Follicular dysfunction induced by autoimmunity to zona pellucida

Koji Koyama, Akiko Hasegawa, Nahoko Mochida, Gunena Calongos

Department of Obstetrics and Gynecology, Hyogo College of Medicine,
Laboratory of Developmental Biology and Reproduction, Institute for Advanced Medical Sciences, Hyogo College of Medicine

Received: 10 October 2005; accepted: 2 November 2005

SUMMARY

The mammalian zona pellucida is an extracellular matrix that occurs in growing oocytes, ovulated eggs and pre-implantation embryos, and is known to be involved in several important events during ovarian folliculogenesis and fertilization. Since the zona pellucida is formed at an early stage of oocyte growth, circulating antibodies against zona pellucida may impair ovarian function. In this article we discuss whether anti-zona antibodies cause ovarian dysfunction and infertility. The discussion is based on clinical examination and animal experiments including the following approaches: 1/ immunological method using solubilized human zona pellucida detected anti-zona antibody with a high frequency in infertile patients, especially premature ovarian failure syndrome; 2/ in vivo experiment using hamsters showed that some, but not all, animals

---

2 Corresponding author: Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Japan, 663-8501, e-mail: kkoyma@hyo-med.ac.jp

Copyright © 2005 by the Society for Biology of Reproduction
experienced ovarian failure after immunization with hamster recombinant zona proteins; 3/ in vitro experiment using mouse isolated ovarian follicles showed significant inhibitory effects on follicular growth and oocyte development. We concluded that anti-zona antibody may be involved in causing ovarian failure. Reproductive Biology 2005 5 (3):269-278.

**Key words**: anti-zona antibody; premature ovarian failure; follicle culture; infertility

### INTRODUCTION

The mammalian zona pellucida, an extra-cellular matrix surrounding the oocyte, has been extensively studied for its role in fertilization and folliculogenesis [2, 20, 21]. It basically consists of three glycoproteins called ZPA, ZPB and ZPC for which the \(zpA\), \(zpB\) and \(zpC\) genes code respectively in various mammalian species. Recently, the \(zpB'\) gene coding for a fourth zona pellucida protein has been found in the human zona pellucida [11].

Since the zona pellucida has been shown to possess a strong immunogenicity, a number of attempts have been made to develop a contraceptive vaccine using zona pellucida antigens. However, active immunizations with native zona antigens have been found to cause ovarian dysfunction, such as disorder of estrous cycles and histological ovarian disruption [14, 17, 19]. In our experiments, immunization with porcine native zona antigen caused severe auto-immune oophoritis with follicle-like cell clusters in rabbits and hamsters [6]. Figure 1 shows an ovarian section from a hamster immunized with solubilized pig zona pellucida.

The ovarian status of immunized animals is similar to that of premature ovarian-failure (POF) patients [12, 14]. POF occurs in approximately 1% of women of reproductive age. This syndrome is characterized by amenorrhea due to cessation of ovarian function before age 40 associated with a high FSH level. Antibodies against the zona pellucida may interfere with oocyte maturation and follicular development because some zona components are expressed in the early stages of follicular development in
human follicles [5]. Bidirectional communications which traverse the zona pellucida between oocyte and granulosa cells through microvilli and gap junctions are essential for oocyte and follicular development [1]. It is still controversial whether the anti-zona antibody detected in some infertile women is actually pathological in POF patients. This article describes the relationship between anti-zona antibodies and ovarian dysfunction and the influence of the antibodies on follicle growth in our animal experimental systems in vivo and in vitro.

DETECTION OF ANTI-ZONA PELLUCIDA ANTIBODIES IN INFERTILE PATIENTS

Sera from patients with POF often contain anti-ovarian antibodies including anti-zona antibodies [3, 10, 13]. Since antibodies against pig zona pellucida were shown to cross-react with human zona pellucida [8, 16], pig zona pellucida has been used for detecting of anti-zona antibodies in infertile patients. Immunofluorescent tests [18], passive hemagglutination tests [9] and enzyme-linked immunosorbent assay [7] have been developed to detect anti-zona antibodies in patients’ sera, but the positive results obtained by these means do not always correlate to infertility. Indeed, the biochemical
and immunological properties of pig and human zona pellucida are not identical. The human zona pellucida contains antigens that are not present in pig zona pellucida. Consequently, pig zona antigens can generate false negative results in the detection of anti-zona antibodies in humans. Moreover, as natural antibodies sometimes react with heterologous animal antigens, it is difficult to exclude nonspecific antibodies bound to pig zona antigens.

On the basis of these observations, we conducted experiments to detect anti-zona auto-antibodies by dot immunoassay using human zona pellucida. The human zona pellucida was collected by pipetting from unfertilized oocytes in a clinical program of IVF-ET under informed consent. The preparation was solubilized at 70°C for 30 min in phosphate buffer saline (PBS). One µl containing 5 µg of protein was dotted onto a nitrocellulose membrane. After blocking with bovine serum albumin (BSA) in PBS, the membrane was incubated with the patients’ sera (1:3 dilution) and subsequently treated with an anti-human IgG antibody conjugated with horseradish peroxidase. Color development was performed by chloronaphthol and H₂O₂. Densitometric values showing more than twice the SD values of the control group were assessed as positive. We found that 5 of 10 POF patients and 2 of 17 infertile women were positive for anti-zona antibodies. It is therefore concluded that anti-zona antibodies are involved in causing POF.

**IN VIVO EXPERIMENT**

This experiment was conducted to examine whether isologous zona pellucida antigens could disrupt normal ovarian function in hamsters (*Mesocricetus auratus*). Recombinant proteins of hamZPA (583 amino acids) and hamZPC (325 amino acids) were used for immunization. To isolate cDNA encoding for hamZPA, a degenerate RT-PCR technique was adopted. Primers were designed from highly similar regions already reported in various mammals and mRNA was prepared from 3-week-old hamster ovaries. Hamster cDNA for ZPA was cloned in our laboratory
(NCBI accession #AY876920). A recombinant hamZPA protein (rec-hamZPA) was produced in *Escherichia coli* using the pET21 system. cDNA coding for hamZPC was isolated by RT-PCR using primers designed from the NCBI database (gi:191389) and used for construction of a recombinant protein expression vector. Hamsters were immunized by subcutaneous injection of the antigen (500 µg/injection) with Freund’s adjuvant 4 times at 2-week-intervals. Ovaries from the immunized hamsters were removed for histological examination at ten weeks after the initial immunization.

*Figure 2.* Detection of antibodies on the zona pellucida in immunized hamsters. Frozen sections prepared from hamsters immunized a/ with adjuvant only, b/ recombinant hamZPA and c/ hamZPC were incubated in FITC-conjugated anti-hamster IgG and observed under a fluorescent microscope. In the two latter sections (b, c) the zona pellucida showed positive reactions; bar: 50 µm.

(Figure 3. Histological examination of ovarian sections from hamsters immunized with recombinant b/ hamZPA and c/ hamZPC proteins. Significant decreases, compared with a/ control section, in the number of follicles were observed in the sections b/ and c/. Hemorrhage (big arrow) and many atretic follicles (small arrows) were detected in section b and c, respectively; bar: 50 µm.)
Antibodies reactive to isologous recombinant antigens were produced in both the hamZPA and hamZPC-immunized hamsters. These antibodies were found to be present in the ovarian zona pellucida in immunized animals (fig. 2). Two of five animals immunized with hamZPA showed a disrupted estrus cycle. Significant decreases in the number of follicles at every stage of development including primordial follicles were observed by histological examination (fig. 3b). Similarly, decreases in the number of normal follicles and increases in the number of atretic follicles were observed in the hamZPC-immunized group (fig. 3c).

Immunization of hamsters with the isologous recombinant protein resulted in the production of self-reactive antibodies that were bound to their own zona pellucida. The antigen-antibody complex probably induced ovarian dysfunction by impairing the communication between oocyte and granulosa cells, thereby leading to POF. Other researchers have also reported induction of ovarian dysfunction by immunization with recombinant ZPA and ZPC proteins in both heteroimmunization [14, 19] and isoimmunization [4, 15]. Taken together, these findings support a hypothesis that the zona pellucida contains pathogenic antigens that induce POF and subsequently cause infertility. However, another factor(s) may be also involved in inducing ovarian failure.

**IN VITRO EXPERIMENTS USING OOCYTE-GRANULOSA CELL COMPLEXES (OGCS)**

In this section, we discuss the influence of anti-zona antibodies on the growth, maturation and fertilization abilities of oocytes *in vitro*. The protocols used are shown in fig. 4. In experiment 1, the oocyte maturation and fertilizing ability were evaluated using OGCs. In experiment 2, the development of preantral follicles into antral follicles was evaluated using intact follicles.

Antiserum was prepared from rabbits immunized with synthetic peptides of mouse ZPA (TDVRYKDDMYHFFPAIQAC: 121-139) conjugated diphtheria toxoid. In experiment 1, OGCs were collected from 16-day-old mice by mechanical and enzymatic treatments and incubated at
10% antiseraum for six days. Normal rabbit serum was used for a control group. After the in vitro growth culture, the OGCs were transferred to the maturation medium without antisera to assess oocyte maturation, and then the oocytes were subjected to in vitro fertilization.

There were no significant differences in morphological appearance of grown OGCs at the optical microscopic level between the control and antibody-treated groups. However, after in vitro maturation, the OGCs-mucification and oocyte-maturation rates in the antibody-treated group were significantly lower than those of the control group. The oocyte fertilization rate was also significantly lower in the antibody-treated group compared with the control (tab. 1).

Similarly, in experiment 2, antral formation from preantral follicles during follicle culture was interfered with anti-ZPA antibody (tab. 2). These results indicated that antibodies to the zona pellucida inhibited not only the growth and maturation of
Table 1. Effects of anti-ZPA synthetic peptide antiserum on in vitro culture of oocyte- granulosa cell complexes (OGCs)

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Used OGCs</th>
<th>Mucified OGCs (%)</th>
<th>Matured oocytes</th>
<th>Fertilized oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120</td>
<td>62 (51.7%)</td>
<td>54 (45.0%)</td>
<td>43 /54 (79.6%)</td>
</tr>
<tr>
<td>Anti-ZPA</td>
<td>111</td>
<td>15 (13.5%)*</td>
<td>14 (12.6%)*</td>
<td>2 /14 (14.3%)*</td>
</tr>
</tbody>
</table>

*p<0.01 (compared with control, \(\chi^2\)-test)

Table 2. Effect of anti-ZPA synthetic peptide antiserum on in vitro culture of intact preantral follicles

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Used follicles</th>
<th>Antral formation</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47</td>
<td>20</td>
<td>42.6</td>
</tr>
<tr>
<td>Anti-ZPA</td>
<td>47</td>
<td>11</td>
<td>23.4*</td>
</tr>
</tbody>
</table>

*p<0.05 (compared with control, \(\chi^2\)-test)

oocytes (and subsequently interfered with in vitro fertilization) but also follicular growth. It is possible that if anti-zona antibodies are present in the follicular fluid, both, oocyte growth and follicular development may be impaired.

CONCLUSIONS

The zona pellucida is important for oocyte growth and follicular development. Anti-zona antibodies caused ovarian dysfunction through the impairment of oocyte and follicular development in the animal experiments. The present results suggest that we must consider the adverse effect of auto-immunity to zona pellucida on ovarian function as well as on fertilization.
ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid of Scientific Research (No. 14370536) from MEXT (Ministry of Education, Science, Culture, Sports and Technology), 2002-2004 and by the “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT, 2004-2008. The authors appreciate the useful conversation with our colleagues and technical assistances of the laboratory staff.

REFERENCES

Anti-zona antibody and follicular dysfunction


