Mechanisms of thrombosis in the antiphospholipid syndrome

G. Espinosa¹, R. Cervera¹, J. Font¹, J.C. Reverter², Y. Shoenfeld³

¹Department of Autoimmune Diseases, Institut Clinic d’Infeccions i Immunologia (ICII), Hospital Clinic, Barcelona, Catalonia, Spain.
²Haemostasis and Haemotherapy Department, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clinic, Barcelona, Catalonia, Spain.
³Department of Medicine «B» and Center of Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, and Sackler Faculty of Medicine, Tel-Aviv University, Israel.

MECANISMOS DE TROMBÓTICOS EN EL SÍNDROME ANTIFOSFOLÍPIDICO

INTRODUCTION

The antiphospholipid syndrome (APS) is diagnosed when arterial or venous thrombosis or recurrent miscarriages occur in a person in whom laboratory tests for antiphospholipid antibodies (aPL) (anticardiolipin antibodies [aCL], lupus anticoagulant [LA], or both) are positive⁴. This syndrome is considered primary if it is not associated with any other underlying disease⁴, or secondary if it appears in association with other autoimmune disorders, mainly systemic lupus erythematosus (SLE)⁴.

ABSTRACT

Patients with antiphospholipid syndrome (APS) have an increased risk of venous and arterial thrombosis, recurrent pregnancy loss, and/or thrombocytopenia. These clinical manifestations are associated with the presence of antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL) and lupus anticoagulant (LA). Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. It is known that aPL are directed against phospholipid-binding proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets. The involvement of aPL in clinically important normal procoagulant and anticoagulant reactions and on certain cells altering the expression and secretion of various molecules may offer a basis for definitive investigations of possible mechanisms by which aPL may develop thrombotic events in patients with APS. In this article, we review the mechanisms by which aPL may develop thrombotic events in patients with APS.

KEY WORDS: Antiphospholipid syndrome / Antiphospholipid antibodies / Pathogenic mechanisms / Thrombosis.
It is known that aPL are directed against phospholipid-binding proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets\(^5\). The main protein associated with aCL activity is \(\beta\)-glycoprotein I (\(\beta\)-GPI) bound to phospholipids\(^9\). \(\beta\)-GPI is a highly glycosylated single-chain protein that is present in plasma and avidly binds to negatively charged phospholipids such as cardiolipin, phosphatidylserine, or phosphatidylethanolamine\(^8\).

Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. Several pathogenic mechanisms have been proposed, including inhibition of endothelial release of prostacyclin\(^9\), alterations in protein C-protein S pathway\(^10\), a direct procoagulant effect on platelets\(^11\), and impairment of fibrinolysis\(^10\). The aPL appear to play a direct pathogenic role and the APS is now widely accepted as an example of an autoantibody-mediated disease\(^12\). Given the heterogeneity of clinical manifestations in APS it is likely that more than one pathophysiological process may play a role. A wide variety of procoagulant mechanisms have been proposed and the major candidates are listed in Table I. Proposed pathophysiological mechanisms may be categorised into two types. First, aPL may act in vivo by disrupting haemostatic reactions occurring on cell membranes. The aPL may alter the kinetics of the normal procoagulant and anticoagulant reactions by cross-linking membrane-bound proteins, by blocking protein-protein interactions, and/or by blocking the access of other proteins to the phospholipid membrane. Second, aPL may stimulate certain cells thereby altering the expression and secretion of various molecules. In this article, we review the mechanisms by which aPL may develop thrombotic events in patients with APS (Fig. 1). It should be kept in mind that this is an area of active research and new data may point to other directions in the near future.

### INHIBITION OF ANTICOAGULANT REACTIONS

**Inhibition of the Protein C Pathway**

Protein C, a vitamin K-dependent plasma glycoprotein, is the precursor of a serine protease. Protein C becomes activated when thrombin binds to thrombomodulin, a constitutively expressed protein on the surface of vascular endothelial cells. Activated protein C (APC) is a physiological anticoagulant through its potential to inactivate clotting factors Va and VIIIa, which results in inhibition of further thrombin formation. Protein S, another vitamin K-dependent protein, amplifies the activity of APC. Protein S forms a 1:1 complex with APC on phospholipid surfaces. Protein S circulates in plasma in two forms: as a free protein and in a bimolecular complex with C4b-binding protein. Only the free form of protein S has APC cofactor activity\(^13\).

The assembly of the APC-protein S complexes on anionic phospholipid surfaces is essential for the catalytic activity. It is logical to assume that when aPL inhibit the binding of the clotting factors, they also inhibit the binding of protein C and protein S and thereby their activity. Inhibition of both protein C activation and the function of APC have been observed in association with APS\(^14\). The aPL can interfere with the protein C system in different ways\(^15\). Smirnov et al.\(^14\) showed that aPL required the presence of phosphatidylethanolamine to observe anti-APC activity. Purified \(\beta\)-GPI inhibits the binding of protein C to phospholipids much better than the binding of prothrombin, resulting in a prothrombotic state\(^15\). The aPL recognise protein C only in the presence of \(\beta\)-GPI\(^14\). These results suggest that aPL-induced protein C dysfunction is mediated by \(\beta\)-GPI. \(\beta\)-GPI itself can inhibit protein C activation by thrombin/thrombomodulin using thrombomodulin incorporated in cardiolipin vesicles\(^15\). However, \(\beta\)-GPI has little or no effect on protein C activation occurring on endothelial cells, in the presence or absence of anti-\(\beta\)-GPI antibodies\(^14\). Autoantibodies directed against components
of the protein C pathway have been detected in some APS patients. These included antibodies to thrombomodulin, protein C, and protein S. Additionally, autoantibodies to C4b-binding protein have been reported.

There are a number of publications describing acquired protein C or S deficiencies in isolated patients with APS, although studies in larger populations of aPL-positive patients failed to show a correlation between decreased protein C plasma levels and the presence of aPL.

Inhibition of Antithrombin Activity

Antithrombin is the major inhibitor of factors IXa, Xa, and thrombin. Heparan sulphate proteoglycan is expressed on vascular endothelium and plays an important role in vascular structure and function. Vascular heparan sulphate proteoglycan is required for the activation and optimal anticoagulation activity of antithrombin. Autoantibodies to heparan sulphate proteoglycan have been detected in plasma of patients with SLE. An APS patient with normal antigenic levels of antithrombin, but low functional activity, has also been reported.

Displacement of Annexin A5

Annexin A5 is a potent anticoagulant protein whose activity is consequence of its high affinity for anionic phospholipids and the inhibition of phospholipid-dependent coagulation reactions. Annexin A5 appears to play a thrombomodulatory role in the placental circulation where it is necessary for maintenance of placental integrity. Some patients with the APS have evidence for antibodies that specifically recognise annexin A5 and the presence of these antibodies has also been reported to be increased in patients with thrombosis. However, other studies did not find this association, and one group found no significant associations between anti-annexin A5 antibodies and any clinical manifestation. Additionally, the presence of antibodies against annexin A5 has been reported to be increased in patients with recurrent miscarriages.

Since both aPL and annexin A5 have affinity for anionic phospholipids, it was hypothesised that the aPL, without anti-annexin A5 specificities, might interfere with the assembly of the antithrombotic annexin A5 shield over phospholipids on membranes. IgG fractions from APS patients reduce the quantity of annexin A5 on cultured trophoblasts and endothelial cells and also accelerate the coagulation of plasma which is incubated with these cells following their exposure to the antibodies. This aPL-mediated reduction of annexin A5 also occurs in noncellular phospholipid surfaces and appears to occur through displacement by aPL in a β2GPI-dependent manner.
Recently, Hanly et al. demonstrated that aPL reduce the binding of annexin A5 to phospholipid-coated microtitre plates. This reduction is dependent upon anti-β2GPI antibodies and correlates with clinical thrombosis.

Inhibition of β2GPI Anticoagulant Activity

β2GPI is a highly glycosylated single-chain-protein present in plasma that avidly binds to negatively charged phospholipids such as cardiolipin, phosphatidylserine or phosphatidylinositol. The physiological function of β2GPI is uncertain. This protein exhibits anticoagulant properties; it inhibits platelet aggregation induced by ADP, intrinsic coagulation pathways, the prothrombinase activity of platelets, and the activation of protein C in the presence of phospholipids. However, familial deficiency of β2GPI is not a risk factor for thrombosis. It has been suggested that β2GPI may contribute to the pathogenesis of APS-associated thrombosis. In physiological conditions, β2GPI may inhibit phospholipid-dependent haemostasis reactions, but β2GPI is by itself only a weak anticoagulant. However, aPL may enhance the affinity of β2GPI to phospholipids and, then, β2GPI may become a real competitor to phospholipid-dependent haemostasis reactions. Therefore, since β2GPI has strong binding properties to negatively charged proteins or phospholipids involved in coagulation processes, it is likely that aPL may hamper β2GPI-associated coagulation steps. However, decreased levels of β2GPI are not usually found in APS. In conclusion, more studies to elucidate the physiological role of β2GPI are needed.

CELL-MEDIATED EVENTS

On Monocytes: Expression of Tissue Factor

Tissue factor is a single chain transmembrane protein composed of 263 amino acid residues that is widely accepted to be a major physiological initiator of blood coagulation in vitro. Tissue factor is normally not expressed by intravascular cells but can be induced in monocytes and endothelial cells by different physiological or nonphysiological stimuli, such as bacterial lipopolysaccharides, tumour necrosis factor, interleukin-1, or immune complexes. Induced tissue factor forms a tissue factor/activated factor VII complex in the presence of phospholipids and activates rapidly factors IX and X leading to thrombin generation. The tissue factor pathway is modulated by the tissue factor pathway inhibitor. Tissue factor pathway inhibitor inhibits factors VIIa and Xa by forming a quaternary complex and may also inhibit factor Xa directly in a phospholipid-independent manner.

Our group has found an increased procoagulant activity attributed to tissue factor expression on monocytes induced by murine monoclonal aCL that can induce APS. Subsequently, attention has focused on increased tissue factor expression and procoagulant activity on circulating blood monocytes. Cuadrado’s group has shown that tissue factor-related procoagulant activity and tissue factor mRNA levels in monocytes are increased in primary APS patients with thrombosis when compared with those without thrombosis and with healthy controls. Clinically, increased tissue factor was associated with IgG aCL and a history of thrombosis. Our group has shown that purified IgG aCL from three APS patients with previous thrombotic episodes induce a significant increase in both monocyte procoagulant activity and tissue factor expression, as compared with purified IgG aCL from two SLE individuals without thrombosis. Moreover, we have also reported an increase on tissue factor expression on normal monocytes using affinity-purified IgM aCL (with anti-β2GPI activity) from two APS patients with a history of thrombosis.

Inhibition of tissue factor pathway inhibitor is another mechanism by which autoantibodies may upregulate the tissue factor pathway in APS. Functional anti-tissue factor pathway inhibitor activity was detected in a subset of APS patients, and anti-β2GPI antibodies have been implicated. Rouhey’s group has recently detected autoantibodies directed against tissue factor pathway inhibitor in APS patient sera and found an association between these antibodies and arterial thrombosis and stroke.

On Endothelial Cells

Enhanced procoagulant activity: Expression of tissue factor and adhesion molecules

Endothelium is now emerging as a major site of regulation of haemostasis. Unperturbed endothelial cells maintain blood fluidity through several anticoagulant mediators. However, when perturbed, endothelial cells serve as a surface that can support many steps in the coagulation cascade by producing tissue factor and plasminogen-activators inhibitors and synthesising specific binding sites for several coagulation factors. Potentiation of human umbilical vein endothelial cells (HUVEC) procoagulant activity by aPL-containing sera from SLE patients is strongly decreased after depleting IgG from their sera. Fractions of human APS sera containing monomeric IgG, IgM, or IgA, as well as high molecular weight IgG, each cause HUVEC to increase the procoagulant activity characteristic of tissue factor. These results indicate that the factor responsible for the induction of tissue
factor activity resides, at least in part, in the serum IgG fraction. More recently, human anti-β2-GPI IgM monoclonal antibodies as well as polyclonal anti-β2-GPI antibodies have been shown to induce tissue factor at both protein and mRNA level in HUVEC monolayers in vitro.

Endothelial cells incubated in the presence of aPL preparations were shown to up-regulate adhesion molecule (E-selectin, ICAM-1 and VCAM-1) expression and pro-inflammatory cytokine (IL-1β and IL-6) secretion. Mononuclear leukocytes, adhering to activated endothelium, can be activated by endothelial inflammatory cytokines and induced to display a procoagulant phenotype. Indirect evidence that the endothelial activation does occur also in vivo comes from recent observations. Increased plasma levels of soluble VCAM-1 were found in primary APS patients with recurrent thrombotic events, and elevated levels of tissue plasminogen activator and von Willebrand factor (as endothelial perturbation markers) were associated with aPL in SLE.

In contrast to these findings, Fijns et al. did not find any significant difference in soluble adhesion molecule, soluble thrombomodulin and von Willebrand factor plasma levels in APS secondary to SLE.

Impaired Fibrinolysis

The different population of autoantibodies observed in APS may disturb fibrinolysis and contribute thereby to vascular complications. Thrombosis may therefore result from an impaired or insufficient fibrinolytic cellular response to vascular injury provoked by autoantibodies. Some reports demonstrated the existence of impaired fibrinolysis associated with thrombosis and aPL in SLE. In recent studies, it has been shown that the hypofibrinolysis detected in these patients is most probably a manifestation of endothelial cell dysfunction, as indicated by increased plasma levels of PAI-1 and t-PA antigen. These manifestations of endothelial cell dysfunction have been found in association with antibodies directed against endothelial cells, or with the presence of immune complexes, thus suggesting that endothelial cells are important sites of action for antibodies that have a role in the pathogenesis of thrombosis.

Similar manifestations of endothelial dysfunction have also been found in primary APS, though at present there are no solid arguments to propose a direct association between aPL and impaired fibrinolysis in the thrombotic manifestation of APS.

Koike’s group investigated the effect of both β2-GPI and aPL on the activity of extrinsic fibrinolysis. The remaining tissue-plasminogen activator/α2-antiplasmin (t-PA) of the sample consisting of β2-GPI, two-chain recombinant t-PA, PAI-1 was measured by a chromogenic assay. Without PAI-1, β2-GPI did not affect t-PA activity. When PAI-1 was added to t-PA, the remaining t-PA activity was increased from 49% to 60% by the addition of β2-GPI. The effect of β2-GPI did not require phospholipids. The β2-GPI seems to protect t-PA activity from the inhibition by PAI-1. When monoclonal aCL from a patient with APS were further added to the mixture with a diluted phospholipid to investigate the influence of aPL, the remaining t-PA activity decreased to 50% and 81%. Monoclonal aCL appeared to inhibit the effect of β2-GPI, that is, these monoclonals inhibited the fibrinolytic activity by an elevation in PAI-1 activity. These results suggest the possibility that the impairment of fibrinolytic activity by aCL is one of reasons for the increased incidence in thrombosis in patients with aCL.

Dysregulation of Eicosanoids: Decreased Endothelial Cell Prostacyclin Production and Increased Platelet Thromboxan A2 Production

Decreased endothelial cell prostacyclin (PGI2) production and increased thromboxane A2 (TXA2) production by platelets have both been implicated as mechanisms predisposing to thrombosis in patients with APS.

The biosynthesis of eicosanoids depends primarily on the liberation of unesterified arachidonic acid from membrane phospholipids as a consequence of the activation of phospholipase A2 by different cell stimulation agents. Unesterified arachidonic acid is rapidly transformed into unstable intermediates such as prostaglandin (PG) H2 by PGH-synthase (also referred to as cyclo-oxygenase), and leukotriene A4 by a lipooxygenase. Although practically all cells are capable of synthesising PGH2 intermediates, their further metabolism depends on the specific enzymes that are present in each tissue. Platelets contain thromboxane-synthase generating TXA2 whereas endothelial cells have prostacyclin-synthase transforming PGH2 into PGI2. The biosynthesis of eicosanoids in vivo can be measured by evaluating the urinary excretion of platelet and vascular cell metabolites.

PGI2 is the most important natural inhibitor of platelet aggregation and it is also a vasodilator, being considered as one of the antithrombotic mechanisms of vascular endothelium. Its effect is contrary to that of TXA2. It was suggested that LA could interfere with arachidonic acid release from phospholipid membranes because the effect was abolished in the presence of arachidonic acid. In addition, the inhibition of PGI2 synthesized by endothelial cells has been associated to arterial but not to venous thrombosis. In contrast, some authors obtained discrepant results.
Enhanced Platelet Activation/Aggregation

Platelets play a central role in primary haemostasis involving adhesion to the injured blood vessel wall, followed by platelet activation, granule release, shape change, and rearrangement of the outer membrane phospholipids and proteins transforming them into a highly efficient procoagulant surface.

The interaction of aPL with platelets can occur in at least three different ways. First, immunoglobulins may bind through the Fab fragment with specific platelet antigens in a classic antigen-antibody reaction; second, immune complexes may bind to platelets via FeγRII receptor; and third, aPL, like other immunoglobulins, may bind to platelets in a non-specific manner by mechanisms not well characterised but probably related to platelet membrane injury. The last mechanism does not seem to have a pathophysiological role in APS-related thrombosis.

Some studies have demonstrated that aPL may bind to platelet surface, and this binding is higher on activated or damaged platelets than in resting ones. Shi et al. observed that human aCL only bind to activated platelets but not to resting platelets and this binding was ß2GPI-dependent way. Our group demonstrated that monoclonal aCL obtained from patients with APS increased platelet interaction with the subendothelium under flow conditions. Moreover, it was demonstrated that both Fab and Fc fragments of the antibodies were essentials for this activity. The only FeγR molecules present on platelets are the FeγRII. Activation of the FeγRII receptor causes platelet activation and granule release. With these results, the following hypothesis has been proposed. Small initial platelet activation is produced by physiological or pathological conditions resulting in the expression of phospholipids on the platelet surface. The binding of ß2GPI to these phospholipids may occur. The aPL subsequently may bind to the formed ß2GPI-phospholipid complexes, and then, interact through their Fc portion with the platelet surface FeγRII receptors. Through this interaction, platelets may be activated and a vicious circle of cellular activation may be created finally ending in a thrombotic event. Activation of the FeγRII receptor by the aPL bound to ß2GPI causes platelet activation and TXA2 generation.

Recently, we demonstrated that three monoclonal aCL with anti-ß2GPI activity promoted platelet interaction with collagen-rich subendothelium under flow conditions with a clear ß2GPI dependence.

The FeγRII receptor has high affinity for the Fc portion of IgG, either contained in immune complexes or bound to an antigen on the platelet surface. For this reason, it is probable that the monoclonal aCL bound to ß2GPI-phospholipid complexes on platelet membranes exert their action through complement activation, besides of FeγRII receptor activation. The complement generated in presence of aPL bound to negatively charged phospholipids may cause platelet activation and, eventually, platelet destruction. This hypothesis has been supported by some findings.
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On the other hand, increased levels of inactivated terminal membrane attack complex (C5b-9) were found in patients with aPL(100). On the other hand, it is known that C5b-9 causes platelet activation(101,102), and complement activation by aPL bound to cardiolipin liposomes may cause platelet activation. In addition, it has been demonstrated that C5b-9 action may increase the transbilayer migration of phosphatidyliosine in the platelet membrane(103), causing increased binding of β2GPI, and, then, C5b-9 activation, and, in a vicious circle, platelet activation. In this case, an initial activation of platelet is needed. Then, negatively charged phospholipids are exposed in a small extent on the platelet surface and aPL may bind to these exposed phospholipids or to proteins bound to these phospholipids. The aPL fixed on the platelet surface may induce complement activation in a Fe-independent manner causing more platelet activation(103).

CONCLUSIONS

Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. Recent data indicate that many of the autoantibodies associated with APS are directed against a number of plasma proteins and proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets. The involvement of aPL in clinically important normal procoagulant and anticoagulant reactions and on certain cells altering the expression and secretion of various molecules may offer a basis for definitive investigations of possible mechanisms by which aPL may develop thrombotic events in patients with APS.

CORRESPONDENCE TO:

Ricard Cervera, MD PhD
Department of Autoimmune Diseases
Hospital Clinic
Villarroel 170, 08036 Barcelona, Spain.
Phone: 34 93 2275774. Fax: 34 93 2275774.
e-mail: rcervera@clinic.ub.es

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