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Low Molecular Weight Heparin and Aspirin Exacerbate Human Endometrial Endothelial Cell Responses to Antiphospholipid Antibodies

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Abstract

Problem—Women with antiphospholipid antibodies (aPL) are at risk for pregnancy complications despite treatment with low molecular weight heparin (LMWH) or aspirin (ASA). aPL recognizing beta₂ glycoprotein I can target the uterine endothelium, however, little is known about its response to aPL. This study characterized the effect of aPL on human endometrial endothelial cells (HEECs), and the influence of LMWH and ASA.

Method of Study—HEECs were exposed to aPL or control IgG, with or without low dose LMWH and ASA, alone or in combination. Chemokine and angiogenic factor secretion was measured by ELISA. A tube formation assay was used to measure angiogenesis.

Results—aPL increased HEEC secretion of pro-angiogenic VEGF and PlGF; increased anti-angiogenic sFlt-1; inhibited basal secretion of the chemokines MCP-1, G-CSF and GRO- α ; and impaired angiogenesis. 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.

Conclusions—By aPL inhibiting HEEC chemokine secretion and promoting sFlt-1 release, the uterine endothelium may contribute to impaired placentation and vascular transformation. LMWH and ASA may further contribute to endothelium dysfunction in women with obstetric APS.

Keywords

Endothelium; Pregnancy; Antiphospholipid Antibody; Angiogenic; Chemokine

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Introduction

Antiphospholipid syndrome (APS) is an acquired autoimmune thrombophilia characterized by the presence of at least one of three types of antiphospholipid antibodies (aPL): anti-cardiolipin; anti-beta₂ glycoprotein I (β_2 GPI); and lupus anticoagulant. Systemic manifestations of this disease are a hyper-coagulable state which leads to increased risk of recurrent venous or arterial thrombosis. In women, recurrent pregnancy loss (RPL) is a hallmark of obstetric APS. During pregnancy, women with APS are at further increased risk of uteroplacental insufficiency, intrauterine growth restriction (IUGR), and preeclampsia^{1, 2}. Furthermore, women with anti- β_2 GPI antibodies are at highest risk of fetal and late-term complications because the placental trophoblast synthesizes and expresses β_2 GPI, making this organ a specific target for aPL³⁻⁶.

Pregnant women with APS are routinely treated with low molecular-weight heparin (LMWH), either alone or in combination with acetyl-salicylic acid (aspirin, ASA), with practice varying amongst physicians⁷. Studies regarding each medication's effectiveness for preventing RPL in the setting of aPL are controversial⁸⁻¹², especially in light of histological studies which confirm inflammation rather than intraplacental thrombosis at the maternal-fetal interface⁶. Moreover, despite treatment, pregnant patients with APS continue to have high rates of preeclampsia and preterm birth¹³⁻¹⁶. Experimental studies have further confirmed the lack of thrombosis, and instead demonstrated inflammation and placental insufficiency as the underlying pathogenesis for obstetric APS¹⁷⁻²⁰. Thus, any beneficial effects of LMWH may be through mechanisms distinct of its anticoagulant properties^{18, 21-23}. The majority of human *in vitro* studies have focused on how aPL impact the placental trophoblast. Anti- β_2 GPI aPL induce a pro-inflammatory, anti-angiogenic, and anti-migratory profile in trophoblast cells, leading to their diminished ability to interact with and replace the endothelial cells found within the uterine spiral arteries¹⁸⁻²⁰. Furthermore, a role for the innate immune Toll-like receptor 4 (TLR4) in initiating aPL-induced trophoblast inflammation has been demonstrated²².

The serum glycoprotein, β_2 GPI, localizes not only to the placental trophoblast but also to the uterine endothelium⁴. As result, aPL recognizing β_2 GPI can also affect the maternal side of the utero-placental interface by binding directly to the endometrial endothelial cells²⁴⁻²⁶; the same cells that the trophoblast interacts with and replaces during normal spiral artery transformation²⁷. However, much about the action of aPL on these human endometrial endothelial cells (HEECs) is still unclear. Most aPL-endothelial interactions 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 trophoblast²², aPL recognizing β_2 GPI are able to bind HUVEC TLR4, activate the TLR4/MyD88 signaling pathway²⁸⁻³⁰, and induce an inflammatory cytokine/chemokine response^{29, 31}. The objective of this study was to elucidate the influence that anti- β_2 GPI aPL have on the maternal uterine vasculature using human endometrial endothelial cells (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.

Materials & 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 microvasculature 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^{32–36}. Primary human umbilical vein endothelial cells (HUVECs) were obtained from Yale University's tissue culture core laboratory³⁷. Cell culture was performed as previously described^{32, 35} 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 the aPL, IIC5, which is a mouse IgG1 anti-human β_2 GPI monoclonal antibody. This aPL has been previously characterized. Like patient-derived polyclonal aPL, IIC5 binds β_2 GPI when it is immobilized on a negatively charged surface such as phospholipids, cardiolipin, phosphatidyl serine or irradiated polystyrene³⁸. IIC5 also has pronounced lupus anticoagulant activity and thus represents a triple positive aPL³⁹. IIC5 reacts specifically with an epitope within domain V of β_2 GPI, which may be more important than domain I binding aPL for pregnancy morbidity in APS patients⁴⁰. Moreover, IIC5 binds to human first trimester extravillous trophoblast cells^{3, 22, 41}, and alters their function in a similar fashion to patient-derived polyclonal aPL-IgG⁴², and polyclonal IgG aPL recognizing β_2 GPI^{22, 43}. Mouse IgG1 clone 107.3 (BD Biosciences) was used as an isotype control.

Angiogenic factor and chemokine studies

HEECs (5×10^5) or HUVECs (5×10^5) 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 (60 μ g/ml) or control IgG (60 μ g/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 studies²⁶. To study the role of TLR4, HEECs were treated for 72hrs with NT or aPL in the presence of either media or LPS-RS (10 μ g/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 setting²³. 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 (PIGF); 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 described⁴⁴: 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 (IFN γ); IFNγ -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). All factors were detected within the range of the assays.

Angiogenesis assay

An endothelial tube formation assay was used to measure angiogenesis as previously described^{24, 25}. HEECs (5×10^4) 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 either mean $\pm$ SEM of pooled experiments for bar charts, or median and quartiles $\pm$ SD for box plots 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.

Results

aPL alter HEEC angiogenic factor and chemokine secretion

The first objective of this study was to measure the effects of aPL 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 to the NT control (Figure 1). Treatment of HEECs with aPL significantly reduced basal MCP-1 secretion by $44.8 \pm 7.3\%$; G-CSF by $31.0 \pm 12.8\%$; and GRO- α by $37.5 \pm 4.6\%$ 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).

aPL induce a distinct angiogenic factor and chemokine profile in HUVECs

Since HUVECs are commonly used as a model for the study of the endothelium in APS^{28–31}, we sought to determine if their response to aPL recognizing β_2 GPI 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 2). The IgG control had no significant effect on HUVEC secretion of VEGF, PlGF or sFlt-1 (Figure 2). 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 2). 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 $28.0 \pm 20.4\%$, while the IgG control had no significant effect (Figure 2). 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 2).

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

Having established the HEEC angiogenic and chemokine profile induced by aPL, 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 3). 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 3). Although LPS-RS slightly, yet significantly, increased basal G-CSF levels by 1.2 ± 0.0 -fold, there was no significant effect of LPS-RS on the aPL-mediated inhibition of G-CSF or GRO- α secretion by HEECs (Figure 3).

aPL inhibit HEEC angiogenesis

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, aPL significantly reduced the number of HEEC tubes formed by $19.1 \pm 9.9\%$, while the IgG control had no significant effect (Figure 4).

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

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 aPL 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 5).

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 $19.8 \pm 3.8\%$ and G-CSF by $16.4 \pm 4.3\%$. 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 $31.3 \pm 5.0\%$ and $31.3 \pm 6.5\%$, respectively. Compared to the NT/media control, LMWH alone significantly decreased basal levels of HEEC GRO- α secretion by $9.7 \pm 3.1\%$ (Figure 5 & 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 $25.9 \pm 8.1\%$. 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 $24.6 \pm 13.5\%$. 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 1.6 ± 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 $25.6 \pm 4.8\%$ and $17.4 \pm 8.9\%$, respectively, while LMWH alone had no effect. LMWH alone further reduced the aPL-mediated inhibition of HEEC GRO- α secretion by $14.4 \pm 5.0\%$ (Figure 5 & Table 1).

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

When compared to the NT/media control, HEEC tube formation was significantly reduced by $27.9 \pm 15.1\%$ in the presence of ASA alone, by $30.2 \pm 14.2\%$ in the presence of LMWH alone, and by $24.1 \pm 14.5\%$ in the presence of combination LMWH and ASA (Figure 6). 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 $26.3 \pm 9.1\%$ 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 6).

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 remodeling⁶. One major way in which aPL negatively impact pregnancy is by targeting the placenta; altering trophoblast function^{18–20}. During pregnancy, aPL recognizing β_2 GPI can also affect the maternal side of the interface by binding directly to the uterine endothelium^{4, 24–26}, which provides the scaffolding upon which trophoblast cells transform the maternal spiral arteries²⁷. 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 aPL modulates HEEC angiogenic factor and chemokine production, in part through TLR4 activation, and disrupts angiogenesis. Furthermore, LMWH and ASA, in general, exacerbates rather than protects against these aPL-mediated changes in HEEC function.

Previous studies found that aPL inhibit HEEC VEGF secretion; STAT3 phosphorylation; NF κ B activity; and their ability to form vessel tube-like structures *in vitro*^{24–26}. However, little else is known about their responses to aPL or the mechanisms involved. What these studies did highlight was a role for domain V of β_2 GPI since the peptide TIFI, that shares homology with the aPL-binding site of domain V, blocked these responses²⁶. In our current study, using an aPL that binds to domain V of β_2 GPI⁴⁰, 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 preeclampsia⁴⁵, there is also evidence that sFlt-1 contributes to poor placentation by impairing endothelial function, impacting uterine vessel remodeling^{46, 47}, and blocking the action of VEGF and PlGF, which promote trophoblast differentiation and invasion⁴⁸, and angiogenesis^{49–51}. Thus, although HEEC production of pro-angiogenic VEGF and PlGF were also increased, the elevated sFlt-1 response may contribute to the impaired HEEC angiogenesis that we, and others^{24–26}, have observed. Moreover, the aPL-induced upregulation of HEEC VEGF production may drive the sFlt-1 response⁵¹. 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^{24, 25}.

In this study we also found that aPL suppressed HEEC secretion of chemokines that promote trophoblast invasion (G-CSF)⁵², and recruit macrophages and natural killer cells (MCP-1, GRO- α) that are necessary for normal spiral artery remodeling^{53–56}. This data suggests that aPL may contribute to the shallow trophoblast invasion and disrupted spiral artery transformation seen in obstetric APS⁶ by directly impacting the uterine endothelium, in addition to targeting the placental trophoblast^{57, 58}. 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 remodeling⁵⁹. 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^{60, 61}, and in aPL-mediated trophoblast inflammation²². In studies using HUVECs, β_2 GPI interacts directly with TLR4 and mediates aPL-induced endothelial cell activation^{28–30, 62}. HEECs express functional TLRs, including TLR4³⁶. 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 TLR2⁶³ or ApoER2⁶⁴.

LMWH is commonly used as anti-coagulant treatment in systemic APS, and early initiation of aspirin (prior to 16 weeks of gestation) is thought to be beneficial for fetal development⁶⁵. Both therapies are given empirically to women with recurrent miscarriage, whether it is due to APS or not^{13, 66, 67}. 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^{8–12}. Furthermore, despite treatment, women with APS continue to have high rates of late gestational complications^{13–16}. 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^{18, 23, 43, 68}, LMWH induced the release of sFlt-1, confirming this observation in endothelial cells from other sources⁶⁹. LMWH also reduced HEEC angiogenesis, potentially as a consequence of the overwhelming sFlt-1 release^{46, 47}, which again has been observed in other endothelial cell types⁶⁹. Interestingly, ASA had a similar effect by inhibiting HEEC tube formation, an observation also seen in HUVECs^{49, 50}. As a result, combination 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^{21–23}; and ASA is an anti-inflammatory agent^{70, 71}.

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, in order to establish the adequate placentation and vascular development needed for a healthy pregnancy. aPL dysregulate 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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Figure 1. Effect of aPL on HEEC angiogenic factor and chemokine secretion

HEECs were treated with no treatment (NT), aPL, or control IgG. Data are pooled from 9 independent experiments. Box plots 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.

Figure 2. Effect of 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. Box plots 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.

Figure 3. Effect of TLR4 inhibition on aPL-induced HEEC angiogenic factor and chemokine secretion

HEECs were treated with no treatment (NT) or aPL 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.

Figure 4. Effect of aPL on HEEC tube formation

HEECs were seeded on Matrigel-coated plates and treated with no treatment (NT), aPL, or control IgG. 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.

Figure 5. Effect of ASA and LMWH on HECC aPL-induced HECC 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.

Figure 6. 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 aPL. After 8hrs, tube-like vessels were imaged and quantified. Images are from one representative field of one representative experiment (magnification 10X). Barchart 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.

Table 1

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

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 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