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Glycosphingolipids of Rabbit Uterine Tissue and Changes During the Peri-Implantation Period

Tracy A. Bochantin*, M.J.K. Harper**, and Marjorie A. Jones*
*Chemistry Department
Illinois State University
Normal, Il. 61790-4160

** Obstetrics and Gynecology
University of Texas Health Science Center
San Antonio, TX 78284

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ABSTRACT

Glycosphingolipids (cerebrosides and gangliosides), which are involved in cell receptor functions and cell-cell recognition, may play an important role in the selection of the blastocyst implantation site. Therefore isolation and characterization of glycosphingolipids from uterine tissue is of interest. Uteri from estrous, day-6-pregnant and day-6-pseudopregnant rabbits were examined. Total lipids from uterine epithelial scrapings were extracted by the method of Bligh and Dyer (1959). The glycosphingolipids were obtained following saponification of the total extract and thin layer chromatography. The glycosphingolipids were identified using lipid specific reagents, relative to TLC migration of authentic standards, and quantified using densitometry. The uterine epithelial scrapings of day-6-pregnant and day-6-pseudopregnant tissues contained a greater concentration of cerebrosides and gangliosides than scrapings from estrous rabbits. The gangliosides were 10 to 20-fold higher concentrations than cerebrosides. Of the four gangliosides isolated, the ratios varied with reproductive state studied. Ganglioside 1 was a lower concentration in day-6-pseudopregnant tissue while ganglioside 2 was lowest in day-6-pregnant and gangliosides 3 and 4 were lowest from estrous tissue. These results indicate that amounts of glycosphingolipids do vary with respect to the reproductive state of the rabbit.

Key Words: gangliosides, cerebrosides, uterus

INTRODUCTION

Reproduction is a complex process. During the peri-implantation period an interaction between embryonic and maternal tissue must be established and maintained for pregnancy to be successful (Newton et al., 1988). This interaction between the blastocyst and the uterus is believed to be controlled by hormones over long range distances or distances greater than one to two cells. However, communication over short distances (cell to cell) is believed to be controlled by a not well understood association of the blastocyst with the epithelial surface of the endometrium (Surani, 1977). Blastocyst implantation is considered to be a short range cell-cell recognition interaction. Therefore molecules, including glycoconjugates such as glycosphingolipids and glycoproteins, which are located at the maternal-embryo interface, may play a role in establishing pregnancy. While a number of researchers have chosen to study the role of glycoproteins in implantation, glycosphingolipids have been largely ignored. Therefore, this research has involved isolation and characterization of the two major types of glycosphingolipids, (glycolipids) cerebrosides and gangliosides. A cerebroside is a sphingosine derivative where the carbohydrate residue is galactose or glucose. A ganglioside is a sphingosine derivative where there are multiple carbohydrate residues which usually include a sialic acid.

Glycosphingolipids are important cell membrane components, present primarily on the outer surface of the plasma membrane (Lehninger, 1982). The high glycosphingolipid content on the outer bilayer suggests two possibilities: glycosphingolipids contribute to the structural rigidity of the membrane surface and/or glycosphingolipids are well suited to interact with exogenous ligands through their carbohydrate moieties (Hakomori, 1981). These molecules are believed to affect a variety of cell functions including cell recognition and cell receptor functions (Kanfer, 1983). A change in glycolipids has been associated with cellular interactions and differentiation, and cell contact enhances glycolipid synthesis (Hakomori, 1981).

Glycoconjugates have been identified as being present on a number of reproductive structures. Previous studies, using carbohydrate specific dyes, have revealed rapid, stage-specific changes in the surface glycoconjugates of uterine epithelium (Schlafke and Enders, 1975; Sherman and Wudl, 1976; Hewitt et al., 1979; Chavez and Enders, 1981; Chavez and Anderson, 1985). These studies are limited in that they failed to quantitate and identify chemically the molecular species of the glycoconjugates involved and to distinguish between glycolipids and glycoproteins. A more recent study by Zhu et al. (1990) focused specifically on glycosphingolipids and showed that these lipids were present in human uterine tissues. They also demonstrated that the relative proportions of glycolipids changed during the menstrual cycle, pregnancy, and aging. This study did not specifically focus on the peri-implantation period. Therefore, the purpose of the current study was to isolate and quantify the glycosphingolipids present in rabbit uteri and compare the types and relative concentrations of glycolipids found at estrus, and day-6 of pregnancy and pseudopregnancy. As these studies were in progress, Zhu et al. (1992) published observations on glycosphingolipids of rabbit endometrium during pregnancy. They used a different lipid extraction procedure, a different isolation procedure, and a different thin layer chromatographic system to evaluate their extracts. The work by Zhu et al. (1992) is very interesting since they demonstrate that there was a 70% decrease in

ganglioside content during early pregnancy (day-6) and a 2.5-fold increase in neutral glycolipid during later pregnancy (day-26). They did not, however, evaluate pseudopregnant rabbits. The studies using pseudopregnant tissue can give some insight into the maternal and embryo control of uterine surface changes which occur during peri-implantation.

MATERIALS AND METHODS

Materials

New Zealand White-Cambridge rabbits (body weight greater than 3.0 kg) were obtained from Penn Acres, Wimberly, Texas. Porcine follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG), bovine serum albumin (Fraction V), standard gangliosides (Type IV), standard galactocerebrosides (Type IV), cholesterol standard (tlc grade), resorcinol, molybdenum blue reagent, and diphenylamine were purchased from Sigma Chemical Company (St. Louis, MO). High performance thin layer chromatography (hptlc) plates (silica gel 60, 0.2mm, 10 cm x 10 cm) were purchased from Alltech (Deerfield, IL.) and Redi-prep thin layer chromatography (tlc) plates (Silica Gel G) were obtained from Fisher Scientific Company (Pittsburg, PA). Dimethylaminobenzaldehyde was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). The Image Analyzer Spectrophotometer was obtained from Sun Microsystems, Inc. (Mountain View, CA) and programs for the Image Analyzer Computer were obtained from Bio Image Corporation (Ann Arbor, MI).

Methods

Mature, female rabbits were caged individually in a controlled environment with a 14 hour light:10 hour dark cycle. They were fed 170 g of rabbit chow daily and provided with fresh water *ad libitum*. The rabbits were sacrificed by an intravenous overdose of sodium pentobarbital. The uterine tissues were rapidly removed, flushed with Tyrode's buffer (Jones and Harper, 1984) and frozen. Tissues were stored at -80ºC until used. Estrous rabbits were injected with 0.5 IU of FSH, subcutaneously, twice a day for three days (Mukherjee et al., 1978). On day 0 they were artificially inseminated with sperm from fertile bucks. After insemination, the animals were then injected IV with 100 IU of hCG. For pseudopregnant rabbits only the injections of FSH and hCG were given. Rabbits were sacrificed at 144 hours post hCG injection (day-6).

Glycosphingolipid Isolation: Each uterus was thawed on ice and a wet weight determined. The uterus was then cut open along the mesometrial line and the endometrial lining was scraped with a stainless steel spatula and transferred to a chilled polypropylene tube. The wet weight of the scraping was obtained before the material was lyophilized for 24 hours then re-weighed. Nine ml of ice-cold saline (0.9% NaCl, wt/v) were added to the dry tissue scraping and this was homogenized, for one 30 second period, using an OMNI International 1000 polytron at high setting. The homogenate was then extracted twice for total lipids by the method of Bligh and Dyer (1959). The chloroform from both extracts was pooled, dried under nitrogen and then resuspended in 1 ml of chloroform:methanol (1:1). To evaluate the completeness of this extraction, tlc was done with the methanol/water phase. No glycolipids were detected in this fraction.

High Performance Thin Layer Chromatography (hptlc): Extracted lipids were evaluated, relative to authentic standards (gangliosides, galactocerebrosides, cholesterol, sphingomyelin, phosphatidylcholine, and palmitic acid) by hptlc using several solvent systems. Chloroform/methanol/water (65:25:4) was selected as the solvent of choice since lipids extracted from the tissues had comparable Rf values with the standards and resulted in a good separation of the various lipids. To identify the lipids separated by hptlc several reagent sprays were used. These reagents were sprayed onto the developed places using an all-glass atomizer operating off a compressed air line. A solution of 5% sulfuric acid in ethanol (v/v) was used as a general detection reagent for carbon containing compounds. After spraying, the plates were gently heated using a hot plate so that the carbon containing molecules charred and appear as black spots (Kates, 1988). Other reagents used were 4-dimethylaminobenzaldehyde-HCl for gangliosides and other lipids with sialic acid (Mehlitz et al., 1963), diphenylamine for glycolipids (Jatzkewitz and Mehl, 1960), resorcinol for gangliosides (Miettinen and Takki-Luukainen, 1959), and molybdenum blue (Dittmer and Lester, 1964) for detection of phospholipids. The molybdenum blue reagent clearly indicated that the lipid extract contained substantial amounts of phospholipids which were masking some of the gangliosides. A saponification reaction was therefore done by heating the lipid extract at 37ºC in methanolic KOH (0.1M KOH in methanol, freshly prepared) for 30 min then extracting with chloroform (Kates, 1988). Glycosphingolipids are stable to this treatment and therefore are found in the chloroform layer. After saponification, separation of the cerebrosides and gangliosides was done using Silica gel G Redi-Prep tlc plates (fig 1). After chromatography using chloroform/methanol/water (65:25:4) the positions of the cerebroside and ganglioside standards were marked. Those sections of the plate containing the lipid extracts were then scraped and re-extracted with the same solvent system. The extracts were filtered using a ground glass funnel to remove the silica and the sample was collected and the solvent evaporated under nitrogen gas; the samples were stored at -80ºC until assayed for glycosphingolipids.

Determination of Protein: Protein content per uterine tissue scraping was evaluated by the method of Lowry et al., 1951 using bovine serum albumin as standard.

Determination of Glycospingolipid: Glycosphingolipid content per uterine tissue scraping was evaluated using a Sun Image Analyzer (densitometer). After treating the hptlc plates with the 5% sulfuric acid/ethanol spray and heating, the spots were evaluated by integrating the area of the curve generated spectrophotometrically. Authentic standards of known concentrations were also evaluated with this procedure.

Statistical Analysis: Statistics were performed using linear regression and analysis of Variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post test as applicable to determine significance between reproductive states. Data are reported as mean $\pm$ SD for 4 rabbits per group and considered to be significantly different at $p < 0.05$.

RESULTS

The mean $\pm$ SD of uterine weights, normalized wet endometrial scraped weights, normalized dry weights and protein are presented in Table 1.

Table 1. Uterine, endometrial scraping, and protein weights (mean ± SD).

REPRODUCTIVE STAGE	UTERINE WEIGHT(g) OF TISSUE USED	mg WET ENDOMETRIAL SCRAPING / g TISSUE	mg DRY ENDOMETRIAL SCRAPING / g TISSUE	mg PROTEIN PER g WET TISSUE
Day-6-Pregnant	4.4 ± 1.1a	168.0 ± 22.1d	31.0 ± 4.4g	15.3 ± 2.5j
Day-6-Pseudo-pregnant	2.9 ± 0.4b	88.9 ± 61.7e	18.9 ± 12.3h	11.8 ± 7.9j
Estrus	1.5± 0.4c	25.8 ± 8.3f	4.7 ± 1.7i	3.2 ± 0.8k

Values with the same letter are not significantly different at $p < 0.05$ (ANOVA, followed by Fisher's LSD)

As shown, the amounts of tissue available for these studies varied between the reproductive states used. Therefore, data have been normalized per g of tissue weight to adjust for these differences. The pregnant tissue exhibited the highest amount of endometrial scraping per g of tissue followed by day-6-pseudopregnant and then by estrous samples which were significantly lower than the other two groups. The amount of the estrous scraping was approximately six-fold less than the pregnant material and three-fold less than the pseudopregnant material. The same trend was observed when data were normalized on a tissue dry weight basis. In addition, both the day-6-pregnant and day-6-pseudopregnant tissue scrapings contained approximately 5-fold more protein than estrous tissue scrapings. This was expected as a result of the increase in uterine tissue size in preparation of a receptive state (Psychoyos, 1973; Finn and Marti, 1974). These data clearly show differences in total mass between the reproductive stages monitored and are not, therefore, simply due to differences in the wet weights of the starting tissues. It is of interest to note that although the day-6-pregnant and day-6-pseudopregnant scrapings had significantly different values of mg wet or dry endometrial scrapings per g tissue, the mg of protein per g of tissue was not significantly different. This suggests that the presence of the embryo affects other classes of biological polymers than proteins in the uterine endometrial layer.

Using tlc, two major cerebrosides and four gangliosides were detected in each reproductive stage tissue and normalized glycosphingolipid content is reported in Table 2.

Table 2. Normalized glycosphingolipid content (mean ± SD).

REPRODUCTIVE STAGE	ug CEREBROSIDE PER g WET WT TISSUE	ug GANGLIOSIDE PER g WET WT TISSUE
Day-6-Pregnant	66 ± 11a	998 ± 245c
Day-6-Pseudo-pregnant	80 ± 50a	650 ± 427c
Estrus	12 ± 8b	223 ± 65d

Values with the same letter are not significantly different at $p < 0.05$ (ANOVA followed by Fisher's LSD).

To calculate these values, each glycosphingolipid mass, determined from Image analyzer data collected from tlc, was divided by the appropriate tissue weight. The mass of the cerebrosides was detected by comparing the absorbance values of standard concentrations (0.1 to 100 ug) with those of the unknowns. Overall, the hormonally stimulated tissue scrapings exhibited approximately 6-fold more cerebrosides and approximately 4-fold more gangliosides than estrous scrapings. These increases in glycosphingolipid content between estrus and day-6 after ovulation were significant. Since the tissue sizes increase only two to three fold, these increases in cerebrosides reflect *in vivo* increases. There were two major neutral glycosphingolipids (cerebrosides) detected in the endometrial epithelial scrapings. The apparent types of the cerebrosides did not change as a result of the reproductive state. This corresponds with the findings of Zhu et al. (1990) who identified the major cerebrosides of both human myometrium and endometrium as GB3 and GB4 using high performance tlc. Their report is one of the few studies that has looked specifically at glycosphingolipids in reproductive tissues. However, they did not evaluate the peri-implantation period. They did not observe any changes in cerebroside concentrations during the human menstrual cycle, pregnancy, or aging. Zhu et al. (1992) did report changes in neutral glycolipid content as evaluated by sphingosine content in early pregnancy (day-6) in rabbit. Since Zhu et al. (1990; 1992) did not include Rf values, it is difficult to determine if these cerebrosides are the same as reported here. The tlc migration relative to each other, however, appears to be the same. There were four main gangliosides detectable with specific reagents in the lipid extracts of the endometrial tissue scrapings. The total mass of gangliosides was detected by comparing the absorbance values of standard concentrations (0.1 to 100 ug) with those of the unknowns. Since glycosphingolipids are metabolically expensive for cells to synthesize (Elbein, 1987), these lipids must have some important biological roles in the endometrium of the rabbit uterus.

Comparisons of glycosphingolipid ratios within the same uterine scrapings are shown in Table 3. These data were obtained by dividing the total cerebroside weight per scraping into the total ganglioside weight per scraping.

Table 3. Glycosphingolipid ratios (mean ± SD).

REPRODUCTIVE STAGE	GANGLIOSIDE PER CEREBROSIDE	GANGLIO-SIDE 1 PER TOTAL GANGLIO-SIDES	GANGLIO-SIDE 2 PER TOTAL GANGLIO-SIDES	GANGLIO-SIDE 3+4 PER TOTAL GANGLIO-SIDES
Day-6-Pregnant	15 ± 13a	0.48 ± 0.15b	0.28 ± 0.11d	0.24 ± 0.06f
Day-6-Pseudo-pregnant	14 ± 14a	0.19 ± 0.18c	0.48 ± 0.04e	0.32 ± 0.14f
Estrus	21 ± 50a	0.46 ± 0.02b	0.48 ± 0.01e	0.05 ± 0.03g

All values are in ug units
Values with the same letter are not significantly different at $p < 0.05$ (ANOVA followed by Fisher's LSD).

While there was a trend for a higher ratio of ganglioside per cerebroside for estrus relative to the other two stages, this was not a statistically significant trend. This is likely due to the large standard deviations especially for the estrus data. This is likely due to the limited amount of endometrial tissue which can be collected from estrous animals. Zhu et al. (1990) did not look at the total ganglioside weights but at each individual ganglioside weight per total ganglioside weight. Therefore, comparisons of some individual gangliosides relative to the total gangliosides (ug per total ug) were calculated. While gangliosides 1 and 2 could be easily resolved by tlc, gangliosides 3 and 4 could not and were therefore were treated as one pool. The average ug per g wet weight of tissue for ganglioside 1 was 482, 122, and 103 for day-6-pregnant, day-6-pseudopregnant and estrus, respectively. The average ug per g wet weight of tissue for ganglioside 2 was 281, 314, and 108 for day-6-pregnant, day-6-pseudopregnant and estrus, respectively. The average ug per g wet weight of tissue for ganglioside 3+4 was 235, 210, and 12 for day-6-pregnant, day-6-pseudopregnant and estrus, respectively. There were significant changes in individual gangliosides relative to total gangliosides as a function of the reproductive status of the animal. For ganglioside 1, day-6-pregnant and estrous tissues contained about 4 times more than did the day-6-pseudopregnant tissue. The ratios of ganglioside 2 relative to total gangliosides for the estrus and day-6-pseudopregnant animals were not different, but were significantly higher than the day-6-pregnant by a factor of approximately two. For gangliosides 3 plus 4, the hormonally stimulated tissues had about 5-fold more than the estrous samples. Thus, the ratios of ganglioside types in uterine scrapings appear very dependent upon the reproductive status of the rabbit. Zhu et al. (1990) also found four gangliosides, two major and two minor tlc bands, using human myometrium and endometrium. Using high performance tlc they identified the gangliosides as GM3, GD3, GM1, and GD1a. As with the cerebrosides, they also reported that the apparent types of gangliosides did not change as a function of the reproductive state. They did find that during pregnancy, GD3 content declined whereas GM3 content increased. A similar pattern of change was seen in both of our hormonally stimulated tissues when compared to the estrous scrapings. Using rabbit tissues, Zhu et al. (1992) reported a substantial decrease in ganglioside content (as measured by lipid-

bound sialic acid) during pregnancy relative to estrus. This is in contrast to the substantial increases which we found after ovulation in rabbits. Their data do suggest that total ganglioside changes are not similar humans and rabbits. Yet, Zhu et al. (1992) did report that several specific gangliosides, especially GM3 and GD3 were the major species in rabbit endometrium. They report that GM3 content increases during early pregnancy whereas GD3 content decreases.

DISCUSSION

The present study was designed to isolate and quantitatively evaluate glycosphingolipids from rabbit uterine tissue and determine if there were variations with the reproductive state of the animal. Since different size uterine horns were obtained from the different animals the data were normalized. Although there were larger amounts of tissue for the day-6-pregnant group, we found that each group contained the same percentage of water (82%) with varying amounts of some specific organic materials (as shown in Tables 1 and 2) relative to g of initial material.

In our study we found 234 ug of glycosphingolipids per g tissue weight in the estrous rabbit tissue. This conflicts with the study by Chavez and Anderson (1985) where they found few carbohydrate residues in the estrous animals. This could be due to the sensitivity of the assay which they used. The lectin dye, *Ricinus communis* agglutinin, has a recognition specificity for beta-linked D-galactose. If the sugar ending of the glycolipid or glycoprotein was not beta-linked D-galactose then it would not be detected. Also binding studies can be misleading if the sites are sterically hindered.

The ganglioside ratios reported here show a complex relationship. There was no single trend evident when comparing the three reproductive stages and no systematic comparisons which could be made. The variation of the ganglioside mass relative to reproductive state reveals that gangliosides have a complex, yet not well understood, role in implantation. Zhu et al. (1992) suggest that the rapid changes in glycosphingolipid content and composition during early pregnancy may enhance embryo-maternal interactions. Also our observation that the gangliosides are in much higher relative concentrations than cerebrosides for all samples evaluated is supportive of this enhancement role. There appears to be approximately 14 to 20-fold more mass of ganglioside per cerebroside on a weight basis (ug/ug) and 8-fold increase on a mole to mole basis (assuming the average MW of a cerebroside is 800 and that of a ganglioside is 1500). Since gangliosides would contribute to a negative tissue surface change, due to the sialic acid moiety, this ratio is interesting especially since there was no apparent ganglioside/cerebroside ratio difference with reproductive state.

The apparent total amounts of glycolipids detected in this study by tlc followed by spectroscopy can be estimated as the sum of ganglioside plus cerebroside per tissue. The values were calculated to be 234 ug, 1064 ug, and 730 ug per g wet wt of tissue for estrus, day-6 of pregnancy and pseudopregnancy, respectively. Thus there is apparently fifteen fold less glycolipid (ug/ug) than protein per g tissue. However, since most of the glycolipid would be expected to be associated with the plasma membrane while a much smaller porportion of the total protein would be similarly associated, this suggests that the epithelial plasma membrane is very rich in glycosphingolipid.

The same apparent types of glycosphingolipids (two cerebrosides and four gangliosides) were found in the rabbit uterine epithelial scraping regardless of the reproductive stage of the animal. Although the chromatographic pattern did not change, small changes in the glycosphingolipid structure could have occured such as an increased degree of glycosylation. According to a review by Johnson (1991), glycosylation of a protein or lipid changes with the physiological state of the animal; for example, the glycosylation of two human hormone-binding serum glycoproteins (transcortin and thyroxine-binding globulin) changes during pregnancy. An increased amount of galactosyltransferase is directly related to an elevated fertilization ability (Johnson, 1991). Since glycosylation is an important aspect of glycoprotein and glycolipid activity, it should be further investigated to evaluate possible changes in carbohydrate content. The apparent amounts of both cerebroside and ganglioside, as detected from the tlc, showed significant differences due to the reproductive state. The hormonally stimulated tissues had a significantly higher amount of glycosphingolipid than the estrous samples by at least a factor of three. This signals that there is a correlation between the steroid hormones released by the maternal system and glycosphingolipid concentration in preparation for implantation and pregnancy. Since we did not see a difference between day-6-pregnant and day-6-pseudopregnant samples, this suggests that the embryos have little influence on maternal glycosphingolipid metabolism and that the ovary is the controlling factor. Reflecting on the study by Zhu et al. (1992), which is the only recent study done on glycosphingolipids in rabbit reproductive tissue, our findings do not correspond with their findings during early pregnancy relative to ganglioside content. This is of interest especially since different extraction techniques and quantitative methods were used. They, also, report that the ratio of neutral glycolipids to gangliosides increased during pregnancy (even on day-6) while we show that the ganglioside to cerebroside (one type of neutral glycolipid) ratio does not change significantly on day-6 relative to estrus. Since Zhu et al. (1992) were measuring sphingosine content (which reflects total neutral glycolipids) and we were measuring specifically cerebrosides, it may be that some neutral lipids increase while others decrease. Clearly, more works remains in this area.

The data do suggest that the rabbit is a good model for evaluating the events which lead to changes in glycosphingolipids during the peri-implantation period. Further studies are now necessary to extend these findings among additional days of reproduction in the rabbit and also to look specifically at the implantation and interimplantation sites of day-7-pregnant rabbits. Since the apparent types of glycosphingolipids did not change, a closer detailed look at the structures of these glycosphingolipids must be done using gas chromatography and mass spectrometry to see if subtle changes in structure occur.

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Figure 1. A representative high performance tlc of glycosphingolipids extracted from epithelial scrapings then saponified. Solvent used was 65/25/4 chloroform/methanol/water (v/v/v); spots were detected after spray with 5% H_2S0_4 in methanol then gentle heating of the tlc plate

Lane 1: standard galactocerebrosides
Lane 2: standard gangliosides
Lane 3: standard cholesterol
Lane 4: standard sphingomyelin
Lane 5: saponified lipid extract.

Figure 1 is not available in this on-line edition. Please contact a library or the author for a hard-copy of the figure.