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Alteration of acidic lipids in human sera during the course of pregnancy: characteristic increase in the concentration of cholesterol sulfate

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Abstract

In this study, we determined the concentrations of acidic lipids, including cholesterol sulfate (CS), sulfatide and GM3 ganglioside, in human sera of non-pregnant state and during the course of pregnancy. In human sera of non-pregnant women, GM3 was present at a concentration of 8 nmol/ml and the concentrations of CS and sulfatides were less than 20% of that of GM3. The concentration of sulfatides in sera at the second trimester of gestation was decreased, but CS gradually increased from the first to the third trimester of gestation with a correlation coefficient of 0.66, and a correlation between the concentration of CS and weeks of gestation ($p<0.01$). CS was also contained in the placental villi, and its concentration increased from the first to the third trimester of gestation, suggesting that placental CS is one of the source of CS in the blood by shedding. © 1997 Elsevier Science B.V.

Keywords: Lipids; Cholesterol sulfate; Sulfatide; GM3 ganglioside

1. Introduction

In previous studies [3–6], we found that sulfated lipids, sulfatide and cholesterol sulfate (CS), were characteristically expressed in the uterine endometria of humans during the luteal phase, and of rabbits during pregnancy, respectively and their metabolisms were strictly regulated by steroid hormones responsible for the functional modification of gynecological organs. Sulfated lipids, particularly CS, in the plasma membrane are known to function not only as a donor group of negative charge under physiological condition, but also as a biomodulator for enzymes related to the signal transduction – such as protein kinases C eta (η) [7], epsilon (ϵ) and zeta (ζ) [8], and phosphatidylinositol 3-kinase [9] – suggesting that sulfated lipids expressed in the endometria are involved in the functional establishment of endometria during pregnancy. In addition, CS activated factor XII and prekallikrein in plasma [10] and the synthesis of thromboxan B-2 in platelets [11], and inhibited the activities of hydroxymethylglutaryl-CoA reductase, a key enzyme of cholesterol metabolism [12] and of acrosin, an acrosomal protease in sperm [13]. To elucidate the role of CS, we com-

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pared the concentrations of acidic lipids[1] in human sera during the course of pregnancy, in which the level of steroid hormones was dramatically altered to maintain the fetus, and found an elevated concentration of CS in sera of pregnant women, as well as in the placental tissues. To our knowledge, this is the first report to describe a characteristic alteration in the concentration of CS among the acidic lipids in human sera during pregnancy.

2. Experimental

2.1. Materials

Human sera from non-pregnant and pregnant women without any pathological conditions including hyperthyroidism, were obtained from Keio University Hospital, Keio University School of Medicine, and were stored at −80°C until use. Human placental tissues, at 5–40 weeks of the gestational period, were obtained from patients after normal spontaneous deliveries, premature deliveries and artificial curettings without any complication. Whole placental tissues were divided into chorionic villous and decidual tissues by scraping and washed with phosphate-buffered saline (PBS) to remove blood. The tissues at various gestational periods were stored at −20°C until the lipid analysis was performed. The ion-exchange resin, DEAE–Sephadex A-25, was provided by Pharmacia, Uppsala, Sweden, and was converted to the acetate form by washing it successively with 0.5 mol sodium hydroxide in water, water, 1.0 mol acetic acid in water, water, methanol and chloroform–methanol (1:1, v/v). Iatrobeads (6RS-8060) for column chromatography, glass-coated high-performance thin-layer chromatography (HPTLC) plates (Silica Gel 60) for thin-layer chromatography (TLC), and plastic-coated TLC plates for TLC-immunostaining were supplied by Iatron (Tokyo, Japan), E. Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA), respectively. Murine monoclonal anti-sulfatide antibody (IgM) was generated in our laboratory by immunizing sulfatide from human brain and hybridizing the splenocytes with myeloma cells (P3.X63.AG8.653), and then monitoring the antibody-producing hybridomas by ELISA with sulfatide as the antigen. The epitope structure required for the binding of the monoclonal antibody was essentially identical with that reported by Fredman et al. [14], reacting with glycolipids having galactose substituted at the 3-position with a sulfate group, GalCer-I^3-sulfate and LacCer-II^3-sulfate, but not with CS.

2.2. Extraction of acidic lipids from human sera and placental villi

By the modified method reported previously [15], total lipids were extracted directly from sera successively with three volumes of chloroform–methanol (1:4, 1:1 and 1:1, v/v) at 40°C, and the combined lipid extracts were directly applied to a DEAE–Sephadex column (acetate form). The column was eluted stepwise with three volumes of chloroform–methanol (1:1, v/v), one volume of methanol, and five volumes of 0.15 *M* sodium acetate in methanol. The acidic lipid fraction, which was eluted from the column with the last solvent, was incubated with 0.5 *M* sodium hydroxide in methanol at 40°C for 1 h to cleave ester-containing lipids and after neutralization with 1.0 *M* acetic acid in methanol and dialysis for removal of the salts, the solution was evaporated to dryness. The extraction of acidic lipids from human placental villi was carried out according to the method described above [15].

2.3. TLC and TLC-immunostaining of acidic lipids from human sera and placental villi

Acidic lipids were chromatographed on a HPTLC plate with solvent systems of chloroform–methanol–water (65:35:8, v/v) for sulfatide, (55:45:10, v/v) for GM3 ganglioside, and chloroform–methanol–acetone–acetic acid–water (8:2:4:2:1, v/v) for CS, and the spots were visualized with 35 m*M* oricinol in 3 *M* sulfuric acid for carbohydrates, 1.8 m*M* ferric chloride in 0.9 *M* acetic and 1.8 *M* sulfuric acid for steroids and 0.2 *M* cupric acetate in 8% phosphoric acid for organic compounds, respectively. The acidic lipids, corresponding to 0.4 ml of serum, were

[1]The nomenclature used for acidic glycosphingolipids follows the recommendations by IUPAC–IUB Commission [1]. The nomenclature used for gangliosides is based on the system of Svennerholm [2].

diluted with 2 ml of chloroform–methanol (2:1, v/v), and the contaminating steroid sulfates were removed by partitioning with 0.5 ml of water. The resultant lower phase containing CS was applied on the TLC plate. Sulfatide isolated from human brain, ganglioside GM3 isolated from human erythrocytes, and chemically synthesized CS were used as standards for quantitative determination, and were detected at 420 nm, 500 nm and 500 nm, respectively, with a TLC-densitometer (CS-9000; Shimadzu, Kyoto, Japan). The standard curves were linear from 0.1 μg to 2.0 μg of sulfatide and GM3, and 0.1 μg to 1.0 μg of CS, respectively. For TLC-immunostaining of sulfatide, the acidic lipids were chromatographed on a plastic-coated TLC plate with chloroform–methanol–water (65:35:8, v/v), and then the plate was incubated with the blocking buffer [1% bovine serum albumin (BSA) in PBS] at 4°C overnight. Then the plate was incubated with about 3.8 ng protein of murine anti-sulfatide monoclonal antibody in 10 ml of 1% BSA in PBS at 37°C for 2 h. After washing the plate five times with 0.1% Tween 20 in PBS, it was incubated again with the blocking buffer at 37°C for 15 min, and then the antibody bound on the plate was detected with peroxidase-conjugated anti-mouse IgM+G antiserum (Jackson Immuno Research Labs., USA) diluted 1:1000 with 1% BSA in PBS, and the enzyme substrates (H_2O_2 and 4-chloro-1-naphthol), as described in Ref. [16].

2.4. Structural characterization of sulfatide and CS in human sera

To determine the structures of sulfatide and CS, they were purified from the acidic lipids on a column (45 cm×1 cm I.D.) packed with 16 g of Iatrobeads (6RS-8060, Iatron, Tokyo, Japan) with a linear gradient system formed from chloroform–methanol–water (70:30:0.5 and 70:30:5, v/v), as described previously [17]. Then the mobilities of the purified sulfolipids on the TLC plate were compared with those of standard lipids, and sulfatide was confirmed by TLC-immunostaining with murine monoclonal anti-sulfatide antibody as described above. Further identification of sulfatide and cholesterol sulfate was performed by negative-ion fast atom bombardment mass spectrometry (FAB-MS), as follows. About 5 μg of the sulfolipids purified from human serum in 5 μl of chloroform–methanol (1:1, v/v) were mixed with about 5 μl of triethanolamine, and then the resultant mixture was put on a stainless-steel sample holder for FAB-MS and analysis was performed by bombardment with a neutral xenon beam, with a kinetic energy of 4 KeV and detection of negative ions with a mass spectrometer (JMS HX-110; Jeol, Tokyo, Japan), as described elsewhere [15].

Furthermore, the sulfate groups were removed from CS and sulfatide by solvolysis with dimethylsulfoxide–methanol (9:1, v/v) containing 9 m*M* sulfuric acid at 80°C for 1 h, and after partitioning by Folch's procedure, and the resultant products, cholesterol and galactosylceramide (GalCer), were examined by TLC with chloroform–methanol–acetic acid (100:2:1, v/v) and chloroform–methanol–water (65:35:8, v/v), respectively.

3. Results

3.1. Acidic lipids in human sera

In human sera, GM3 ganglioside was the major acidic lipid (Fig. 1), and as already reported in the literature [18], sulfatide (GalCer-I^3-sulfate) was detected to a lesser extent (Fig. 1). But the serum sulfatides migrated on TLC to a position lower than that of brain sulfatides, which contained lignoceric (24:0) and cerebronic (24h:0) acids as the major fatty acids. They were isolated by Iatrobeads column chromatography (Fig. 2A). Among the bands around

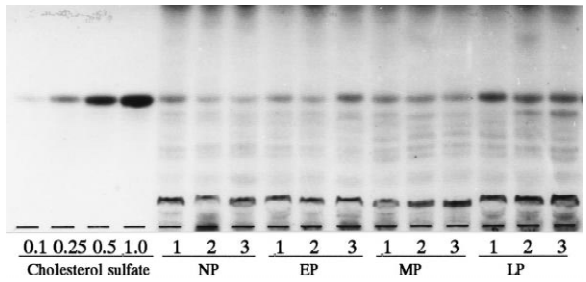

Fig. 1. HPTLC of acidic lipids from human sera during the course of pregnancy. The acidic lipids were chromatographed with chloroform– methanol–acetone–acetic acid–water (8:2:4:2:1, v/v) and were detected with cupric acetate–phosphoric acid reagent. EP, first trimester; MP, second trimester; LP, third trimester; NP, non-pregnancy.

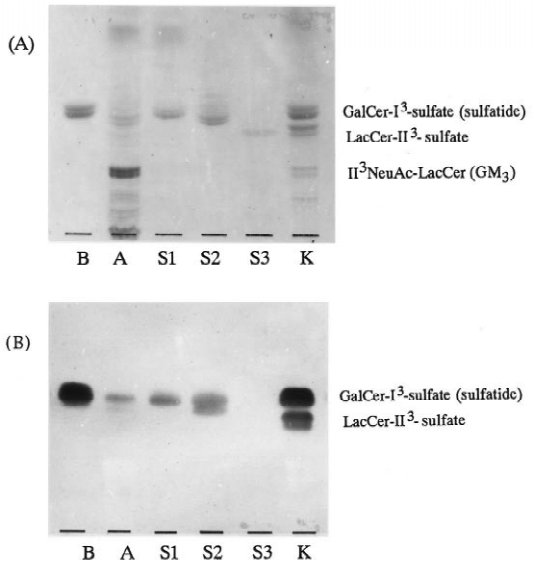

Fig. 2. HPTLC and TLC immunostaining of sulfatides isolated from human sera. The sulfatides purified by Iatrobeads column chromatography were designated as S1, S2 and S3, which were compared with brain (B) and kidney (K) sulfatides as to position on HPTLC and reactivity with anti-sulfatide antibody. HPTLC (A; upper plate) and plastic TLC (B; lower plate) were developed with chloroform–methanol–water (65:35:8, v/v), and the spots were visualized with orcinol–H_2SO_4 reagent and anti-sulfatide antibody, respectively. S3 migrated close to the position of LacCer-II^3- sulfate, but its color on staining with orcinol–H_2SO_4 was not that of a glycolipid and it was negative with the anti-sulfatide antibody. A, total acidic lipid fraction from human sera.

the position of sulfatides, bands which were designated S1 and S2, were positive with the anti-sulfatide antibody, but the band which was designated S3, which migrated close to the position of LacCer-II^3-sulfate, was negative with the anti-sulfatide antibody (Fig. 2B) and orcinol–H_2SO_4 reagent. On negative-ion FAB-MS, the major molecular species of S1 and S2 were found to be palmitic (16:0) and α-hydroxy palmitic acid (16h:0)-containing sulfatides, respectively, indicating that the difference in the migration positions on TLC between serum and brain sulfatides is due to the chain length of fatty acids (Fig. 3). On the other hand, CS, which gave a positive reaction with ferric chloride–H_2SO_4 reagent, was readily detected as shown in Fig. 1, and its structure was confirmed by negative-ion FAB-MS and solvolysis. As shown in Fig. 3, an intense molecular ion corresponding to CS, $[M-H]^-$, and the fragment

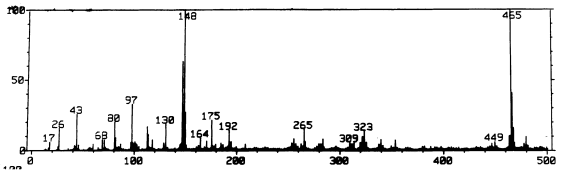

Fig. 3. Negative-ion FAB-MS spectrum of cholesterol sulfate isolated from human sera.

ions $[HSO_4]^-$, yielded at m/z 465 and 97, respectively, and cholesterol was detected in the products after solvolysis of CS isolated from human serum (data not shown).

3.2. Changes in the concentrations of acidic lipids in human sera during the gestational period and placental villi

The concentrations of GM3, sulfatide and CS were determined densitometrically after staining the spots with orcinol–H_2SO_4 and cupric acetate–phosphoric acid reagents. The detection limit of these acidic lipids was 0.1 μg and the standard curves were linear up to 2.0 μg of acidic lipids. The concentration of GM3 in sera was not significantly different between non-pregnant and pregnant women, but that of sulfatide of pregnant women was rather decreased in comparison with that of non-pregnant women, the lowest concentration being observed at the second trimester of gestation (Table 1). In contrast to the changes in the concentrations of GM3 and sulfatide, a gradual increase in the concentration of CS was observed during the course of gestation, the concentration in sera at the third trimester of gestation being twice than that in sera of non-pregnant women (Table 1). The correlation coefficient between the concentration of CS and weeks of gestation was 0.66, and the significance of the difference was $p<0.01$ (Fig. 4). Since the CS increase during the course of gestation was postulated to be derived from the placental tissue, its concentration in the placenta was measured by the same procedure. As shown in Table 1, a significantly high concentration of CS was detected in the placenta, and its concentrations in the second and third trimesters of gestation were six- and eight-fold that in the first trimester of gestation. The concentrations at the second and the third trimesters were 18 and 23 pmol per mg of dry mass. In the

Table 1
Concentrations of acidic lipids in sera from non-pregnant and pregnant women at different periods of gestation and in placental villi

	Sera (nmol/ml)			Placental villi (pmol/mg dry mass)
	Sulfatide (Mean±S.D.)	Cholesterol sulfate (Mean±S.D.)	GM_3 (Mean±S.D.)	Cholesterol sulfate (Mean±S.D.)
Non-preganancy (n=5)	1.21±0.27	1.56±0.30	8.31±3.31	
First trimester (n=5)	0.90±0.25[a]	1.85±0.62	6.87±0.93	3.93±1.90 (n=5)
Second trimester (n=5)	0.68±0.28[a]	2.23±0.60[a]	7.89±1.82	18.35±6.34[b] (n=5)
Third trimester (n=5)	0.96±0.33	3.09±0.62[a]	8.27±3.30	23.75±7.19[c] (n=6)

[a] $p<0.05$, compared with the concentration in sera from non-pregnant women.
[b] $p<0.01$, compared with the concentration in the first trimester placental villi.
[c] $p<0.001$, compared with the concentration.

placental villi, no sulfatide was detected and GM3 was kept at a relatively constant concentration during the gestation (172±89 pmol/mg of dry tissue).

4. Discussion

As shown in this paper, among the acidic lipids in human sera, CS only was found to be altered in the concentration during the course of gestation, and the phenomenon was thought to be due to the change in the steroid hormones during the pregnancy. Both the anabolic and catabolic enzymes for steroid sulfate are known to be under the regulation of steroid hormones, that is, the level of mRNA of hydroxysteroid sulfotransferase-a in female rat liver is four- to six-times higher than that in male rat liver [19], activation of cholesterol sulfotransferase and concurrent inhibition of CS sulfatase were observed in the uteri of pseudopregnant rabbits induced with estradiol and gonadotropin [6], and steroid sulfate sulfatase was inhibited by progesterone [20]. Thus, alteration of the hormonal levels due to pregnancy might enhance the synthesis of CS in the tissues including the placenta. Since the concentration of CS in the placental villi was gradually increased from the first to the third trimester of gestation in a similar manner as that in the sera, placental CS was thought to be one of the source of CS in serum. In fact, an increased concentration of CS in human sera has been reported in patients suffering from recessive X-linked ichthyosis [21], liver cirrhosis [22], hypercholesterolemia [22] and hypothyroidism [23], in which CS has accumulated in tissues due to a deficiency of steroid sulfatase [21], and enhanced synthesis of CS [22,23]. On the other hand, CS circulating in the plasma was shown to be present in low density lipoprotein and chylomicrons [24,25], and was postulated to modify the biological functions as follows. CS in plasma has been readily transferred to erythrocytes in proportion to its concentration in plasma, and has stabilized the erythrocyte membrane against hypotonic hemolysis [26]. It has shortened the clotting time of human plasma through the activation of factor XII and prekallikrein [10] and has stimulated the synthesis of thrombin-induced thromboxane B2 in the platelet [11]. In addition, CS has been shown to regulate the ac-

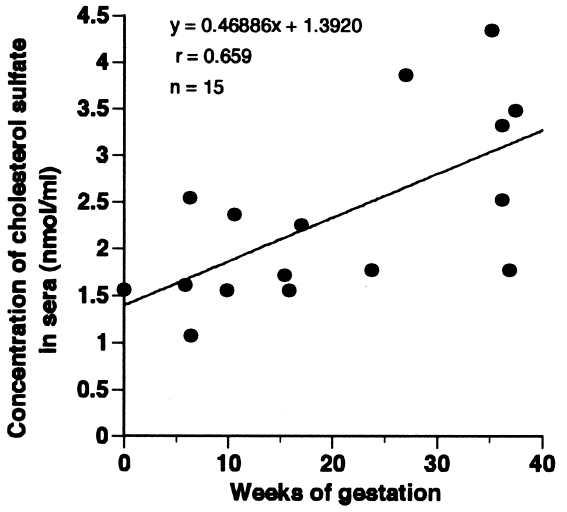

Fig. 4. Correlation between the cholesterol sulfate levels in sera and weeks of gestation.

tivities of several enzymes related to the signal transduction systems such as protein kinases C η [7], ϵ and ζ [8], and phosphatidylinositol 3-kinase [9], and to the sterologenesis such as 3-hydroxy-3-methylglutaryl CoA reductase [12]. These were expected to be modified in the third trimester of gestation by the increased concentration of cholesterol sulfate in the plasma to maintain the homeostasis during the pregnancy, and the altered bioactivities related to cholesterol sulfate in the sera of pregnant women are now being investigated in our laboratory.

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