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Study on the pathogenesis of autoimmune-type recurrent spontaneous abortion by establishing a new mouse model

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ABSTRACT

Objective: To establish a new mouse model for autoimmune-type recurrent spontaneous abortion (AI-RSA) and demonstrate the potential role of intrauterine immunization with β2GP-1-like antigen in AI-RSA, we performed an intrauterine injection of human β2GP-1 in BALB/c mice and unrelated protein, adjuvants, and normal saline (NS) as controls. The mean number of embryos implanted (MNEI), embryo loss rate (ELR), mean embryo bulk (MEB), and mean placental weight (MPW) were analyzed. Compared with the control mice, BALB/c mice injected with human β2GP-1 showed increased anti-β2GP-1 and MPW. Moreover, BALB/c mice immunized with human β2GP-1 exhibited hypercoagulability and vascular thrombus formation in the placenta. Electron microscopy confirmed the existence of platelet aggregation, mitochondrial swelling, and endothelial cell necrosis in the placentas of BALB/c mice immunized with human β2GP-1. These finding indicated that intrauterine immunization with β2GP-1 successfully induced AI-RSA in mice. Increased anti-β2GP-1 antibody could independently induce hypercoagulability, vascular endothelial injury, and vascular thrombus formation in the placenta, which led to AI-RSA.

Introduction

Anti-phospholipid antibody syndrome (APS) has been established as one of the most frequent causes of autoimmune-type recurrent spontaneous abortion (AI-RSA). APS is characterized by vascular thrombosis and/or pregnancy morbidity, in association with anti-cardiolipin antibodies (ACAs) and/or lupus anti-coagulant (LA) and/or anti-β2GP-1 antibodies [1].

Anti-β2GP-1 antibody is a biomarker for the diagnosis of APS. More than 10 studies have confirmed that an increased IgG anti-β2GP-1 antibody level is an independent risk factor for venous thrombosis and fetal loss [2–4]. "Intra-placental thrombosis" has traditionally been presumed to play a role in the fetal loss in APS patients [5], but no direct evidence has been reported. To clarify the pathogenic role of anti-β2GP-1 antibodies in both thrombosis and fetal loss, several animal models have been established for the study of APS since the 1990s [1]. In a previous study, mouse footpad immunization with human β2GP-1 resulted in elevated antibodies against negatively charged phospholipids, fetal loss, prolonged activated partial thromboplastin time (APTT), and thrombocytopenia, without evidence for thrombus formation [2]. Recently, Arad et al. found that anti-β2GP-1 antibodies from patients with APS induced arterial thrombus formation in a mouse model [3]. However, no available animal model reproduces the full range of clinical manifestations observed in the human syndrome. Most studies have shown either fetal loss or thrombosis, not both.

Herein, we immunized BALB/c mice by intrauterine injection with human β2GP-1 and included unrelated protein, adjuvants, and normal saline (NS) as controls. BALB/c mice injected with β2GP-1 developed high concentrations of anti-β2GP-1 antibodies. Moreover, they also showed both fetal loss and thrombus formation in the placenta. This study demonstrates that anti-β2GP-1 antibodies independently induce fetal resorption and thrombus formation in the placenta, which may be the primary mechanism of recurrent abortion.

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Mice

Six- to 8-week-old female BALB/c mice were purchased from the Laboratory Animal Center of Shanghai, The Chinese Academy of Science. Genetic monitoring confirmed that the mice were up to the international standard of quality. The study was approved by the Institutional Review Board of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

Intrauterine injection method

A self-designed injection syringe was used for intra-uterine injection. The TIP (Axygen, USA) was heated for 3–5 s using a Spirit Lamp, and the melted tips were then elongated to 3 cm and a diameter of 1 mm (Fig. 1A). We termed it an intrauterine injection syringe when it was attached to a 1 ml injection syringe. Using this intrauterine injection syringe, the antigen and the control formulations were injected into the uterine cavity through the vagina and cervix (Fig. 1B). Dye injection was first used to test this injection method. We observed that the uterus and not the peritoneal tissue filled with dye when the needle was manipulated gently (Fig. 1C).

Immunization

Fifteen BALB/c female mice were immunized in the uterine cavity with 10 μg of human β2GP-1 (Cell Sciences, USA) mixed with CFA (Sigma, USA). One week later, the mice were immunized again with 10 μg of human β2GP-1 in IFA (Sigma, USA). The unrelated protein and adjuvants were injected into the uterine cavity as controls (6–15 mice/group). Mice immunized with β2GP-1 were observed in four replicate groups. Ten days after active immunization, BALB/c female mice were paired overnight with males. Mating was evidenced by the appearance of a vaginal plug the following morning.

Evaluation of pregnancy outcome

All plugged females were sacrificed by cervical dislocation on days 12–14, the uteri were removed, and the total number of implantations and resorption sites were recorded, as described by Bertoja et al. [4]. The MNEI was calculated as the number of total fetuses divided by the total number of mice. The ELR was calculated as the number of resorbed fetuses divided by the number of resorbed and full-term fetuses. The MPW (g) was calculated as the total placenta weight divided by the number of full-term fetuses. The MEB was calculated using the following formula: MEB (mm^3) = 0.5 × length-diameter × short $diameter^2$.

ELISA

Blood samples were collected on day 14, and the serum levels of ACA and anti-β2GP-1 antibodies were measured by ELISA. The ACA ELISA (EUROIMMUN, Germany) was performed according to the manufacturer's protocol, The intra- and inter-assay coefficients of variation are respectively 7.5% and 10.5%. The minimum detectable amount of the assayed substance is 2 U/ml. Anti-β2GP-1 activity was detected by a solid-phase ELISA similar to a previous report [4]. Plates were coated with purified β2GP-1 (5 μg/ml) in PBS overnight. The coated plates were then blocked with 2% BSA and incubated with serial dilutions of test serum. After washing, the plates were incubated with a diluted goat anti-mouse IgG HRP conjugate (1/5000) and developed with 1 mg/ml p-nitrophenyl-phosphate in diethanolamine buffer for 15–20 min. The intra- and inter-assay coefficients of variation are respectively 8.0% and 11.3%, the minimum detectable amount of the assayed substance is 2 U/ml.

Blood cell counts and coagulation studies

Platelets from mouse blood samples were counted using the Sysmex XT-1800i Automated Hematology Analyzer. Anticoagulant activity was evaluated by the APTT, prothrombin time (PT), and fibrinogen (FIB) level obtained using the Sysmex CA-7000 Automated Blood Coagulation Analyzer.

Placental pathology

Pathological changes in the placenta were observed by H&E staining and electron microscopy (PHILIP CM-120). The presence of thrombus was evaluated independently by two pathologists (Dr. Yao and Dr. Yan).

Statistical analysis

Data are expressed as the mean ± SD. ANOVA followed by post hoc test was carried out for the statistical analysis. The p values < 0.05 were considered statistically significant.

Characterization of serological features of BALB/c mice following intrauterine injection

Six- to eight-week-old female BALB/c mice were immunized with β2GP-1 via intrauterine injection. Fourteen days after the injection, we examined the concentrations of anti-β2GP-1 antibody and ACAs in the serum from mice in the CFA/β2GP-1 group and the control group. The concentration of anti-β2GP-1 antibodies in the CFA/β2GP-1 group was significantly higher compared to the control group ($p < 0.05$), whereas there were no significant differences in the ACA concentrations among all groups ($p > 0.05$) (Fig. 1D). These results indicated that intrauterine injection successfully induced a high anti-β2GP-1 antibody concentration in BALB/c mice.

Intrauterine injection with human β2GP-1 leads to a high rate of abortion

To identify the effect of increased anti-β2GP-1 antibody on embryo resorption, all pregnant mice were sacrificed 12–14 days after plug formation, and the embryos and the placenta were further analyzed. We found that the rate of resorption (23.36%) in the CFA/β2GP-1 group was significantly higher compared to the control groups (4.23–10.19%). Moreover, BALB/c mice immunized with 10 μg of β2GP-1 developed a significantly lighter and smaller placenta and embryos compared to the CFA/BSA, CFA, and NS groups ($p < 0.05$) (Fig. 1E). Representative resorbed fetuses are shown in Fig. 1E. No significant difference in the MNEI was observed among all groups (Table 1). These results suggested that the intrauterine injection of β2GP-1 induced fetal resorption and had no obvious effect on fertility or embryo implantation. Interestingly, a Pearson correlation analysis demonstrated no significant correlation between the anti-β2GP-1 antibody concentration and the rate of embryo loss in the CFA/β2GP-1 group ($r = 0.369$, $p = 0.176$).

Coagulation state

Previous reports on the effect of anti-β2GP-1 antibodies on PT, FIB, and platelet count were controversial [2,3]. In our intrauterine injection mouse model, we found that pregnant mice injected with β2GP-1 had a higher level of FIB ($p < 0.05$) (Table 2). However, the PT, APTT and platelet count did not significantly differ between β2GP-1-immunized mice and the other groups (Table 2).