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Specific Binding of $17D_3$ Anti-pZP mAb to Porcine and Human Zona Pellucida but not to Other Tissue Antigens Identified by ABC Immunohistochemistry

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Objective *To characterize the binding effects of $17D_3$ anti-pZP monoclonal antibody (mAb) on porcine and human ZP, ovary and other important tissues*
Methods *The $17D_3$ anti-pZP mAb was produced by immunizing mouse with porcine zona pellucida and hybridoma and monoclonal antibody preparation. An ABC immunohistochemistry was used to evaluate reaction of mAb $17D_3$pZP to porcine and human ZP antigens as well as important human tissue antigens.*
Results *mAb $17D_3$pZP specifically bound to porcine and human ZP antigen, but not to other cells of ovaries and other important human tissue antigens.*
Conclusion *$17D_3$ anti-pZP mAb can recognize porcine and human ZP antigen without cross-reaction with other human tissue antigens including ovary, so it may further help us to abstract and purify corresponding target antigen and settle basis for producing human ZP contraceptive vaccine.*

Key words: ZP (zona pellucida); monoclonal antibody (mAb); immunohistochemistry

The mammalian zona pellucida (ZP) is a transparent, porous sulfated glycoprotein extracellular matrix that surrounds growing and mature oocytes as well as the fertilized ovum before implantation. It mediates the initial spermatozoon-egg interaction involved in recognition, binding and penetration of spermatozoon to oocyte in a relatively species-specific manner, and plays an important role in the subsequent activation events including induction of the acrosome reaction as well as the primary block to polyspermy fertilization during fertilization process [1-3]. Because of the critical role of ZP glycoproteins in mammalian fertilization, together with their powerful tissue-specific nature has led to it being considered as a potential candidate antigen for immunocontraception. The proposed vaccine is the induction in female subjects of effective, sustained, but reversible levels of ZP-specific antibodies that inhibit sperm-egg binding and/or prevent sperm penetration into the ZP.

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Immunization of female with ZP glycoproteins leads to an anti-fertility effects in several animal models. Therefore, induction of anti-zona pellucida antibodies appears to be a potential promising and feasible approach of immunocontraception[4,5]. Because of common epitopes with human ZP antigens, porcine ZP has long been considered to be a promising immunogen in the development of human immunocontraceptive vaccine. However, immunization of animals directly with ZP antigen usually is invariably associated with either a transient or an irreversible alteration in the cyclicity, hormonal profile and follicular development, leading to the dysfunction of ovary, so revealed to be one of the major stumbling blocks for their use in humans[6,7]. The technology of monoclonal antibody has revealed a novel and bright promising field for screen and development of ZP contraceptive vaccine for anti-fertilization. We prepared anti-porcine ZP mAb, $17D_3$ anti-pZP mAb, in our laboratory, and studies *in vitro* had demonstrated that it could effectively inhibit the sperm-egg binding capacity, and the biological effect presented positive dose-dependent[6,8]. In the present study, we investigated its immunological reaction on porcine and human ZP, oocyte, granular cells as well as the other important organ antigens by ABC immunohistochemistry assay. Our aim was to further help in extracting and purifying the target antigen which does not cross-react to ovaries and other important tissue antigens by using the mAb as a probe.

Materials & Methods

Preparation of $17D_3$ anti-pZP mAb

BALB/c mice of 8 weeks old were immunized with heat-solubilized porcine ZP, and then spleen cells were taken. The hybridoma cells were prepared by fusing spleen and SP2/0 murine myeloma cells. A total of 144 strains of the hybridoma cells secreting anti-porcine ZP monoclonal antibodies were obtained by assaying the supernatant with ELISA. We selected 6 promising strains of strong positive cells, and then cloned the hybridoma cells by limiting dilution. The celiac fluids were obtained by inoculating intraperitoneally the hybridoma cells to the BALB/c mice. The monoclonal antibody, $17D_3$ mAb, was selected by screening with indirect immunofluorescent assay and purified by $(NH_4)_2SO_4$ salting out method.

Preparation of tissue specimen and sections

Porcine ovaries were collected before preoestrus, stored frozen after emasculated, and recovered before preparation of tissue sections. Human ovaries were freshly isolated, and collected from patients receiving total hysterectomy at prophase of ovulation with the patients' consent. Human tissues of heart, liver, spleen, lung, kidney and thymus were freshly isolated and collected from female fetus by induction of labor with water bag at metaphase. Appropriate informed consent and Ethic Committee approval were obtained for collection of ovary and other humankind materials. The tissue specimens were fixed in 10% formaldehyde for 24 h and embeded in paraffin at low temperature of 52℃−54℃. The longitudinal thickness of sections was 4 μm.

ABC enzyme-linked immunohistochemical assay

The ABC enzyme-linked immunohistochemical assay kit was product by Vector

Laboratories, Burlingame, CA, USA. The tissue sections were dewatered using dimethybenzene according to the routine protocol, followed by sequential hydration by treating with ethanol. The slides were incubated in methanol solution containing 0.3% H_2O_2 at room temperature for 30 min to eliminate the endogenetic peroxidase activity. After washed with phosphate-buffered saline (PBS), the slides were added by the diluted normal horse serum solution, and then incubated for 20 min at room temperature to block the non-specific binding. The slides were then sequentially treated with serial diluted primary monoclonal antibodies as well as negative and positive murine controls, respectively, and then incubated for 20 min at room temperature. After washing, the biotinylated secondary antibody was added and followed by 30 min incubation at room temperature. The ABC enzyme compounds were added after washing, incubated for 45 min at room temperature. The enzyme substrate DAB was added after thoroughly washing, and incubated for 5 min at room temperature. The slides were rinsed with tap water to stop the reaction. The sections were counterstained and fixed in routine.

Results

Immunohistochemical results on porcine ovary tissue

After the porcine ovary sections containing ZP had been treated with the $17D_3$ anti-pZP mAb of higher concentration, the whole layer of ZP appeared to brown with the outer darkest, the color deepness observed was outer layer >inner layer > middle layer, respectively. Meanwhile, oocyte cytoplasm was shallow brown, but not the granular cells, Graafian follicle membrane or connective tissue (Figure 1). With the increase of dilution of the monoclonal antibody, such as 1∶800 and 1∶1 600, only the outer layer of ZP was apparently coloured, while the brown color reactions of other layers including ovarian cytoplasm were vanished (Figure 2,3). Negative control sections showed no color on ZP, ovarian cytoplasm or the granular cells, Graafian follicle membrane and connective tissue (Figure 4).

Immunohistochemistry results on human ovary tissue

After human ovary sections containing ZP had been treated with the $17D_3$ anti-pZP mAb at 1∶100, both the outer and the inner layer of human ZP were shallow brown, with the inner layer more apparent (Figure 5); only the inner layer of ZP was brown if the concentration was 1∶200 (Figure 6). In spite of different dilutions of mAb (1∶100 and 1∶200), no positive reactions were observed on the granular cells, ovarian cytoplasm , Graafian follicle membrane and connective tissue, respectively.

Immunohistochemistry results on other important tissues of human

No brown reactions were observed on human tissues of heart, lung, thymus, nephridium, spleen, pituitary and liver after having been treated with $17D_3$ anti-pZP mAb (Figure 7).

Discussion

The control of human fertility would be revolutionised by the development of a safe, effective, long-acting and reversible contraceptive vaccine. The potential utility of a ZP

Figure 1 Treating porcine ovary with 17D$_3$pZP mAb (1:20), the whole layer of the ZP was brown with the outer part darkest; the ovarian cytoplasm was partly coloured while the granular cells were not coloured (400 ×)

Figure 2 Treating porcine ovary with 17D$_3$pZP mAb (1:800), the outer layer of ZP was dark brown, while the ovarian cytoplasm and the granular cells were not coloured (400 ×)

Figure 3 Treating porcine ovary with 17D$_3$pZP mAb (1:1600), the outer layer of ZP was brown, while the ovarian cytoplasm and the granular cells were not coloured (400 ×)

Figure 4 Negative control of porcine ovary (normal mice serum 1:8), neither of the ZP, ovarian cytoplasm nor the granular cells were coloured brown (400 ×)

Figure 5 Treating human ovary with 17D$_3$pZP mAb (1:100), the inner layer of ZP was apparently coloured, while the granular cells were not coloured (400 ×)

Figure 6 Treating human ovary with 17D$_3$pZP mAb (1:200), the inner layer of ZP was brown, while the granular cells were not coloured (400 ×)

antigen in development of contraceptive vaccine is contingent on at least several advantages as described below: ZP is highly immunogenic, both active and passive immunization with ZP antigen result in induction of antibodies, and the administration of monoclonal antibodies to ZP results in blocking of fertilization and implantation afterward. The crossing-antigenicity of ZP exists among different genetic species, such as porcine and human ZP. Anti-ZP antibody is highly sensitive, only a low titer is needed to exert its function[9]. Because of these unique immunological and species-specific characteristics of porcine ZP, it has long been regarded as a promising and potential immunogen for developing immunocontraceptive vaccine, and the efficacy of ZP as an antigen is well documented. However, the major challenges are the incompleteness of the contraceptive effect and the assurance of vaccine safety. The safety surfaced when animals injected with heterologous or even homologous form of the ZP or its glycoprotein was found to develop ovarian damage with loss of oocytes as well as ovarian function. The pursuit of this objective has involved the selection of appropriate targets within

Figure 7 Treating fetal tissues of heart(A), lung(B), thymus(C), nephridium(D), spleen(E), pituitary(F) and liver(G) with $17D_3$pZPmAb (1:100), ABC enzyme-linked immunohisto-chemical assay showed no brown reaction

the reproductive process that are amenable to interference with antibodies. Polyclonal antibody to porcine ZP extracted and purified by biochemistry methods could cross-reacted with Graafian follicle and other tissues of ovary except for ZP[10]. Pathological examination on ovary indicated that there was unreversible dysfunction of ovary characterized by loss of oocytes and attenuation of granular cell layer[11]. Meanwhile, studies based on porcine ZP glycoprotein immunization showed that anti-ZP antibodies were induced in most animal species and led to reversible infertility, but the effects were usually accompanied by prostration of ovary function[12]. Accordingly, preparation of the anti-ZP monoclonal antibody cross-reacted with human ZP by means of hybridoma techniques is therefore a significant inevitable approach to explore the potential ZP immunocontraceptive vaccine.

The analysis of immunohistochemistry showed that $17D_3$ anti-pZP mAb bonded specifically the inner and middle layer of porcine ZP as well as oocyte cytoplasm when concentration of the mAb was relatively higher, the colour of oocyte cytoplasm vanished with concentration of antibody decreasing. This phenomenon probably may be caused by "contamination" of neighboring tissue by excessive antibody. The immunohistochemical results of the mAb to human ovarian tissue and other important organs indicated that it could recognize the cross epitopes of human ZP without reaction with the other important organ

tissues. This demonstrated that the mAb could identify ZP antigen without causing dysfunction to other viscera. Accordingly, it could be considered as a passive immunogen in ZP immuno-contraception research.

Porcine ZP is composed of three biochemical and immunological distinct glycoproteins, termed ZP1(subdivided as ZP2 and ZP4), ZP3α and ZP3β, respectively, under the condition of deoxidized SDS-PAGE. The ZP1 may act as the secondary sperm receptor, exert its function during egg-sperm binding process[13]. Hasegawa[14] demonstrated that hamsters immunized with porcine ZP4 showed reversible anti-fertility effect with comparatively weak ovarian damage, and could be rehabilitated so suggested that pZP4 could be considered to be a prospecting ideal target antigen. Purification and identification of the target antigen recognized by $17D_3$ anti-pZP mAb turned out to belong to ZP4 with the molecular weight of 25 kD. Moreover, anti-ZP antibody against human fertility has been confirmed as IgG class, and the mAb $17D_3$ anti-pZP is IgG1[15]. From this point of view, it is hopeful to identify the corresponding ZP protein constituents by using this designated monoclonal antibody for developing human ZP contraceptive vaccine.

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