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TESTIS - SPECIFIC PROTEINS AND THEIR ROLE IN CONTRACEPTIVE VACCINE DEVELOPMENT

Rajesh K. Naz and Cynthia M. Vanek

Division of Research, Department of Obstetrics and Gynecology, Medical College of Ohio, Richard D. Ruppert Health Center, 3120 Glendale Avenue, Toledo, OH 43614-5809, USA

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1. ABSTRACT

Development of a vaccine(s) based on sperm antigens represents a promising approach for contraception. The utility of an antigen in immunocontraception is contingent upon its testis/sperm specificity and involvement in spermatogenesis and/or fertilization. The aim of the present article is to review the information regarding the proteins that have been reported to be testis/sperm-specific and may have an important function in spermatogenesis and/or fertilization. The potential role of these proteins in the development an antisperm contraceptive vaccine(s) is discussed.

2. INTRODUCTION

Sperm antigens are attractive candidates for the development of a contraceptive vaccine. The rationale and feasibility of using sperm antigens in immunocontraception is provided by the following findings. Deliberate immunization of male or female animals of various species (1-3) including humans (4, 5) with autologous or isologous spermatozoa results in infertility. The data of vasectomy and involuntary infertility in humans also provide strong evidence for the antisperm contraceptive approach. Up to 70% of vasectomized men form antisperm antibodies (ASA) (6) and up to 30% of infertility may be associated with the presence of ASA in the male and/or female partner of an infertile couple (7). These data indicate that the spermatozoon has both auto- as well as isoantigenic potentials, and when sufficient antibody titers are present, it can cause infertility in humans.

Although whole spermatozoon can produce an antibody response that is capable of inducing infertility in humans, they cannot *per se* be employed for the development of a vaccine (8,9). Besides the presence ofnumerous 'internal' antigens common with somatic cells, there are several proteins on the sperm cell surface that are likely to be shared with various somatic cell plasma membrane. The spermatozoon has antigens that can be shared with antigens on brain (10, 11), kidney (10), erythrocyte (12), lymphocyte (13), embryo (14) and oncofetal antigens (15). Sperm antigens can also react with soluble proteins such as lactoferrin and other proteins present in body fluids such as milk, serum, and saliva (16). Some of these cross-reacting antigens have been characterized. A nervous system antigen (NS-6) has been delineated on the cell surface of brain, kidney, and sperm cell (10); D_2 adhesion protein is present on both brain and testicular cells (18); and F-9 antigen is present on sperm, embryo, and teratocarcinoma cells (19).

Thus, only those proteins that are testis/sperm-specific can be employed for the development of an antisperm contraceptive vaccine (ACV). The utility of a sperm antigen in immunocontraception is contingent upon its: 1. Testis/sperm-specificity, 2. Role in spermatogenesis and/or fertilization, and 3. Accessibility to the antibody action. Also, the antigen should be capable of raising enough antibody titer, especially in the genital tract, to have a contraceptive effect. The aim of the present article is to review and update the information regarding the proteins that have been reported to be testis/sperm-specific and may have potential application in the ACV development. Specifically, the tissue-specific antigens expressed on mature sperm cell and developed during later stages of spermatogenesis will be reviewed in this article. The proteins that are added onto spermatozoon during its transit through the epididymis and vas deferens and through exposure to secretions of various accessory glands are not included in this review.

Table 1. Testis-specific proteins involved in sperm-egg interaction

Protein	Molecular size[a]	Expression stage	Species studied	Identification method	Function
1. SP-10[37, 38]	18-34 kD, 256 aa, 1117 bp (human)	Primarily in round spermatids stages I, II, III; ↓ in stages IV, V, VI (mRNA expression); All six stages and all steps of spermiogenesis (protein expression)	Human	*In situ* hybridization with cDNA probe	Acrosomal antigen
2. Mouse sperm antigen (MSA-63)[39] (60% homology with human SP-10 at protein level)	24-28 kD, 1.5 kb	Postmeiotic germ cells	Mouse	Indirect immunofluorescence assay of frozen sections of developing mouse testis with antibody probe	Intraacrosomal protein
3. SP17[40, 41]	17 kD, 149 aa, 1.3 kb (human, mouse); 17 kD, 0.9 kb, 1.1 kb (rabbit)	Spermatocytes, spermatids, testicular spermatozoa	Human, rabbit, mouse	Immunofluorescence and immunocytochemistry assay	Participates in zona pellucida (ZP) binding in vitro
4. SP38[42]	38 kD, 299 aa (boar)	Testis-specific, exact stage of expression not known	Boar	____	Participates in sperm binding to ZP
5. NZ-1[43]	14-18 kD, 151aa, 1395 bp	Testis-specific, exact stage of expression not studied	Mouse	Northern blot analysis	Involved in ZP binding and has tyrosine phosphorylation activity
6. Fertilization antigen-1 (FA-1)[43, 44]	23 kD (monomer), 51± 2 kD (dimer)	Testis specific, secondary spermatocyte stage onward	Mouse	Immunocytochemistry, Northern blot analysis	Involved in ZP binding and sperm capacitation
7. Zonadhesin[45]	7.5-8 kb	Haploid spermatids	Pig	*In situ* hybridization with RNA probe	Mediates sperm adhesion to ZP
8. Fertilin, beta subunit[46-49]	85 kD, 2.9 kb (human); 55 kb (mouse); 919 aa, 751 aa (rabbit)	Pachytene spermatocytes to late elongating spermatids	Guinea pig	Immunoblotting with PH-31 mAb	Binding of sperm to oocyte plasma membrane

[a] amino acids (aa), base pairs (bp), kilobase (kb), kilodalton (kD)

3. DISCUSSION

3.1. Testis-specific proteins involved in sperm-egg interaction

Fertilization is a complex process requiring the spermatozoon to undergo a cascade of events before it can fuse with the egg plasma membrane. This chain of events include capacitation, binding to the zona pellucida (ZP), acrosome reaction, penetration through the ZP, and fusion with the plasma membrane of the oocyte, which subsequently cleaves, develops and implants. These events are not clearly understood at the molecular level (19). One of the crucial steps in the fertilization process is the attachment of the spermatozoon to the ZP of the ovum, which requires specific recognition and interaction between the complementary molecules (19,20). The sperm-ZP interaction constitutes the most important event in the fertilization process, and because of the tissue/cellular specificity of this event, the molecules involved at this site are the most attractive candidates for the development of an ACV.

The non-enzymatic testis-specific proteins involved in sperm-egg interaction/binding that have been proposed as potential candidates for the development of an ACV are described in table 1. Although over fifty

molecules have been delineated by various monoclonal antibodies and described in the literature (9, 21), the antigens included in table 1 are more characterized for

their molecular structure and function, tissue specificity, and have been proposed as candidates for the ACV development. These molecules have been characterized in spermatozoa of various mammalian species. The human counterparts of many of these molecules have also been delineated or are under investigation. Active immunization with many of these molecules such as MSA-63, SP17, and FA-1 antigens have been shown to have contraceptive effects in various species of animals (9, 22). Other molecules, such as SP-10 and the beta subunit of fertilin, although are specifically expressed during later stages of spermatogenesis, have shown limited to no contraceptive effect in the active immunization studies (23, 24). SP-10 is present in the inner acrosomal membrane and may be accessible to antibodies only after the acrosome reaction. All eight molecules described in table 1 have been shown to have testis-specific expression during later stages of spermatogenesis.

3.2. Testis-specific enzymes present in sperm

The acrosome is a unique organelle present in the sperm head that is required for fertilization. Several enzymes are present in the acrosome, including acid hydrolases and other enzymes specific to spermatogenic cells. The acrosome has characteristics of both the lysosome and the secretory vesicle. During the acrosome reaction, its contents are released by calcium-mediated exocytosis that helps the sperm cell to penetrate the ZP surrounding the oocyte (21). Over twenty enzymes have been described that are present specifically in the acrosome, and multiple enzymes of the glycolytic pathway are also present in sperm cell that are common to somatic cells.

In table 2, eight molecules have been described that either have enzymatic activity or are associated with some enzyme function. These molecules are specifically expressed in the testis during later stages of development. Although some of these enzymes are also present in various somatic cells, the testis has expression of their unique isomeric form. Three of these molecules (acrosin/LDH-C_4/PH-20) have also been investigated for their immunocontraceptive effects. Active immunization with acrosin did not affect fertility of ewes and female rabbits, in spite of the presence of high titers of antibodies in the serum (25). Active immunization with LDH-C_4 causes a reduction in fertility of various species of animals including baboons (26). Active immunization of male and female guinea pigs with the guinea pig sperm protein PH-20 causes fully effective contraception (27). The contraceptive effect of other enzymatic molecules needs to be explored.

3.3. Testis-specific nuclear proteins

The extreme condensation and distinctive shape of the sperm nucleus is due to the presence of unique chromosomal basic protein, designated protamines, which replace the somatic histones during spermatogenesis. The protamines have been isolated from sperm of several mammalian species. By comparison to the somatic histones, the mammalian protamines are smaller, contain a higher molar content of arginine and cysteine, and have distinct primary sequences. Although not highly conserved phylogenetically, the known protamines do have a relatively invariant N-terminal region and central arginyl domains. The change from histone to protamine is a gradual process involving several variants of histones and protamines. Six of these proteins that have been well characterized are described in table 3.

These nuclear proteins, although specifically expressed in the testis, may not provide ideal targets for ACV development because they are present inside the cell, thus inaccessible to antibodies. However, these nuclear proteins are strongly immunogenic and species cross-reactive. Antibodies to protamine cross-react with sperm of various species including salmon, rabbit, and human (28, 29). Also, antibodies to protamine are present in the serum and seminal plasma of vasectomized men (30), and in sera of infertile men and women (28). However, these antibodies did not block the sperm function *in vitro* and *in vivo,* as they were inaccessible to the protamine (28). Thus, the nuclear proteins cannot be used for the development of an ACV. However, interfering with their synthesis in the testis using the antisense oligonucleotide approach may provide an alternative method of contraception to control male fertility.

3.4. Testis-specific transcription and translation factors and structural proteins

The expression of many proteins is temporally regulated during spermatogenesis (31). Boitani (32) examined the synthesis of testicular polypeptides in seminiferous tubules and quasi-homogeneous germ cell populations by radiolabeling with ^{3}[H] leucine. Analysis of *de novo* synthesized radiolabeled proteins by 2-D gel electrophoresis showed different polypeptide patterns in meiotic versus post-meiotic cells. The polypeptide profile further changed during the successive stages of spermatid differentiation. Kramer and Erickson (33) analyzed stage-specific protein synthesis during spermatogenesis using testicular cells isolated by centrifugal elutriation. They concluded that approximately 15% of the soluble and 20% of the particulate proteins seen on 2-D gels showed stage-specific synthesis. Subsequently, it has been well documented that each cell type contains a small but reproducible number of polypeptides that appear to be cell and stage-specific. The expression of these proteins is regulated at the transcription and/or translation levels by various specific factors. Expression of several of these cell/stage-specific proteins regulate growth, division, differentiation, and structure of developing spermatozoon during spermatogenesis and spermiogenesis.

Table 2. Testis-specific enzymes present in sperm

Protein	Molecular size[a]	Expression stage	Species studied	Identification method	Function
1. Acrosin[50-52]	417 aa, 1.8 kb (mouse)	Pachytene spermatocytes to round spermatids (mRNA expression); Spermatozoa (protein expression)	Human, mouse	*In situ* hybridization, Northern blot analysis	Serine protease localized in sperm acrosome
2. Lactate dehydrogenase-C_4 (LDH-C_4)[53]	140 kD, 331 aa, 1171 bp	Midpachytene spermatocytes to spermatids	Mouse	*In situ* hybridization	Participates in enzymatic reactions of glycolysis
3. PH-20[49, 54]	64 kD, 509 aa, 1683 bp (human)	Testis-specific, exact stage of expression not known	Guinea pig	____	Primary role: Hyaluronidase activity, sperm penetration of the layer of cumulus cells surrounding oocyte; Secondary role: sperm binding to ZP
4. Phosphoglycerate kinase-2 (PGK-2)[55, 56]	900 bp	Preleptotene spermatocytes to round spermatids (mRNA expression)	Mouse	*In situ* hybridization, reverse transcription-PCR	Specific function unknown; a key enzyme involved in metabolism of glucose or fructose
5. Cytochrome c_t[57, 58]	12.1 kD	Zygotene to pachytene spermatocytes to spermatozoa	Mouse, rat	Immunocytochemistry, immunofluorescence	Electron transport protein of the mitochondrial respiratory chain
6. Calpastatin[59, 60]	17.5 kD, 186 aa	Postmeiotic haploid stage of spermatogenesis (mRNA expression)	Human	*In situ* hybridization	Possibly participates in acrosome reaction
7. MC41 antigen[61]	165 kD	Step 2 to 19 spermatids, mature spermatozoa	Rat	Immunochemistry	Intraacrosomal protein
8. Glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS) protein[62]	438 aa	Step 9 spermatogenesis (maximal mRNA expression); Steps 12-13 (protein expression)	Mouse	Immunochemistry	Regulation of glycolysis and energy production for sperm motility

[a] amino acids (aa), base pairs (bp), kilobase (kb), kilodalton (kD)

The fourteen of the transcription and translation factors and structural proteins whose expression is specifically modulated during spermatogenesis/spermiogenesis are included in table 4. These factors/proteins have been selected because they have recently drawn special attention and have

Table 3. Testis-specific nuclear proteins

Protein	Molecular size[a]	Expression stage	Species studied	Identification method	Function
1. Histone H1t[63-65]	207 aa	Late and mid-pachytene spermatocytes stage VII-XII (mRNA expression)	Rat, mouse	*In situ* hybridization	Binds to linker DNA between nucleosomes
2. Histone TH2A[66]	Not clear	Pachytene spermatocytes	Rat	Electrophoretic pattern comparison of histones from nuclei of immature and adult testes	Part of octamer core which binds DNA to form nucleosomes
3. Histone TH2B[67-69]	17 kD, 126 aa	Preleptotene or leptotene spermatocytes to mid- or late pachytene spermatocytes	Human, rat	*In situ* hybridization	Part of octamer core which binds DNA to form nucleosomes
4. Protamines (PRM1, PRM2) [70-72]	PRM1: 0.6 kb; PRM2: 0.9 kb	Postmeiotically in round and elongating spermatids (mRNA expression); Stage I throughout rest of spermiogenesis, late step 11-19 (protein expression)	Mouse, rat, human, horse	*In situ* hybridization	Replaces transition proteins to compact DNA during late spermiogenesis
5. Transition protein-1 (TP-1)[71]	54 aa	Initial expression at stage XII, peaking at stage XIII- I, Steps 12-15 (protein expression)	Rat	Immunocytochemical analysis	Replaces histones to compact DNA during late spermiogenesis (appears at time of chromatin compaction)
6. Transition protein-2 (TP-2)[71, 72]	115 aa	Round and elongating spermatids (mRNA expression); Stages IX-XI faint band, ↑ levels stages XII-XIV, steps 9-14 (protein expression)	Rat	*In situ* hybridization, immunocytochemical analysis	Replaces histones to compact DNA during late spermiogenesis (associated with onset of nuclear elongation)

[a] amino acids (aa), base pairs (bp), kilobase (kb), kilodalton (kD)

probable testis-specific expression. These are very interesting molecules and some of them have been shown to have crucial roles in spermatogenesis, for example, the mice lacking CREM gene show severe impairment of spermatogenesis (34-36). However, unless these molecules are also expressed on the sperm surface, they will not be accessible to the antibodies.

4. PERSPECTIVE

In conclusion, the testis-specific proteins that are expressed on the mature sperm cell surface and have a crucial role in sperm function and fertilization are interesting candidates for the development of a contraceptive vaccine. The testis-specific proteins that are present inside the cell but have an important role in spermatogenesis/spermiogenesis, unless expressed on the mature sperm surface will have limited applications in the ACV development. However, inhibition of the synthesis by antisense oligonucleotide approach may provide a viable method for contraception in men. The oligonucleotides because of their smaller size should be able to cross the blood-testis barrier as well as the membrane of the developing germ cells. Further studies are needed to test this hypothesis and feasibility of the antisense oligonucleotide approach.

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Table 4. Testis-specific transcriptional and translational factors and structural proteins

Protein	Molecular size[a]	Expression stage	Species studied	Identification method	Function
1. Cyclic AMP- responsive element modulator ($CREM_t$)[34-36]	Not clear	Pachytene spermatocytes onwards (mRNA expression); Post-meiotic spermatids (protein expression)	Mouse	*In situ* hybridization	Activator of testis-specific genes (protamine/transition protein genes)
2. Zinc finger protein-35 (Zfp-35)[73]	24 kb	Pachytene spermatocytes	Mouse	*In situ* hybridization	Translational control during pachytene stage
3. Heat shock cognate 70t (HSC70t)[74]	70 kD, 630 aa, 2.7 kb	Postmeiotic stages	Mouse	Slot-blot hybridization	Transcriptional control of spermatogenesis
4. Megl gene[75]	Not clear	Leptotene ↓, then ↑ through zygotene and pachytene stages	Mouse	*In situ* hybridization	Transcriptional control of spermatogenesis
5. Testis enhanced gene transcript (Tegt)[85,86]	1.0 kb	Postmeiotic germ cells	Human, rat	Southern blot analysis	Structural protein of the nucleus and/or transcription factor
6. Testis-specific protein, Y-encoded (TSPY)[78]	Not clear	Early spermatogenesis	Human	Immunostaining	Transcriptional control of spermatogenesis
7. t complex polypeptide-3 (TCP-3)[79]	Not clear	Postmeiotic germs cells (highest in round spermatid stage)	Mouse	2D gel electrophoresis	Transcriptional control of spermatogenesis
8. t complex polypeptide-7 (TCP-7)[79]	Not clear	Postmeiotic germs cells (highest in round spermatid stage)	Mouse	2D gel electrophoresis	Transcriptional control of spermatogenesis
9. t complex polypeptide-10b[t] ($TCP\text{-}10b^1$)[80, 81]	Not clear	Spermatocyte stage onward	Mouse	Northern blot analysis	Transcriptional control of spermatogenesis
10. Antigen 1C9 (AG-1C9)[82]	103 kD (hamster, rat); 101 kD (mouse)	Middle pachytene spermatocytes to maturation phase spermatids (step 15)	Rat, mouse, hamster	Immunocytochemistry and immunoblotting	Exact function unknown; localized to ER and nuclear envelope
11. Protamine-1 RNA-binding protein (Prbp)[83]	7785 bp	Late stage meiotic cells and haploid round spermatids	Mouse	Immunocytochemical localization	Repressor of protamine-1 translation
12. Spermatid A kinase anchor protein-84 (S-AKAP84)[84, 85]	84 kD, 593 aa, 3.2 kb	Round spermatids and elongating spermatids	Human, mouse	*In situ* hybridization	Participates in subcellular shift of protein kinase
13. Testicular differentiation antigen (TDA95)[86]	95 kD	Zygotene and early pachytene spermatocytes	Mouse	Immunohistochemistry	Cell surface glycoprotein
14. Outer dense fiber protein (ODF)[87-89]	27 kD, 90 aa, 1.0-1.1 kb	Round spermatids: steps 1-5 few transcripts, steps 6-7 ↑ transcripts, steps 8-10 ↑↑ transcripts, steps 11-15 ↑ transcripts, steps 16-18 ↑↑ protein synthesis and ↓ transcripts, step 19 no transcripts	Rat	*In situ* hybridization	Coarse cytoskeletal fibers, each associated with one microtubular doublet along axoneme of sperm tail

[a] amino acids (aa), base pairs (bp), kilobase (kb), kilodalton (kD)

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Send correspondence to: Dr Rajesh K. Naz, Division of Research, Department of Obstetrics and Gynecology, Medical College of Ohio, Richard D. Ruppert Health Center, 3120 Glendale Avenue, Toledo, OH 43614-5809, Tel: (419)-383-3502, Fax: (419)-383-4473, E-mail: rnaz@gemini.mco.edu