasma concentration in samples with abnormal APCR (mean ± SEM = 176.7 ± 14.2; median = 190) and normal APCR (mean ± SEM = 103.5 ± 8.0; median = 110). Linear regression analysis (± CI 95%) between APCR and FVIII results allowed us to estimate that FVIII values greater than 153.0 U/dl could induce an abnormal response to APC (Fig. 1). Six out of 10 samples with FVIII >153 U/dl (range: 160–250 U/dl) showed abnormal APCR values while the rest (4/10) (FVIII: 160–180 U/dl) had APCR results (2.5–2.6) near the cut-off value.

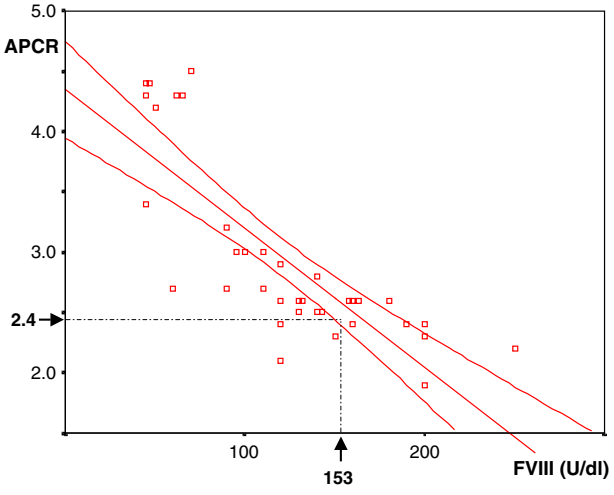

Figure 1 APCR and endogenous FVIII. Linear regression analysis (± CI 95%) between APCR and endogenous FVIII values. Thirty-six samples from patients before and after DDAVP infusion were analyzed. Dotted lines show the FVIII level from which an abnormal response to APC may be expected.

Endogenous FII

There was no significant correlation between endogenous FII concentration and APCR results in plasmas from patients with FII levels between 100 and 125 U/dl ($r_{Spearman} = 0.423$; $p = 0.117$).

Exogenous FII

Fig. 2 shows APCR values obtained in each normal plasma pool aliquot after the addition of purified FII. A significant ($p_{MW} = 0.018$) increase in the resistance between samples with 100 and 120 U/dl was detected, with APCR mean values ± SEM of 3.3 ± 0.002 and 3.1 ± 0.025 respectively. At higher FII concentration the difference in the response lost statistical significance ($p_{MW} > 0.1$) ($APCR_{140} = 3.1 \pm 0.013$; $APCR_{180} = 3.1 \pm 0.027$; $APCR_{220} = 3.0 \pm 0.032$). Abnormal values of APCR were not detected, in spite of the inverse significant correlation with FII levels ($r_{Spearman} = -0.843$; $p < 0.001$).

Endogenous FX

FX plasma values in samples from patients with concentration ranging from 100 to 125 U/dl did not show correlation with APCR results ($r_{Spearman} = -0.169$; $p = 0.548$).

Exogenous FX

Fig. 2 shows APCR values obtained from the different aliquots of normal plasma pool with increasing amounts of purified FX. The increase of the resistance was significant not only between samples with 100 and 120 U/dl ($APCR_{100} = 3.9 \pm 0.014$, mean ± SEM; $APCR_{120} = 3.5 \pm 0.026$; $p_{MW} = 0.020$), but also between 120 and 140 U/dl ($APCR_{140} = 3.5 \pm 0.023$; $p = 0.021$), 140 and 180 U/dl ($APCR_{180} = 3.3 \pm 0.006$; $p = 0.020$) and between 180 and 220 U/dl ($APCR_{220} = 3.2 \pm 0.022$; $p = 0.028$). APCR inversely correlated with FX concentration in the

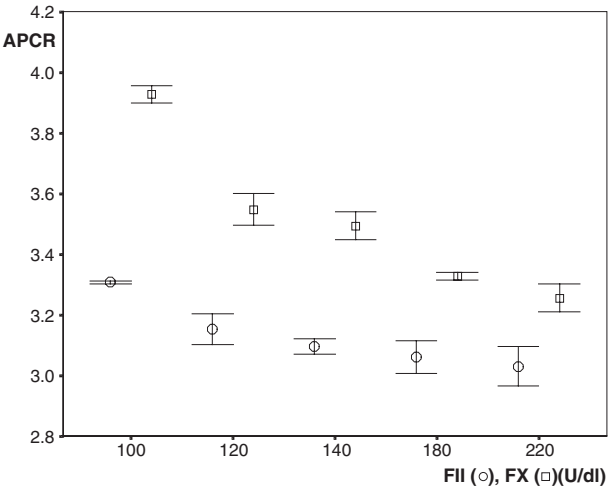

Figure 2 APCR and exogenous FII or FX. APCR values in normal plasma pool after the addition of purified FII or FX (final concentration: 100, 120, 140, 180 and 220 U/dl). APCR was measured fourfold in each aliquot. Circles (○) indicate the mean value in each FII aliquot and squares (□) FX values. Bars indicate 2 standard error of the mean.

samples ($r_{Spearman} = -0.958$; $p < 0.001$); but without reaching abnormal APCR results.

Discussion

Factor VIII [12] or FII [13] increase has been described as an independent risk factor for thrombosis; unlike FX elevation, in which the risk disappears after adjustment for the other vitamin K-dependent coagulation factors [17]. FVIII or FII increase has also been associated with the APC resistant phenotype [5–7]; this abnormal response is not due to the presence of FVR506Q mutation and could be the mechanism accounting for the thrombotic risk associated with elevated coagulation factor levels.

Risk associated with FVIII would not be related to its acute phase reactant condition, but to genetic and environmental variations [22]. The proposed mechanism of action would be the influence on the response to APC, determined by an APTT system, particularly sensitive to variations in the concentration of FVIII, that lead to a high procoagulant activity through an increase in the thrombin generation [6]. We verified not only that the increased plasma levels of FVIII in response to the administration of DDAVP affect the response to APC (Fig. 1), but also that FVIII concentration associated to an abnormal response (FVIII >153 U/dl) would be similar to that reported in vitro (FVIII >150 U/dl) [10]. However, other factors in addition to FVIII levels must determine the APCR, since we found samples with high FVIII (>153 U/dl) that still displayed normal values (although not far from the abnormal cut-off point) (Fig. 1).

It has been postulated that FII would also play a role affecting protein C system, but through a different mechanism from that of FVIII [7]. In this case, FII increase will appear to modulate APC anticoagulant activity, inhibiting its ability to inactivate FVa [11]. Correlation between FII plasma levels and APCR values was previously described by Tripodi et al. in a series of 285 individuals without FVL, in which 12% of the subjects with levels of FII >110 U/dl (range: 110–170 U/dl), presented APCR values below the normal limit [7]. We did not find a significant correlation between APCR results and FII plasma concentrations (100 to 125 U/dl), but a significant effect was detected when purified FII was added at concentrations ≥120 U/dl (Fig. 2). Perhaps, it would be necessary to analyze samples from patients in a higher range of FII concentration, in order to verify a significant effect on APC response. According to the number of patients analyzed the likelihood of having abnormal APCR values was low (1–2 of 15; 12%); in fact, we did not find patients with abnormal results. Moreover, a different sensitivity in the method applied to evaluate the response to APC in both studies can be suspected. Although in both cases the original technique was applied, based on APTT, Tripodi et al. [7] used a modified assay by Faioni et al. [23], while we employed a commercial kit. In the latter, the final concentration of APC is higher, which could determine the necessity of higher concentrations of FII to inhibit the APC present in the assay. The fact that we did not observe an abnormal APCR value in a wide range of purified FII (up to 220 U/dl) may account for the latter assumption.

The FX increase would predict the development of thrombosis in a non-independent manner, but the possible mechanism of action is unknown [17]. It was reported that individuals with FX concentrations higher than 126 U/dl present 1.6 times higher risk for venous thrombosis; however this estimated risk disappears after adjusting for the other vitamin K-dependent factors (FII, FVII and FIX) [17]. A correlation between FX and the response to APC was reported in cancer patients but not in the normal controls [18]. This suggests a potential role of FX on acquired APCR in cancer. The effect of FX on APCR is not clear since Kluft et al. referred a low but significant correlation in normal volunteers [19]. According to our results, FX plasma levels (between 100 and 125 U/dl) did not show a significant correlation with the APCR values. However, a progressive reduced response to APC was observed when purified FX was added, but not

enough to induce abnormal APCR results (Fig. 2). Moreover, this effect was less evident in comparison with FII at the same concentrations (Fig. 2).

In the current conditions, it would not be feasible to associate, at least directly, the thrombotic risk by the increased FII or FX plasma concentrations with a defect in the protein C system. However, we believe that the suitability of the system employed to evaluate the response to APC should be considered in order to analyze the apparent lack of agreement with previous reports for FII [7,11]. The commercial assay, based on APTT, sensitive to increases in procoagulant activity caused by FVIII, could not be the most appropriate to detect the resistance caused by increases in FII. If its mechanism of action is through the direct inhibition of APC, as has been postulated [11], the final concentration of APC in the system has to be critical; higher amount of FII should be required to inhibit the higher APC activity present in the reagent.

Acknowledgements

This work was partially supported by Fundación "Alberto J. Roemmers", and Fundación "René Baron", Buenos Aires, Argentina.

References

[1] Dahlbäck B, Carlsson M, Svensson PJ. 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**:1004–8.

[2] Dahlbäck B. Activated protein C resistance and thrombosis: molecular mechanisms of hypercoagulable state due to FVR506Q mutation. *Semin Thromb Hemost* 1999;**25**:273–89.

[3] Bertina RM. Molecular risk factors for thrombosis. *Thromb Haemost* 1999;**82**:601–9.

[4] Esmon NL, Safa O, Smirnov MD, Esmon CT. Antiphospholipid antibodies and the protein C pathway. *J Autoimmun* 2000; **15**:221–5.

[5] Henkens CMA, Bom VJJ, van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. *Thromb Haemost* 1995;**73**:402–4.

[6] Laffan MA, Manning R. The influence of factor VIII on measurement of activated protein C resistance. *Blood Coagul Fibrinolysis* 1996;**7**:761–5.

[7] Tripodi A, Chantarangkul V, Mannucci PM. Hyperprothrombinemia may result in acquired activated protein C resistance. *Blood* 2000;**96**:3295–6.

[8] de Visser MCH, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood* 1999;**93**:1271–6.

[9] Gennari L, Blanco A, Bermejo E, Salviú J, Grosso S, Lazzari MA. Activated protein C (APC) resistance: considerations about the importance of using the original and modified methods in thrombophilic disease. *Thromb Haemost* 2000;**84**:138–9.

[10] Henkens CMA, Bom VJJ, van der Meer J. Lowered APC-sensitivity ratio related to increased factor VIII-clotting activity. *Thromb Haemost* 1995;**74**:1198–9.

[11] Smirnov M, Safa O, Esmon N, Esmon CT. Inhibition of activated protein C anticoagulant activity by prothrombin. *Blood* 1999;**94**:3839–46.

[12] Rosendaal FR. High levels of factor VIII and venous thrombosis. *Thromb Haemost* 2000;**83**:1–2.

[13] Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996;**88**:3698–703.

[14] van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. *Blood* 2000;**95**:3678–82.

[15] Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. *N Engl J Med* 2000;**342**: 696–701.

[16] Tripodi A. Levels of coagulation factors and venous thromboembolism. *Haematologica* 2003;**88**:705–11.

[17] de Visser MCH, Poort S, Vos H, Rosendaal FR, Bertina RM. Factor X levels, polymorphisms in the promoter region of factor X, and the risk of venous thrombosis. *Thromb Haemost* 2001;**85**:1011–7.

[18] Sarig G, Michaeli Y, Lanir N, Brenner B, Haim N. Mechanisms for acquired activated protein C resistance in cancer patients. *J Thromb Haemost* 2005;**3**:589–90.

[19] Kluft C, Meijer P, Gram J, Jespersen J. Acquired activated protein-C resistance. *Lancet* 1999;**353**:1882.

[20] Chitolie A, Lawrie AS, Mackie IJ, Harrison P, Machin SJ. The impact of oral anticoagulant therapy, factor VIII level and quality factor V-deficient plasma on three commercial methods for activated protein C resistance. *Blood Coagul Fibrinolysis* 2001;**3**:179–86.

[21] Gennari L, Blanco A, Alberto MF, Grosso S, Peirano A, Lazzari MA. Antiphospholipid antibodies impact the protein C (PC) pathway behavior. *Am J Hematol* 2002;**71**:128–30.

[22] O'Donnell J, Mumford AD, Manning RA, Laffan M. Elevation of FVIII:C in venous thromboembolism is persistent and independent of the acute phase response. *Thromb Haemost* 2000;**83**:1–2.

[23] Faioni EM, Franchi F, Asti D, Sacchi E, Bernardi F, Mannucci PM. Resistance to activated protein C in nine thrombophilic families: interference in a protein S functional assay. *Thromb Haemost* 1993;**70**:1067–71.