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Aspirin and Heparin Effect on Basal and Antiphospholipid Antibody Modulation of Trophoblast Function

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Abstract

OBJECTIVE—Low molecular weight (LMW) heparin, with or without aspirin (acetylsalicylic acid [ASA]), is used to prevent complications in antiphospholipid syndrome in pregnancy. Our objective was to elucidate the actions of low-dose LMW heparin and ASA on basal and antiphospholipid antibody -induced modulation of trophoblast function.

METHODS—The human first-trimester trophoblast cell line (HTR-8) was treated with or without antiphospholipid antibody in the presence of: 1) no medication, 2) low-dose LMW heparin, 3) low-dose ASA, or 4) combination therapy. Interleukin (IL)-6, IL-8, IL-1 β , growth-regulated oncogene-alpha, vascular endothelial growth factor (VEGF), placental growth factor (PIGF), soluble FMS-like tyrosine kinase-1 (sFlt-1) and soluble endoglin were measured in the supernatant. Cell migration was performed using a two-chamber assay.

RESULTS—LMW heparin improved basal trophoblast migration, and induced potent increases in growth-regulated oncogene-alpha and sFlt-1. ASA did not affect basal function. Combined therapy promoted migration, but did not reverse the LMW heparin-induced sFlt-1 effect. Antiphospholipid antibody increased IL-8, IL-1 β , growth-regulated oncogene-alpha, VEGF, PIGF, and soluble endoglin secretion, while decreasing cell migration and IL-6 and sFlt-1 secretion. The antiphospholipid antibody-induced cytokine changes were best reversed with LMW heparin, with partial reversal of IL-8 and IL-1 β up-regulation. The antiphospholipid antibody-induced angiogenic changes were worsened by LMW heparin, with increased sFlt-1 secretion. The therapies did not reverse antiphospholipid antibody-induced decrease in migration.

CONCLUSION—In the absence of antiphospholipid antibodies, LMW heparin induces potentially detrimental proinflammatory and antiangiogenic profile in the trophoblast. In the presence of antiphospholipid antibodies, single-agent LMW heparin may be the optimal therapy to counter trophoblast inflammation, but also induces an antiangiogenic response. These findings may explain the inability of current therapies to consistently prevent adverse outcomes.

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INTRODUCTION

Pregnancies affected by antiphospholipid syndrome (APS) are managed with heparin, either alone or in combination with acetyl-salicylic acid (ASA), with prevention of maternal and fetal adverse outcomes as the goal (1,2). Although low molecular weight (LMW) heparin is standard with a history of thromboembolism, studies regarding its effectiveness for the prevention of APS-associated adverse pregnancy outcomes have been fraught with contradiction (3–8). Furthermore, late pregnancy complications remain prevalent despite heparin therapy (3,4).

Similarly, some studies of ASA in APS found increased live birth rate (7), while others did not (9,10). Nonetheless, ASA has gained favor as prophylaxis for recurrent intrauterine growth restriction (IUGR) or preeclampsia in non-APS pregnancies. A recent meta-analysis reported that ASA initiated before 16 weeks of gestation decreases risk of recurrent preeclampsia (RR 0.47, 95% CI: 0.34–0.65), severe preeclampsia (RR 0.09, 95% CI 0.02–0.37), and IUGR (RR 0.44, 95% CI 0.30–0.65), whereas ASA started after 16 weeks yielded no benefit (11).

The effects of these medications on third trimester trophoblasts had previously been reported (12); however, dysregulation of first trimester trophoblast function is a stronger mediator in the development of adverse pregnancy outcomes (13,14). Previous studies by our group have demonstrated that antiphospholipid antibodies (aPL) adversely affect human first trimester trophoblast function by reducing cell migration and inducing a potentially unfavorable pro-inflammatory cytokine and altered angiogenic factor milieu (15, 18, 24). Therefore, given the controversies, we sought to elucidate the action of these compounds on human first trimester trophoblast function in the absence and presence of aPLs.

MATERIALS AND METHODS

Reagents

Sterile low molecular weight heparin (LMWH), enoxaparin sodium injection, 100 mg/mL, was purchased from Aventis Pharmaceuticals, Inc. (Bridgewater, NJ). Acetyl-salicylic acid (ASA) was obtained from Sigma-Aldrich (St. Louis, MO), reconstituted in diluted ethanol, and filter-sterilized prior to use. No significant differences in measured outcomes were seen with ethanol controls.

Antiphospholipid Antibody

A mouse anti-human β_2 -GPI monoclonal IgG1, designated ID2, was chosen to mimic APS in pregnancy *in vitro*, due to its well-characterized properties (12,13,16). Similar to human polyclonal aPL, ID2 binds β_2 -GPI when immobilized on a suitable negatively charged surface (17), binds to human first trimester trophoblast (15,23), and alters first trimester trophoblast function (15,18, 24).

Trophoblast Cells

The human first trimester extravillous cytotrophoblast cell line, HTR-8, was used (19), and was a gift from Dr. Charles Graham (Queens University, Kingston, ON, Canada). Cells were cultured in RPMI 1640 media (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10 mM Hepes, 0.1 mM minimal essential medium (MEM) non-essential amino acids, 1 mM sodium pyruvate, and 100 nM penicillin/streptomycin (Gibco). Cells were maintained at 37°C with 5% CO₂. These cells respond to aPL similarly to primary first trimester trophoblast cultures (15, 24).

Cytokine and Angiogenic Factor Studies

HTR-8 cells were treated with or without ID2 (20 µg/mL), in the presence and absence of: 1) media-only, no treatment (NT) control, 2) low-dose LMWH (10 µg/mL); 3) low-dose ASA (10 µg/mL); or 4) combination low-dose LMWH+ASA (10 µg/mL each) in OptiMEM (Invitrogen, Carlsbad, CA). As control, cells were also treated with ethanol, which had no effect (data not shown). Cell-free supernatants were collected after 72-hour culture, centrifuged at $1500 \times g$ for 10 min, and stored at -80°C . The concentrations of ASA and LMWH used in this study were based on a previous report and equivalent to low dose medications used in the clinical setting (12).

Cytokines and angiogenic factor concentrations in the supernatant were evaluated by ELISA (Assay Designs, Ann Arbor, MI) and multiplex assay (Luminex, Austin, TX). Pro-inflammatory cytokines assayed were interleukin (IL)-6, IL-8, IL-1 β , and growth-regulated oncogene- α (GRO- α , a monocyte chemoattractant). Pro-angiogenic factors assayed were vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), and anti-angiogenic factors assayed were soluble FMS-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng). We have previously demonstrated that aPL modulates first trimester trophoblast secretion of all of these factors (15, 18, 24)

Migration Assay

A two-chamber assay was used for the migration studies to measure the spontaneous migratory property of the trophoblast that occurs during normal implantation, which has been previously described by our group (15). The lower chamber for this assay consisted of 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ), which contained 800 µL of treatment media. Trophoblast cells (1×10^5 cells in 200 µL of respective treatment media) were then seeded into a cell culture well insert with 8-µm pore size membrane (BD Biosciences), which served as the upper chamber. Cells were treated with the same combinations of ID2, ASA, and LMWH as described above. Following a 48-hour incubation, trophoblast migration across the membranes were determined using the QCM 24-Well Colorimetric Cell Migration Assay (Chemicon International, Temecula, CA). The resulting colored mixture were read in triplicate at 560 nm using a BioRad plate reader (Hercules, CA), and compared to a 100% cell control to determine relative percent migration.

Statistical Analysis

Experiments were performed three times and assayed in duplicate. Data were then pooled and expressed as mean $\pm$ standard deviation (S.D.). Statistical significance ($p < 0.05$) was determined using analysis of variance (ANOVA).

RESULTS

LMWH and ASA alter basal cytokine/chemokine production

LMWH and ASA are both known to have anti-inflammatory properties (20–23). The first objective of this study was, therefore, to determine the effects of these therapies on basal cytokine/chemokine production by first trimester trophoblast. As shown in Fig. 1, LMWH significantly increased basal GRO- α secretion by $269\% \pm 18.5\%$ ($p=0.006$), without affecting secretion of IL-6, IL-8 and IL-1 β . ASA alone did not have any significant effect on the basal levels of secreted IL-8, IL-6, GRO- α or IL-1 β . Treatment of trophoblast with both LMWH and ASA fully reversed the up-regulation of GRO- α induced by single-agent LMWH therapy.

LMWH, but not ASA, alters basal angiogenic factor production

The next objective of this study was to determine the effect of the therapies on basal trophoblast secretion of pro-angiogenic (VEGF and PlGF) and anti-angiogenic factors (sFlt-1 and sEng). Although a previous report by our group showed that high-dose LMWH (100 $\mu\text{g/mL}$) up-regulated HTR-8 PlGF and sFlt-1 secretion (18), the effects of a low-dose LMWH (10 $\mu\text{g/mL}$) on these factors had not yet been evaluated. As shown in Fig. 2, treatment with low-dose LMWH induced an anti-angiogenic state, with a $262.5\% \pm 43.6\%$ increase in sFlt-1 ($p < 0.001$) and $34.0\% \pm 6.3\%$ reduction in VEGF ($p < 0.001$). The pro-angiogenic PlGF also increased by $230.2\% \pm 85.4\%$ ($p < 0.001$). ASA had no effects on the production of these angiogenic factors by trophoblasts. Combination treatment resulted in the same effects as single-therapy LMWH.

LMWH and ASA alter basal trophoblast migration

The next objective of this study was to determine the effects of the therapies on basal trophoblast migration. We previously reported that high-dose LMWH augmented basal trophoblast migration (24). As shown in Fig. 3, single-agent low-dose LMWH also induced this pro-migratory phenomenon, with a $133.6\% \pm 9.9\%$ increase in migration ($p < 0.001$). Since ASA is also used routinely for prophylaxis against recurrent preeclampsia and IUGR (11), we speculated that ASA would have an overall pro-migratory effect on the first-trimester trophoblast. Treatment with low-dose ASA, however, did not induce any changes in the migratory capacity of HTR-8 trophoblasts. Combination therapy, however, modestly augmented the LMWH-dependent increase in migratory capacity by an additional $4.6\% \pm 2.1\%$ ($p < 0.001$).

LMWH and ASA modify the aPL-induced cytokine/chemokine response

Having determined the effects of the treatment regimens on basal trophoblast function, we next sought to determine the effects of LMWH and ASA on aPL-mediated alterations in the trophoblast function, beginning with cytokine/chemokine production. Consistent with our previous reports (24), treatment of the HTR-8 cell line with the anti- $\beta_2\text{GPI}$ monoclonal antibody, ID2 (20 $\mu\text{g/mL}$), significantly decreased IL-6 secretion by $40.7\% \pm 11.7\%$ ($p < 0.001$) and increased the secretion of IL-8 by $641.3\% \pm 376.3\%$ ($p < 0.001$), GRO- α by $541.1\% \pm 52.8\%$ ($p < 0.001$), and IL-1 β by $508.8\% \pm 10.5\%$ ($p < 0.001$) (Fig. 4).

Treatment with LMWH did not reverse the aPL-induced down-regulation of IL-6, but attenuated the aPL-induced elevations of IL-8 and IL-1 β secretion (Fig. 4). The augmentation of GRO- α levels with LMWH treatment (Fig. 1C) was maintained in the presence of aPL, although the response was more pronounced (Fig. 4C). Single-agent ASA therapy did not affect any of the aPL-induced cytokines or chemokine responses (Figs 4A–D). Combination LMWH and ASA treatment did minimally reverse the GRO- α effect (Fig. 4C), but did not offer any other additional anti-inflammatory effect.

LMWH, but not ASA, alter aPL-mediated modulation of angiogenic factor production

We next turned our attention to the effects of the therapies on aPL-mediated modulation of angiogenic factor production. As previously reported (25), aPL significantly increased HTR-8 secretion of VEGF by $145.5\% \pm 18.1\%$ ($p < 0.001$), PlGF by $224.5\% \pm 45.4\%$ ($p < 0.001$), and sEng by $278.7\% \pm 192.5\%$ ($p < 0.001$), while decreasing sFlt-1 by $28.7\% \pm 12.4\%$ ($p = 0.012$). (Fig. 5)

None of the treatment regimens altered the aPL-induced changes in levels of VEGF or sEng (Fig. 5A & D). Treatment with single-agent LMWH further augmented the aPL-induced PlGF secretion by $159.7\% \pm 19.5\%$ ($p < 0.001$), whereas ASA had no significant effect. The addition of ASA to LMWH in combination therapy yielded a similar effect on PlGF as

treatment with LMWH alone (Fig. 5B). As with the basal state, LMWH caused a significant elevation of sFlt-1 accumulation in supernatant, even though the aPL alone reduced sFlt-1 levels, and combination therapy with ASA was unable to reverse this profound response (Fig. 5C).

LMWH and ASA do not affect aPL-mediated modulation of trophoblast migration

The final objective was to determine the effects of the therapies on aPL-mediated modulation of trophoblast migration. As seen in Fig. 6, and in our previous report (24), aPL significantly reduced trophoblast migration by $59.2\% \pm 2.4\%$ ($p < 0.001$). None of the therapies were able to reverse the effect (Fig. 6).

DISCUSSION

In spite of conflicting data on the efficacy of heparin and ASA therapies in preventing adverse pregnancy outcomes, pregnant women with APS are routinely treated with LMWH, either alone or in combination with ASA (9–19). While several studies have reported on the anti-inflammatory properties of LMWH and ASA (3, 28–30), there is a paucity of literature on the effects of these therapies on first trimester trophoblast, a major player in the pathogenesis of a spectrum of obstetrical disorders (13). Given the heterogeneity in clinical outcomes with heparin and ASA therapy, it is increasingly apparent that the action of these therapies, as single agents or in combination, need to be better elucidated in order to individualize and optimize therapies.

We focused our current study on human first trimester trophoblast given their unique susceptibility to aPL with β_2 GPI activity, and their role in the development of pregnancy complications (13,14). Trophoblasts synthesize their own β_2 -GPI (12), and while most cells will only bind β_2 -GPI on their cell surface under pathologic conditions when there is exteriorization of negatively charge phospholipids on the outer surface of the plasma membrane, the rapid proliferation and differentiation of trophoblasts renders them able to express endogenous and bind exogenous β_2 -GPI on the cell surface under normal physiological conditions (13). Thus, anti- β_2 -GPI antibodies target the trophoblast surface, establishing the pathogenic placental environment in APS (13).

The present study demonstrates the multiplicity and complexity of the effects of LMWH and ASA on trophoblast function, in both the presence and absence of aPL. Contrary to previous studies demonstrating anti-inflammatory effects of LMWH (3, 28–30), using our trophoblast *in vitro* model, single-agent LMWH was actually shown to have both pro-inflammatory and anti-inflammatory effects. Our finding that LMWH increased basal and aPL-induced trophoblast secretion of GRO- α , a potent monocyte chemoattractant, is novel, although the potential clinical significance and mechanism are currently unknown. While basal IL-6, IL-8 and IL-1 β were not affected by either LMWH or ASA, the aPL-mediated elevations in IL-8 and IL-1 β were partially reversed by single-agent LMWH. Interestingly, while ASA alone had no effect on basal or aPL-induced cytokine production, in combination with LMWH, GRO- α levels in the absence of aPL were brought back to baseline, suggesting that ASA can counteract the pro-inflammatory actions of LMWH.

These combined cytokine/chemokine findings may explain the inability of LMWH and ASA to consistently reverse the inflammation-induced endpoints seen in APS. None of the therapies proved beneficial to the trophoblast in the absence of aPL, although combination therapy is the least detrimental by minimizing the adverse increase in GRO- α . Although the *in vitro* model is an isolated system and cannot replicate the complex *in vivo* milieu in APS patients, these findings underscore the need to better understand the effects of our therapies

on the inflammatory milieu and to identify better-targeted therapy in the treatment of pregnancies complicated by APS.

As previously described by our group (18), some derangements in angiogenic factor production in APS mimic those seen in preeclampsia, such as elevated sEng. In preeclampsia, circulating levels of VEGF and PlGF are markedly reduced, while placental-derived sFlt-1 and sEng are elevated (26–28). Alterations in the angiogenic factor balance may be associated with the dysfunctional low-capacitance, high-resistance placenta seen with preeclampsia. A similar effect is paradoxically induced with exposure to single-agent LMWH, with profound increases in sFlt-1 in the basal and aPL-induced states. A recent mechanistic study implicates placental heparanase in the regulation of sFlt-1 release (29). LMWH also decreased VEGF, further inducing an overall anti-angiogenic milieu. The potentially beneficial PlGF response may be counteracted by the profound anti-angiogenic sFlt-1 response.

These changes in the angiogenic milieu may explain the inability of LMWH and ASA to prevent adverse outcomes in late gestation, such as preeclampsia or IUGR. LMWH may prevent early loss by altering the inflammatory milieu in APS patients (13), while setting the foundation for impaired placentation by worsening angiogenesis. In the basal *in vitro* model, mimicking non-APS patients at high-risk for recurrent adverse outcomes, ASA, the current accepted standard of care, did not affect the angiogenic milieu. More importantly, the paradoxical development of an anti-angiogenic milieu mirroring that seen in preeclampsia speaks to a need for judicious use of LMWH in conditions that are not absolute indications for anticoagulation.

Lastly, trophoblast migration and invasion into spiral arteries is necessary to establish an adequate placental vascular system. We previously reported that aPL limit trophoblast migration by reducing IL-6 production (24). Despite unchanged IL-6 levels with LMWH therapy, LMWH increased basal trophoblast migration, with further improvement when combined with ASA, albeit marginally. This contradictory up-regulation of migration indicates that trophoblast migration is likely regulated by a complex network of factors. Possible factors that merit further investigation include the hypoxia-inducible microRNA-210 (30) or Nodal (31), a member of the transforming growth factor- β superfamily. However, none of the treatments provided protection against aPL-induced down-regulation of migration.

While this *in vitro* model does not fully mimic the complex milieu *in vivo*, the strength of our system is that it allows for an uncomplicated way to test the actions of the current treatment regimen on the trophoblast, a crucial player in placental health. The use of a monoclonal anti-human β_2 -GPI antibody, instead of patient-derived polyclonal aPL, and the first trimester trophoblast cell line instead of primary trophoblast cells, because of the large scale of the experiments, should also be noted. However, we believe these are valid alternatives since our previous studies have shown the monoclonal to behave in a similar manner to patient-derived antibodies and the trophoblast cell line to respond to aPL similarly to primary first trimester cultures (15, 18). Nonetheless, further evaluation of the effects of LMWH and ASA on primary trophoblasts from different gestational ages may further shed light on the effects of these medications. Lastly, LMWH was chosen for this model, instead of unfractionated heparin, since LMWH is the standard of care in many clinical settings, due to ease of administration and improved patient adherence. However, we have found that unfractionated heparin and LMWH have similar effects on the trophoblast (15, 24).

In summary, LMWH, ASA, and combination therapy each impart a distinct action on the function of human first-trimester trophoblasts, with both beneficial and potentially

detrimental cellular effects. Their mixed impact on the aPL-induced pathological state may explain the inability of current therapies to fully reverse the detrimental effects of APS and the conflicting data seen *in vivo*. Our findings highlight the need for additional investigation into the use of heparin and ASA in the treatment of APS-induced pregnancy complications, and the need for new targeted therapeutics.

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Figure 1.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on basal trophoblast cytokine/chemokine production. HTR-8 cells were treated for 72 hours with: 1) media-only, no treatment control; 2) LMW heparin (10 μ g/mL); 3) ASA (10 μ g/mL); or 4) combination LMW heparin and ASA (10 μ g/mL each). Supernatants were analyzed for (A) interleukin (IL)-6, (B) IL-8, (C) growth-regulated oncogene- α , and (D) IL-1 β . (* P <.001; † P <.05; N =3).

Figure 2.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on basal trophoblast angiogenic factor production. HTR-8 cells were treated for 72 hours with: 1) media-only, no treatment control, 2) LMW heparin (10 μ g/mL); 3) ASA (10 μ g/mL); or 4) combination LMW heparin and ASA (10 μ g/mL each). Supernatants were analyzed for (A) vascular endothelial growth factor (VEGF), (B) placental growth factor, (C) soluble FMS-like tyrosine kinase-1 (sFlt-1), and (D) soluble endoglin. * P <.05; N=3.

Figure 3.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on basal trophoblast cell migration. HTR-8 cells were treated for 72 hours with: 1) media-only, no treatment control, 2) LMW heparin (10 $\mu\text{g/mL}$); 3) ASA (10 $\mu\text{g/mL}$); or 4) combination LMW heparin and ASA (10 $\mu\text{g/mL}$ each). Cell migration was assessed using a colorimetric assay. Bar charts show changes in the percentage of cell migration compared to the no-treatment control. * $P < .05$; $N=3$.

Figure 4.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on antiphospholipid antibody-mediated modulation of trophoblast cytokine/chemokine production. HTR-8 cells were treated with no treatment (NT), antiphospholipid antibodies (ID2, 20 μ g/mL), or antiphospholipid antibodies in the presence of: 1) LMW heparin (10 μ g/mL); 2) ASA (10 μ g/mL); or 3) combination LMW heparin and ASA (10 μ g/mL each). Supernatants were analyzed for (A) interleukin (IL)-6, (B) IL-8, (C) growth-regulated oncogene- α , and (D) IL-1 β . * P <.05; † P <.05 when compared to antiphospholipid antibodies; N=3.

Figure 5.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on antiphospholipid antibody-mediated modulation of trophoblast angiogenic factor production. HTR-8 cells were treated with no treatment (NT), antiphospholipid antibodies (ID2, 20 μ g/mL), or antiphospholipid antibodies in the presence of: 1) LMW heparin (10 μ g/mL); 2) ASA (10 μ g/mL); or 3) combination LMW heparin and ASA (10 μ g/mL each). Supernatants were analyzed for (A) vascular endothelial growth factor (VEGF), (B) placental growth factor, (C) sFlt-1, and (D) sEng by enzyme-linked immunosorbent assay (ELISA). * $P < .05$; † $P < .05$ when compared to antiphospholipid antibodies; N=3.

Figure 6.

Effects of low molecular weight (LMW) heparin, acetylsalicylic acid (ASA), and combination therapy on antiphospholipid antibody-mediated modulation of trophoblast cell migration. HTR-8 cells were treated with no treatment (NT), antiphospholipid antibodies (ID2, 20 $\mu\text{g/mL}$), or antiphospholipid antibodies in the presence of: 1) LMWH (10 $\mu\text{g/mL}$); 2) ASA (10 $\mu\text{g/mL}$); or 3) combination LMW heparin and ASA (10 $\mu\text{g/mL}$ each). Cell migration was assessed using a colorimetric assay. Bar charts show changes in the percentage of cell migration compared to the no-treatment control. * $P < .05$; † $P < .05$ when compared to mouse anti-human $\beta 2$ -GPI monoclonal IgG1; $N=3$).