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REVIEW

Antiphospholipid syndrome treatment beyond anticoagulation: are we there yet?

SS Pierangeli[1] and D Erkan[2]
[1]Division of Rheumatology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA; and
[2]Barbara Volcker Center for Women and Rheumatic Diseases, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, NY, USA

Persistently positive antiphospholipid antibodies in association with thromboses and/or pregnancy morbidity is the hallmark of the antiphospholipid syndrome. The management of antiphospholipid antibody-positive patients has been focused on utilizing anti-thrombotic medications such as heparin or warfarin. Given that our understanding of the molecular mechanisms of antiphospholipid antibody-mediated thrombosis has been growing, it is highly likely that the current 'anti-thrombotic' approach to these patients will be replaced by an 'immunomodulatory' approach in the near future. This review article will address the experimental and/or clinical evidence behind some of these potential 'immunomodulatory' approaches (tissue factor inhibition, P38 mitogen-activated protein kinase inhibition, nuclear factor-κB inhibition, platelet glycoprotein receptor inhibition, hydroxychloroquine, statins, inhibition of β_2GPI and/or anti-β_2GPI binding to target cells, complement inhibition, and B cell inhibition) in antiphospholipid syndrome. *Lupus* (2010) **19,** 475–485.

Key words: anticardiolipin antibodies; antiphospholipid antibodies; endothelial cells; lupus anticoagulant; targeted therapies; thrombosis

Introduction

Persistently positive antiphospholipid antibodies (aPL) in association with thromboses and/or pregnancy morbidity is the hallmark of the antiphospholipid syndrome (APS).[1] The treatment and prevention of thrombosis in aPL-positive patients has been focused on utilizing anti-thrombotic medications such as heparin or warfarin. Despite long-term anticoagulation, recurrent thrombosis can occur in patients with APS and oral anticoagulation can be associated with bleeding. Furthermore, warfarin treatment is problematic, with the need for frequent blood monitoring as well as patient compliance with diet and lifestyle recommendations. Another debated issue is the management of persistently aPL-positive patients without previous thromboses; there are no evidence-based controlled data supporting that low dose aspirin is sufficient for primary thrombosis prophylaxis.[2]

Given that aPL might be present in the serum for long periods but thrombotic events do occur only occasionally, it has been suggested that aPL increase the thrombophilic threshold as the 'first hit' (induce a pro-thrombotic and pro-inflammatory phenotype in endothelial cells), and then clotting takes place only when a 'second hit' (a triggering event such as an infection, a surgical procedure, use of estrogens, or prolonged immobilization) exists.[3] In this context, current treatments in APS are directed to modulate the final event or 'second hit'. However, treatments that modulate early effects ('first hit') of aPL on target cells (monocytes, endothelial cells, or platelets) would be more beneficial and potentially less harmful than current treatments. This approach also would reduce the risk for thrombosis in the case that a 'second hit' does occur. Thus, it is important to investigate new treatment strategies in APS.

In this review article, we will address the experimental and/or clinical evidence behind some of the potential 'immunomodulatory' approaches in APS (Table 1).

Correspondence to: Silvia S Pierangeli, Ph.D., Division of Rheumatology. Department of Internal Medicine, 301 University Boulevard, Brackenridge Hall 2.108, Galveston, TX 77555-0883, USA. Email: sspieran@utmb.edu

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Table 1 Potential immunomodulatory approaches in antiphospholipid-antibody (aPL)-positive patients

Target or medication	*Support based on in vitro and/or animal studies*	*Support based on aPL(+) human studies*
Tissue Factor (TF)	Dilazep inhibits aPL-induced TF upregulation in monocytes and endothelial cells (EC)	No
Nuclear Factor (NF)-κB	NF-κB inhibition decreases aPL-induced upregulation of TF in EC and aPL-enhanced thrombosis in mice	No
p38 Mitogen Activated Protein Kinase (MAPK)	p38MAPK inhibition decreases aPL-induced upregulation of TF in EC, platelet activation, and aPL-enhanced thrombosis in mice	No
Platelet Glycoprotein (GP) Receptors	GP receptor antagonists decrease the aPL-mediated enhancement of platelet activation and abrogate aPL-induced thrombus formation in mice	No
Hydroxychloroquine (HCQ)	HCQ decreases aPL-induced platelet activation, inhibits aPL-mediated thrombosis in mice, and protects aPL-induced displacement of annexin A5 from phospholipids bilayers	No (possibly protective against thrombosis in lupus patients)
Statins	Statins reverse aPL-induced endothelial cell activation and TF upregulation, and abrogate enhanced thrombus formation in mice	Statins decrease pro-inflammatory and pro-thrombotic markers (pilot data, small number of patients)
β_2GPI and/or anti-β_2GPI binding to Target Cells	Peptides that mimic domains of β_2GPI or β_2GPI receptor blockers (e.g. anti-annexin A2, anti-TLR4, aPOER2 antagonists) inhibit aPL-induced EC activation and/or aPL-mediated thrombosis in mice	No
Complement	Anti-C5 monoclonal antibody decreases aPL-mediated thrombus formation in mice; anti- C5aRA peptide inhibits aPL-mediated thrombosis and TF expression in mice	No
B Cells	B-cell activating factor (BAFF) blockage can prevent the disease onset in antiphospholipid syndrome mouse model	Rituximab is effective for non-criteria aPL manifestations, based on anecdotal reports

Tissue factor inhibition

Tissue factor (TF) upregulation has been advocated as an important mechanism to explain the pro-thrombotic effects of aPL. Endothelial cells and monocytes treated with aPL demonstrate upregulation of TF expression and function, which is accompanied by an increase in interleukin (IL)-6 and IL-8 secretion by those cells. Monocytes isolated from APS patients exhibit increased expression and transcription of TF mRNA and antigen; enhanced TF expression has been observed also in monocytes isolated from healthy individuals that are incubated with serum, plasma, and purified total immunoglobulin (Ig)G from APS patients (in particular, anti-β_2-glycoprotein-I (anti-β_2GPI) human monoclonal antibody derived from APS patients enhance monocyte TF mRNA and TF activity).[4–7] Hence, pro-coagulant cell activation, accompanied with TF expression and TF pathway upregulation, is one of the key pathophysiologic events in the development of thrombosis in aPL-positive patients.

TF-specific human studies demonstrate that patients with APS have: (a) elevated plasma levels of soluble TF;[8] (b) antibodies against TF pathway inhibitor, suggesting the impairment of the negative regulation of TF;[9] and (c) elevated plasma levels of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) (the most relevant proteins involved in the development of the vasculature),[6,10] which is associated with elevated TF expression (of note, monocytes from APS patients express increased levels of VEGF and VEGF receptor Flt-1 compared with monocytes from healthy donors,[6] suggesting that VEGF might act as a regulatory factor in aPL-mediated monocyte activation and TF expression).

Therapeutic approaches targeting TF are limited. Zhou et al. demonstrated that dilazep, an antiplatelet agent, inhibits in vitro aPL-induced monocyte and endothelial cell TF expression at a post-transcriptional level (probably via its effect as an adenosine uptake inhibitor, since increased extracellular concentration of adenosine also inhibits TF expression).[7] Other therapeutic possibilities for TF inhibition include dipyridamole (adenosine uptake inhibitor), pentoxifylline (inhibits lipopolysaccharide (LPS)-induced monocyte TF expression),[11] defibrotide (an adenosine receptor agonist that inhibits monocyte TF expression),[12] and angiotensin-converting enzyme inhibitors such as captopril (inhibits LPS-induced monocyte TF activity, antigen expression, and gene transcription).[13] However, there are no clinical studies in APS patients.

In summary, TF is emerging as a potential biomarker of thrombosis in APS. Given the relationship between increased TF activity and upregulation of pro-inflammatory cytokines, pharmacological agents that block TF activity may be a novel and attractive therapeutic approach in APS.

Nuclear factor-κB or P38 mitogen-activated protein kinase inhibition

Although there is convincing evidence that aPL can stimulate monocytes and endothelial cells, relatively little is known about the cell surface receptors and intracellular signaling pathways involved in this process. Espinola et al. first reported that aPL-induced upregulation of adhesion molecules in endothelial cells induce activation of nuclear factor-κB (NF-κB) in vitro,[14] a finding also confirmed by others.[15,16]

NF-κB is a complex group of heterodimeric and homodimeric transcription factors that are trapped in the cytoplasm as an inactive complex by IκB. Cell activation through cytokine stimulation, engagement of toll-like receptors (TLRs), or stress initiates a host-defense signaling pathway that can converge on an enzyme complex containing two IκB kinases. Upstream kinases, including members of the mitogen-activated protein kinase (MAPK) family and NF-κB-activating kinase (NAK), can phosphorylate the IκK signalsome and initiate the NF-κB cascade. This process is initiated within minutes of surface receptor ligation, releases NF-κB, and leads to its nuclear translocation, followed by initiation of gene transcription. 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. For instance, the transcription of many cytokine genes, including IL-6, IL-8, tumor necrosis factor (TNF)-α and IL-1β is initiated by NF-κB activation. Induction of TF and adhesion molecules on endothelial cells (VCAM-1, E-selectin and ICAM-1) as well as recruitment of inflammatory cells to extravascular sites are also mediated by this transcription factor. Activation of NF-κB has also been shown to be a critical mediator in some autoimmune diseases such as rheumatoid arthritis.

p38MAPK is also an important component of intracellular signaling cascades that initiate various inflammatory cellular responses. For instance, p38MAPK is an important regulator of the coordinated release of cytokines by immunocompetent cells and the functional neutrophil response to inflammatory stimuli. 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. p38MAPK 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.[17]

In platelets, p38MAPK is activated by stress (heat and osmotic shock, arsenite, H_2O_2, α-thrombin, collagen) and by thromboxane (TX) analogs. Moreover, p38MAPK is involved in the phosphorylation of cytosolic phospholipase A2 (cPLA2) with subsequent production of TXB2. Vega-Ostertag et al. demonstrated that aPL-mediated platelet activation involves phosphorylation of p38MAPK,[17] and both p38MAPK phosphorylation and NF-κB activation are required for in vitro aPL-induced TF upregulation.[18] These in vitro effects of aPL mediated by p38MAPK and NF-κB were confirmed also in monocytes.[16]

The effects of the specific p38MAPK inhibitor (SB 203580) (4-(4 fluorophenyl)-2 (4methylsulfinylphenyl)-(4pyridyl) 1 imidazole) and a specific NF-κB inhibitor (MG132) (carbobenzoxyl-leucinyl leucinylleucinal) were evaluated in vitro on aPL-induced TF expression of endothelial cells. Simoncini et al. showed that IgG aPL from 12 APS patients caused a large and sustained increase in reactive oxygen species (ROS),[19] which acted as a second messenger by activating the p38MAPK and its subsequent target (the stress-related transcription factor activating transcription factor-2 (ATF-2)). ROS also controlled the upregulation of VCAM-1 expression by IgG aPL-stimulated human umbilical vein endothelial cells (HUVEC) and the increase in THP-1 monocytic cells adhesion. In another study, Vega-Ostertag et al. showed that treatment of mice with aPL significantly increased TF function in peritoneal cells and in homogenates of carotid artery in vivo, when compared with control mice. This increased activity, which correlated with enhanced thrombosis formation and endothelial cell activation in vivo,[20] was inhibited by SB203580 and MG132.[20,21]

In summary, based on in vitro and vivo models, both p38MAPK and NF-κB are extensively involved in pro-inflammatory and pro-thrombotic pathways. Given that p38MAPK inhibitors are

currently tested in clinical trials for diseases such as septic shock, asthma or multiple myeloma, inhibition of these pathways could be a novel therapeutic strategy in aPL-positive patients.

Platelet glycoprotein receptor inhibition

Antiphospholipid Antibodies enhance the expression of platelet membrane receptors (particularly GPIIb/IIIa and GPIIIa) when platelets are pre-treated with low doses of ADP, thrombin, or suboptimal doses of thrombin receptor agonist peptide[22–24] (of note, the pre-stimulation of platelets by these agonists leads to the exposure of phosphatidylserine on the outer cell membrane, a requirement for the effects of aPL). Furthermore, aPL-enhanced thrombosis in vivo can be abrogated by infusions of a GPIIb/IIIa antagonist (1B5) monoclonal antibody, and aPL-mediated thrombosis is not observed in GPIIb/IIIa-deficient mice.[23]

Recently, Jiménez et al. also reported that double heterozygosity polymorphisms for platelet GPIa/IIa and GPIIb/IIIa increase arterial thrombosis risk in APS patients.[25] These data indicate that GPIIb/IIIa antagonists or platelet membrane GPIIb/IIIa receptor inhibitors may prove to be useful in the treatment of an acute thrombotic event – particularly arterial – in patients with APS.

Abciximab (a GPIIb/IIIa receptor inhibitor) has been used in the treatment of acute thrombotic syndromes, such as myocardial infarctions and strokes.[26] In addition, the combination of GPIIb/IIIa and ADP receptor antagonists, such as ticlopidine, is an attractive therapeutic strategy that provides fast and continuous platelet inhibition.

In summary, specific platelet glycoprotein receptor inhibitors may be important to inhibit aPL-induced platelet aggregation. However, no data on the use of these inhibitors in APS patients exist, apart from experimental data that hydroxychloroquine (HCQ) might inhibit aPL-induced GPIIb/IIIa receptor expression.[22]

Hydroxychloroquine

In addition to anti-inflammatory effects, HCQ possesses an anti-thrombotic effect by inhibiting platelet aggregation and arachidonic acid release from stimulated platelets.[27] Other immunomodulatory effects of HCQ include increasing the pH of intracellular vacuoles and interfering with antigen processing,[28] and inhibiting T-cell receptor- and B-cell antigen receptor-induced calcium signaling.[29]

In aPL-injected mice, HCQ decreases the thrombus size and the time of thrombus in a dose-dependent manner.[30] Furthermore, HCQ inhibits the aPL-induced platelet GPIIb/IIIa receptor expression (further discussed above) in a dose-dependent fashion.[22] Rand et al. recently demonstrated that HCQ reverses the binding of aPL-β_2GPI complexes to phospholipid bilayers[31] and protects the annexin A5 anticoagulant shield from disruption by aPL.[32]

In humans, HCQ was historically used as a prophylactic agent against deep venous thrombosis and pulmonary embolism after hip surgeries.[33] Wallace first observed that HCQ-receiving systemic lupus erythematosus (SLE) patients had had fewer thrombotic events.[34] Subsequently, multivariate analyses of large lupus cohorts demonstrated that HCQ decreases the risk of thrombosis,[35–37] except in the LUMINA (Lupus in Minorities: Nature versus Nurture) multiethnic lupus cohort where HCQ was not protective against thrombosis.[38] In a Cox multiple failure time analysis, Ruiz-Irastorza et al. demonstrated that antimalarial medications protect against thrombosis and increase survival in patients with SLE.[39] A cross-sectional study in which Erkan et al. compared 77 APS patients with vascular events (65% had no other systemic autoimmune diseases) with 56 asymptomatic aPL-positive (no history of thrombosis or fetal loss) patients (18% had no other systemic autoimmune diseases) suggested that HCQ is protective against thrombosis in aPL-positive individuals.[40] Given that HCQ has other beneficial effects in lupus patients, such as suppression of the disease activity and lowering the cholesterol levels, it is highly likely that the protection against thrombosis in HCQ-receiving lupus patients is multi-factorial.

McCarty and Cason reported that aCL titers decrease in patients treated with HCQ 200 mg twice daily and aspirin 81 mg once daily;[41] however, when Erkan et al. analyzed the degree of variation of aPL levels over time in a large cohort of aPL-positive patients, there was no relationship between HCQ treatment and change in aCL titers.[42]

In summary, although there is experimental and clinical evidence (mostly in lupus patients) that HCQ may decrease the incidence of thrombosis, controlled studies are needed to determine the effectiveness of HCQ for primary and secondary thrombosis prevention in APS. Even though there are not enough data to recommend HCQ as the only treatment for thrombosis prevention, it may

be reasonable to add HCQ to anticoagulation in APS patients who develop recurrent thrombosis despite optimal anticoagulation.

Statins

Statins are potent inhibitors of cholesterol synthesis in the mevalonate pathway. In the general population, clinical trials have demonstrated beneficial effects of statins in primary and secondary prevention of coronary heart disease as well as ischemic stroke; the beneficial effects of statins are not limited to lowering cholesterol levels.

Pleiotropic effects of statins include: (a) decreasing the expression of cellular adhesion molecules (CAM) in monocytes and affecting leukocyte–endothelial interactions; (b) downregulating inflammatory cytokines in endothelial cells; (c) increasing fibrinolytic activity; (d) decreasing TF mRNA expression and activity in cultured human monocytes obtained from healthy individuals; and (e) reversing TF upregulation induced by TNF-α and LPS in a dose-dependent manner.[24,43,44] Furthermore, cerivastatin, simvastatin, pravastatin, and fluvastatin substantially reduce TF expression in atherosclerotic lesions, along with suppression of inflammation in atheroma, independently of lipid lowering in animal models.[45]

These findings have been corroborated in the general population by multiple randomized controlled trials. In a double-blind, placebo-controlled study ATROCAP (Atorvastatin and Thrombogenicity of the Carotid Atherosclerotic Plaque), 4–6 months of treatment with atorvastatin (20 mg/day) was associated with 29% lower TF antigen levels and 56% lower TF activity in atherosclerotic plaques compared with placebo.[46] In healthy persons with normal low-density lipoprotein (LDL) levels of less than 130 mg/dL and elevated C-reactive protein levels greater than 2.0 mg/dL, rosuvastatin 20 mg daily significantly reduced the occurrence of the first major cardiovascular event and symptomatic venous thromboembolism.[47,48]

Statins interfere with aPL-mediated thrombosis by preventing the expression of CAMs and IL-6 in aPL-treated endothelial cells.[49] Ferrara et al. showed that the thrombogenic and pro-inflammatory effects of aPL in vivo could be abrogated in mice fed with fluvastatin for 15 days independent of the cholesterol-lowering effects.[50] Fluvastatin also inhibits the effects of aPL on TF expression on endothelial cells in vitro at doses utilized to reduce cholesterol levels in patients.[51] Martínez-Martínez et al. demonstrated that rosuvastatin decreases VCAM-1 expression by HUVEC exposed to APS serum in an in vitro model.[52] More recently, statins reversed the TF-mediated effects of aPL in a mouse model.[53]

Mechanistic studies in statin-receiving APS patients are limited. Our ongoing mechanistic study (ClinicalTrials.gov Identifier: NCT00674297) examines whether pro-inflammatory/pro-thrombotic markers are elevated in aPL-positive patients and whether treatment with fluvastatin is effective in decreasing these markers. Based on a preliminary analysis of nine APS patients (eight primary APS and one with concomitant SLE) who received 30 days of 40 mg daily fluvastatin, seven out nine, three out nine, and six out nine patients showed a variable but significant decrease in VEGF, soluble (s)TF, and TNF-α titers, respectively (of note, in a separate cross-sectional study, mean levels of TNF-α, VEGF, and sTF were significantly elevated in the sera of 93 APS patients when compared with 60 healthy controls).[54] Utilizing a proteomic analysis, Cuadrado et al. also showed that inflammatory proteins can be reversed in aPL-positive patients (25 with APS and 10 asymptomatic) following one month of 20 mg daily fluvastatin.[55]

In summary, although statins have been used in primary and secondary cardiovascular disease prevention in the general population, no controlled clinical data exist for thrombosis prevention in aPL-positive patients. Experimental evidence in APS models and a limited number of small mechanistic studies in APS patients justifies well-designed large-scale clinical studies of statins in non-pregnant aPL-positive patients (statins are teratogenic and therefore their use in pregnancy is contraindicated). The concomitant use of statins in the management of selected APS patients with recurrent thrombosis despite therapeutic anticoagulation can be considered.

Inhibition of β_2GPI and/or anti-β_2GPI binding to target cells

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

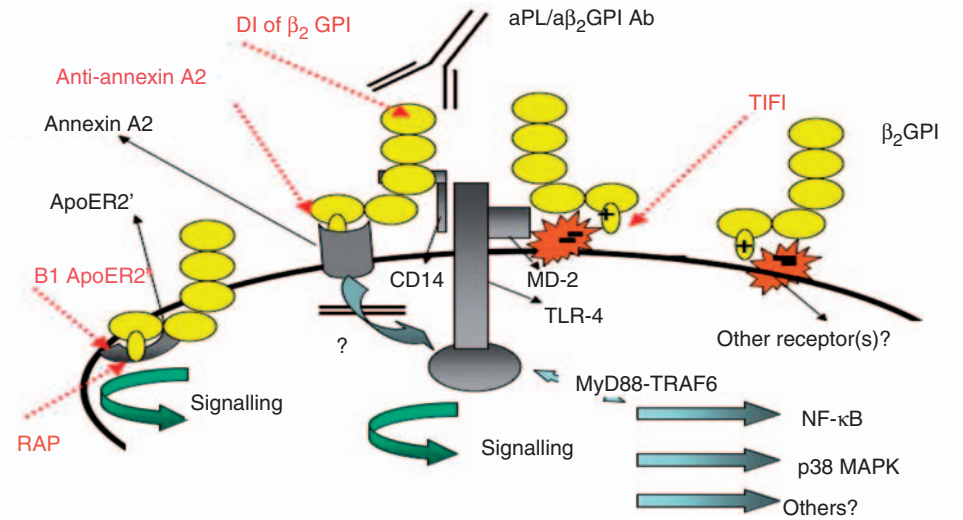

Figure 1 Diagrammatic representation of by β_2GPI receptors on the endothelial cell membranes and their signaling pathways. The dashed arrows indicate proposed ways to inhibit the binding of β_2GPI to receptor(s) and of anti-β_2GPI to β_2GPI. RAP: receptor associated protein; B1 ApoER2': binding domain I of apoER2'. Reproduced with permission from *Blood*. Romay-Penabad Z et al. *Blood* 2009; 114:3074–3083 © the American Society of Hematology.

PL-binding region.[56] β_2GPI interacts with diverse cell types, receptors, and enzymes (endothelial cells and monocytes, trophoblasts, and decidual cells).

Pathogenic aPL recognize β_2GPI bound to the above mentioned cells and 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. Anti-β_2GPI binding to domain I (DI) of β_2GPI triggers cell activation upon clustering and formation of complexes. Hence, blocking anti-β_2GPI binding to β_2GPI or β_2GPI binding to target cells may be the most specific approach to ameliorate their pathogenic effects without interrupting any important physiologic mechanism (Figure 1).

Ostertag et al. demonstrated that a 20 amino acid peptide (TIFI) mimics the phospholipid-binding region in domain V of β_2GPI and significantly decreases thrombus size in IgG-aPL-injected mice by displacing the binding of β_2GPI to target cells. TIFI also inhibited, in a dose-dependent fashion, the binding of fluoresceinated β_2GPI to endothelial cells, murine macrophages, and trophoblasts in vitro (Figure 1).[57]

The nature of the receptor(s) recognized by β_2GPI in target cells has been a subject of interest in recent years. Annexin A2, a receptor for tissue plasminogen activator, has been shown to mediate endothelial cell activation, which is followed by anti-β_2GPI binding to β_2GPI.[58,59] In addition, aPL-mediated pathogenic effects are ameliorated in annexin A2-deficient mice and diminished in vitro/vivo by an anti-annexin A2 antibody,[60] hence confirming the importance of annexin A2 in aPL-mediated thrombosis.

As annexin A2 does not span the cell membrane, this interaction may require an 'adaptor' protein(s) that is able to transduce intracellular signaling. Raschi et al. demonstrated that Myeloid Differentiation Factor 88 (MyD88) signaling cascade (an adaptor molecule for TLR-4 used to transduce TLR-mediated intracellular signaling such as translocation of NF-κB, phosphorylation of p38MAPK, or upregulation of pro-inflammatory cytokines) is triggered by anti-β_2GPI on human endothelial cells in vitro.[61] TLR-4 may be involved also as a co-receptor for endothelial cell signaling when anti-β_2GPI recognize β_2GPI bound to annexin A2 on the cell membrane. Zhang et al. recently identified a protein of 83 kD that appeared to be TLR-4 among those that bound to immobilized β_2GPI by affinity-purification in Affi-Gel HZ columns followed by elution, SDS-PAGE and LC-MS analysis.[62] Subsequently, Sorice et al. confirmed the involvement of TLR-4 and annexin A2 as a receptor for anti-β_2GPI in the cell surface of lipid rafts of monocytes.[63]

In order to evaluate the role of TLR-4 in aPL-mediated endothelial cell activation and thrombosis in vivo, Pierangeli et al. carried out experiments in LPS-non-responsive (−/−) and LPS-responsive (+/+) mice. LPS −/− mice displayed a point mutation of the *tlr4* gene leading to the expression of a TLR-4 which does not

recognize LPS. IgG aPL isolated from two APS patients produced significantly larger thrombi and induced increased TF activity in carotid artery homogenates. In addition, there was an increased number of adhering leukocytes to endothelial cells in the microcirculation of the cremaster muscle of LPS +/+ mice when compared with control IgG-NHS (as an indication of endothelial cell activation). These effects were abrogated after removal of the anti-β_2GPI activity from the IgG-aPL preparation. The IgG-aPL induced significantly smaller thrombus size, lower number of leukocytes adhering to endothelial cells, and TF activity in LPS −/− mice compared with LPS +/+ mice.[64] Altogether, the data demonstrate the involvement of TLR-4 in aPL-mediated in vivo pathogenic effects in mice. In recent studies, a TLR-4 ligand antagonist inhibited the binding of anti-β_2GPI and the upregulation of ICAM-1 in decidual cells and in human endothelial cells (Figure 1).

In recent experiments carried out in Pierangeli's laboratories, cultured HUVEC were treated with IgG-aPL (200 μg/ml), with IgG-aPL plus 1 mg/ml anti-TLR-4 (Santa Cruz Biotechnologies, sc-10741), IgG-normal human serum (NHS), or culture medium for 4 hours. TF activity was determined in lysates of the cells using a commercial kit that detects the conversion of Factor X into Factor Xa (Actichrome TF, American Diagnostica, Inc.) IgG-aPL induced a significant upregulation of TF activity in HUVEC when compared with cells treated with IgG-NHS (180.8 pM/mg/ml vs. 69.8 pM/mg/ml, respectively). The treatment with anti-TLR-4 decreased that activity by 38% (112.0 pM/mg/ml). In another set of experiments, cultured HUVEC were treated with 200 μg/ml IgG-APS or IgG-NHS in the presence or in the absence of 1 μg/ml TLR-4 ligand antagonist (Invivogen, cat# tlrl-mklps) or 4 μg/ml anti-TLR-4 Ab (Santa Cruz Biotechnologies, cat# sc-10741), for 4 hours. ICAM-1 and E-selectin expression were determined by cyto-ELISA. The data showed significant inhibition of aPL-induced ICAM-1 expression by TLR-4 antagonist (30%) and by anti-TLR4 antibody (39%). Similarly, E-selectin expression was inhibited by 23% and 42%, by those inhibitors.

Other molecules such as apoER2' also might act as receptors for β_2GPI. ApoER2' is a member of the LDL receptor family and is present in endothelial cells.[65] In addition to its function as a scavenger receptor for lipoproteins, apoER2' induces intracellular signaling.[66] In platelets, dimers of β_2GPI – that mimic β_2GPI/anti-β_2GPI complexes – bind to apoER2', leading to phosphorylation of p38MAPK, thromboxane production, and cell activation. Van Lummel et al. showed that domain V of β_2GPI is involved in both binding of β_2GPI to anionic phospholipids and also in interaction with apoER2' with subsequent activation of platelets.[67] Lutters et al. also demonstrated that when the apoER2' receptor on platelets is blocked using receptor-associated protein, the anti-β_2GPI-β_2GPI-induced increase in platelet adhesion to collagen is lost.[68] 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' is involved in the activation of platelets. As apoER2' is found in other cells, including endothelial cells and monocytes, it is hypothesized that β_2GPI binds to endothelial cells through a multi-protein receptor and intracellular signaling is started when aPL bind to β_2GPI bound to endothelial cells.

The bulk of the evidence favors that anti-β_2GPI binding epitopes are located within the N-terminal domain I (DI) of β_2GPI. Furthermore, the ability of anti-β_2GPI purified from APS patients to bind DI of β_2GPI is correlated with the occurrence of thrombosis.[69] The variants of β_2GPI lacking DI or with point mutations in DI have reduced ability to bind aPL derived from patients with APS. The same is not true for changes in the other domains. Ioannou et al. have developed the first (and so far the only) system for expressing DI in bacterial periplasm;[70] the investigators used this system to create a series of site-directed mutations in DI, which showed that two distinct areas of DI are important in binding IgG aPL extracted from APS patients.[71] 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 eight human aPL samples tested. In a recent study, Ioannou et al. also showed that soluble recombinant domain I of β_2GPI abrogates the in vitro and in vivo effects of anti-β_2GPI in a dose-dependent fashion. 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, underscoring the possibility of utilizing decoy peptides that are part of β_2GPI to abrogate the binding of pathogenic aPL to target cells in the treatment of APS patients.[72] Human studies are needed to establish the safety and efficacy of such treatment.

In summary, 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. Based on in vitro and animal studies, it is possible to speculate that inhibiting the binding of β_2GPI to the putative receptor proteins on target cells and/or the interaction of anti-β_2GPI to β_2GPI may be effective approaches to ameliorate aPL-mediated effects. Clinical studies are needed confirm these findings.

Complement inhibition

Complement activation has emerged as a common event in the pathogenesis of certain diseases, many of them associated with endothelial activation due to the presence of complement receptors on endothelial cells. Investigators have shown that complement activation is a critical early mediator in APS by linking aPL to thrombosis and fetal injury; C3, C5, and the membrane attack complex (MAC) are involved in aPL-mediated thrombosis, endothelial cell activation, and fetal loss in mice.[73,74]

Complement activation by aPL can contribute to thrombosis by increasing TF expression in various cell types, specifically through C5 and MAC binding to specific receptors in endothelial cells and upregulation of TF. Pierangeli and Fischetti demonstrated that inhibition of C5 activation (using anti-C5 monoclonal antibodies) prevents aPL-induced thrombophilia in aPL-treated mice, suggesting that C5a–C5aR interactions are critical mediators of aPL–induced thrombotic complication.[3,75,76]

Currently, a C5aR-antagonist peptide (C5aR-AP) also known as PMX-53 is in phase 2 clinical trials for the treatment of rheumatoid arthritis and psoriasis. In both studies, PMX-53 was found to be safe and well tolerated.[77] In a mouse model of APS pregnancy loss, PMX-53 was able to block the C5a–C5aR interaction, inhibiting the deleterious effects of aPL; Carrera-Marín et al. demonstrated that C5aR-AP can abrogate aPL-mediated thrombosis as well as TF activity and expression in aPL-treated mice (in carotid arteries homogenates and peritoneal macrophages), (Figure 2).[78]

In summary, a growing body of evidence shows that the complement pathway acts upstream of important effector mechanisms in aPL-associated thrombosis. Given that heparin prevents aPL-induced complement activation in animal models[79] and that low complement levels have been demonstrated in primary APS patients,[80–82] we speculate that the complement system could be a potential therapeutic target in aPL-positive patients.

B-cell inhibition and other anti-cytokine therapy

Based on the in vitro experience: (a) B cells are involved in aPL-related clinical events;[83] (b) B-cell activating-factor (BAFF) blockage can prevent disease onset and prolongs survival in APS murine models;[84] and (c) cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4-Ig) prevents initiation, but not development, of APS, in the NZW × BXSB F1 APS mouse model.[85]

Rituximab is an anti-CD20 chimeric monoclonal antibody that is effective against B-cell non-Hodgkin's lymphomas. Rituximab is also approved by the Food and Drug Administration for the treatment of rheumatoid arthritis. Based on limited number of case reports, rituximab can be effective for thrombocytopenia and hemolytic anemia in aPL-positive patients.[86–89] However, it is unknown if rituximab is effective against aPL-mediated-thrombosis or eliminates persistently and significantly positive aPL. An open-label Phase IIa descriptive pilot study (RITAPS) is ongoing, in which the effectiveness and safety of rituximab has been assessed in 20 patients with anticoagulation-resistant manifestations of aPL. The inclusion criteria of the RITAPS trial are (in addition to the persistent aPL-positivity): persistent thrombocytopenia; persistent autoimmune hemolytic anemia; cardiac valve disease; chronic skin ulcers; aPL-nephropathy; and/or cognitive dysfunction (confirmed by neuropsychological batteries with/without white matter changes). We believe that the RITAPS trial will provide systematic data on the clinical, laboratory, and serologic parameters of rituximab-receiving aPL-positive patients (Clinical Trials.gov Identifier: NCT00537290); see Table 1.

IL-6 is an important cytokine in atherothrombotic disorders, and IL-6 levels can help predict atherosclerosis and future cardiovascular risk.[90,91] In addition to inducing a pro-inflammatory and pro-thrombotic state, IL-6 also inhibits endothelial vasodilation and is associated with arterial stiffness. IL-6 has been suggested to induce aPL production by B lymphocytes,[92] and increased levels of IL-6 were demonstrated in APS patients.[8,93] Salobir et al. also showed an association between IL-6

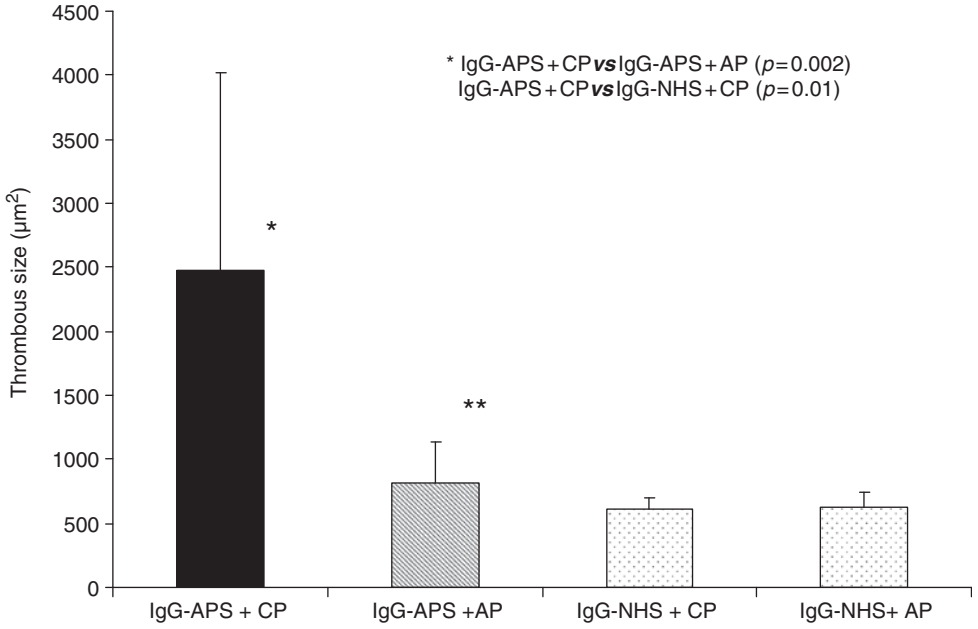

Figure 2 Effects of C5aR-AP on aPL-mediated thrombosis in mice. Mice in groups of five were injected with IgG aPL from an APS patient, control IgG (IgG-NHS) plus C5aR-AP, or control peptide (CP) twice, 48 h apart. Thrombus size was measured (in μm^2) in the femoral vein of mice 72 h after the first injection with aPL and the peptides. *Statistically significant difference between IgG-NHS and CP-treated mice. **Statistically significant difference between IgG-aPL and CP-treated mice.

and aβ_2GPI titers in aPL-positive patients who were on oral contraceptives at the time of thrombosis.[94] The potential effect of IL-6 inhibition in APS patients is unknown.

Conclusion

Barriers to the development of new treatment strategies in APS include the multifactorial nature of thrombosis, controversies about the strength of association between aPL antibodies and thrombotic events, and the fact that the mechanisms of aPL-induced thrombosis are not well understood. Despite these barriers, it is highly likely that the current 'anti-thrombotic' approach to aPL-positive patients will be replaced by an 'immunomodulatory' approach in the near future as our understanding of the molecular mechanisms of aPL-mediated thrombosis grows. We hope that this paper will guide basic and clinical researchers while designing future collaborative trials of aPL-positive patients.

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