A Comprehensive Review of Thrombogenic Mechanisms in APS

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Abstract: The antiphospholipid syndrome (APS) is an acquired thrombophilia, 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-β2-glycoprotein I (β2-GPI) 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.

ANTIPHOSPHOLIPID 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 heterogeneous and bind to various protein targets, among them the plasma protein β2Glycoprotein I (β2-GPI) [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 β2-GPI [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: β2GPI, 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 β2-GPI and PT [17-25]. β2-GPI 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]. β2-GPI interacts with diverse cell types, receptors and enzymes [27]. β2-GPI 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 β2-GPI have been identified. Hulstein et al found that
β2-GPI inhibits von Willebrand factor (VWF)-induced platelet aggregation. β2-GPI binds to the A1 domain of VWF but preferentially 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-β2-GPI 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**

β2-GPI is a cell surface-binding plasma protein. The affinity of β2-GPI for the cellular surfaces appears to be low. However, in the presence of Abs against β2-GPI, that affinity increases significantly. The increase in the affinity of β2-GPI 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 prothrombinase 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]. β2-GPI is proteolytically cleaved by plasmin in domain V (nicked β2-GPI) and becomes unable to bind to phospholipids, reducing antigenicity against aPL Abs. Nicked β2-GPI binds to plasminogen and suppresses plasmin generation in the presence of fibrin, plasminogen, and tPA, thus, nicked β2-GPI 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 β2-GPI *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-kappaB (NF-κB) 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 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 IkB signalosome 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 example, 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, H2O2, 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 (carboxbenzoxyl-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 monocyctic 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-β2-GPI Abs purified from patients with APS to bind DI of β2-GPI 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 β2-GPI has been shown by several groups using different techniques. These include the demonstration that variants of β2-GPI 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 them 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 β2-GPI to ameliorate thrombosis in APS. Clinical studies will be needed to confirm these observations in animal models.

**Receptor for β2-GPI on Endothelial Cells**

Studies with β2-GPI 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 β2-GPI, reversed thrombogenic effects of aPL/anti-β2-GPI Abs in mice and displaced the binding of fluorescinated (FITC) β2-GPI to human endothelial cells and murine peritoneal macrophages [101]. The data indicate that TIFI inhibits thrombogenic properties of human aPL/anti-β2-GPI Abs in mice by competing with β2-GPI and by preventing its binding to target cells. There is evidence that β2-GPI 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].

β2-GPI 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 placentaion and fetal loss [103-105]. Pathogenic aPL Abs recognize β2-GPI bound to these cells and were shown to affect cell functions leading to endothelial perturbation and trophoblast differentiation inhibition. Furthermore, endothelial 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 β2-GPI binding. Accordingly, studies have recently shown that annexin A2 mediates EC activation by aPL/anti-β2-GPI Abs after binding to β2-GPI [106,107]. Because annexin A2 does not span the cell membrane, this interaction may require an "adapter" 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-β2-GPI 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-β2-GPI Abs recognize β2-GPI bound to annexin A2 on the cell membrane. Zhang and colleagues recently were able to identify a protein of 83 kD that appeared to be TLR-4 among those that bound immobilized β2-GPI 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 antibodies can be diminished by anti-annexin A2 antibodies in mice in vivo [110]. Furthermore, Sorice et al recently demonstrated the involvement of TLR-4 and annexin A2 as a receptor for aPL/anti-β2-GPI 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 expression of a TLR-4, which does not recognize LPS. IgG isolated from APS patients (IgG-APS; n=2) produced significantly larger thrombi. Induced higher TF activity in carotid artery homogenates and number of adhering leukocytes (WBC) to EC in the microcirculation of the cremaster muscle of LPS +/- mice when compared to control IgG-NHS. These effects were abrogated after removal of the anti-β2-GPI activity from IgG-APS. The two IgG-APS induced significantly smaller thrombus size, lower number of WBC adhering to endothelial cells and TF activity in LPS -/+ compared to LPS +/- mice. Altogether, the data demonstrate involvement of TLR-4 in aPL-mediated in vivo pathogenic effects in mice [112].

It is also possible that other molecules might act as receptors for β2-GPI, 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 scavenger receptor for lipoproteins, it has been shown to induce intracellular signalling [114]. In platelets, apoER2’ has been shown to bind dimers of β2-GPI – that mimic β2-GPI - aPL/anti-β2-GPI Abs complexes - leading to phosphorylation of p38MAPK, thromboxane production and cell activation induced by aPL/anti-β2-GPI Abs [115,116]. Van Lummel et al showed that domain V of β2-GPI is involved in both binding β2-GPI 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 $\beta_2$GPI–aPL anti-$\beta_2$GPI was lost [115]. The apoER2 was able to co-precipitate with dimerized $\beta_2$GPI providing evidence for a direct interaction between $\beta_2$GPI 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 $\beta_2$GPI binds to EC through a multi-protein receptor and intracellular signalling is started when for aPL Abs bind to $\beta_2$GPI 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 A2 (cPLA2); 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)$^\prime_2$ fragments resulted in a significant increase in phosphorylation of p38MAPK. Neither IgG aPL nor their F(ab)$^\prime_2$ 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 other 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 Thrombogenic Mechanisms Mediated by aPL Antibodies**

Based on the information discussed before and on data available, the following thrombogenic and pro-inflammatory mechanism mediated by aPL antibodies can be proposed. We propose the following mechanism for the pathogenic effects of aPL/anti-$\beta_2$GPI Abs on thrombosis. First, aPL/anti-$\beta_2$GPI 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-$\beta_2$GPI Abs 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-$\beta_2$GPI Abs 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
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 β2GPI to the receptor or the binding of aPL/anti- β2GPI Abs to the β2GPI, – 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 β2GPI 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 thrombophilia, 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 devise 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.

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Thrombotic Effects of Antiphospholipid Antibodies

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