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Factor V Leiden and antibodies against phospholipids and protein S in a young woman with recurrent thromboses and abortion

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Abstract

We describe the case of a 39-year-old woman who suffered two iliofemoral venous thromboses, a cerebral ischemic infarct and recurrent fetal loss. Initial studies showed high levels of antiphospholipid antibodies (APAs) and a moderate thrombocytopenia. After her second miscarriage, laboratory diagnosis revealed that the woman was heterozygous for the factor V Leiden mutation and had a functional protein S deficiency as well as anti-protein S and anti-β_2-glycoprotein I antibodies. The impairment of the protein C pathway at various points could well explain the recurrent thromboses in the patient and supports the role of a disturbed protein C system in the pathophysiology of thrombosis in patients with APAs.
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Key words: factor V Leiden, activated protein C resistance, antiphospholipid antibodies, protein S deficiency, recurrent abortion, thrombosis

Thrombophilia is ever more often considered to be the result of a combination of two or more risk factors, either hereditary or acquired, in an individual. One of these factors, usually acquired, is the presence of antiphospholipid antibodies (APAs). APAs are a family of closely related immunoglobulins that interact with negatively charged phospholipids[1] and include lupus anticoagulants (LAs) and anticardiolipin antibodies (ACAs).[2] In many cases both types of antibodies occur concurrently, but in other cases only one type of antibody is detected in an individual. Although in some patients ACA and LA cannot be separated into two different immunoglobulin fractions, in others ACA IgG can be separated from LA IgG,[3,4] showing that ACAs and LAs recognize different epitopes. Moreover, they require different protein cofactors for their activity: ACAs require β_2-glycoprotein I (β_2GPI),[5,6] whereas LAs need protein cofactors such as prothrombin, thrombomodulin, protein C or protein S,[7-9] although a subgroup of LAs can need β_2GPI as cofactor.[10] In some individuals, the antibodies are transient. The persistence of APAs at a high titer is often associated with recurrent fetal losses, arterial and/or venous thrombosis or thrombocytopenia.[11] The origin of the presence of APAs is uncertain, but they usually appear following infections, drug consumption or an autoimmune disease (for a review of the pathophysiology of APAs, see ref. #12).

Abnormalities in components of the protein C pathway are a common risk factor for venous thrombosis. Several polymorphisms and mutations in the genes for protein C and protein S predisposing to thrombosis have been reported.[13,14] Resistance to the anticoagulant effect of activated protein C (APC-R) has been described as an important cause of venous thrombosis.[15] This abnormality is associated with the presence of a guanidine to adenine substitution at nucleotide 1691 of the factor V gene (factor V Leiden).[16] A high prevalence of this mutation has been found in patients with venous thrombosis, whereas the prevalence of the mutation in the general European population is only about 3-5%.[16]

Associations between APAs and factor V Leiden mutation[17] or acquired protein S deficiency[18] have recently been described in patients with thrombotic diatheses. In this report, we describe the case of a 39-year-old woman who suffered several thrombotic events and recurrent abortions, and had a combination of factor V Leiden, APAs and acquired protein S deficiency.

Case report

The patient was a Caucasian female who in March 1989, at the age of 29 years, developed iliofemoral venous thrombosis in both legs with a marked inflammatory component and cyanosis, one month after her first miscarriage. She was treated with streptokinase (250,000 IU in 30 min and 100,000 IU for 24 hours), followed by oral anticoagulation with acenocoumarol for 22 months, keeping the INR between 2 and 3. Thereafter, an evaluation of her hypercoagulable state revealed no alteration in the levels of antithrombin III, protein C, protein S or type 1 plasminogen activator inhibitor. Five years later, in January 1994, the patient suffered a sudden feel-

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ing of dizziness, right motor deficiency and loss of consciousness. Over the following days she developed a complete flaccid right hemiplegia, hyporeflexia with right Babinski's sign, right hemihyposthesia and mixed aphasia, and was diagnosed by a CAT scan as having right Sylvian ischemic infarction. Since neither the ECG nor the EEG showed alterations, a potential embolic etiology was ruled out. Laboratory analysis at that point showed an APTT of 61.2 sec (normal range, 26-36 sec). The diluted Russell viper venom test gave a ratio of 1.75 (normal index <1.2), and the correction with a mixture of platelet phospholipids was 40% (normal range <10%), which indicated the presence of LA. ACAs were GPL 160 U (normal level <15 U), MPL 29 U (normal level <13 U). The patient had a moderate thrombocytopenia (103,000 platelets/μL). Underlying systemic lupus erythematosus disease was ruled out. These laboratory and clinical findings led to the diagnosis of antiphospholipid syndrome. Initially, the patient was anticoagulated with therapeutic doses of sodium heparin (1200 IU/hr) and then with long-term acenocoumarol to maintain the INR between 3.0 and 4.0. Her clinical progress was partially satisfactory. She regained movement in the right arm and leg and began to utter some phonemes.

The woman was again brought to our Department in August, 1996 for a new study of thrombophilia because she had another spontaneous abortion. The oral anticoagulation treatment was replaced by low molecular weight heparin therapy and, after two weeks, a new analytical study was carried out with additional assays including LAs, ACAs, anti-β_2GPI, antiphosphatidylethanolamine and anti-protein S antibodies, protein C, circulating activated protein C, protein S, C4b-binding protein, antithrombin III, tissue plasminogen activator, plasminogen activator inhibitor type 1, β-thromboglobulin, APC-R, factor V Leiden and the prothrombin gene G20210A variant. The heparin treatment was replaced by life-long acenocoumarol therapy, intended to keep the INR between 3 and 4.

The only noteworthy aspect of the medical history of the patient's family is that her mother suffered two cerebral ischemic infarctions, and died as a result of the second one. Her father, her two brothers and her two children, aged 15 and 19, have never had thrombotic events. The analytic studies run on her children showed no alterations.

Unless specified to the contrary, basic analytical assays were performed following standard clinical laboratory procedures. IgG and IgM ACAs were assayed using standardized ELISAs (Cheshire Diagnostic Ltd., Chester, England). Results were expressed in either GPL or MPL arbitrary units derived from the activity of affinity purified sera.[19] A positive ACA value was considered to be IgG ≥15 GPL or IgM ≥13 MPL. The β_2GPI concentration was measured by a standardized ELISA (Inova Diagnostics Inc, San Diego, CA, USA). Antiphosphatidylethanolamine antibodies were assayed as reported elsewhere.[20] The modified APC-R assay was performed by previous dilution of the patient's plasma with factor V-depleted plasma as reported earlier[21] with slight modifications.[22] The platelet neutralization assay was a diluted Russell viper venom time in which one volume of plasma sample was first mixed with one volume of a commercial platelet preparation (Platelet extract reagent, Biodata Corporation, Horsham, PA, USA) to neutralize the patient's APAs.[23] Protein S was purified as previously reported.[24] IgG antibodies to protein S were detected essentially as reported by Sorice *et al*.[25] Briefly, 70 μg of purified protein S was subjected to SDS-polyacrylamide gel electrophoresis on one-well slab gel (8×10 cm) formed by 4% stacking gel and 10% running gel. Protein was transferred to a nitrocellulose membrane and the membrane was cut into strips. Each strip was incubated for 2 h with different dilutions of the patient's plasma or pooled normal plasma, and then with horseradish peroxidase-labeled rabbit anti-human IgG (Sigma Chemical Co., St. Louis, MO, USA). Finally, the bands were visualized with 4-chloro-1-naphthol (BioRad, Richmond, CA, USA). Circulating activated protein C was assayed as reported earlier[26] (PCR analyses for the factor V Leiden mutation and for the prothrombin gene G20210A variant were performed as indicated in previous reports.[27,28]

The study done in our laboratory in 1996, after the patient's second miscarriage, confirmed that the thrombocytopenia and APAs persisted (Table 1). Fur-

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Table 1. Laboratory data of the patient.

	Platelets (x 1,000/μL)	LAs	ACAs GPL	ACAs MPL	anti-β_2GPI IgG Units	APC-R R_{FV}	APC-R R_{PL}	FV Leiden	Protein S (%) Total	Protein S (%) Free	Protein S (%) Functional
Patient	113	Positive	>140	36	26	1.39	1.84	heterozygous	91	90	51
Control	150-300	Negative	<15	<10	<20	2.7±0.2	2.9±0.4	–	75-108	61-137	60-130

LAs: Lupus anticoagulants; ACAs: anticardiolipin antibodies; β_2GPI: β_2-glycoprotein-I; APC-R: activated protein C resistance; RFV: APC-R ratio evaluated by diluting the patient's plasma 1/5 with factor V-depleted plasma; RPL: APC-R ratio evaluated by diluting the patient's plasma 1/1 with a suspension of platelet phospholipids.

thermore, abnormal levels of anti-β_2GPI were detected (26 U; normal levels, <20 U). Additional studies showed the presence of APC-R (APTT+ APC/APTT ratio below the cut-off point of 2.3), as evaluated with a modified test using a previous 1/5 dilution of the patient's plasma with factor V-depleted plasma.[21,22] When the APAs were neutralized with a suspension of platelet phospholipids the ratio remained below the cut-off point, suggesting the presence of a molecular abnormality. PCR analysis showed that the patient was heterozygous for the factor V Leiden mutation but did not show the recently described G20210A mutation in the prothrombin gene. Functional protein S was decreased. Antibodies to protein S were detected by immunoblotting. The blots showed a single band at about 70 kDa (Figure 1). The patient was screened for antiphosphatidylethanolamine and was negative. The circulating activated protein C/total protein C ratio (0.681) was within normal limits (0.61-1.3, n=35), suggesting normal protein C activation. All other hemostatic parameters studied were found to be normal. It is not uncommon to find extensive venous thrombosis, arterial thrombosis or recurrent abortions in patients with APAs. What makes the present case unusual is the co-existence of all these clinical manifestations in the same patient. This can be explained by the coincidence in this patient of several laboratory abnormalities: presence of LAs, ACAs, anti-β_2GPI antibodies, anti-protein S antibodies and the factor V Leiden mutation.

The presence of anti-β_2GPI autoantibodies in patients with ACAs and LAs has been associated with an increase in thrombotic risk,[29] although Horbach *et al.*[30] have recently speculated that anti-β_2GPI antibodies are only relevant in assays to detect APAs, but have no role in *in vivo* thrombosis. Similarly, acquired protein S deficiency could also contribute to the development of thrombosis.[25] Co-occurrence of APAs, anti-protein S and anti-β_2GPI antibodies has been reported previously.[12,31] Morange *et al*. described a similar case of a 26-year-old woman with venous thrombosis and the combination of APAs, anti-β_2GPI and anti-protein S autoantibodies, factor V Leiden mutation and systemic lupus erythematosus.[31] However, their patient did not develop arterial thrombosis or suffer recurrent abortions.

APAs are seen as a heterogeneous population of antibodies that, depending of their specificity, can interfere with different anticoagulant reactions. In 7 patients with a history of thrombotic complications, Oosting *et al*. showed the presence of IgGs which inhibited APC activity.[32] Phospholipids are known to participate in the activation and function of protein C. Specifically, phosphatidylethanolamine appears to play an important role both in protein C activation[33] and in the anticoagulant function of activated protein C.[34,35] *In vitro* studies have provided some clues to explain the thrombotic tendency observable in patients with APAs. Plasma or immunoglobulin fractions were used to show that both thrombomodulin-dependent activation of protein C[36,37] and activated protein C anticoagulant function[32,38,39] were inhibited by APAs. Studies by Berard *et al.*[20] showed a high frequency of patients with clinical symptoms suggesting the occurrence of an antiphospholipid syndrome in patients whose only APA was antiphosphatidylethanolamine. They also showed a strong association between antiphosphatidylethanolamine and thrombosis, suggesting that inhibition of activated protein C function was the pathogenic mechanism. Galli *et al*.[40] recently studied the differential effects of anti-β_2GPI and anti-prothrombin antibodies on the anticoagulant activity of activated protein C. They found that anti-β_2GPI, but not anti- prothrombin antibodies, hampered the inactivation of factor Va by endogenous activated protein C. They also found that 20 of the 24 APA patients with anti-β_2GPI had reduced inactivation of factor Va whereas only six of the 18 patients with anti-prothrombin antibodies showed abnormal factor Va inactivation. Moreover, an abnormal rate of factor Va inactivation was found in 73%

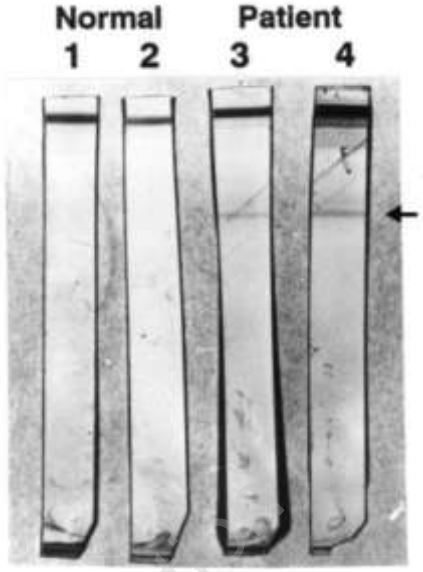

Figure 1. Detection of IgG anti-protein S antibodies by immunoblotting. Purified protein S (70 µg) was subjected to 10% SDS-polyacrylamide gel electrophoresis in a 8×10 cm gel. After transferring the protein to a nitrocellulose membrane, the latter was cut into strips and each portion incubated for 2 h with different dilutions of pooled normal plasma (lanes 1 and 2) or patient's plasma (lanes 3 and 4). The strips were then incubated for 2 h with rabbit anti-human IgG labeled with horseradish peroxidase followed by the addition of a chromogenic substrate. Plasma dilution: lanes 1 and 4, 1/50; lanes 2 and 3, 1/10. The arrow indicates the band at 70 kDa corresponding to the IgG anti-protein S antibodies from patient's plasma but not from normal plasma bound to purified protein S.

of APA patients with venous thrombosis versus 56% of patients without venous thrombosis. The authors conclude that anti-β_2GPI antibodies are associated with thromboembolic events that occur in patients with APAs and suggest that acquired APC-R resulting from the impairment of the anticoagulant activity of the protein C system may represent one of the possible pathogenetic mechanisms responsible for the increased thrombotic risk in some APA patients. Our patient did not have antiphosphatidylethanolamine antibodies, which is consistent with the finding of a normal activated protein C/total protein C ratio since the presence of these antibodies would induce a decrease in protein C activation and hence a reduced APC/protein C ratio. However, the relatively low APC level and the presence of anti-β_2GPI could hamper the anticoagulant function of activated protein C.

The presence of antibodies to protein S in the patient's plasma strongly suggest that the reduced functional protein S level is due to inhibition of protein S activity by specific anti-protein S antibodies. However, functional assays for protein S can give falsely low levels in patients with APC-R.[41] Since we did not demonstrate directly that the patient's anti-protein S IgG inhibits protein S activity, the presence of a true functional protein S deficiency in the patient remains uncertain.

The co-presence of factor V Leiden mutation and anti-protein S antibodies in our patient may, therefore, have contributed to the severe clinical manifestations since the patient's protein C system is impaired at different levels: reduced anticoagulant function of activated protein C as a consequence of decreased protein S function due to the presence of anti-protein S antibodies, and poor anticoagulant response of the activated protein C due to the presence of the factor V Leiden mutation and anti-β_2GPI.

Contributions and Acknowledgments

YM and AV were responsible for the clinical care of the patient and commented on the draft of the manuscript. MR and FE performed protein S and activated protein C studies. SG and AE performed genetic studies and commented on the draft. PV was responsible for all other laboratory analyses. FE wrote the manuscript and JA revised it and gave final approval. The authors thank Drs. E. Balada and J. Ordí for the evaluation of antiphosphatidylethanolamine antibodies.

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Disclosures

Conflict of interest: none.

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