ORIGINAL ARTICLE

Phytosphingosine-containing Neutral Glycosphingolipids and Sulfatides in the Human Female Genital Tract: Their Association in the Cervical Epithelium and the Uterine Endometrium and Their Dissociation in the Mucosa of Fallopian Tube with the Menstrual Cycle

Kiyoshi Takamatsu

Department of Obstetrics and Gynecology, School of Medicine, Keio University, Tokyo, Japan

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Abbreviations: Glycolipids are designated in the text according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature.¹ FABMS; fast-atom bombardment mass spectrometry, TLC; thin-layer chromatography, GLC; gas-liquid chromatography, SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abstract. In human cervical epithelium and uterine endometrium, globo-series neutral glycosphingolipids with N- α -hydroxy fatty acyl phytosphingosine (4-D-hydroxysphinganine) as the ceramide and sulfatide ($\text{I}^3\text{SO}_3\text{-GalCer}$), which were contained in trace amount at the follicular phase, significantly increased in concentration at the luteal phase, comprising about 20% of the individual neutral glycosphingolipids and about 15% of the total acidic glycosphingolipids, respectively. However, in the mucosa of fallopian tube, neutral glycosphingolipids with the same polarity as those in the cervical epithelium and uterine endometrium at the luteal phase and sulfatide remained at a constant and higher level independently of the menstrual cycle. The structures of neutral glycosphingolipids in the fallopian tube, having the same polarity as that of N- α -hydroxy fatty acyl phytosphingosine-containing molecules appeared in the cervical epithelium and uterine endometrium at the luteal phase, were determined to be N- α -hydroxy palmitoyl 4-sphingenine-containing ones by negative-ion FABMS. Also, laminin, but not collagen type IV, was found to be contained in the concentration correlated well with that of sulfatide in the genital tract, when determined by western blotting with monoclonal anti-laminin and anti-collagen type IV antibodies, indicating a possible function of sulfatide as a receptor for laminin in the human female genital tract. (Keio J Med 41 (3): 161–167, September 1992)

Key words: ceramide, hydroxy fatty acid, carbohydrate, hydroxylation, luteal phase

Introduction

Glycosphingolipids, which consist of an oligosaccharide chain linked to a hydrophobic ceramide chain, are ubiquitous membrane components in mammalian tissues and cells, and are associated primarily with the outer leaflet of the lipid bilayer in the plasma membrane. The specific structure of the oligosaccharide chain formed by the different sequence, linkage position, and anomeric

configuration of the carbohydrate serves an antigenic determinant or a receptor in the plasma membrane of cells,^{2,3} and the ceramide is believed to regulate the activity of the oligosaccharide chain, as well as to fix the oligosaccharide in the lipid bilayer of the plasma membrane. For expression of bioactivities, the ceramide moiety as well as the oligosaccharide of glycosphingolipids has been shown to play an essential role. For example, glycosphingolipids have been recently shown to regulate

高松 潔

Reprint requests to: Dr Kiyoshi Takamatsu, Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan

cell growth and to induce cell differentiation.^{4,5} Also, receptor functions of glycosphingolipids have been proved in cell-to-cell and cell-to-extracellular matrix interactions, such as sulfatide (I^3SO_3 -GalCer) for laminin⁶ and sialyl Le^x for ELAM-1.⁷

In a recent study, we showed that the concentration of sulfatide increased greatly from the follicular to the luteal phase in the human uterine endometrium.⁸ This finding strongly indicates that glycolipid metabolism in the female genital tract is probably controlled by sex steroid hormones during the menstrual cycle and that the newly synthesized glycolipid is involved in the embedment process of the fertilized egg in the endometrium at the luteal phase. To characterize glycosphingolipids whose metabolism is affected by hormonal changes during the menstrual cycle, we analyzed the glycosphingolipids in the cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular and the luteal phases, as well as determined the concentration of laminin in the tissues.

Materials and Methods

Tissues

Human cervical epithelium, uterine endometrium and fallopian tubes were obtained at Keio University Hospital from patients suffering from myoma uteri. Stage of the menstrual cycle of the tissues was determined by microscopic examination of tissue sections stained with hematoxylin and eosin. All tissue samples were rinsed with 0.9% (w/v) NaCl and stored at -70°C until used or processed immediately as described below.

Preparation of glycosphingolipids

Extraction and preparation of crude lipids from tissues were carried out according to the method reported previously.⁹ Briefly, the tissues were minced, homogenized, and then lyophilized. Total lipids were extracted from the lyophilized powders with chloroform/methanol/water (20:10:1 and 10:20:1 (v/v/v)) and chloroform/methanol (1:1 (v/v)) at 45°C , and were fractionated into neutral and acidic lipids by DEAE-Sephadex A-25 (acetate form) column chromatography. Neutral glycosphingolipids were prepared from the neutral lipid fraction by successive acetylation, Florisil column chromatography, deacetylation, and dialysis. While acidic glycosphingolipids were prepared from the acidic lipid fraction by mild alkaline hydrolysis to cleave the ester-containing lipids, followed by dialysis. The neutral and acidic glycosphingolipids thus obtained were developed on TLC plates with chloroform/methanol/water (65:35:8 (v/v/v)) and chloroform/methanol/0.5% CaCl_2 in water (55:45:10 (v/v/v)), respectively, and the spots on the

plate were visualized with orcinol- H_2SO_4 reagent.

Quantitative determination of glycosphingolipids

The concentrations of sulfatide and individual neutral glycosphingolipids were determined densitometrically with a dual-wavelength TLC densitometer (CS-9000: Shimadzu Co., Kyoto, Japan) after location of the spots with orcinol- H_2SO_4 reagent. A standard curve for quantification was prepared with GalCer from human brain, ranging from $0.2\mu\text{g}$ to $2\mu\text{g}$, on the same plate. The analytical and control wavelengths for quantification of the orcinol-positive spots were set at 420 and 710 nm, respectively.¹⁰

Purification of neutral glycosphingolipids

The neutral glycosphingolipids were further purified with a Iatrobead (6RS8060, Iatron Lab, Tokyo, Japan) column with a gradient elution of chloroform/methanol/water (85:15:0.2 (v/v/v) and 40:60:2 (v/v/v)). Homogeneity of the isolated glycosphingolipids was examined by TLC with orcinol- H_2SO_4 reagent.

Fast-atom bombardment mass spectrometry (FABMS)

The structure of the glycosphingolipids was determined by negative-ion FABMS (JMS HX-110: JEOL Ltd, Tokyo, Japan) as follows.¹¹ $5\mu\text{g}$ of the purified glycosphingolipid in $5\mu\text{l}$ of chloroform/methanol (1:1 (v/v)) was mixed with $5\mu\text{l}$ of triethanolamine on a sample holder for FABMS.

Analysis was performed by bombardment with a neutral xenon beam with a kinetic energy of 4 keV, and the negative ions were detected with a mass spectrometer. Assignment of the mass numbers was achieved by comparison of the spectrum with that of perfluoroalkyl phosphazine (Ultra Mark: PCR, Fa., USA).

SDS-PAGE and western blotting

The proteins from the uterine endometrium and the mucosa of fallopian tube were analyzed by SDS-PAGE and western blotting as follows: Tissues were homogenized with an aid of ultrasonication in 10 vol of PBS at 4°C . After filtration of the homogenate through a filter ($500\mu\text{m}$ mesh), protein in the solution was determined by the blue-dye method at 595 nm with bovine serum albumin as the standard¹² and analyzed by SDS-PAGE according to the procedure described by Laemmli.¹³ The gels were stained with Coomassie brilliant blue. Also, the proteins separated on the gels were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). After incubation of the membrane with 3% bovine serum albumin in Tris-

buffered saline (0.15M NaCl in 10mM Tris-HCl (pH 7.5)) at 37°C for 1 hr to block nonspecific binding sites, the membrane was reacted with anti-laminin and anti-collagen type IV (CHEMICON, Temecula, CA, USA) antisera diluted 1:500 with 3% bovine serum albumin in Tris-buffered saline for 2 hr at 37°C and then with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antiserum for 1 hr at 37°C after a wash with 0.1% Tween 20 in PBS. The activity of peroxidase remaining on the plate was visualized with 4-chloro-1-naphthol and hydrogen peroxide as the substrate. The spots were quantitatively determined with a dual-wavelength densitometer at 550 nm and 700 nm for the analytical and control wavelengths, respectively.

Results

Neutral glycosphingolipids in the cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular and luteal phases

TLCs of neutral glycosphingolipids from the cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular and luteal phases are shown in Fig 1-A and Fig 1-B, respectively. Neutral glycosphingolipids in these tissues consisted of the globo-series glycosphingolipids, GlcCer, LacCer, Gb₃Cer, and Gb₄Cer, and their concentrations were varied in the different tissues (Table 1). On TLC, these glycosphingolipids were divided further into two or three bands due to the difference in the molecular heterogeneity of the ceramide portion. In particular, although neutral glycosphingolipids in the mucosa of fallopian tube were comprised three bands at both the follicular and luteal phases, two bands in the cervical epithelium and the uterine endometrium at the follicular phase became three bands

Fig 1 TLCs of neutral glycosphingolipids from cervical epithelium (1), uterine endometrium (2) and the mucosa of fallopian tube (3) at the follicular (A) and luteal (B) phases. Neutral glycosphingolipids, corresponding to 2.5 mg of dry tissue weight, were developed on TLC with chloroform/methanol/water (65:35:8, by vol.) and the spots were visualized with orcinol-H₂SO₄ reagent. In cervical epithelium and uterine endometrium, neutral glycosphingolipids at the follicular phase were divided into two bands, and those at the luteal phase were divided into three. In the mucosa of fallopian tube, those at both phases were divided into three bands.

Fig 2 Relative concentration of the upper, middle and lower bands of neutral glycosphingolipids from cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular (F) and the luteal (L) phases. The lower bands existed at the luteal phase in the cervical epithelium and uterine endometrium, and at both phases in the mucosa of fallopian tube.

Table 1 Concentration of Neutral Glycosphingolipids in the Cervical Epithelium, Uterine Endometrium, and the Mucosa of Fallopian Tube

	Cervical Epithelium		Uterine Endometrium		Mucosa of Fallopian Tube	
	Follicular Phase	Luteal Phase	Follicular Phase	Luteal Phase	Follicular Phase	Luteal Phase
GlcCer	0.276	0.803	0.504	0.781	0.508	0.969
LacCer	0.230	0.632	0.448	0.380	0.862	1.134
Gb ₃ Cer	0.222	0.268	0.286	0.397	0.726	1.458
Gb ₄ Cer	0.224	0.295	0.369	0.344	0.828	1.491

at the luteal phase, the lowest-migrating band of LacCer, Gb₃Cer, and Gb₄Cer appearing at the luteal phase. As clearly shown in Fig 2, the lowest band of LacCer, Gb₃Cer and Gb₄Cer, which was present in a concentration of less than 1% of the total for each class in the cervical epithelium and uterine endometrium at the follicular phase, became 8.1%, 6.4%, and 28.9%, and 24.3%, 27.6%, and 31.3% of the total LacCer, Gb₃Cer, and Gb₄Cer, respectively, in the cervical epithelium and uterine endometrium at the luteal phase.

These results indicate that the lowest band of each of these three neutral glycosphingolipids in the cervical epithelium and uterine endometrium were induced at the luteal phase by alteration of the hormonal circumstance, whereas neutral glycosphingolipids with the same polarity as those of the lowest bands were constantly contained in the fallopian tube independently of the menstrual cycle.

Structures of the lowest-migrating bands of neutral glycosphingolipids in the uterine endometrium and the fallopian tube

The individual bands of neutral glycosphingolipids from the mucosa of fallopian tube were isolated by Iatrobeds column chromatography as shown in Fig 3, and were directly analyzed by negative-ion FABMS. The lowest band of LacCer and Gb₃Cer gave intense molecular ions, (M-H)⁻s, at m/z 876 and 1038, corresponding to glycosphingolipids with 4-sphingenine and α -hydroxy palmitic acid (Fig 4). The relative intensity of each ion was in good agreement with the fatty acid compositions determined by GLC. Thus, the lowest-migrating bands of neutral glycosphingolipids in the mucosa of fallopian tube were concluded to be exclusively constructed with N- α -hydroxy palmitoyl 4-sphingenine as the ceramide.

On the other hand, the lowest-migrating bands of LacCer and Gb₃Cer from the cervical epithelium and uterine endometrium, which migrated to the same position as those of LacCer and Gb₃Cer from the fallopian tube mucosa (Fig 1), yielded intense molecular ions at m/z 1006, 978 and 922 for LacCer, and at m/z 1168, 1140, and 1084 for Gb₃Cer, corresponding to LacCer and Gb₃Cer with phytosphingosine and α -hydroxy fatty acids with chain lengths of C24h:0, C22h:0, and C18h:0, respectively, along with ions at m/z 960 and 876 for LacCer and at m/z 1122 and 1038 for Gb₃Cer, corresponding to those with 4-sphingenine and α -hydroxy fatty acids with chain lengths of C22h:0 and C16h:0, respectively. And phytosphingosine was detected in the methanolizates of GlcCer, LacCer, Gb₃Cer, and Gb₄Cer from the cervical epithelium and uterine endometrium at the luteal phase, but not in those at the follicular phase or in those from the mucosa of fallopian tube at either phase. Thus, neutral glycosphingolipids

Fig 3 TLC of the individual bands of neutral glycosphingolipids purified from the mucosa of fallopian tube by Iatrobeds column chromatography. U, upper band; M, middle band; L, lower band; T, total neutral glycosphingolipids. The developing solvent and detection reagent were the same as those in Figure 1.

Fig 4 Negative-ion FABMS spectra of the lower band of LacCer (1) and Gb₃Cer (2) purified from the uterine endometrium at the luteal phase (A) and the mucosa of fallopian tube (B).

with high polarity due to N- α -hydroxy palmitoyl 4-sphingenine were constantly contained in the mucosa of fallopian tube independently of the menstrual cycle, but those due to N- α -hydroxy fatty acyl phytosphingosine and N- α -hydroxy palmitoyl 4-sphingenine in the uterine endometrium and cervical epithelium were only detected at the luteal phase and were probably induced by alteration of the hormonal circumstance.

Acidic glycosphingolipids in the cervical epithelium, uterine endometrium and the mucosa of fallopian tube at the follicular and luteal phases

Fig 5 TLCs of acidic glycosphingolipids from cervical epithelium (1), uterine endometrium (2), and the mucosa of fallopian tube (3) at follicular (A) and luteal (B) phases. Acidic glycosphingolipids, corresponding to 5mg of dry tissue weight, were developed with chloroform/methanol/0.5% CaCl₂ (55:45:10, by vol.), and the spots were visualized with orcinol-H₂SO₄ reagent. In cervical epithelium and uterine endometrium, the concentration of sulfatide significantly increased from the follicular to the luteal phase. However, in the mucosa of fallopian tube, no significant difference was observed.

By comparison of their mobilities on TLC with those of authentic standards and examination of the reactivities with orcinol-H₂SO₄ for carbohydrate-containing lipids, H₂SO₄ for sterol-containing lipids, and resorcinol-HCl for sialic acid-containing lipids, I³SO₃-GalCer (sulfatide) and II³NeuAc-LacCer (GM₃) were detected in the cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular and the luteal phases as shown in Fig 5. Also several polar gangliosides that migrated in the positions lower than that of II³NeuAc-LacCer were also detected. As already reported previously,⁸ the concentration of I³SO₃-GalCer in the uterine endometrium at the luteal phase was significantly higher than that at the follicular phase (Fig 6),

Fig 6 Relative concentrations of I³SO₃-GalCer, II³NeuAc-LacCer and polar gangliosides in cervical epithelium, uterine endometrium, and the mucosa of fallopian tube at the follicular (F) and the luteal (L) phases.

Table 2 Concentrations of I³SO₃-GalCer and Gangliosides, and Molar Ratio of I³SO₃-GalCer to Gangliosides in the Mucosa of Fallopian Tube at the Follicular and the Luteal Phases

		I ³ SO ₃ -GalCer	Gangliosides	Molar Ratio of I ³ SO ₃ -GalCer to Gangliosides
		μmoles/g dry Weight		
Follicular Phase	1	0.822	1.021	0.81
	2	0.286	1.008	0.30
	3	0.377	2.024	0.19
	4	0.415	1.329	0.31
	5	0.962	1.244	0.77
	6	0.730	1.038	0.70
	7	0.063	0.213	0.30
Mean ± SD		0.522 ± 0.299	1.125 ± 0.497	0.48 ± 0.24
Luteal Phase	8	0.393	1.572	0.25
	9	0.124	1.141	0.11
	10	0.711	1.450	0.49
	11	0.082	0.663	0.12
	12	0.063	0.283	0.22
	13	0.619	1.818	0.34
	14	0.738	1.519	0.48
Mean ± SD		0.390 ± 0.280	1.207 ± 0.509	0.29 ± 0.14

amounting to 1.0% and 18.0% of the total acidic glycosphingolipids at the follicular and the luteal phases, respectively. In the cervical epithelium, I^3SO_3 -GalCer at the luteal phase was also significantly increased in concentration when compared with that at the follicular phase. Whereas no significant difference in the concentration of I^3SO_3 -GalCer in the mucosa of fallopian tube between the follicular and luteal phases was observed, as shown in Table 2 and Fig 6. The molar ratios of I^3SO_3 -GalCer to sialoglycosphingolipids in the fallopian tube mucosa at the follicular and luteal phases were 0.48 and 0.29, respectively, and were in contrast with the dramatic changes of the ratios, 0.01 and 0.22 in the uterine endometrium, and 0.00 and 0.16 in the cervical epithelium at the follicular and the luteal phases, respectively. Thus, although the synthesis of I^3SO_3 -GalCer in both cervical epithelium and uterine endometrium seemed to be affected by sex steroid hormones, that in the mucosa of fallopian tube seemed to be activated at either phase.

Concentration of laminin in the uterine endometrium and the mucosa of fallopian tube during the menstrual cycle

To elucidate a possible function of I^3SO_3 -GalCer as a receptor for laminin, the relative concentrations of laminin and collagen type IV were determined by western blotting with anti-laminin and anti-collagen type IV antibodies and were compared with the level of I^3SO_3 -GalCer in the uterine endometrium and the mucosa of the fallopian tube at the day just after menstruation, and at the follicular, early-luteal, and late-luteal phases (Fig 7). The relative concentrations of collagen type IV in the uterine endometrium and the fallopian tube were not significantly altered during the periods examined. However, although the relative concentration of laminin in the fallopian tube mucosa was not different among the periods just after menstruation, at the follicular and the luteal phases, that in the uterine endometrium at the luteal phase was higher than that in the periods at the follicular phase and just after the menstruation, indicating good correlation of the concentration of laminin with that of I^3SO_3 -GalCer in the uterine endometrium and the mucosa of fallopian tube.

Discussion

In human uterine endometrium, we found that I^3SO_3 -GalCer dramatically increased in concentration at the luteal phase. This result suggests to us that steroid hormones, particularly estrogen and progesterone, affect the metabolism of glycosphingolipids and that the newly expressed glycosphingolipids might be involved in the function of the luteal endometrium, in which a fertilized egg was embedded or in the case of unsuccessful

Fig 7 Comparison of the concentrations of I^3SO_3 -GalCer and laminin in the uterine endometrium and the mucosa of fallopian tube at each stage of the menstrual cycle. Sulfatide and laminin showed the same gradient during the menstrual cycle.

fertilization, which is shed at the menstruation.

Accordingly, we analyzed glycosphingolipids in the female genital tract including the cervical epithelium, uterine endometrium, and the mucosa of fallopian tube to clarify the metabolic pathway of glycosphingolipids modified during the menstrual cycle, and then determined the concentration of laminin to assess a possible interaction with I^3SO_3 -GalCer. As clearly shown in this paper, the concentrations of I^3SO_3 -GalCer and phyto-sphingosine-containing neutral glycosphingolipids were significantly altered between the follicular and the luteal phases in the cervical epithelium and uterine endometrium, but not in the mucosa of the fallopian tube.

Thus, even in the cervical epithelium, which is not discarded at menstruation, menstrual cycle-associated alteration of glycosphingolipid was detected, and therefore a sulfuric acid-derived negative charge is lost in the cervical epithelium and uterine endometrium at the follicular phase. At the luteal phase, I^3SO_3 -GalCer comprised 30–40% of the sialic acid-bound glycosphingolipids in these tissues and simultaneously the relative concentration of laminin in the endometrium showed a higher level than that at the follicular phase.

If I^3SO_3 -GalCer is associated with the receptor for

laminin, it should be distributed at the basal side, rather than at the apical side, and might be involved in regulation of cellular attachment of the endometrial and epithelial cells during the menstrual cycle. In contrast, the concentrations of I³SO₃-GalCer and laminin in the fallopian tube remained constantly at higher levels independently of the menstrual cycle. For further understanding of the meaning of the menstrual cycle-associated alteration of I³SO₃-GalCer in the cervical epithelium, the uterine endometrium and the mucosa of fallopian tube, histochemical examination of I³SO₃-GalCer, along with analysis of the receptor activities for steroids, is required and is now in progress in our laboratory. In addition, neutral glycosphingolipids with the higher polarities due to α -hydroxy palmitic acid, and combination with phytosphingosine and α -hydroxy longer chain-fatty acids, which were not contained in the cervical epithelium or uterine endometrium at the follicular phase, appeared in the tissues at the luteal phase.¹⁴ Sialic acid of gangliosides with shorter-chain fatty acids in the ceramide moiety was more cryptic to neuraminidase than that with longer-chain fatty acids,¹⁵ sialyl Le^a immunoprecipitated with its monoclonal antibody was restricted to the molecule with N- α -hydroxy fatty acyl phytosphingosine,¹⁶ and *Neisseria gonorrhoeae* could characteristically bind to LacCer with N- α -hydroxy fatty acyl 4-sphingenine, but not to LacCer with N-nonhydroxy fatty acyl 4-sphingenine.¹⁷ Thus, the presence of a more polar structure in the hydrophobic ceramide of the neutral glycosphingolipids at the luteal phase might be important for regulation of the reactivity of glycosphingolipids responsible for recognition in the process of fertilization.

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