EXTENDED REPORT

Pathogenic role of anti- β 2-glycoprotein I antibodies in antiphospholipid associated fetal loss: characterisation of β 2-glycoprotein I binding to trophoblast cells and functional effects of anti- β 2-glycoprotein I antibodies in vitro

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Background: Antiphospholipid antibodies reacting with β 2-glycoprotein I (β 2GPI) have been associated with recurrent fetal loss and pregnancy complications.

Objective: To investigate whether specific mutations in the phospholipid binding site of β 2GPI might affect its binding to trophoblast and in turn the anti- β 2GPI antibody induced functional effects.

Methods: β 2GPI adhesion to trophoblast was evaluated as human monoclonal IgM or polyclonal IgG anti- β 2GPI antibody binding to trophoblast monolayers cultured (1) in complete medium; (2) in serum-free medium; (3) after serum starvation in the presence of purified human β 2GPI; or (4) in the presence of β 2GPI with single or multiple mutations in the amino acid loop Cys²⁸¹-Lys-Asn-Lys-Glu-Lys-Lys-Cys²⁸⁸. The effect of anti- β 2GPI binding to trophoblast was evaluated as chorionic gonadotropin (hCG) mRNA expression, and protein release by RT-PCR and radioimmunoassay, respectively.

Results: β 2GPI adhesion to trophoblast and its consequent recognition by the specific antibodies were inversely proportional to the mutation number in the phospholipid binding site. Anti- β 2GPI antibodies reduced gonadotropin release, hormone dependent hCG mRNA expression, and protein synthesis in the presence of β 2GPI, while the addition of the mutants or the absence of β 2GPI had no effect.

Conclusions: β 2GPI binds to trophoblast in vitro through its fifth domain, as reported for endothelial cells, and can be recognised by anti- β 2GPI antibodies; the antibody binding downregulates trophoblast hCG synthesis and secretion. Such a mechanism might contribute to defective placentation in women with fetal loss associated with the antiphospholipid syndrome.

Human β 2-glycoprotein I (β 2GPI) is a phospholipid binding plasma protein that is one of the main antigenic targets for antiphospholipid antibodies (aPL), the formal diagnostic tools for the antiphospholipid syndrome.¹⁻⁵ β 2GPI dependent aPL not only have diagnostic value but also play a pathogenic role in the syndrome.⁶

β 2GPI is composed of five highly conserved subunits called sushi domains or complement control protein repeats.⁷ The molecule binds to negatively charged structures through a major phospholipid binding site located in the fifth domain, and identified as a highly positively charged amino acid sequence: Cys²⁸¹-Lys-Asn-Lys-Glu-Lys-Lys-Cys²⁸⁸.⁸ Anti- β 2GPI antibodies recognise different epitopes located in all five domains; however, several investigators have reported that most of the antibodies are directed against domain I (reviewed by de Groot *et al*).⁹⁻¹¹ Recent findings suggest that the antibodies have low avidity and react with the native β 2GPI when the molecule is available at increased antigenic density.⁹⁻¹¹

β 2GPI binds not only to negatively charged substances such as phospholipids, heparin, DNA, and lipoproteins, but also to surface membranes of cell types directly involved in the pathogenic mechanisms of the antiphospholipid syndrome, such as activated platelets and endothelial cells.^{7 12 13} It has been reported recently that dimeric β 2GPI binds to anionic phospholipids exposed on activated platelets and interacts with apolipoprotein E receptor 2, a member of the

low density lipoprotein receptor family expressed on both platelets and trophoblast.^{14 15} The phospholipid binding site in the fifth domain of the molecule has also been shown to be involved in the binding of the molecule to the endothelial cell membranes.¹⁶

More recently, we found that β 2GPI can adhere to human trophoblast cells in vitro.¹⁷ Our results are consistent with the hypothesis that the exposure of anionic phospholipids on the external cell surface during intertrophoblastic fusion might offer a useful substrate for the cationic phospholipid binding site.^{18 19} This finding is in line with the previous immunohistological demonstration of the in vivo binding of β 2GPI to trophoblast structures.^{20 21} Once bound, the molecule offers suitable epitopes for both human polyclonal and monoclonal anti- β 2GPI antibodies obtained from patients with antiphospholipid syndrome. The antibody binding was found to affect trophoblast differentiation in vitro by inhibiting gonadotrophin release and matrigel invasiveness, which might represent mechanisms potentially involved in the defective placentation reported in the antiphospholipid syndrome.¹⁷

Abbreviations: APL, antiphospholipid antibodies; β 2GPI, β 2 glycoprotein I; CL, cardiolipin; GnRH, gonadotropin releasing hormone; hCG, human chorionic gonadotropin; NHS, normal human sera; RT-PCR, reverse transcriptase polymerase chain reaction

By using recombinant β2GPI mutants we investigated whether β2GPI could bind to trophoblast cells in a manner comparable to that shown for cardiolipin (CL) coated plates and endothelial monolayers, and whether such binding is strictly required for the antibody mediated biological effects on the trophoblast.

METHODS

Human β2GPI purification

Human β2GPI was purified from human serum and characterised as previously described.²²

Human β2GPI mutants

Site directed mutagenesis was carried out to assess the role of individual amino acids in the Cys²⁸¹–Cys²⁸⁸ loop for phospholipid binding and cofactor activity as detailed.^{8, 23} Four mutants were obtained: 1K, with a single amino acid change from Lys²⁸⁶ to Glu²⁸⁶; 2K and 2Ka, with a double amino acid change (from Lys^{286, 287} to Glu^{286, 287} and from Lys^{284, 287} to Glu^{284, 287}, respectively); and 3K with a triple amino acid change (from Lys^{284, 286, 287} to Glu^{284, 286 and 287}). In comparison with human purified β2GPI, mutant 1K showed reduced phospholipid binding activity, lower cofactor activity for β2GPI dependent antibodies in a CL-ELISA (enzyme linked immunosorbent assay), and decreased inhibition of the binding of iodinated human purified β2GPI to CL coated plates. Double or triple mutants 2K, 2Ka, and 3K lost the phospholipid binding activity and did not show any cofactor activity for anti-β2GPI antibodies in CL-ELISA or the ability to inhibit iodinated native β2GPI binding to CL. While mutations in the Lys^{282–287} loop altered the properties of the phospholipid binding site, they did not affect the recognition of all the mutants by purified polyclonal anti-β2GPI antibodies.²³ Comparable experiments carried out with the monoclonal antibodies gave similar results (data not shown).

Human monoclonal anti-β2GPI antibodies

We used two human monoclonal antibodies of the IgM isotype obtained from hybridised Epstein-Barr virus (EBV)-induced B cell lines from patients with antiphospholipid syndrome. TM1G2 has been shown to recognise β2GPI, both when complexed with anionic phospholipids (in CL coated plates) and alone in γ irradiated β2GPI coated plates. TM1B9 did not display any anti-β2GPI reactivity and was used as a negative control. The characterisation of the monoclonal antibodies had been reported previously in detail.²⁴

Human polyclonal anti-β2GPI antibodies

Whole IgG fractions from two patients suffering from primary antiphospholipid syndrome diagnosed according to the Sapporo criteria²⁵ and from two normal human sera (NHS) were purified on protein-G-sepharose.²² The final protein IgG concentration was evaluated by nephelometry

and the specific reactivity with β2GPI coated plates was confirmed as previously described.²²

Cell cultures

Placentas were obtained from healthy women immediately after uncomplicated vaginal delivery at 36 weeks' gestation. Cytotrophoblast cells were isolated as detailed elsewhere.²⁶ The enriched (95%) cytotrophoblast cells (5×10^5 cells/ml) were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% FCS (fetal calf serum) in 96-well plates at 37°C in 5% CO₂/95% air. The purity and the maturation of the cell preparations were evaluated using a panel of antisera directed against fibroblasts, macrophages, cytocheratin, and human chorionic gonadotrophin (hCG) as previously described.^{17, 26} Ninety five per cent of the cell preparations tested positive for anti-cytocheratin antibodies. Cytotrophoblasts at different times of culture were further assayed for the cytoplasmic presence of hCG as a marker for syncytial trophoblast. Cell cultures were carried out for 72 hours in standard medium, washed three times with HBSS (Hank's balanced salt solution, Flow Laboratories, Irvine, Strathclyde, UK), and cultured in serum-free (HyQ-CCMTM 1, HyClone, Laboratories, Logan, Utah, USA) to remove adherent β2GPI.¹⁷

Binding assays

On day 3 (72 hours) of culture in 96-well plates, the medium was removed and the cells were washed and cultured in serum-free medium. A cell ELISA was carried out to

Figure 1 Human anti-β2 glycoprotein I (β2GPI) monoclonal antibody (mAb) binding to trophoblast cell monolayers. Cell cultures were incubated with TM1G2 mAb (50 μg/ml) in the presence of human purified β2GPI or 1K, 2K, 2Ka, or 3K mutants at serial protein concentrations (from 5 to 1.25 μg/ml). Binding values are expressed as mean optical density (OD) units $\times 10^{-3}$; error bars = SD (n = 3). Washed trophoblast cell monolayers cultured in serum-free medium in the absence of β2GPI or 1K, 2K, 2Ka, and 3K mutants and incubated with TM1G2 gave mean (SD) background values of 201 (70) OD units (n = 6 experiments). Comparable experiments carried out with TM1B9 mAb (50 μg/ml) gave background binding values only (values $< 200 \times 10^{-3}$ OD units). TM1G2 mAb binding to trophoblast was significantly greater in the presence of human purified β2GPI than in the presence of mutants (*p < 0.05).

Table 1 Sequence and expected fragment size for synthetic oligonucleotides used for polymerase chain reactions and Southern blotting

Oligonucleotide sequence	Primer sequence	Size (bp)
β-hCG5'	ATG-CCA-CCC-TGG-CTG-TGG-AGA-A	367
β-hCG3'	GGG-AGT-CGG-GAT-GGA-CTT-GGA-A	
β-hCG-Southern	CAA-CTA-CCG-CGA-TGT-GCG-C	
Aldolase5'	CGC-AGA-AGG-GGT-CCT-GGT-GA	176
Aldolase3'	CAG-CTC-CTT-CTT-CTG-CTC-CGG-GGT	
Aldolase-Southern	GAA-CTT-GCT-ACT-ACC-AGC-A	

determine whether the polyclonal and monoclonal anti- β 2GPI antibodies bound to trophoblast cells through the adherent β 2GPI and β 2GPI mutants, as previously described.¹⁷ Serum-free trophoblast cell cultures were incubated for one hour at 37°C with different protein concentrations of: (1) human purified β 2GPI (5, 2.5, 1.25 μ g/ml); (2) recombinant β 2GPI mutants 1K, 2K, 2Ka, and 3K (5, 2.5, 1.25 μ g/ml); or (3) serum-free medium. Polyclonal or monoclonal anti- β 2GPI antibodies (50 μ g/ml) were added to the wells. After a two hour incubation followed by three washes, the plates were incubated with alkaline phosphatase conjugated goat anti-human IgM or anti-IgG (Sigma Chemicals, St Louis, Missouri, USA) for 90 minutes. After two further washes, p-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine buffer, pH 9.8, was added to each well and incubated for 30 minutes. Optical density (OD) was read at 405 nm by a microplate photometer (Platereader; Bio-Rad Laboratories, Milan, Italy).¹⁷

Hormone secretion

Human purified β 2GPI or β 2GPI mutants (5 μ g/ml) were added to primary trophoblast cells cultured in serum-free medium. After a one hour incubation, 50 μ g/ml of polyclonal or monoclonal anti- β 2GPI antibodies or appropriate controls were added. The culture media were changed daily from the first day of incubation. After 72 hours of culture, the cells were then treated for 24 hours with 10^{-7} M gonadotropin releasing hormone (GnRH; Lutrelf, Ferring, Milan, Italy). At the end of the incubation, the media were removed and stored at -20°C for hCG determination. The assay was carried out with a commercial radioimmunoassay kit (Radim, Rome, Italy) as previously described.¹⁷ The intra-assay and

inter-assay coefficients of variation were $<12\%$ and $<8\%$, respectively.

Semiquantitative RT-PCR analysis

A comparable experimental protocol was followed to evaluate the hCG mRNA expression on trophoblast cell cultures. Confluent cells were collected, centrifuged, and washed and total RNA was isolated by lysing cells with TrizolTM reagent (Gibco BRL) according to the manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining, as well as by monitoring absorbance at 260/280 nm. The RNA concentration was determined by spectrophotometric analysis and before each RT-PCR (reverse transcriptase polymerase chain reaction) experiment. The Perkin-Elmer Gene Amp Gold RNA PCR kit was used for all the RT-PCRs, which employed the Gene Amp PCR system 9600 (Perkin-Elmer/Cetus, San Diego, California, USA). After removal of contaminating chromosomal DNA with DNase I treatment, 1 μ g of RNA was reverse transcribed with 25 units of Moloney murine leukaemia virus at 42°C for 20 minutes. Three microlitres of cDNA products were used in each PCR cycle. Primer sequence and location as well as expected product size are listed in table 1.

The hCG cDNA was co-amplified with aldolase-A as internal control with 1 unit of AmpliTaq Gold DNA polymerase in 1.75 mM MgCl_2 , and 32 cycles of 20 seconds at 94°C, 60 seconds at 62°C, followed by seven minutes at 72°C. The PCR products were loaded onto 2% agarose gels and stained with ethidium bromide. The images of the gels were acquired with a Cohu charged coupled device camera, and quantification was carried out with a Phoretix ID (Phoretix International Ltd, Newcastle upon Tyne, UK). The relative

Figure 2 Human chorionic gonadotropin (hCG) secretion induced by anti- β 2 glycoprotein I (β 2GPI) polyclonal IgG (panel A) and by monoclonal antibodies (mAb) (panel B). (A) Trophoblast cell monolayers were incubated in the presence of polyclonal anti- β 2GPI IgG (50 μ g/ml) with either serum-free medium, human purified β 2GPI (5 μ g/ml), or 3K mutant (5 μ g/ml). Two sets of cultures were carried out, in the presence or absence of gonadotropin releasing hormone (GnRH) (10^{-7} M). hCG values are expressed as mIU/ml. hCG secretion was significantly reduced in cultures done in the presence of human β 2GPI in comparison with cultures carried out with serum-free or 3K mutant (* $p < 0.05$). (B) Trophoblast cell monolayers were incubated in the presence of GnRH (10^{-7} M) together with TM1G2 mAb (50 μ g/ml) plus native β 2GPI, 1K mutant, 2Ka mutant, or 3K mutant (5 μ g/ml). In the control cultures, TM1B9 mAb (50 μ g/ml) replaced TM1G2 mAb. hCG secretion by cultures carried out in the presence of native β 2GPI or 1K mutant plus TM1G2 was significantly reduced in comparison with control cultures carried out with TM1B9 mAb (* $p < 0.05$). No difference in hCG secretion was found in the cultures done with the other mutants. Additional controls were trophoblasts in medium alone (mean (SD): 12.3 (1.5) mIU/ml hCG; $n = 6$ experiments) and in the presence of GnRH alone (28.3 (1.5) mIU/ml hCG; $n = 6$ experiments). Cells incubated with mutants alone or in the presence of the mAbs but without GnRH stimulation gave background values (<13.5 mIU/ml hCG).

concentration of each hCG mRNA was determined by densitometric scanning and normalisation to the aldolase-A signal for each sample. The number of cycles and the reaction conditions were chosen so that none of the target cDNAs reached a plateau and so that the two pairs of primers did not compete with each other. The PCR product identity was verified by Southern blotting using synthetic oligonucleotides located internal to the two PCR primers (table 1).

Statistical analysis

For statistical analyses we used Student's *t* test and two way analysis of variance for multiple comparisons. Probability (*p*) values of <0.05 were considered significant.

RESULTS

Binding of human purified β2GPI and β2GPI mutants to trophoblast cells

In order to demonstrate the role of β2GPI or mutants in the anti-β2GPI antibody trophoblast binding, cell cultures were extensively washed and cultured in serum-free medium to remove adherent β2GPI. Under such conditions, TM1G2 showed background binding values comparable to those found with the irrelevant control TM1B9. Addition of exogenous human β2GPI restored TM1G2 (50 µg/ml) binding but did not affect TM1B9 (50 µg/ml) binding (data not shown). When trophoblast cells were incubated with 1K, 2K, and 2Ka mutants (5 µg/ml), mean (SD) TM1G2 binding values were 59 (3)%, 41 (5)%, and 35 (3)%, respectively (*n* = 3 experiments) of those obtained in the presence of human purified β2GPI. Lower TM1G2 binding (26 (2)%; *n* = 3 experiments) was observed when trophoblast cells were incubated in the presence of the 3K mutant (fig 1). TM1G2 monoclonal antibody binding to trophoblast was dependent

Figure 3 Human chorionic gonadotrophin (hCG) mRNA expression induced by anti-β2 glycoprotein I (β2GPI) polyclonal IgG. RT-PCR amplified products (top panel): 1, markers; 2, H₂O; 3, PCR w/o RT; 4, control; 5, with GnRH; 6, with β2GPI + anti-β2GPI Abs; 7, with β2GPI + NHS IgG; 8, with 3K mutant + anti-β2GPI Abs; 9, with 3K + NHS IgG; 10, with β2GPI + anti-β2GPI Abs and GnRH; 11, with β2GPI + NHS IgG and GnRH; 12, with 3K mutant + anti-β2GPI Abs and GnRH; 13, with 3K mutant + NHS IgG and GnRH. The hCG/aldolase ratio in trophoblast cultures was determined in serum-free conditions, with human β2GPI or 3K mutant, and in the presence of anti-β2GPI polyclonal IgG (50 µg/ml). Two sets of cultures were done: with GnRH (black bars) and without GnRH (white bars). The results are the mean of five different experiments. Significantly higher hCG/aldolase ratios were found in cultures of trophoblasts in serum-free medium or in the presence of 3K mutant than in cultures carried out in the presence of human β2GPI (**p* < 0.05). Abs, antibodies; GnRH, gonadotropin releasing hormone; NHS, normal human sera; RT-PCR, reverse transcriptase polymerase chain reaction.

on the final amount of human purified β2GPI or recombinant mutants added to the cultures (fig 1).

Comparable results were found by using whole polyclonal IgG fractions (50 µg/ml) with anti-β2GPI activity but not with NHS IgG (data not shown).

All the human antibodies were used at a final protein concentration of 50 µg/ml, which has previously been shown to display optimal binding.¹⁷

Modulation of hCG protein secretion by anti-β2GPI antibodies

In accordance with previous findings, the addition of GnRH (10⁻⁷ M) to trophoblast cells significantly increased hCG secretion.¹⁷ The incubation of trophoblast cells with NHS IgG in the presence of human β2GPI (5 µg/ml) did not modify either basal or GnRH induced hCG production (data not shown). In contrast, polyclonal anti-β2GPI IgG (50 µg/ml) significantly reduced GnRH induced hCG secretion when the cells were cultured in the presence of human purified β2GPI (5 µg/ml); no changes in basal or GnRH induced hormonal placental secretion were observed in the presence of the 3K mutant (5 µg/ml) or in serum-free conditions (fig 2A). Experiments carried out with the human anti-β2GPI IgM monoclonal antibody (TM1G2, 50 µg/ml) gave comparable results (31 (4), 12 (4), and 28 (3) mIU/ml hCG in triplicate experiments for cultures in the presence of GnRH plus serum-free medium, native β2GPI, or 3K mutant, respectively; 11 (3), 10 (2), and 10 (4) mIU/ml hCG in triplicate experiments for comparable cultures carried out without GnRH). Cultures in the presence of the irrelevant control (TM1B9; 50 µg/ml) gave values ranging from 29 to 32 mIU/ml hCG in the presence of GnRH and <13.5 mIU/ml hCG in the absence of GnRH.

Figure 2B gives the results on the hCG secretion by trophoblast monolayers incubated in the presence of monoclonal antibodies and the mutants. TM1G2 monoclonal antibody recognised the 1K mutant and induced an inhibition of hCG secretion less than that shown in the presence of native β2GPI but still statistically significant in comparison with the control. Neither mutant 2Ka nor 3K was recognised, and no inhibition of hCG secretion was found.

Incubation of trophoblast cells with human β2GPI or 1K, 2Ka, and 3K mutants (5 µg/ml) alone did not modify basal (<13.5 mIU/ml hCG) or GnRH induced hCG production (28 (3), 29 (2), 31 (5), and 30 (4) mIU/ml hCG, respectively).

Modulation of hCG mRNA expression by polyclonal anti-β2GPI IgG

Because it has been shown that both polyclonal and monoclonal anti-β2GPI monoclonal antibodies downregulate the GnRH induced hCG secretion after trophoblast binding, we investigated whether such an effect might also be reproducible at the hCG mRNA level.

Addition of GnRH (10⁻⁷ M) to trophoblast cells significantly increased hCG mRNA expression. As shown in fig 3, the presence of both anti-β2GPI IgG (50 µg/ml) and human purified β2GPI (5 µg/ml) significantly reduced GnRH-induced hCG mRNA expression. In parallel experiments done with 3K mutant, GnRH-induced hCG mRNA was not inhibited by the addition of anti-β2GPI IgG. Incubation with NHS IgG did not modify hCG mRNA expression in any of the conditions (data not shown).

Comparable results were found using TM1G2 anti-β2GPI monoclonal antibody and its irrelevant control (TM1B9) (data not shown).

DISCUSSION

Our results show for the first time that β2GPI adhesion to trophoblast cell membranes involves the same phospholipid

binding site used for the binding to negatively charged structures.

It has been suggested that the highly positively charged amino acid sequence, Cys²⁸¹-Lys-Asn-Lys-Glu-Lys-Lys-Cys²⁸⁸, in the fifth domain of the molecule is the putative phospholipid binding site responsible for the β 2GPI binding to CL coated plates. Single or multiple amino acid substitutions of Lys with Glu progressively decrease the ability of the molecule to bind to anionic structures.^{8, 23} Interestingly, the same phospholipid binding site is involved in the adhesion of β 2GPI to human endothelial cell monolayers, because Lys substitution with Glu significantly decreases the presence of β 2GPI on endothelial monolayers, as shown by the lack of anti- β 2GPI antibody binding.¹⁶

We confirmed our previous results by showing that human purified β 2GPI binds to trophoblast cells and is recognised by both monoclonal and polyclonal anti- β 2GPI antibodies from antiphospholipid syndrome sera.¹⁷ When trophoblast cells were incubated with serial protein concentrations of mutant 1K (single amino acid substitution from Lys²⁸⁶ to Glu²⁸⁶) there was approximately a 50% reduction in anti- β 2GPI antibody binding to the cells in comparison with trophoblasts cultured with comparable protein concentrations of purified β 2GPI. Much lower antibody binding was found by using 2K (substitution from Lys^{286,287} to Glu^{286,287}) and 2Ka (substitution from Lys^{284,287} to Glu^{284,287}) mutants, and the lowest antibody binding was detected with the mutant 3K (substitution from Lys^{284,286,287} to Glu^{284,286,287}).

We previously reported that, once bound to trophoblast-adhered β 2GPI, anti- β 2GPI antibodies might significantly inhibit GnRH induced hCG protein release from trophoblast cell cultures.¹⁷ We confirmed and extended the previous data by showing that anti- β 2GPI antibody mediated hCG inhibition might also occur at the mRNA level. Moreover, the present findings indicate that a large alteration to the phospholipid binding site on the fifth domain of the molecule does not allow efficient β 2GPI adhesion, antibody binding, and, in turn, antibody mediated cell function modulation. Experiments carried out with trophoblast cells incubated with anti- β 2GPI antibodies and mutant 3K show hCG mRNA expression comparable to those found in control or serum-free cultures.

Cytotrophoblast cells from term placentas were used for ethical reasons. Although these have a lower percentage of mitotic figures and lower in vitro invasiveness than cells taken from the first or second trimester placentas, nevertheless they were previously shown to be a suitable in vitro experimental tools for investigating aPL mediated effects.¹⁷

The presence of β 2GPI on the trophoblast cell membranes could be one of the main targets for β 2GPI dependent aPL in the placental circulation.²⁰ Such a finding is a prerequisite for a pathogenic role for these antibodies, as suggested by the clinical association between recurrent fetal loss and β 2GPI dependent aPL or anti-human β 2GPI antibodies themselves.^{5, 27-30} At the same time, it also might explain the aPL placental tropism that has been described in experimental animal models of aPL associated fetal loss. In fact, when exogenous human aPL are passively infused in pregnancy-naïve mice they undergo rapid plasma clearance.^{31, 32} It has been suggested that the rapid clearance could be related to aPL binding to placental structures as the same antibodies can be eluted from the placenta.³² Moreover, there is also evidence from immunohistochemical studies that β 2GPI is expressed in higher quantity on the trophoblastic villi of placentas from women with antiphospholipid syndrome who have had fetal loss than from control placentas, and an immunoglobulin deposition with a comparable immunohistochemical pattern is also detectable.²¹ These findings suggest that most of the circulating β 2GPI dependent aPL (or

even the anti- β 2GPI antibodies themselves) might be bound to placental β 2GPI in vivo. The fact that aPL can be "absorbed" by placental structures has been thought to be pivotal for allowing the potential pathogenic effect of the antibodies on the placenta and for explaining—at least in part—why maternal IgG aPL do not often cause thrombotic events in fetuses or neonates.³³

Our results are consistent with the hypothesis that β 2GPI binds to trophoblastic cell membranes through the phospholipid binding site in the fifth domain of the molecule, being able to trap or increase antigen density. The exposure of anionic phospholipids on the cell membranes during inter-trophoblastic fusion might represent the natural substrate for placental β 2GPI adhesion.^{18, 19}

One could speculate that the clustering effect of anti- β 2GPI antibodies on trophoblast adhered β 2GPI might be the molecular event that signals the hCG mRNA downregulation as well as the impaired invasiveness,¹⁷ eventually contributing to the defective placentation in antiphospholipid syndrome.³⁴ It has been reported that an antiphosphatidylserine monoclonal antibody is capable of inhibiting intracellular fusion of a choriocarcinoma cell line, and we obtained comparable results by incubating polyclonal IgG from patients with antiphospholipid syndrome with anti- β 2GPI binding activity on cytotrophoblast cells.^{35, 36} Thus it is possible that anti- β 2GPI antibodies may also affect placental development by interfering with normal syncytiotrophoblast formation, the reduced hCG secretion in the presence of anti- β 2GPI antibodies being the result of inhibition of the trophoblast fusion process.

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