ORIGINAL ARTICLE

Biochemical Analysis of Glycosphingolipids in Murine Uterus and Human Uterine Endometrium with Special Reference to the Sexual Cycle

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Abstract. Uterus is an organ in which cellular proliferation and differentiation are strictly controlled by sex steroid hormones. Since we found a characteristic expression of sulfatide ($\text{I } ^3\text{SO}_3\text{-GalCer}$) in the human endometrium during its secretory phase, we decided to analyze developmental changes of glycolipids in murine uteri with special reference to the estrous cycle. Moreover, we analyzed sulfotransferase activity for the synthesis of sulfatide in the endometria in the proliferative and secretory phases and that in the epithelial and stromal cells derived from the human uterine endometrium. In the murine uterus, contents of GlcCer, LacCer, and Gb₄Cer were relatively constant both during estrus and diestrus, but those of Gb₃Cer and Gg₄Cer during estrus were significantly greater than those during diestrus. In contrast, GM₃ and sulfatide contents during diestrus were higher than those during estrus. This increase in GM₃ and sulfatide during diestrus was characteristically observed in the uterus after sexual maturity. In human uterine endometrium, sulfotransferase activity for the synthesis of sulfatide was found only in the epithelial cells, but not in the stromal cells. Furthermore, the synthetic potential of sulfatide in the human endometrium was higher in the secretory phase than in the proliferative phase. Thus, sulfatide metabolism in human endometrial epithelial cells as well as in the murine uterus is supposed to be well controlled under hormonal regulation, and sulfatide may participate in several reproductive functions of the uterus. (Keio J Med 40 (2): 82–89, June 1991)

Key words: glycolipids, sulfatide, endocrinological environment

Introduction

Glycosphingolipids are ubiquitous membrane components in mammalian tissues and cells, and are mainly located at the exterior surface of the plasma membrane. Their composition is known to change in association with cellular differentiation, proliferation, and morphogenesis, and this change is also species- and tissue-specific. Although their functional significance has not yet been clarified, they are somehow involved in the differentiation-, proliferation-, and transformation-associated antigens and receptors for various hormones, bacterial toxins and lectins.^{1,2} Therefore they are thought to act as a recognition molecule or modulator for the functional membrane components that regulate and carry out various cellular functions.^{3,4} In our previous study,⁵ we

found the characteristic expression of sulfatide in human uterine endometrium during its secretory phase. Since cellular proliferation and differentiation in the endometrium are strictly controlled by sex steroid hormones, the glycolipid metabolism would appear to be under the periodic control of hormonal regulation. To further evaluate this finding, we analyzed the glycolipids in the murine uterus, even though the uterine structure and the sexual cycle in the murine uterus are different from those of the human. Moreover, in order to evaluate the functions of sulfatide in the human endometrium, we investigated the localization and anabolism of sulfatide in the epithelial and stromal cells isolated from the human uterine endometrium. The glycosphingolipid nomenclature used follows the IUPAC-IUB recommendations.⁶

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Materials and Methods

Analysis of glycosphingolipids in murine uterus

Tissues: Mice (BALB/c, 2-to-8-week-old females) were purchased from Charles River Inc. (Tokyo, Japan). The estrous cycle was determined by staining of the vaginal smears with Sheaffer red ink/blue ink/water (2:20:110, by vol); and mice over 5 weeks of age were found to undergo the cycle. All of the uterine tissues at each age and during the estrous cycle in Table 1 were combined, respectively, and were stored at -20°C until the glycolipid analysis was performed.

Extraction and preparation of glycosphingolipid from murine uterus: The extraction and preparation of crude lipids were carried out according to the method reported previously.⁷ Briefly, the tissues were homogenized and lyophilized, and total lipids were extracted from the lyophilized powder successively with chloroform/methanol/water (20:10:1 and 10:20:1, by vol) and chloroform/methanol (1:1, by vol) at 40°C . The extracts were combined, and then cholesterol content in the total lipid extract was determined by gas liquid chromatography (GLC) with 5 α -cholestane as an internal standard.⁸ The total lipids were fractionated into neutral and acidic fractions by DEAE-Sephadex A-25 (acetate form) column chromatography. Neutral glycosphingolipids were prepared from the neutral lipid fraction by successive acetylation, Florisil column chromatography and deacetylation,⁹ while acidic glycosphingolipids were prepared from the acidic lipid fraction by mild alkaline hydrolysis to cleave the ester-containing lipids, followed by dialysis. The acidic and neutral glycosphingolipids thus obtained were developed on thin-layer chromatography (TLC) plates with chloroform/methanol/0.5% CaCl_2 in water (55:45:10, by vol) and chloroform/methanol/water (65:35:8, by vol), respectively, and the spots were visualized with orcinol- H_2SO_4 reagent.

Quantitative determination of the lipid components: Lipid-bound sialic acid in the acidic glycolipid fraction was measured by the resorcinol-HCl method,¹⁰ and the contents of sulfoglycolipids and individual neutral glycolipids were determined densitometrically with a dual-wavelength TLC densitometer (CS-9000, Shimadzu Co, Kyoto) after location of the spots with orcinol- H_2SO_4 reagent. The analytical and control wavelengths for quantification of the orcinol-positive spots were 420 and 700 nm, respectively. Calibration curves for the densitometric quantification were made with standard glycolipids, GM_3 and GlcCer , prepared from human erythrocyte and horse kidney, respectively. In addition, TLC immunostaining with carbohydrate-specific antibodies was used for the quantitative determination of

Gg_4Cer and Forssman antigen as follows:¹¹ The neutral glycolipids obtained were chromatographed on TLC plates (Polygram, Macherey-Nagel, Duren, FRG) with chloroform/methanol/water (65:35:8, by vol), and the plates were incubated with a blocking buffer (1% polyvinylpyrrolidone (PVP), 1% ovalbumin and 0.02% NaN_3 in phosphate-buffered saline (PBS), pH 7.3) at 37°C for 1 hr, and then incubated at 37°C for 2 hr with a rabbit anti- Gg_4Cer or an anti-Forssman antibody diluted 1:500 with 3% PVP in the PBS. After washing the plates 5 times with 0.1% Tween 20 in the PBS, the antibody bound on the plates was detected with a peroxidase-conjugated anti-rabbit immunoglobulin (Cappel Lab, Cochranville, PA, USA) antiserum and the peroxidase substrates (H_2O_2 and 4-chloro-1-naphthol). Gg_4Cer was prepared from GM_1 , which was purified from human brain by treatment with 1M formic acid at 80°C for 1 hr. Forssman antigen was prepared from horse kidney.¹² Anti- Gg_4Cer or anti-Forssman antisera were prepared as follows:^{12,13} 2 mg of Gg_4Cer or the Forssman antigen and 1 mg of bovine serum albumin (fraction V, Sigma Chemicals, St Louis, Mo, USA) were dissolved in 1 ml of the PBS and emulsified with 1 ml of Freund's complete adjuvant (Iatron Chemicals, Tokyo). When 1 ml of this antigen emulsion was injected into the footpads of a rabbit, the highest titer of antiserum was obtained after 4 or 5 weeks without a booster injection. The titers of anti- Gg_4Cer and anti-Forssman antisera were found to be 30,000 and 160,000, respectively, and the antibodies did not react with other glycosphingolipids, such as GlcCer , LacCer , Gb_3Cer and Gb_4Cer , on immunodiffusion or by the ELISA technique.¹⁴

PAPS: *GalCer* sulfotransferase in the cells derived from human uterine endometrium and endometria in the proliferative and secretory phases

Tissues: Normal human uterine endometrium was obtained by curetting the tissue at hysterectomy or from outpatients, and the tissues were immediately transferred in Ham's F-12 medium containing 10% fetal calf serum (FCS) under sterile conditions.

Separation of the epithelial and stromal cells from human uterine endometrium: The endometria used in this experiment were at the mid-to-late secretory phases as confirmed by histological examination. The tissues were first washed several times with Ham's F-12 medium containing 10% FCS, minced into cubes (approximately 1 mm^3) and were incubated with 0.25% collagenase (Sigma, St Louis, MO, USA) in the same medium (0.5 cm^3 tissue/5 ml) by the modified procedure as described previously.^{15–17} At 15-min intervals, the tissue was mixed by pipetting several times to facilitate the enzyme reaction and was examined by phase contrast microscopy. Within

1 to 2 hours, the glands containing the epithelial cells were liberated in a form free from nonepithelial cells. After separation of the epithelial cells, the stromal cells still adhering to the epithelial clumps were removed by shaking vigorously and were allowed to stand for 5 min. After removal of the top two-thirds of the suspension, fresh medium was added to the remaining portion and mixed well. This procedure was repeated several times to separate the stromal cells, which were allowed to attach to a plastic dish. Next, the cells were cultivated with a fresh medium in a plastic flask (25 cm², Falcon) in a 5% CO₂ atmosphere at 37°C for 45 min. Then the flasks were inverted, the attached cells were separated from the free cells, and the procedure was repeated several times to increase the purity.

PAPS: GalCer sulfotransferase in the epithelial and stromal cells from human uterine endometrium: The glandular epithelial cells and stromal cells separated from human uterine endometrium as above were cultivated for 96 hrs in Ham's F-12 medium, and 5 µCi/ml of ³⁵S-PAPS (New England Nuclear) was added to the medium. After cultivation for 20 hrs, the medium was removed and the cells were washed three times with PBS, pH 7.2, and harvested with a rubber policeman. Lipids were extracted with 1 ml of chloroform/methanol (1:1) at 45°C for 30 min. The combined extracts were washed by Folch procedure¹⁸ to remove the water-soluble radioactive materials and were chromatographed on a TLC plate (0.25 mm thick, Merck, FRG) with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol). Radioautograms of the TLC plate were made with X-ray film (X-OMAT, Kodak).

PAPS: GalCer sulfotransferase in human uterine endometrium in the proliferative and the secretory phases: The human endometria in the proliferative and the secretory phases obtained were cut into about 2-mm cubes under sterile conditions and were incubated in 1 ml of Ham's F-12 medium containing 10% FCS and 5 µCi ³⁵S-PAPS at 37°C for 20 hrs. Three specimens in each of the proliferative and the secretory phases were used in the experiments. The extraction of lipids was carried out as described above and the extracts corresponding to 2 mg of wet tissue weight were chromatographed on a TLC plate (0.25 mm thick, Merck, FRG) with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol). Radioautograms of the TLC plate were made with X-ray film (X-OMAT, Kodak).

Results

Analysis of glycosphingolipids in murine uterus

Changes in tissue weight and cholesterol content in

Table 1 Dry Weight and Cholesterol Content of Murine Uterus at Various Ages and during the Estrous Cycle

Age (weeks)	Estrous Cycle	Number of Uteri	Dry Weight Tissue (mg/mouse)	Cholesterol (µmol/g dry weight tissue)
2	/	19	1.7	33.3
3	/	18	1.4	26.4
4	/	17	5.4	25.8
5	estrus	7	15.0	22.7
	diestrus	11	8.0	26.0
6	estrus	7	18.8	24.3
	diestrus	6	9.2	21.4
7	estrus	6	14.7	30.9
	diestrus	7	7.9	26.9
8	estrus	5	15.3	33.9
	diestrus	6	9.0	27.2

All of the uterine tissues at each age and during estrous cycle shown in Table 1 were combined, respectively, and examined.

murine uterus during the estrous cycle and the development: The estrous cycle of the murine uterus was clearly discriminated by staining of vaginal smears. Fully cornified smears were observed for the estrus, and atrophic leukocytic smears for the diestrus. The estrous cycle did not begin until approximately 5 weeks after birth; that is, after the onset of sexual maturity. At this turning point, the dry weight of the uterus significantly increased as shown in Table 1, and the weight during estrus was almost twice that during diestrus. The cholesterol content also increased after the sexual maturity, and that during estrus was higher than that during diestrus.

Neutral glycosphingolipids of murine uterus during the estrous cycle and the development: Figure 1 shows a TLC chromatogram of neutral glycosphingolipids obtained from 2.5 mg of dry weight of the murine uterus. Four neutral glycosphingolipids, whose mobilities on the TLC plate were identical with those of GlcCer, LacCer, Gb₃Cer and Gb₄Cer, respectively, were detected in the extracts of all the uteri examined. Among them, the content of Gb₃Cer in the tissue during estrus was always higher than that during diestrus, whereas the amounts of GlcCer, LacCer, and Gb₄Cer were relatively constant during the estrous cycle (Table 2). Gg₄Cer and Forssman antigen were also detected by TLC immunostaining with carbohydrate chain-specific antibodies (Fig 2 and Fig 3). However, by this procedure, Gg₄Cer was found to appear in estrus as well as in diestrus uterus only after the onset of sexual maturity (5 weeks after birth), but the Forssman antigen was present throughout the ages examined (Fig 4).

Acidic glycosphingolipids of murine uterus during the estrous cycle and the development: As shown in Fig 5,

Fig 1 TLC chromatogram of neutral glycosphingolipids from murine uterus at different ages and during the estrous cycle. The neutral glycosphingolipids from 2.5 mg dry weight tissue were chromatographed with chloroform/methanol/water (65:35:8, by vol), and the spots were located by spraying with orcinol-H₂SO₄ reagent and heating at 110°C for 10 min. The spots indicated by * in the figure were not glycolipids. e, estrus; d, diestrus. Numbers refer to age (weeks) of animals examined. The lane s is the standard glycolipid.

Table 2 Contents of Neutral Glycosphingolipids of Murine Uterus at Various Ages and during the Estrous Cycle

Age	Estrous Cycle	GlcCer	LacCer	Gb ₃ Cer	Gb ₄ Cer	Forssman Antigen	Gg ₄ Cer
(weeks)		(μmol/g dry weight tissue)					
2	/	0.33	0.09	0.03	0.35	0.22	ng
3	/	0.27	0.12	0.03	0.38	0.22	ng
4	/	0.22	0.13	0.06	0.36	0.22	0.008
5	estrus	0.22	0.12	0.11	0.33	0.24	0.024
	diestrus	0.32	0.12	0.04	0.58	0.27	0.020
6	estrus	0.24	0.12	0.08	0.32	0.32	0.043
	diestrus	0.32	0.13	0.03	0.34	0.21	0.024
7	estrus	0.33	0.15	0.13	0.45	0.29	0.040
	diestrus	0.28	0.15	0.04	0.30	0.19	0.023
8	estrus	0.23	0.14	0.12	0.35	0.28	0.042
	diestrus	0.31	0.05	0.03	0.20	0.19	0.007

ng: negligible amount

Fig 3 TLC immunostaining of neutral glycosphingolipids in murine uterus at different ages and during the estrous cycle with anti-Forssman antibodies. Glycosphingolipids from 0.25 mg of dry tissue were spotted on the plate. The content of Forssman antigen, determined by TLC densitometry is shown in Table 2. e, estrus; d, diestrus. Numbers refer to age (weeks) of animals examined.

Fig 2 TLC-immunostaining of neutral glycosphingolipids in murine uterus at different ages and during the estrous cycle with anti-Gg₄Cer antibodies. Glycosphingolipids from 0.25 mg of dry tissue were spotted on the plate. The content of Gg₄Cer, determined by TLC densitometry is shown in Table 2. e, estrus; d, diestrus. Numbers refer to age (weeks) of animals examined.

Fig 4 Contents of Gb₃Cer, Forssman antigen, and Gg₄Cer in murine uterus at different ages and during the estrous cycle. ●, before sexual maturity; ○, estrus; ×, diestrus.

GM₃ was found to be the major acidic glycosphingolipid in the murine uterus at all ages examined, as well as the tissues during estrus and diestrus of sexual maturity. In contrast with the findings that Gb₃Cer, Gg₄Cer, and Forssman antigen were always at higher contents during estrus than during diestrus, the level of GM₃ during diestrus was higher than that during estrus (Fig 6). A significant increase in GM₃ content was observed during diestrus at 6 weeks of age and during estrus at 8 weeks of age. The age-dependent increase in the sulfatide content was quite similar to that of GM₃. During estrus, sulfatide was present only in a trace amount.

Fig 6 Developmental changes of GM₃ and sulfatide in murine uterus at different ages and during the estrous cycle. ●, before sexual maturity; ○, estrus; ×, diestrus.

Fig 5 TLC chromatogram of acidic glycosphingolipids from murine uterus at different ages and during the estrous cycle. The acidic glycosphingolipids from 5 mg dry weight tissue were chromatographed with chloroform/methanol/water (60:35:8, by vol), and the spots were located by spraying with orcinol-H₂SO₄ reagent and heating at 110°C for 10 min. e, estrus; d, diestrus. Numbers refer to age (weeks) of animal examined. The lane s is the standard glycolipid.

PAPS: GalCer sulfotransferase in the cells derived from human uterine endometrium and endometria in the proliferative and secretory phases

Separation of the epithelial and stromal cells from human uterine endometrium: The enriched gland preparation obtained by the collagenase treatment showed a typical feature of the mature epithelial cells, which consisted of tightly packed polygonal or tadpole-shaped cells that grew in a swirl shape around the epithelial clumps. On the other hand, the single-cell suspension formed the cells of spindle and stellate morphology, which were typical to the endometrial stromal cells after attachment on the dish. The morphologies of the epithelial and stromal cells thus obtained fit into the categories reported previously.¹⁴⁻¹⁶

Fig 7 TLC autoradiogram of the lipids extracted from the epithelial and the stromal cells of human uterine endometria cultured in the presence of ³⁵S-PAPS(5 μ Ci/ml) for 20 hrs. The lipid extracts from the cells were applied on a TLC plate, which was chromatographed with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol). E, epithelial cells; S, stromal cells. Numbers refer to each endometrial tissue obtained from each patient.

PAPS: GalCer transferase in the epithelial and the stromal cells from human uterine endometrium: Figure 7 shows a radioautogram of the total lipid extracts from endometrial epithelial and stromal cells after radiolabeling with ³⁵S-PAPS as described in Materials and Methods. ³⁵S-labeled glycolipids were detected only in the epithelial cells, but not in the stromal cells even in trace amounts. As shown in Fig 7, a single sulfoglycolipid in the epithelial cell was labeled with ³⁵S and its mobilities on the TLC plate were identical with that of I³SO₃GalCer, sulfatide.

Fig 9 Incorporation of ³⁵S into sulfatide in human uterine endometrium in the proliferative and the secretory phases. Lane 1, 2 and 3 are the tissues in the proliferative phase, and lane 4, 5 and 6 are the tissues in the secretory phase, respectively.

Fig 8 TLC autoradiogram of the lipids extracted from human uterine endometria in the proliferative and the secretory phases cultured in the presence of ³⁵S-PAPS(5 μ Ci/ml) for 20 hrs. The extracts corresponding to 2 mg of wet weight were applied on a TLC plate, which was chromatographed with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol). Lane 1, 2 and 3 are the tissues in the proliferative phase, and lane 4, 5 and 6 are the tissues in the secretory phase, respectively.

PAPS: GalCer transferase in human uterine endometrium in the proliferative and the secretory phases: Figure 8 shows a radioautogram of the total lipid extracts from human uterine endometrium in the proliferative and the secretory phases after incubation with ^{35}S -PAPS. ^{35}S -labeled glycolipids were observed in both the proliferative and secretory phases and a single sulfoglycolipid, whose mobility on the TLC plate was identical with that of sulfatide, was labelled with ^{35}S . The rate of incorporation of ^{35}S per wet weight of tissue in the secretory phase was greater by 5–20-fold in the secretory phase than that in the proliferative phase (Fig 9).

Discussion

Developmental changes of glycosphingolipids in various animal organs have been reported in several papers^{19–22} and these changes are thought to reflect the organization as well as the function of the tissues. Mice enter puberty, which is characterized by opening of the vagina and cornification of the vaginal epithelium at 4 to 5 weeks of age, at which point they reach sexual maturity. During this period from puberty to the sexual maturity, cells in the uterus actively proliferate and differentiate due to stimulation by sex steroid hormones for pregnancy. As clearly shown in this communication, drastic changes in the glycosphingolipid content as well as a large increase in uterine tissue weight in the murine uterus occurred just during this period. In particular, the contents of Gb₃Cer, Gg₄Cer, GM₃, and sulfatide significantly increased during this period.

The sexual cycle of rodents is different from that of the human. After ovulation without sexual intercourse, the corpus luteum, which produces progesterone, is not formed, and as a result, the uterine endometrium does not shift to the secretory phase. Thus, the estrous cycle of rodents is controlled only by estrogen.²³ Therefore, the difference in the glycosphingolipid composition of the murine uterus between the estrus and the diestrus should be explained by the serum level of estrogen.

The mode of the developmental changes of glycosphingolipid composition in the murine uterus clearly differed between the estrus and the diestrus. The increases in neutral glycosphingolipids, Gb₃Cer and Gg₄Cer, during estrus were higher than those during diestrus, whereas the increase in the acidic glycosphingolipids, GM₃ and sulfatide, was greater during diestrus. Probably, these characteristic changes in glycosphingolipid pattern are associated with the alternation of the cellular populations in the murine uterus, since Gg₄Cer, Forssman antigen and IV $^2\text{FucGg}_4\text{Cer}$ in the murine intestine have been shown to be distributed in different regions.^{24–26}

In our previous paper, we reported that only sulfatide among the glycosphingolipids in human uterine endometrium increased in the content during the course of

the menstrual cycle from the proliferative phase to the secretory phase.⁵ From this observation, we presumed that sulfatide might serve as a receptor for trapping the fertilized eggs in the human uterine endometrium in the secretory phase. The higher contents of sulfatide and GM₃ in the murine uterus during diestrus may possibly be related to a role similar to that of the sulfatide in the human uterus in the secretory phase.

It is known that the tissues rich in sulfatide in the human are the kidney, small intestine and brain.^{27–29} As for the localization, rabbit kidney sulfatide was histochemically demonstrated to be localized in the luminal cell membranes at the ascending limbs of the loops of Henle, suggesting that sulfatide is implicated in the passive diffusion of sodium chloride from the lumen of the tubule. It is important to clarify the localization of sulfatide in order to evaluate its functional significance in the human endometrium. In the present study, to characterize the localization of sulfatide in human endometrium, we separated the epithelial and stromal cells, and sulfatide was found to be localized in the glandular epithelial cells of the endometrium. Therefore, sulfatide and sulfotransferase could be used as biochemical markers of endometrial epithelial cells. Moreover, the epithelial cells isolated from the endometrium may provide a valuable tool for studying the hormonal regulation of sulfatide metabolism and the functional significance of sulfatide in human endometrium.

Furthermore, we were able to prove the higher activity of PAPS: GalCer sulfotransferase in the secretory phase than in the proliferative phase and the finding was coincident with the high expression of sulfatide in the secretory phase, presuming that sulfotransferase activities responsible for the synthesis of sulfatide are activated or induced through the cooperative action of estrogen and progesterone. To clarify these observations, an investigation on the effect of estrogen and progesterone on sulfotransferase in the human endometrium is now being carried out using the epithelial cells cultured in our laboratory.

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