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Investigating The Effect And Mechanism Of Antiphospholipid Antibodies On Human Endometrial Endothelial Cell Function And The Impact Of Current Standard Therapies

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Investigating the effect and mechanism of antiphospholipid antibodies on human endometrial endothelial cell function and the impact of current standard therapies

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Abstract

Women with pathologic levels of antiphospholipid antibodies (aPL) are at high risk for recurrent pregnancy loss and preeclampsia. Despite treatment with low molecular weight heparin (LMWH), either alone or with low dose aspirin (ASA), the incidence of late-term complications remains high for these patients. aPL recognizing β_2 glycoprotein I (β_2GPI) are particularly harmful during pregnancy as they target the placenta causing insufficient spiral artery transformation and inflammation, giving rise to obstetric APS. There have been extensive studies into the mechanisms by which aPL affect the placental trophoblast which constitutively synthesizes and expresses β_2GPI. However, aPL specific for β_2GPI can also bind to maternal uterine endothelial cells, but much less is known about the impact aPL have on the maternal side of the maternal-fetal interface. This study sought to characterize the effects of anti-β_2GPI aPL on human endometrial endothelial cells (HEECs); the role of toll-like receptor-4 (TLR4) in the mechanism of aPL-HEEC interactions; and the influence of LMWH and ASA on aPL-mediated HEEC responses using in vitro culture systems. aPL, but not control IgG, significantly increased HEEC secretion of pro-angiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF); increased anti-angiogenic soluble fms-like tyrosine kinase receptor-1 (sFlt-1); inhibited basal secretion of the chemokines monocyte chemoattractant protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF) and growth related oncogene- α (GRO-α); and impaired angiogenesis. The aPL-triggered inhibition of HEEC MCP-1 was mediated by activation of TLR4. LMWH and ASA, alone and in combination, exacerbated the aPL-induced changes in the HEEC angiogenic factor and chemokine profile. There was no reversal of the aPL-inhibition of HEEC

angiogenesis by either single or combination therapy. In conclusion, HEECs produce chemokines necessary for normal trophoblast invasion, immune cell recruitment, and spiral artery remodeling. By aPL inhibiting HEEC chemokine secretion and promoting sFlt-1 release, the uterine endothelium may contribute to impaired placentation and vascular transformation in obstetric APS. Combination LMWH and ASA may further contribute to this endothelium dysfunction in women with obstetric APS.

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Introduction

Diagnosis of APS

Antiphospholipid syndrome (APS) is an acquired autoimmune disease in which patients have circulating autoantibodies called antiphospholipid antibodies that aberrantly recognize certain phospholipid-binding proteins leading to a hypercoaguable state and increased risk of recurrent venous and arterial thrombosis. These patients have increased risk of stroke and myocardial infarction, among other complications and disease manifestations. In women, APS causes increased risk of recurrent fetal loss in addition to complications such as hypertension and preeclampsia, preterm labor, premature rupture of membranes, and intrauterine growth restriction [1, 2].

The Sapporo Classification Criteria Guidelines for diagnosis of this disease include both clinical and laboratory criteria. Criteria for clinical diagnosis rely on patient history while laboratory criteria relies on the presence of certain antibodies. The current clinical criteria include: arterial, venous, or small vessel thrombosis, in any organ or tissue; and pregnancy morbidity consisting of: 1) one or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation; or 2) one or more premature births of morphologically normal neonate at or before the 34^{th} week of gestation because of severe preeclampsia, eclampsia or severe placental insufficiency; or 3) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. The updated laboratory criteria include: medium to high serum titers (greater than 40 GPL/MPL or > 99th percentile) of IgG or IgM isotype of anticardiolipin on two or more occasions, at least 12 weeks apart measured by standardized ELISA; lupus anticoagulant present in plasma on two or more occasions at least 12 weeks apart

detected by the guidelines of the International Society of Thrombosis and Haemostasis; and anti-beta $_2$ glycoprotein-I (anti-β_2GPI) antibody of IgG or IgM isotype in serum or plasma (>99th percentile), also measured at 12 week intervals [1].

Given the fact that diagnosis of this disease in women oftentimes relies on repeated pregnancy loss, it may require years of symptoms and/or several episodes of high-risk gestation before patients receive proper treatment. During pregnancy, women with APS are at further increased risk of late-term complications in addition to the systemic cardiovascular risks of the disease. Furthermore, women with anti-β_2GPI antibodies are at highest risk of fetal loss and late-term complications because the placental trophoblast synthesizes and expresses β_2GPI, making this organ a specific target for aPL [3-6].

Antiphospholipid antibodies in APS

There are several antibodies that are pathogenic in APS, but, as previously stated, the most deleterious in pregnancy are anti-β_2GPI aPL. Anti-β_2GPI antibodies are a heterogeneous group of polyclonal antibodies, which bind to different epitopes of β_2GPI, a glycosylated, anionic phospholipid-binding glycoprotein that belongs to the complement control protein (CCP) superfamily. This protein's function is still a subject of study, but its structure is known. β_2GPI has five CCP domains. The first four domains have the regular, conserved sequences, but the fifth domain is different. These additional amino acids in domain V are responsible for a unique feature of this CCP domain, because they constitute a large positively charged patch that determines the affinity for anionic phospholipids [7]. Domain V is thought to interact with various cell surface

receptors such as apoER2, Toll-like receptors (TLRs), and other negatively charged molecules such as heparin, DNA, low density oxidized lipoproteins and annexin II [8]. In the serum, β_2GPI is circular with domain V binding to the cell surface causing a conformational change, thus exposing the other CCP domains. β_2GPI localizes to the syncytiotrophoblast cells that are in direct contact with maternal blood [3, 9] as well as the invasive cytotrophoblast and extravillous trophoblast cells. In addition, serum β_2GPI can also localizes to human endometrial endothelial cells, which the invasive trophoblasts invade and replace during normal spiral artery transformation [10]. Thus, both the trophoblast and endometrial endothelial cells are a target for anti-β_2GPI aPL. Studies have shown that different epitopes on β_2GPI are recognized by different aPL, with only some of these populations having pathological significance in aPL [11-13], though there is debate between which domains and epitopes are more pathogenic than others. Depending on the epitopes to which they bind, aPL can have varying functional and pathogenic effects on the cells they attack [14]. aPL recognizing β2GPI do not only bind trophoblasts, but can also affect the maternal side of the uteroplacental interface by binding directly to the endometrial endothelial cells [15-17]. Animal models have supported a role for anti-β_2GPI aPL in the pathogenesis of pregnancy failure and complications. Immunization of animals with β_2GPI or passive transfer of aPL to mice lead to pregnancy complications often seen in APS, namely fetal demise and IUGR, as well as reduced litter size and fetal resorption equivalent to implantation failure and pregnancy loss in humans [18, 19].

Normal Placentation

To form a normal placenta, a fertilized egg, a zygote, undergoes a process of cleavages into a collection of cells called a blastula and differentiates into what is called a blastocyst. The blastocyst contains a blastocoel, or fluid-filled blastocyst cavity, which contains an inner mass of cells called the embryoblast, that will form the fetus. The blastocyst also contains an outer layer of cells called the trophoectoderm, which forms the placenta. The blastocyst interacts with and implants into the maternal decidua. After contact and adhesion of the blastocyst to the uterine epithelium, trophoblast cells differentiate into a fusion lineage, the syncytiotrophoblast, and an invasive lineage, the cytotrophoblast. The invasive lineage of trophoblasts penetrate deep into the maternal decidua, differentiating into extravillous trophoblasts, through to the smooth muscle layer called the myometrium. This lineage of trophoblasts is also responsible for invading the uterine spiral arteries where they differentiate into endovascular trophoblast. Eventually they interact with and replace the endometrial endothelial cells in the spiral arteries order to form large caliber vessels for a well-rooted placenta with a large-volume blood supply in a process called vascular transformation [20].

In order for this placentation to occur, endothelial and decidual cells must adequately promote trophoblast invasion, immune cell recruitment, and spiral artery remodeling via the secretion and excretion of factors, cytokines, and chemokines, to establish the adequate placentation and vascular development needed for a healthy pregnancy. Normal placentation relies on successful vascular transformation and angiogenesis which leads to the expansion in size and number of vasculature at the maternal-fetal interface and of the placenta and decidua. Extravillous trophoblasts invade

the spiral arteries disrupting the smooth muscle cells, and interacting with and ultimately replacing the endothelial cells. This transforms them into high flow, low resistance vessels necessary to support the fetus through the term of pregnancy. If this process is aberrant and there is a failure of trophoblast contact, adhesion, invasion, and vascular transformation, pregnancy complications associated with insufficient placentation may result. In obstetric APS, preeclampsia is the most common complication associated with inadequate placentation. There are many proposed mechanisms for this observed pathology.

Preeclampsia and abnormal placentation in APS

Impaired angiogenesis and vascular development/remodeling.

Gestational hypertension and preeclampsia are likely due to insufficient placentation and poor spiral artery transformation. Since APS is a syndrome which affects the caliber of placentation, obstetric APS shares many features of preeclampsia. Clinical signs of inadequate placentation are placental hypoperfusion and ischemia which cause increased resistance in placental vessels and vascular endothelial stress. Clinical signs of preeclampsia in the mother are characterized by the onset of high blood pressure and often a significant amount of protein in the urine in addition to end-organ damage as a result of the hypertension after 20 weeks of pregnancy. In severe cases, preeclampsia can lead to eclampsia, which is characterized by maternal seizures and can progress to more severe liver, kidney, and retinal damage, systemic edema, and thrombocytopenia [21]. There are many potential causes of preeclampsia, but it is clear that some sort of placental injury or insults create the hypertensive phenotype of the disease and immediate delivery

of the fetus and placenta is the therapy of choice. Preeclampsia presents risks to the mother and fetus as immediate delivery due to potential end-organ damage of a potentially premature fetus represents a significant deviation from normal uncomplicated pregnancy. Premature births are at risk of cerebral hemorrhage and inadequate lung development. Women with preeclampsia are also at risk of further cardiovascular complications after delivery [22].

Preeclampsia is hypothesized to be as result of early aberrations in placental vascular remodeling, though it often presents as a late-term complication. Altered vascular reactivity, impaired trophoblast invasion, increased oxidative stress, deregulation of vasoactive substances, immune response and deregulation of angiogenic factor secretion have all been attributed to causing preeclampsia [21, 23, 24]. Similarly in obstetric APS, inadequate placentation and deregulation of angiogenic factor secretion has been shown to cause preeclampsia [25-27]. Furthermore, mouse models have suggested that two placental anti-angiogenic proteins, circulating sFlt1 and soluble endoglin (sEndoglin), play a causal role in the pathogenesis of preeclampsia [28, 29]. sEndoglin is a truncated, soluble form of Eng, which antagonizes transforming growth factor-β (TGF-β). TGF-β has many functions, but it is also a potent angiogenic factor and upregulates VEGF, in early gestational trophoblasts [29]. sFlt1 is a soluble, truncated variant of the VEGF receptor, Flt1. sFlt1 acts as a potent antagonist to VEGF and PlGF. VEGF and PlGF are both pro-angiogenic factors [30]. Placental growth factor is pro-angiogenic as it enhances the activity of VEGF by competitively binding to the VEGF receptor-1, allowing VEGF to bind then to VEGF receptor-2 which has stronger tyrosine kinase activity [31]. Studies have also shown that heterodimers of VEGF and PlGF act as

pro-angiogenic factors [32, 33]. Both VEGF and PlGF are made by the placenta and circulate in high concentration during pregnancy. There is an observed downregulation of circulating vascular endothelial growth factor, VEGF, and placental growth factor, PlGF and a marked increase in sFlt-1 and sEndoglin [21] in preeclampsia. Furthermore, a study by Levine *et al.* showed that rising circulating serum levels of sEndoglin and ratios of sFlt1:PlGF predict the onset of preeclampsia in pregnant patients [34]. In patients with APS, Cuadrado *et al*. demonstrated that VEGF may stimulate monocyte tissue factor expression through its receptor, Flt-1, contributing to the proinflammatory-prothrombotic phenotype of obstetric APS patients [25].

Reduced cytotrophoblast invasiveness

sFlt1 has also been shown to decrease cytotrophoblast invasiveness *in vitro* [35]. Reduced cytotrophoblast invasion is a shared characteristic between preeclampsia and obstetric APS. Di Simone *et al*. found that direct action of polyclonal patient derived aPL on cytotrophoblasts has been associated with reduced placental human chorionic gonadotropin (hCG) and placental placental lactogen (hPL) secretion and a complete block of trophoblast invasiveness and adhesion [36]. Similar findings were replicated by Katsuragawa *et al.* which found that monoclonal aPLs decrease trophoblast hCG and hPL secretion and block trophoblast invasiveness [37].

Trophoblast adhesion is also negatively affected by the trophoblast. Zhou *et al.* found that in normal placental development the cytotrophoblasts assume an endothelial phenotype in a process called vascular mimicry, or pseudovasculogenesis, by downregulating the expression of adhesion molecules characteristic of their epithelial cell

origin and adopting an endothelial cell surface adhesion phenotype [38]. In preeclampsia, cytotrophoblasts do not undergo switching of cell-surface molecules and are unable to invade the myometrial spiral arteries [39]. In obstetric APS, Di Simone *et al.* were able to demonstrate that patient derived aPLs are able to change the expression of trophoblast adhesion molecules [40]. Furthermore, they were able to show that aPL are able to mediate trophoblast gonadotropin secretion and reduced invasion through direct binding to adhered β_2GPI. Our group also found two domain V anti-β_2GPI Abs (ID2 and IIC5) inhibit first trimester trophoblast cell migration by downregulating IL-6 production. This inhibition leads to decreased signal transducer and activator of transcription 3 (STAT3) activity. By using a blocking anti-IL-6R antibody, IL-6 was shown to be partly responsible for trophoblast cells' spontaneous migratory capacity [41].

Dysfunctional immune cell recruitment and inflammation

Although systemic APS is known and studied as a pro-thrombotic disorder, obstetric APS has been increasingly been shown to be a disorder of inflammation. Previously, the prevailing hypothesis was that, similar to the pathophysiology of stroke, myocardial infarction, and venous thromboembolism in patients with systemic APS, the pathophysiology of recurrent pregnancy loss in pregnant patient with APS was due to clotting at the maternal-fetal interface [42-44]. This hypothesis was supported by the apparent success of the anti-thrombotic heparin combined with anti-platelet aspirin at increasing live birth weight. However, histological studies of placentas with APS confirm more prevalent inflammation rather than intraplacental thrombosis at the maternal-fetal interface including inflammatory cytokine expression, immune cell infiltration and

complement deposition [6, 45, 46]. Sebire *et al.* found that defective trophoblast invasion and spiral artery transformation was more often seen in aPL positive placentas, while there was no significant increase in thrombotic events between aPL positive and aPL negative placentas [47]. Similarly, Stone *et al.* showed that the placentas of these patients become infiltrated with inflammatory immune cells such as macrophages [46]. Salmon and Girardi showed using *in vivo* mouse models that aPL activate both classical and alternative complement pathways. Complement activation propagates injury to the fetus by recruiting inflammatory neutrophils, inducing their expression of tissue factor (TF), and overwhelming the trophoblast's normal inhibitory mechanisms [48]. This also potentiates inflammation in the decidua and leading to miscarriages [49]. Thus, while immune cells are necessary for promoting trophoblast recruitment, spiral artery remodeling, angiogenesis, and thus, successful implantation [50, 51], unregulated inflammation can cause defective placentation.

Mechanisms of placental inflammation in obstetric APS

It is hypothesized that anti-B_2GPI aPL bind to membrane-bound β_2GPI complexed with anionic transmembrane receptors and intracellular ligands on the surface of cells such as monocytes, trophoblasts, endothelial cells, and others. In this way, aPL are able to effect mechanisms of innate immunity, trophoblast proliferation, and cellular regulation of homeostasis [52]. However, the mechanisms of thrombotic and non-thrombotic aPL-mediated cellular changes are unclear, especially in the case of obstetric APS which is seen as a subset of APS and may have distinct pathogenic mechanisms.

Alone, one of β_2GPI's alleged functions is as a component of innate immunity. Agar et al. showed that binding of β_2GPI to LPS caused a conformational change in β_2GPI that led to binding of the β_2GPI-LPS complex to monocytes and ultimately clearance of this complex [53]. β_2GPI, once it is bound to aPL, has also been shown to bind and augment its interaction with transmembrane and intracellular ligands similarly to microbial and murine studies have shown that bacteria partially homologous with β_2GPI structure are able to induce the generation of pathogenic anti-β_2GPI antibodies along with some APS manifestations [54, 55]. Anti- β_2GPI antibodies have been shown to interact with many innate immune receptors such as toll-like receptors 2 and 4, most commonly, which recognize and bind bacterial LPS, in addition to annexin A2, an extracellular receptor for plasminogen and tissue type plasminogen activator, and apolipoprotein E receptor-2 (apoER2), involved in endocytosis of ligands and signal transduction [7, 56]. Thus, microbial mimicry may be the mechanism by which aPL are able to alter cellular response, not by inducing thrombosis through the complement cascade or interfering with thrombin and antithrombin III, as has previously been hypothesized [57].

Toll-like receptors have increasingly been associated with increased inflammatory cytokine release in systemic APS and more recently in obstetric APS [58]. In systemic APS, TLRs have been found to facilitate activation of monocytes and endothelial cells by aPL *in vitro* and *in vivo* [59, 60]. And a recent study by Laplante *et al.* showed that aPL-mediated effects on the arterial model of thrombosis require TLR4 activation [61]. Since TLR4 has been established as a necessary receptor in LPS signaling receptor in mice studies [62] and has been found to activate endothelial cells in APS [55], homology

between LPS and other microbes is seen as the mechanism by which aPL induce an inflammatory immune response. This theory was further supported in a study by Sorice et al., where it was demonstrated that in monocytes, anti-β2GPI binding Abs causes β2GPI and TLR4 to co-localize in lipid rafts and induce interleukin-1 receptor associated kinase (IRAK) phosphorylation and nuclear factor-κB (NF-κB) translocation and a subsequent release of tumor necrosis factor-α (TNF-α) and TF [63]. TNF-α is a central regulator of inflammation and tissue factor is a pro-coagulant. Tissue factor has also been implicated in aPL mouse studies where TF blockade prevented aPL antibody–induced inflammation and pregnancy loss. TF expression in neutrophils contributes to respiratory burst and subsequent trophoblast injury and pregnancy loss induced by aPL antibodies [64].

In the trophoblast, our group has shown that aPL induces a pro-inflammatory and anti-angiogenic response in first trimester trophoblast via the TLR4/MyD88 pathway [65]. Through TLR4 activation, anti-β_2GPI antibodies trigger increased trophoblast secretion of IL-8, MCP-1, GRO-α, and IL-1β. IL-8 is a potent neutrophil chemoattractant cytokine produced by several cells. In vivo, intracutaneous application of IL-8 induces local exudation and a massive, long-lasting accumulation of neutrophils [66]. IL-1β is a potent pro-inflammatory cytokine important in mediating host immune responses towards infection [67]. Not only has IL-1β upregulation been associated with autoimmune disease [68], but has also been associated with pre-eclampsia and pre-term birth [69, 70]. MCP-1 is a chemoattractant for human monocytes (which mature into macrophages) and may also activate cellular functions related to host defense [50, 71]. GRO-α is expressed on macrophages, neutrophils, and epithelial cells and has chemotactic properties for

neutrophils and is important in promoting successful spiral artery transformation and implantation [51].

Effect of aPL on endothelial cells in obstetric APS

The effect of aPL on the trophoblast has been well studied, however, much about the action of aPL on HEECs, the cells which trophoblasts bind to and replace during spiral artery transformation, is still unclear. Most aPL-endothelial interactions in pregnancy and placentas have been studied using fetus-derived human endothelial cells from umbilical veins (HUVECs) as a model for systemic vascular events in APS. Similar to findings in the trophoblast [65], aPL recognizing β_2GPI are able to activate the TLR4/MyD88 signaling pathway in HUVECs [72, 73] and induce an inflammatory cytokine/chemokine response [74]. Another study by Raschi *et al.* also using HUVECs showed that both human anti-β_2GPI IgM monoclonal antibodies and polyclonal affinity-purified anti-β_2GPI IgG antibodies induce a pro-inflammatory phenotype in endothelial cells, most likely via the involvement of TLRs and activation of the MyD88 pathway [55]. Di Simone *et al.* have investigated the influence of aPL on human endometrial endothelial cells and confirmed a direct effect of aPL on maternal cells. A 2010 report found that human polyclonal aPL significantly decreased the number and length of tubules formed by HEECs and reduced newly formed vessels in a murine model. aPL also significantly reduces VEGF and matrix metalloproteinase (MMP) production and suppressed NFκB DNA binding activity[16]. In a 2012 study by the same group, low-molecular weight heparin mitigated the aPL inhibited effects on NFκB and STAT-3 activity, VEGF secretion and MMP activity in HEECs [15]. Finally, Di Simone *et al.*

found that a synthetic peptide (that shares an amino acid sequence with domain V of β_2GPI) competes with aPL binding to HEECs to restore aPL-mediated effects on angiogenesis [17]. These studies indicate that aPL have a specific effect in HEECs and further investigation is needed to define the spectrum of ways angiogenesis and endometrial activity are altered by different aPL.

Preeclampsia, APS, and the use of Therapeutic Low molecular Weight Heparin and Aspirin

In the absence of aPL, heparin, both LMWH and unfractionated (UFH), and low dose ASA, have been proposed as potential prophylaxis for preeclampsia and other hypertensive disorders like HELLP syndrome in addition to being used to prevent unexplained fetal loss [75]. Although LMWH (and UFH) and low dose ASA have long been prescribed empirically, their effectiveness has only been demonstrated in cases of obstetric APS [76, 77]. However, LMWH and low dose ASA have also been suggested in second-trimester pregnancy loss [78] and in pregnancies deemed to be at high risk for poor perinatal outcome [79, 80].

Pregnant women with APS are routinely treated with LMWH, either alone or in combination with acetyl-salicylic acid (aspirin, ASA), as soon as possible in pregnancy and 6 weeks post-partum, with practice varying amongst physicians and with regard to patient disease severity and history [81]. In women with aPL, without a history of previous clot, or pregnancy loss, low dose ASA alone is deemed sufficient. In women with a history of previous thromboembolism, therapeutic doses of unfractionated heparin or LMWH are recommended. In women with medium titers of anti-cardiolipin

antibodies, anti-β_2GPI antibodies or lupus anticoagulant, and who have a history of 2 or more early pregnancy losses, fetal deaths or >1 preterm births, low dose ASA along with prophylactic doses of UFH or LMWH is the standard of care [82]. However, studies regarding each medication's effectiveness for preventing RPL in the setting of aPL are controversial [83, 84]. Heparin has been shown in previous studies to increase incidence of live birth weight and reduced rates of fetal loss. A study by Girardi *et al.* showed that heparin prevents fetal loss by inhibiting complement and not through its anticoagulant effects [85]. On the other hand, there is evidence that heparin increases the release of sFlt-1 and impairs VEGF signaling in placental villi, thereby potentially damaging and inhibiting placentation [86]. However, LMWH treatment also resulted in increased release of PlGF, whereas sEndoglin release remained unaltered and VEGF was undetected. Similarly, a study in 11 preeclamptic women in the third trimester showed that administration of LMWH resulted in a 26% mean increase in serum sFlt1 though this antiangiogenic effect was partially counterbalanced by a smaller increase in PlGF levels, resulting in a less pronounced difference in the ratio between the two concentrations [87]. Additionally, heparin does not reverse aPL-mediated changes in trophoblast angiogenic factor secretion but instead promotes sFlt-1 release [88]. However, LMWH can dampen aPL-induced trophoblast inflammation and complement activation, as shown by Girardi et al. in a murine model of APS-mediated pregnancy losses [85]. It must be noted that both UFH and LMWH are prescribed and studied, but LMWH has improved pharmacokinetics and bioavailability and is less likely to cause heparin-induced thrombocytopenia. It is also demonstrably more successful in some studies than unfractionated heparin [81], though the difference in pregnancy outcome between UFH

and LMWH in APS is also controversial [89]. UFH has also been shown to reduce trophoblast invasion in extravillous trophoblasts [90]. Aspirin is used as an adjunct medication, but has since been shown to have no significant beneficial effect and may no longer be indicated [91]. However, combination low molecular weight heparin and aspirin are still administered for obstetric APS and recent studies contend that early administration of aspirin in the first trimester is more beneficial than 2nd or 3rd trimester administration due to potential benefit in early stages of implantation [63] and beneficial effects of LMWH may be through mechanisms distinct of its anticoagulant properties.

Summary

The impact aPL have on trophoblast function and the mechanisms involved has been well studied. Much about the action of aPL on these human endometrial endothelial cells (HEECs) is still unclear. Most aPL-endothelial interactions in pregnancy and placentas have been studied using fetus-derived HUVECs as a model for systemic vascular events in APS. However, to better understand the placental endothelium and the nature of the maternal decidua's interaction with aPL, an additional set of fetally-derived cells are not as appropriate to detect key differences and, perhaps, similarities between aPL-trophoblast and aPL-endometrium interactions. The objective of this study was to elucidate the influence that domain I and V anti-β_2GPI aPL have on the maternal uterine vasculature using HEECs, and to investigate the role of TLR4. This study also sought to evaluate the current therapeutic strategy of LMWH and ASA in counteracting any influences aPL had on HEEC function.

Hypothesis

In previous studies, aPL recognizing domain V of β_2GPI have been shown to induce elevated levels of pro-inflammatory cytokines and chemokines [65]; inhibit spontaneous trophoblast migration[41]; and modulate trophoblast angiogenic factor secretion [47]. The inflammatory response of trophoblasts to aPL has been confirmed to be mediated by innate immune receptor TLR4. Similarly, researchers have studied the effect of aPL on systemic APS using HUVECs. This research also found that TLR4 may be influencing HUVEC response to aPL. Finally, there is conflicting evidence as to whether domain I or domain V anti-β_2GPI antibodies are more deleterious in APS pregnancies [73, 92]. Thus, my hypothesis ***is that aPL recognizing domain V, but not domain I induce a pro-inflammatory and anti-angiogenic profile in HEECs, disrupting their tube formation, via activation of TLR4. Furthermore, I hypothesize that LMWH and ASA do not attenuate aPL effects on HEECs.***

Specific Aims

1. To characterize the effect of aPL recognizing domain I or domain V of β_2GPI on HEEC cytokine, chemokine, and angiogenic factor secretion and tube formation.
2. To determine the role of TLR4 in HEEC responses to aPL.
3. To determine the effect of LMWH and/or ASA on HEEC responses to aPL.

Materials and Methods

Reagents

Sterile low molecular weight heparin (LMWH) (enoxaparin sodium injection; 100mg/ml), was purchased from Aventis Pharmaceuticals, Inc. (Bridgewater, NJ). Acetyl-salicylic acid (ASA) was obtained from Sigma-Aldrich (St. Louis, MO), reconstituted in ethanol, and filter-sterilized prior to use. Ethanol alone was included as a vehicle control for ASA. The TLR4 antagonist, LPS-RS (Lipopolysaccharide isolated from *Rhodobacter sphaeroides*), was purchased from Invivogen (San Diego, CA).

Human endothelial cells

The human endometrial endothelial cells (HEECs) used in this study were originally isolated from the microvascular of cycling endometrium of multiple women, and all stages of cycles were pooled. The primary cells were characterized to express a range of adhesion molecules and endothelial markers, and to form endothelial tubes when seeded in Matrigel. Subsequently these cells were telomerase immortalized and have been characterized to express the same markers and function as the primary cells [93-97].

Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Yale University's tissue culture core laboratory, courtesy of Dr. Seth Guller [98]. Cell culture was performed as previously described [93, 96] and both cell types were cultured in Endothelial Basal Medium-2 (EBM-2) supplemented with 2% fetal bovine serum (FBS) (Lonza; Allendale, NJ).

Antiphospholipid antibodies

This study used two mouse IgG1 anti-human β_2GPI monoclonal antibodies (mAbs), ID2 and IIC5. Like patient-derived polyclonal aPL, both IIC5 and ID2 bind β_2GPI in a similar manner to human aPL when they are immobilized on negatively charged surfaces such as phospholipids, cardiolipin, phosphatidyl serine or irradiated polystyrene [99]. The binding of both ID2 and IIC5 to β_2GPI is inhibited by patient-derived aPL, and both antibodies have been shown to alter trophoblast function similarly to patient-derived polyclonal aPL-IgG [100] and polyclonal IgG aPL recognizing β_2GPI [65, 88] upon binding to first trimester extravillous trophoblast cells, whereas a mouse IgG1 isotype control has no effect [65, 88]. IIC5 and ID2 react specifically with an epitope within domain V of β_2GPI, which may be more important than domain I binding aPL for pregnancy morbidity in APS patients [101]. IIC5 also has pronounced lupus anticoagulant activity and thus represents a triple positive aPL [102]. This study also used one anti-human β_2GPI Ab (P1-117) that reacts specifically with an epitope within domain I of β_2GPI [103, 104]. Mouse IgG1 clone 107.3 (BD Biosciences) was used as an isotype control.

Angiogenic factor and chemokine studies

HEECs ($5x10^5$) or HUVECs ($5x10^5$) were plated in 60mm tissue culture plates pre-coated with 2% gelatin in EBM-2 media supplemented with 2% FBS. The next day the media was replaced and the cells treated. HEECs or HUVECs were treated for 72hrs with either no treatment (NT), aPL (60μg/ml) or control IgG (60μg/ml) to establish the baseline effect of aPL. The aPL dose was determined by preliminary dose response experiments

(data not shown) and are similar to other studies [17]. To study the role of TLR4, HEECs were treated for 72hrs with NT or aPL in the presence of either media or LPS-RS (10μg/mL). To study the effect of current therapeutics on HEEC responses to aPL, cells were treated for 72hrs with NT, LMWH (10μg/ml), ASA (10μg/ml) or both LMWH and ASA (each at 10μg/ml) in the presence of either media or aPL (60μg/ml). The concentrations of ASA and LMWH used in this study were based on a previous study, and equivalent to low dose medications used in the clinical setting [105]. After treatment, cell-free supernatants were collected and stored at -80°C. Supernatants were then measured by ELISA and/or multiplex analysis. The pro-angiogenic factors: vascular endothelial growth factor (VEGF) and placental growth factor (PlGF); and the anti-angiogenic factors: soluble FMS-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEndoglin) were measured by ELISA (R&D Systems; Minneapolis, MN). The chemokines, monocyte chemoattractant protein 1 (MCP-1/CCL2) and granulocyte colony-stimulating factor (G-CSF/CSF3) were also measured by ELISA (R&D Systems). Multiplex analysis (Bio-Rad, Hercules, CA, USA) was performed for the following analytes as previously described [106]: interleukin (IL)-1β; IL-6; IL-8 (CXCL8); IL-10; IL-12 (p70); IL-17; growth-regulated oncogene-alpha (GRO-α/CXCL1); granulocyte macrophage-colony stimulating factor (GM-CSF/CSF2); interferon gamma (IFNγ); IFN γ inducible protein 10 (IP-10/CXCL10); macrophage inflammatory protein 1 alpha (MIP-1α/CCL3); MIP-1β (CCL4); Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES/CCL5) and tumor necrosis factor alpha (TNFα). ELISA was used for subsequent measurements of IL-8 (Assay Designs/Enzo Life Sciences; Farmingdale, NY, USA) and GRO-α (R&D Systems).

Angiogenesis assay

An endothelial tube formation assay was used to measure angiogenesis as previously described [15, 16]. HEECs ($5x10^4$) were seeded into 24-well tissue culture plates over undiluted reduced growth factor Matrigel (BD-Biosciences; San Jose, CA) with or without aPL (60μg/ml) or control IgG (60μg/ml). In separate experiments, HEECs were plated with NT, LMWH (10μg/ml), ASA (10μg/ml) or both LMWH and ASA (each at 10μg/ml) in the presence of either media or aPL (60μg/ml). Following an 8hr incubation, the formation of vessel tube-like structures was monitored by light microscopy (Carl-Zeiss Observer Z1). Four fields per well were recorded using OpenLab software (Perkin Elmer). The number of tubes per field were counted manually by two individuals, independently, and the two sets of data for each field were averaged.

Statistical analysis

Each experiment was performed at least three times. All analyses were performed at least in duplicate. All data are reported as mean ± standard error of the mean (SEM) of pooled experiments. Statistical significance was set at $p<0.05$ and determined using Prism Software (Graphpad, Inc; La Jolla, CA). For normally distributed data, significance was determined using either one-way analysis of variance (ANOVA) for multiple comparisons or a t-test. For data not normally distributed, significance was determined using a non-parametric multiple comparison test for multiple comparisons or the wilcoxon matched-pairs signed rank test.

Statement of Contribution

The author was responsible for execution of all cell culture maintenance, experiments, and data analysis with the exception of secondary tube counts which were completed by lab member Mancy Tong, and Multiplex analysis which was prepared by the author, but analyzed by Paulomi Aldo of the Gil Mor Lab.

RESULTS

Domain V aPL, IIC5, alter HEEC angiogenic factor and chemokine secretion

Figure 1

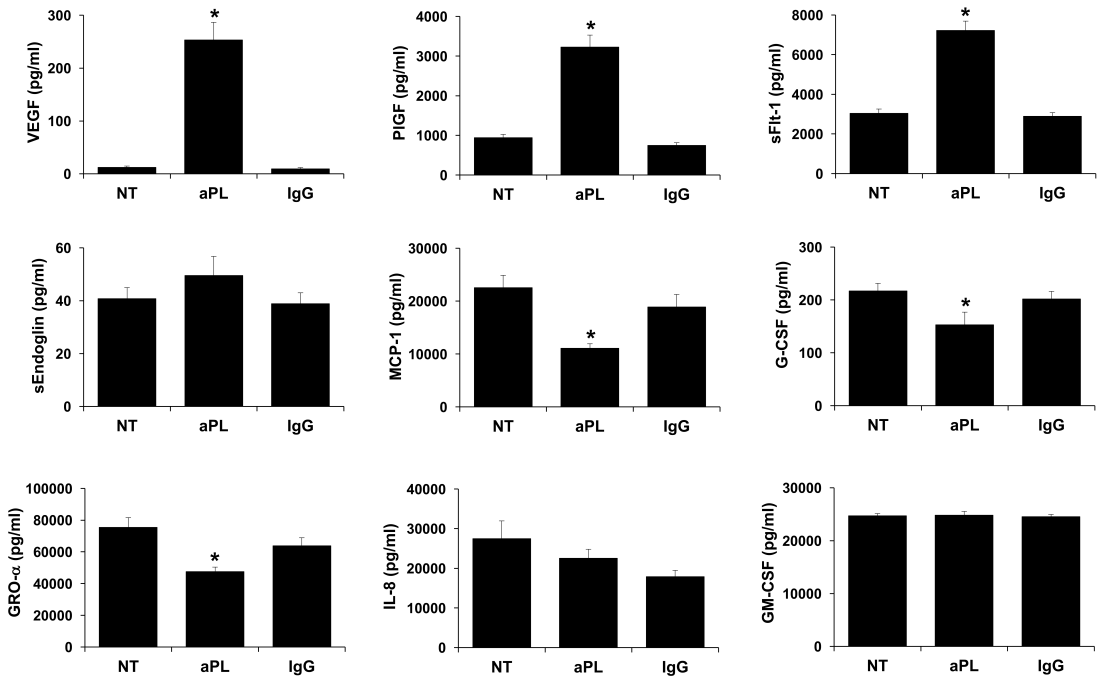

Figure 1. Effect of aPL on HEEC angiogenic factor and chemokine secretion. HEECs were treated with no treatment (NT), aPL (IIC5), or control IgG. Data are pooled from 9 independent experiments. Bar charts show secreted levels of VEGF; PlGF; sFlt-1; sEndoglin; MCP-1; G-CSF; GRO-α; IL-8; and GM-CSF as determined by ELISA and multiplex analysis. *$p<0.05$ relative to the NT control.

The first objective of this study was to measure the effects of the anti-β_2GPI aPL, IIC5, that recognizes domain V of β_2GPI on the HEEC angiogenic and cytokine/chemokine factor profile. Compared to the no treatment (NT) control, aPL significantly increased HEEC secretion of: pro-angiogenic VEGF by 20.5±8.7-fold; pro-angiogenic PlGF by 3.7±0.8-fold; and anti-angiogenic sFlt-1 by 2.4±0.4-fold (Figure 1). The IgG control had

no significant effect on HEEC secretion of VEGF, PlGF, or sFlt-1 (Figure 1). Neither aPL, nor the IgG control had any significant effect on HEEC production of sEndoglin compared tp the NT control (Figure 1). Treatment of HEECs with aPL significantly reduced basal MCP-1 secretion by 44.8±7.3%; G-CSF by 31.0±12.8%; and GRO-α by 37.5±4.6% when compared to the NT control. The IgG control had no significant effect on HEEC secretion of MCP-1, G-CSF, or GRO-α (Figure 1). Neither aPL, nor the IgG control, had any significant effect on HEEC production of IL-8 or GM-CSF compared to the NT control (Figure 1), and all other factors tested by multiplex were below the assay's detection limit (data not shown).

Domain V aPL, ID2, and Domain I aPL, PI-117, do not alter HEEC angiogenic factor and chemokine secretion

Figure 2

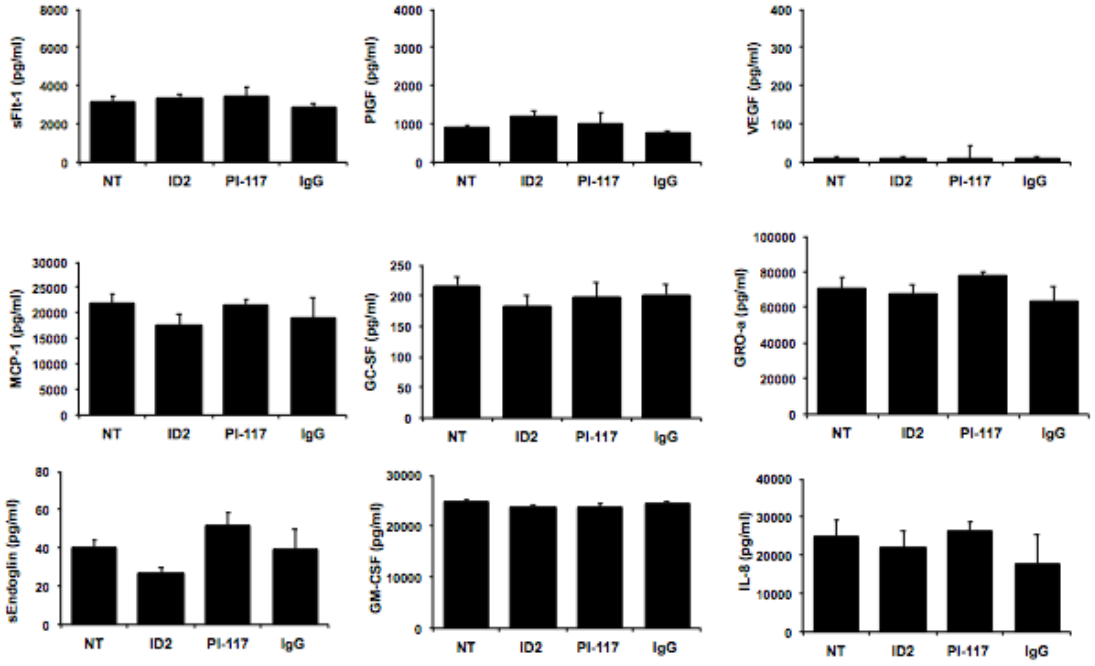

Figure 2. Effect of Domain V Abs, ID2, and Domain I Ab, PI-117 on HEEC angiogenic factor and chemokine secretion. HEECs were treated with no treatment (NT), ID2, PI-117 or control IgG. Data are pooled from 9 independent experiments. Bar charts show secreted levels of sFlt-1; PlGF; VEGF; MCP-1; G-CSF; GRO-α; sEndoglin; IL-8; and GM-CSF as determined by ELISA and multiplex analysis. *$p<0.05$ relative to the NT control.

The next objective was to determine whether the domain I aPL, PI-117, or domain V aPL, ID2, also influenced HEEC angiogenic and cytokine/chemokine factor profile. Compared to the no treatment (NT) control, ID2, PI-117, and IgG control had no

significant effect on HEEC secretion of sFlt-1, PlGF, VEGF, MCP-1, G-CSF, GROα, sEndoglin, GM-CSF, or IL-8 (Figure 2).

Domain V aPL, IIC5 induce a distinct angiogenic factor and chemokine profile in HUVECs

Figure 3

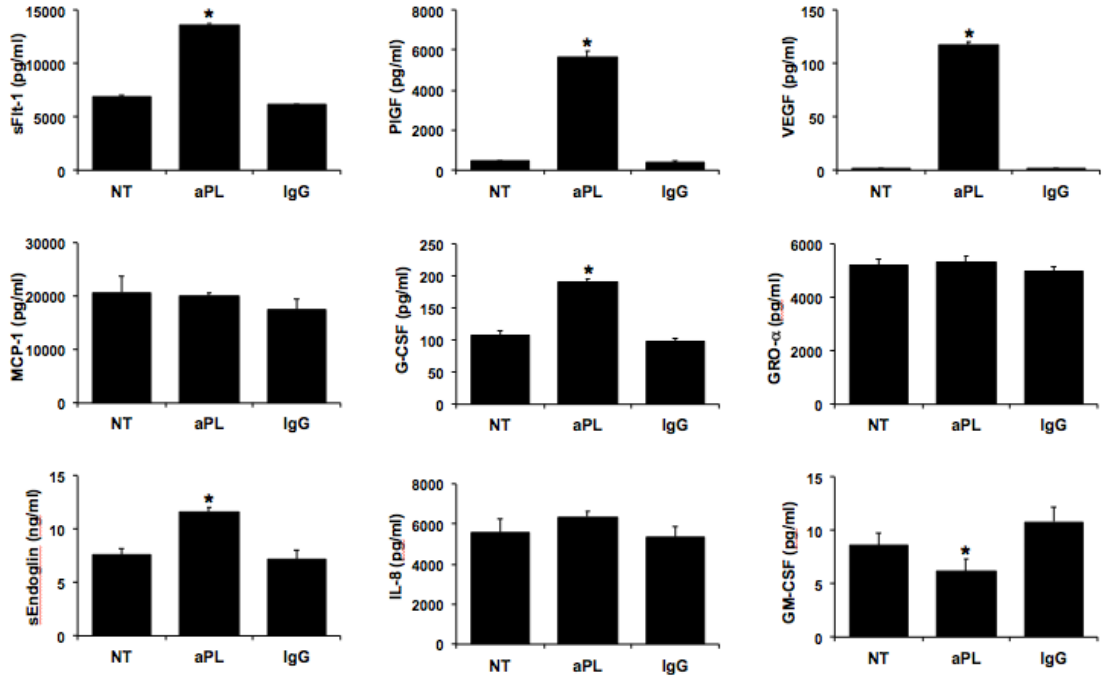

Figure 3. Effect of IIC5 aPL on HUVEC angiogenic factor and chemokine secretion. HEECs were treated with no treatment (NT), aPL, or control IgG. Data are pooled from 3 independent experiments. Barcharts show secreted levels of VEGF; PlGF; sFlt-1; sEndoglin; MCP-1; G-CSF; GRO-α; IL-8; and GM-CSF as determined by ELISA. *$p<0.05$ relative to the NT control.

Since HUVECs are commonly used as a model for the study of the endothelium in APS, (27-30), we sought to determine if their response to aPL recognizing β_2GPI was similar to the HEECs. Similar to the HEECs, aPL significantly increased HUVEC secretion of pro-angiogenic VEGF by 80.4±11.6-fold; pro-angiogenic PlGF by 12.1±0.3-fold; and anti-angiogenic sFlt-1 by 2.0±0.0-fold when compared to the NT control (Figure 3). The IgG control had no significant effect on HUVEC secretion of VEGF, PlGF or sFlt-1 (Figure 3). In contrast to the HEECs, aPL significantly increased HUVEC secretion of the anti-angiogenic sEndoglin by 1.6±0.2-fold compared to the NT control, while the IgG control had no effect (Figure 3). The aPL-modulated chemokine profile was also distinct. Compared to the NT control, aPL significantly increased HUVEC secretion of G-CSF by 1.8±0.2-fold and significantly reduced GM-CSF secretion by 28.0 ±20.4%, while the IgG control had no significant effect (Figure 3). Neither aPL, nor the IgG control, had any significant effect on HUVEC production of MCP-1, GRO-α or IL-8 compared to the NT control (Figure 3).

Domain V aPL, ID2, and Domain I aPL, PI-117, do not induce a distinct angiogenic factor and chemokine profile in HUVECs

Figure 4

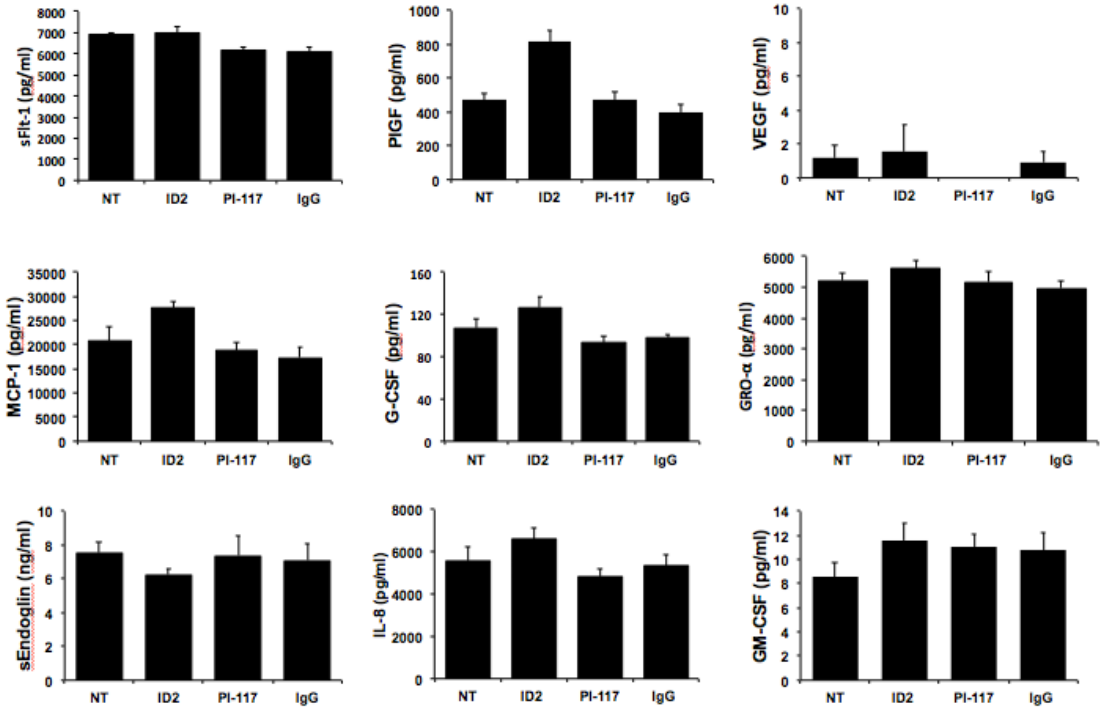

Figure 4. Effect of Domain V Ab, ID2, and Domain I Ab, PI-117 on HUVEC angiogenic factor and chemokine secretion. HEECs were treated with no treatment (NT), ID2, PI-117 or control IgG. Data are pooled from 9 independent experiments. Bar charts show secreted levels of sFlt-1; PlGF; VEGF; MCP-1; G-CSF; GRO-α; sEndoglin; IL-8; and GM-CSF as determined by ELISA and multiplex analysis. *$p<0.05$ relative to the NT control.

Again, since HUVECs are commonly used as a model for the study of the endothelium in APS, we sought to determine if their response to ID2 and PI-117 Abs recognizing domain V and domain I β2GPI epitopes, respectively, were similar to the HEECs. Compared to

the no treatment (NT) control, ID2, PI-117, and IgG control had no significant effect on HEEC secretion of sFlt-1, PlGF, VEGF, MCP-1, G-CSF, GRO-, sEndoglin, GM-CSF, or IL-8 (Figure 4).

aPL inhibition of HEEC MCP-1 secretion is mediated by TLR4

Figure 5

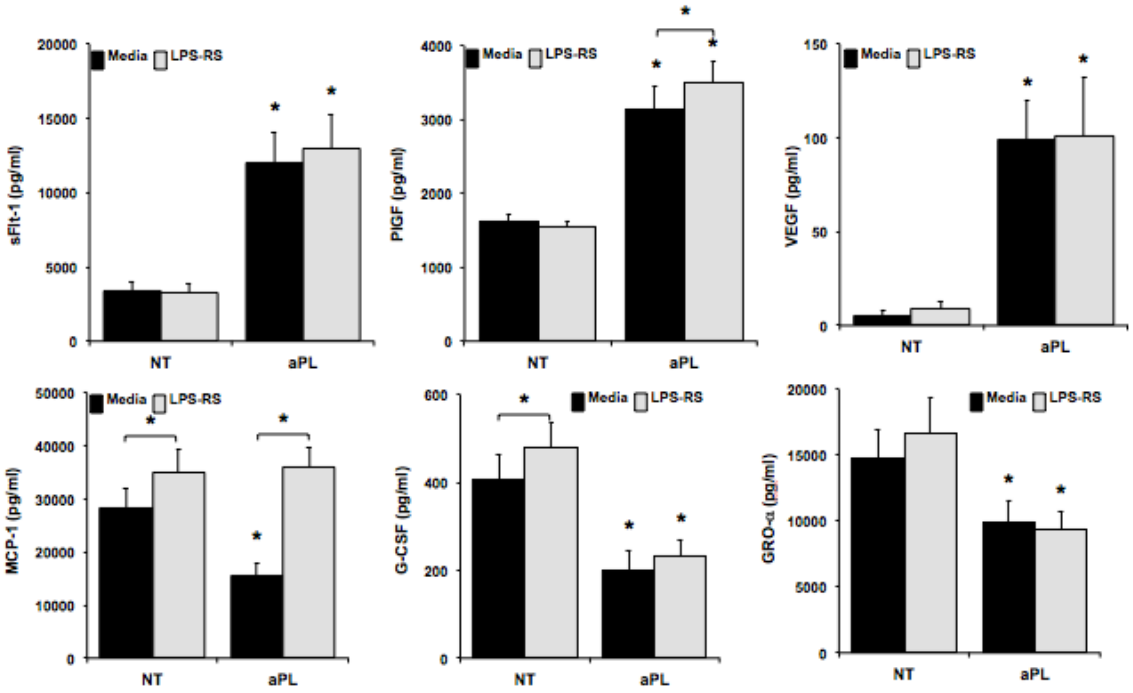

Figure 5. Effect of TLR4 inhibition on aPL-induced HEEC angiogenic factor and chemokine secretion. HEECs were treated with no treatment (NT) or aPL (IIC5) in the presence of media or LPS-RS. Supernatants were measured by ELISA for: VEGF; PlGF; sFlt-1; MCP-1; G-CSF; and GRO-α. Data are pooled from 3 independent experiments. *$p<0.05$ relative to the matching NT control for each condition (media or LPS-RS) unless otherwise indicated.

Having established the HEEC angiogenic and chemokine profile induced by the domain V aPL, IIC5, the next objective was to investigate the mechanism involved by blocking TLR4 function using the antagonist, LPS-RS. aPL-induced secretion of VEGF, PlGF and sFlt-1 was not reversed by the presence of LPS-RS, although aPL-induced PlGF was slightly yet significantly increased by 1.1±0.1-fold (Figure 5). While LPS-RS significantly increased basal MCP-1 levels by 1.3±0.0-fold, in the presence of aPL, MCP-1 levels were significantly increased by 2.4±0.1-fold, bringing levels back to baseline. Thus, the presence of LPS-RS, completely reversed the inhibition of HEEC MCP-1 secretion induced by aPL (Figure 5). Although LPS-RS slightly, yet significantly, increased basal G-CSF levels by 1.2±0.0-fold, there was no significant effect on the ability of aPL to inhibit HEEC secretion of either G-CSF or GRO-α (Figure 5).

Effect of LMWH and ASA on HEEC angiogenic factor and chemokine secretion in the presence and absence of aPL

Figure 6

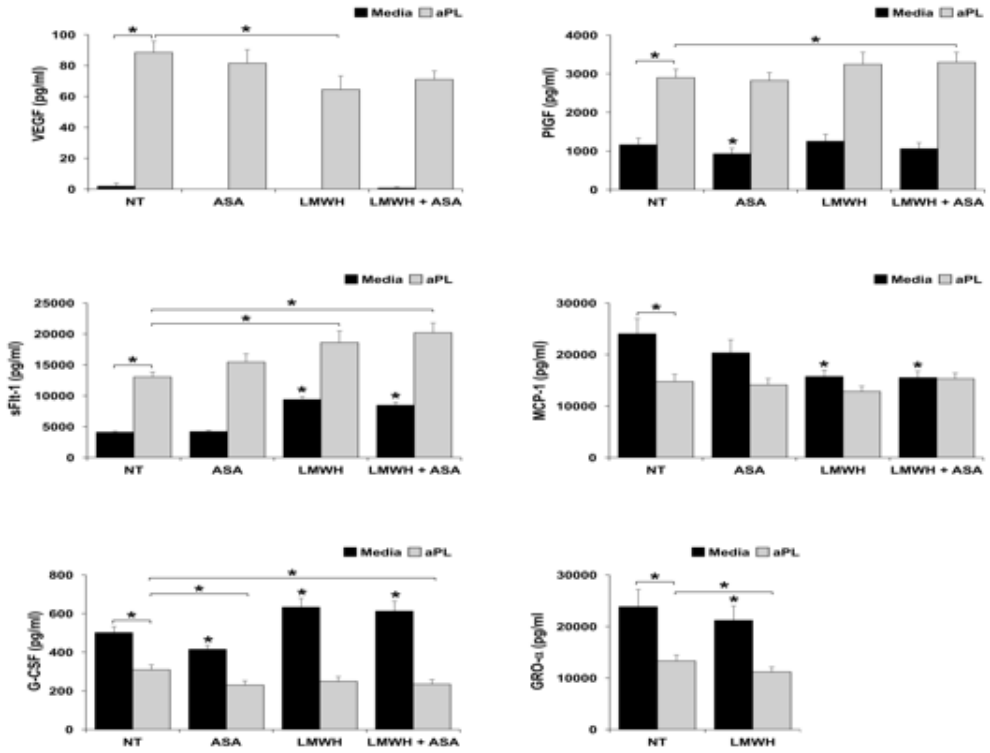

Figure 6. Effect of ASA and LMWH on HEEC aPL-induced HEEC angiogenic factor and chemokine secretion. HEECs were treated no treatment (NT), ASA, LMWH or both (LMWH + ASA) in the presence of media or aPL. Supernatants were measured by ELISA for: VEGF; PlGF; sFlt-1; MCP-1; G-CSF; and GRO-α. Data are pooled from 3 independent experiments. *$p<0.05$ relative to the NT/media control unless otherwise indicated.

Since our understanding of the actions of LMWH and ASA in obstetric APS are incomplete, the effects of these therapies, alone and in combination, on HEEC function in the presence and absence of aPL were investigated. The findings are summarized in

Table 1. As already determined, treatment of HEECs with the domain V aPL, IIC5, significantly increased VEGF, PlGF, and sFlt-1; and significantly inhibited MCP-1, G-CSF and GRO-α secretion compared to the NT/media control (Figure 6).

LMWH and ASA, either alone or in combination, had no effect on basal VEGF secretion. When compared to the NT/media control ASA alone significantly reduced basal PlGF by 19.8±3.8% and G-CSF by 16.4±4.3%. Compared to the NT/media control, LMWH alone, or in combination with ASA, significantly increased basal levels of HEEC sFlt-1 by 2.3±0.1-fold and 2.1±0.1-fold, respectively; increased basal levels of G-CSF by 1.3±0.1-fold and 1.2±0.1-fold, respectively; and decreased basal levels of MCP-1 by 31.3±5.0% and 31.3±6.5%, respectively. Compared to the NT/media control, LMWH alone significantly decreased basal levels of HEEC GRO-α secretion by 9.7±3.1% (Figure 6 & Table 1). Ethanol alone was run as a control for ASA and had no effect on any of the factors tested (data not shown), with the exception of GRO-α which was significantly inhibited by 25.9±8.1%. Thus, ASA, either alone or in combination with LMWH, was excluded from the GRO-α analysis.

LMWH alone significantly reduced the aPL-induced upregulation of HEEC VEGF secretion by 24.6±13.5%. However ASA, either alone or in combination, had no effect on VEGF production in the presence of aPL. LMWH in combination with ASA significantly augmented the aPL-induced upregulation of HEEC PlGF by 1.1±0.0-fold. However LMWH alone or ASA alone had no effect on PlGF production in the presence of aPL. LMWH either alone, or in combination with ASA, significantly augmented the aPL-

induced upregulation of HEEC sFlt-1 by 1.4±0.1-fold and 1.6s±0.1-fold, respectively, while ASA alone had no effect. LMWH and ASA, either alone or in combination, had no effect on the ability of aPL to reduce HEEC MCP-1 secretion. ASA either alone, or in combination with LMWH significantly and further reduced the aPL-mediated inhibition of HEEC G-CSF by 25.6±4.8% and 17.4±8.9%, respectively, while LMWH alone had no effect. LMWH alone further reduced the aPL-mediated inhibition of HEEC GRO-α secretion by 14.4±5.0% (Figure 6 & Table 1).

Table 1

Baseline effect on HEECs	ASA	LMWH	LMWH + ASA
Increased		sFlt-1, G-CSF	sFlt-1, G-CSF
Decreased	PlGF, G-CSF	MCP-1, GRO-α	MCP-1
No effect	VEGF, sFlt-1, MCP-1	VEGF, PlGF	VEGF, PlGF
Effect on HEECS in the presence of aPL	**ASA**	**LMWH**	**LMWH + ASA**
Further increased		sFlt-1	sFlt-1, G-CSF
Further decreased	G-CSF	VEGF, GRO-α	MCP-1
No effect	VEGF, PlGF, sFlt-1, MCP-1	PlGF, MCP-1, G-CSF	VEGF, MCP-1

Table 1: Summary of the effects of ASA and LMWH on HEEC angiogenic factor and chemokine secretion in the presence and absence of aPL.

aPL inhibit HEEC angiogenesis

Figure 7

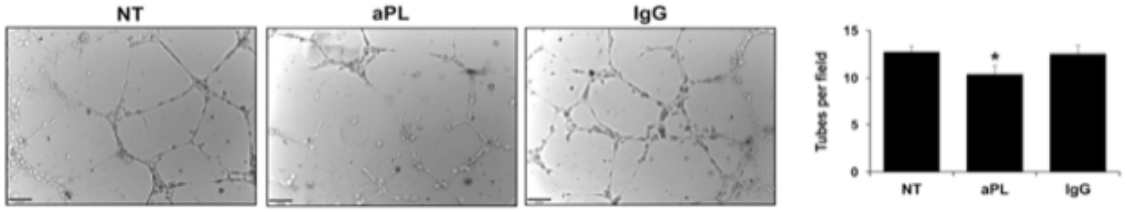

Figure 7. Effect of aPL on HEEC tube formation. HEECs were seeded on Matrigel-coated plates and treated with no treatment (NT), the domain V aPL, IIC5, or control IgG control. After 8hrs, tube-like vessels were imaged and quantitative analysis performed. Images are from one representative field of one representative experiment (magnification 10X). Bar chart shows the number of tubes counted per field and pooled from 5 independent experiments. *$p<0.05$ relative to the NT control.

The next objective was to determine whether aPL affected vascular development. For this, an angiogenesis assay was utilized in which the formation of HEEC vessel tube-like structures in Matrigel was measured. When compared to the NT control, the domain V aPL, IIC5, significantly reduced the number of HEEC tubes formed by 19.1±9.9%, while the IgG control had no significant effect (Figure 7).

Effect of LMWH and ASA on HEEC angiogenesis in the presence and absence of aPL

Figure 8

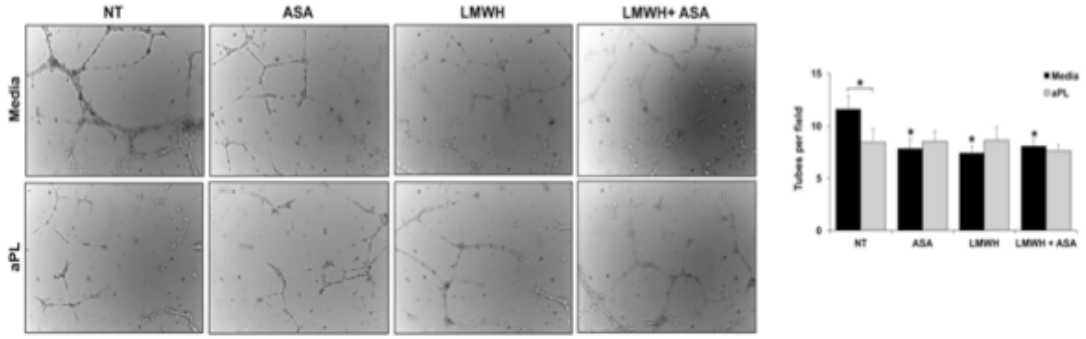

Figure 8. Effect of ASA and LMWH on HEEC tube formation in the presence and absence of aPL. HEECs were seeded on Matrigel-coated plates and treated with no treatment (NT), ASA, LMWH or both (LMWH + ASA) in the presence of media or the domain V aPL, IIC5. After 8hrs, tube-like vessels were imaged and quantified. Images are from one representative field of one representative experiment (magnification 10X). Bar chart shows the number of tubes counted per field and pooled from 4 independent experiments. *$p<0.05$ relative to the NT/media control unless otherwise indicated.

When compared to the NT/media control, HEEC tube formation was significantly reduced by 27.9±15.1% in the presence of ASA alone, by 30.2±14.2% in the presence of LMWH alone, and by 24.1±14.5% in the presence of combination LMWH and ASA (Figure 8). The ethanol control had no effect on the basal HEEC tube formation (Data not shown). As already determined, treatment of HEECs with aPL significantly inhibited HEEC tube formation by 26.3±9.1% compared to the NT/media control. LMWH and

ASA, either alone or in combination, had no additional effect on the numbers of HEEC tubes formed in the presence of aPL (Figure 8).

DISCUSSION

Obstetric APS, once thought of as a thrombotic disease, is now known to be inflammatory in origin and associated with placental insufficiency and reduced vascular development and remodeling [6]. One major way in which aPL negatively impact pregnancy is by targeting the placenta, via alteration of trophoblast function [107-109]. During pregnancy, aPL recognizing β_2GPI can also affect the maternal side of the interface by binding directly to the uterine endothelium [4, 15-17] which provides the scaffolding upon which trophoblast cells transform the maternal spiral arteries [10]. However, little is known about how aPL influence the function of these human endometrial endothelial cells (HEECs). Furthermore, little is known about the impact the current therapeutics for obstetric APS have on HEEC function. Herein, we report that an aPL recognizing domain V of β_2GPI modulates HEEC angiogenic factor and chemokine production, in part through TLR4 activation, and disrupts angiogenesis. Furthermore, LMWH and ASA, in general, exacerbate rather than protect against these aPL-mediated changes in HEEC function.

Previous studies found that aPL inhibits HEEC VEGF secretion; STAT3 phosphorylation; NFκB activity; and their ability to form vessel tube-like structures *in vitro* [107-109]. However, little else was known about their responses to aPL or the mechanisms involved. What these studies did highlight was a role for domain V of β_2GPI as the peptide TIFI, that shares homology with the aPL-binding site of domain V, blocked

these responses [17]. In our current study, using an aPL that binds to domain V of β_2GPI, [101] we found that HEEC pro-angiogenic (VEGF, PlGF) and anti-angiogenic factor production (sFlt-1) was augmented, while basal chemokine secretion (MCP-1, G-CSF, GRO-α) was inhibited. In addition, HEEC angiogenesis was inhibited. While anti-angiogenic sFlt-1 is associated with promoting hypertension and proteinuria in preeclampsia [110] there is also evidence that sFlt-1 contributes to poor placentation by impairing endothelial function, impacting uterine vessel remodeling [111, 112] and blocking the action of VEGF and PlGF, which promote trophoblast differentiation and invasion [113] and angiogenesis [114-116]. Thus, although HEEC production of pro-angiogenic VEGF and PIGF was also increased, the elevated sFlt-1 response may contribute to the impaired HEEC angiogenesis that we, and others [15-17], have observed. Moreover, the aPL-induced upregulation of HEEC VEGF production may drive the sFlt-1 response [116]. The differences in our studies demonstrating elevated HEEC VEGF secretion in response to aPL, while Di Simone's group showed a reduction in this pro-angiogenic factor, may be explained by differences in culture conditions or in the use of our monoclonal domain V aPL compared to their use of polyclonal aPL [15, 16].

In this study, we also found that aPL suppressed HEEC secretion of chemokines that promote trophoblast invasion (G-CSF) [117], and recruit macrophages and natural killer cells (MCP-1, GRO-α) that are necessary for normal spiral artery remodeling [50, 51, 118, 119]. These data suggest that aPL may contribute to the shallow trophoblast invasion and disrupted spiral artery transformation seen in obstetric APS by directly impacting the uterine endothelium, in addition to targeting the placental trophoblast [41,

120]. Furthermore, we observed that under basal conditions, in general, HEECs produced higher levels of chemokines compared to the angiogenic factors. This may reflect the normal function of uterine endothelial cells in playing an important role in immune cell recruitment at the maternal-fetal interface for successful vascular remodeling [121]. Our studies also highlight that being of fetal origin, and generating a distinct angiogenic and chemokine profile in response to aPL, HUVECs may not be the best model for studying the endothelium in either systemic or obstetric APS.

A role for TLR4 has been demonstrated in aPL-mediated thrombosis [58, 61] and in aPL-mediated trophoblast inflammation [65]. In studies using HUVECs, β_2GPI interacts directly with TLR4 and mediates aPL-induced endothelial cell activation [55, 72, 73, 122]. HEECs express functional TLRs, including TLR4 [97]. In this current study, TLR4 was found to mediate the aPL-induced suppression of HEEC MCP-1 secretion, but was not involved in any of the other responses tested, suggesting a role for additional cell surface receptors that aPL may recruit. These could include TLR2 [59] or ApoER2 [123].

Finally, our studies confronted the debate amongst domain specificity and aPL pathogenicity. Domain V ID2 and IIC5 have previously been shown to mediate changes in trophoblast secretion [92] and PI-117 has demonstrated lupus anticoagulant activity [104]. However, the reaction of HEECs and HUVECs, both endothelial cells, show that specific epitopes, even more specific than the general domain to which aPL bind, indicate what types of aPL are more deleterious to endothelial cells.

LMWH is commonly used as an anticoagulant treatment in systemic APS, and early initiation of aspirin (prior to 16 weeks of gestation) is thought to be beneficial for

fetal development [124]. Both therapies are given empirically to women with recurrent miscarriage, whether it is due to APS or not [75, 125, 126]. In women with aPL, while LMWH, either alone or in combination with aspirin, has been shown to decrease rates of fetal loss, results are conflicting [83, 89, 91, 127, 128]. Furthermore, despite treatment, women with APS continue to have high rates of late gestational complications [84, 125, 129, 130]. Given these controversies, we sought to elucidate the action of LMWH and ASA either alone or in combination on HEEC function in the absence and presence of aPL.

In the absence of aPL, LMWH and ASA, alone and in combination, induced potentially detrimental angiogenic and chemokine effects on HEECs, somewhat akin to aPL treatment. Similar to what has been found in the trophoblast [88, 105, 107, 131]. LMWH induced the release of sFlt-1, confirming this observation in endothelial cells from other sources [132]. LMWH also reduced HEEC angiogenesis, potentially as a consequence of the overwhelming sFlt-1 release [111, 112], which again has been observed in other endothelial cell types [132]. Interestingly, ASA had a similar effect by inhibiting HEEC tube formation, an observation also seen in HUVECs [114, 115]. As a result, combination of LMWH and ASA increased HEEC sFlt-1 release and limited angiogenesis. Both single and combination therapies also reduced basal HEEC chemokine production.

This may be because LMWH can act through non-thrombotic pathways, by inhibiting inflammation [65, 85, 105] and ASA is an anti-inflammatory agent [133, 134]. In the context of aPL, LMWH alone further augmented aPL-induced HEEC sFlt-1 release, further reduced GRO-α secretion, and reduced aPL-induced VEGF, while ASA

alone further reduced HEEC G-CSF secretion. In combination, LMWH and ASA further augmented aPL-induced HEEC sFlt-1 and PlGF release, and further reduced G-CSF secretion. Thus, the net effect of LMWH and ASA was the exacerbation of aPL-induced changes in the HEEC angiogenic factor and chemokine profile. Additionally, there was no reversal of the aPL inhibition of HEEC angiogenesis by either single or combination therapy.

In summary, HEECs produce a number of chemokines and pro-angiogenic factors that may promote trophoblast invasion, immune cell recruitment, and spiral artery remodeling, to establish the adequate placentation and vascular development needed for a healthy pregnancy. An aPL recognizing domain V of β_2GPI dysregulates this HEEC angiogenic factor and chemokine profile, and limit angiogenesis. Thus, the uterine endothelium may contribute to impaired placentation and vascular transformation seen in women with obstetric APS. LMWH and ASA, either alone or in combination, may further contribute to endothelial dysfunction in the setting of aPL, which may explain the inability of current therapies to prevent adverse pregnancy outcomes in women with APS.

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