Glycosphingolipids of rabbit endometrium and their changes during pregnancy

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Summary. The glycolipids of nonpregnant and pregnant rabbit endometrium were characterized using a combination of biochemical and immunochemical techniques. Quantitative analyses indicated a 70% decline in acidic glycolipid (ganglioside) content during early pregnancy (day 6), and a 2.5-fold increase in neutral glycolipid content during later pregnancy (day 26). The major gangliosides of rabbit endometrium were identified by thin-layer chromatography as GM3 and GD3, with minor amounts of GM1, GD1a and GT1b. The major neutral glycolipids were identified similarly as globo-series structures Gb3 and Gb4. Monoclonal antibodies (mAbs) directed to glycolipid antigens permitted the detection of additional glycolipid species, including sialylated, sulfated and fucosylated lacto-series structures. Difucosyl Le^x structure (defined by mAb AH-6) and sulfated-galactosyl structure (defined by mAb VESP 6-2) were identified by indirect immunofluorescence along the luminal surface of the endometrium during the implantation period. Rapid changes in the glycolipid composition of endometrial cells during early pregnancy may facilitate embryo adhesion and trophoctoderm outgrowth during implantation.

Keywords: endometrium; glycolipids; pregnancy; immunohistochemistry; rabbit

Introduction

The carbohydrate chains of cell surface glycolipids, glycoproteins and proteoglycans may play a crucial role in regulating maternal–fetal interactions during implantation and early development. For example, uterine receptivity to blastocyst attachment is known to be associated with changes in (i) the glycoprotein composition of the epithelium (Ricketts *et al.*, 1984; Anderson *et al.*, 1986), (ii) the surface charge of the apical glycocalyx (Hewitt *et al.*, 1979; Murphy & Rogers, 1981; Anderson & Hoffman, 1984) and (iii) the profile of glycoproteins secreted into the luminal fluid (Bell *et al.*, 1986). The preimplantation embryo also undergoes a complex programme of glycosylation changes (reviewed by Fenderson *et al.*, 1990). Together, these changes in surface carbohydrate may regulate contacts between the cell surface membranes of trophoctoderm and endometrium. Indeed, blood group H antigen, which is highly expressed on the luminal surface of mouse endometrium (Kimber *et al.*, 1988; Kimber & Lindenberg, 1990), has been implicated as a ligand for trophoctoderm adhesion (Lindenberg *et al.*, 1988). In addition, lactosaminoglycans have been identified as receptors for endometrial cell adhesion (Dutt *et al.*, 1987) and trophoctodermal cell migration (Hathaway *et al.*, 1989).

Glycolipids are ubiquitous components of the plasma membrane. They consist of ceramide embedded in the outer leaflet of the lipid bilayer and oligosaccharide chains extending into

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the aqueous environment at the cell surface (reviewed by Thompson & Tillack, 1985). There is considerable variation in the oligosaccharide sequence and ceramide composition of membrane glycolipids: over 300 neutral and acidic glycolipid species have been described (Hannum & Bell, 1989). This structural diversity may contribute to the specialization and function of the plasma membrane. For example, glycolipids are hypothesized to enhance the stability of the lipid bilayer (Pascher, 1976), regulate transmembrane signalling (Hanai *et al.*, 1988; Nojiri *et al.*, 1991), and mediate cell interaction through carbohydrate–protein (Springer & Lasky, 1991) and carbohydrate–carbohydrate (Eggers *et al.*, 1989; Kojima & Hakomori, 1989) interactions.

We described changes in the glycolipid composition of human myometrium and endometrium during the menstrual cycle, pregnancy and ageing (Zhu *et al.*, 1990). The results indicated an overall decline in polysialoganglioside during early pregnancy, with a corresponding increase in neutral glycolipid. We suggested that the appearance of novel glycolipids in the uterus could provide receptors for various hormones, and that the overall increase in neutral glycolipid during pregnancy was important for maternal–fetal interactions. In this report, we extend our analyses to a second species, the rabbit, and use immunohistochemical procedures to localize specific glycolipid antigens within the uterus.

Materials and Methods

Tissues

Uteri were removed from New Zealand White rabbits at specific reproductive stages: nonpregnant (oestrus), early pregnancy (day 6), mid-pregnancy (day 18) and late pregnancy (day 26). The day of mating was considered day 0 of pregnancy. For glycolipid analyses, endometrium was isolated by scraping the uterine lumen. Implantation sites were excluded from analysis, but no attempt was made to distinguish mesometrium from anti-mesometrium. Samples were collected from three rabbits at each reproductive stage. For immunohistochemical analyses, segments of uteri were transferred to embedding compound (Miles Laboratories, Naperville, IL, USA) and frozen immediately on dry ice.

Glycolipid extraction and characterization

Glycolipids were extracted from 1–2 g of rabbit endometrium according to the method of Chien *et al.* (1978). In brief, an acetone powder of each sample was extracted sequentially using chloroform:methanol (CM) 2:1, CM 1:1 and CM 1:2. Neutral and acidic glycolipids were separated by ion-exchange chromatography using DEAE-Sephadex A25 (Pharmacia, Uppsala, Sweden), as described previously (Ledeen & Yu, 1982). Gangliosides were purified further by alkaline hydrolysis, dialysed against water, and lyophilized. Neutral glycolipids were separated from phospholipids and cholesterol by chromatography (Vance & Sweeley, 1967) using Bio-Sil silica gel columns (Bio-Rad, Richmond, CA, USA). Total lipid-bound sialic acid was determined by the method of Aminoff (1961): in brief, glycolipid samples were oxidized with periodic acid, reduced with sodium arsenate, reacted with thiobarbituric acid at 100°C and monitored for colour reaction at 549 nm. Sphingosine content was determined by the method of Naoi *et al.* (1974): in brief, free sphingosine base was released from glycolipid samples by acid hydrolysis and ether extraction, reacted with fluorescamine and monitored for fluorescence using a fluorometer. Gangliosides were separated by high-performance thin-layer chromatography (HPTLC) on silica gel plates (Merck, Darmstadt, Germany) in a solvent system of chloroform:methanol:water (CMW) 60:40:9 containing 0.2% CaCl₂. Gangliosides were visualized by resorcinol–HCl spray, and individual bands were quantified by scanning densitometry using a Shimadzu CS-910 scanner. Immunostaining of neutral glycolipids separated on Whatman HP-KF silica gel plates was performed as described previously (Fenderson *et al.*, 1987).

Immunohistochemistry

Frozen sections (5 µm) of rabbit uterus were post-fixed with acetone for one minute, air dried and rehydrated with phosphate-buffered saline (PBS) containing 5% (w/v) bovine serum albumin (fraction V; Sigma, St Louis, MO, USA). After 30 min, sections were treated with monoclonal antibody (mAb) for 1–2 h at 4°C, followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG plus IgM (Dakko, Carpinteria, CA, USA) for 1 h at 4°C. Sections were then washed with PBS, mounted with 70% glycerol in 50 mmol Tris–HCl l⁻¹ (pH 9.6), and examined using a Zeiss fluorescence microscope with epi-illumination. The following mAbs that recognize glycolipid antigens were used: AH-6 directed to Le^x (Abe *et al.*, 1983), 1A4-E10 directed to Gb3 (Fenderson *et al.*, 1987), SSEA-3 directed to Gb4 (Kannagi *et al.*, 1983), FE-A5 directed to nLc4 (Fenderson *et al.*, 1986a), BE2 directed to H type 2 chain (Young

et al., 1981), SH-1 directed to Le^x (Singhal *et al.*, 1990) and VESP 6-2 directed to sulfated galactosyl glycolipids (Petry *et al.*, 1988).

Results

Sphingosine and lipid-bound sialic acid analysis

The glycolipid content of rabbit endometrium was determined quantitatively at various reproductive stages. Neutral and acidic glycolipid fractions corresponding to normalized wet weight of tissue were assayed for sphingosine and lipid-bound sialic acid, respectively. The lipid-bound sialic acid content of endometrium of nonpregnant rabbits (representing total gangliosides) declined by 70% during the first week of pregnancy, day 6 (Fig. 1). There were no significant changes in lipid-bound sialic acid content during later pregnancy. By contrast, the sphingosine content of rabbit endometrium (representing total neutral glycolipids) was unchanged during early pregnancy (day 6), but increased 2.5 fold during middle (day 18) and late (day 26) pregnancy. Thus, the ratio of neutral glycolipids to gangliosides in endometrium increased dramatically during pregnancy.

Fig. 1. Quantitative analysis of glycolipid expression in rabbit endometrium during pregnancy: (a) lipid-bound sialic acid content of the acidic glycolipid (ganglioside) fraction; (b) sphingosine content of the neutral glycolipid fraction. Endometrium samples include nonpregnant oestrous stage (1); day 6 early pregnancy (2); day 18 mid-pregnancy (3); and day 26 late pregnancy (4). Data points represent the mean $\pm$ SD of three separate assays. Changes in glycolipid content were evaluated for statistical significance using a two-sample *t* test.

HPTLC analysis

The major gangliosides of nonpregnant and pregnant rabbit endometrium were identified using pure ganglioside standards as GM3 and GD3, with minor amounts of GM1, GD1a and GT1b (Fig. 2). Quantitative changes in ganglioside composition during pregnancy were determined by scanning densitometry on replicate HPTLC plates (Table 1). GM3 and GD1a content were found to increase dramatically during early pregnancy (day 6), whereas GD3 and GT1b content declined. GD3 was the major ganglioside during mid-pregnancy (day 18), accounting for 45% of total glycolipid. GD1a content declined during mid-pregnancy, but increased during late pregnancy (day 28).

The major neutral glycolipids of rabbit endometrium during early pregnancy (day 6) were similarly identified using pure glycolipid standards, lactosylceramide (CDH), Gb3 and Gb4 (Fig. 3;

Fig. 2. HPTLC analysis of ganglioside composition in rabbit endometrium during pregnancy. Samples were normalized to contain 1.5 µg sialic acid and included day 6 early pregnancy (1), day 18 mid-pregnancy (2), day 26 late pregnancy (3), and oestrus, nonpregnancy (4). A standard set of glycolipids was included (5). After streaking, HPTLC plates were developed by ascending chromatography in a solvent system of chloroform:methanol:water 60:40:9 containing 0.2% CaCl₂. Gangliosides were visualized by resorcinol spray. Note that individual gangliosides may resolve as doublets on HPTLC plates owing to discrete differences in fatty acid chain length.

Table 1. Ganglioside composition of rabbit endometrium (percentage of total) at specific stages of pregnancy

Ganglioside ^a	Oestrous stage	Day 6 (early pregnancy)	Day 18 (mid-pregnancy)	Day 26 (late pregnancy)
GM3	28 ± 3 ^b	52 ± 10	39 ± 1	35 ± 2
GD3	29 ± 5	15 ± 3	45 ± 3	23 ± 2
GD1a	5 ± 2	21 ± 9	2 ± 2	20 ± 1
GD1b	9 ± 3	not detected	7 ± 1	6 ± 2

^aGlycolipids 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).

^bData represent percentages (mean ± SD) of total resorcinol-positive glycolipid identified by scanning densitometry in three to six separate assays.

orcinol). During later pregnancy (day 26) there was an accumulation of CDH and the appearance of several new glycolipids of unknown structure. The presence of globo-series glycolipids in rabbit endometrium was confirmed by HPTLC immunostaining using mAbs specific for Gb3 and Gb4 (Fig. 3; 1A4E10 and SSEA-3).

The sensitivity of the HPTLC immunostaining method permitted the detection of additional, lacto-series glycolipids in rabbit endometrium (Fig. 3). For example, difucosyl Le^y was clearly detected by mAb AH-6 in samples of endometrium from day 6 and day 26 pregnant rabbits. Extended Le^y structures, also detected by mAb AH-6, were present during early pregnancy (day 6), but lost during later pregnancy (day 26). In contrast, nonsialylated poly-*N*-acetylglucosamine chains recognized by mAb FE-A5, and monofucosylated poly-*N*-acetylglucosamine chains recognized by mAb SH-1, both appeared in endometrium during late pregnancy (day 26). The

Fig. 3. HPTLC immunostaining analysis of neutral glycolipid composition in rabbit endometrium during pregnancy. Samples were normalized to 2 μ g sphingosine and included day 6 (implantation stage) and day 26 (late pregnancy). An appropriate standard was included (S). Replicate HPTLC plates were developed in a solvent system of chloroform:methanol:water 50:40:10 containing 0.05% CaCl_2 , and stained for carbohydrate by (a) orcinol/sulfuric acid spray (orcinol), or labelled with monoclonal antibodies (b) 1A4-E10, (c) SSEA-3, (d) FE-A5, (e) SH-1 or (f) AH-6. Results represent autoradiographs of an ^{125}I -labelled protein A detection system.

neutral glycolipids of nonpregnant (oestrus-stage) endometrium were not examined by HPTLC immunostaining.

Immunohistochemical analysis

The distribution of specific glycolipid antigens within the uterus of nonpregnant and pregnant rabbits was examined by the indirect immunofluorescence method (Figs 4 and 5). Of the several mAbs directed to neutral glycolipid antigens used in this study, mAb AH-6 (anti- Le^y) showed the strongest immunohistochemical reaction with endometrium. MAb AH-6 strongly labelled surface epithelium and glandular epithelium in uteri of both nonpregnant (oestrus stage) and early pregnant (day 6) rabbits (Fig. 4a–d). Epithelial cells appeared to be less-strongly labelled during later pregnancy (day 26); however, the epithelium is sparse and thin at this stage, making interpretation of results difficult. Of several mAbs directed to acidic glycolipids, VESP 6.2 (anti-sulfated-galactosyl glycolipid) showed the strongest immunohistochemical reaction. MAb VESP

Table 2. Summary of monoclonal antibody reactivity with epithelium and stroma of rabbit endometrium at different stages of pregnancy

Stage	VESP 6-2		AH-6		SH-1		BE2	
	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma
Nonpregnant (oestrus)	+	++	+++	—	+	—	—	—
Pregnant								
Day 6	++	+	+++	—	+	—	—	—
Day 8	—	—	+++	—	+	—	—	—
Day 26	—	—	+	—	+	—	—	—

Scale is negative (—) to strong positive (+++). Epithelium staining refers to both glandular and luminal components.

6-2 labelled endometrial stromal cells in uteri of both nonpregnant and early pregnant (day 6) rabbits (Fig. 5a–d). Stromal cells were not labelled during later pregnancy (days 8 and 26). MAb VESP 6-2 also labelled the glandular and surface epithelium of the endometrium on day 6 (Fig. 5c, d), but not on day 8 (Fig. 5e, f) of pregnancy. Thus, the VESP 6-2 antigen is expressed by endometrial epithelial cells for only a few days, during the time of uterine receptivity to blastocyst attachment. MAb SH-1 (anti-Le^x) weakly labelled the endometrial epithelium at all stages of oestrus and pregnancy, whereas mAb BE2 (anti-H type 2 chain) did not label the rabbit uterus at any stage. Results of indirect immunofluorescence assays are summarized (Table 2).

Fig. 4. Indirect immunofluorescence analysis of Le^x antigen expression in rabbit endometrium during pregnancy. Cryostat sections of (a, b) nonpregnant oestrous stage and (c, d) day 6 early pregnant uteri were labelled with monoclonal antibody AH-6 (anti-Le^x). Photomicrographs were taken using phase (a, c) or fluorescence (b, d) microscopy. E: epithelium.

Fig. 5. Indirect immunofluorescence analysis of sulfated galactose antigen expression in rabbit endometrium during pregnancy. Cryostat sections of (a, b) nonpregnant oestrous stage, (c, d) day 6 early pregnant, and (e, f) day 8 early pregnant uteri were labelled with monoclonal antibody VESP-6-2. Photomicrographs were taken using phase (a, c, e) or fluorescence (b, d, f) microscopy. Abbreviations: E: epithelium; GE: glandular epithelium; S: stroma.

Discussion

Analysis of glycolipid composition and localization in endometrium provides a first step towards understanding the functional roles of membrane glycolipids in implantation and pregnancy. The results presented here are summarized as follows: (i) the ganglioside content of rabbit endometrium declined rapidly during the first week of pregnancy (by 70%), whereas neutral glycolipid content increased approximately threefold during mid- and late pregnancy; (ii) the major gangliosides of rabbit endometrium were identified as GM3 and GD3, and the major neutral glycolipids were identified as globo-series structures Gb3 and Gb4; (iii) additional glycolipid structures, including difucosyl Le^y and sulfated-galactose, were localized using monoclonal antibodies along the luminal surface of the endometrium during the implantation stage (day 6). The structures of the major glycolipid antigens of rabbit endometrium are summarized in Table 3.

The glycolipids of rabbit endometrium and their changes during pregnancy are remarkably similar to the glycolipid changes that were identified in human endometrium (Zhu *et al.*, 1990). In

Table 3. Major neutral and acidic glycolipids of rabbit endometrium

Glycolipid structure		Designation
<i>Neutral glycolipids</i>		
	Galβ1→4Glcβ1→Cer	CDH
	Galα1→4Galβ1→4Glcβ1→Cer	Gb3
	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer	Gb4
	Fuca1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer	Le ^y
	3	
	↑	
	Fuca1	
<i>Acidic glycolipids</i>		
	NeuAca2→3Galβ1→4Glcβ1→Cer	GM3
	NeuAca2→8NeuAca2→3Galβ1→4Glcβ1→Cer	GD3
	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer	GD1a
	3	
	↑	
	NeuAca2	
	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer	GT1b
	3	
	↑	
	NeuAca2	
	8	
	↑	
	NeuAca2	

Glycolipid structures were identified by: (i) co-migration on HPTLC plates with pure glycolipid standards; and (ii) HPTLC immunostaining using specific anti-glycolipid monoclonal antibodies.

both species, ganglioside expression declines dramatically during early pregnancy, whereas neutral glycolipid expression increases gradually during the course of pregnancy. As discussed previously (Zhu *et al.*, 1990), these changes in glycolipid expression may facilitate the close apposition of maternal and embryonic cell membranes during implantation by reducing electric charge at the cell surface. A decrease in sialic acid charge has previously been identified in the rabbit uterus at the time of implantation, and postulated to play an important role in regulating trophoblast adhesion (Anderson & Hoffman, 1984; Anderson *et al.*, 1986). However, regional specificity of blastocyst attachment within the uterus suggests that local changes in charged or other molecules may be more important than the global changes described here.

Stage-specific changes in the glycoprotein composition of the rabbit endometrium have been reported previously. By analysing the surface iodination patterns of isolated uterine epithelial cells, Ricketts *et al.* (1984) noted quantitative changes in the expression of two proteins (molecular masses 38 and 42 kDa) between day 4 and day 6.5 of pregnancy. Anderson *et al.* (1986) noted an apparent loss of sialic acid and a corresponding increase in terminal galactose in the rabbit uterine epithelium during the period of receptivity to implantation. In addition, these workers characterized the appearance of three new glycoproteins (molecular masses 24, 42, and 58 kDa) during this time. More recently, Bukers *et al.* (1991) have described regional differences in glycoconjugate expression in the rabbit uterus: differences in lectin staining were observed between the mesometrial and anti-mesometrial surface of the uterine lumen, and between the implantation chamber and the interblastocyst segments.

The specific fucosylated and sulfated antigens that we have identified in rabbit endometrium by indirect immunofluorescence (Figs 4 and 5) have been identified previously in the uteri of other mammalian species. For example, Le^y, Le^x and blood group H antigens are known to be expressed by epithelial cells of mouse endometrium (Babiarz & Hathaway, 1988; Kimber *et al.*, 1988; Kimber & Lindenberg, 1990), and the Le^y antigen has been shown to appear on the mouse blastocyst upon exposure to the uterine environment (Fenderson *et al.*, 1986b). In addition, sulfatide

(sulfated-galactosyl ceramide) has been found to increase dramatically in human endometrial cells during the secretory stage (i.e. the receptive phase) of the menstrual cycle (Kubushiro *et al.*, 1989), and sulfated glycoproteins have been identified as secretory products of the mouse oviduct (Erickson-Lawrence *et al.*, 1989). In this connection, the carrier molecule for the sulfated-galactosyl epitope recognized by mAb VESP 6:2 in rabbit endometrium could be a glycoprotein, as only trace amounts of sulfated glycolipid were detected in this study by HPTLC analysis (data not shown).

The presence of sulfated galactose epitopes on the surface epithelium of rabbit endometrium during the time for implantation (Fig. 5) focuses attention on sulfated glycolipids or sulfated glycoproteins or both of these molecules as possible recognition signals for trophoctoderm–endometrium adhesion. Although negatively charged, sulfatide is known to interact strongly with the extracellular matrix protein laminin (Roberts *et al.*, 1988). In addition, poly-sulfate groups of the zona pellucida are considered to mediate fertilization by providing high-affinity receptors for sperm acrosomal proteins (Jones, 1991). The presence of laminin on the blastocyst surface could provide one mechanism for specific recognition during implantation; however, immunochemical studies have not detected laminin on trophoctodermal cells at this stage (Carnegie, 1991). The results presented here suggest that the expression of other sulfatide-binding proteins, such as thrombospondin and properidin (see Holt *et al.*, 1989), by trophoctodermal cells and their possible role in implantation should be investigated.

In addition to proteins, recognition molecules for carbohydrates may include carbohydrates. For example, we have reported previously that fucosylated $\alpha 1 \rightarrow 3$ lactosamine (i.e. Le^x) structures self-aggregate in the presence of Ca²⁺ (Eggers *et al.*, 1989), a property which may underlie the role of Le^x in mediating interactions between embryonic cells during morula compaction (reviewed by Fenderson *et al.*, 1990). These results have been extended by the demonstration of other specific glycolipid–glycolipid interactions, including GM3–asialo-GM2 interaction (Kojima & Hakomori, 1989) and sulfatide–galactosylceramide interaction (Kojima & Hakomori, unpublished). By analogy, Le^y, sulfated-galactosyl structures or both on the surface of uterine epithelial cells (Figs 4 and 5) may provide multivalent ligands for cell surface carbohydrates present on the surface of trophoctodermal cells of the implanting blastocyst. It should be possible to substantiate this hypothesis using liposome adhesion assays (e.g., Eggers *et al.*, 1989) to identify matching carbohydrate ligands for Le^y and sulfatide. In addition, it should be possible to test the general role of carbohydrate recognition in implantation using monoclonal anti-carbohydrate antibodies (e.g. AH-6 and VESP 6:2) as blocking agents, in *in vitro* and *in vivo* implantation assays.

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