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The Differential Distribution of Ganglioside GM3 in Atretic Follicles During Follicular Development of Adult Rat Ovary

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Gangliosides are ubiquitous membrane components in mammalian cells and are suggested to play essential roles in cellular phenomena such as cell-cell interaction, differentiation, and signal transduction. Rat ovary contained GM3 as major ganglioside. In order to study GM3 distribution in the atretic follicles and its possible changes during follicular development, frozen sections were stained with specific monoclonal antibodies against eleven ganglio-series gangliosides including GM3. In the atretic follicles, GM3 was expressed in a spatio-temporally different manner during follicular development, but GM1 and other gangliosides were not immunohistochemically detected. In atretic follicle from primary follicle stage, GM3 was expressed in all the theca cells and some granulosa cells adjacent to oocyte. In atretic follicle from secondary follicle stage, GM3 were expressed in all theca cells and granulosa cells. In atretic follicles from developing Graafian follicle stages, GM3 was similarly expressed as in secondary follicle stage.

KEY WORDS: Ganglioside, Follicular Development, Rat Ovary, Atretic Follicle

Gangliosides, Glycosphingolipids (GSLs) containing sialic acid, are ubiquitous membrane components of essentially all eukaryotic cells including ovarian cells and the majority are assumed to be present at the outer leaflet of plasma membranes (Hakomori, 1981). Although evidence has been accumulated that GSLs are involved in cell proliferation and differentiation (Okada *et al.*, 1985; Bremer *et al.*, 1986; Hanada *et al.*, 1992) and cell-cell interactions (Huang, 1978; Blackburn and Schnaar, 1983), the exact organization and the function of GSLs in the plasma membrane still remain to be established.

In all the adult animal tissues, a balance between cell proliferation and cell loss must be established. This is particularly important in the ovary, where the tissue mass is maintained throughout fertile life during the rapid proliferation of follicular cells. Cohorts of growing follicles are continuously generated, from which dominant follicles are selected during each estrous cycle for development to the late Graafian follicle stage (Schwartz *et al.*, 1972). To compensate for the growth of follicles and to maintain the size of the ovary, many follicles are eliminated by the process of atresia. The fundamental reproductive units of the ovary are the primordial follicles. Each primordial follicle consists of an oocyte arrested in the dictyate stage of meiotic prophase, a single layer of granulosa cells and a basement membrane. At various times throughout the estrous cycle, some primodial follicles are selected to initiate growth, but compared to the total number of primordial follicles, only a small number are stimulated to develop at any one time.

In the primary follicle, the oocyte is surrounded by two or more layers of cuboidal granulosa cells and then theca cells. Although atresia, or follicle degeneration, is very rare in primordial follicles, once a primordial follicle begins to grow and

reaches the primary follicle stage, there occurs a sharp increase in the percentage of atretic follicles found in the ovary (Richards and Midgley, 1976). In response to the FSH stimulation, the granulosa cells begin working in concert with the steroid-secreting cells of the theca interna to produce a hormonal milieu that initiates and supports the differentiation of a early secondary follicle into a preovulatory follicle. By definition a secondary follicle is an ovarian follicle with an antrum or fluid-filled cavity.

During estrous cycle, only a small number of the primordial and primary follicles develop to the Graafian follicle and undergo ovulation, indicating that 99.9% of all the follicles in the ovary undergo atresia. Atresia of the primordial and primary follicles is suggested to be important for ovulation, although the regulatory mechanism of cell atresia is still unclear. In the subsequent stage of development, the connection between granulosa cells is gradually loosened with the development of a new, liquid-filled intercellular space. The follicles at this stage are called Graafian follicles in which the oocyte is surrounded by loosened granulosa cells, now called cumulus cells. From the puberty until the menopause, only one of the secondary follicles that develops during each cycle is selected to continue its differentiation into a preovulatory follicle; the rest degenerate.

Recently, Choo *et al.* reported that GM3, GM1 and GD1a during follicular development and luteinization in the adult rat ovaries were spatiotemporally expressed; however, little information is available about the direct relationship between follicular degeneration and the expression of gangliosides. In fact, cultured granulosa cells derived from immature rat ovary express GM3 and GM1 in response to insulin and follicle stimulating hormone (FSH) *in vitro* (Hattori and Horiuchi, 1992). In addition, a role for ovarian steroids in blocking (estrogens) or inducing (androgens) apoptosis in ovarian granulosa cells of the estrogen implanted, hypophysectomized, immature rat has also been reported (Billig *et al.*, 1993). Despite the rapidly growing list of hormones reported to regulate granulosa cell differentiation and death in various model systems, it remains unknown whether gangliosides are expressed in granulosa cells of atretic follicles during estrous cycle *in vivo*. Recently, a series of mouse monoclonal antibodies (MAbs) specific for different ganglio-series gangliosides have been established (Kotani *et al.*, 1992; Ozawa *et al.*, 1992).

These MAbs enabled us to examine the spatial and temporal expression of ganglio-series gangliosides in atretic follicles of the adult rat ovaries. This paper describes immunohistochemical analyses of spatio-temporal expressions of GM3, a major ganglioside in atretic follicles during follicular development.

Materials and Methods

Monoclonal antibodies

Eleven MAbs specific for one of the following gangliosides, GM3, GM2, GM1, GD3, GD2, GD1b, O-Ac-GD3, GT1b, GQ1b, GD1a and GT1a were used. Only atretic follicles were appreciably positive to GM3. Therefore GM3 MAb was used for further experiments. The production and characterization of these MAbs has been described previously (Kotani *et al.*, 1992, 1993; Ozawa *et al.*, 1992).

Tissue preparation

Female Wistar rats were housed under controlled conditions of 14 hr light and 10 hr darkness and fed a standard diet of pellets and water ad libitum. Vaginal smears were taken daily to examine the duration and regularity of the estrous cycles, and only the rats having regular cycles were used. Administration time of PMSG was determined in the metestrus stage, as judged by vaginal smear examination. Superovulation was induced in 12-week female rats by intraperitoneal injection of 30 I.U. PMSG, followed 50 hr later by 30 I.U. hCG. The ovulatory response began ~11 hr after the injection of hCG. At various times after the administration of hCG, animals were decapitated and the ovaries were immediately removed and mixed with O.C.T. compound (Miles Inc., USA), then frozen in liquid nitrogen and stored at −80°C until use. The time intervals chosen were 5 hr and 9 hr after hCG injection.

Ovaries were also obtained from non-injected controls.

Staining and immunofluorescence microscopy

The distribution of ganglioside GM3 in mature rat ovaries was determined by indirect immunofluorescence microscopy of frozen sections. Serial sections (6 μm thick) cut with a cryostat microtome were thaw-mounted on albumin coated glass slides. The mounted sections were dried in air for 2 hr and fixed with acetone at -20°C for 5 min (Graus *et al.*, 1984). When frozen sections were not fixed with acetone, immunofluorescence staining was much poorer. Sections were washed twice with PBS at room temperature for 10 min and then incubated with 5% BSA in PBS for 15 min at room temperature. After washed with PBS twice, they were incubated with mouse MAb diluted in PBS containing 5% BSA overnight at 4°C. They were washed with cold PBS four times, and then incubated with FITC-conjugated goat anti-mouse IgM antibody diluted in PBS to 1:100 for 1 hr. After washed with PBS five times, the sections were sealed with a coverslip. To identify nuclei, 1 μg/ml of a DNA-specific fluorescent dye (Hoechst 33342) was added. All fluorescent samples were observed with a Nikon Microphot-FX microscope equipped for epifluorescence and photographed on a 35 mm film (Presto 400, Fuji Film). A control section was incubated without primary antibodies.

Results

Immunofluorescence staining

Atresia in primary follicle stage

Images by nuclear staining and Nomarski differential interference contrast microscopy show typical architecture of the developing primary and atretic preantral follicles constructed of an oocyte, granulosa cells and theca cells (Fig. 1 E). An atretic preantral follicle was found, located predominantly in around of the healthy primary follicle. The early stage of preantral atretic follicle was recognized by degeneration of the oocyte; subsequent stages in atretic process were recognized by disorganization of the layers of granulosa cells and evidence of degenerating granulosa cells nuclei (Byskov, 1978).

In the developing primary follicle, anti-GM3 Mab stained whole cytoplasm of all the theca and interstitial cells (Fig. 1A), but not granulosa cells. However, at atretic preantral follicle from this stage, some granulosa cells adjacent to oocyte were positive to anti-GM3 Mab. These results indicate that granulosa cells in the atretic follicle start expressing GM3 before secondary follicle stage.

Atresia in Secondary follicle stage

Concomitant with the development from a primary follicle into a secondary follicle, the granulosa cells and the theca cells proliferate and an antrum is formed (Fig. 1F). An atretic preantral follicle was frequently found in the secondary follicle stage. In this stage, the staining patterns of the secondary follicle and preantral atretic follicle were quite similar to those of the primary follicle stage, except that all granulosa cells of atretic preantral follcle became positive to anti-GM3 Mab (Fig. 1B).

Atresia in Graafian follicle stage

It is difficult to collect ovaries which contain developing Graafian follicles without hormonal injection. In order to specify the timing of ovulation more precisely, ovaries were examined at various times after injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). Fig. 2 shows immunohistochemical images of GM3 in developing Graafian follicles and preantral atretic follicles at different stages of development. At the developing Graafian follicle stages, the granulosa cells surrounding an oocyte are collectively called cumulus cells, and the connection between the oocyte and cumulus cells is loosened (Fig. 2E and F). In the middle Graafian follicle from 5 hr after hCG injection, GM3 was predominantly distributed in the theca cells and granulosa cells near the theca layer as shown in Fig. 2A. Figure 3A shows the overall staining pattern of the whole Graafian follicle from 5 hr after the hCG injection.

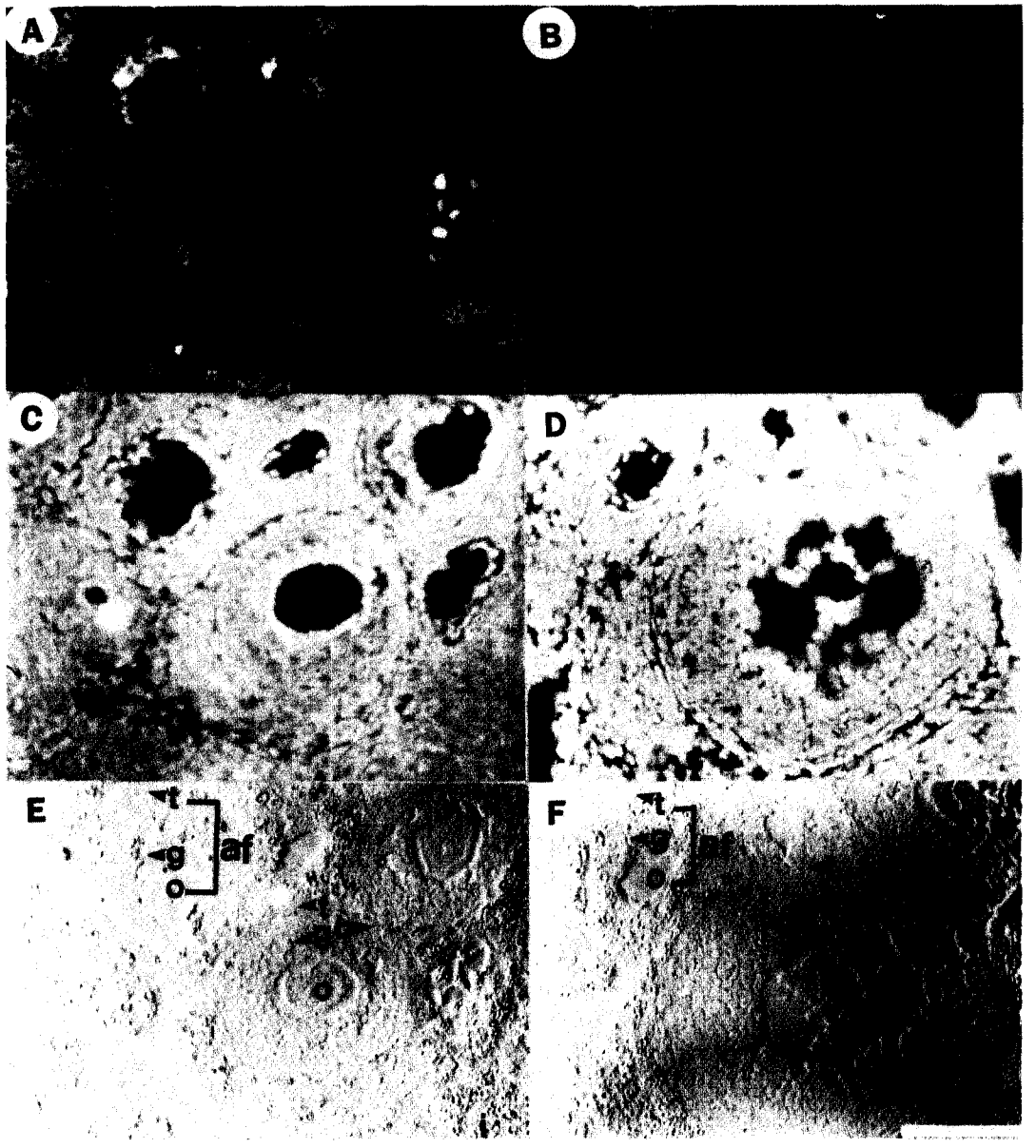

Fig. 1. Localization of GM3 by indirect immunofluorescence microscopy in preantral atretic follicles from the primary and secondary follicle stages of an adult rat ovary. Serial sections were immunostained with GMR 6 (anti-GM3 Mab) and FITC-labeled goat anti-mouse IgM antibody, and then DNA-stained with Hoechst 33342. (A) and (B) are images with immunostaining; (C) and (D) are images with DNA staining; (E) and (F) are images with Nomarski optics showing normal morphology of primary and secondary follicles. In (E) and (F), each small follicles are mildly atretic (upper left; still possessing many layers of granulosa cells) af, atretic follicle; i, interstitial tissue; t, theca layer; b, basement membrane; g, granulosa layer; o, oocyte. The bar represents 100 μm.

By 9 hr after injection of hCG (2 hr before ovulation), all granulosa and cumulus cells were expressed GM3 (Fig. 2B). Throughout these Graafian follicle stage, intense staining for GM3 of

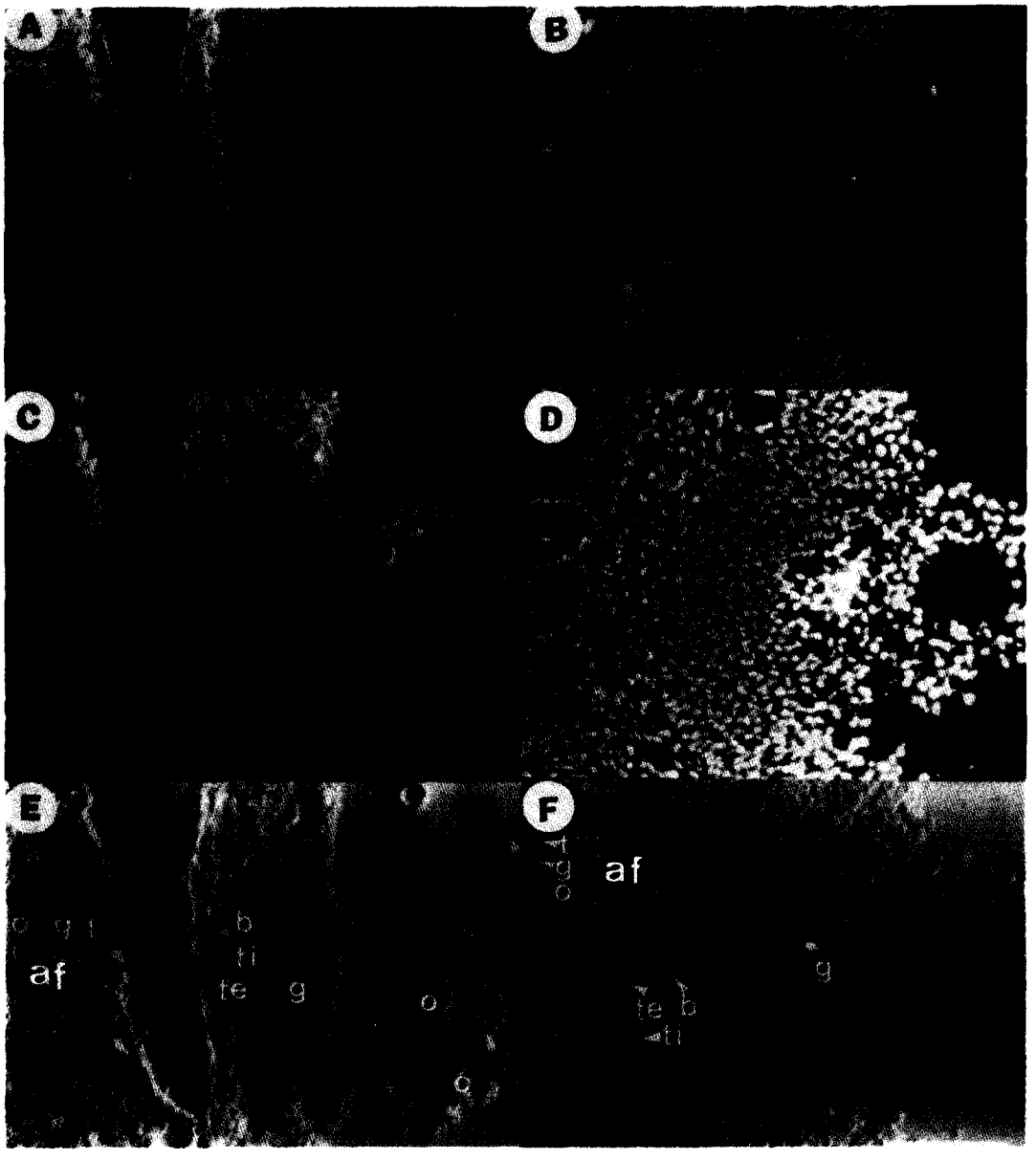

Fig. 2. Localization of GM3 by indirect immunofluorescence microscopy in preantral atretic follicles from developing Graafian follicles after hCG injection. Ovaries were fixed and frozen at 5 hr (left) and 9 hr (right) after hCG injection. Serial sections were immunostained with GMR6 (anti-GM3 MAb) and FITC-labeled goat anti-mouse IgM antibody, and then stained with Hoechst 33342 for DNA. (A) and (B) are images with immunostaining; (C) and (D) are images with DNA staining; (E) and (F) are images with Nomarski optics showing normal morphology of the Graafian follicles. In (A) and (B), each small follicles are mildly atretic (upper left; still possessing many layers of granulosa cells) af, atretic follicle; te, theca externa layer; ti, theca interna layer; b, basement membrane; g, granulosa layer; c, cumulus oophorus; o, oocyte. The bar represents 100 μm.

preantral atretic follicles persisted in the granulosa cells. Furthermore, these atretic preantral follicles were not change much from secondary follicles in the expression pattern of GM3. Table I.

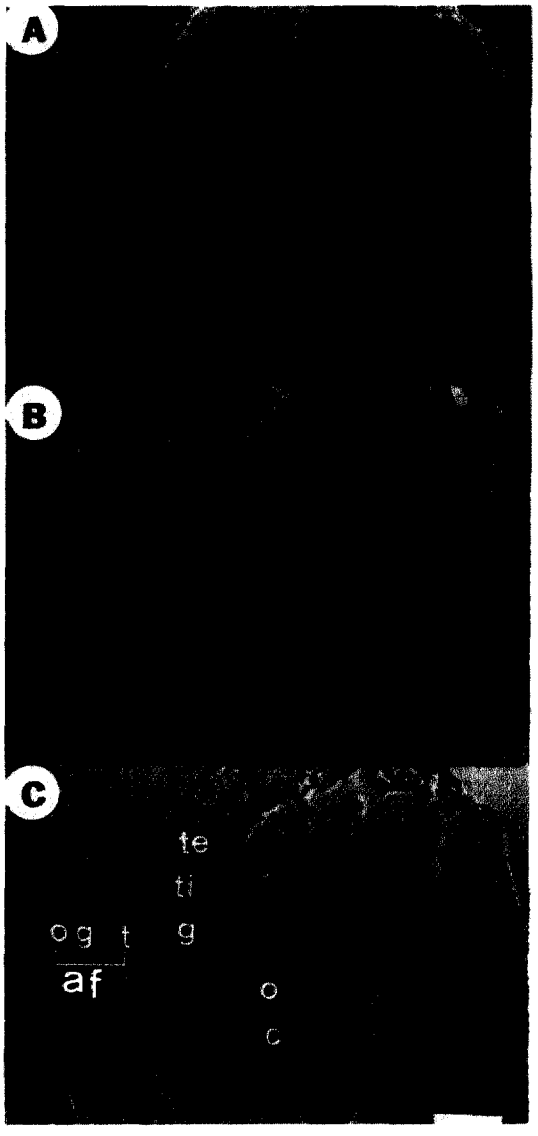

Fig. 3. Localization of GM3 by indirect immunofluorescence microscopy in preantral atretic follicle from the whole Graafian follicle from 5 hr after hCG injection. Ovary was fixed and frozen at 5 hr. The section was immunostained with GMR6 (anti-GM3 MAb) and FITC-labeled goat anti-mouse IgM antibody, and then stained with Hoechst 33342 for DNA. (A) immunostaining; (B) DNA staining; (C) Nomarski optics image showing normal morphology of the Graafian follicle. In (C), small follicle is mildly atretic (upper left; still possessing many layers of granulosa cells) af, atretic follicle; te, theca externa layer; ti, theca interna layer; b, basement membrane; g, granulosa layer; c, cumulus oophorus; o, oocyte. The bar represents 100 μm

summarizes the immunohistochemical patterns of GM3 in atretic follicles from various stages of developing follicles. These results indicate that the intense staining for anti-GM3 Mab in granulosa cells of atretic follicle from secondary follicle stage was not influenced by the timing of developing Graafian follicle for ovulation.

Discussion

The present study is the first immunohistochemical report showing the ganglioside expression in atretic follicles during follicular development. The expression pattern in this context reflects the immunoreactivity of ganglioside GM3 concerned, but not their presence or absence. One of the major gangliosides in degenerating follicles, namely GM3, was well detected by immunohistochemistry.

In the preantral atretic follicle from primary follicle stage, GM3 was predominantly distributed in the theca cells and in some granulosa cells near the oocyte (Fig. 1A), suggesting that GM3-positive cells are the granulosa and theca cells. However, not granulosa cells of developing normal primary follicle were GM3 positive, suggesting that GM3 in granulosa cells may play an important signal for cell survival and cell death.

In the secondary and Graafian follicle stages, all granulosa cells of preantral atretic follcles expressed GM3 (Fig. 1B, 2A and B). There is a possibility that, as discussed above, GM3 was present in all granulosa cells of primary follicles, yet immunohistochemically undetectable by unknown mechanism. If this is the case, the immunohistochemically distinct change in the GM3 expression during follicular development may support the hypothesis that this ganglioside plays important roles in follicular growth and atresia.

Sex steroids are important intraovarian regulators of follicle atresia, and the profile of sex steroid production in healthy follicles differs from that in atretic ones. In rats and hamsters, the production of both estrogens and androgens decreases in atretic follicles (Uilenbroek *et al.*,

Table I. Distribution of ganglioside GM3 in atretic follicles from various stages of developing follicles

Cell Type	Degree of Expression[a]		
	primary follicle	secondary follicle	Graafian follicle[b]
Theca cells	++	+++	+++
Granulosa cells	+	+++	+++
Oocyte	–	–	–

Ganglioside GM3 was detected by GMR6 MAb with indirect immunofluorescence microscopy. [a] ++, strong; ++, moderate; +, weak; –, negative. [b] 5 hr and 9 hr after hCG injection

1980; Braw and Tsafriri, 1980; Braw *et al.*, 1981; Terranova, 1981). In human, ovine, and porcine species, estradiol production by atretic follicles is also decreased, but the production of androgen is increased (Carson *et al.*, 1981; Maxson *et al.*, 1985; Moor *et al.*, 1978). However, a common denominator for all of these species is decreased estrogen production in atretic follicles. In general, changes in steroidogenesis can be observed before morphological signs of atresia (Uilenbroek *et al.*, 1980; Braw and Tsafriri, 1980; Bill and Greenwald, 1981). In degenerating follicles, granulosa cells expressed GM3. Indeed, it is suggested that some gangliosides play an essential role for cell growth (Hakomori, 1981). Thus, it is likely that the expression, or at least immunohistochemical reactivity, of GM3 is related to the atretic process of follicles with sex steroids.

In conclusion, the expression and/or the architecture of ganglioside GM3 in the preantral atretic follicles is spatio-temporally regulated during estrous cycle throughout follicular development. It is currently under investigation in our laboratory to elucidate the functions of gangliosides in these biologically important events.

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Abbreviations

BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MAb, monoclonal antibody; PBS, phosphate-buffered saline; PMSG, pregnant mare serum gonadotropin. Gangliosides are named according to the nomenclature of Svennerholm (1964).

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성숙한 Rat 난소의 난포발달이 진행되는 동안 폐쇄난포에서의 Ganglioside GM3의 서로 다른 분포

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Ganglioside는 포유동물세포에 편재하는 막성분으로서, 이들은 세포상호간의 접착, 분화 및 정보전달과정에 관여하는 것으로 알려지고 있다. Rat난소는 주요한 Ganglioside로서 GM3를 함유하고 있으며, 본 연구에서는 폐쇄난포에서 이들의 분포여부와 난포의 발달과정에서의 변화여부를 조사하기 위하여, Rat 난소의 동결절편을 이용해 GM3를 포함 11종류의 Ganglio-series Ganglioside에 대해 특이한 단일항체로서 염색시켰다. 폐쇄난포들에서 GM3는 난포발달이 진행되는 동안 시간적, 공간적으로 서로 다른 양식으로 발현하였다. 그러나 GM1을 포함한 다른 종류의 Ganglioside들은 면역조직화학적으로 검출되지 않았다. 일차난포에서 관찰되는 폐쇄난포들에서 GM3는 모든 교막세포와 난자에 인접한 과립막세포의 일부에서 발현 하였다. 이차난포의 시기에서 이들 폐쇄난포의 GM3는 모든 교막세포와 과립막세포들에서 발현 하였다. 이어서 발달하고 있는 그라프난포의 시기에서 관찰되는 폐쇄난포의 GM3 발현은 이차난포에서의 분포패턴과 유사함을 보여 주었다.