Heparin and low-dose aspirin restore placental human chorionic gonadotrophin secretion abolished by antiphospholipid antibody-containing sera

N.Di Simone, S.Ferrazzani, R.Castellani, S.De Carolis, S.Mancuso and A.Caruso

Department of Obstetrics and Gynecology, Universita Cattolica S. Cuore, Largo Gemelli 8, 00168 Rome, Italy

Introduction

Antiphospholipid syndrome (APS) is a multisystem disease with the predominant clinical features of venous and arterial thrombosis, recurrent pregnancy loss, fetal death and the presence of antiphospholipid antibodies (aPL) (Love and Santoro, 1990; McNeil et al., 1991). Although it had been assumed that aPL are directed against anionic phospholipid molecules, current advances in the field suggest that the target antigens are phospholipid-binding proteins (Balestrieri et al., 1994; Kutteh, 1996). This combination of aspirin and heparin may promote successful embryonic implantation in the early stages of pregnancy and protect against thrombosis of the utero–placental vasculature after placentaation (Rai et al., 1997). However, the mechanism by which aPL cause recurrent miscarriage is still unknown. It is possible that a direct immune mechanism may be involved.

Lyden et al. (1992) supported the possibility of functional damage to the trophoblastic layer by aPL; monoclonal aPL reacted directly with the trophoblastic region of the human placenta. aPL-induced obstetric complications may be mediated by inhibition of the intercytotrophoblast fusion process (Adler et al., 1994) or by hormonal alterations, such as changes in the HCG secretion (Gleicher et al., 1992; Shurtz-Swirski et al., 1993; Di Simone et al., 1995).

In a previous study (Di Simone et al., 1995), we suggested that aPL could interfere with gonadotrophin-releasing hormone (GnRH)-induced signal transduction. When GnRH was added to human trophoblast cells pre-incubated with normal serum, human chorionic gonadotrophin (HCG) secretion increased significantly, while GnRH-induced HCG stimulation was abolished in the presence of aPL-containing sera.

The present study was designed to investigate whether drugs used to treat pregnancies complicated by APS (heparin, corticosteroids and acetylsalicylic acid) would be able to restore, in vitro, GnRH-induced placental HCG secretion.

Materials and methods

Sera

Table I summarizes hormonal values of the five women who provided sera for this study during the early follicular phase of their menstrual cycle. Hormonal assays were performed by commercial radioimmunoassay kits (Radim, Rome, Italy). For all hormones, the intra- and interassay coefficients of variation were <14 and <9% respectively.

Lupus anticoagulant was detected with a standard activated partial thromboplastin time (aPTT), a diluted phospholipid aPTT (Alving et al., 1985), a kaolin clotting time (Exner et al., 1978) and tissue thromboplastin inhibition test (Schleider et al., 1976). Lupus anticoagulant was defined by prolongation of coagulation times (patient:control ratio of 1:3 or more) persisting after the addition of control plasma (patient:control ratio 1:1) in at least two tests.

Enzyme-linked immunosorbent assay (ELISA)

All the women selected were evaluated for the presence of aPL using the ELISA method as described by Harris (1990). Individual 96-well microtitre plates were coated with 30 µl of cardiolipin at a concentration of 50 µg/ml (Sigma Chemical, St Louis, MO, USA). The plates were air-dried overnight and blocked with 200 µl of 10% fetal calf serum (FCS, Gibco, Long Island, NY, USA) in 1× phosphate-buffered saline (PBS), and then incubated at room temperature for 1 h with 50 µl of patient’s serum diluted 1/50 in 10% FCS in PBS. The plates were then washed to remove unbound antibodies and proteins. A secondary antibody, alkaline phosphatase-conjugated antihuman IgG (Sigma Immunochemicals, St Louis, MO, USA) was added to the plate. After incubation and washing, p-nitrophenylphosphate substrate
Dispersion and culture of trophoblast cells

Placentae were obtained immediately after spontaneous vaginal term delivery from five normal women delivering at the Obstetrics and Gynecology Department of the Catholic University, Rome, Italy. Trophoblast cells were isolated and incubated as previously described (Di Simone et al., 1995).

Placental tissue was debrided of the membranes and decidua. After mincing, the tissues were submitted to repeated enzymatic digestions in Ringer-bicarbonate buffer containing 0.25% trypsin-10 IU/ml DNase I at 37°C in a shaking water bath. Pooled cell supernatants were filtered through a 42 µM mesh filter and centrifuged in a 40% Percoll gradient for 20 min at 1200 g.

For multiple comparisons. Absence and with increasing amounts of heparin or low molecular weight heparin (Efacast; Crinos, Villaguardia, Como, Italy; 5–500 IU), low molecular weight heparin (Clexane; Glaxo Wellcome, Verona, Italy; 0.05–50 nM) were made in 1 ml of PBS. PBS (100 µl) as a control or PBS with different concentrations of heparin, aspirin or betamethasone was added to the ELISA plate immediately before patient’s serum. All assays were carried out in triplicate.

Methylene blue assay

We used a colorimetric technique modified by Oliver et al. (1989) to study cell number. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% normal or aPL serum in 96-well culture plates. Low molecular weight heparin (200 IU/ml), betamethasone (50 nM) and acetylsalicylic acid (1.6 µM) were added at the time of plating to the three replicate wells. Cells were treated for 5 days with the same serum (patient 1 or 3) and drug concentrations; the medium was changed every 24 h. On the fifth day, the culture medium in each well was removed. After washing with 0.15% saline the cell layer was fixed by adding 100 µl of 10% formal saline to each well for at least 30 min. The fixative was shaken off each plate and 100 µl of filtered 1% (w/v) Methylene Blue in 0.01 M borate buffer (pH 8.5) was added to each well. After 30 min excess dye was removed and the remaining dye was then washed off with 0.01 M borate buffer. The absorbance at 650 nm (A 650) was measured from each well by a microplate photometer (Biorad platereader Model 405).

Results

Table II summarizes clinical presentations and aPL specificities of the five women who provided sera for this study. The three aPL-antibody-positive patients satisfied the criteria for APS (patients 3, 4 and 5; Alargon-Segovia and Sanchez-Guerrero, 1989; Harris, 1990).

Antiphospholipid antibody binding in ELISA: effects of heparin, acetylsalicylic acid and betamethasone

Serum with elevated concentrations of aPL was assayed in the absence and with increasing amounts of heparin or low molecular weight heparin (Efacast; Crinos, Villaguardia, Como, Italy; 5–500 IU), low molecular weight heparin (Clexane; Glaxo Wellcome, Verona, Italy; 0.05–50 nM) were made in 1 ml of PBS. PBS (100 µl) as a control or PBS with different concentrations of heparin, aspirin or betamethasone was added to the ELISA plate immediately before patient’s serum. All assays were carried out in triplicate.
Heparin and low-dose aspirin: effect on HCG secretion

APL-containing sera (patients 3, 4 and 5)
When GnRH was added to the culture medium pre-incubated with aPL-containing sera (Table III), the HCG stimulation was abolished. Low molecular weight heparin appeared to restore the hormonal placental secretion. As shown in Table III the GnRH (10^{-7} M) addition in placental cells cultured with low molecular weight heparin (25–200 IU/ml) increased HCG production in comparison with untreated (control) cells and with GnRH or heparin treatment alone (P < 0.05).

Table IV shows that acetylsalicylic acid (0.03 µM) slightly increased GnRH-induced HCG secretion (P < 0.05). A higher dose (1.6 µM) reduced both basal and GnRH-induced HCG production.

Betamethasone increased basal and GnRH-induced HCG secretion in a dose-dependent way (Table V), in both normal or aPL-containing sera treated cells. Its effect was significant at concentrations of 0.5 nM and above (P < 0.05), reaching a maximum at 50 nM (P < 0.01).

**Methylene Blue assay**
Since heparin, betamethasone and acetylsalicylic acid modified the placental HCG secretion, the effect of these drugs on cell number was investigated. Acetylsalicylic acid (1.6 µM) resulted in a significant inhibition of cell number (e.g. patient 1: untreated cells: 0.48 ± 0.1 OD units versus acetylsalicylic acid treatment: 0.26 ± 0.07 OD units, P < 0.05; patient 3: untreated cells: 0.39 ± 0.05 OD units versus acetylsalicylic acid treatment: 0.20 ± 0.03 OD units, P < 0.05). No effect on cell number was observed in the presence of heparin, betamethasone and lower doses of aspirin.

**Discussion**
The reproductive history in patients with APS is characterized by recurrent spontaneous abortions, intrauterine growth retardation and stillbirths. A pathogenic mechanism has been postulated by several authors (Bulletti et al., 1996). The majority of scientific evidence suggests a thrombotic tendency in this syndrome.

Another hypothesis proposed is that aPL have a detrimental effect on the trophoblastic layer resulting in severe placental dysfunction and pregnancy loss.

Studies on human tissue and in mice suggest that aPL cause pregnancy loss by binding to phospholipids expressed on the trophoblast, thereby inhibiting successful embryonic implantation into the endometrium (Rai et al., 1997). Heparin, in addition to its anticoagulant action, might act to reduce fetal loss by binding to aPL, thereby protecting the trophoblast phospholipids from attack and promoting successful implantation in early pregnancy (Rai et al., 1997).

We found that, in vitro, heparin treatment of cytotrophoblast cells was able to restore the GnRH-induced HCG secretion, suggesting direct interference in the binding of aPL to cytotrophoblast–synctiotrophoblast membranes.

Recently, Adler et al. (1994) provided direct evidence that monoclonal aPL prevent intracellular fusion and differentiation of choriocarcinoma cells. In our system, the failure of placental cells to respond to GnRH might be due to reduced syncytium...
Betamethasone treatment versus untreated cells (CTR): c

Acetylsalicylic acid treatment versus untreated cells (CTR): c

induced HCG secretion in presence of antiphospholipid antibodies containing sera (patients 3, 4 and 5).

more effective at pharmacological and lower concentrations logical concentration that should be found in the plasma

b GnRH (10 \text{e} 7 M) treatment versus untreated cells (control

6

31 4

6

21 9

11 5

no.

Patient CTR Betamethasone (nM) GnRH Betamethasone (nM) and GnRH

Table V.

Effect of betamethasone on basal and gonadotrophin-releasing hormone (GnRH)-induced human chorionic gonadotrophin (HCG) production by human trophoblast cells

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CTR</th>
<th>Betamethasone (nM)</th>
<th>GnRH</th>
<th>Betamethasone (nM) and GnRH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
<td>0.50</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>15 \pm 2^c</td>
<td>20 \pm 3</td>
<td>32 \pm 9</td>
<td>42 \pm 9^d</td>
</tr>
<tr>
<td>2</td>
<td>19 \pm 4</td>
<td>23 \pm 2</td>
<td>39 \pm 4^d</td>
<td>69 \pm 3^e</td>
</tr>
<tr>
<td>3</td>
<td>14 \pm 3</td>
<td>29 \pm 12</td>
<td>47 \pm 13^e</td>
<td>56 \pm 3^e</td>
</tr>
<tr>
<td>4</td>
<td>9 \pm 1</td>
<td>13 \pm 4</td>
<td>27 \pm 4^d</td>
<td>33 \pm 4</td>
</tr>
<tr>
<td>5</td>
<td>14 \pm 4</td>
<td>17 \pm 5</td>
<td>39 \pm 4^d</td>
<td>44 \pm 9</td>
</tr>
</tbody>
</table>

4HCG: human chorionic gonadotrophin (IU/l). Data are means \pm SD of five experiments.

4GnRH (10^{-7} M) treatment versus untreated cells (control = CTR): patient 2, \( P < 0.05 \); patient 1, \( P < 0.01 \) LMWH treatment significantly increases GnRH-induced HCG secretion in presence of antiphospholipid antibodies containing sera (patients 3, 4 and 5).

4P < 0.05 versus untreated cells (control = CTR), GnRH and LMWH treatment alone.

formation. It is possible that the morphology and the differentiation state of the trophoblast is different between untreated cultures and heparin-treated cultures. Studies are ongoing to investigate whether aPL can modify the differentiation of normal placental cells.

Furthermore, low molecular weight heparin seems to be more effective at pharmacological and lower concentrations than regular heparin. Dawes et al. (1986) demonstrated that low molecular weight heparin may be more effective than unfractionated heparin, because it is more effectively absorbed after s.c. administration and has a longer half-life in the circulation. According to our study, low molecular weight heparin has some advantage over heparin in having more significant effects. This represents an important role for low molecular weight heparin in the treatment of APS in pregnancy, because it causes less bleeding in both vaginal and abdominal deliveries (Dulitzki et al., 1997).

The hormonal response (HCG) restored by low-dose aspirin could open a new field of research. The dose of aspirin (0.03 \mu M) used in our experiments is close to the pharmacological concentration that should be found in the plasma of low-dose (100 mg) aspirin-treated patients (Dekker and Sibbax, 1993).

Low-dose aspirin might improve the pregnancy outcome in women with aPL by irreversibly blocking the action of cyclooxygenase in platelets, thereby inhibiting platelet thromboxane synthesis and preventing thrombosis of the placental vasculature (Rai et al., 1997; Christiansen, 1996).
However, no prospective, placebo-controlled trial has been carried out to compare aspirin therapy with placebo (Christiansen, 1995). Fishman et al. (1995) showed that low-dose aspirin (0.03 μM) is able to act as potent stimulator of interleukin-3 (IL-3) through its ability to raise leukotriene production. Only low-dose aspirin stimulates IL-3 production, while higher doses fail to do so. Cytokines have been shown to have a role in the endocrine and immune systems (Lim et al., 1996); and there seems to be some interaction between cytokines and the endocrine system in endometrial development during implantation (Lim et al., 1996). We observed in trophoblast cells, that low aspirin doses are able to restore, at least partially, the hormonal secretion GnRH-induced, whereas high doses significantly decrease HCG production.

These observations may provide attractive explanations to understand the effects of these treatments on the syndrome; studies are ongoing to clarify the mechanisms of action of aPL positive sera on placental function.

Acknowledgements
Partially supported by grant 95.00784.PF41(*) from Targeted Project FATMA, National Research Council (CNR-Targeted Project ‘Prevention and Control Disease Factors’). Subproject ‘Study of haemodynamic parameter and endocrine factors in placental fetal development’

References
Dawes, J., Bara, L., Billaud, E. et al. (1986) Relationship between biological activity and concentration of a low molecular-weight heparin (PK 10169) and unfractionated heparin after intravenous and subcutaneous administration. Haemostasis, 16, 116–122.