Glycosphingolipids of human myometrium and endometrium and their changes during the menstrual cycle, pregnancy and ageing*

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Summary. The glycolipid composition of human myometrium and endometrium was examined at various stages of maturation and reproduction. The major neutral glycolipids of both myometrium and endometrium were identified by high-performance thin-layer chromatography as globo-series glycolipids, Gb₃ and Gb₄. The major acidic glycolipids (gangliosides) were identified similarly as GM₃ and GD₃, with lesser amounts of GM₁, GD_{1a}, and GT_{1b}. During pregnancy, GD₃ expression declined in both myometrium and endometrium, whereas GM₃ expression increased. Reciprocal changes in GM₃/GD₃ expression were mirrored by appropriate changes in the glycosyltransferases required for their synthesis; $\alpha 2 \rightarrow 3$ sialyltransferase activity increased approximately 3-fold during pregnancy, while $\alpha 2 \rightarrow 8$ sialyltransferase activity declined to about 20%. The results focus attention on the glycolipids of uterine tissues, their regulation, and their possible role in reproduction and fertility.

Keywords: myometrium; endometrium; glycolipids; pregnancy; human

Introduction

The surface properties of embryonic and maternal tissues are believed to play an important role in regulating complex cell interactions associated with implantation and pregnancy. Previous studies using lectins and cationic dyes have revealed rapid, stage-specific changes in the surface glycoconjugates of both the uterine epithelium and trophoctoderm (Schlafke & Enders, 1975; Sherman & Wudl, 1976; Hewitt *et al.*, 1979; Chavez & Enders, 1981; Chavez & Anderson, 1985). More recently, there has been some elucidation of the biological functions of specific membrane glycoconjugates. The histo-blood group H antigen has been implicated as a ligand for blastocyst–endometrium recognition (Lindenberg *et al.*, 1988), and high molecular weight poly-*N*-acetyllactosaminoglycans have been reported to mediate endometrium cell–cell adhesion (Dutt *et al.*, 1987).

Glycolipids are important membrane components, associated primarily with the outer leaflet of the plasma membrane lipid bilayer. They consist of an oligosaccharide chain linked via glucose to ceramide (N-fatty acyl sphingosine). Glycolipids are believed to modulate a variety of cell biological functions, including cell recognition, growth, and differentiation (reviewed by Hakomori & Kannagi, 1983; Igarashi *et al.*, 1989). In addition, glycolipids have been identified as receptors for various lectins, toxins, and viruses (reviewed by Kanfer, 1983). The oligosaccharide chains of glycolipids are synthesized in the Golgi apparatus by the sequential action of multiple glycosyltransferases; each enzyme catalyses the addition of a monosaccharide to an acceptor with precise specificity. Over 100 different glycolipid structures are known. This diversity arises from the cells' use of multiple monosaccharides (glucose, N-acetylglucosamine, galactose, N-acetylgalactosamine,

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fucose, sialic acid), α and β linkage arrangements, and multiple linkage positions (1 $\rightarrow$ 2, 1 $\rightarrow$ 3, 1 $\rightarrow$ 4, 1 $\rightarrow$ 6). Despite their enormous diversity, glycolipids can be classified as globo-, lacto- or ganglio-series depending upon their oligosaccharide core structure: globo-series glycolipids contain Gal α 1 $\rightarrow$ 4Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ ceramide, lacto-series glycolipids contain GlcNAc β 1 $\rightarrow$ 3 Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ ceramide, and ganglio-series glycolipids contain Sia α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ ceramide. The globo-, lacto- and ganglio-series glycolipids therefore represent different pathways for lactosylceramide chain elongation.

As a first step towards understanding the functional roles of membrane glycolipids in female reproductive biology, we have examined the quantity and composition of glycolipids in human myometrium and endometrium, at different functional stages, using quantitative spectrophotometric and thin-layer chromatography techniques. We have also investigated the enzymic basis for changes in glycolipid expression during pregnancy.

Materials and Methods

Abbreviations. Glycolipids are designated in the text according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1978), but the suffix OseCer is omitted. Gangliosides (acidic glycolipids containing N-acetylneuraminic acid) are designated according to the nomenclature of Svennerholm (1964).

Tissues. Human neonatal myometrium (6–20 days of age) was obtained from autopsy. Myometrium from women at reproductive (30–40 years) and perimenopausal (52–55 years) stages was obtained at hysterectomy. Full-term pregnancy myometrium was obtained from Caesarean section. These tissues were identified as normal by standard pathological examination. Endometrium was obtained from dilatation surgery. Stages of the menstrual cycle (i.e. proliferative and secretory) were determined by microscopic examination. Decidua were obtained from early pregnancy (first trimester) by suction curettage. Decidua from full-term pregnancy were obtained from Caesarean section. All tissue samples were rinsed with 0.9% (w/v) NaCl to remove excess blood, blotted between layers of filter paper, and either stored at -30°C or processed immediately as described below.

Reagents. The following reagents and chemicals were used for glycolipid extraction and analysis: DEAE-cellulose (Whatman, Clifton, NJ, USA), DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden), Sep-pak C18 reverse phase column (Waters, San Francisco, CA, USA), Iatrobeads 6RS-8060 (Iatron, Tokyo, Japan), Bio-sil silica gel (BioRad, Richmond, CA, USA), and HPTLC plates (Merck, Darmstadt, W. Germany). Resorcinol, thiobarbituric acid, sphingosine, N-acetyl sialic acid, fluorescamine, *Clostridium perfringens* sialidase, Triton CF-54, Tween-80, nitroblue tetrazolium, 5-bromo-4-chloro-3-indole phosphate, sodium azide and cacodylate were purchased from Sigma (St Louis, MO, USA). Glycolipid standards were either isolated in this laboratory or provided as gifts from Dr Gu, Department of Biochemistry, Shanghai Medical University, China. Organic solvents were obtained from local suppliers and redistilled before use. Other reagents were of analytical grade, used without further purification. [^3H]-CMP sialic acid was a gift from Dr Gu. Sialidase from *Vibrio cholera* was obtained from Lanzhou Bioproducts Manufacturing Inst., Beijing, China.

Glycolipid extraction and characterization. Glycolipids were extracted from 4–5-g samples of reproductive- and perimenopausal-age myometrium according to the method of Chien *et al.* (1978). In brief, total lipid extracts in methanol were subjected to alkaline hydrolysis, dialysed against water, lyophilized, and fractionated into neutral and acidic (ganglioside) fractions by DEAE-cellulose chromatography. Glycolipids were extracted from 0.1–0.5 g samples of endometrium and myometrium (neonatal and full-term pregnant) according to the method of Irwin & Irwin (1979), except that Sep-pak C18 reverse-phase columns were used for desalting instead of dialysis. Neutral glycolipids were purified further according to the method of Vance & Sweeley (1967). Total lipid-bound sialic acid was determined by the method of Aminoff (1961), and sphingosine content was determined by the method of Naoi *et al.* (1974). Glycolipids were separated by t.l.c. on silica gel plates. Ganglioside mixtures equivalent to 2 μg sialic acid and neutral glycolipid mixtures equivalent to 2.5 μg sphingosine were developed by ascending chromatography in a solvent system of chloroform:methanol:water (60:35:8, by vol.). Gangliosides were visualised by resorcinol-HCl spray and neutral glycolipids by diphenylamine spray. Individual glycolipid bands were quantified by scanning densitometry using a Shimadzu CS-910 scanner. Sialidase digestion of gangliosides was performed using 50 mM-sodium acetate buffer, pH 5.0, containing 0.25% (w/v) sodium taurocholate. The t.l.c. immunostaining using monoclonal antibodies 1A4-E10 (anti-Gb $_3$), SSEA-3 (anti-Gb $_4$ and -Gb $_5$) and R24 (anti-GD $_3$) was performed as described previously (Fenderson *et al.*, 1987).

Glycosyltransferase assays. The activities of CMP-sialic acid:lactosylceramide α 2 $\rightarrow$ 3sialyltransferase (α 2 $\rightarrow$ 3SiaTase) and CMP-sialic acid:GM $_3$ α 2 $\rightarrow$ 8sialyltransferase (α 2 $\rightarrow$ 8SiaTase) were determined under optimal assay conditions (Richardson *et al.*, 1977; Keenan & Morre, 1973). Endometrium samples (0.5–1.0 g) were cut into small pieces and placed in 1.5 ml Buffer I (50 mM-Tris/HCl, pH 6.35, containing 50 mM-KCl, 5 mM-MgCl $_2$, 250 mM-sucrose, and 10 μM -2-mercaptoethanol), and sonicated for 30 sec to obtain a suspension. After centrifuging for 12 min

at 200 g, the supernatant was removed and used as the enzyme source. Protein content was determined by the method of Hartree (1972), using bovine serum albumin as a standard. Reaction mixtures contained 0.05 μmol lactosylceramide or GM_3 , 6×10^4 d.p.m. [^3H]CMP sialic acid, approximately 100 μg protein and 10 μl Buffer I (50 μl total volume). After 1 h at 37°C, enzymic reactions were stopped by addition of 50 μl methanol, applied to filter paper, and developed (descending chromatography) with 1% sodium borate overnight to separate the unreacted [^3H]CMP sialic acid from the reaction product which stayed at the origin. The origin was cut off, dried at 100°C, and counted for 1 min using a β -scintillation counter. Lactosylceramide or GM_3 were omitted from controls. Enzyme activity was expressed as nmol/h/mg protein.

Statistical analyses. The significance of changes in glycolipid content was evaluated using a two-sample, Student's *t* test. The significance of differences in ganglioside GM_3 and GD_3 expression was determined using a one-sample *t*-test. A negative correlation between the percentages of GM_3 and GD_3 was assumed, thus underestimating the true significance value. Null hypothesis (H_0) was no difference and the alternative (H_1) was positive difference; $\text{GD}_3 > \text{GM}_3$ for reproductive- and perimenopausal-age myometrium, and $\text{GM}_3 > \text{GD}_3$ for the remainder of the samples.

Results

Sphingosine and lipid-bound sialic acid analysis

The glycolipid content of human myometrium and endometrium at various developmental stages was determined quantitatively as follows. Uterine samples were extracted and partly purified as described in 'Materials and Methods'. Neutral and acidic glycolipid fractions corresponding to normalized wet weight amounts were assayed for sphingosine and lipid-bound sialic acid, respectively. As shown in Fig. 1(a), the sphingosine content of human myometrium (representing neutral glycosphingolipids) was unchanged during ageing, but increased approximately 3-fold during pregnancy. The sphingosine content of human endometrium was significantly higher than that of non-pregnant myometrium at each stage of the menstrual cycle and pregnancy examined (i.e. $> 80 \mu\text{g/g}$ wet weight (Fig. 1b). In contrast, the lipid-bound sialic acid content of myometrium and endometrium (representing total gangliosides) declined to about 30% during pregnancy (Fig. 1c). A 50% decline in lipid-bound sialic acid was also noted in the endometrium during the receptive (secretory) phase of the menstrual cycle. The ratio of neutral glycolipids to gangliosides therefore increased dramatically during the menstrual cycle and pregnancy.

Analysis by t.l.c.

The major neutral glycolipids of myometrium and endometrium (Fig. 2a, b) were identified, using pure glycolipid standards, as CTH and CQH (i.e. Gb_3 and Gb_4). This identification was confirmed by t.l.c. immunostaining using monoclonal antibodies directed to Gb_3 and Gb_4 (data not shown). Lactosylceramide (CDH) was present only in trace amounts in samples of neonatal and full-term pregnancy myometrium. Within the endometrium, glucosylceramide (CMH) and CDH accumulated during pregnancy. A pentasaccharide-containing glycolipid (CPH; oligosaccharide sequence unknown) also accumulated in the endometrium during the receptive (secretory) stage of the menstrual cycle and during pregnancy. In contrast, CPH was present in only trace amounts in reproductive-age myometrium.

The major gangliosides of myometrium and endometrium were identified, using pure ganglioside standards, as GM_3 and GD_3 , with minor amounts of GM_1 , GD_{1a} , and GT_{1b} (Figs 2c, d). The apparent complexity of gangliosides in myometrium declined during ageing (i.e. $\text{N} \rightarrow \text{R}$). The identity of GD_3 as the major ganglioside of reproductive-age myometrium was confirmed by purifying this component and finding that its core structure (obtained by enzymic desialylation) co-migrated with authentic CDH (Fig. 3). The identity of GD_3 was also confirmed by t.l.c. immunostaining using monoclonal antibody directed to GD_3 (data not shown). Monoclonal antibodies specific for GM_1 , GD_{1a} , and GT_{1b} are not available; therefore, the identification of these minor glycolipid components is based solely on co-migration with standards.

Fig. 1. Quantitative analysis of glycolipid expression in human myometrium (a, c) and endometrium (b, d) during the menstrual cycle, pregnancy, and ageing. (a, b) Sphingosine content of the neutral glycolipid fraction. (c, d) Lipid-bound sialic acid content of the acidic glycolipid (ganglioside) fraction. Myometrium samples were from neonatal (N), reproductive (R), full-term pregnancy (FP), and perimenopausal (M) uteri. Endometrium samples were from proliferative (P), secretory (S), early pregnancy (EP), and full-term pregnancy (FP) uteri. Values are mean $\pm$ s.d. for 3–6 separate assays. Changes in glycolipid content were evaluated for statistical significance using a two-sample *t* test.

The quantity of individual gangliosides in uterine samples was determined by scanning densitometry on replicate t.l.c. plates. Monosialoganglioside GM_3 and disialoganglioside GD_3 exhibited striking, reciprocal patterns of expression during the menstrual cycle, pregnancy, and ageing (Fig. 4). In myometrium, GD_3 predominated during reproductive and perimenopausal stages, whereas GM_3 predominated during neonatal and full-term pregnancy stages. In endometrium, GM_3 predominated at all stages, and the GM_3/GD_3 ratio increased progressively during pregnancy. Quantitative analyses also revealed a 2-fold increase in GT_{16} expression in myometrium during pregnancy (i.e. $9.8 \pm 3.0 \rightarrow 19.4 \pm 1.5$; data represent the percentage of total ganglioside identified by scanning densitometry in 3–4 separate assays).

Enzymic basis for GM_3/GD_3 modulation during pregnancy

Gangliosides GM_3 and GD_3 are synthesized by the sequential addition of sialic acid to a common core structure, i.e. $\text{CDH} \rightarrow \text{GM}_3 \rightarrow \text{GD}_3$. The sialyltransferases involved in these steps include the $\alpha 2 \rightarrow 3\text{SiaTase}$ (for GM_3 synthesis) and the $\alpha 2 \rightarrow 8\text{SiaTase}$ (for $\text{GM}_3 \rightarrow \text{GD}_3$ conversion). Changes in the expression or activities of these enzymes would provide a mechanism for GM_3/GD_3 modulation during pregnancy.

To investigate this hypothesis, the activities of $\alpha 2 \rightarrow 3\text{SiaTase}$ and $\alpha 2 \rightarrow 8\text{SiaTase}$ enzymes in endometrial (decidual) tissues were measured during pregnancy. As shown in Fig. 5, $\alpha 2 \rightarrow 3\text{SiaTase}$ activity increased approximately 3-fold during pregnancy, while $\alpha 2 \rightarrow 8\text{SiaTase}$ activity declined to about 20%. These changes in enzyme activity broadly parallel the pregnancy-associated changes in GM_3 and GD_3 expression observed by t.l.c. analysis (see Figs 2 & 4). Sialylhydrolase activities in decidua were not determined.

Fig. 2. Thin-layer chromatography analysis of glycolipid composition in human myometrium (a, c) and endometrium (b, d) during the menstrual cycle, pregnancy, and ageing. (a, b) Neutral glycolipids; samples were normalized to contain 2.5 µg sphingosine. (c, d) Acidic glycolipids (gangliosides); samples were normalized to contain 2.0 µg sialic acid. A standard (Std) set of glycolipids was included. Myometrium samples were from neonatal (N), reproductive (R), full-term pregnancy (FP), and perimenopausal (M) uteri. Endometrium samples were from proliferative (P), secretory (S), menstrual (MS), early pregnancy (EP), and full-term pregnancy (FP) uteri. After streaking, t.l.c. plates were developed by ascending chromatography in a solvent system of chloroform/methanol/water (60:35:8, by vol., for neutral glycolipids or 60:40:9, by vol., containing 0.2% CaCl_2 for gangliosides). Neutral glycolipids and gangliosides were visualized by diphenylamine spray and resorcinol spray, respectively.

Discussion

The uterus is the principal target organ of female sex hormones and undergoes significant changes in morphology and function during maturation and reproduction. The endometrium exhibits extensive changes in growth and differentiation during each menstrual cycle (Schlafke & Enders, 1975), and provides a substratum for embryo attachment and trophoblast invasion during pregnancy (Finn & Martin, 1972; Schlafke & Enders, 1975; Glasser & McCormack, 1979).

In the present report, we have identified changes in glycolipid content and composition that accompany various functional stages of the human uterus. The following results were obtained. (1) During pregnancy, the neutral glycolipid content of myometrium increased approximately 3-fold, whereas the ganglioside content of myometrium and endometrium declined to about 30%. (2) The

Fig. 3. Identification of GD_3 as the major ganglioside of reproductive-stage myometrium. Ganglioside was purified to homogeneity by DEAE-Sephadex, ion-exchange chromatography and subjected to enzymic hydrolysis using *Vibrio cholera* sialidase (see 'Materials and Methods'). The t.l.c. lanes are as follows: (1) and (2), glycolipid standards; (3) purified ganglioside; (4) incomplete (i.e. partial) sialidase digestion; (5) complete sialidase digestion. After streaking, t.l.c. plates were developed by ascending chromatography in a solvent system of chloroform/methanol/water (60:35:8, by vol.). Glycolipids were visualized by diphenylamine spray. As expected for the disialyl ganglioside GD_3 , sialidase digestion products are identified as GM_3 and CDH .

major neutral glycolipids of adult myometrium and endometrium were identified as globo-series glycolipids Gb_3 and Gb_4 ; the major gangliosides were identified as GM_3 and GD_3 . The structures of these glycolipids, as well as minor glycolipid components, are shown in Table 1. (3) During pregnancy, GM_3 expression in myometrium and endometrium increased, while GD_3 expression declined. (4) This shift in ganglioside expression was accompanied by corresponding changes in the activities of the glycosyltransferases responsible for their synthesis.

Glycolipid composition is known to change rapidly during development (Kannagi *et al.*, 1983) and differentiation (Fenderson *et al.*, 1987). Recent enzymic studies indicate that these phenotypic changes are mediated largely by appropriate changes in the expression of glycosyltransferases (Chen *et al.*, 1989). The results presented here are consistent with this mechanism for glycolipid regulation; however, a role for glycosylhydrolases (glycosidases) in altering cellular glycolipid patterns cannot be ruled out. In this connection, oestrogen and progesterone have been reported to stimulate the activities of lysosomal glycosidases in rabbit endometrium (Munakata *et al.*, 1986). The uterus should provide an excellent system for future studies on the roles of sex hormones in regulating lipid and protein glycosylation.

The biological significance of changes in glycolipid metabolism and cell surface glycolipid expression is largely unknown; however, it is believed that glycolipids are instrumental in regulating membrane physiology and cell social behaviour. For example, GM_3 and its catabolic derivatives are known to be potent modulators of growth factor receptors (reviewed by Igarashi *et al.*, 1989). Similarly, disialogangliosides GD_2 and GD_3 have been implicated in the adhesion of human cells to extracellular matrix proteins (Cheresh *et al.*, 1986). In addition, some glycolipids may act as

Fig. 4. Reciprocal changes in ganglioside GM₃ (●) and GD₃ (○) expression in human myometrium (a) and endometrium (b) during the menstrual cycle, pregnancy, and ageing. Myometrium samples were from neonatal (N), reproductive (R), full-term pregnancy (FP), and perimenopausal (M) uteri. Endometrium samples were from proliferative (P), secretory (S), early pregnancy (EP), and full-term pregnancy (FP) uteri. Data points represent the percentage of total resorcinol-positive glycolipid identified by scanning densitometry in 3–6 separate assays (mean $\pm$ s.d.). Levels of GM₃ and GD₃ expression in myometrium and endometrium are significantly different at each developmental stage ($P < 0.05$, one-sample t test; see 'Materials and Methods').

Fig. 5. Reciprocal changes in glycolipid-glycosyltransferase activity in human endometrium (decidua) during the menstrual cycle and pregnancy. Samples were from proliferative (P), secretory (S), early pregnancy (EP), mid-term pregnancy (MP), and full-term pregnancy (FP) uteri. The activities of $\alpha 2 \rightarrow 3$ SiaTase (responsible for GM₃ synthesis) and $\alpha 2 \rightarrow 8$ SiaTase (responsible for GD₃ synthesis) were assayed under optimal conditions, as described in 'Materials and Methods'. Data represent enzyme activity in single tissue samples at each developmental stage. ND, not determined.

second messengers, stimulating specific pathways of cellular differentiation (Nojiri *et al.*, 1986; Nakajima *et al.*, 1986).

The results presented here focus particular attention on (i) a specific increase in GT_{1b} expression in myometrium during full-term pregnancy, and (ii) an overall decline in polysialoganglioside expression in the myometrium and endometrium during pregnancy, with a corresponding increase in neutral glycolipid expression. The appearance of novel polysialogangliosides in the uterus, including GT_{1b}, could provide receptors for hormones such as oxytocin, vasopressin, or prostaglandins (Kanfer, 1983). The increase in neutral glycolipids during pregnancy may be viewed

Table 1. Major neutral and acidic glycolipids of human myometrium and endometrium

	Glycolipid structure*	Designation
Neutral	Glcβ1 → Cer	CMH
	Galβ1 → 4Glcβ1 → Cer	CDH
	Galα1 → 3Galβ1 → 4Glcβ1 → Cer	CTH (Gb ₃)
	GalNAcβ1 → 3Galα1 → 3Galβ1 → 4Glcβ1 → Cer	CQH (Gb ₄)
Acidic (gangliosides)	Siaα2 → 3Galβ1 → 4Glcβ1 → Cer	GM ₃
	Siaα2 → 8Siaα2 → 3Galβ1 → 4Glcβ1 → Cer	GD ₃
	Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glcβ1 → Cer	GM ₁
	3 ↑ Siaα2	
	Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glcβ1 → Cer	GD _{1a}
	3 ↑ Siaα2	
	Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glcβ1 → Cer	GT _{1b}
	3 ↑ Siaα2	
	8 ↑ Siaα2	

*Glycolipid structures were identified in this report by: (i) co-migration on t.l.c. plates with pure glycolipid standards; (ii) analyses of sialidase cleavage products; (iii) t.l.c. immunostaining using specific anti-glycolipid monoclonal antibodies.

in terms of altered membrane contacts necessary for maternal–fetal interactions. Although the glycolipids of the human uterus undergo a complex series of changes during maturation and differentiation, the biological significance of these changes remains to be determined.

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