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Differential Distribution of Ganglioside GM3 in Seminiferous Tubule and Epididymis of Adult Rats

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Gangliosides are ubiquitous membrane components in mammalian cells and are suggested to play important roles in various functions such as cell-cell interaction, adhesion, cell differentiation, growth control and signaling. Among all ganglio-series gangliosides, GM3 has the simplest carbohydrate structure, and has been shown as a major ganglioside in male reproductive system. To study GM3 distribution in the seminiferous tubule and epididymis, frozen sections were stained with specific monoclonal antibody (MAb) against ganglioside GM3. In the seminiferous tubule of testis, pachytene spermatocytes and spermatids expressed ganglioside GM3, but not in spermatogonia and sertoli cells. Spermatogonia and sertoli cells near the basement membrane were negatively reacted to anti-GM3. In the epididymis, GM3 was expressed only in some interstitial cells. Taken together, these results suggest that the expression of ganglioside GM3 in rat seminiferous tubule and epididymis is spatio-temporally regulated during spermatogenesis.

Key words: Gangliosides, Spermatogenesis, Seminiferous tubule, Epididymis

INTRODUCTION

Gangliosides are sialic acid (NeuAc)-containing glycosphingolipids found widely in the plasma membranes of all vertebrate tissues and are particularly abundant in the central nervous system (CNS) (Svennerholm, 1980; Ji *et al.*, 1999; 2000). They play important roles in a large variety of biological processes such as cell-cell interaction, adhesion, cell differentiation, growth control, and receptor function (Hanada *et al.*, 1992; Choo *et al.*, 1995; 1999a; 1999b). Among the gangliosides, GM3 has the simplest carbohydrate structure and is known to be involved in induction of HL-60 differentiation (Nojiri *et al.*, 1986), modulation of cell proliferation (Hakomori, 1990; Choo *et al.*, 1995, 1999b), signal transduction (Hakomori *et al.*, 1998), maintenance of fibroblast morphology (Meivar-Levy *et al.*, 1997), and integrin-mediated cell adhesion (Kojima and Hakomori, 1996).

Spermatogenesis has been studied most extensively in the mammalian testis (Fawcett, 1975; Eddy and OBrien, 1994). Mammalian spermatozoa are produced by a process known as spermatogenesis in the testes in coiled tubes called seminiferous tubules. These seminiferous tubules consist of two types of somatic cells: the myoid or smooth muscle-like cells and the sertoli cells, and five types of germ cells: spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa. From each testis, spermatozoa pass into a coiled tube called the epididymis, which stores spermatozoa while they develop motility and fertilizing ability. Spermatozoa leave the epididymis during ejaculation, the expulsion of sper-

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matozoa-containing fluid from the penis. At that time, muscular contractions propel the sperm from the epididymis through another duct called the vas deferens. Spermatogenesis in seminiferous tubules of adult testis is regulated by several hormones as FSH and LH and cell cell interactions, implying the importance of surface membrane components including gangliosides. Actually, GM3 synthase was expressed in somatic and early germ line cells and epididymal epithelium of seminiferous tubules (Stern *et al.*, 2000). However, it remains unknown whether ganglioside GM3 is expressed in somatic cells and developing germ cells in rat seminiferous tubules.

A series of mouse monoclonal antibodies specific for different ganglio-series ganglioside have been established (Kotani *et al.*, 1992; Ozawa *et al.*, 1992). These monoclonal antibodies enable us to examine expression of ganglioside GM3 in adult rat seminiferous tubule and epididymis. The present study indicates that expression of GM3 is developmentally regulated in tissue- and cell specific-manners during spermatogenesis in seminiferous tubules and epididymis of adult rats.

MATERIALS AND METHODS

Monoclonal antibody

Rat seminiferous tubules and epididymis were appreciably positive to MAb against GM3, namely GMR6. Therefore, GMR6 was used for further experiments. The production and characterization of GMR6 have been described previously (Kotani *et al.*, 1992, 1993; Ozawa *et al.*, 1992).

Glycosphingolipids

GM3, GM1 and GD1a were kindly provided by Dr. I. Kawashima (Tokyo Metropolitan Institute of Medical Science). Other GSLs were prepared in our laboratory.

Glycolipid extraction and purification

Since blood cells are rich in gangliosides, testes for GSL analysis were collected from rats after perfusion with phosphate-buffered saline (PBS). Collected testes were then washed in PBS. Testis gangliosides were extracted and fractionated according to the procedure described by Choo *et al.* (1995). Briefly, two pairs of testes obtained from 12-week-old Wistar rats (Samyuk Laboratory Animals Inc., Korea) were homogenized in 0.2 ml ice-cold distilled water, and 10 ml of chloroform/methanol (1/1, v/v) were added to the homogenate. After centrifugation at 2000 g for 10 min, the supernatant was saved, and the pellet was re-extracted twice with the same solvent. Pooled supernatant was dried at 30°C with a rotary evaporator. The residue was dissolved in 7.5 ml of chloroform/methanol/water (30:60:8, v/v/v) and then charged onto a DEAE-Sephadex A-25 column to remove neutral lipids. The column was washed 7.5 ml of the same solvent to eliminate neutral lipids and then adsorbed acidic lipids were eluted with 15 ml of chloroform/methanol/0.8 M aqueous sodium acetate (30:60:8, v/v/v). Lipid fractions were separately evaporated to dryness and then subjected to alkaline treatment with 10 μl ammonia (12 N) in 0.2 ml of chloroform/methanol (1/1, v/v) overnight at room temperature. The reaction mixture was evaporated to dryness, and the residue was dissolved in distilled water and then applied to a Sep-Pak C18 cartridge (Millipore, Milford, MA) to eliminate salts. GSLs were eluted from the cartridge with 2 ml of methanol and 4 ml of chloroform/methanol (2:1, v/v) successively. The eluted material was concentrated and then analyzed for silica acid content (Svennerholm, 1957). One rat testis of 100 mg fresh weight contained 8 μg lipid-bound sialic on average.

HPTLC

For high-performance thin-layer chromatography (HPTLC) of gangliosides, HPTLC plates 5651 (Merck, Darmstadt, FRG) were used. The solvent system used for developing chromatograms was chloroform/methanol/0.2% $CaCl_2$ in water (55:45:10, v/v/v). Gangliosides were detected with resorcinol average.

HPTLC immunostaining

Immunostaining on TLC plates was performed as previously described by Choo (1999a). Briefly, HPTLC aluminium sheets 5547 (Merck, Darmastadt, FRG) were coated with 0.1% polyisobutylmethacrylate in cyclohexane for 80 s. They were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min and incubated with MAb solution in PBS for 2 h at room temperature. The sheets were washed PBS over 10 min, followed by the addition of biotin-conjugated goat anti-mouse IgM and IgG antibodies (Cappel), appropriately diluted with 1% BSA in PBS. After 2 h incubation, the sheets were washed in PBS and incubated with avidin- and biotin-linked horseradish peroxidase for 1 h at room temperature. After washing with PBS, they were incubated in 400 mg/ml o-phenylenediamine (Sigma) in 80 mM citrate-phosphate buffer (pH 5.0) containing 0.12% H_2O_2. Sheets were then washed in PBS to stop the reaction.

Tissue preparation

Male Wistar rats (10 weeks old; Samyuk Laboratory Animal Inc., Korea) were housed under controlled 14 h light and 10 h darkness, and fed a standard diet of pellets and water ad libitum. At 12 weeks, animals were decapitated and the testis and epididymis were removed immediately and mixed with Tissue-Tek® O.C.T. compound (Miles Inc., USA), then frozen in liquid nitrogen, and stored at -80°C until use.

Staining and immunofluorescence microscopy

The distribution of ganglioside in mature rat testes and epididymis was determined by indirect immnunofluorescence microscopy of frozen sections. Serial sections (6 μl thick) cut with a cryostat microtome were thaw-mounted on albumin-coated glass slides. The mounted sections were dried in air for 2 h and fixed with acetone at -20°C for 5min (Graus *et al.*, 1984). Sections were washed twice with PBS for 10 min and then incubated with 5% BSA in PBS for 15 min at room temperature. After washing with PBS twice, they were incubated with mouse monoclonal antibody diluted in PBS containing 5% BSA overnight at 4°C. They were washed with cold PBS four times, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody diluted in PBS to 1:100 for 3 h. After washing with PBS five times, the sections were sealed with a coverslip. To identify nuclei I μl/ml of a DNA-specific fluorescein dye (Hoechst 33342) was added. All fluorescent samples were observed with a Carl Zeiss Axiovert 25 microscope equipped for epi-fluorescence and photographed on a 35 mm film (Presto 400, Kodak Film). A control section was incubated without primary antibody. For delipidated controls, the section was treated prior to the staining, first with methanol and then with chloroform/methanol (1:1, v/v) for 10 min each (Suzuki and Yamakawa, 1981; Umesaki, 1984).

RESULTS

Thin-layer chromatography of rat testis gangliosides

Fig. 1A shows a profile of gangliosides in adult rat testes, suggesting that GM3 and GD3 are the major components. The presence of GM3, which was immunohistochemically detected in the testes, was further confirmed by TLC immunostaining (Fig. 1B). Ganglioside profiles were not significantly changed in the epididymis (data not shown).

Immunofluorescence staining of seminiferous tubules

Images by nuclear staining and Nomarski differential interference contrast microscopy show typical architec-

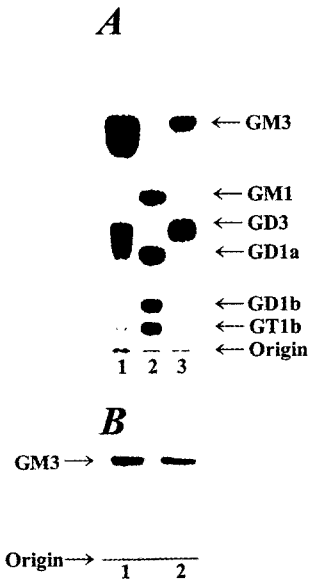

Fig. 1. TLC profiles of mature rat testis gangliosides. (A) Ganglioside fraction (1 μg sialic acid/lane) of the mature rat testis was developed on an HPTLC silica gel plate with chloroform/methanol/0.22% $CaCl_2$ (55:45:10, v/v/v) and detected with resorcinol spray. Lane 1, rat testis gangliosides; lanes 2 and 3, bovine brain gangliosides and a mixture of purified GM3 and GD3 as the standard, respectively. (B) TLC immunostaining of GM3. Lane 1, standard sample of GM3 (2 μg/lane); lane 2, rat testis ganglioside (10 μg/lane of sialic acid/lane). It was developed on an HPTLC aluminium sheet silica gel with the same solvent. Primary antibody used was GMR6 (anti-GM3) for lanes 1 and 2.

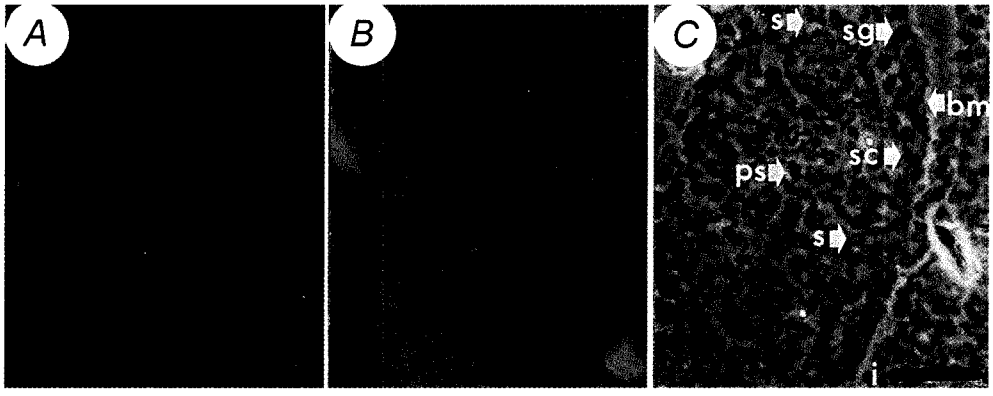

Fig. 2. Localization of GM3 by indirect immunofluorescence microscopy in the seminiferous tubules. Serial sections were immunostained with GMR6 (anti-GM3 MAb) and FITC-labeled goat anti-mouse IgM antibody (A), and then stained with Hoechst 33342 for DNA (B). Images with Nomarski optics (C) showing normal morphology of seminiferous tubules in the rat testis. bm, basement membrane; i, interstitial cells; ps, pachytene spermatocytes; s, spermatids; sc, sertoli cells; sg, spermatogonia. The bars present 200 μm.

ture of the seminiferous tubules of testis constructed of germ cells, a basement membrane and sertoli cells in the testis. The germ cells are further surrounded by interstitial cells (Fig. 2). GM3 was predominantly distributed in pachytene spermatocytes, spermatids, interstitial cells and in some sertoli cells (Fig. 2A). GM3 stained whole cytoplasm of these cells, but not the nucleus. Spermatogonia and almost sertoli cells near basement membrane were negative to anti-GM3 (Fig. 2A).

Immunofluorescence staining of epididymis

Images by nuclear staining and Nomarski differential interference contrast microscopy show typical architecture of the epididymis constructed of epithelial cells, interstitial cells and spermatozoa (Fig. 3B and C). Anti-GM3 monoclonal antibody was expressed only in some interstitial cells. Spermatozoa and epithelial cells were negative to the anti-GM3 antibody (Fig. 3A). Table I summarizes the immunohistochemical data on the differential localization of ganglioside GM3 in seminiferous tubules and epididymis.

Table I. Distribution of GM3 in the seminiferous tubules and epididymis

Organs	Cell types	Degree of expression
Seminiferous tubules	Spermatogonia	-
	Pachytene spermatocytes	+
	Spermatids	+
	Sertoli cells	$+_s$
Epididymis	Epithelial cells	-
	Interstitial cells	$+_s$
	Spermatozoa	-

Ganglioside GM3 was detected by GMR6 with indirect immunoflouresence.
+, positive; -, negative; $+_s$, positive not all the cells.
Scores were from one experiment representative of three similar repeats.

Delipidation treatment for tissue sections and control staining

Images by nuclear staining and Nomarski differential interference contrast microscopy show typical architecture of the seminiferous tubules (Fig. 4B and C) and epididymis (Fig. 4E and F). When serial sections were delipidated with chloroform/methanol (1:1, v/v) before immunostaining, they became negative to anti-GM3 monoclonal antibody (Fig. 4A). Controls without the primary and antibody did not show significant staining (Fig. 4D). Taking these results together, we conclude that GM3 MAb reacts specifically with the ganglioside GM3, but not with the carbohydrate portions of glycoproteins in seminiferous tubules and epididymis.

DISCUSSION

The present study is, to our knowledge, the first immunohistochemical report showing the expression pattern of gangliosides in the seminiferous tubule and epididymis of adult rats. The expression pattern in this context reflects the immunoreacivity of the ganglioside GM3 concerned. In the seminiferous tubules, GM3 was detected in pachytene spermatocytes and spermatids, suggesting that GM3-positive germ cells are pachytene spermatocytes and spermatids. However, no spermatogonia were GM3 positive, suggesting that GM3 is likely to be a signal molecule for differentiation between the spermatogonia and pachytene spermatocytes.

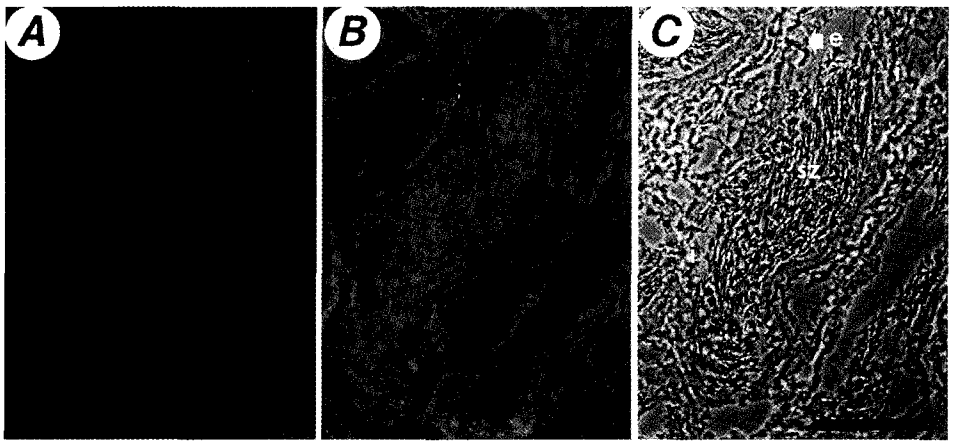

Fig. 3. Localization of GM3 by indirect immunofluorescence microscopy in the epididymis. Serial sections were immunostained with GMR6 (anti-GM3 MAb) and FITC-labeled goat anti-mouse IgM antibody (A), and then stained with Hoechst 33342 for DNA (B). Images with Nomarski optics (C) showing normal morphology of in the rat epididymis. e, epithelial layer; i, interstitial cells; sz, spermatozoa. The bars present 200 μm.

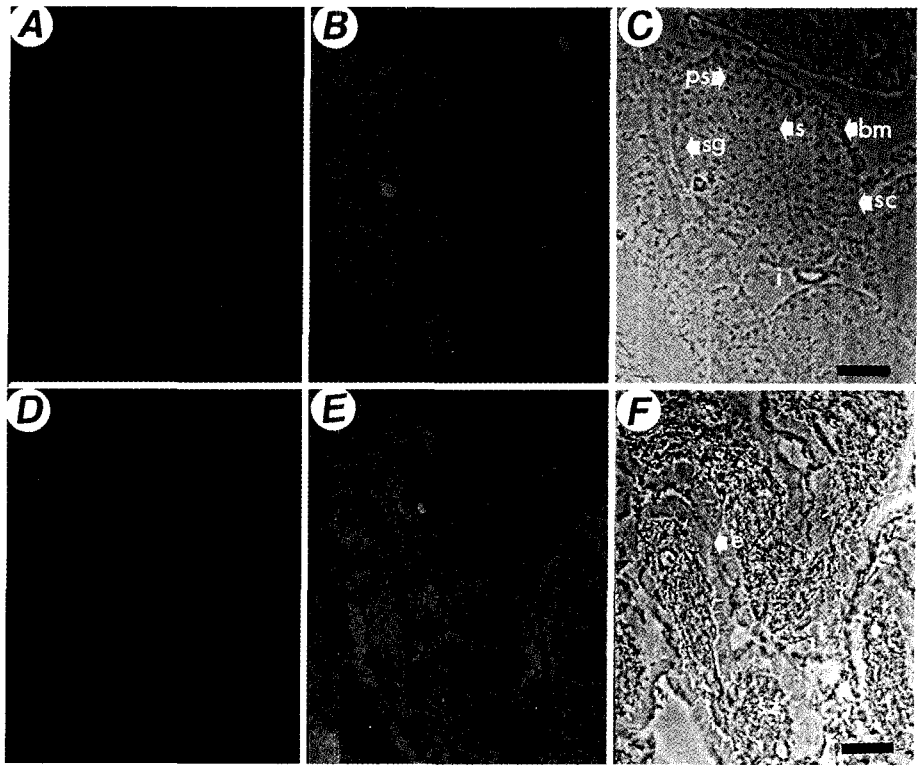

Fig. 4. Indirect immunofluorescence analysis of delipidated sections. The seminiferous tubules (A-C) were delipidated by chloroform/methanol treatment and immunostained with GMR6 (anti-GM3 MAb) and FITC-labeled goat anti-mouse IgM antibody, and then stained with Hoechst 33342 for DNA. (D-F) control without primary antibody in epididymis. (A) and (D) are images with Immunostaning; (B) and (E) are images with DNA staining; (C) and (F) are images of Nomarski optics showing normal morphology of the seminiferous tubules (C) and epididymis (F). bm, basement membrane; i, interstitial cells; ps, pachytene spermatocytes; s, spermatids; sc, sertoli cells; sg, spermatogonia; sz, spermatozoa. The bars present 200 μm.

One of the major gangliosides in rat seminiferous tubules, namely GD3, was not detected by immunohistochemistry. This kind of discrepancy between immunohistochemical and biochemical detection is occasionally found with gangliosides (Hakomori, 1981). With this particular ganglioside, GD3, Seyfried and Yu (1985) reported such a discrepancy in rat oligodendroglia. The nature of this discrepancy still remains to be clarified.

Several studies have demonstrated that sertoli cells are involved in the RNA and DNA synthesis of germ cells (Rivarola *et al.*, 1985) and spermatogonial divisions and signaling to the developing sperm (Meehan *et al.*, 2000; Meng *et al.*, 2000). However, to date, the testicular gangliosides from sertoli cells associated with spermatogenesis are unknown. Ganglioside GM3 was predominantly distributed in some sertoli cells of seminiferous tubules (Fig. 2A), suggesting that the testicular-specific ganglioside GM3 may play an important signal for mitosis, meiosis and spermiogenesis of germ cells during spermatogenesis. Anti-GM3 monoclonal antibody stained the entire cytoplasm of germ cells, but not the nucleus, suggesting that it may play some additional roles in the cytoplasm. Interestingly, in the previous biochemical study, GM3 synthase is found in somatic and early germ line cells of seminiferous tubules (Stern *et al.*, 2000). These localization patterns of GM3 synthase were similar to that of germ cells stained by anti-GM3 monoclonal antibody (Fig. 1). Thus, these results indicate that anti-GM3 monoclonal antibody used in present study is specific monoclonal antibody against ganglioside GM3.

It is generally accepted that spermatozoa are passed into epididymis for developing motility and fertilizing ability. In the epididymis, GM3 was not expressed in spermatozoa, suggesting that ganglioside GM3 plays an important role in maturation of germ cells and obtain of fertilizing ability.

We conclude that the expression of gangliosides in the seminiferous tubules and epididymis is spatio-temporally regulated during spermatogenesis. The present study could not clarify the biological functions of gangliosides in germ cells. To clarify the pathophysiological role of the testicular gangliosides and ultrastructural localization of GM3 at various times of spermatogenesis in the germ cells, further work is needed.

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