A Comprehensive Review of Thrombogenic Mechanisms in APS

Renán Aguilar-Valenzuela, Laura Aline Martínez-Martínez and Silvia S. Pierangeli*

Division of Rheumatology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA

Abstract: The antiphospholipid syndrome (APS) is an acquired thombophilia, which is characterized by one or more thrombotic episodes and obstetric complications in the presence of antiphospholipid (aPL) antibodies (Abs). aPL Abs are detected by laboratory tests such as lupus anticoagulant (LA), anticardiolipin (aCL) and anti-β_2-glycoprotein I (β_2GPI) Abs. This article reviews the most current pathophysiological aspects of APS with emphasis in thrombotic and pro-inflammatory mechanisms mediated by aPL antibodies.

Keywords: Antiphospholipid syndrome, acquired thrombophilia, pathogenesis, thrombosis.

ANTIPHOSPHOLIPD SYNDROME: DEFINITION AND EPIDEMIOLOGY

Antiphospholipid Syndrome (APS) is an autoimmune and multisystem disorder of recurrent thrombosis, pregnancy loss, and thrombocytopenia associated with the presence of antiphospholipid (aPL) antibodies (Abs), a persistently positive anticardiolipin (aCL) and/or lupus anticoagulant (LA) tests [1, 2]. It is now well established that aPL Abs are heterogenous and bind to various protein targets, among them the plasma protein β_2Glycoprotein I (β_2GPI) [3, 4]. Historically, aPL Abs were classified based on the clinical laboratory test in which they were detected (i.e. LA and aCL Abs). This classification is problematic in light of current understanding of the specificities of aPL Abs. Most of the antibodies detected in aCL and LA assays do not, in fact, recognize anionic phospholipids [5, 6]. A large body of data indicates that, in patients with APS, the majority of autoantibodies detected in aCL assays are directed against β_2GPI [3-6].

APS was first described in patients with systemic lupus erythematosus (SLE), more specifically in a subset of patients with SLE that had abnormal "LA" test [7-9]. APS was then classified as "secondary" (SAPS) in the presence of SLE and "primary" (PAPS) in the absence of SLE or other autoimmune disorders. In the general population, PAPS is the most common cause of acquired thrombophilia and accounts for 15-20% of all episodes of deep vein thrombosis with or without pulmonary embolism, one third of new strokes occurring in patients under the age of 50 and 10-15% of women with recurrent fetal loss [9-14]. It has been estimated that from 2 to 5% of the general population have experienced an episode of deep vein thrombosis, suggesting that the prevalence of venous thrombosis associated with PAPS may be as high as 0.3 to 1% of the general population [9-14]. Thus APS may be one of the most common autoimmune diseases. APS also accounts for a significant proportion of thromboembolic disease and recurrent fetal loss in patients with SLE. aPL Abs are present in 30-40% of SLE patients and approximately one third of those with Abs, or 10-15% of all SLE patients, have clinical manifestations of APS [9-14]. Since thrombosis can affect any arterial or venous site in the body, the consequences of the disorder are often debilitating. Stroke, myocardial infarction, gangrene of the extremities, deep vein thrombosis, or occlusion of renal veins and inferior vena cava are some of the complications that can occur. Patients are usually young and often otherwise well. A recent study of patients with SLE showed that aCL positivity preceded the onset of a more severe form of SLE, as well as SLE complicated with thrombosis, pregnancy loss and thrombocytopenia [15]. Studies have found no difference between PAPS and SAPS with respect to the clinical complications, the timing of those complications, the prognosis or frequency of positive aCL, LA or other autoantibody tests. In addition, management of PAPS and SAPS is the same and prognosis does not appear to differ [16].

ANTIGENS RECOGNIZED BY aPL ANTIBODIES

aPL Abs owe their name to the fact that initially these Abs were believed to recognize anionic phospholipids. Nowadays, it is known that aPL Abs have specificity against some proteins with affinity for these phospholipids. Several target antigens have been described as being recognized by these Abs including: β_2GPI, prothrombin (PT), several components of the protein C system, annexin A5, tissue factor pathway inhibitor (TFPI); proteins of the fibrinolytic system and other proteins of the coagulation cascade, such as: Factor XII, XI, VII. Of these antigens, the most studied are β_2GPI and PT [17-25]. β_2GPI is a 54 kDa plasma glycoprotein that consists of five homologous domains. Domains I-IV each consist of 82 amino acids due to a 6-residue insertion and one 19-residue C-terminal extension cross-linked by an additional disulfide bond. Domain V is unique in its high content of lysine residues that has been shown to contribute to the formation of a positively charged PL-binding region [26]. β_2GPI interacts with diverse cell types, receptors and enzymes [27]. β_2GPI is synthesized mainly in the liver, which has a noticeable affinity for negatively charged molecules, such as anionic phospholipids, heparin, lipoproteins, and activated platelets [28]. Potential antithrombotic properties of β_2GPI have been identified. Hulstein *et al* found that

*Address correspondence to this author at the Division of Rheumatology, Department of Internal Medicine, University of Texas Medical Branch, Texas, 301 University Boulevard Galveston, TX 77555-0883, Brackenridge Hall 2.124, USA; Tel: 409-772-0222; Fax: 409-772-0223; E-mail: sspieran@utmb.edu

β_2GPI inhibits von Willebrand factor (VWF)-induced platelet aggregation. β_2GPI binds to the A1 domain of VWF but preferably when the A1 domain is in its active glycoprotein Ib alpha-binding conformation [29]. This mode of action could contribute to the thrombosis and consumptive thrombocytopenia observed in patients with anti- β_2GPI Abs.

PATHOGENIC MECHANISMS INVOLVED IN aPL ANTIBODIES THROMBOSIS

There is strong evidence that aPL Abs are pathogenic *in vivo* from studies that utilized animal models of thrombosis, EC activation and pregnancy loss [30-33]. However, the mechanisms by which aPL Abs mediate disease are only partially understood and our knowledge is limited by the apparent polyspecificity of the Abs, the multiple potential end-organ targets and the variability of clinical context that disease may present. aPL Abs are heterogeneous and it is known that more than one mechanism may be involved in causing thrombosis [34-40]. In fact, *in vitro* studies have reported that aPL Abs may cause thrombosis by interfering with activation of protein C, or inactivation of factor V by activated protein C, by inhibiting endothelial prostacyclin production, by impairment of fibrinolysis, by activating EC, monocytes and by exerting stimulatory effects on platelet function [34-40]. There is now also convincing data indicating that activation of complement mediates aPL-induced fetal loss, thrombosis and EC activation [41-47].

Alterations of the Coagulation and Fibrinolysis Systems by aPL Antibodies

β_2GPI is a cell surface-binding plasma protein. The affinity of β_2GPI for the cellular surfaces appears to be low. However, in the presence of Abs against β_2GPI, that affinity increases significantly. The increase in the affinity of β_2GPI for the plasmatic membrane can modify its function and affect the coagulation/fibrinolysis rate on the cellular surface when interfering with others proteins that bind to phospholipids such as coagulation factors and C protein [48].

Yang *et al* have shown that 28% of APS patients have Abs that react with plasmin that interfere with the plasmin-mediated lysis of fibrin clots, suggesting that plasmin may be an important driving Ag for some aPL specific B cells in APS patients [49]. Then, the induced anti-plasmin Ab may act either directly, by binding to plasmin and inhibiting its fibrinolytic activity, or indirectly, by cross-reacting with other homologous proteins in the coagulation cascade to promote thrombosis [49].

Recently, Chen *et al* have found that five out of seven patient-derived IgG monoclonal aCL Abs react with thrombin, activated protein C, and plasmin [50]. All three proteins are trypsin-like serine proteases (SP), and are highly homologous in their catalytic domains. Importantly, among these SP autoantigens, the reactive aCL Abs bind to plasmin with the highest affinity, and thus plasmin serve as a major driving autoantigen for some aCL Abs in approximately 30% of APS patients who are positive for IgG anti-plasmin Ab [50]. Lu *et al* studied plasmin-reactive aCL and has shown that these antibodies may bind to tissue plasminogen activator (tPA) and that some of the tPA-reactive aCL may inhibit tPA activity and, thus, may be prothrombotic in the host [51].

The Abs against PT may either have an anticoagulant or a procoagulant activity, based in their ability to interfere with the action of the prothombinase complex, and to act at level of the lipid surface [52]. The procoagulant activity of these Abs is based on: (i) increase of the binding of prothrombin to anionic phospholipids, which favors the formation of thrombin and (ii) interference on the action of the antithrombin (natural anticoagulant) [19].

In a significant percentage of the patients with APS, Abs against tissue factor pathway inhibitor (TFPI) activity have been found [21, 53]. IgG fractions of these Abs interfere with the TFPI favoring the generation of thrombin.

In addition, impaired fibrinolysis has been reported in patients with APS. Lower activity of intrinsic fibrinolysis in euglobulin fractions from APS patients has been demonstrated [54]. β_2GPI is proteolytically cleaved by plasmin in domain V (nicked β_2GPI) and becomes unable to bind to phospholipids, reducing antigenicity against aPL Abs. Nicked β_2GPI binds to plasminogen and suppresses plasmin generation in the presence of fibrin, plasminogen, and tPA, thus, nicked β_2GPI plays a role in the extrinsic fibrinolysis [55].

Effects of aPL Abs on Endothelial Cells and Monocytes

Investigators have shown that endothelial cells expressed significantly higher amounts of cellular adhesion molecules (CAMs) such as intercellular cell adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM-1) and E-selectin when incubated with aPL Abs and β_2GPI *in vitro* [56-58]. Our group has shown that aPL Abs activate endothelium *in vitro* and in mouse models and this correlated with enhancement of thrombus formation *in vivo* [33]. Utilizing ICAM-1, E-selectin and P-selectin knock out mice and specific anti-VCAM-1 monoclonal Abs, we demonstrated that endothelial cell-activating properties of aPL Abs are mediated by these CAMs [59, 60]. Accordingly, some investigators have shown increased levels of soluble adhesion molecules such as VCAM-1, P-selectin, etc in patients with aPL Abs and thrombosis [61, 62].

Tissue factor (TF) is a transmembrane protein present on the surface of activated cells and a member of the class II cytokine and hematopoietic growth factor receptor family [63]. It is located on the surface of a number of cell types, primarily monocytes, vascular EC and smooth muscle cells. When the integrity of the vasculature is breached, endothelial cells are induced to express cell surface TF, and TF may then interact with factor VIIa and initiate blood coagulation [63]. Inflammatory mediators and events, such as vascular injury and repair, induce expression of TF on surface of cells. Pro-inflammatory cytokines such as TNF-α and also bacterial lipopolysaccharides (LPS) induce activation of endothelial cells and expression of TF involving: translocation of nuclear factor-kappaβ (NF-$\kappa\beta$) to the nucleus of the cell and upregulation of adhesion molecules and TF expression [63, 64]. In endothelial cells, TF expression has been reported *in vivo* in association with neoplastic disease and cytokine activation in association with sepsis [65]. This inappropriate expression of TF may be responsible for thrombotic disorders and fibrin deposition as seen in disseminated intravascular coagulation and thromboembolic disease. TF upregulation has been advocated as an important mechanism to ex-

plain the pro-thrombotic effects of aPL Abs [63-68]. Studies have shown upregulation of TF expression and function in endothelial cells and monocytes treated with aPL Abs [66-68]. Moreover, studies have reported higher plasma levels of TF in APS patients than controls [69]. One study showed that both PAPS and SAPS patients have higher plasma levels of TF than do healthy controls [70-72]. Furthermore, patients with APS have increased surface expression of TF and function or procoagulant activity (PCA) in blood mononuclear cells [71, 72]. Fractions of APS sera containing monomeric IgG, IgM or IgA, as well as fractions containing IgG complexes, stimulate endothelial cells to produce more PCA than similar fractions of normal sera [73]. Kornberg *et al* reported that IgG aCL monoclonal Abs directly stimulated monocytes to generate PCA, whereas monoclonal Abs lacking aCL activity did not [74]. More recently, Zhou *et al* demonstrated that IgG from patients with APS significantly increased TF function and transcription in monocytes. Interestingly, dilazep, a platelet inhibitor and an adenosine uptake inhibitor known to block the induction of monocyte TF expression by LPS, inhibited aPL-mediated TF activity but did not show any effect on TFmRNA expression [68]. Hence, there is convincing evidence that aPL Abs induce endothelial cells and monocyte activation and a pro-coagulant and pro-inflammatory phenotype *in vitro* and *in vivo*.

Intracellular Events Induced by aPL Abs in Endothelial Cells and in Monocytes

Although there is convincing evidence that aPL Abs can stimulate monocytes and endothelial cells, relatively little is known about the cell surface receptors and intracellular signaling pathways involved. We first reported that aPL-induced upregulation of adhesion molecules (i.e. E sel) in endothelial cells induce activation of NF-κB *in vitro* [60]. These findings were subsequently confirmed by others [75, 76]. NF-κB is a complex group of heterodimeric and homodimeric transcription factors that are trapped in the cytoplasm as an inactive complex by IkB. Cell activation through cytokine stimulation, engagement of toll-like receptors (TLRs) or stress initiates a host-defense signalling pathway that can converge on an enzyme complex containing two IkB kinases. Upstream kinases, including members of the MAPK family, and NF-κB–activating kinase (NAK) can phosphorylate the IkK signalsome and initiate the NF-κB cascade. This process is initiated within minutes of surface receptor ligation, releases NF-κB and lead to its nuclear translocation, followed by initiation of gene transcription [77, 78]. The specific genes that are activated depend on the various NF-κB binding sequences in promoter regions as well as the components of the NF-κB dimers, inflammatory and immune responses. For instance, the transcription of many cytokine genes, including interleukin-6 (IL-6), IL-8, TNF-α and IL-1b is initiated by NF-κB activation. Induction of adhesion molecules on endothelial cells (VCAM-1, E-sel and ICAM-1) and TF and recruitment of inflammatory cells to extravascular sites is also mediated by this transcription factor [77-80]. Activation of NF-κB has also been shown to be a critical mediator in some autoimmune diseases such as rheumatoid arthritis [81].

P38 mitogen activated protein kinase (p38MAPK) is an important component of intracellular signaling cascades that initiate various inflammatory cellular responses. For exam-

ple, p38MAPK has been implicated as an important regulator of the coordinated release of cytokines by immunocompetent cells and the functional response of neutrophils to inflammatory stimuli [82]. Different stimuli can activate p38MAPK, including LPS and other bacterial products, TNF-α and IL-1, growth factors, and stresses such as heat shock, hypoxia, and ischemia/reperfusion. In addition, p38MAPK positively regulates a variety of genes involved in inflammation, such as TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2 and collagenase [82, 83]. P38 MAPK also activates transcriptional factors such as activating transcriptional factor-2, which forms a heterodimer with JUN family transcriptional factors and associates with the activator protein-1 (AP-1)-binding site. The promoter region of the TF gene contains two AP-1 binding sites and one NF-κB binding site, and these transcription factors have been proven required for maximal induction of TF gene transcription [82]. In platelets, p38MAPK is activated by stress such as heat and osmotic shock, arsenite, H_2O_2, α-thrombin, collagen and a thromboxane analog and is involved in the phosphorylation of cytosolic phospholipase A2 (cPLA2), with subsequent production of TXB2 [84]. In a recent study, we demonstrated that aPL-mediated platelet activation involves phosphorylation of p38 MAPK [85]. We also examined the involvement of NF-κB and p38 MAPK on aPL induced transcription, expression and function of TF on endothelial cells. The effects of the specific p38MAPK inhibitor SB 203580 (4-(4 fluorophenyl)-2 (4methylsulfinylphenyl)-(4pyridyl) 1 imidazole) and of MG132 (carbobenzoxyl-leucinyl leucinylleucinal), a specific inhibitor of NF-κB, on aPL-induced TF expression and function were evaluated *in vitro*. We showed that aPL Abs induce significant TF transcription, function and expression on EC, pronounced increase in pro-inflammatory cytokines (IL-6 and IL-8) and phosphorylation of p38 MAPK. By utilizing SB203580 and MG132, we demonstrated that both p38MAPK phosphorylation and NF-κB activation are required for *in vitro* aPL-induced TF upregulation [86]. These effects were significantly diminished by fluvastatin [87, 88]. These *in vitro* effects of aPL Abs, mediated by p38MAPK and NF-κB, were confirmed in monocytes by Bohgaki *et al* [76]. Subsequently, Simoncini *et al* showed that IgG from 12 patients with APS caused a large and sustained increase in reactive oxygen species (ROS) [89]. ROS acted as a second messenger by activating the p38 MAPK and its subsequent target, the stress-related transcription factor activating transcription factor-2 (ATF-2). ROS controlled the up-regulation of VCAM-1 expression by IgG-APS-stimulated HUVEC and the increase in THP-1 monocytic cells adhesion [89]. In another recent study, we showed that treatment of mice with aPL Abs induced significantly increased TF function in peritoneal cells and in homogenates of carotid artery *in vivo*, when compared to control mice and this correlated with enhanced thrombosis and EC activation *in vivo* [90]. These effects were inhibited *in vivo* by SB203580 and MG132 [90, 91]. Hence, there is convincing evidence that aPL/anti-β2GPI Abs induce endothelial cell activation and a pro-inflammatory/pro-coagulant phenotype *in vitro* and *in vivo*.

Interactions of aPL/Anti-β2GPI Abs with β2GPI

The bulk of the evidence favors aPL/anti-β2GPI Ab binding epitopes located with the N-terminal domain I (DI) [92-

94]. Furthermore, the ability of aPL/anti-β_2GPI Abs purified from patients with APS to bind DI of β_2GPI has been shown to be strongly correlated with the occurrence of thrombosis in those patients [95, 96]. The fact that pathogenic aPL bind primarily to epitopes in DI of β_2GPI has been shown by several groups using different techniques. These include the demonstration that variants of β_2GPI lacking DI or with point mutations in DI have reduced ability to bind aPL Abs derived from patients with APS. The same is not true for changes in the other domains. Drs. Rahman and Giles at University College London have developed the first (and so far the only) system for expressing DI in bacterial periplasm [97, 98]. This was achieved by creating a synthetic gene that encoded human DI by using codons that are preferentially expressed in bacteria. The gene was synthesised by recursive polymerase chain reaction and differed in nucleotide sequence from the human DI gene at 67% of positions. They used this expression system to create a series of site-directed mutations in DI, which allowed us to show that two distinct areas of DI are important in binding IgG aPL extracted from the blood of patients with APS. These regions were aspartic acid residues at positions 8 and 9 (D8-D9) and the region between arginines at 39 and 43 (R39-R43). In particular they found that the variant in which D8 and D9 were mutated to serine and glycine respectively (D8S,D9G) bound more strongly than wild-type DI to all 8 human aPL samples tested [96]. We then wanted to test whether DI and DI (D8S,D9G) inhibit the ability of aPL to induce thrombosis in-vivo. As shown in a recent publication by our group, intra-peritoneal injection of aPL enhances size and longevity of a femoral vein thrombus caused by a standard traumatic stimulus [99]. Both DI and DI (D8S,D9G) inhibited this aPL-induced enhancement of thrombosis in a dose-dependent manner and DI (D8S,D9G) was a more potent inhibitor than DI. These data underscore the possibility of using decoy peptides that from DI of β_2GPI to ameliorate thrombosis in APS. Clinical studies will be needed to confirm these observations in animal models.

Receptor for β_2GPI on Endothelial Cells

Studies with β_2GPI mutants and synthetic peptides showed that the molecule binds to endothelial cell membranes through that "putative PL-binding site" [100]. We subsequently showed that TIFI – a 20 synthetic peptide – that mimics the PL-binding domain (in region V) of β_2GPI, reversed thrombogenic effects of aPL/anti-β_2GPI Abs in mice and displaced the binding of fluorescinated (FITC) β_2GPI to human endothelial cells and murine peritoneal macrophages [101]. The data indicate that TIFI inhibits thrombogenic properties of human aPL/anti-β_2GPI Abs in mice by competing with β_2GPI and by preventing its binding to target cells. There is evidence that β_2GPI binding to endothelial cells through that region may involve, at least in part, heparan sulphate (HPS), a negatively charged structure on endothelial cell membranes [101,102].

β_2GPI has been shown to bind to different types of endothelial cells – the main tissue targets for thrombosis - and to trophoblasts and decidual cells – the main target for defective placentation and fetal loss [103-105]. Pathogenic aPL Abs recognize β_2GPI bound to these cells and were shown to affect cell functions leading to endothelial perturbation and trophoblast differentiation inhibition. Furthermore, endothe-lial cells are heterogeneous, displaying different phenotype and function depending on their different anatomical origin. In addition, it has been suggested that endothelial cell structures other than HPS might be also responsible for β_2GPI binding. Accordingly, studies have recently shown that an-nexin A2 mediates EC activation by aPL/anti-β_2GPI Abs after binding to β_2GPI [106,107]. Because annexin A2 does not span the cell membrane, this interaction may require an "adaptor" protein(s) able to transduce intracellular signalling. Raschi *et al* previously shown that Myeloid Differentiation Factor 88 (MyD88) signalling cascade - an adaptor molecule for toll-like receptor (TLR)-4 that is used to transduce TLR-mediated intracellular signaling (i.e. translocation of NF-κB, phosphorylation of p38MAPK, upregulation of pro-inflammatory cytokines, CAMs and TF - is triggered by aPL/anti-β_2GPI Abs on human endothelial cells *in vitro* [108]. There is also some indication that TLR-4 is involved as co-receptor for endothelial cells signalling when aPL/anti-β_2GPI Abs recognize β_2GPI bound to annexin A2 on the cell membrane. Zhang and colleagues recently were able to iden-tify a protein of 83 kD that appeared to be TLR-4 among those that bound immobilized β_2GPI by affinity-purification in Affi-Gel HZ columns followed by elution, SDS-PAGE and LC-MS analysis [109]. We recently demonstrated that annexin A2 deficient mice are partially protected from aPL-induced thrombosis and those pathogenic effects of aPL an-tibodies can be diminishes by anti-annexin A2 antibodies in mice *in vivo* [110]. Furthermore, Sorice *et al* recently dem-onstrated the involvement of TLR-4 and annexin A2 as a receptor for aPL/anti-β_2GPI Abs in monocytes cell surface lipid rafts [111]. In order to evaluate the role of TLR-4 in aPL-mediated endothelial cell activation/thrombosis *in vivo*, we carried out experiments in lipopolysaccharide (LPS) non-responsive (-/-) and LPS responsive (+/+) mice. LPS -/- mice display a point mutation of the *tlr4* gene leading to the ex-pression of a TLR-4, which does not recognize LPS. IgG isolated from APS patients (IgG-APS; n=2) produced sig-nificantly larger thrombi. Induced higher TF activity in ca-rotid artery homogenates and number of adhering leukocytes (WBC) to EC in the microcirculation of the cremaster mus-cle of LPS +/+ mice when compared to control IgG-NHS. These effects were abrogated after removal of the anti-β_2GPI activity from IgG-APS. The two IgG-APS induced signifi-cantly smaller thrombus size, lower number of WBC adher-ing to endothelial cells and TF activity in LPS -/- compared to LPS +/+ mice. Altogether, the data demonstrate involve-ment of TLR-4 in aPL-mediated *in vivo* pathogenic effects in mice [112].

It is also possible that other molecules might act as recep-tors for β_2GPI, such as the apoER2'. ApoER2' is a member of the low density lipoprotein (LDL) receptor family and is also present in EC [113]. In addition to function as a scaven-ger receptor for lipoproteins, it has been shown to induce intracellular signalling [114]. In platelets, apoER2' has been shown to bind dimers of β_2GPI – that mimic β_2GPI - aPL/anti-β_2GPI Abs complexes - leading to phosphorylation of p38MAPK, thromboxane production and cell activation induced by aPL/anti-β_2GPI Abs [115,116]. Van Lummel *et al* showed that domain V of β_2GPI is involved in both bind-ing β_2GPI to anionic PL and in interaction with apoER2' and subsequent activation of platelets [116]. Lutters *et al* also showed that when they blocked the apoER2' receptor on

platelets using receptor-associated protein (RAP), the increased adhesion of platelets to collagen induce by the β_2GPI–aPL anti-β_2GPI was lost [115]. The apoER2' was able to co-precipitate with dimerized β_2GPI providing evidence for a direct interaction between β_2GPI and the receptor. These findings suggest that the apoER2'mediates a role in the activation of platelets. ApoER2' is found in many other cell types including endothelial cells and monocytes. Hence, it can be hypothesized that β_2GPI binds to EC through a multi-protein receptor and intracellular signalling is started when for aPL Abs bind to β_2GPI bound to endothelial cells.

Interaction of aPL Antibodies with Platelets

aPL Abs harness the platelet activation. The platelets of patients with APS display greater expression of CD63 and they release larger amounts of P-selectin to the plasma than the platelets of normal individuals. Also aPL Abs increase the expression of the GPIIb-IIIa, stimulate the platelet aggregation in the presence of subaggregating concentrations of platelets agonists and increase the synthesis of thromboxane A2 *in vitro* [117-121]. Vega-Ostertag *et al* showed that theses effects of aPL Abs on platelets are also mediated by p38MAPK [122]. In order to study intracellular pathways activated by aPL Abs, Vega-Ostertag *et al* examined their effects on: phosphorylation of p38MAPK, ERK1/ERK2 and cytosolic phospholipase A$_2$ (cPLA$_2$); intracellular Ca^{2+} mobilization; and TXA2 production [122]. The effects of the specific inhibitor for SB203580 on aPL-mediated enhancement of platelet aggregation and on TXB2 production were also determined. Treatment of the platelets with IgG aPL Abs or with their F(ab`)$_2$ fragments resulted in a significant increase in phosphorylation of p38MAPK. Neither IgG aPL nor their F(ab`)$_2$ significantly increased the phosphorylation of ERK1/ERK2. Furthermore, pretreatment of the platelets with SB 203580 completely abrogated aPL-mediated enhanced platelet aggregation. Platelets treated with F(ab)$_2$ derived from aPL produced significantly larger amounts of TXB2 when compared to controls, and this effect was completely abrogated by treatment with SB 203580. cPLA2 was also significantly phosphorylated in platelets treated with thrombin and F(ab`)$_2$ derived from aPL Abs [122]. The data strongly indicates that aPL Abs induce TXB2 production mainly through the activation of p38MAPK and subsequent phosphorylation of cPLA2, and that the ERK1/ERK2 pathway does not seem to be involved, at least in early stages of aPL-mediated platelet activation.

Activation of the Complement System and its Relationship with aPL-Mediated Thrombosis and Endothelial Cell Activation

Some studies have recently suggested the involvement of the complement system in APS. aPL Abs may activate the complement system and may favor the generation of C5a, a molecule that attracts and activates neutrophils and monocytes and that leads to the release of inflammatory mediators and others molecules [43, 45]. Using specific complement inhibitors or mice deficient in several complement components, Girardi *et al* has shown that C4, C3, C5 and C5a-C5aR are required to induce fetal injury by aPL Abs [43]. Furthermore, our group showed that mice deficient in complement C3 and C5 are resistant to the enhanced thrombosis and EC activation that is induced by aPL Abs [45].

A proposed mechanism for aPL-induced fetal damage is that when these Abs act on the placenta they may generate C5a, which attracts and activates neutrophils and monocytes that in turn stimulate the release of inflammatory mediators and other molecules, such as proteolytic enzymes, chemokines, cytokines, C3a and C5a. Neutrophils have been implicated in pregnancy loss in an antibody-independent form, and C5a could enhance this effect in APS [43]. Furthermore, Fischetti *et al.* showed that in C6-deficient rats and in animals treated with an anti-C5 miniantibody and aPL Abs. the number of intravascular platelet-leukocyte aggregates and thrombotic occlusions is markedly reduced, suggesting the contribution of the terminal complement complex to the aPL antibody-mediated intravascular thrombosis [46].

Given the participation of the complement system in thrombosis and fetal loss, it is tempting to speculate that the inhibition of complement activation may be beneficial for the treatment of thrombosis and pregnancy complications in women with APS. Further studies in humans are needed to confirm these postulated mechanisms.

Summary of Proposed Thromboghenic Mechanisms Mediated by aPL Antibodies

Based on the information discussed before and on data available, the following thrombogenic and pro-inflammatoty mechanism mediated by aPL antibodies can be proposed. We propose the following mechanism for the pathogenic effects of aPL/anti-β_2GPI Abs on thrombosis. First, aPL/anti-β_2GPI Abs bind to endothelial cells, induce their activation and a procoagulant state, as demonstrated *in vivo* and *in vitro* studies. These include upregulation of adhesion molecules and TF expression. APL/anti-β_2GPIAbs also induce platelet activation and interact with elements of the coagulation cascade. This activity however does not seem to be sufficient to cause thrombosis. Activation of the complement cascade by aPL/anti-β_2GPIAbs may amplify these effects by stimulation of the generation of potent mediators of platelet and endothelial cell activation, including C3a and C5a and possibly the C5b-9 MAC (Fig. **1**).

New Targeted Therapies for aPL-Induced Pathogenic Effects?

The recurrence of thrombosis in patients with aPL Abs is high. APS occurs predominantly in young women and the recurrence of the symptoms combined with high morbidity calls for an adequate treatment. In addition, this disease is associated with a significant socio-economical impact, often involving long-term disability and costly treatments. APS may be considered a manifestation of SLE - since it is present in a significant proportion of lupus patients - for which there is no current good management. An understanding of the pathobiology of this syndrome is clearly an important step towards designing novel therapeutics.

Thrombosis *per se* is a devastating consequence in PAPS and SAPS and may affect any organ. APS is a severe manifestation of SLE for which the best treatment is still matter of debate. Like in most autoimmune conditions including APS, therapy modalities include steroids and immunosuppressive cytotoxic agents that are counterbalanced by the

Inhibition of natural anticoagulants

Protein C inhibition

Inhibition of the extrinsic pathway   *PROCOAGULANT*
                                                                            EFFECTS
Annexin A5

Inhibition of the fibrinolytic system

Cellular activation

Endothelial cells

Monocytes   *PROINFLAMMATORY / PRO*
                                                              THROMBOTIC EFFECTS
Platelets

Activation of the complement system
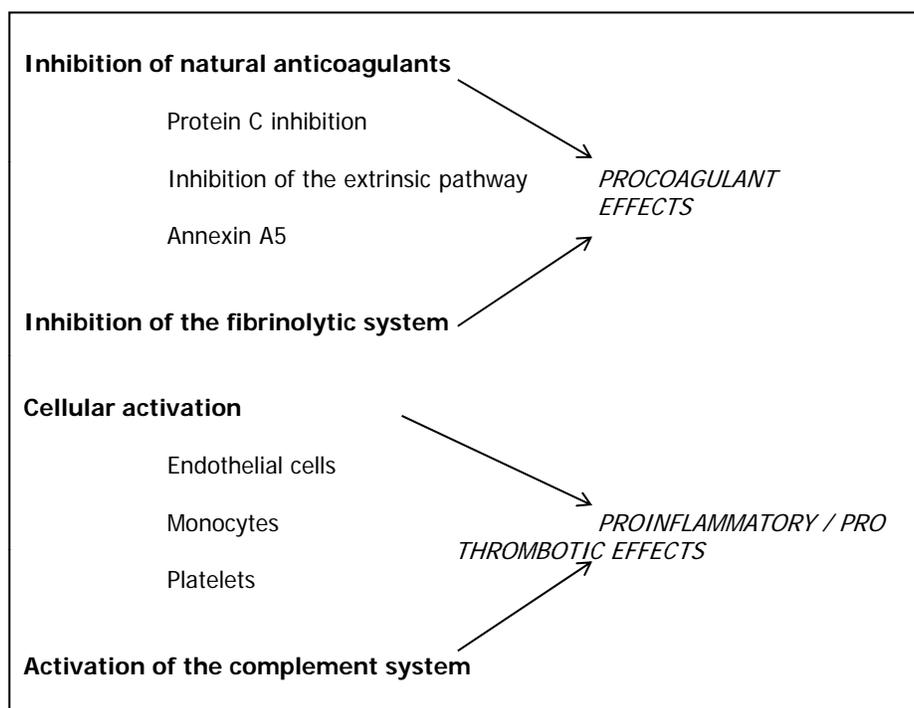
Fig. (1). Thrombogenic mechanisms mediated by antiphospholipid antibodies.

toxicity and side effects of these medications. In the case of thrombotic manifestations, treatment has been focused on preventing thromboembolic events utilizing anti-thrombotic medications or modulating the immune response itself. Recurrences in spite of treatment have been reported and the use of oral anticoagulation at a relative high international normalized ratio (INR) for a long period of time has also been associated with a high risk of bleeding, with the need for frequent monitoring and patient compliance with diet and lifestyle to optimize the therapy [123-126]. Moreover, still debated is the approach to patients with aPL Abs without a previous thrombotic event. The opinions of the clinicians are divided. Some would recommend prophylaxis with low dose aspirin, while some others would advise a more aggressive treatment and still others would recommend no treatment at all. Prophylaxis with low dose aspirin has been suggested but its efficacy is not certain, since it may not be very beneficial in preventing venous events [127]. Therefore we need to develop therapeutic agents that, unlike warfarin, target the pathogenic actions of aPL specifically in order to achieve greater efficacy with fewer side-effects.

It has been reported that aPL Abs may increase the threshold activation in EC, monocytes and platelets [128] (first hit) and the clinical event (i.e. thrombosis or "second hit") happens sporadically possibly associated with another triggering event (infection, trauma, surgery, etc.). Current treatments in APS are directed to the "second hit" (thrombosis) and include aggressive anticoagulation and immunosuppression, both associated with considerable side effects. Treatments that modulate early effects of aPL Abs on target cells, (first hit) would be more beneficial and potentially less harmful than what is currently used. Hence, agents able to inhibit the binding of β_2GPI to the receptor or the binding of aPL/anti- β_2GPI Abs to the β_2GPI, – as discussed earlier - may act directly on the putative "first hit" reducing the risk

for developing the clinical events in the case that a "second hit" does occur. This is even more important taking into account that "second hits" (such as common infectious processes) cannot be easily prevented. Knowing the molecular interactions induced by aPL Abs and the nature of the receptor and its interaction with β_2GPI and the specific Abs may help to identify useful surrogate markers (biomarkers) of thrombotic risk and to devise new targeted, more specific modalities for treatment and prevention of thrombosis with fewer adverse effects in patients with aPL Abs. A better knowledge of APS pathogenesis, particularly at the molecular level, is needed in order to address new therapeutic strategies. Furthermore, targeting the specific interactions of pathogenic autoantibodies to antigens and or the binding of the antigen to target cells provides a far more specific means of abrogating the pathogenic effects.

SUMMARY

APS is an acquired thombophilia, which is characterized by recurrent thrombotic events and obstetric complications in the presence of aPL Abs. The diagnostic of APS is based in the discovery of one clinical and one laboratory criteria at least. The treatment of APS is based fundamentally in the use of oral anticoagulants with or without aspirin. Pathogenic mechanisms include effects on the coagulation cascade, cellular activation and complement activation. Lately much has been advanced in the knowledge of cellular receptors that participate in signaling transduction. Further studies are needed to clarify how aPL Abs affect cell surface molecules and how signal transduction events occur. Understanding intracellular events in aPL-mediated EC, platelet and monocyte activation may help in designing new targeted therapies for thrombosis in APS. Understanding molecular events triggered by aPL Abs may help to device new modalities of treatment for clinical manifestations of APS (i.e. use

of specific inhibitors, antibodies, etc.). *In vivo* studies in animal models followed by clinical trials in humans will need to be performed to determine the safety and effectiveness of specific inhibitors to be used in the treatment of complications of APS.

ACKNOWLEDGEMENTS

REFERENCES

[1] Harris EN. Syndrome of the black swan. Br J Rheumatol 1987; 26: 324-6.

[2] Miyakis S, Lockshin MD, Atsumi T, *et al*. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006; 4: 295-306.

[3] Galli M, Comfurius P, Maassen C, *et al*. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet 335: 1544-7.

[4] Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. Lancet 1990; 336: 177-8.

[5] Roubey RA. Antiphospholipid syndrome: antibodies and antigens. Curr Opin Hematol 2000; 7: 316-20.

[6] Miyakis S, Ginnakopoulos B, Krilis SA. β_2 glycoprotein I – function in health and disease. Thromb Res 2004; 114: 335-46.

[7] Boey ML, Colaco CB, Gharavi AE, Elkon KB, Loizou S, Hughes GR. Thrombosis in SLE: striking associations with the presence of circulating "lupus anticoagulant". Br Med J 1983; 287: 1021-3.

[8] Elias M, Eldor A. Thromboembolism in patients with the "lupus" like circulating anticoagulant. Arch Int Med 1984; 144: 510-5.

[9] Bowie WEJ, Thompson JH, Pascuzzi CA, Owen CA. Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. J Clin Invest 1963; 62 :416-30.

[10] Levine JS, Branch DW, Rauch J. The antiphospholipid syndrome. N Engl J Med 2002; 346: 752-63.

[11] Kittner SJ, Gorelick PB. Antiphospholipid antibodies and stroke: an epidemiological perspective. Stroke 1992; 23 (Suppl I): 19-I-22.

[12] Love PE, Santoro SA. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and non-SLE disorders. Prevalence and clinical significance. Ann Intern Med 1990; 112: 682-98.

[13] Coon WW, Willis PW, Keller JB. Venous thromboembolism and other venous disease in the Tecumseh Community Health Study. Circulation 1973; 48: 839-46.

[14] Gjores JE. The incidence of venous thrombosis and its sequelae in certain districts in Sweden. Acta Chir Scand Suppl1956; 206: 1-88.

[15] McClain MT, Arbuckle MR, Heinlen LD, *et al*. The prevalence, onset and clinical significance of antiphospholipid antibodies prior to diagnosis of systemic lupus erythematosus. Arthritis Rheum 2004; 50: 1226-32.

[16] Weber M, Hayem G, De Band M, *et al*. The family history of patients with primary or secondary antiphospholipid antibody syndrome (APS). Lupus 2000; 9: 258-63.

[17] Matsuura E, Igarashi Y, Yasuda T, Triplett DA, Koike T. Anticardiolipin antibodies recognize β_2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J Exp Med 1994; 179: 457-62.

[18] Bevers E, Galli M, Barbui T, Comfurius P, Zwaal RF. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb Haemost 1991; 66: 629-32.

[19] Galli M, Willems G, Rosing J, *et al*. Anti-prothrombin IgG from patients with anti-phospholipid antibodies inhibits the inactivation of factor Va by activated protein C. Br J Haematol 2005; 129: 240-7.

[20] Rand J, Wu X. Antibody-mediated interference with annexins in the antiphospholipid syndrome. Thromb Res 2004; 114: 383-9.

[21] Adams M, Breckler L, Stevens P, Thom J, Baker R, Oostryck R. Anti-tissue factor pathway inhibitor activity in subjects with antiphospholipid syndrome is associated with increased thrombin generation. Haematologica 2004; 89: 985-90.

[22] Lopez-Lira F, Rosales-Leon L, Martinez V, Ruiz-Ordaz BH. The role of $\beta_2$glycoprotein I (β_2GPI) in the activation of plasminogen. Biochim Biophys Acta 2006; 1764: 815-23.

[23] Harris S, Jones D, Gallimore M, Nicholls PJ, Winter M. The antigenic binding site(s) of antibodies to factor XII associated with the antiphospholipid syndrome. J Thromb Haemost 2005; 3: 969-75.

[24] Sugi T, McIntyre J. Certain autoantibodies to phosphatidylethanolamine (aPE) recognize factor XI and prekallikrein independently or in addition to the kininogens. J Autoimmun 2001; 17: 207-14.

[25] Bidot C, Jy W, Horstman L, *et al*. Factor VII/VIIa: a new antigen in the anti-phospholipid antibody syndrome. Br J Haematol 2003; 120: 618-26.

[26] Bouma B, De Groot PhG, Van Der Elsen JMH, *et al*. Adhesion mechanism of human b2glycoprotein I to phospholipids based on its crystal structure. EMBO J 1999; 18: 5166-74.

[27] Giannakopoulos B, Passam F, Rahgozar S, Krilis SA. Current concepts on the pathogenesis of Antiphospholipid syndrome. Blood 2007; 109: 422-30.

[28] Koike T, Matsuura E. β_2Glycoprotein I and antiphospholipid syndrome. Isr J Med Sci 1997; 33: 225-38.

[29] Hulstein J, Lenting P, de Laat B, Derksen RH, Fujnheer Rm de Groot PG. Beta2-Glycoprotein I inhibits von Willebrand factor dependent platelet adhesion and aggregation. Blood 2007; 110: 1483-91.

[30] Branch DW, Dudley DJ, Mitchell MD, *et al*. Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. Am J Obstet Gynecol 1990; 163: 210-6.

[31] Pierangeli SS, Liu XW, Anderson GH, Barker JH, Harris EN. Induction of thrombosis in a mouse model by IgG, IgM and IgA immunoglobulins from patients with the antiphospholipid syndrome. Thromb Haemost 1995; 74: 1361-7.

[32] Jankowski M, Vreys I, Wittevrongel C, *et al*. Thrombogenicity of b2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. Blood 2003; 101: 157-62.

[33] Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GH, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells *in vitro* and *in vivo*. Circulation 1999; 99: 1997-2000.

[34] Esmon NL, Safa O, Smirnov M, Esmon CT. Antiphospholipid antibodies and the protein C pathway. J Autoimmun 2000; 15: 221-5.

[35] Campbell AL, Pierangeli SS, Wellhausen S, Harris EN. Comparison of the effect of anticardiolipin antibodies from patients with the antiphospholipid syndrome and with syphilis on platelet activation and aggregation. Thromb Haemost 1995; 73: 529-34.

[36] Arvieux J, Rousell B, Pouzol P, Colomb MG. Platelet activating properties of murine monoclonal antibodies to b2glycoprotein I. Thromb Haemost 1993; 70: 336-41.

[37] Forastiero R, Martinuzzo M, Carreras LO, Maclouf J. Anti-β_2 glycoprotein I antibodies and platelet activation in patients with antiphospholipid antibodies: association with increased excretion of platelet-derived thromboxane urinary metabolites. Thromb Haemost 1998; 79: 42-5.

[38] Martinuzzo ME, Maclouf J, Carreras LO, Levy-Toledano S. Antiphospholipid Antibodies enhance thrombin-induced platelet activation and thromboxane formation. Thromb Haemost 1993; 70: 667-71.

[39] Lin YL, Wang CT. Activation of human platelets by the rabbit anticardiolipin antibodies. Blood 1992; 80: 3135-43.

[40] Khamashta MA, Harris EN, Gharavi AE, *et al*. Immune mediated mechanism for thrombosis: antiphospholipid antibody binding to platelet membranes. Ann Rheum Dis 1988; 47: 849-54.

[41] Holers VM, Girardi G, Mo L, *et al*. C3 activation is required for anti-phospholipid antibody-induced fetal loss. J Exp Med 2002; 195: 211-20

[42] Salmon JE, Girardi G, Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. Ann Rheum Dis 2002; 61: 46-50.

[43] Girardi G, Berman J, Redecha P, *et al*. Complement C5a receptors and neutrophils mediate fetal injury in the antiphophospholipid syndrome. J Clin Invest 2003; 112: 1644-50.

[44] Girardi G, Redecha P, Salmon J. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat. Med. 2004; 10: 1222-6.

[45] Pierangeli SS, Girardi G, Vega-Ostertag ME, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. Arthritis Rheum 2005; 52: 2120-4.

[46] Fischetti F, Durigutto P, Pellis V, *et al*. Thrombus formation induced by antibodies to b2-glycoprotein I is complement-dependent and requires a priming factor. Blood 2005; 106: 2340-6.

[47]　Romay-Penabad Z, Liu X, Montiel-Manzano G, *et al*. C5a receptor-deficient micer are protected from thrombophilia and endothelial cell activation induced by some antiphospholipid antibodies. NY Acad Sci 2007; 1108: 554-66.

[48]　Safa O, Esmon C, Esmon N. Inhibition of APC anticoagulant activity on oxidized phospholipid by anti-β₂-glycoprotein I monoclonal antibodies. Blood 2005; 106: 1629-35.

[49]　Yang C, Hwang K, Yan W, *et al*. Identification of anti-plasmin antibodies in the antiphospholipid syndrome that inhibit degradation of fibrin. J Immunol 2004; 172: 5765- 73.

[50]　Chen X, Gu Y, Li S, *et al*. Some plasmin-induced antibodies bind to cardiolipin, display lupus anticoagulant activity and induce fetal loss in mice. J Immunol 2007; 278: 5351-6.

[51]　Lu C, Horizon A, Hwang K, *et al*. Identification of plyclonal and monoclonal antibodies against tissue plasminogen activator in the antiphospholipid syndrome. Arthritis Rheum 2005; 52: 4018-27.

[52]　Bevers E, Zwaal R, Willems G. The effect of phospholipids on the formation of immune complexes between autoantibodies and β₂-glycoprotein I or prothrombin. Clin Immunol 2004; 112: 150-60.

[53]　Adams M, Donohoe S, Mackie I, Machin SJ. Anti-tissue factor pathway inhibitor activity in patients with primary antiphospholipid syndrome. Br J Haematol 2001; 114: 375-9.

[54]　Yasuda S, Bohgaki M, Atsumi T, Koike T. Pathogenesis of antiphospholipid antibodies: impairment of fibrinolysis and monocyte activation *via* the p38 mitogen-activated protein kinase pathway. Immunobiology 2005; 240: 775-80.

[55]　Yasuda S, Atsumi T, Ieko M, Koike T. β₂-glycoprotein I, anti-β₂-glycoprotein I, and fibrinolysis. Thromb Res 2004; 114: 461-5.

[56]　Del Papa N, Guidali L, Sala A, *et al*. Endothelial cell target for antiphospholipid antibodies. Human polyclonal and monoclonal anti-b₂glycoprotein I and induce endothelial cell activation. Arthritis Rheum 1997; 40: 551-61.

[57]　Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GH, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells *in vitro* and *in vivo*. Circulation 1999; 99: 1997-2000.

[58]　Simantov E, LaSala J, Lo SK, *et al*. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. J Clin Invest 1995; 96: 2211-9.

[59]　Pierangeli SS, Espinola RG, Liu X, Harris EN. Thrombogenic effects of antiphospholipid antibodies are mediated by intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and P-selectin. Circ Res 2001; 88: 245-50.

[60]　Espinola RG, Liu X, Colden-Stanfield M, Hall J, Harris EN, Pierangeli SS. E-selectin mediated pathogenic effects of antiphospholipid antibodies. J Thromb Haemost 2002; 1: 843-8.

[61]　Kaplanski G, Cacoub P, Farnarier C, *et al*. Increased soluble vascular cell adhesion molecule 1 concentrations in patients with primary or systemic lupus erythematosus-related antiphospholipid syndrome: correlations with the severity of thrombosis. Arthritis Rheum 2000; 43: 55-64.

[62]　Williams FMK, Parmar K, Hughes GRV, Hunt BJ. Systemic endothelial cell markers in primary antiphospholipid syndrome. J Thromb Haemost. 2000; 84: 742-6.

[63]　Nemerson Y. Tissue factor and hemostasis. Blood 1988; 71: 1-8

[64]　Semerato N, Colucci M. Tissue factor in health and disease. Thromb Haemost 1997; 78: 759-64.

[65]　Osterud B, Bjorklid E. The tissue factor pathway in disseminated intravascular coagulation. Semin Thromb Haemost 2001; 27: 605-17.

[66]　Clemens N, Frauenknecht K, Katzav A, Sommer C, von Landenberg P. *In vitro* effects of antiphospholipid síndrome-IgG fractions and human monoclonal antiphospholipid IgG antibody on human umbilical vein endotelial cells and monocytes. Ann NY Acad Med 2009; 1173: 805-13.

[67]　Amengual O, Atsumi T, Khamashta MA, Hughes GRV. The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. Thromb Haemost 1998; 79: 276-81.

[68]　Zhou H, Woldberg AS, Roubey RA. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood 2004; 104: 2353-8.

[69]　Forastiero RR, Martinuzzo ME, De Larranaga G. Circulating levels of tissue factor and proinflammatory cytokines in patients with primary antiphospholipid syndrome or leprous related antiphospholipid antibodies. Lupus 2005; 14: 129-36.

[70]　Amengual O, Atsumi T, Khamashta MA, Hughes GRV. The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. Thromb Haemost 1998; 79: 276-81.

[71]　Cuadrado MJ, Lopez-Pedrera C, Khamashta MA, *et al*. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. Arthritis Rheum 1997; 40: 834-41.

[72]　Dobado-Barrios M, Lopez-Perrara C, Velasco F, Aguirre MA, Torres A, Cuadrado MJ. Increased levels of TF mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. Thromb Haemost 1999; 82: 1578-82.

[73]　Reverter JC, Tassies D, Font J, *et al*. Effects of human monoclonal anticardiolipin antibodies on platelet function and on tissue factor expression on monocytes. Arthritis Rheum 1998; 41: 1420-7.

[74]　Kornberg A, Blank M, Kaufman S, Shoenfeld Y. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. J Immunol 1994; 153: 1328-32.

[75]　Dunoyer-Geindre S, de Moerloose P, Galve-de Rochemonteiz B, Reber G, Kruithof EKO. NFkB is an essential intermediate in the activation of endothelial cells by anti-β₂glycoprotein I antibodies. Thromb Haemost 2002; 88: 851-7.

[76]　Bohgaki M, Atsumi T, Yamashita Y, *et al*. The p38 mitogen-activated protein kinase (MAPK) pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-b₂glycoprotein I antibodies. Int Immunol 2004; 16: 1632-41.

[77]　Xie H, Ray PE, Short BL. NF-kappa B activation plays a role in superoxide-mediated cerebral endothelial dysfunction after hypoxia/reoxygenation. Stroke 2005; 36: 1047-52.

[78]　Morgan EN, Pohlman TH, Vocelka C, *et al*. Nuclear factor kappa B mediates a procoagulant response in monocytes during extracorporeal circulation. J Thorac Cardiovasc Surg 2003; 125: 165-71.

[79]　Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kB, a ubiquitous transcription factor in the initiation of diseases. Clin Chem 1999; 45: 7-17.

[80]　Saccani S, Pantano S, Natoli G. p38-dependent marking of inflammatory genes for increasing NF-kappa B recruitment. Nat Immunol 2002; 3: 16-7.

[81]　Firestein GS. NF-kB: holy Grail for rheumatoid arthritis. Arthritis Rheum 2004; 50: 2381-6.

[82]　Cohen P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell Biol 1997; 7: 353-8.

[83]　McGilvray ID, Tsai V, Marshall JC, Dackiw APB, Rotstein OD. Monocyte adhesion and transmigration induce tissue-factor expression: role of the mitogen-activated protein kinases. Shock 2002; 18: 51-7.

[84]　Borsch-Haubold AG, Ghomanschi F, Pasquet S, Goedert M, Cohen P, Gelb MH, Watson SP. Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple-stress activated protein kinase pathways. Eur J Biochem 1999; 265: 195-203.

[85]　Vega-Ostertag ME, Harris EN, Pierangeli SS. Intracellular events in platelet activation induced by antiphospholipid antibodies in the presence of low doses of thrombin. Arthritis Rheum 2004; 50: 2911-9.

[86]　Vega-Ostertag M, Casper K, Swerlick R, Ferrara D, Harris EN, Pierangeli SS. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum 2005; 52: 1545-54.

[87]　Ferrara DE, Liu X, Espinola RG, *et al*. Inhibition of the thrombogenic and inflammatory properties of antiphospholipid antibodies by fluvastatin in an *in vivo* animal model. Arthritis Rheum 2003; 48: 3272-9.

[88]　Ferrara DE, Swerlick R, Casper K, *et al*. Fluvastatin inhibits upregulation of tissue factor expression by antiphospholipid antibodies on endothelial cells. J Thromb Haemost 2004; 2: 1558-63.

[89]　Simoncini S, Sapet C, Camoin-Jau L, *et al*. Role of reactive oxygen species and p38 MAPK in the induction of the pro-adhesive endothelial state mediated by IgG from patients with anti-phospholipid syndrome. Int Immunol 2004; 17: 489-500.

[90]　Vega-Ostertag ME, Ferrara DE, Romay-Penabad Z, *et al*. Role of p38 mitogen-activated protein kinase in antiphospholipid antibody-mediated thrombosis and endothelial cell activation. J Thromb Haemost 2007; 5: 1828-34.

[91]　Montiel-Manzano G, Romay-Penabad Z, Papalardo de Martinez E, *et al*. *In vivo* effects of an inhibitor of nuclear factor-kappa B on thrombogenic properties of antiphospholipid antibodies. Ann NY Acad Sci 2007; 1108: 540-53.

[92]　Iverson GM, Victoria EJ, Marquis DM. Anti-b₂glycoprotein I autoantibodies recognize an epitope on the first domain of b₂GPI. Proc Natl Acad Sci 1998; 95: 15542-6.

[93]　Reddel SW, Wang YX, Sheng YH, Krilis SA. Epitope studies with anti-b₂glycoprotein I antibodies from autoantibody and immunized sources. J Autoimmun 2000; 15: 91-6.

[94]　Iverson GM, Reddel SW, Victoria EJ, *et al*. Use of single point mutations in domain I of b₂glycoprotein I to determine fine antigen

specificity of antiphospholipid autoantibodies. J Immunol 2002; 169: 7097-103.

[95] Giles IP, Isenberg DA, Latchman DS, Rahman A. How do antiphospholipid antibodies bind beta-2-glycoprotein I? Arthritis Rheum 2003; 48: 2111-21.

[96] DeLaat B, Derksen RH, Urbanus RT, deGroot PG. IgG antibodies that recognize epitope Gly40-Arg 43 in domain I of $\beta_2$glycoprotein I cause LAC and their presence correlates strongly with thrombosis. Blood 2005; 105: 1540-5.

[97] Ioannou Y, Giles I, Lambrianides A, *et al*. A novel expression system of domain I of human b₂glycoprotein I in *Escherichia coli*. BMC Biotechnol 2006; 6: 8.

[98] Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg D, Rahman A. Binding of Antiphospholipid antibodies to discontinuous epitopes on domain I of human b₂glycoprotein I. Mutation studies including residues R39 to R43. Arthritis Rheum 2007; 56: 280-90.

[99] Ioannou Y, Romay-Penabad Z, Periclerous C, *et al*. *In vivo* inhibition of antibody-induced pathogenicity utilizing the antigenic target peptide domain I of b₂glycoprotein I: proof of concept. J Thromb Haemost 2009; 7: 833-42.

[100] Del Papa N, Sheng YH, Raschi E, *et al*. Human b₂glycoprotein I binds to endothelial cells through a cluster of lysine residues that are critical for anionic phospholipids binding and offers epitopes for anti-b₂glycoprotein I antibodies. J Immunol 1998; 160: 5572-8.

[101] Vega Ostertag M, Liu X, Henderson V, Pierangeli SS. A peptide that mimics the Vth region of b₂glycoprotein I reverses antiphospholipid-mediated thrombosis in mice. Lupus 2006; 15: 358-65.

[102] Sheng Y, Sali A, Herzog H, Lahnstein J, Krilis SA. Site-directed mutagenesis of recombinant human b₂glycoprotein I identifies a cluster of lysine residues that are critical for phospholipids binding and anti-cardiolipin antibody activity. J Immunol 1996; 157: 3744-51.

[103] Di Simone N, Raschi E, Testoni C, *et al*. Pathogenic role of anti-b₂glycoprotein I antibodies in antiphospholipid associated fetal loss: characterization of b₂glycoprotein I binding to trophoblasts cells and functional effects of anti-b₂glycoprotein I antibodies *in vitro*. Ann Rheum Dis 2005; 64: 462-7.

[104] Di Simone N, Meroni PL, Del Papa N, *et al*. Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered b₂glycoprotein I. Arthritis Rheum 2000; 43: 140-51.

[105] Meroni PL, Tincani A, Sepp N, *et al*. Endothelium and the brain in CNS lupus. Lupus 2003; 12: 1-10.

[106] Ma K, Simantov R, Zhang J, Silverstein R, Hajjar K, McCrae K. High affinity binding of b₂glycoprotein I to human endothelial cells is mediated by Annexin II. J Biol Chem 2000; 275: 15541-8.

[107] Zhang J, McCrae KR. Annexin A2 mediates endothelial cell activation by antiphospholipid/b₂Glycoprotein I antibodies. Blood 2005; 105: 1964-9.

[108] Raschi E, Testoni C, Bosisio D, *et al*. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. Blood 2003; 101: 3495-500.

[109] Zhang J, Lieske K, McCrae B, McCrae K. Activation of endothelial cells by $\beta_2$glycoprotein I (β_2GPI) antibodies is mediated by annexin II cross linking and may involve TLR4. Blood 2004; 104: 83.

[110] Romay-Penabad Z, Montiel-Manzano G, Shilagard T, *et al*. Annexin A2 is involved in antiphospholipid antibody-mediated pathogenic effects in v itro and *in vivo*. Blood 2009; 114: 3074-83.

[111] Sorice M, Longo A, Capozzi A, *et al*. Anti-$\beta_2$glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and

tissue factor by signal transduction involving lipid rafts. Arthritis Rheum 2007; 56: 2687-97.

[112] Pierangeli SS, Vega-Ostertag ME, Raschi E, *et al*. Toll like receptor-4 is involved in antiphospholipid-mediated thrombosis: *in vivo* studies. Ann Rheum Dis 2007; 66:1327-33.

[113] Andersen OM, Benhayon D, Curran T, Willnow TE. Differential binding of ligands to the apolipoprotein E receptor 2'. Biochemistry 2003; 42: 9355-64.

[114] Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. An LDL-receptor-related protein mediates Wnt signaling in mice. Nature 2000; 407: 535-8.

[115] Lutters BCH, Derksen RHWM, Tekelenburg WL, Lenting PJ, Arnout J, de Groot PH. Dimers of $\beta_2$glycoprotein I increase platelet deposition to collagen *via* interaction with phospholipids and the apolipoprotein E receptor 2'. J Biol Chem 2003; 278: 33831-8.

[116] Van Lummel M, Pennings MTT, Derksen RHWM, *et al*. The binding site of $\beta_2$glycoprotein I for apoER2' on platelets is located in Domain V. J Biol Chem 2005; 280: 36729-36.

[117] Joseph J, Harrison P, Mackie I, *et al*. Increased circulating platelet-leucocyte complexes and platelet activation in patients with antiphospholipid síndrome, systemic lupus erythematosus and rheumatoid arthritis. Br J Haematol 2001; 115: 451-9.

[118] Kubota T, Fukuya Y, Hashimoata R, *et al*. Possible involvement of chemokine-induced platelet activation in thrombophilic diathesis of antiphospholipid síndrome. Ann NY Acad Med 2009; 1173: 137-45.

[119] Bidot C, Jy W, Horstman L, *et al*. Antiphospholipid antibodies and platelet activation as risk factors for thrombosis and thrombocytopenia. Hematology 2005; 10: 451-6.

[120] Espinola R, PIerangeli S, Gharavi A, *et al*. Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid ant ibodies. Thromb Haemost 2002; 87: 518-22.

[121] Robbins D, LEung S, Miller-Blair D, *et al*. Effect of anticardiolipin/b2glycoprotein I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid síndrome. J Rheumatol 1998; 25: 51-6.

[122] Vega-Ostertag M, Harris E, Pierangeli S. Intracellular events in platelet activation induced by antiphospholipid antibodies in the presence of low doses of thrombin. Arthritis Rheum 2004; 50: 2911-9.

[123] Khamashta MA, Cuadrado MJ, Mujic T, Taub NA, Hunt BM, Hughes GRV. The management of thrombosis in the antiphospholipid antibody syndrome. N Engl J Med 1995; 332: 993-7.

[124] Krnic-Barrie S, O'Connor CR, Looney SW, Pierangeli SS, Harris EN. A retrospective review of 61 patients with antiphospholipid syndrome. Arch Intern Med 1997; 157: 2101-8.

[125] Crowther MA, Ginsberg JS, Julian J, *et al*. A comparison of two intensities of warfarin for the prevention of recurrent thrombosis in patients with the antiphospholipid antibody syndrome. N Engl J Med 2003; 349: 1133-8.

[126] Finazzi G, Marchioli R, Brancaccio V, *et al*. A randomized clinical trial of high-intensity warfarin vs conventional antithrombotic therapy for the prevention of recurrent thrombosis in patients with the antiphospholipid syndrome (WAPS). J Thromb Haemost 2005; 3: 848-53.

[127] Erkan D, Harrison MJ, Levy R, *et al*. Aspirin for primary thrombosis prevention in the antiphospholipid syndrome: a randomized, double-blind, placebo-controlled trial in asymptomatic antiphospholipid positive individuals. Arthritis Rheum 2007; 56: 2382-91.

[128] De Groot PG, Derksen RHW, Pathophysiology of the antiphospholipid syndrome J Thromb Haemost 2005; 3: 1854-60.