

Clinical Study

Circulating Anti-PLAC1 Antibodies during Pregnancy and in Women with Reproductive Failure: A Preliminary Analysis

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The aims of this study were to determine the prevalence of anti-PLAC1 antibodies in normal pregnant women and in women with infertility or recurrent pregnancy loss (RPL). Secondary outcomes were the development of complications associated with anti-PLAC1 seropositivity and the rate of seroconversion during pregnancy. Sera from 103 healthy pregnant women and 45 women with unexplained infertility or RPL were analyzed by ELISA. The prevalence of anti-PLAC1 antibodies was 2% in healthy pregnant women and 4.5% in women with unexplained infertility or RPL ($P = 0.355$). There was no detectable association of seropositivity with increased risk of pregnancy complications. Finally, 2% of women seroconverted during pregnancy. The prevalence of anti-PLAC1 antibodies in women with unexplained infertility or RPL is not significantly higher than the prevalence in normal pregnant women. However, the sample size in this study was too small. The exposure to the PLAC1 antigen during pregnancy can lead to the spontaneous development of antibodies.

1. Introduction

PLAC1 (PLACenta-specific 1) is an X-linked gene that encodes a protein localized to membranous compartments of cells of trophoblast lineage. It maps to a locus on the X-chromosome known to be important for placental development [1, 2]. Although its expression is highly restricted to the trophoblast [2, 3], its exact function in the placenta remains unclear. A significant role for *PLAC1* in normal placental development was suggested by aberrant placentation, fetal growth retardation, and neonatal death in mice that have large deletions of the X-chromosome in the region where *PLAC1* maps [4, 5]. Additionally, clinical studies in pregnant women reported that markedly decreased concentration of *PLAC1* mRNA in maternal plasma was associated with vaginal bleeding during the first twenty weeks of pregnancy [6].

While *PLAC1* expression is restricted to the placenta in normal tissues [1–3], it is also ectopically expressed in a variety of human cancers, including breast, lung, gastric, colon,

and ovarian [7–11]. Evidence from *in vitro* studies using the MCF-7 breast cancer cell line suggests that *PLAC1* promotes cell proliferation and motility, processes essential to tumor survival [12]. Independent studies from Tchabo et al., Dong et al., and Silva et al. reported the presence of anti-*PLAC1* autoantibodies in some cancer patients [9–11]. Additionally, Liu et al. reported that the *PLAC1* protein also elicits a CD8-mediated cytotoxic response that appeared to improve survival in a small cohort of colorectal patients [13]. Collectively, these studies suggested that expression of the *PLAC1* antigen can elicit a spontaneous antibody/immunologic response capable of altering tumor cell biology. In the study by Silva et al., anti-*PLAC1* autoantibodies were also detected in a small percentage of healthy control patients. Interestingly, the observed immunoreactivity was restricted to females, suggesting that they were likely exposed to the *PLAC1* antigen during a prior pregnancy [11]. The ability of these antibodies to interact with trophoblast-derived *PLAC1* and alter placental function under normal physiological circumstances is unknown. We therefore hypothesized that

the presence of anti-PLAC1 autoantibodies would adversely affect PLAC1 function during the early development of the placenta, resulting in increased reproductive loss or pregnancy complications.

In order to begin examining this question, our initial objectives were to define the prevalence of anti-PLAC1 seropositivity in a population of healthy pregnant women and compare it to the prevalence of anti-PLAC1 seropositivity in women with a history of unexplained infertility or RPL.

2. Materials and Methods

This study was approved by the Institutional Review Board of the University of South Florida. Informed consent was obtained from each patient.

2.1. Subjects. 103 primigravid or multigravid women were screened at their first prenatal visit at the obstetric clinic at the South Tampa Center, University of South Florida College of Medicine. Blood was obtained if the first visit occurred prior to 20 weeks' gestation and the patients were undergoing a blood draw as part as their routine prenatal evaluation. Subsequent samples were also obtained during pregnancy as part of routine clinical testing, up to five times.

Blood samples were also obtained from 45 patients with either unexplained infertility or recurrent pregnancy loss from the infertility clinic at the South Tampa Center, University of South Florida College of Medicine. Unexplained infertility patients were infertile for at least a year and had undergone a standard clinical evaluation including semen analysis, prolactin levels, thyroid stimulating hormone (TSH) levels, and tubal patency. Patients with recurrent pregnancy loss (RPL) had suffered at least two unexplained previous miscarriages before 20 weeks' gestation. All presumptive etiological factors (anatomic, hormonal, chromosomal, infections, and thrombophilic) had been ruled out. Exclusion criteria for both groups included the presence of significant underlying disease, that is, cardiac disease, renal disease, chronic hypertension, chromosomal abnormalities, and cancer.

The blood sample was obtained by peripheral venipuncture and placed into a yellow-top BD vacutainer tube for later determination of the presence of anti-PLAC1 autoantibodies in serum. Serum was separated by centrifugation after clot formation and stored at -20°C until analysis.

2.2. ELISA Assay for Determination of Serum Anti-PLAC1 Autoantibodies. His-tagged, rPLAC1 was produced and purified using a baculovirus expression system as previously described [2]. The presence of anti-PLAC1 autoantibodies in serum was measured by an ELISA assay using recombinant PLAC1 (rPLAC1) as coating antigen. Briefly, microtiter medium binding 96-well plates (CostarR 3591, NY 14831) were coated with PLAC1 protein by adding 150 μL of a solution of rPLAC1 (1 $\mu\text{g}/\text{mL}$) to each well and incubated overnight at 4°C . Wells were washed with a solution of PBS containing 0.1% of TWEEN 20 (Fisher Scientific, NJ

07410) three times and then blocked by incubation for 1.5 hours with the Blocking Solution (CANDOR Bioscience GmbH, Germany). After decanting the blocking buffer, a 150 μL aliquot of patient serum (diluted 1:100 in PBS) was added to each well. Normal human serum diluted to 1:100 (Jackson ImmunoResearch, PA 19390) was used as a negative control. Recombinant rabbit anti-PLAC1 antibody, diluted to 1:5,000, was initially used as a positive control until a positive patient sample was identified. The plates were incubated for 1.5 h at room temperature. The wells were then washed three times with PBS-TWEEN 20 at 0.1% and incubated with a peroxidase-linked goat anti-human IgG (Jackson ImmunoResearch, PA 19390) diluted 1:20,000 in blocking solution or with a peroxidase-linked goat anti-rabbit IgG (Millipore, MA 01821) diluted 1:15,000 in PBS for 1 hour. After washing, 100 μL of TMB substrate (Thermo Scientific, IL 61101) were added to each well as colorimetric substrate. The reaction was terminated with 100 μL of Stop Solution (Thermo Scientific, IL 61101). The absorbance was read at 450 nm on an automatic micro-ELISA plate reader. All samples were run in triplicate. Normal ranges were calculated using serum values obtained from healthy pregnant women. The cut-off representing a positive antibody response was defined as the mean optical density (OD) + 4SD of the healthy pregnant women after discarding all OD above the 95th percentile. The specificity of serum samples determined to be positive for anti-PLAC1 autoantibodies was tested by using insulin, transforming growth factor- α , and endothelin as coating antigens.

2.3. Statistical Analysis. All patient data were stored on a secure server. Statistical analysis was performed using SPSS 17.0 program (SPSS Inc., Chicago, IL, USA). We determined our sample size based on a previous study, which had shown a prevalence of 5% in a small cohort ($n = 78$) healthy female volunteers [11]. Based on the assumption that the prevalence of the antibody in the low-risk population was 5% and that the prevalence would be 25% in the high-risk population, 120 subjects (60/group) were needed in order to have at least 80% power at 0.05 significant level with one sided test.

Quantitative data were described as mean \pm SD and the differences between values were determined by Student's t -test when normally distributed. If the distribution was non parametric, differences were analyzed using Mann-Whitney comparisons by ranks and the data described as median and range. Frequency data were compared using χ^2 or Fisher's exact test. Statistical tests were considered significant whenever P -value was <0.05 .

3. Results

A cohort of normal pregnant women and women with histories of infertility/RPL agreed to participate in the study. Table 1 summarizes the demographic and clinical characteristics of the patients included in the study. Women with histories of infertility/RPL were significantly older than the healthy pregnant women; 22 (48.9%) of them had a history of infertility and 23 (51.1%) had a diagnosis of RPL.

TABLE 1: Demographics of the patients included in the study.

	Women with unexplained infertility or RPL ($n = 45$)	Healthy pregnant women ($n = 103$)	P
Age at inclusion in study, mean (SD)	35 (± 4.46)	30.6 (± 5.5)	0.00
Body mass index (kg/m^2) ^a , mean (SD)	28 (± 8)	27 (± 7)	0.43
Ethnicity, n (%)			
Caucasian	35 (78)	73 (71)	
African-American	1 (2.5)	11 (12)	
Hispanic	6 (13.5)	13 (13)	
Asian	2 (4.4)	1 (1)	
Missing	1 (2.2)	5 (5)	
Smokers (n , %)	11 (24)	15 (14.5)	0.15
Gravida (median, range)	1.5 (0–10)	2 (0–6)	0.18
Abortions (median, range)	1 (0–6)	0 (0–2)	0.00

^aPrepregnancy weight was used to determine BMI in healthy pregnant women.

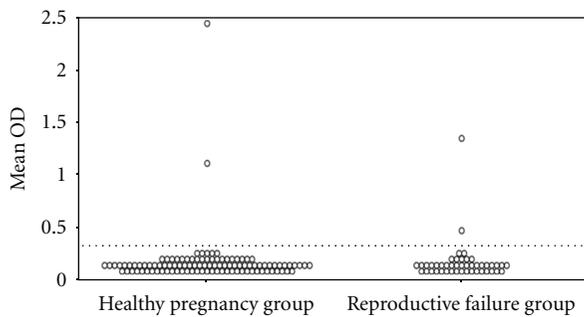


FIGURE 1: Mean OD readings of anti-PLAC1 antibodies in patients with history of infertility/RPL versus healthy pregnant women. The dotted line shows the mean OD + 4SD of healthy pregnant women.

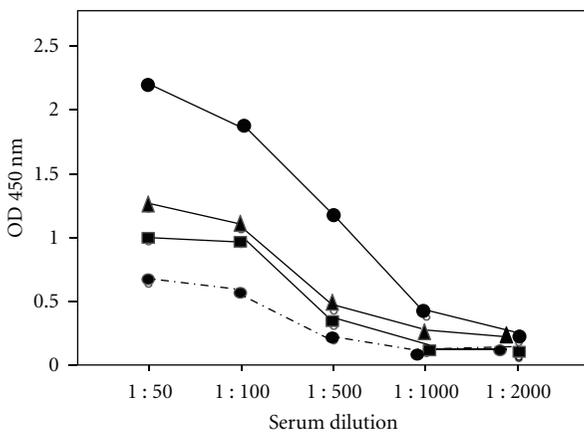


FIGURE 2: Antibody titer of positive serum samples.

Anti-PLAC1 autoantibodies were detected in 2% ($n = 2$ of 103) of healthy pregnant women. By contrast, a prevalence of 4.5% ($n = 2$ of 45) was obtained for the cohort of women with a history of infertility/RPL (Figure 1). There was no statistical difference in anti-PLAC1 seropositivity between these groups ($P = 0.355$). In the 4 positive sera for anti-PLAC1 antibodies, the titers ranged from 1:50 to 1:500 (Figure 2). Based on the current data, a sample size of 1141 patients per

group would be required to demonstrate a significant two-fold increase in the prevalence of seropositivity in women with unexplained infertility/RPL. Approximately 39% (41 of 104) of healthy pregnant women had subsequent blood samples drawn during their pregnancy. Of these, 1 patient exhibited seroconversion on a second sample tested (data not shown).

Of the 5 women positive for anti-PLAC1 autoantibodies, 4 were Caucasian and 1 was African-American. Of the four cases that were positive at the initial sampling (2 normal pregnant women, 2 infertility/RPL), three had histories of prior pregnancies and/or miscarriages and one was a primigravida. Interestingly, the primigravida patient had a history of a loop electro-excision procedure (LEEP procedure) performed secondary to an abnormal papanicolaou smear raising the intriguing possibility that PLAC1 may have been ectopically expressed by dysplastic cervical epithelial cells. The patient that exhibited seroconversion on her second serum sample had two previous pregnancies, one of them associated with the intrauterine fetal demise of twins.

At the time of the submission of this manuscript, 2 of the 3 anti-PLAC1 seropositive patients in the healthy cohort of pregnant women had delivered a term infant by spontaneous vaginal delivery. The only complication reported during the pregnancy for one of them was gestational diabetes. In the group of women with a history of infertility/RPL, one of the seropositive patients became pregnant and received her prenatal care outside of the USF clinics. Complications during the pregnancy and/or delivery for women negative for anti-PLAC1 antibodies are described in Table 2. No detectable signal indicating an increased risk for adverse events emerged from these data.

4. Discussion

Reproductive failure including recurrent spontaneous abortion and infertility can result from multiple causes: chromosomal abnormalities, infection, uterine abnormalities, hormonal factors, and systemic disease. After evaluation for these causes, about 10% of all cases remain unexplained [14–17]. Recently, the search for etiologic factors

TABLE 2: Pregnancy outcomes of the women included in the study.

	Women negative for antiPLAC1 (n = 86)*	Women positive for antiPLAC1 (n = 5)
Preeclampsia	4 (5%)	0
Maternal PIH	10 (12%)	0
Preterm labor	1 (1.2%)	0
Fetal distress	3 (3.5%)	0
Chorioamnionitis	5 (6%)	0
Gestational diabetes	7 (8%)	1
IUGR	0	0
Clotting abnormalities	2 (2.4)	0

*Outcomes missing in 17 patients due to loss of follow-up or pending deliveries.

contributing to unexplained infertility and recurrent abortions has included the possible role of immune-mediated processes. Autoantibodies that appear to be associated with reproductive failure include anti-phospholipid (anti-cardiolipin, lupus anticoagulant, anti-2 glycoprotein1), anti-laminin, antibodies against nuclear antigens (ANAs), anti-prothrombin antibodies (aPTs), antisperm antibodies, and antisaccharomyces cerevisiae antibodies among others. However, these antibodies are not pathognomonic of pregnancy failure. Their role remains controversial due to differing prevalence, lack of controls, and small sample sizes among studies [18–21].

The initial observation by Silva et al. [11] that autoantibodies directed against PLAC1 expressed by human cancer also circulated in a small number of normal women without cancer raised some intriguing questions. First, it suggested that these women may have been exposed during a previous pregnancy since no antibodies were detected in the men who were part of the same control group. Secondly, and more important, it raised the question of whether or not anti-PLAC1 autoantibodies could potentially interact with PLAC1 in the trophoblast to alter placental function and adversely affect pregnancy outcome. The highly restricted expression of PLAC1 by the placenta and its potential importance for placental development make it an attractive target for autoantibodies that may have functional significance in reproduction.

Since this study began, a role for *Plac1* at the fetal-maternal interface has been confirmed by our laboratory using a mouse mutant model [22, manuscript in preparation]. Deletions of the *Plac1* gene resulted in placentomegaly and intrauterine growth retardation suggesting diminished functional capacity of the placenta. Although the *Plac1* mutants were capable of survival, they exhibited decreased viability prior to weaning. The basis for this diminished placental function (and viability) has not been determined but may be related to diminished implantation, alterations in genes important for nutrient transport, or perhaps secondary to physical constraints imposed on the labyrinth (where nutrient transport occurs) by contiguous areas of overgrowth. *Plac1* expression has been demonstrated in all

of the trophoblast lineages in the mouse including the labyrinth and in trophoblast giant cells that possess invasive characteristics and are important during the early stages of placentation. Using a breast cancer cell line, Koslowski et al. [12] demonstrated the importance of PLAC1 in modulating cell proliferation and invasion. Although there is no direct experimental evidence, the presence of anti-PLAC1 antibodies could potentially alter placentation during the initial phases of trophoblast invasion of maternal decidua, myometrium, and blood vessels. Given its expression in the labyrinth of the mouse placenta and the analogous chorionic villus syncytiotrophoblast of the human placenta, *Plac1* may also play a more direct role in nutrient and gas exchange throughout gestation.

In the current study, we detected anti-PLAC1 antibodies in 2% of healthy pregnant women early in pregnancy. This was lower than the prevalence reported by Silva et al. for their group of control, noncancer patients [11]. This could be explained by the fact that our control population consisted of a stringently selected group of pregnant women while the characteristics of the control population in Silva et al. study were selected solely on the presence or absence of cancer. Approximately 4.5% of women with infertility/RPL had detectable anti-PLAC1 autoantibodies. This suggests a trend toward a twofold increase in the prevalence of antibodies in women with documented reproductive problems compared to normal pregnant women. However, the sample size used for this report was designed to test for a fivefold difference in prevalence and therefore too small to adequately test the significance of the twofold difference observed here. Given the relatively small sample size of our study, a *post hoc* power calculation was performed that showed that our study only had a power of 9% to show a twofold difference in the prevalence rate between the groups.

In summary, we demonstrated that the prevalence of anti-PLAC1 antibodies is approximately 2% in healthy pregnant women, early in gestation. We also demonstrated, for the first time, the spontaneous development of anti-PLAC1 antibodies during pregnancy. Finally, although there is a trend towards an increased prevalence of anti-PLAC1 antibodies in women with infertility/RPL, more patients need to be studied to determine the significance of this observation. In view of the observation that *Plac1*-null mice are capable of survival [22], it is not surprising that the presence of anti-PLAC1 antibodies did not appear to be associated with dramatic adverse outcomes in our relatively small patient sample. The functional impact of these antibodies may involve specific pathways that are important for optimal placental function but not absolutely required for pregnancy maintenance. Future studies will be directed towards characterizing the PLAC1-antibody interaction. Specifically, the antigenic epitopes eliciting the antibody response will be determined for each patient to determine if they are directed against the same part of the protein and to determine if they interact with epitopes involved in specific PLAC1-protein interactions relevant to its function at the cellular level. This will permit the interaction of anti-PLAC1 antibodies with specific signaling pathways to be examined.

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