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Characteristic Expression of Cholesterol Sulfate in Rabbit Endometrium during the Implantation Period

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SUMMARY: Sialic acids and sulfate residues are the major negative-charged cellular components, which are considered to be crucial in embryonal adhesion to the endometrium. To explore the mechanism of implantation, we examined the change in the amounts of these substances in the rabbit endometrium during the implantation period. Gangliosides and sulfatides were present in very small quantity in the endometrium irrespective of the reproductive stage. Though the content of cholesterol sulfate was relatively low in the nonpregnant endometrium, it abruptly increased at day 5 of pregnancy, i.e. at the beginning of implantation, followed by a gradual decline toward day 9. Cholesterol sulfate level in the interimplantation sites was about twice as much as that in the implantation sites and was comparable with that in the pseudopregnant endometrium. These results demonstrate that cholesterol sulfate is a major negative-charged lipid in the peri-implantation endometrium in rabbits. We further point to the difference in the concentration of cholesterol sulfate between implantation and interimplantation sites, thus suggesting cholesterol sulfate as a major participant in the process of implantation.

The earliest significant event of the implantation process in mammals is the adhesion of the blastocyst to the uterine epithelium. Several reports have appeared in the literatures which suggest the involvement of the reduction in the electrostatic repulsion between trophoblast and epithelium in facilitating the juxtaposition of the two surfaces, and thereby aiding the adhesive process. Along with this line of notion, the intensity of surface charge on uterine epithelial cells has been shown to be greatly reduced at the time of implantation in mice(1) and in rabbits (2). On the other hand, mouse blastocysts exhibit a loss of negative charge on the surface membrane during the implantation period (3).

Sialic acid and sulfate residues are considered to be the major cellular components which contribute to surface negative charge. Therefore, in this study, we attempted to analyze these components in the rabbit endometrium during the implantation period to get insight into the biochemical mechanisms underlying the implantation process. The purpose of the present study was to seek the difference in the concentrations of these components between implantation and interimplantation sites. We further examined their concentrations in the endometrium obtained from pseudopregnant rabbits where hormonal environment and epithelial morphology are essentially the same as those in pregnant animals.

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Here we demonstrated that cholesterol sulfate (CS) is a major negative-charged component occurring in the rabbit endometrium at peri-implantation. We further presented evidence for a significant reduction in the concentration of CS in implantation sites as compared with interimplantation sites, thus providing a clue in the elucidation of the adhesive mechanisms during implantation.

MATERIALS AND METHODS

Animals and Treatments. Virginal 6-month-old female New Zealand White rabbits weighing approximately 4.2 kg were obtained from Japan Biological Materials Co., Tokyo. In five rabbits, pregnancy was induced by copulation with two males. The day of coitus was defined as day 0 of pregnancy. In another rabbit, pseudopregnancy was induced by intravenous injection of 100 IU of human chorionic gonadotropin (hCG) in 1 ml of saline solution 3 days after subcutaneous administration of 50μg of 17β-estradiol in 0.2ml sesame oil. The day of hCG injection was taken as day 0 of pseudopregnancy.

Preparation of Endometrium. Nonpregnant, pregnant at days 3, 5, 6, 7 and 9, and pseudopregnant at day 6 rabbits were hysterectomized under intravenous pentobarbital anesthesia (25mg/kg). Then the endometrium was separated from the myometrium by scraping after removing embryonic tissues. In rabbits at day 6, 7 and 9 of pregnancy, the endometrium underlying the implantation sites was collected separately from interimplantation sites. The tissues, after washing with ice-cold phosphate-buffered saline (Ca^{2+}-free and Mg^{2+}-free, pH 7.4), were homogenized in distilled water and then lyophilized.

Lipid Extraction. Total lipids were extracted from the lyophilized endometrium sequentially with chloroform/methanol/water (20:1:1, 10:20:1, 20:10:1, 10:20:1, 1:1:0 v/v) at 40°C. To separate neutral and acidic lipids, the combined lipid extracts were directly applied to DEAE-Sphadex A25 column (acetate form, Pharmacia LKB Biotechnology) and eluted stepwise with 5 vol. of chloroform/methanol (1:1, v/v), 1 vol. of methanol and 10 vol. of 0.3 M sodium acetate in methanol. The acidic lipids fraction, which was eluted from the column with last solvent, was incubated with 0.5M sodium hydroxide in methanol at 40°C for 1 hour to cleave the ester-containing lipids. After neutralizing with 1.0M acetic acid in methanol, salts were removed by dialysis and the solution was evaporated to dryness.

Identification of Cholesterol Sulfate. CS was purified by column chromatography on Iatrobeads (6RS-8060, Iatron Laboratories., Inc.) with stepwise solvent system, chloroform/methanol (1:0, 9:1, 8:2, 6:4, 1:9 and 0:1 v/v). Then the purified CS was identified by comparing the mobility with that of chemically synthesized cholesterol sulfate by TLC with a HPTLC-plate (silica gel 60, Merck) before and after solvolysis. The plate was developed with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v) and the materials on the plate were located by spraying with cupric acetate-phosphoric acid reagent and heating at 110°C for 5 minutes. For solvolysis, CS purified above was dissolved in a mixture of dimethylsulfoxide/methanol (9:1 v/v), containing sulfuric acid in the concentration of 9mM, and heated at 80°C for 1 hour. After cooling the mixture, the liberated cholesterol was recovered by Folch's partition.

Further identification of CS was performed by the negative-ion FAB mass spectrometric analysis. It was performed on a JEOL HX-110 mass spectrometer, and data processing was controlled by a JMA DA-5000 data system. The primary bombarding xenon beam was operated at 6keV. The ion acceleration voltage was 10kV. Samples were dissolved in chloroform/methanol (1:1, v/v) and admixed with a small volume of triethanolamine as a matrix. Mass spectra were recorded in the mass range of m/z 0-600, and acquired data were offset to nominal masses.

Determination of Cholesterol Sulfate. CS was determined by TLC as described above following densitometry at wavelength 420nm with a CS-9000 flying-spot scanner (Shimadzu). The sodium salt of CS, which was synthesized in this laboratory according to Mumma(4), was used as a standard.

RESULTS

Gangliosides and sulfatides, candidates to contribute to surface negative charge due to their sialic acid and sulfate residues respectively, were hardly detected in the endometrium at any reproductive stage by TLC. On the other hand, considerable amount of an acidic lipid was detected on TLC in endometria at days 5, 6, 7 and 9 of pregnancy and day 6 of pseudopregnancy (Fig. 1). This acidic lipid component was eluted from an Iatrobeads column with chloroform/methanol (8:2 v/v) and the Rf value was consistent

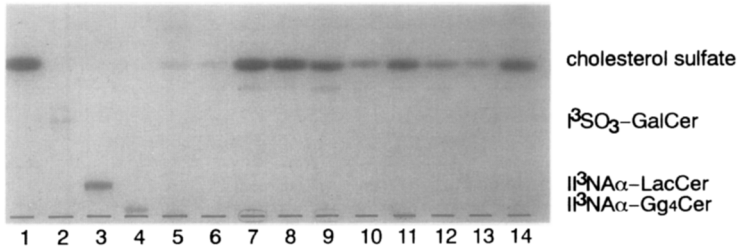

Fig. 1. *TLC of acidic lipids from the rabbit endometrium during the implantation period.* The developing solvent was chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v) and the spots were located by spraying with cupric acetate-phosphoric acid reagent. Lanes 1, 2, 3 and 4; standards of cholesterol sulfate, I^3SO_3-GalCer, $II^3NA\alpha$-LacCer and $II^3NA\alpha$-Gg_4Cer. Lane 5; nonpregnant endometrium. Lanes 6 and 7; pregnant endometrium on days 3 and 5. Lanes 8, 9 and 10; interimplantation sites of pregnant endometrium on days 6, 7 and 9. Lanes 11, 12 and 13; implantation sites of pregnant endometrium on days 6, 7 and 9. Lane 14; pseudopregnant endometrium on day 6.

with that of chemically synthesized CS. After solvolysis, the band of the lipid shifted to the Rf value compatible with cholesterol, thus indicating that the acidic lipid is CS. Further identification of this lipid was performed by negative-ion FAB mass spectrometry. The FAB mass spectrum clearly demonstrated $[M-H]^-$ at m/z 465 and sulfate-derived ions at m/z 80 and 97 (Fig. 2), indicating that the lipid is certainly CS. From these results, this lipid component was concluded to be CS.

Endometria of nonpregnant rabbit and pregnant rabbit at day 3 contained relatively low amounts of CS, concentration of which was approximate 0.06 μmol/g of dry tissue weight (d.wt.). Its concentration rose abruptly on day 5, the day before egg attachment, amounting to 1.63 μmol/g d.wt. Then, the concentration of CS declined stepwisely from day 6 to day 9, at which it was less than one tenth of that at

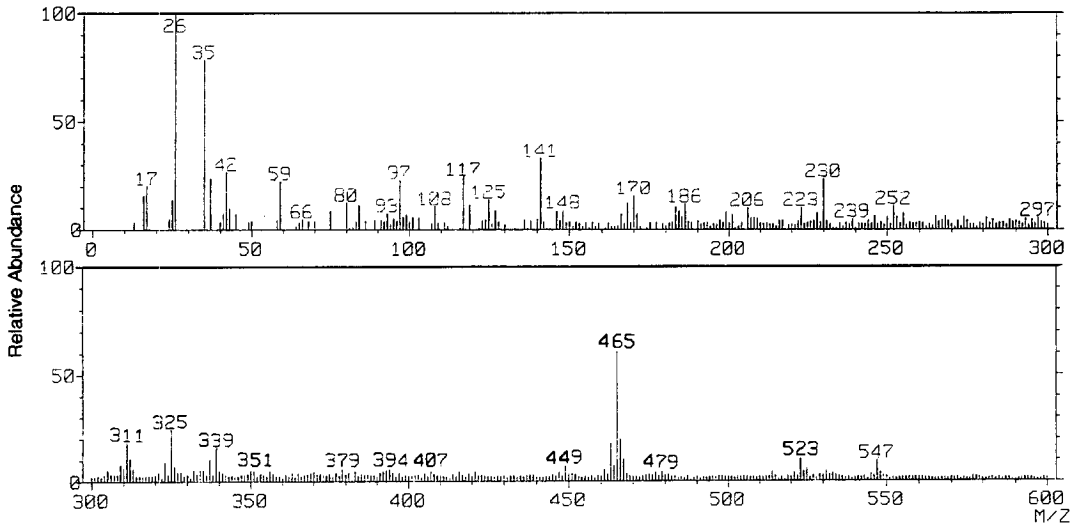

Fig. 2. *FAB mass spectrum of the dominant acidic lipid component in pregnant rabbit endometrium on day 6.* This component was eluted from an Iatrobeads column with chloroform/methanol (8:2 v/v) and the Rf value was consistent with that of chemically synthesized CS. The FAB mass spectrum demonstrated $[M-H]^-$ at m/z 465 and sulfate-derived ions at m/z 80 and 97.

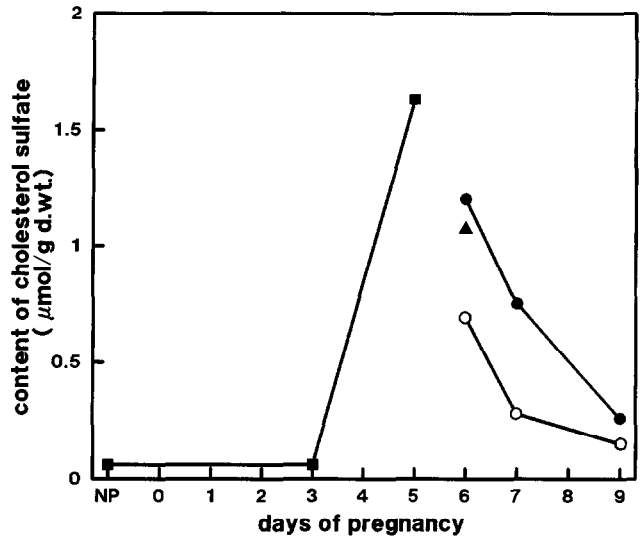

Fig. 3. *Content of cholesterol sulfate in rabbit endometrium during the implantation period.*
NP; nonpregnant endometrium. ■; endometrium before implantation. ●; interimplantation site of pregnant endometrium. ○; implantation site of pregnant endometrium. ▲; pseudopregnant endometrium.

day 5. To determine whether CS is uniformly distributed or not in the endometrium, we measured the CS concentrations in the implantation sites and in the interimplantation sites separately after day 6, the first day to recognize implanted embryos. CS concentration in the interimplantation sites were 1.20, 0.75, and 0.26 μmol/g d.wt. at day 6, 7 and 9 respectively. On the other hand, CS concentrations in the implantation sites were 40–60% of those in the interimplantation sites. The concentration in the pseudopregnant endometrium at day 6 was essentially the same as that in the interimplantation sites at the same day (Fig. 3).

DISCUSSION

An initial step of implantation is the interaction between trophoectoderm and uterine epithelium which results in adhesion of two different cell populations. Complexity of cellular and molecular mechanisms is thought to be involved in this initial event. Thus far, several studies have been concerned with the charges in cell-surface molecules in the expectation of elucidating the adhesion mechanisms. In this regard, change in surface negative charge has been the subject of extensive investigations. In this study, during the search for substances contributing to surface negative charge, CS was shown to be a major negative-charged lipidic substance in the rabbit endometrium occurring at the discrete period i.e. peri-implantation. It is of interest to note that concentrations of CS in the implantation sites were lower as compared with those in interimplantation sites. Thus, these results lead us to suggest that CS may play a role in the early process of implantation.

In experiments using pregnant hamsters, increased vascular permeability as determined by the localized accumulation of a macromolecule dye from the circulation occurs 15–20 hours before morphological signs of trophoblastic invasion (5). Furthermore, an increase in prostaglandin synthesis in localized areas where blastocysts are to be implanted is documented (6). These findings provide evidence for certain changes in localized areas of the endometrium prior to apparent initiation of implantation. In this context, an interesting question arises as to whether a reduction in CS concentration in the implantation sites precedes the adhesion of blastocysts to the endometrium or not. Alternatively, the reduction may be a consequence of the invasion of trophoblasts. Studies are now in progress to address this issue.

From the fact that the CS concentration in the endometrium from the pseudopregnant rabbit is comparable with that in the interimplantation sites, the maternal hormonal environment seems to be responsible for a significant increase in CS concentration in both pregnant and pseudopregnant animals. However, hormonal influence cannot solely account for the difference in the CS concentration between implantation and interimplantation sites. Instead, the presence of embryos may somehow affect the concentration and the metabolism of CS in the endometrium. In recent studies, attention has been paid to embryonic signals which induce local changes in the endometrium, such as steroid hormones (7, 8), histamine (9, 10) or prostaglandins (11, 12). Therefore, it is conceivable that the reduction in CS concentration in the implantation sites may be caused by such embryonic signals.

The CS content is regulated by two enzymes, i.e. cholesterol sulfotransferase and cholesterol sulfatase. Our preliminary studies revealed that cholesterol sulfotransferase was significantly activated in the pregnant rabbit endometrium (Momoeda et al. unpublished data). On the other hand, cholesterol sulfatase has been shown to be present in the human endometrium and to be far higher in the endometrium than in fallopian tubes (13). The activity of sterol sulfatase was reported to be inhibited by progesterone (14), which may in part explain the general increase in CS concentration observed in both pregnant and seudopregnant animals. Further precise studies on the regulatory mechanism of these enzyme activities during the implantation period are needed for better understanding the biochemical basis of the reduction in CS concentration in the restricted area of the endometrium.

At present, several physiological roles of CS in various cells have been postulated, including stabilization of erythrocyte membranes (15), and inhibition of the fusion of Sendai virus with the plasma membrane of target cells (16). The latter effect of CS is interpreted as a result of its ability to stabilize target cell membranes by altering the physical properties of membranes. CS is further shown to inhibit the fertilization by acting on spermatozoa. Spermatozoa have abundant CS on their heads (17). It is suggested

that CS may function as a stabilizer of acrosomal membranes as well and reduction in the content of CS by sulfatase in the female reproductive tract results in an acrosome reaction (18). Here we show the reduced concentration of CS in implantation sites. It is tempting to speculate that reduction in CS concentration may render endometrial cells more fusogenic, and thus ensure implantation, a phenomena of cell fusion between trophoblasts and endometrial cells.

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REFERENCES

1. Chavez, D.J., and Anderson, T.L. (1985) Biol. Reprod. 32, 1135-1142.
2. Anderson, T.L., and Hoffman, L,H. (1984) Am. J. Anat. 171, 321-33.
3. Nilsson, O., Lindqvist, I., and Ronquist, G. (1973) Exp. Cell Res. 83, 421-423.
4. Mumma, R.O., (1969) Lipids 1, 221-223.
5. Orsini, M.W. (1963) Delayed Implantation, pp. 155-166, University of Chicago Press.
6. Evans, C.A., and Kennedy, T.G. (1978) J. Reprod. Fertil. 54, 255-261.
7. Heap, R.B., Flint, A.P.F., Gadsby, J.E., and Rice, C. (1979) J. Reprod. Fertil. 55, 267-275.
8. Sauer, M.J. (1979) J. Reprod. Fertil. 56, 725-743.
9. Dey, S.K., Johnson, D.C., and Santos, J.G. (1979) Biol. Reprod. 21, 1169-1173.
10. Dey, S.K. (1981) Biol. Reprod. 24, 867-869.
11. Racowsky, C., and Biggers, J.D. (1983) Biol. Reprod. 29, 379-388.
12. Harper, M.J., Norris, C.J., and Rajkumar, K. (1983) Biol. Reprod. 28, 350-362.
13. Lalumière, G., Bleau, G., Chapdelaine, A., and Roberts, K.D. (1976) Steroids 27, 247-260.
14. Bleau, G., Chapdelaine, A., and Roberts, K.D. (1972) Can. J. Biochem. 50, 277-286.
15. Bleau, G., Bodley, F.H., Longpre, J., Chapdelaine, A., and Roberts, K.D. (1974) Biochim. Biophys. Acta 352, 1-9.
16. Cheetham, J.J., Epand, R.M., Andrews, M., and Flanagan, T.D. (1990) J. Biol. Chem. 265, 12404-12409.
17. Langlais, J., Zollinger, M., Plante, L., Chapdelaine, A, Bleau, G., and Roberts, K.D. (1981) Proc. Natl. Acad. Sci. USA 78, 7266-7270.
18. Cheetham, J.J., Chen, R.J.B., and Epand, R.M. (1990) Biochim. Biophys. Acta 1024, 367-372.