Pravastatin does not prevent antiphospholipid antibody-mediated changes in human first trimester trophoblast function

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STUDY QUESTION: What is the effect of pravastatin on antiphospholipid antibody (aPL) modulation of human first trimester trophoblast function?

SUMMARY ANSWER: Pravastatin does not prevent the effects of aPL on human first trimester trophoblast cell function.

WHAT IS KNOWN ALREADY: Antiphospholipid syndrome (APS) is associated with recurrent pregnancy loss and late pregnancy complications, such as pre-eclampsia, owing to direct targeting of the placenta by aPL. While treatment with heparin reduces the rate of pregnancy loss, the risk for severe pre-eclampsia remains high. Thus, there is a need to find alternative treatments for the prenatal management of patients with APS. Statins have recently been shown to prevent aPL-mediated fetal loss in mice but their effects on a human pregnancy model of APS have not yet been studied.

DESIGN, DATA COLLECTION, METHODS: The human first trimester trophoblast cell line, HTR8, and human first trimester trophoblast primary cultures were incubated with or without a mouse anti-human beta 2 glycoprotein I (β2GPI) monoclonal antibody in the presence or absence of pravastatin. Cytokine and angiogenic factor secretion were measured by enzyme-linked immunosorbent assay and multiplex analysis. Cell migration was measured using a colorimetric two-chamber migration assay.

MAIN FINDINGS: Using the human first trimester trophoblast cell line, HTR8, pravastatin significantly augmented, compared with no treatment, aPL-dependent secretion of interleukin (IL)-8 (P < 0.05), IL-1β (P < 0.05) and soluble endoglin (P < 0.01) but had no effect on aPL-induced up-regulation of vascular endothelial growth factor, placenta growth factor or growth-related oncogene alpha secretion. Furthermore, pravastatin alone limited basal HTR8 cell migration (P < 0.01), and did not mitigate the adverse effect of aPL on trophoblast migration. Pravastatin also had no impact on the secretion of pro-inflammatory cytokines and angiogenic factors by primary human first trimester trophoblast cells exposed to aPL.

LIMITATIONS AND WIDER IMPLICATIONS OF THE FINDINGS: While our in vitro findings suggest that pravastatin may not be effective in preventing pregnancy complications in patients with APS, the in vivo condition may be more complex, and thus, more studies are needed to determine the effectiveness of pravastatin in the prevention of aPL-associated pregnancy complications in humans.

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Introduction

Women with antiphospholipid syndrome (APS) are at high risk of recurrent pregnancy loss and late gestational pregnancy complications, such as pre-eclampsia, HELLP syndrome (hemolytic anemia, elevated liver enzymes and low platelet counts), premature delivery and intrauterine growth restriction (George and Erkan, 2009). In patients with APS who have a history of fetal loss, pregnancy morbidity can be as high as 90% in the absence of treatment (Biggiogero and Meroni, 2010). Therefore, early prevention of pregnancy complications is a priority in APS. Current management includes treatment with low-molecular weight heparin (LMWH), either alone or in combination with aspirin (Miyakis et al., 2006). While this treatment regime may be beneficial at preventing antiphospholipid antibody (aPL)-associated miscarriage, it has not been shown to reduce the risk of late gestational pregnancy morbidities, such as pre-eclampsia (Backos et al., 1999; Branch and Khamashta, 2003). Furthermore, both clinical and experimental studies have raised questions about the efficacy of these therapeutics, in particular LMWH, in preventing aPL-associated pathology during pregnancy (Backos et al., 1999; Erkan et al., 2002; Farquharson et al., 2002; Branch and Khamashta, 2003; Stephenson et al., 2004; Cohn et al., 2010; Han et al., 2011). As a result there has been growing interest in finding alternative treatments for the prenatal management of patients with APS.

While the mechanism of aPL-associated pregnancy failure and pregnancy complications was initially attributed to pro-thrombotic events at the fetomaternal interface, clinical and experimental studies now support the view that inflammatory factors and processes, such as cytokines, complement and immune cell activation, are the primary cause for placental dysfunction and pregnancy failure in APS (Sebire et al., 2002; Girardi et al., 2003; Berman et al., 2005; Bose et al., 2006; Mulla et al., 2009, 2010; Carroll et al., 2011). Consequently, therapeutic interventions that target such mechanisms may provide better protection against the detrimental effects of aPL, thus improving pregnancy outcome in patients with APS. One such potential therapeutic is statins (143-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors), which in addition to lowering cholesterol also have anti-inflammatory and immunomodulatory properties (Blum and Shamburek, 2009; Bu et al., 2011). Statins have been shown to be useful in animal models of pre-eclampsia (Costantine et al., 2010; Fox et al., 2011; Kumasawa et al., 2011; Singh et al., 2011) and can prevent fetal loss in aPL-treated mice (Redecha et al., 2008; Girardi, 2009). However, the beneficial effects of pravastatin in this mouse model have yet to be recapitulated in a human pregnancy model of APS. Moreover, emerging evidence suggests that statins may adversely impact the human placenta by inhibiting trophoblast migration and proliferation, and by increasing apoptosis (Kenis et al., 2005; Tartakover-Matalon et al., 2007; Forbes et al., 2008).

We previously demonstrated that aPL directly impacts normal human first trimester trophoblast function by inducing a pro-inflammatory (increased IL-8, IL-1β and growth-related oncogene alpha (GRO-α) secretion), anti-angiogenic (increased soluble endoglin secretion) and anti-migratory profile (Mulla et al., 2009, 2010; Carroll et al., 2011; Han et al., 2011). In the current study, we sought to determine whether pravastatin could prevent any of these aPL effects on the trophoblast.

Materials and Methods

Trophoblast cells

The human first trimester extravillous trophoblast cell line, HTR8, was used in these studies. The HTR8 cells, immortalized by SV40 (Graham et al., 1993), were a kind gift from Dr Charles Graham (Queens University, Kingston, ON, Canada). HTR8 cells were cultured in RPMI 1640 (Gibco; Grand Island, NY, USA) which was supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA), 10 mM HEPES, 0.1 mM minimum essential medium (MEM) non-essential amino acids, 1 mM sodium pyruvate, 100 ng/mL penicillin/streptomycin (Gibco). For primary cultures, first trimester placentas (8–12 weeks of gestation) were obtained from elective terminations of normal pregnancies performed at the Yale-New Haven Hospital. The use of patient samples was approved by the Yale University’s Human Investigation Committee. Primary cytotrophoblast cells were isolated as previously described (Mulla et al., 2009, 2010; Carroll et al., 2011; Han et al., 2011). In brief, placental villous tissue specimens were washed with cold Hanks Balanced Salt Solution (Gibco) to remove excess blood. Cells were scraped from the membranes, transferred to trypsin EDTA (Invitrogen, Carlsbad, CA, USA) digestion buffer and incubated at 37°C for 40 min with shaking. The mixture was then passed through a nylon strainer and then layered over lymphocyte separation media (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged at 400g for 25 min. The cellular interface containing the trophoblast cells was collected and resuspended in Dulbecco’s modified Eagle’s medium with d-valine (Caissin Labs, North Logan, UT, USA) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA, USA). All cells were cultured under normoxic conditions in 5% CO2 at 37°C.

Antiphospholipid antibodies

The mouse immunoglobulin G1 anti-human beta 2 glycoprotein I (β2GPI) monoclonal antibody (anti-β2GPI mAb), IICS, was used in these studies. This antibody was produced by one of us (L.W.C.), under sterile conditions, and was filter sterilized prior to use. IICS was cloned from mice immunized with purified human β2GPI and has been previously characterized (Chamley et al., 2001). Like human aPL, IICS binds β2GPI but only when it is immobilized on a suitable negatively charged surface, such as the phospholipids, cardiolipin or phosphatidyl serine or irradiated polystyrene (Chamley et al., 1999). IICS binds to first trimester trophoblast cells and, similar to patient-derived polyclonal aPL, alters trophoblast function (Mulla et al., 2009; Carroll et al., 2011).

Cell viability studies

Trophoblast cell viability was determined using the CellTiter 96 viability assay (Promega, Madison, WI, USA). Cells were plated in wells of a 96-well plate at 1 × 104 cells per well in the growth medium, as described above, and cultured until they were 70% confluent. The medium was then replaced with serum-free Opti-MEM (Invitrogen), and cells were cultured for another 4 h. Cells were then treated with camptothecin, an inhibitor of nucleic acid synthesis (CPT; Sigma Aldrich, St Louis, MO, USA) at 4 µM or pravastatin (Sigma Aldrich) at 0.1, 1, 2.5 and 5 µg/ml for 72 h. Pravastatin was dissolved in endotoxin-free water and subsequent dilutions made using Opti-MEM prior to treatment. CellTiter 96Tm substrate was added to all wells and cells were then incubated for 2 h at 37°C. Optical densities were read at 490 nm. All samples were assayed in triplicate, and cell viability was presented as a percentage of the untreated control.
Cytokine and angiogenic factor secretion
Trophoblast cells were treated with or without IIC5 (aPL) at 20 μg/ml in OptiMEM (Invitrogen) in the presence and absence of pravastatin at either 0.5 or 2.5 μg/ml. After 72 h, cell-free supernatants were collected by centrifugation at 400g for 10 min and stored at −80°C until analysis. The concentrations of interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble Endoglin (sEndoglin) were evaluated by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). IL-8, IL-1β, GRO-α and VEGF levels were also determined by multiplex analysis using the BioPlex assay (Bio-Rad, Hercules, CA, USA) with detection and analysis using the Luminex 100 IS system (Upstate Biotechnology, Charlottes-ville, VA, USA).

Cell migration studies
To assess trophoblast migration, a two-chamber colorimetric assay was employed. The top chamber was an 8-μm pore size cell culture insert (BD Biosciences, Franklin Lakes, NJ, USA) while the lower chamber was a 24-well tissue culture plate (BD Falcon, Franklin Lakes, NJ, USA). The lower chamber was filled with 800 μl OptiMEM, while the top chamber was seeded with 1 x 10^5 HTR8 cells suspended in 200 μl of medium plus the treatments. Following 48 h incubation, the 8-μm inserts on the top chambers were removed and trophoblast migration across the membrane was determined using the QCM 24-Well Colorimetric Cell Migration assay, according to the manufacturer’s direction (Chemicon International, Temecula, CA, USA). Briefly, migrated cells were stained, collected and lysed. The resulting colored mixture was transferred to a 96-well plate and optical density read in triplicate at 560 nm. A 100% migration control consisted of 196-well plate and optical density read in triplicate at 560 nm. A 100% migration control consisted of 196-well plate and optical density read in triplicate at 560 nm. The relative percentage of cells exposed to aPL (Fig.3). The presence of pravastatin modestly decreased cell viability by 22.9 ± 33.1%.

Statistical analysis
All experiments were performed at least three times. Data are expressed as mean ± SD. Statistical significance (P < 0.05) was determined using either the one-way analysis of variance, or the student’s t-test using GraphPad InStat software (GraphPad Software, Inc., La Jolla, CA, USA).

Results
Dose-dependent effect of pravastatin on trophoblast viability
The first objective was to determine the concentration of pravastatin that confers trophoblast cytotoxicity. As shown in Fig. 1A, treatment of HTR8 cells, a first trimester trophoblast cell line, with 5 μg/ml pravastatin for 72 h significantly decreased cell viability by 22.9 ± 33.1%.

Pravastatin augments the aPL-induced inflammatory response in HTR8 trophoblast
We next sought to determine the effect pravastatin had on the ability of the mouse anti-human β2GPI mAb, IIC5 to induce human first trimester trophoblast inflammatory response (Mulla et al., 2009; Han et al., 2011). Pravastatin, at 0.5 or 2.5 μg/ml, induced a slight, but not significant, increase in basal IL-8 or GRO-α secretion by the HTR8 trophoblast cell line, but did significantly elevate basal IL-1β secretion at the higher concentration (Fig. 2). Pravastatin at both doses significantly augmented the anti-β2GPI mAb-dependent IL-8 secretion as well as, at least at the lower concentration, the induction of IL-1β (Fig. 2). In contrast, pravastatin had no significant effect on the levels of GRO-α induced by anti-β2GPI mAb.

Pravastatin enhances the anti-angiogenic actions of aPL on HTR8 trophoblast
We next evaluated the effect pravastatin had on the ability of the mouse anti-human β2GPI mAb to trigger an anti-angiogenic response in first trimester trophoblast (Carroll et al., 2011; Han et al., 2011). Pravastatin had no effect on basal VEGF, PlGF or sEndoglin secretion, but at high concentration (2.5 μg/ml) increased sFlt-1 production (Fig. 3). Pravastatin had no effect on the down-regulation of sFlt-1 secretion in cells exposed to aPL (Fig. 3). The presence of pravastatin modestly,

Figure 1 High-dose pravastatin reduces the viability of a human trophoblast cell line. (A) The first trimester trophoblast cell line (HTR8) was incubated with either no treatment (NT), camptothecin (CPT, 4 μM) as a positive control for cell death, or pravastatin (0.1, 1, 2.5, 5 μg/ml) for 72 h. Cell viability is shown as a percentage of the NT control. Data are from four independent experiments. *P < 0.05; **P < 0.001 versus NT control. (B) Human primary first trimester trophoblast cells either had no treatment (NT) or were incubated with pravastatin (0.1, 1, 2.5, 5 μg/ml) for 72 h.
yet significantly, reduced the ability of the anti-β₂GPI mAb to increase trophoblast VEGF and PlGF secretion. However, at both high and low concentrations, pravastatin significantly augmented anti-β₂GPI mAb-induced sEndoglin secretion (Fig. 3).

**Figure 2** Pravastatin augments aPL-induced secretion of IL-8 and IL-1β secretion in a human trophoblast cell line. HTR8 cells either had NT or were incubated with the β₂GPI mAb, IICS (20 μg/ml) in the absence (media) or presence of pravastatin (0.5 or 2.5 μg/ml) for 72 h. Supernatants were assayed for IL-8, IL-1β and GRO-α. Data are from three independent experiments. *P < 0.05; **P < 0.01 versus media/NT unless otherwise indicated. a: P < 0.05 relative to pravastatin 0.5/NT and b: P < 0.05 versus pravastatin 2.5/NT.

**Figure 3** Pravastatin augments aPL-induced up-regulation of sEndoglin secretion in a human trophoblast cell line. HTR8 cells either had NT or were incubated with the anti-β₂GPI mAb, IICS (20 μg/ml) in the absence (media) or presence of pravastatin (0.5 or 2.5 μg/ml) for 72 h. Supernatants were assayed for VEGF, PlGF, sFlt-1 and sEndoglin. Data are from three independent experiments. *P < 0.05; **P < 0.01 versus media/NT unless otherwise indicated. a: P < 0.05 versus pravastatin 0.5/NT and b: P < 0.05 versus pravastatin 2.5/NT.

Pravastatin does not reverse the inhibitory effect of aPL on trophoblast migration

As shown in Fig. 4, pravastatin failed to reverse the inhibition of trophoblast migration induced by the anti-β₂GPI mAb. In fact, at a low concentration (0.5 μg/ml), pravastatin significantly reduced basal trophoblast migration, although its effect was modest.

**Figure 4** Pravastatin does not reverse the inhibitory effect of aPL on trophoblast migration. HTR8 cells either had NT or were incubated with the anti-β₂GPI mAb, IICS (20 μg/ml) in the absence (media) or presence of pravastatin (0.5 or 2.5 μg/ml) for 72 h. Data are from three independent experiments. *P < 0.05; **P < 0.01 versus media/NT unless otherwise indicated.

Pravastatin does not reverse aPL responses in primary first trimester trophoblast

We established primary first trimester trophoblast cultures to validate our assertion that pravastatin does not reverse the adverse effects of aPL on human placental cells. As shown in Fig. 5, pravastatin alone had no effect on basal cytokine or angiogenic factor production by primary human trophoblast, apart from a significant reduction in basal...
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Figure 4 Pravastatin has no effect on aPL-inhibition of human trophoblast migration. The first trimester trophoblast cell line (HTR8) either had NT or was incubated with the anti-β2GPI mAb, IIC5 (20 μg/ml) in the absence (media) or presence of pravastatin (0.5 or 2.5 μg/ml) for 48 h. Cell migration is shown as a percentage of the starting total number of cells. Data are from three independent experiments. *P < 0.05; **P < 0.01 versus media/NT unless otherwise indicated. a: P < 0.05 versus pravastatin 0.5/NT and b: P < 0.05 versus pravastatin 2.5/NT.

sEndoglin levels. Moreover, there was no significant augmentation or reversal of any of the effects of the anti-β2GPI mAb on trophoblast cytokine or angiogenic factor secretion (Fig. 5).

Discussion

While women with APS are routinely treated with LMWH during pregnancy, clinical and experimental studies have highlighted the potential lack of efficacy of this regimen, as well as potential problems resulting from the treatment (Backos et al., 1999; Erkan et al., 2002; Farquharson et al., 2002; Branch and Khamash, 2003; Stephenson et al., 2004; Cohn et al., 2010; Han et al., 2011). Consequently, there is a need for alternative preventative strategies in the management of pregnant women with APS. Recent studies in mice have demonstrated that pravastatin prevents the onset of symptoms of pre-eclampsia brought about either by the overexpression of human sFlt-1 (Costantine et al., 2010; Fox et al., 2011; Kumasawa et al., 2011) or the genetic background (Ahmed et al., 2010; Singh et al., 2011). Furthermore, using mouse models, pravastatin has been shown to prevent aPL-mediated pregnancy loss in vivo (Redecha et al., 2008; Girardi, 2009). These findings have led to an interest in the use of statins to treat pregnancy complications, such as pre-eclampsia (Ahmed, 2011) and those associated with APS (Girardi, 2010). Indeed, a clinical trial on statins for the prevention of early-onset pre-eclampsia is currently underway (Ahmed, 2011). Since the impact of statins have yet to be tested in a human model of aPL-associated pregnancy problems, and studies using normal human placental explants have raised concerns over the use of statins during pregnancy (Kenis et al., 2005; Tartakover-Matalon et al., 2007; Forbes et al., 2008), we sought to determine whether pravastatin could prevent aPL-mediated effects using a human trophoblast in vitro system. In this study we report that pravastatin reduces basal human first trimester trophoblast migration, and fails to prevent aPL-mediated alterations in trophoblast function.

In our preliminary experiments we found that while pravastatin had no adverse effect on the cell viability of primary first trimester trophoblast cultures, pravastatin at 5 μg/ml, reduced human first trimester trophoblast cell viability, an observation also made by other groups using human first trimester placental explants (Kenis et al., 2005; Forbes et al., 2008). Hence, we used pravastatin at concentrations (0.5 and 2.5 μg/ml) that did not induce cell death. At these lower doses, we found that pravastatin modestly enhanced sFlt-1 and IL-1β secretion and reduced the migratory capacity of HTR8 trophoblast. Our findings are in line with the observation by others, demonstrating that statins reduce trophoblast cell migration and outgrowth from first trimester explant cultures (Kenis et al., 2005; Tartakover-Matalon et al., 2007).

We also failed to observe unequivocal beneficial effects of pravastatin on HTR8 trophoblast exposed to anti-β2GPI mAb. Pravastatin was unable to prevent the up-regulation of the pro-inflammatory cytokines, IL-8, IL-1β and GRO-α; the induction of sEndoglin or the reduction in cell migration upon aPL exposure. Pravastatin did, however, reduce aPL-induced VEGF and PGF secretion. While these effects reached significance, they were modest and, therefore, it is unclear whether such changes would be physiologically relevant. Moreover, this finding does not support the observation in the mouse model of pre-eclampsia where pravastatin increases PIGF expression (Kumasawa et al., 2011). Where we did see a marked effect by pravastatin was in its ability to augment aPL-induced secretion of IL-8, IL-1β and sEndoglin by the HTR8 trophoblast cell line. Indeed studies using immune cells have shown that statins can enhance lipopolysaccharide-induced inflammation, including IL-8 and IL-1β production (Matsumoto et al., 2004; Yilmaz et al., 2006; Kuijk et al., 2008). Since aPL-induced IL-8 and IL-1β secretion is mediated by toll-like receptor 4 (Mulla et al., 2009), the mechanism by which pravastatin enhances the trophoblast inflammatory response to aPL may be similar. However, when the effect of pravastatin was tested using primary human first trimester trophoblast cultures, we found no positive or negative effects on either basal or aPL-stimulated cytokine and angiogenic factor production. The difference for these observations between the trophoblast cell line and primary cells may be because the primary cultures are a more heterogeneous population. One other possibility is that cell cultures were maintained, and experiments performed, under normoxic, rather than hypoxic conditions. However, a recent study demonstrated that changes in culture oxygen levels has no effect on cytokine production, including IL-1β and IL-8, in basal first trimester placental explants in vitro (Chen et al., 2011, #496). Alternatively, since we see similar effect of the aPL on both the primary trophoblast and the trophoblast cell line (Mulla et al., 2009; Carroll et al., 2011), our results may indicate that pravastatin has differential effects on the two types of culture. Indeed, the augmentation of aPL-induced IL-1β, IL-8 and sEndoglin secretion by pravastatin in the HTR8 cell line may be a reflection of pravastatin’s ability to slightly elevate the cell line’s basal production of these factors, an observation not seen in the primary cultures.

Based on our observations we can conclude that while pravastatin may not be overtly detrimental to the trophoblast in the absence of aPL, in terms of cytokine and angiogenic factor production, alone it still might impair placental cell function by limiting migration, which is critical for normal placentation; and it fails to protect the trophoblast from aPL effects. Thus, our findings might be interpreted as not supporting the use of pravastatin in patients with APS. Why this is in contrast to the in vivo studies in mice showing that pravastatin prevents...
aPL-induced pregnancy loss may simply be a consequence of species differences. Alternatively, it might be the result from methodological differences. In the mouse model of aPL-induced pregnancy failure, pravastatin was administered prior to aPL exposure, whereas in our in vitro model, trophoblast cells were treated simultaneously with aPL and pravastatin. Furthermore, the mouse model explains the pathogenesis of APS-associated pregnancy failure, and the success of pravastatin in preventing this is based on its ability to prevent aPL-induced neutrophil tissue factor and proteinase-activated receptor-2 expression (Redecha et al., 2008; Girardi, 2009), rather than the direct effects of aPL on the trophoblast, as in our model (Mulla et al., 2009, 2010; Carroll et al., 2011; Han et al., 2011). Thus, we can conclude that in our study, pravastatin fails to prevent the direct effects of aPL on the placenta. Whether pravastatin can prevent aPL-associated pregnancy complications, such as pregnancy loss or pre-eclampsia, still remains to be determined.

In conclusion, our findings demonstrate that, in vitro, pravastatin does not prevent the effects of anti-β2GPI Abs on human first trimester trophoblast cell function and, therefore, may not be beneficial as a therapeutic for pregnant women with APS. However, since the in vivo condition may be more complex, more studies are needed to determine the effectiveness of pravastatin in the prevention of aPL-associated pregnancy complications in humans.

**Authors’ roles**

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Conflict of interest
None declared.

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