Detection of antizona pellucida antibodies in the sera from premature ovarian failure patients by a highly specific test

Satoru Takamizawa, M.D., Hiroaki Shibahara, M.D., Ph.D., Tamaho Shibayama, M.D., and Mitsuaki Suzuki, M.D., Ph.D.

Department of Obstetrics and Gynecology, School of Medicine, Jichi Medical University, Tochigi, Japan

Objective: To develop a highly specific test for the detection of antizona pellucida (ZP) antibodies in the sera from premature ovarian failure (POF) patients.
Design: Laboratory study.
Setting: University hospital.
Patient(s): Twenty-seven idiopathic POF patients, 30 control women, and 30 healthy males.
Intervention(s): Anti-ZP antibodies were detected by the microdot assay using a very small amount of human ZP or porcine ZP. The effect of anti-ZP antibodies on sperm-ZP binding was examined by hemizona assay.
Main outcome measure(s): Results from the microdot assay and hemizona assay.
Result(s): By the microdot assay using human ZP, the sera from POF patients reacted significantly stronger than those of control women and healthy males. However, no obvious difference could be found by the same assay using porcine ZP among these three groups. Anti-ZP antibodies against sera from some POF patients showed significant blocking effects on sperm-ZP binding assessed by hemizona assay. Anti-ZP antibodies were detected in 7 of 27 POF patients, while none were detected in control women and healthy males.
Conclusion(s): Some idiopathic POF patients have anti-ZP antibodies in their sera, which were detected with high specificity by a newly developed microdot assay using a very small amount of human ZP. (Fertil Steril® 2007;88:925–32. ©2007 by American Society for Reproductive Medicine.)

Key Words: Antizona pellucida (ZP) antibody, human ZP, premature ovarian failure, sperm-ZP binding

Premature ovarian failure (POF) is a syndrome characterized by development of amenorrhea before the age of 40 years with elevated serum gonadotropin levels and low serum estrogen levels (1). It occurs in 1% of women, in 10%–28% of women with primary amenorrhea, and in 4%–18% of those with secondary amenorrhea (2, 3).

The etiology of POF is thought to involve a wide spectrum of pathogenic mechanisms including chromosomal, genetic, environmental (radiation or medicine), metabolic, and autoimmune factors (4). In the past 3 decades, some investigators have reported the clinical association of POF with other autoimmune disorders. Approximately 20%–40% of patients with POF have associated autoimmune disorders (4–7). In a large proportion of cases no cause has been identified, and these cases are classified as idiopathic POF. Autoimmune mechanisms are involved in the pathogenesis of up to 30% of cases in idiopathic POF (8, 9).

The presence of organ-specific autoimmune antibodies may support the role of autoimmune mechanisms in endocrine diseases. For example, antiovarian antibodies in patients with POF were detected by different methods, the most common being indirect immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA). Numerous investigators have tested POF patient groups using IF, ELISA, and other methods. The prevalence of antiovarian antibodies ranges widely, between 2.2% and 69% of patients (10–16). Considering these variable results, the pathogenic roles of antiovarian antibodies in POF are still uncertain in terms of their specificity. Such conflicting results could be explained by the methodologic differences, relatively small number of patients, the different stages of the disease when tested, the differences of antigen sources, and the multiplicity of potential immune targets that comprised various steroidogenic enzymes, gonadotropins, and their receptors: the corpus luteum, oocyte, and zona pellucida (ZP) (9, 17).

Zona pellucida consists of glycoproteins that have strong antigenic potency. Therefore, anti-ZP antibodies may be one of the causes of autoimmune POF. Evidence showing that antibodies directed to ZP could cause POF has been demonstrated. Ovarian failure could be induced in rabbits immunized with porcine ZP proteins (18). Ovarian autoimmune disease was induced in B6AF1 mice by 15-amino acid peptides from mouse ZP3 (19). Because ZP proteins are conserved among mammals (mouse and human ZP3 proteins are 67% identical), those animal models may lead to a better understanding of the pathogenesis of human autoimmune oophoritis. Circulating antibodies against ZP were consistently detected by immunohistochemical assay in mice with autoimmune oophoritis that could be induced with high incidence by thymectomy at 3 days of age and caused great oocyte loss

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Reprint requests: Hiroaki Shibahara, M.D., Ph.D., Department of Obstetrics and Gynecology, School of Medicine, Jichi Medical University, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan (FAX: 81-285-44-8505; E-mail: sibahara@jichi.ac.jp).

(20, 21). Smith and Hosid (22) reported two cases of POF associated with antibodies directed against ZP .

After antigenic crossreactivity between human and porcine ZP was revealed (23, 24), porcine ovaries and oocytes have been generally used to detect anti-ZP antibodies because they are easy to obtain in large quantities. Anti-ZP antibodies have also been discussed as a possible cause of infertility in women for the blocking effect on sperm-ZP binding. Some studies reported that anti-ZP antibodies were detected with high incidence in infertile women by IF using porcine oocytes and ZP (24–26). However, the specificity of immune reaction with porcine oocytes has been questioned because of the nonspecific reaction of porcine ZP. To have better specificity in the detection of anti-ZP antibodies using porcine ZP, passive hemagglutination reaction or absorptive treatment techniques were developed (27–29). Although such passive hemagglutination reaction or absorption techniques contributed to better specificity and reliability for detecting anti-ZP antibodies, the significance of these antibodies remains unclear because of possible nonspecific reactions.

Therefore, we concluded that it is necessary to develop a highly specific test for the detection of anti-ZP antibodies using human ZP. However, one limitation is that it is still difficult to obtain human ZP in large quantities, even in the era of modern assisted reproductive technology. In the present study, a microdot assay using a very small amount of human ZP was developed. Moreover, sera containing anti-ZP antibodies from patients with idiopathic POF patients were used to investigate the blocking effect on sperm-ZP binding.

MATERIALS AND METHODS

Approval for the study was obtained from the Institutional Ethics Committee of Jichi Medical University Hospital, and informed consent was obtained from all patients.

Serum Samples

Serum samples were collected from 27 women diagnosed with idiopathic POF. Criteria for POF included secondary amenorrhea, age <40 years at the onset of ovarian failure, persistent high serum gonadotropin levels, and low serum estrogen levels (1). In addition, to classify as idiopathic POF, patients with abnormal karyotype, previous pelvic irradiation, operative castration, and previous cytotoxic chemotherapy were excluded, and patients with no cause identified were selected (8).

As the control, sera from 30 fertile females without disorder on ovulation and fertilization were obtained. Women with regular ovulatory cycle and experience of conception, who had undergone at least two cycles of IVF treatment with total number of oocytes retrieved >10 and 100% fertilization rate in their IVF cycles, were defined as the control women. Sera from 30 normally healthy volunteer males were also obtained as the negative control. Because anti-ZP antibodies were autoimmune antibodies, there was a possibility that normal cycling women had these antibodies. On the other hand, healthy males were not expected to have these antibodies logically and suitable for the true negative control.

Whole serum was diluted 1:4 in phosphate-buffered saline (PBS) without calcium containing 3% bovine serum albumin as the test serum sample.

Preparation of Human and Porcine ZP

Under informed consent, human oocytes that failed to fertilize in vitro or that were not used because of being immature were obtained from infertile couples that had undergone assisted reproductive technology. They were stored until use at 4°C in a solution of 0.5 M ammonium sulfate with 1 M magnesium chloride and 0.1% dextran. After hundreds of human oocytes were stored, they were put to use.

Porcine ovaries obtained from a local slaughterhouse were frozen and stored. The porcine oocytes were collected from thawed porcine ovaries by aspiration using syringes and needles. Several sets of ten oocytes from one ovary could be collected at one time.

Human and porcine ZP were individually separated from their cytoplasm mechanically by pipetting with a narrow glass pipette with a caliber smaller than the oocyte diameter. They were then placed in PBS (100 μg/mL = 100 ZP/30 μL PBS) at 75°C for 30 minutes.

Microdot Assay

The microdot assay is an immunostaining method, and anti-ZP antibodies in the sera can be detected when combined with soluble ZP proteins used for antigen.

A small piece of nitrocellulose membrane measuring 1.5 cm in width and 1.0 cm in length was divided into six areas with two vertical lines and one horizontal line. On each of the upper panels, a microdot was made with serial dilution ($\times$1, $\times$3, $\times$9) of 0.2 μL soluble human ZP, while on that of the lower panels, it was made with soluble porcine ZP. Approximately 0.7 ZP, 0.2 ZP, and 0.07 ZP were contained in one dot of 0.2 μL soluble human and porcine ZP with serial dilution $\times$1, $\times$3, and $\times$9, respectively. After drying, they were blocked in PBS containing 3% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 10 minutes. As a primary antibody reaction, 180 μL of the test serum was added on the nitrocellulose membrane. After incubation of the nitrocellulose membranes with patients' sera in a moist chamber at 4°C overnight, they were washed three times in PBS containing 0.02% Tween 20 (Kanto Chemical Co., Inc., Tokyo, Japan, 40350-32, polyoxyethylenesorbitan monolaurate, nonionic detergent) for 5 minutes. The excess PBS containing 0.02% Tween 20 was aspirated off, then the nitrocellulose membranes were incubated in horseradish peroxidase-conjugated antihuman IgG (Sigma Chemical Co.) diluted 1:1,000 in PBS as a second reaction at room temperature for 1 hour. Then, they were washed three times in PBS containing 0.005% Tween 20 for 5 minutes. The dots on the nitrocellulose membranes were colored and visualized by chloronaphthol, 3 mg of which was dissolved in 1 mL of methanol with 5 mL of PBS and 2 μL of H_2O_2 (Kanto