Monoclonal Antibody TSd-1 is Specific to Elongating and Matured Spermatids in Testis of Common Tree Shrew (Tupaia glis)

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ABSTRACT. The monoclonal antibody (MAb), named TSd-1, specific to spermatogenic cells of the common tree shrew (Tupaia glis) was established and characterized using immunohistochemistry and immunoblotting. MAb TSd-1 reacted with elongating and matured spermatids in a stage-dependent manner. TSd-1 recognized a 94 kilodalton (kDa) peptide in the plasma membrane and cytosol. Additionally, an extremely weak 107 kDa band was detected only in the cytosol. The reactions were not detected in round spermatids. In elongating Stage VI spermatids, the plasma membrane and the granular structure within the cytoplasm were intensely positive, and most intense after the appearance of new round spermatids in the lower layer (Stage I). The reactions were observed neither in the other organs of the common tree shrew nor in the testes of other animals, indicating that TSd-1 antigen is specific to the spermatogenic cells of the common tree shrew, and may act on elongating or matured spermatids. — KEY WORDS: monoclonal antibody, spermatogenesis, spermatozoa, tree shrew.


Tree shrews (Order Scandentia) are small animals distributed mainly in Southeast Asia. Although they possess some similarities to primates, rodents, or insectivores, their taxonomical status has been controversial. Morphology of the reproductive system is occasionally used to obtain information on taxonomy among species. Eckstein [6, 7] concluded that the reproductive system of tree shrews exhibits more primate-like characteristics than other species on the basis of the detailed anatomical description of the common tree shrew (Tupaia glis) [9] and the feather-tailed tree shrew (Ptilocercus lowi) [12]. Martin [15] discussed on the taxonomical significance of the location of the testis and scrotal disposition of the common tree shrew. He showed that the anatomical observation of the reproductive tract of the tree shrew is quite different from that of all living primates, especially in the male; the testis in the tree shrew is located anterior to the penis as in marsupialia and lagomorpha, but not as it is in primates, and tree shrews lack the os penis, typical of male primates. Collins and his coworkers also described the anatomical location of reproductive tract [5], and histological and physiological postnatal differentiation [3, 4] of the male reproductive system of the common tree shrew, and concluded that the tree shrew resembles the primate. However, the discoidal sperm head and spermatogenesis (different stages did not appear in one cross-sectioned tubule) in the common tree shrew were obviously different from those in primates [14]. There is little biological information on the taxonomy and property of other tree shrews.

Immunological cross-reactivity and specificity often provide taxonomical information. Therefore, molecular-level information on reproductive organs is valuable for assessing the illusive reproductive system of the common tree shrew. From this viewpoint, a MAb specific to the spermatogenic cells of the common tree shrew was established. In the present study, the MAb named TSd-1 was characterized using immunohistochemistry and immunoblotting.

MATERIALS AND METHODS

Animals: Six adult male common tree shrews, captured in Thailand, were used for immunization, immunohistochemistry, and immunoblotting. For immunization and immunoblotting in the mouse, testes and epididymides were excised from common tree shrews under pentobarbital anesthesia and stored at -80°C. For immunohistochemistry, common tree shrews were perfused with Ringer's solution followed by Bouin's fixative. Then the heart, thymus, spleen, salivary gland, esophagus, stomach, small intestine, large intestine, liver, pancreas, lung, kidney, testis, ovary, epididymis, prostate, penis, uterus, thyroid, and adrenal gland was removed from each tree shrew.

In addition to the common tree shrew, ten other kinds of animals (northern smooth-tailed tree shrew, musk shrew, Finlayson squirrel, Japanese monkey, striped skunk, mouse, dog, goat, Japanese quail, and habu) were used. The testes were removed from anesthetized animals and fixed in Bouin's fixative followed by paraffin embedding.
**Production of monoclonal antibody:** The monoclonal antibody was obtained by a basic procedure [10]. The testes of common tree shrews were homogenized in 0.01 M phosphate buffered saline (PBS) and mixed with the same volume of Freund’s complete adjuvant for immunogen. The immunogen was injected into mice twice at an interval 2 weeks, and boosted with PBS-homogenized testes at 2 weeks after the last injection. After 2.5 days, the immunized mice was killed by anesthetization and the spleen was excised. Splenocytes were collected and fused with a myeloma cell line in 50% polyethylene glycol 3500, and incubated with hypoxantine-aminoprine-tymidine selective medium containing 10% fetal bovine serum. Hybridoma screening was performed using immunohistochemistry on a section of the common tree shrew testis. Hybridoma secreting the testis-positive MAb were selected by limiting dilution and one of them, named TSd-1, was injected into the mice intraperitoneally. After several weeks, the ascitic fluid was collected from the mice and stored at 4°C.

**Immunohistochemistry:** The organs embedded in paraffin were sectioned at 4 to 5 mm; they were then deparaffinized and permeated with 0.01 M PBS containing 0.03% Tween 20 (PBS/T). The specimens were blocked with Block Ace (Dainihon-seiyaku, Inc., Japan) for 30 min at room temperature (RT). They were incubated with diluted MAb Tsd-1 ascitic fluid (1:500) for 1 hr and washed with PBS/T at RT. After incubation with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:100) for 1 hr at RT, reactions were developed with diaminobenzidin containing H2O2. Staging of the tree shrew seminiferous epithelium was in accordance with a previous study [14].

**Immunoblotting:** The testes of common tree shrews were homogenized in the hypotonic buffer (10 mM HEPES (pH 7.5), 1 mM dithiothreitol, and 0.1% aprotinin). The specimen was centrifuged at 600 g for 7 min, then the supernatant was removed as Sup I and stored at 4°C until use. Lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% deoxicholate, and 0.1% sodium dodecyl sulfate (SDS)) was added to the pellet, gently homogenized and stored for 30 min on ice. Then it was centrifuged at 15,000 g for 30 min and the supernatant as Sup II was removed and stored at 4°C. Sup I and Sup II were added to a sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptethanol, 10% glycerol and 0.05% bromophenolblue) and denatured in a boiling water bath for 10 min. They were separated by SDS-polyacrylamide gel (10%) electrophoresis (SDS-PAGE) [11] and electrically transferred to a polyvinylidene difluoride (PVDF) membrane [20]. The blotted PVDF membrane was first blocked with Block Ace. The membrane was rinsed briefly with PBS/T and then incubated with diluted MAb Tsd-1 (1:10,000) in PBS/T containing 1% Block Ace for 2 hr. After washing with PBS/T, the membrane was incubated with HRP conjugated secondary antibody (diluted 1:5,000 with PBS/T containing 1% Block Ace) for 2 hr. After washing again, the membrane was treated with enhanced chemiluminescent detection system (Amersham, UK) and exposed to X-ray film in a dark room.

**RESULTS**

In the present study, MAb TSd-1 was established by fusing myeloma cells with splenocytes from a mouse immunized with a homogenate of the common tree shrew testis. The MAb isotype was IgG1 with a κ-light chain, determined using a MAb isotyping kit (Amersham, UK). Immunohistochemical localization of Tsd-1 was examined in the common tree shrew testis. As the result, immunoreactivity with MAb Tsd-1 was localized in elongating and matured spermatids (Figs. 1 and 2). At higher magnification, it was observed intensely in the plasma membrane and intracellular aggregated granule of elongating spermatids, while it was weak throughout the cytoplasm (Fig. 2). The reaction to matured spermatids was limited in the mid-piece (Figs. 2 and 3). Stage-dependent changes in the reaction were observed. A weak reaction first appeared in elongating spermatids at stage VI (Fig. 2E). It was localized in the plasma membrane and intracellular aggregated granule of spermatids, and persisted until stage XII (Fig. 2E to I). After the meiotic phase, an intense reaction was observed in the plasma membrane and intracellular aggregated granule of elongating spermatids (Fig. 2A to C), though it was weak throughout the cytoplasm. No reactivity was observed within round spermatids (Fig. 2A to D). At stage IV, the flagellar mid-piece of matured spermatids and residual bodies were positive (Figs. 2C and 3). The plasma membrane of residual bodies was intensely positive. Immediately after spermiation, residual bodies were still situated on the top of the seminiferous epithelium. Some positive granules were observed in Sertoli cells. In all likelihood, they were taken into Sertoli cells by phagocytosis (Fig. 2D). Flagellar mid-

![Fig. 1. MAb Tsd-1 reaction in the seminiferous epithelium of common tree shrew (× 50). The reaction, appearing in a stage-dependent manner, is restricted to the germ cells near the lumen. There are some non-reactive seminiferous tubules (arrows).](image-url)
pieces of spermatozoa in the epididymis were still positive (Fig. 4), similar to matured spermatids at stage IV (Fig. 2C).

Other organs of the common tree shrew were also examined by immunohistochemistry, but none of them contained MAb TSd-1 positive cells except for the spermatozoa in the epididymis (data not shown). This finding indicated that TSd-1 is specific to spermatogenic cells. Testes of other kinds of animals were also examined, and none of these animals cross-reacted (data not shown), indicating that the TSd-1 reaction is species-specific.

In the course of the characterization of the MAb TSd-1-recognized molecule, TSd-1 antigen, immunoblotting analysis was examined in the extract of the common tree shrew testis. The Sup I with cytosolic extract and the Sup II with membrane-associated extract exhibited a 94 kDa

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**Fig. 2.** MAb TSd-1 reaction appearing in a stage-dependent manner (× 525). A) Stage I. The reaction is detected in the plasma membrane of elongated spermatids (arrows). Round spermatids in the lower layer show no reaction (arrowheads). B) Stage III. The reaction in elongated spermatids is stronger and more condensed. C) Stage IV. Mid-piece (arrows) of matured spermatids and residual bodies (arrowheads) are positive. D) Stage V. Matured spermatids are released. Positive granules (arrows), probably corresponding to residual bodies, are observed in Sertoli cells. Round spermatids are negative (arrowheads). E) Stage VI. Spermatids weakly react with TSd-1 (arrows). F) Stage VIII. The reaction is increased in intensity but still weaker than that of later stages, and elongation of the nucleus progresses. Intensely-positive granular particles are observed in the cytoplasm of spermatids (arrows). G and H) Stage IX and XI. The reaction gradually increases in intensity. I) Stage XII (meiotic stage). The reaction is still weak until this step.
band (Fig. 5), while a weak 107 kDa band was detected only in Sup I (Fig. 5; left lane).

**DISCUSSION**

MAb Tsd-1 recognized a specific molecule of elongating and matured spermatids of the common tree shrew in a stage-dependent manner. Expression of Tsd-1 antigen, recognized by MAb Tsd-1, first occurred in spermatids at stage VI. Tsd-1 began to appear together with initiation of spermatid elongation, and reached the maximum intensity in matured spermatids at stage IV. The antigen may be functional to elongate spermatids.

On the other hand, a number of mid-piece antigens have been reported [1, 2, 8, 13, 16–18, 21]. For example, sp42 tyrosine kinase is localized in the mid-piece of spermatozoa in the boar [1]. This tyrosine kinase is a germ cell-specific gene product with highly conserved tissue expression extended to other mammalian species, such as the human, mouse, and rat. It is suggested that the cytoplasmic tyrosine kinase may play a role in a cell signaling network specific to haploid male germ cells [1]. MPM-2, a centrosomal material related to aster formation of oocytes, also exists in the neck and mid-piece of spermatozoa [13, 19]. It has been suggested that dephosphorylation of sperm mid-pieces by alkaline phosphatase can be rephosphorylated after injecting spermatozoa into oocytes, thus this centrosomal material is involved in fertilization events. Calsequerin-like calcium-binding protein is also localized in the mid-piece of human spermatozoa [2]. Therefore, such amorphous and structural materials must be present in the residual cytoplasm of the mid-piece of spermatozoa. The Tsd-1 antigen is also expressed in the mid-piece of matured spermatids and spermatozoa of the epididymis, indicating that the antigen may play a role in fertilization or related phenomena.

Tsd-1 is specific to the spermatogenic cells of the common tree shrew. If the Tsd-1 antigen acts in general fertilization, analogous molecules will be present in other animals. In this case, MAb Tsd-1 recognizes the molecule with a specific epitope of the common tree shrew. If Tsd-1 is specific to the common tree shrew, its function may be
suitable for the specificity of tree shrew reproduction. It is necessary to obtain more information on the TSd-1 antigen in the reproductive system of common tree shrew.

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