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RESISTANCE TO ANNEXIN A5 ANTICOAGULANT ACTIVITY IN WOMEN WITH HISTORIES FOR OBSTETRIC ANTIPHOSPHOLIPID SYNDROME

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Abstract

Objectives—To investigate whether resistance to annexin A5 anticoagulant activity (AnxA5) occurs in women with histories for obstetric complications of antiphospholipid syndrome (Obs-APS) and whether this correlates with antibody recognition of domain 1 of β 2-glycoprotein.

Study Design—136 women with antiphospholipid antibodies, including 70 with histories for Obs-APS, and 30 controls, were investigated.

Results—Women with Obs-APS showed resistance to AnxA5 activity (median (range) 216% (130–282%) vs. controls 247% (217–283%), $p < 0.0001$) and elevated levels of anti-domain I IgG (OD: median (range) 0.056 (0.021–0.489) vs. 0.042 (0.020–0.323); $p = 0.002$). Those in the lowest tertile of AnxA5 anticoagulant ratios had an OR for Obs-APS of 58.0 (95% CI 3.3–1021.5). There was an inverse correlation between levels of annexin A5 anticoagulant activity and anti-domain I IgG.

Conclusions—Resistance to AnxA5 anticoagulant activity is associated with antibody recognition of domain I of β 2GPI and identifies a subset of women with histories for Obs-APS.

Keywords

annexin V; obstetric; Antiphospholipid antibodies; Antiphospholipid syndrome; Annexin A5; β 2-glycoprotein I; Pregnancy loss

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Resistance to annexin A5 anticoagulant activity correlates with antibody recognition of domain I of β 2GPI and is associated with obstetric complications in antiphospholipid syndrome.

INTRODUCTION

The antiphospholipid syndrome (APS) is defined by the association of a persistently abnormal antiphospholipid antibody (aPL) assays – i.e. elevated immunoassays anticardiolipin and/or anti- β 2glycoprotein I IgG or IgM antibodies or a positive lupus anticoagulant test - with a history of thrombosis or specific pregnancy complications¹. The currently available antiphospholipid assays are empirically-derived tests that do not measure a disease mechanism; the immunoassays were derived from the biologic false positive syphilis phenomenon and the lupus anticoagulant from the observation an inhibitor to the activated partial thromboplastin time, both described over 50 years ago. The pathogenic mechanism for obstetric APS has remained enigmatic.

The syndrome is referred to as primary APS (PAPS) when it occurs without other autoimmune disease, and secondary APS when it is associated with another autoimmune disease, usually systemic lupus erythematosus. In this paper, the term obstetric APS applies to aPL associated with the pregnancy complications that were defined by consensus diagnostic criteria; these include a previous unexplained recurrent first trimester loss and/or mid trimester and third trimester intrauterine death and/or severe pre-eclampsia, placental abruption or intrauterine growth retardation¹.

The purpose of this study was to investigate whether women with histories of obstetric APS might have evidence for resistance to annexin A5 (AnxA5) anticoagulant activity in their blood. AnxA5 is a placental anticoagulant protein that is highly expressed on the apical surfaces of syncytiotrophoblasts² where the protein is in an anatomic position to play a thrombomodulatory role and contribute to the fluidity of the maternal circulation through the intervillous space. The protein is also expressed in a number of other cell types including, among others, vascular endothelial cells, renal tubular epithelial cells and bile duct epithelial cells. The protein's potent anticoagulant activities result from its forming two-dimensional crystals over anionic phospholipids that shield the phospholipids from contributing to critical phospholipid-dependent coagulation enzyme reactions. aPL antibodies have been shown to reduce the quantity of AnxA5 on cultured placental trophoblasts^{3,4} and accelerate the coagulation of plasma that is exposed to these cells⁵. Furthermore aPL antibodies reduce the binding of AnxA5 to phospholipid bilayers⁶⁻⁹ and create significant defects in the ordered crystallization of this protein^{10,11} that expose unshielded phospholipids, thereby accelerating coagulation enzyme reactions¹⁰.

We previously reported that patients with APS-associated vascular thrombosis had resistance to AnxA5 anticoagulant activity^{7,12}, and that this reduced AnxA5 anticoagulant activity correlated strongly with antibody-mediated displacement of AnxA5 from binding to phospholipids⁷ and with antibody recognition of a specific epitope on domain 1 of β 2-glycoprotein I (β 2GPI)¹³. We also previously reported that women with a history of recurrent spontaneous pregnancy losses - not screened for aPL antibodies – had reduced AnxA5 anticoagulant activity¹⁴. However, the specific question of whether there may be evidence for resistance to annexin A5 anticoagulant activity in the bloods of women with aPL-associated pregnancy complications has never been previously investigated. Nor has the question of whether anti-domain 1 IgG antibodies might correlate with obstetric APS been previously investigated.

Therefore, the aim of this study was to measure these specific parameters in women with histories of obstetric APS. Due the inflammatory state induced by systemic lupus erythematosus, the study was confined to patients with PAPS.

MATERIALS AND METHODS

Patients

After obtaining Local Ethical committee approval at Guy's & St Thomas' Trust, blood specimens were collected with informed consent from healthy non-pregnant women who had a history of obstetric PAPS, and both men and women with a diagnosis of thrombotic PAPS or isolated aPL antibodies. All PAPS patients satisfied the Miyakis criteria for the diagnosis of aPL and APS¹.

In total 136 patients with aPL antibodies were classified into three groups:

1. Women, not currently pregnant but with a past history of obstetric PAPS (n=70).
2. Subjects without obstetric APS, but with a history of thrombotic PAPS, with their last thrombotic event more than 6 months previously (n=50).
3. Subjects with isolated aPL antibodies who had not sustained any thrombotic or pregnancy events (n=16).

The demographic, aPL characteristics including types of obstetric APS and treatment details are summarized in Table 1. There was no significant difference in ages between groups, but obviously, those with obstetric PAPS were all female, and the majority of the other groups were also female. As described in Table 1, 29 of the 70 women with obstetric APS had histories for 3 or more spontaneous first trimester losses, 39 of the women had a history for intrauterine fetal demise; and 26 had histories for placental insufficiency.

A minority of the obstetric PAPS group (n=23, 30%) also had a thrombotic history. Those in the thrombotic PAPS group had similar rates of venous and arterial previous events with 9 (18%) having had both venous and arterial thrombotic events.

In addition, 30 plasmas from disease free non-pregnant women (group D) were obtained from a commercial vendor (George King Bio-Medical Inc, Overland Park, KS) as normal healthy controls. The plasma samples were sent as coded samples to the Pathology Department of the Montefiore Medical Center for AnxA5 resistance assay and to the Hematology Department of Utrecht University Hospital for the anti-domain I immunoassays.

Annexin A5 Resistance Assay

AnxA5 was purified from human placentas as previously described¹⁵. The effects of patient plasmas on AnxA5 anticoagulant activity were determined using a two-stage assay as previously described¹³. Briefly, EDTA (0.5 M) was added to recombinant human tissue factor (Innovin; Dade Behring Inc. Newark, DE) to a final concentration of 10 mM. The Innovin-EDTA was then mixed with aPTT reagent-phospholipids (Actin FSL; Dade Behring Inc. Newark, DE, USA) at 1:1 ratio. The mixture of Innovin-EDTA-actin FSL (200 µL) was incubated with citrated test plasma (50 µL) for 5 minutes at room temperature. The plasma-treated mixture was then centrifuged with a microcentrifuge (Eppendorf centrifuge 5417R, Brinkmann Instrument, Westbury, NY) for 15 minutes at 20 800g at 25°C. The pellets were washed once in HEPES buffer saline (HBS; 0.01 M HEPES, 0.14M NaCl, pH 7.5) and resuspended in HBS (220 µL). The suspension (50 µL) was incubated with pooled normal plasma (50 µL) at 37°C in a ST4 Coagulation Instrument (American Bioproducts, Parsippany, NJ) for 30 seconds. The plasma was then recalcified with 50 µL of 0.02 M calcium or 0.02 M calcium containing AnxA5 (30 µg/mL). The coagulation times, in the presence and absence of AnxA5, were determined and the mean times of duplicate tests were recorded. The anticoagulant activity of AnxA5 was calculated as follows: AnxA5 anticoagulant ratio= (coagulation time in the presence of AnxA5/ coagulation time in the

absence of AnxA5) $\times 100\%$ Plasma samples were considered to demonstrate resistance to AnxA5 anticoagulant activity when the ratios were below the mean minus two standard deviations (SD) of the 30 normal healthy controls.

Anti-Domain I IgG ELISA

Anti- $\beta 2$ GPI IgG antibodies with reactivity toward domain I were assayed as previously described^{13,16}. Briefly, hydrophobic micro titer plates (Costar, catalog# 2595, New York, NY) were coated with domain I IgG of $\beta 2$ -GPI (10 $\mu\text{g/ml}$ in TBS consisting of 50 mM Tris and 100 mM NaCl) for 1 hour at 37°C. The plates were blocked with 150 μl of blocking solution (4% bovine serum albumin/TBS/0.1% Tween) for one hour at 37°C, and subsequently incubated with patient plasma (diluted 1:100 in the blocking solution) containing anti- $\beta 2$ GPI IgG antibodies for 1 hour. After every incubation step the plates were washed 4 times with washing solution (0.1% Tween/TBS). The bound IgG antibodies were detected by a goat-anti-human IgG alkaline-phosphatase-labeled antibody (Invitrogen Corp, Carlsbad, CA), followed by staining with para-nitrophenyl phosphatase (Sigma, St. Louis, MO). The reaction was stopped by 2.4 M NaOH, and absorbance was measured at 405 nm with a micro titer plate reader. Plasma samples were regarded as having elevated levels of antidomain I IgG when the absorbed value exceeded the cut-off value (mean +3 SD of the 30 normal healthy controls).

Study and statistical analysis

Differences between the four groups of patients were analyzed with the non-parametric Mann-Whitney test (GraphPad Software, San Diego California USA, www.graphpad.com) because of departures of data from normal distribution.

To compute odds ratios and 95% confidence intervals (CIs) for those with obstetric PAPS, data were categorized into tertiles, using the frequency distribution of patients and controls combined. The data were analyzed using unconditional logistic regression and odds ratios were computed relative to the highest tertile for AnxA5 resistance and to the lowest tertile for antidomain I IgG. Likelihood ratio tests were used to calculate p-values for trends. All reported p-values were two-tailed and $p < 0.05$ was considered statistically significant. Correlation coefficients between AnxA5 resistance assay and domain I IgG assay among the groups were calculated using the Spearman rank correlation and the odds ratio among the two assays were analyzed using Fisher's exact test.

RESULTS

Characteristics of subjects

The demographic, aPL characteristics and anticoagulant treatment details of the subjects are shown in Table I.

Annexin A5 Resistance Assay

Patients with a history of obstetric PAPS (Figure 1, group A, $n=70$) had a significant reduction of AnxA5 anticoagulant ratios (mean $\pm$ SD: $211 \pm 35\%$; median 216%, range 130-282%) compared to the normal healthy controls (Group D, $n=30$; $247 \pm 19\%$; median 247%, range 217-283%, $p < 0.0001$). It is notable that 41% (29 out of 70) of the patients within group A had resistance to AnxA5 anticoagulant activity (i.e., the AnxA5 anticoagulant ratios were below two SD of the mean of normal healthy controls). The patients in the middle and lowest tertile of AnxA5 anticoagulant ratios had an odds ratio for obstetric APS of 2.4 (95% CI 0.9-6.3) and 58.0 (95% CI 3.3-1021.5), respectively (Table 2).

Patients with thrombotic PAPS (Figure 1, group B) also showed significant reduction of AnxA5 anticoagulant ratios compared to the normal healthy controls (mean $\pm$ SD: $206 \pm 37\%$; median: 208%, range 132-269%; $p < 0.0001$); 52% (26 out of 50) of the patients within group B had reduced AnxA5 anticoagulant ratios. Plasma from those with isolated aPL (Figure 1, group C, $n=16$) also showed significant reduction of AnxA5 anticoagulant ratios (mean $218 \pm 36\%$; median 225%, range 146-270%) compared to healthy controls ($p=0.007$); 38% (6 out of 16) of the patients in this group had reduced AnxA5 anticoagulant ratios. There were no significant differences in AnxA5 anticoagulant ratios between the groups A and B ($p=0.51$), between the groups A and C ($p=0.36$) and between the group B and C ($p=0.24$) (Figure 1).

Anti-domain I Assay

The women with obstetric PAPS (Figure 2, group A, $n=50$) showed significantly elevated levels of anti-domain I IgG (OD: mean $\pm$ SD: 0.122 ± 0.131 ; median 0.056, range 0.021-0.498) compared to the normal healthy controls (Figure 2, group D; OD: mean $\pm$ SD: 0.058 ± 0.057 ; median 0.042, range 0.020-0.323; $p=0.002$); 20% of the patients in this group (14 out of 70) had the IgG levels that were above two SD (i.e., the OD >0.172) of the mean of normal healthy controls. The patients with thrombotic PAPS (Figure 2, group B) also showed significantly elevated levels of the anti-domain IgG (0.142 ± 0.135 ; median 0.073, range 0.018-0.474) compared to the normal healthy controls ($p=0.002$); 30% of the patients in group B (15 out of 50) had elevated levels of anti-domain I IgG. The patients with isolated aPL antibodies (Figure 2, group C) also showed significantly elevated levels of anti-domain I IgG (0.092 ± 0.081 ; median 0.068, range 0.028-0.345) compared to the normal healthy controls (group D; $p=0.03$); two patients (13%) in this group had elevated levels of the IgG that were above two SD of the normal healthy controls. The patients with obstetric PAPS in the middle and highest tertile of anti-domain I IgG had an odds ratio for obstetric PAPS of 3.4 (95% confidence interval of 1.2-9.4) and 6.3 (95% confidence interval of 1.8-21.6), respectively (Table 3). There were no significant differences in the levels of anti-domain I IgG between the groups A, B and C (Figure 2).

Correlation

Analyses were performed to determine whether there might be a correlation between the assays for AnxA5 anticoagulant activity and anti-domain I IgG within the groups (Table 4). There was a significant inverse correlation between AnxA5 resistance and anti-domain I IgG within group A, those with obstetric PAPS ($r=-0.49$), and group B, those with thrombotic PAPS ($r=-0.60$; $p < 0.0001$ for both groups). Within group C, the isolated aPL antibodies, the inverse correlation between the two assays was weaker and was not statistically significant ($r = -0.23$; $p=0.40$), although it was based on only 16 subjects. Similarly, group D, the normal healthy controls showed a weak inverse correlation between these two parameters that was not statistically significant ($r=-0.18$; $p=0.35$). Interestingly, 32 out of 166 tested plasmas had elevated anti-domain IgG, of which, 27 (27/32; 84%) had significantly reduced AnxA5 anticoagulant ratio (Figure 3). 134 out of 166 tested plasmas had anti-domain I IgG levels that were within the mean ± 2 SD, of which 33 (33/134; 25%) had significant reduced AnxA5 anticoagulant ratio (Figure 3).

COMMENT

Although it was previously reported that a significant proportion of otherwise unselected women with histories of unexplained recurrent spontaneous pregnancy losses have reduced AnxA5 anticoagulant activity¹⁴, the prevalence of resistance to AnxA5 anticoagulant activity in women who met the current criteria for antiphospholipid syndrome¹ by having had obstetric complications has not been established. We also compared the results to

individuals with antiphospholipid antibodies and previous vascular thrombosis (thrombotic APS), and individuals with isolated aPL antibodies who did not have histories for obstetrical or thrombotic complications, and normal healthy controls. In addition, since a previous study had reported a negative correlation between AnxA5 anticoagulant activity and elevated anti-domain I β 2GPI IgG levels in patients with thrombotic APS, we investigated there might be a similar correlation in women with histories for obstetric APS.

We found that women with obstetric APS showed a significant reduction in mean AnxA5 anticoagulant activity, compared to controls and patients with isolated aPL. In view of our finding that about 41% of the patients with obstetric APS had AnxA5 anticoagulant ratios that were below two SD of the mean of normal healthy controls, we hypothesize that resistance to AnxA5 may be a mechanism for disease in this subset of APS patients. Although these results are consistent with the hypothesis that antibody-mediated disruption of annexin A5 function may directly affect pregnancy, validity of this idea would need to be tested by direct experimentation – e.g. evidence that the same antibodies that are yielding AnxA5 resistance in a patient's blood test are having a similar effect on AnxA5 function in their placenta.

There is strong evidence that AnxA5 does indeed have a significant role in maintaining placental function. AnxA5 is highly expressed by syncytiotrophoblasts in an apparently constitutive manner² and is localized on the apical membranes of syncytiotrophoblasts² where it faces the maternal blood circulating through the intervillous space. A common haplotype in the promoter region of the AnxA5 gene – designated M2 – results in reduced placental expression of AnxA5^{17,18} and is associated with increased risk for recurrent spontaneous pregnancy losses^{18,19}. This haplotype also appears to have a prothrombotic effect in the systemic vasculature since it is also associated with an increased risk of pregnancy-related venous thromboembolism²⁰. Furthermore, in an animal model, AnxA5 was shown to be necessary for preserving placental integrity²¹; infusion of pregnant mice with anti-AnxA5 antibodies resulted in placental infarction and pregnancy losses²¹.

There has been a question about whether the aPL-mediated mechanism for pregnancy complications might be different from the aPL-mediated mechanism for thrombotic complications. The current results, in keeping with previous studies^{7,12}, indicate that this mechanism is not unique for pregnancy but is also applicable to both forms of APS.

The results of AnxA5 anticoagulant activity correlated with levels of anti-domain I IgG (Figure 3), and are consistent with our previous report in APS patient with previous thrombotic events (thrombotic APS)¹³. However, in contrast to that study, which was done with small, but well-defined, groups of patients, the correlation, though statistically significant, was more modest. Based on the current results, we hypothesize that the patients with decreased AnxA5 anticoagulant ratios but without elevated antibodies against domain I of β 2GPI represent individuals who have antiphospholipid antibodies that recognize other domains on the same protein or possibly against other phospholipid-binding cofactor proteins. Furthermore, the distribution of anti-domain I antibodies in both those with obstetric APS and those with thrombotic APS is not Gaussian, indicating that there may be different antibody populations of clinical interest, one of which has affinity for domain I. We plan to address these questions in future studies.

One of the major practical issues in managing patients with aPL, is the lack of data available to advise asymptomatic individuals who have aPL antibodies of their future risks of complications, for many may never develop either thrombotic or obstetric complications, and others may develop them after several years. It will therefore be interesting to investigate prospectively whether assays for AnxA5 resistance and anti- β 2GPI domain I

antibodies may be risk predictors of obstetric and thrombotic complications in a cohort of asymptomatic patients with isolated aPL.

In summary, women with obstetric APS were more likely to have AnxA5 resistance and also anti- β 2GPI domain I antibodies than the control group. These results are consistent with the hypothesis that AnxA5 resistance is a mechanism for pregnancy losses associated with aPL, and those antibodies with anti-domain I may mediate this effect along with antibodies of other, yet to be described, epitope specificities. These new functional and epitope-specific immunoassays may identify specific mechanisms for aPL-mediated complications in subsets of patients and may thereby open paths toward identifying targeted therapies for this disorder.

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Figure 1. AnxA5 resistance assay

Obstetric PAPS patients (group A) and the thrombotic PAPS patients (group B) showed significant reduction of AnxA5 anticoagulant ratios as compared to the normal healthy controls (both $p < 0.0001$). The patients with isolated aPL antibodies (group C) also showed significant reduction of AnxA5 anticoagulant ratios compared to the normal controls ($p = 0.007$). There were no significant differences in AnxA5 anticoagulant ratio between the groups A and B and between the groups A and C and between the groups B and C. The horizontal lines are shown the mean of each group; the dashed lines are shown the mean plus and minus two standard divisions of the 30 normal healthy controls.

Figure 2. Anti-domain I IgG ELISA

Obstetric PAPS patients (group A) and the thrombotic PAPS patients (group B) had significantly elevated levels of anti-domain I IgG as compared to the normal healthy controls (both $p=0.002$). Those with isolated aPL antibodies (group C) also showed significantly elevated levels of anti-domain I IgG compared to the normal non-pregnant controls ($p=0.03$). There were no significant differences in the levels of antidomain I IgG between the groups A and B, between the groups A and C and between the groups B and C. The horizontal lines are shown median values for each group. The dashed line shows the mean plus two standard divisions of the 30 normal healthy controls.

Figure 3. Correlation between anxA5 anticoagulant ratio results and antidomain I IgG ELISA
 32 out of total 166 tested plasmas had elevated antidomain I IgG, of which, 27 (84%) had AnxA5 anticoagulant ratio that were below 2 standard deviations of the normal healthy controls. 134 out of 166 tested plasmas had antidomain I IgG levels that were within 2 standard deviations of the normal healthy controls, of which, 33 (25%) had AnxA5 anticoagulant ratios that were beneath 2 standard deviations of the normal healthy controls.

Table 1

Demographic, diagnostic and treatment details of the groups of patients studied

	Group A Obstetric APS	Group B Thrombotic APS	Group C aPL only	Group D Healthy controls
Number	70	50	19	30
Age median (range)	40 (25-58)	41 (20-64)	38 (22-68)	
Female: male	70:0	39:11	15:4	30:0
aPL status				
Thrombotic history:	23 (30%)	50 (100%)	0	0
Arterial only	8 (11%)	20 (40%)		
Venous only	9 (13%)	21 (42%)		
Venous & arterial	6 (8%)	9 (18%)		
Previous pregnancy morbidity				
Rec first trimester loss ^a	15			
Death of fetus > 10/40 ^b	18			
Placental insufficiency ^c	15			
Both a & b	11			
Both b & c	8			
Both a & c	1			
a & b & c	2			
Antithrombotic medication				
Aspirin	32 (46%)	6 (12%)	9 (47%)	
Warfarin	27 (39%)	40 (80%)	0	
LMWH	0	1 (2%)	0	
Nil	11 (16%)	8 (16%)	10 (53)	

Key

aPL- antiphospholipid antibody

APS- antiphospholipid syndrome LA- lupus anticoagulant

ACA- anticardiolipin antibody

^a ≥3 consecutive spontaneous abortions at <10/40 gestation, with maternal anatomic or hormonal abnormalities and paternal or maternal chromosomal causes excluded^b ≥ 1 deaths of a morphologically normal fetus ≥ at 10/40 gestation^c ≥ 1 premature births of a morphologically normal neonate before the 34/40 gestation because of: eclampsia or severe pre-eclampsia or placental insufficiency

Table 2

Resistance to AnxA5 Anticoagulant Activity and Odds Ratios of Women Experiencing Obstetric PAPS (n=70 cases vs. 30 normal healthy controls) (relative to the highest tertile of AnxA5 anticoagulant activity values)

Characteristic	Cases <i>n</i> (%)	Controls <i>n</i> (%)	Odds ratios	95% CI
AnxA5 anticoagulant ratio, % tertile				
1 [>233]	18 (26%)	19 (63%)	1.0	Reference
2 [205-233]	25 (36%)	11 (37%)	2.4	0.9-6.3
3 [<205]	27 (39%)	0 (0%)	58.0	3.3-1021.5
<i>p-trend</i>			<0.0001	

Table 3

Levels of Anti-domain I IgG and Odds Ratios of Women Experiencing Obstetric APS

Characteristic	Cases <i>n</i> (%)	Controls <i>n</i> (%)	ORs	95% CI
Anti-domain I IgG, optical density tertile				
1 [<0.041]	18 (26%)	18 (60%)	1.0	Reference
2 [0.041-0.081]	27 (39%)	8 (27%)	3.4	1.2-9.4
3 [>0.081]	25 (36%)	4 (13%)	6.3	1.8-21.6
<i>p-trend</i>			0.001	

Table 4

Spearman correlation between Annexin A5 resistance and anti-domain I IgG antibodies

Group	N	Spearman correlation coefficient	P value
Obstetric APS	70	−0.49	<0.0001
APS without obstetric APS	50	−0.60	<0.0001
aPL antibodies alone	16	−0.23	0.40
Normal healthy controls	30	−0.18	0.35
All subjects	166	−0.49	<0.0001