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Distribution of Molecular Epitope for Ts4, an Anti-Sperm Auto-Monoclonal Antibody in the Fertilization Process

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Abstract. To investigate molecular effects of anti-sperm autoantibodies on fertilization, we previously established anti-mouse sperm-head auto-mono-clonal antibodies (mAbs). Among the mAbs established, one mAb (named Ts4) recognized the sugar moiety of TEX101, a germ cell-marker glycoprotein. In the present study, we examined the immunoreactivity of Ts4 in mouse spermatozoa and fertilized eggs during early embryogenesis to clarify the distribution of the Ts4-reactive antigen in the fertilization process. Similar to TES101 mAb (a specific probe for TEX101), immunopositive staining of Ts4 was observed on spermatocytes, spermatids and spermatozoa within the testis. In contrast to the results obtained with TES101 mAb, Ts4 reacted with the sperm acrosomal region within the cauda epididymis. A Western blot analysis of epididymal sperm extract revealed that Ts4 mainly detected two bands between 100 and 150 kDa, while Ts4 faintly detected a band corresponding to TEX101 at 38 kDa. In addition, Ts4-reactive molecules were observed in the growing early embryo after fertilization. Since Ts4-reactive antigen, potentially a carbohydrate chain, is only observed in reproduction-related areas such as the testis, epididymal sperm-head and early embryo, it is expected to have an effect on fertilization. Therefore, additional studies of this antigen may elucidate the molecular mechanisms underlying the reproductive process.

Key words: Auto-antibody, Fertilization, Monoclonal antibody, Sperm, TEX101

Anti-sperm autoantibodies are present in the sera of some infertile patients [1]; however, the precise mechanism underlying the induction of anti-sperm antibodies remains unclear. One leading hypothesis suggests that cross-reactive immune responses against external antigens (e.g., bacterial or viral infections) may induce an immune response against sperm antigens [2]. Based on this hypothesis, we developed several anti-sperm auto-mono-clonal antibodies (mAbs) using spleen cells from aged mice (over 1 year old) kept under normal conditions. To identify hybridoma cell lines that secrete anti-sperm autoantibodies, culture media from each hybridoma line were screened for antibody activity against mouse epididymal spermatozoa using ELISA and immunofluorescence tests (Fig. 1). To pinpoint the specific antigens recognized by the anti-sperm auto-mAbs, we performed a micro amino acid analysis against testicular proteins that showed an immunopositive reaction for the mAbs using a two-dimensional SDS-PAGE system (Fig. 1). Among the mAbs produced, one specific mAb (named Ts4) reacted with a protein that possessed an N-terminus of TYC-QVSQTLSLEDD [3]; this sequence shares 100% sequence identity with the potential N-terminal sequence of TEX10126,39 [4].

TEX101, a highly N-glycosylated glycosylphosphatidylinositol (GPI)-anchored glycoprotein [5], was originally identified as a protein containing the antigen epitope for a specific mAb, called TES101, produced by immunizing female mice with an allogenic testicular homogenate [4]. Previous studies by our research group have demonstrated that TEX101 is a unique germ cell marker that is expressed during gametogenesis [4–9] and shows sexually dimorphic expression in developing gonadal tissues. In the ovary, oogonia temporarily express TEX101 during the fetal period [6], but the molecule is not detected in the sexually mature ovary [4]. In contrast, during spermatogenesis, TEX101 first appears on the plasma membranes of prospermatogonia within the immature seminiferous cords of the fetal testis [6]. Although it is not expressed on spermatoctyes, spermatids, and testicular sperm following puberty [6]. Finally, TEX101 is shed from the cell surface of epididymal sperm during transport through the caput epididymis [7]. These changes in the TEX101 expression pattern during gametogenesis suggest that TEX101 may play an important role in germ cell development and differentiation within the gonads. However, many points remain unclear as to the involvement of TEX101 in the process of gametogenesis and its regulation of the molecular expression is far from completely understood.

Although Ts4 was unexpectedly established as a mAb reactive with TEX101 (see above), we are interested in whether this mAb can serve as a useful tool in elucidating the functions of TEX101. On the other hand, our preliminary studies have shown that the immunoreactivity of Ts4 against deglycosylated TEX101 with N-glycanase (an endoglycosidase known to cleave all types [high-mannose/hybrid, bi-, tri- and tetraantennary complex-type] of N-linked oligosaccharide (OS) chains [10]) was completely abrogated [3]. These results suggest that the antigenic determinant on
TEX101 for Ts4 is the N-linked OS chain (Fig. 2). Precise Western blot analyses indicate that Ts4 reacts with two additional bands in testicular extracts at an apparent molecular mass between 100 and 150 kDa [3]. These two additional immunopositive bands are not observed when the TES101 mAb is used as a molecular probe [4, 5, 8]. These results suggest that Ts4 also recognizes unidentified testicular proteins that possess presumably an OS chain similar to that of TEX101.

Since immunoreactive molecules for Ts4 are located in cauda epididymal sperm heads, the auto-mAb is expected to have an effect on fertilization. In the present study, we examined the immunoreactivity of Ts4 in mouse spermatozoa and fertilized eggs during early embryogenesis to clarify the distribution of the Ts4-reactive antigen in the fertilization process.

Materials and Methods

Animals

Male ICR mice (8–12-week-old) and male/female C57BL/6N mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained and bred at the Animal facilities of Juntendo University under 12L:12D conditions, and given free access to food and water. All animal experiments were conducted according to the guide for care and use of laboratory animals, Juntendo University.

Antibodies

TES101, a mouse mAb (IgG1) reactive with the peptide portion of TEX101, was produced and purified as previously described [4]. Another anti-TEX101 mAb derived from anti-sperm autoantibodies, Ts4 (mouse IgM), was established and prepared as reported elsewhere [3]. An anti-human GPI-80 mAb, 3H9 (mouse IgG1) [11], was used for control studies. Other antibodies were purchased as follows: normal control mouse IgM and normal goat serum (DAKO, Glostrup, Denmark); Alexa Fluor 488-conjugated goat anti-mouse IgG and IgM polyclonal antibody (pAb) (Molecular Probes, Eugene, OR, USA); and horseradish peroxidase (HRP-) conjugated goat anti-mouse IgM pAb (Chemicon, Temecula, CA, USA).

Preparation of mouse testis, epididymal sperm and embryonic stem (ES) cell extracts

To obtain a mouse testicular Triton® X-100 soluble (TS) fraction, mice testes were extracted and treated as previously described [3]. To isolate epididymal spermatozoa, the epididymides were divided into three regions (caput, corpus and cauda), and the spermatozoa in each portion were isolated. Sperm extracts in the portions were prepared according to a method described previously [7]. A mouse ES cell line, TT2 [12], was cultured, and a cell lysate was prepared for SDS-PAGE using Laemmli’s method [13] under reducing or non-reducing conditions.

Immunoprecipitation

Immunoprecipitation was performed as described elsewhere [8] with some modification. Briefly, prior to immunoprecipitation, 500 μl of solution containing 50 μg total proteins of the TS testicular extract was precleared with 20 μl of Dynabeads Rat anti-Mouse IgM (Invitrogen, Carlsbad, CA, USA) on a rotary shaker for 2 h. After centrifugation for 1 min at 18,000 × g, the supernatant was rotated overnight with Ts4 (10 μg), and Dynabeads Rat anti-Mouse IgM (20 μl) was then added into the identical tubes. After an additional rotation for 3 h, the bound beads were separated by centrifugation at 18,000 × g for 1 min and washed three times. The precipitated beads were boiled for 5 min in buffer for SDS-PAGE under reducing or non-reducing conditions. After centrifugation, the supernatants were used as samples for SDS-PAGE.

Western blot analysis

The protein solutions were electrophoresed using an SDS-PAGE system, and the separated proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane [14]. The reactivity of Ts4 against the blotted proteins was assayed using HRP-conjugated secondary antibodies and visualized as reported previously [3].
Immunofluorescent studies

For male germ cells: Sections (5–6 μm thick) of 4% paraformaldehyde (PFA)-fixed mouse testis and epididymis were prepared as described elsewhere [3]. Spermatozoa from each part of the epididymis were prepared as described previously [7]. The sperm suspension was smeared onto 3-aminopropyltriethoxysilane-coated slide glass, allowed to air dry and then fixed with 1% PFA. Subsequently, the cryosections or isolated epididymal sperms were incubated with either TES101 mAb or Ts4 mAb and then treated for 30 min with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (2 μg/ml). The specimens were sequentially counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) and mounted in ProLong Gold antifade reagent (Molecular Probes). The immunostained samples were observed and analyzed under a BIOTEM BZ-9000 microscope system (KEYENCE, Osaka, Japan). Control sections received the same treatment with the exception that the primary antibody was replaced with an isotype-matched control antibody.

For eggs and early embryos: The immunoreactivity of ovarian eggs was monitored using cryosections as described above. Early embryos from superovulated female mice were collected essentially according to the method described previously [15]. Briefly, 8-week-old female mice were each given an i.p. injection of 5 IU of eCG (G4877: Sigma-Aldrich, St. Louis, MO, USA) followed by an i.p. injection of 5 IU of hCG (C2047, Sigma-Aldrich) 48 h later. Each female animal was housed with a sexually mature male mouse for mating and was examined for the presence of a vaginal plug the next morning. Seventeen (or appropriate) hours after the hCG injection, the oviducts were isolated, and the cumulus masses containing early embryos were recovered from the ampullae. Cumulus-free mouse embryos treated with 0.1% hyaluronidase (from bovine testes, Type IV-S, H4272, Sigma-Aldrich) in M2 medium (M7167, Sigma-Aldrich) were isolated and washed twice in M2 medium. The embryos were then placed into M16 medium (M7292, Sigma-Aldrich) and incubated at 37 °C in 5% CO2 in air. After fixation with 0.5% PFA in PBS (pH 7.2) for 15 min, the embryos were treated with 0.1% Triton® X-100 for 5 min, and then immunostaining with either Ts4 or TES101 was carried out. The immunoreactivity was monitored by a confocal laser microscope (TCS SP2 AOBS; Leica, Heiderberg, Germany) as described previously [8, 16].

Results

Immunohistochemical analysis of male mouse reproductive tissues using TES101 and Ts4 mAbs

To investigate the immunoreactivity of Ts4 in male reproductive tissues, we first performed an immunomorphological analysis. Both TES101 and Ts4 reacted with spermatocytes, spermatids and spermatozoa within the testis (Figs. 3A and B), as demonstrated in our previous reports [3, 4, 6]. The immunostaining patterns of spermatozoa within the epididymis, however, were drastically different for TES101 and Ts4. A slightly patchy pattern of immunopositive staining was observed for TES101 mAb in spermatozoa from the caput epididymis (Fig. 3C). In contrast, the reactivity of Ts4 was clearly observed as a linear pattern in spermatozoa from the caput epididymis including the tail region (Fig. 3D). Throughout the epididymal duct, the immunoreactivity of TES101 decreased in parallel with sperm maturation in the epididymis (Figs. 3E and G). On the other hand, the reactivity of Ts4 remained
observed staining patterns were completely different from those of TES101 mAb in the epithelia of the epididymal ducts or in interstitial cells (Fig. 3).

Immunoreactivity of TES101 and Ts4 in isolated epididymal sperm

Since Ts4 reacted with epididymal spermatozoa and the observed staining patterns were completely different from those of TES101 mAb, we next examined the immunolocalization of Ts4 in spermatozoa isolated from each portion of the epididymis. As previously reported [7], immunopositive staining was only observed for TES101 in the cytoplasmic droplets of caput and corpus epididymal sperm (Figs. 4A and C), and it subsequently disappeared from the cauda epididymal sperm (Fig. 4E). In contrast, Ts4 reacted strongly with the entire flagellum, including the cytoplasmic droplet and acrosomal region of spermatozoa isolated from the caput epididymis (Fig. 4F). During sperm maturation, only the acrosomal regions of cauda epididymal spermatozoa showed immunoreactivity for Ts4 (Fig. 4F). Since these staining samples were not treated with detergent, the Ts4-reactive antigens of epididymal spermatozoa were presumably located on the cell membrane. Interestingly, the immunoreactivity of Ts4 in the cytoplasmic droplets appeared to be linked to that of TES101, whereas immunoreactivity in the acrosomal region was only detected with Ts4 (Figs. 4C, D, E and F). These results suggest that the immunoreactivity of Ts4 in cytoplasmic droplets is the only observed property that reflects its immunospecificity to TES101, since it is the only reactivity linked to that of TES101 mAb (specific for TES101). Taken together, we speculate that the molecule(s) recognized by Ts4 in the acrosomal region may not be TES101.

Western blot analysis of epididymal sperm using Ts4

To confirm that Ts4 recognizes molecules other than TES101 within epididymal sperm, we investigated the immunoreactivity of the mAb against extracts from epididymal sperm using a Western blot analysis. The Ts4 mAb detected a band of approximately 38 kDa that corresponded to TES101 in the testicular extract, which was confirmed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) after immunoprecipitation (see below). In addition, intense immunoreactivity was observed between 100 and 150 kDa as two additional bands (Fig. 5, lane 1), but these bands were not detected with TES101 mAb or other specific mAbs to TES101-recombinant peptide (6002, 6035), as reported previously [3–5]. In the extracts from each epididymal spermatozoon, the main immunoreactivity was observed as two bands at an apparent molecular mass between 100 and 150 kDa (Fig. 5, lanes 2–4). Ts4 also detected a very weak 38-kDa band in each epididymal extract (Fig. 5, lanes 2–4) as compared to the immunoreactivity in the testicular extract (Fig. 5, lane 1).

Biochemical analysis of proteins recognized with Ts4

The results shown in Fig. 5 indicate that two bands between 100 and 150 kDa were detected with Ts4, but they did not appear to be TES101. Therefore, attempts were made to identify the molecule(s) recognized by Ts4 using immunoprecipitation with the mAb. As an initial trial, we used testicular TS extracts to investigate whether the two bands between 100 and 150 kDa could be immunoprecipitated with Ts4. After immunoprecipitation with Ts4, two bands of approximately 140 and 110 kDa were clearly detected with the same mAb (Fig. 6, lane 1). Although a main Ts4 reactive band corresponding to 38 kDa was observed in the immunoprecipitant (Fig. 6A, lane 1), bands were also detected at the apparent molecular masses of 140, 110 and 38 kDa by silver staining (Fig. 6B, lane 1). Our preliminary experiment confirmed that the 38-kDa band in the immunoprecipitant was TES101 by LC-MS/MS and TES101 immunostaining (data not shown). These results show that isolation by immunoprecipitation with Ts4 may be useful for further protein identification.

Ts4 immunoreactivity on fertilized eggs or ES cells

To confirm the tissue distribution of the Ts4 reactive antigen, we...
Fig. 6. SDS-PAGE analysis of proteins in the mouse testicular Triton X-100-soluble fraction (TS), co-immunoprecipitated with Ts4. A: Western blot analysis with Ts4. Proteins immunoprecipitated from the TS with either Ts4 (lane 1) or normal control mouse IgM (n.c.; lane 2) were separated by 5% SDS-PAGE gel under reducing conditions. Control experiments were conducted under the same conditions, except for the absence of the testicular extract (Buf.; Lanes 3 and 4). The TS was used as a positive control (lane 5). Arrows indicate the specific immunoreactive bands observed in lane 1. Mr: Molecular mass. B: Visualized by silver staining. The same immunoprecipitated proteins as used in Fig. 6A were separated by 5% SDS-PAGE gel under reducing conditions. Specific bands by Ts4 immunoprecipitation are boxed.

Fig. 7. Ts4, but not TES101, reacts with the mouse early embryo. Immunostaining of Ts4 in the ovarian follicle (A), 4-cell stage embryo (B) and blastocyst (C), and differential interference contrast (DIC) images for each of the above stages (A’–C’). The control IgM and the TES101 did not react with the ovarian follicles (D, G), 2–4-cell stage embryos (E, H) and blastocysts (F, I). Bars: A’, D and G=60 μm, B’, C’, E, F, H and I=30 μm.

Fig. 8. Western blot analysis of proteins from mouse ES cells. Lane 1: Triton X-100-soluble fraction of mouse testis. Lane 2: Whole cell lysate of TT2 cells. The proteins were separated by SDS-PAGE under reducing (A) or non-reducing (B) conditions and then transferred onto PVDF membrane. Immunoreactivity of Ts4 was monitored with an ECL detection system. Negative control mouse IgM (n.c.) was used for the control experiment. The expression of TEX101 was probed with TES101 mAb.
examined Ts4 immunoreactivity against major mouse organs (i.e., cerebrum, cerebellum, heart, lung, liver, kidney and spleen) using cryosections from 4% PFA-fixed samples. As expected, no positive staining was observed among the tissues examined (data not shown). Immunoreactivity for both Ts4 and TES101 in ovarian follicles was also negative (Figs. 7A and G). TES101 mAb did not react with the early developing embryo (Figs. 7H and I); however, immunoreactivity was clearly observed for Ts4 in the early embryos (Figs. 7B and C). At the 4-cell stage, Ts4 reacted strongly with the nuclear membrane, reacted faintly with its cytoplasm (Fig. 7B) and appeared to disperse into the cytoplasm at the blastocyst stage (Fig. 7C). When ES cells derived from the inner cell mass of the blastocyst were examined by Western blot analysis, immunoreactive bands for Ts4 were found with apparent molecular masses of 68 kDa and 56 kDa (Fig. 8A). Immunoreactivity was not detected in whole ES cell lysates with TES101 mAb (Fig. 8B).

Discussion

As an anti-sperm autoantibody, Ts4 was originally established by hybridizing a myeloma cell with spleen cells from male BDF1 mice that were maintained for over a year without any artificial treatment [3]. Although immunoreactivity against the head portions of spermatozoa in the caput epididymis was used as a screening method to establish a hybridoma cell line, we used testicular extracts (excluding spermatozoa in the epididymis) for further micro amino acid analyses to identify the antigen molecule [3]. This was due to the relative difficulty of sperm collection from the cauda epididymis for micro amino acid sequence analysis. The results of antigen identification for Ts4 was surprising given that the Ts4 reactive (testicular) protein has a completely identical N-linked OS chain(s) in TEX101 (as shown schematically in Fig. 2).

In this study, we examined the immunoreactivity of Ts4 mAb in mouse spermatozoa and fertilized eggs during early embryogenesis to clarify the distribution of the Ts4-reactive antigen in the fertilization process. In contrast to the immunoreactivity patterns observed using TES101 (a mAb specific to a peptide portion within TEX101, CD9, alkaline phosphatase, stage-specific embryonic antigen (SSEA)-1 and Forssman antigen (FA) are well-known molecules that characterize murine ES cells [18–22]. Among these molecules, SSEA-1 and Forssman antigen (FA) are classified as glycolipid antigens. Several other carbohydrate antigens have been discovered (Yoshitake, Yanagida and Araki, unpublished data). At present, however, the identification of these molecules remains inconclusive because specific antibodies against the candidate molecules are not commercially available. Therefore, additional time is necessary to produce specific probes.

It should be noted that Ts4-reactive molecules (but not a portion of TEX101) have been identified in early developing embryos after fertilization (Fig. 7). Although the function and/or physiological significance of the Ts4-reactive molecules are unknown at this time, it is interesting that the Ts4-reactive molecules, presumably OS chains, are not common within mouse organs. The Ts4-reactive antigen is not found in the brain, liver, kidney or any other common organ [this study]. To date, it has only been observed within testicular germ cells in adult mice [3] and the early developing embryo [this study]. Western blot analysis of ES cells with Ts4 suggests that the Ts4-reactive proteins in the early developing embryo seem to differ from that in the testicular acrosome, or TEX101 (Fig. 8), but they might be share a molecular epitope, potentially OS chains on the molecules. In addition, our preliminary experiments indicate that Ts4 has an inhibitory effect on the sperm acrosome reactions induced by calcium ionophore in vitro (Hasegawa and Araki, unpublished data). Since the Ts4-reactive antigen was observed in the early embryo just after fertilization (in addition to the sperm head, Figs. 4 and 7), this autoantibody may have an effect not only on sperm function, but also on early events after fertilization, such as embryo development and implantation. Further studies are necessary to examine these possibilities.

During gametogenesis and early embryogenesis, several molecules have been identified as stage-specific markers. For example, CD9, alkaline phosphatase, stage-specific embryonic antigen (SSEA)-1 and Forssman antigen (FA) are well-known molecules that characterize murine ES cells [18–22]. Among these molecules, SSEA-1 and FA are classified as glycolipid antigens. Several other carbohydrate antigens have been reported as fertilization- or embryogenesis-related antigens [23, 24]. Lectins have frequently been used to identify and characterize novel cell subpopulations. These characterizations are based on the presentation of specific OS chains in cells. Several studies have examined the dynamic temporal and spatial changes of lectin reactivity during early embryogenesis [25–30].

The next step in these studies is identification of the molecular epitope, possibly carbohydrate chain(s) for Ts4 mAb. An analysis of Ts4 immunoreactivity toward various types of authentic glycoproteins, glycolipids, monosaccharides and synthetic OS is one
approach that may provide information concerning identification of the antigen epitope. If we confirm the carbohydrate specificity of Ts4, it may be a useful tool for elucidating the molecular mechanisms underlying fertilization and early embryogenesis.

In summary, anti-sperm head autoantibody Ts4 reacts with sperm proteins located in the acrosomal region. In addition, the antigen epitope is shared with TEX101, a specific germ cell marker. Ts4 reactive molecules are observed in the growing early embryo after fertilization and in reproduction-related areas such as the testis and epididymal sperm head. Therefore, the identity of the specific antigen could provide clues for elucidation of the molecular mechanisms underlying the reproductive process. In conclusion, Ts4 may be helpful in unraveling the role of molecular epitopes, presumably a specific carbohydrate chain(s), in the fertilization process.

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