Relationship Between Fetal Loss and Serum Gonadal Hormones Level in Experimental Antiphospholipid Syndrome Mouse

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Abstract To investigate the effects of antiphospholipid antibodies on establishment of pregnancy and changes in hormones such as estradiol-17 β (E₂) and progesterone (P) levels in circulation. Hence, mice were immunized with human β 2-Glycoprotein I (β 2GPI) and the effect of these antibodies on fetuses weight, placental observation, Serum levels of P and E₂ in pregnant mice, hematological were observed. Immunization of mice with human β 2-GPI resulted in elevated levels of antiphospholipid antibodies. The experimentally induced antiphospholipid syndrome mouse showed higher rate of fetal resorption, low number of viable fetuses, and “placental abnormalities”. In these animals, serum E₂ and P levels were reduced significantly. In addition, the blood cell variation among APS induced and control mice were determined. No significant variations were observed in number of Red Blood Cell count, White Blood Cell count and Hemoglobin content, while platelet number was significantly reduced as compared to control. These results clearly demonstrate that human β 2-GPI might be involved in causing gestational failure in APS by exerting their effect on serum hormones.

Keywords Antiphospholipid syndrome · Anticardiolipin antibodies · SLE · Hormonal imbalance · Fetal loss · Recurrent pregnancy loss

Introduction

Antiphospholipid syndrome (APS) is an acquired autoimmune disease playing a crucial role in immune mediated reproductive failures in women [1, 2]. It is a phenomenon directing antibodies in blood against anionic phospholipid-protein complexes, resulting in recurrent spontaneous abortion and deep venous thrombosis [3, 4]. Many studies have shown that the anticardiolipin antibodies react with the negatively-charged cardiolipin in the presence of a 50 kDa serum co-factor, β 2-glycoprotein-I (β 2GPI) or apolipoprotein-H (APO-H). The β 2GPI has been identified as a major antigenic target for the anticardiolipin antibodies and the association between anticardiolipin antibodies and recurrent miscarriage has been documented previously [5].

Even though APS plays a major role in recurrent spontaneous abortion, its pathogenic mechanisms are unclear. A number of animal models has been developed to understand the mechanism of action of antiphospholipid antibodies in reproductive failure and demonstrating different manifestation of APS including placental dysfunction [6, 7]. So far very little is known regarding the endocrine influence in APS. Initial studies suggested that prostacyclin production was decreased by antiphospholipid antibodies, affecting vascular endothelial cells [8]. Subsequent studies have been inconsistent and the mechanism of action of these antibodies remains in controversial. Steroid hormones such as progesterone and estrogen are necessary for a healthy pregnancy and the association between the sex steroidal hormones and APS has not been clarified yet.

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Since these hormones are required for maintenance of pregnancy, a detailed inference is necessary as obstetric complications are main feature of this syndrome.

It is understood that the placental pathology has been considered an important etiology for pregnancy complication in APS [9]. Since placenta is an essential fetomaternal entity for the overall well-being of the fetus and for the secretion of some steroid hormone, it is possible to expect that levels of these hormones may also be altered. Indeed, there is a paper describing that culturing human placenta in sera from women with or without systemic lupus erythematosus (SLE) produced different pattern of estradiol (E_2), progesterone (P) and human chorionic gonadotropin (hCG). This suggests APS may change the hormonal secretion in vivo, because some of the SLE patients have antiphospholipid antibodies [10]. The precise regulation of these hormones, however, has not been completely established in APS. Hence, it would be interesting to investigate the maternal serum hormone levels. The relevant part of this hypothesis will contribute to the understanding of disease process. Therefore, this study aims at characterizing the effect of APS in reproductive failure in association with hormonal influence and blood cells of experimental APS model in mice.

Materials and Methods

Reagents

All reagents unless otherwise noted were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human serum β_2 GPI was a generous gift from Dr. Larry Chamley, Department of Obstetrics and Gynecology, University of Auckland, New Zealand.

Experimental Animals

Adult healthy female and male (10–12 weeks old) albino mice (*Mus musculus*) were used for the experiment ($n = 60$). All animals of control group ($n = 30$) and experimental group ($n = 30$) were maintained under regular periodic photoperiod (12D/12L). All procedures performed in studies involving animals were in accordance with guidelines of Institutional Animals Ethics Committee (IAEC) of the Bharathidasan University were carried out. This article does not contain any studies with human participants performed by any of the authors.

Active Immunization of β_2 GPI and Assay for Anticardiolipin Antibodies

A total of 30 healthy female mice were immunized intramuscularly with 10 μ g of β_2 GPI along with Complete

Freund's adjuvant (CFA) as described elsewhere [11]. Two weeks later, booster injections were administered, with the same amount of human β_2 GPI with incomplete Freund's adjuvant (IFA). Additional booster injections were given to experimental animals in 2 weeks interval. Control mice were maintained in parallel with Adjuvant alone. Anticardiolipin antibodies in experimental animals was assessed by enzyme linked immunosorbent assay (ELISA) based antibody titre as described previously [12]. Briefly, ELISA microtiter plates were coated with 50 μ l of cardiolipin (47 μ g/ml) in ethanol and incubated overnight and air-dried. The plates were then blocked with 200 μ l of blocking buffer containing 10 % fetal calf serum for 2 h, washed, and incubated with 50 μ l of sera diluted 1:150 in blocking buffer at 37 °C for 2 h. Each unknown sample was run in duplicate. The plates were then washed to remove unbound antibody. Microtiter plates incubated with goat anti-mouse immunoglobulin IgG, labeled with horseradish peroxidase (HRP; MERK, Germany), diluted 1:1000 in PBS for 3 h at room temperature. Following another wash, the substrate (H_2O_2 /Tetra methyl benzidine) was incubated on the plates for 10 min and then the reaction was stopped by adding 100 μ l of stop solution. Results were obtained by reading the optical density (OD) at 450 nm of the samples, caused by the cleavage of the substrate by the enzyme in a BioRad Microplate Reader Model 550 (Need company information).

Evaluation of the Pregnancy Outcome

After 4 weeks of the active immunization in experimental animals, females were allowed for mating with proven stud males. All animals were observed regularly and the day of copulation plug found was considered as day '0' of pregnancy. From the day '0', the female mice were individually housed until the termination of the experiment. On the day 11 of pregnancy, fifteen female mice were sacrificed to observe the effect on early development of fetuses and the remaining fifteen were sacrificed on the day 18 of pregnancy to deduce late pregnancy risk and placental pathology. The control animals were divided into two groups of 15 each and evaluated for the early and late gestational age risk on the day 11 and 18, respectively. The percentage index of resorption of embryos/fetus in the uteri was calculated as described previously [12, 13]. The placenta from the day 18 animals were observed for the weight, vascularity and physiological appearance variation.

Assay for Maternal Serum Steroid Hormones

Quantitative estimation of serum P and E_2 in mice was carried out by ELISA following the procedure of previously described method [14].

Hematological Observations

Blood samples were collected from mice immunized with or without human β_2 GPI by cardiac puncture. Heparinized blood was subjected to hematological observations such as hemoglobin (Hgb, g/dL), red blood cells (RBC), white blood cells (WBC) and platelets. The cells were counted using a Neubauer hemocytometer under the microscope (Carl Zeiss, Germany).

Statistical Analysis

Data are expressed as mean $\pm$ S.D. of duplicate samples. Data are analyzed by either Student's t-test or one-way analysis of variance (ANOVA) using SPSS software, version 11.5 (SPSS, Inc. Chicago, IL, USA). A probability of $p < 0.05$ was considered to be statistically significant.

Results

Pregnancy Index of Experimental Animal

Mice immunized with human β_2 GPI showed significantly higher positive titer for anticardiolipin antibodies when compared to control mice both on the day 11 and 18 of gestation (Table 1). Human β_2 GPI-immunized mice revealed significantly higher number of fetal resorption both on the day 11 and 18 of gestation, while the controls did not (Table 1, Figs. 1, 2). Similarly, as shown in Figs. 1 and 2, number of the viable implants (pups) was significantly reduced when compared to the controls (Table 1). To the contrary, there was no significant difference in weight of fetuses and placenta among the viable implants between immunized and non immunized with β_2 GPI. The

placenta from mice immunized with human β_2 GPI on the day 18 of gestation showed darker appearance (ischemic) than the normal texture found in the control placenta (Fig. 3).

Hematological Count and Hormonal Levels in Experimental APS

Hematological findings revealed that mice immunized with or without human β_2 GPI did not make any significant differences in RBC, WBC and Hgb both on the day 11 and 18 of gestation. On the other hand, mice immunized with human β_2 GPI showed significantly reduced platelets both on the day 11 and 18 of gestation when compared with controls (Table 1). Further, changes in serum hormone such as progesterone and estrogen during APS were assessed in experimental APS animals. Our findings revealed that the serum level of P in mice immunized with human β_2 GPI was significantly lower than that of control only on the day 18 of gestation, whereas the serum level of E_2 was significantly lower than that of control both on the day 11 and 18 of gestation (Table 2).

Discussion

In the current study, immunization of mice with human β_2 GPI resulted in elevated levels of anticardiolipin antibodies and in low platelet number. β_2 GPI-immunized experimental mice showed higher rate of fetal resorption, low number of viable fetuses, and placental abnormalities. These are consistent with previous reports [7, 11–13]. We found, for the first time, both serum E_2 and P levels were reduced significantly in β_2 GPI-immunized mice.

Table 1 Clinical and serological findings in mice immunized with or without human β_2 GPI

	Day 11 of gestation		Day 18 of gestation	
	β_2 GPI- immunized	Control	β_2 GPI- immunized	Control
Anticardiolipin Antibodies (OD _{450 nm})	1.459 $\pm$ 0.4**	0.043 $\pm$ 0.0	1.322 $\pm$ 0.4**	0.043 $\pm$ 0.0
Fetal resorption (%)	28.73**	0.0	36.63**	0.0
Viable implants	6.2 $\pm$ 0.7*	9.1 $\pm$ 1.2	6.4 $\pm$ 0.1*	12.1 $\pm$ 1.0
Embryo weight (g)	ND	ND	1.283 $\pm$ 0.15	1.377 $\pm$ 0.0
Placental weight (g)	ND	ND	0.1006 $\pm$ 0.9	0.0985 $\pm$ 0.0
Platelets ($10^3 \times$ cells/mm ³)	948 $\pm$ 29.6*	1248 $\pm$ 14.1	784 $\pm$ 31.1*	1178 $\pm$ 15.8
WBC (cells/ μ L)	3810 $\pm$ 659	3820 $\pm$ 774	4120 $\pm$ 997	4040 $\pm$ 644
RBC (million/ μ L)	3.64 $\pm$ 0.68	3.68 $\pm$ 0.76	3.46 $\pm$ 0.56	3.66 $\pm$ 0.58
Hb (g/dL)	11.31 $\pm$ 1.02	10.98 $\pm$ 1.42	10.34 $\pm$ 1.92	10.10 $\pm$ 1.42

Values are expressed as mean $\pm$ SD (except fetal resorption)

ND not done

* Significant at $p < 0.05$; ** Significant at $p < 0.001$

Fig. 1 A representative uterus in mice immunized with (b) or without (a) human β_2 GPI on the day 11 of gestation. **a** Uterus with fetuses from the control pregnant mice. **b** Uterus with resorbing fetuses (black arrow) and low number of fetal implants in mice immunized with human β_2 GPI

Although progesterone in mammals rises as pregnancy progresses, the decline of progesterone on the day 18 of gestation in β_2 GPI-immunized mice in the current study suggests the other possible mechanisms of pregnancy complications observed in APS except thrombosis. Previous study in humans demonstrated the decline of progesterone and pregnancy complications in women with SLE [15, 16] or rheumatoid arthritis [16], whereas no human data of progesterone in APS has been reported. Since SLE and APS are the major common interrelated autoimmune diseases associated with recurrent pregnancy loss, it is presumed to expect the similar manifestation between SLE and APS. First of all, progesterone is necessary hormone to maintain pregnancy; decline of progesterone initiates the uterine contraction to make a labor in both human and mice resulting in delivery. This hormone deficit was probably due to placental insufficiency in SLE patients [17]. It is also important to note that the placental trophoblast undergoes alteration in their histoarchitecture [18] and

Fig. 2 A representative uterus in mice immunized with (b) or without (a) human β_2 GPI on the day 18 of gestation. **a** Uterus with fetuses from the control pregnant mice. **b** Uterus with resorbed fetuses in mice immunized with human β_2 GPI

increases in apoptosis [19] in APS. As a consequence of placental insufficiency and trophoblast damage in APS [20], there would be a decrease in progesterone. In terms of an immunosuppressive properties of progesterone [21], it is likely that a subnormal or inadequate production of progesterone might affect the clinical manifestation of APS by producing much more antibodies.

The role of estrogen during the follicular phase of menstrual cycle is crucial in the development of a receptive endometrium capable of supporting implantation and maintaining early pregnancy, which is still controversial in humans [22, 23]. Estrogen is also an important sex steroid hormone produced by the conceptus between days 11 and 12 of gestation in mice which provides the initial signal for maternal recognition of pregnancy [24]. Estrogen also increases during pregnancy and is produced by the placenta to a lesser extent. Among its many other functions,

Fig. 3 A representative appearances of placenta in mice immunized with (A, A1, A2 and A3) or without (C) human β_2 GPI on the day 18 of gestation. β_2 GPI-immunized placenta were darker than that of control

Table 2 Comparison of serum hormonal levels in mice immunized with or without human β_2 GPI

Hormones	Day 11 of gestation		Day 18 of gestation	
	β_2 GPI-immunized	Control	β_2 GPI-immunized	Control
Progesterone (ng/ml)	23.19 $\pm$ 3.9 (9.30–27.26)	21.20 $\pm$ 1.8 (15.80–30.10)	44.60 $\pm$ 3.8* (28.25–59.20)	55.79 $\pm$ 2.6 (39.04–66.79)
Estradiol (pg/ml)	34.26 $\pm$ 1.7* (20.0–41.0)	45.86 $\pm$ 2.7 (32.0–49.0)	37.66 $\pm$ 3.5* (24.0–55.0)	61.60 $\pm$ 2.3 (48.0–71.0)

Data are presented as mean $\pm$ SD

Values in parenthesis are range

* Significant at $p < 0.05$

estrogen increases uterine blood flow. The present study shows moderate decreases of estrogen both on the day 11 (early) and 18 (late) of gestation in β_2 GPI-immunized mice. As far as we know, no earlier studies illustrated the role of estrogen in APS-mediated pregnancy complication. Previous reports only suggest that the patients with SLE exhibit significantly lower values of serum hormones like estrogen and progesterone [25]. From another findings in in vitro study, culturing human placenta in sera from women with SLE suppressed proliferation of placenta but produced the same vales of E_2 and P [10]. These controversial results suggest the possibility that some estrogenic compounds other than E_2 such as estrone and 16- α -hydroxy estrone may relate to the diseases [26].

It would be interesting to study the serum profile of sex steroid hormones in APS during pregnancy, as essential need for evaluating the pathogenesis of pregnancy loss. Further studies (i.e., exogenous hormone support to β_2 GPI-immunized mice or APS patients) are needed to elucidate the detailed mechanisms involved in the disease processes.

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Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interest.

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