A block in the road to fertility: autoantibodies to heat-shock protein 90-β in human ovarian autoimmunity

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Objective: To report autoantibodies to human heat-shock protein 90-β (HSP90β) in sera of women with infertility.
Design: Prospective, controlled observations.
Setting: Major urban infertility referral center and research institution.
Patient(s): Fifty women with premature ovarian failure, 65 infertile women enrolled in the in vitro fertilization–embryo transfer program, and 60 normally menstruating fertile women as controls.
Intervention(s): None.
Main Outcome Measure(s): Identification and complete characterization of a 90-kd protein, the most immunodominant autoantigen.
Result(s): Our previous studies employing a novel blocking demonstrated several cellular and molecular ovarian antigenic targets using patient's serum. Of all these antigens, the 90-kd protein designated as EP90 was found to be conserved across species, was serine–threonine phosphorylated, and was expressed from the primordial stage to the graafian-stage ooplasm of the oocytes during follicular development. Using high-throughput proteomic technologies like liquid chromatography/mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF), and tandem mass spectrometry analysis revealed the identity of this protein to be HSP90β. Commercially available recombinant protein immunoreacted with the sera from patients with anti-ovarian antibodies against the 90-kd antigen. In parallel, using monoclonal antibody to human HSP90, we found that it reacts with the eluted protein from a crude ovarian extract.
Conclusion(s): This is the first report to show the presence of ovarian autoantibodies to human HSP90 in sera of women with infertility. This protein could be involved in human ovarian autoimmunity and thereby be a causative factor in early ovarian failure. (Fertil Steril® 2009;92:1395–409. ©2009 by American Society for Reproductive Medicine.)

Key Words: Ovarian autoimmunity, POF, IVF-ET, immunodominant antigens, autoantibodies, heat-shock protein 90

Premature ovarian failure (POF) is the cessation of ovarian function after the onset of puberty and before the age of 40 years in women with hypergonadotropism (1). Classic POF has a genetic, enzymatic, infectious, or iatrogenic etiology (2, 3); however, in some cases no clear-cut cause can be identified, and these are referred to as idiopathic. Among this group of POF patients, autoimmunity of the ovary and the presence of serum antiovarian antibodies (AOA) is a well-established phenomenon, and in some cases AOA has been considered to be a suitable marker for identification of the immunologic mechanisms involved in autoimmune premature ovarian failure (AI-POF) (4–6). Antiovarian antibodies have been speculated to be factors contributing not only to POF but also to infertility and in vitro fertilization and embryo transfer (IVF-ET) failures (7, 8). These AOA could affect egg development, embryo development, and potentially implantation failures. In some individuals, AOA appear after follicular aspiration; in others, preexisting AOA levels are seen to increase with the number of IVF attempts (7). There are reports showing a higher prevalence of these antibodies in patients with IVF failures than in those with IVF success (9).

Very little is known about the precise nature of the ovarian antigens that are recognized by the antibodies in sera. Identification and characterization of target antigens are prerequisites for elucidation of the underlying immunologic mechanisms and also for devising better approaches for the diagnosis and treatment of ovarian failure leading to infertility. Antigens of oocyte (10), corpus luteum (11), granulosa cells (12), and zona pellucida (13) have been reported to act as autoantigens; however, their pathophysiologic significance remains obscure. The oocyte seems to be the most often targeted cell of AOA detected in cases of ovarian diseases as well as in women with poor assisted reproductive technologies (ART) outcomes (14, 15). Maternal Antigen That Embryos Require (MATER) is one of the candidates among several antigenic targets identified by use of a mouse model for POF (16) or in the sera of patients (14). Although

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preliminary studies indicated that MATER was the most likely candidate, no further reports of its involvement in human ovarian autoimmunity are available.

In addition to MATER, a recent report described another autoantigen, α-enolase, to be a target antigen in patients with autoimmune POF (AI-POF) (17). However, nothing is known about its cell-specific immunolocalization, and no further characteristics of α-enolase have been reported to date. Although 19.1% of that study group was shown to have antibodies against α-enolase, other independent studies have established the involvement of multiple antigens in the development of autoimmune disease (15, 18).

A thorough literature review on infertility with autoimmune involvement has indicated that very few proteins have been formally identified and characterized. Therefore, it would be very useful to identify and characterize these molecules. Once their identity has been established, they could be used for simple, noninvasive diagnostic tests to screen large populations of women with infertility or repeated implantation failures as well as to screen patients before and after enrollment in an IVF-ET program. It is essential to identify and systematically characterize the multiple autoantigenic targets to understand the pathogenesis of the disease (19).

An earlier report from our group showed that 15 out of 50 (30%) and 13 out of 50 (26%) in the POF and IVF-ET groups, respectively, were positive for AOA by Western blot analysis using our novel blocking protocol (15). We further reported in the same study that sera from these women reacted with antigens spanning molecular masses in the range of approximately 30 to 150 kd. Further analysis of the data indicated that a 90-kd protein (EP90) is the predominant antigenic target. In this study, we report the molecular identity and immunochemical characterization of this immunodominant 90-kd molecule.

MATERIALS AND METHODS

Patients

Our study was approved by the institute's clinical ethics review committee. All participants (patients and controls) gave written informed consent for participating in the study. Blood samples were collected to obtain sera from women with POF (n = 50) and from women registered for the IVF-ET program (n = 65) who had been unable to conceive after 1 year of unprotected intercourse and for whom IVF has been determined to be the next treatment. In IVF-ET cases, the blood samples were collected before ovarian hormonal stimulation.

The inclusion and exclusion criteria for patient selection were described previously elsewhere (15). None of the patients had a history of any other autoimmune disease. Regularly menstruating proven fertile women (n = 60) with a median age of 28 years were also enrolled in the study to serve as controls; they had no evidence of any autoimmune disease or fertility problems. Neither the patients nor the controls had received any medical treatment before or during the study.

All the patients and controls enrolled in this study were screened for AOA in their sera by Western blot analysis then by immunohistochemical analysis using rat ovarian protein extracts and sections, respectively. However, for characterization of the target antigen, 10 randomly picked sera from the patient population as well as from the controls were used for further investigations when sufficient volume of sera was available and when good immunoreactivity was seen by Western blot and immunohistochemical analyses.

Animals

Holtzman female rats (aged from day 0 to day 120) and adult Swiss mice were housed in a temperature-controlled room with a 12-hour light cycle. Normal adult female rabbit ovaries were collected under the supervision of a veterinarian. The animals were provided with food and water ad libitum. All animal care practices and experimental procedures complied with the guidelines of the Care and Prevention Society against Cruelty of Experimental Animals (CPSCEA) on animal care, and the study was approved by the institutional animal ethics committee. Ovarian tissue of porcine origin was obtained from the local abattoir and was transported to the laboratory under sterile and chilled conditions.

Preparation of Sample and Tissue Homogenization

Ovarian tissues from rat and pig were resuspended in lysis buffer containing 1% sodium dodecyl sulfate (SDS) in MilliQ water with 0.2% (3-[3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS; Sisco Research Laboratories, Mumbai, Maharashtra, India) and a mixture of protease cocktail inhibitor (Roche Diagnostics GmbH. Mannheim. Germany). Tissue was homogenized on ice using a mechanical homogenizer and centrifuged at 12,000 × *g* for 30 minutes. Supernatant was sonicated for approximately 30 seconds on ice. The protein concentration was estimated by the modified Folin-Lowry protocol (20). Normal human ovarian tissue was obtained commercially from BD-Clontech (Mountain View, CA). This ovarian extract was prepared from the ovaries of a 30-year-old, normal woman who had died from trauma, as stated by the manufacturers.

Immunohistochemical Localization

Ovarian tissues were fixed and processed for immunohistochemistry as per the protocol previously described elsewhere (15, 21). Sections were blocked with our novel blocking recipe (nonfat dry milk in phosphate-buffered saline, pH 7.4 [NFDM-PBS], containing 20% rabbit polyclonal antialbumin antibodies [AAA]), for 2 hours at room temperature. Sera from all controls and patients undiluted or diluted to 1:5 with 2.5 g% NFDM-PBS were added on the slide and incubated at 4°C overnight in a humid chamber. The section serving as the negative control was incubated with 2.5 g% NFDM in PBS. The following day, slides were washed with PBS