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Prolactin-dependent Expression of GD1α Ganglioside, as a Component of Milk Fat Globule, in the Murine Mammary Glands*

Mikio Momoeda[1], Sayaka Fukuta[2], Yuriko Iwamori[2], Yuji Taketani[1] and Masao Iwamori[2,†]

[1]Department of Obstetrics and Gynecology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655; and [2]Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan

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Lactation-associated expression of GD1α ganglioside in murine mammary glands was found to be due to the increasing specific activities of Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases in the glands from 12th day of gestation. The gene for GM1b α2,6-sialyltransferase, mST6GalNAcV, which was not detected in nonpregnant glands, appeared at 12th day of gestation and increased in the following gestational and lactation periods. At 3rd day of lactation, the amounts of lipid-bound sialic acid (LSA) in the mammary glands and milk of HR-1 mice were 99.3 ± 8.5 μg per gram of dried tissue and 2.9 μg per ml, GD1α comprising 64.0% and 80.5% of the total LSA, respectively, and GD1α in milk was found to be preferentially distributed in the fat globule fraction. When the mammary epithelial cells at 15th day of gestation were cultured in prolactin- and epidermal growth factor (EGF)-containing media, the synthesis of fat globules and casein, together with the enhanced synthesis of GD1α, were observed in the cells in prolactin medium, indicating that synthesis of GD1α occurs in association with milk production as a prolactin-dependent event. Thus, GD1α ganglioside, which is characteristically distributed in the cerebellar Purkinje cells of the murine brain, is supplied to neonates through the milk of the mother.

Key words: α-series ganglioside, α2,6-sialyltransferase, mammary epithelial cells, mST6GalNAcV, primary culture.

Abbreviations: FCS, fetal calf serum; PVP, polyvinylpyrrolidone; MFG, milk fat globule; LSA, lipid-bound sialic acid.

The milk fat globule (MFG) membrane has been found to contain gangliosides, which have been implicated in the stability of MFG through hydrogen bonding of their hydrophobic ceramide moieties and protection from aggregation through repulsion due to their negative charge (*3–5*), and are mainly derived from the apical surface of lactating mammary glands on stimulation by several hormones after delivery (*6*).

In MFG of human milk, the ganglioside composition has been observed to characteristically change during the lactation period. That is, GD3 invariably comprises 65% of the total lipid-bound sialic acid (LSA) in the colostrum at 2–6 days postpartum, while GM3 is the major ganglioside in mature human milk after 60 days postpartum. Also, the transition from GD3 to GM3 from 6 to 60 days gradually occurs in a postpartum day-dependent manner, and accordingly the ratio of GM3 and GD3 is a useful index for determining the postpartum day for human milk, including that from women with either their first or third baby. The findings indicate that the activity of α2,8-sialyltransferase as to the synthesis of GD3 in the epithelial cells of mammary glands is strictly regulated during the lactation period (*3*).

In the case of murine mammary glands, GD1α, an α-series ganglioside, characteristically increases during the mid-trimester of gestation and becomes the predominant ganglioside in the lactating glands after delivery (*4*). Since a-series gangliosides GM1a and GD1a are major components in the glands of nonpregnant mice, the expression of α-series gangliosides occurs in association with milk production in murine mammary glands, but the metabolic basis as well as their hormonal regulation remain to be clarified.

Since glycolipids are synthesized through the sequential addition of carbohydrate to the nonreducing terminal of a precursor glycolipid by glycosyltransferases, a set of glycosyltransferases, particularly those at the key step of metabolism, is thought to determine the overall profile of glycolipid composition. In the case of targeted deletion of the fucosyltransferase gene, the precursor glycolipid has been shown to be characteristically accumulated in the respective tissue (*7*). Also, when the

*The glycolipid nomenclature is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*1*). The ganglioside nomenclature of Svennerholm is employed throughout (2), except that GM1a, GM1b and GD1α donate $II^3NeuAc\alpha$-Gg_4Cer, $IV^3NeuAc\alpha$-Gg_4Cer and $III^6NeuAc\alpha$, $IV^3NeuAc\alpha$-Gg_4Cer, respectively.

†To whom correspondence should be addressed. Tel: +81-6-6721-2332, Fax: +81-6-6723-6721, E-mail: iwamori@life.kindai.ac.jp

activity of fucosyltransferase has increased to 20–30 times that in the original cells due to transfection of the fucosyltransferase gene, fucosylation of Lc_4Cer to the H-1 glycolipid prevents the further modification of Lc_4Cer to Le^a and Le^b, and reversely the sialylation of nLc_4Cer and Le^x is restricted by the enhanced synthesis of Le^Y from Le^x in the competitive metabolic pathway (*8*). These findings indicate that the activity of fucosyltransferase responsible for the modification of nonreducing terminal structures affects all glycolipids on the different relevant metabolic routes, as well as those on the same route.

In murine lactating mammary glands, GD1α is the terminal structure of α-series gangliosides and is synthesized from GM1b through the transfer of sialic acid to its *N*-acetylgalactosamine residue through an α2,6-linkage with GM1b α2,6-sialyltransferase mST6GalNAcV, which is only active toward GM1b (*9*, *10*). Therefore, the activity of GM1b α2,6-sialyltransferase together with that of Gg_4Cer α2,3-sialyltransferase, for supplementation of GM1b, are thought to play a key role in determining the ganglioside composition in lactating glands. To evaluate the metabolic background of the expression of GD1α, we determined the sialyltransferases in the mammary glands of mice during different stages of the pregnancy and lactation periods, and cultivated mammary epithelial cells to determine the hormones involved in the expression of GD1α.

MATERIALS AND METHODS

Materials—Standard gangliosides from various sources were purified in our laboratory: GM3 from human erythrocytes, GM1a and GD1a from bovine brain, and GM1b and GD1α from rat hepatoma cells (*11*). LacCer and Gg_4Cer were prepared from GM3 and GM1a by treatment with *Arthrobacter ureafaciens* sialidase, respectively (*12*). Rabbit polyclonal anti-GM1a antibodies were produced in our laboratory, and murine monoclonal anti-GD1α (KA-17) and anti-GM3 (M2590) were kindly donated by Dr Y. Hirabayashi, RIKEN, Saitama (*13*) and Seikagaku Co., Tokyo, respectively. CMP-[456789-^{14}C]-NeuAc (7 GBq/mmol) was purchased from GE Healthcare Bioscience, Piscatway, NJ, USA. Murine milk (Balb/c) was provided by Yakult Co. Tokyo, and fat globules were prepared from it by centrifugation at 750*g* for 20 min, dialysis against distilled water and lyophilization.

Primary Culture—Mice (HR-1, Balb/c) were bred in our animal laboratory, and housed at 25 ± 1°C and 70% humidity. The animal care and experimental protocols were in accord with the guidelines of Kinki University. The mammary glands of mice at 15th day of pregnancy were minced and treated with 0.05% collagenase (300 U/mg, Type 1; Sigma, St. Louis, MO, USA) in Hanks saline solution (Sigma) at 37°C for 6 h. After filtration of the cell suspension through a Nylon filter (100 μm) to remove cell debris, the cells were cultured in medium 199 containing 10% fetal calf serum (FCS) and 5 μg/ml insulin (Sigma) on a collagen-coated dish (Iwaki, Tokyo) at 37°C for 24 h, followed by in basal medium, *i.e*. 5% FCS, 5 μg/ml insulin and 1 μg/ml cortisol in medium 199, and prolactin (5 μg/ml: Sigma)-containing and epidermal growth factor (EGF, 10 ng/ml: Sigma)-containing basal media at 37°C for 24 or 48 h.

Analysis of Gangliosides—The extraction of lipids from freeze-dried mammary glands and cells was performed by incubation with chloroform/methanol/water (20 : 10 : 1, 10 : 20 : 1 and 1 : 1, by vol.), the volume of the combined extracts being adjusted with chloroform/methanol (1 : 1, by vol.). After determination of GM3 and GM1a in the extracts by TLC with resorcinol-HCl reagent, and TLC-immunostaining with anti-GM3 and anti-GM1a antibodies, the extracts were fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience), and gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis (*4*, *8*). The amount of LSA in the fraction thus obtained was determined by the thiobarbituric acid procedure after treatment of the gangliosides with *Arthrobacter ureafaciens* sialidase as described previously (*12*). The partially purified gangliosides were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.), and the spots were visualized with resorcinol-HCl for glass-coated plates (Merck, Darmstadt, Germany), and by immunostaining for plastic-coated TLC plates (Machery-Nagel, Düren, Germany). For TLC-immunostaining, each plate was incubated with a blocking buffer (1% polyvinylpyrrolidone (PVP) and 1% ovalbumin in PBS) at 4°C overnight and then with anti-glycolipid antibodies in 3% PVP in PBS at 37°C for 2 h. Afterwards, the plates were washed 5 times with 0.1% Tween 20 in PBS, and the antibodies bound to the TLC plates were detected using peroxidase-conjugated anti-rabbit IgG (Sigma) and anti-mouse IgG+M (Cappel Laboratories, Cochranville, PA) antibodies, diluted 1 : 1,000 (by vol.) with 3% PVP in PBS, and with enzyme substrates H_2O_2 and 4-chloro-1-naphthol, as described previously (*11*). The density of spots visualized with resorcinol-HCl and by TLC-immunostaining was determined at the analytical wavelength of 580 nm and the control wavelength of 700 nm using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard gangliosides, GM3, GM1a, GD1a, and GD1α (0.1–1.5 μg for TLC and 10–100 ng for TLC immunostaining), were developed on the same TLC plates for preparation of standard curves for quantitation.

Sialyltransferase—Mammary glands and cells were homogenized in 0.25 M sucrose with a Potter-Elvehjem homogenizer to prepare 10% (w/v) homogenates, which were then centrifuged at 1,000*g* for 10 min at 4°C to remove cell debris, followed by centrifugation at 100,000*g* for 60 min to obtain cytosol and microsomal fractions. The microsomal fraction was suspended in 0.25 M sucrose by sonication and its protein concentration was measured by the protein dye binding method with BSA (Sigma) as the standard (*14*). The activities of LacCer α2,3-sialyltransferase for the synthesis of GM3, Gg_4Cer α2,3-sialyltransferase for the synthesis of GM1b, and GM1b α2,6-sialyltransferase for the synthesis of GD1α were determined using CMP-[^{14}C]-NeuAc. The standard assay mixture comprised 10 nmol of LacCer, Gg_4Cer or GM1b, 50 μg of Triton CF54/Tween 80 (2 : 1, by vol.),

0.5 μmol of $MnCl_2$, 0.37 μM CMP-[^{14}C]-NeuAc, 50 mM cacodylate-HCl buffer (pH 6.5), and microsomes (0.4 mg protein) in a final volume of 100 μl. For GM1b α2,6-sialyltransferase, 1 μmol of $MgCl_2$ and 0.5 μmol of $CaCl_2$ were used instead of 0.5 μmol of $MnCl_2$, the other additives being the same as above (*9*). After incubation at 37°C for 2 h, the reaction was terminated with 400 μl of 0.1 M KCl containing 0.05 M EDTA, and the products were separated by reverse phase column chromatography with a C18-SepPak cartridge (Waters, Milford, MA, USA), followed by TLC with chloroform/methanol/0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.). The radioactivity incorporated into GM3, GM1b and GD1α was determined with a liquid scintillation counter (Tri-Carb 1500; Packard) (*15*).

RT-PCR Analysis—Total RNA extracted from the mammary glands and cells by the acid guanidine thiocyanate–phenol–chloroform (AGPC) method was reverse-transcribed to cDNA with reverse transcriptase (M-MuLV; Takara, Kyoto) and random primers, and then subjected to PCR with 0.5 U of Taq DNA polymerase (GoTaq, Promega, Kyoto) under the following conditions: CDH α2,3-sialyltransferase (GenBank Accession Number AF119416), sense primer, ttgaggacaggtacagcatc, antisense primer, gggacttttctgccacttg; CDH β1,4-*N*-acetylgalactosaminyltransferase (L25885), sense primer, aggggaagtaacaggagtga, antisense primer, caatgagtgtccg-tagtcga and GM1b α2,6-sialyltransferase (NM012028), sense primer, cagagtgtgttatccgcatg, antisense primer, aaacggtgatgactgccctt, 35 cycles of 95°C for 15 s, 54°C for 30 s and 72°C for 40 s. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a control. The resulting PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and then examined under a UV transilluminator.

Detection of Casein—Culture media of mammary gland-derived cells cultured in basal, prolactin- and EGF media for 48 h were concentrated 10-fold with a Centriprep concentrator (Amicon, Beverly, MA, USA), and then denatured with a sample buffer (10% glycerol, 2% sodium dodecyl sulphate, 5% 2-mercaptoethanol and 0.125% bromophenol blue in 0.06 M Tris–HCl (pH 6.8) at 95°C for 4 min (*16*). The denatured proteins (about 8 μg) were electrophoresed on an acrylamide gel (4–25%), and then transferred to a nitrocellulose membrane. The immunostaining of the membrane with anti-casein antibodies (Funakoshi, Tokyo) was carried out according to the method for TLC-immunostaining described above.

Thymidine Uptake—Mammary gland-derived cells (5×10^4/ml) were cultured in basal, prolactin- and EGF media for 24 h, and the media containing 18.5 Bq [6-^{3}H] thymidine (0.74–1.1 TBq/mmol, GE Healthcare) for 3 h. After the addition of 1% NaN_3, and washing with PBS containing 0.01% NaN_3 and 1 mM cold thymidine twice, the cells were dissolved with 0.2 N NaOH, and the resulting solution was neutralized with 1.0 N HCl and treated with 10% (w/v) trichloroacetic acid to obtain precipitates, in which the radioactivity incorporated was determined by collection on a glass filter and with a liquid scintillation counter (*17*).

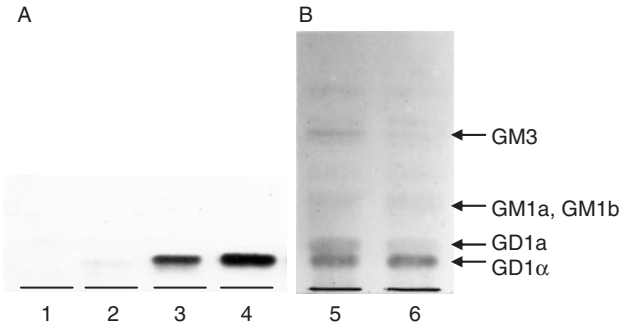

Fig. 1. **TLC-immunostaining (A) and TLC (B) of gangliosides from the mammary glands of mice (HR-1) for nonpregnancy (1), 12th day (2) and 18th day (3) of pregnancy, and 3rd day of lactation (4, 5), and in milk fat globules (6).** Gangliosides, corresponding to 2 mg dry weight, were developed with chloroform/methanol/0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.), and detected by immunostaining with anti-GD1α antibodies (A) and resorcinol-HCl reagent (B).

Table 1. **Distribution of lipid-bound sialic acid in individual gangliosides in the mammary glands and milk fat globules of HR-1 mice at 3rd day of lactation.**

Ganglioside	Mammary glands	Milk fat globules
GM3	23.8	3.0
GM1a	5.6	8.2
GM1b	1.3	4.6
GD1a	5.3	3.8
GD1α	64.0	80.5

Percentage of the total lipid-bound sialic acid.

RESULTS

Gangliosides in Murine Mammary Glands and Milk Fat Globules—In the mammary glands of Balb/c and HR-1 mice, GD1α ganglioside, which was not present in nonpregnancy or before 11th day of gestation even in a trace amount, could be detected at 12th day of gestation, and thereafter its concentration dramatically increased during the late gestational and lactation periods (Fig. 1). The mode of the increase in GD1α, which became a dominant ganglioside in the mammary glands of Balb/c and HR-1 mice after 17th day of gestation, was identical with those in DDD and ICR mice, as reported previously (*4*), indicating that synthesis of GD1α in the mammary glands of several strains of mice occurs as a common event under strict control of hormonal stimuli during the periods of pregnancy and lactation. The amount of LSA in the mammary glands of mice (HR-1) at 3rd day of lactation was 99.3 ± 8.5 μg per gram of dried tissue and GD1α comprised 64.0% of the total LSA (Fig. 1 and Table 1). Also, murine milk at 3rd day of lactation contained LSA at the concentration of 2.9 μg per ml, of which GD1α comprised 80.5% of the total LSA, and was more enriched than in the glands (Fig. 1 and Table 1). After centrifugation of murine milk, 82% of GD1α was recovered in the MFG fraction, indicating that GD1α is a component of MFG. On RT-PCR, the relative intensities of all glycosyltransferase genes examined,

in comparison to that of the housekeeping gene, GAPDH, were found to be strikingly enhanced from 18th day of gestation to lactation period (Fig. 2). In particular, the GM1b α2,6-sialyltransfease (mST6GalNAcV) gene was not detected in the mammary glands of nonpregnant mice, became detectable in the glands at 18th day of gestation, and then increased gradually in the following late gestational and lactation periods (Fig. 2).

As shown in Table 2, although the activity of LacCer α2,3-sialyltransferase did not significantly change in the glands of mice throughout the nonpregnancy, pregnancy and lactation periods, Gg_4Cer α2,3-sialyltransferase at 12th and 18th day of gestation exhibited 9- and 28-fold of the activity in the nonpregnant glands, respectively, and that of GM1b α2,6-sialyltransferase, which was not detected in the nonpregnant or pregnant glands before 11th day of gestation, appeared at 12th day of gestation and increased to 3-fold of the activity at 12th day and 18th day of the gestational and lactation periods, indicating the active synthesis of GD1α in association with lactation in the mammary glands.

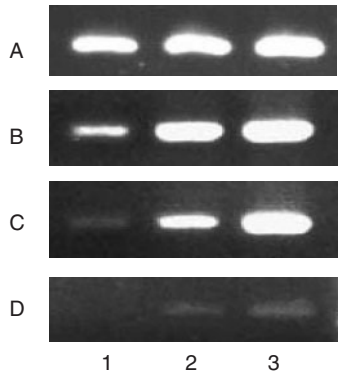

Fig. 2. **RT-PCR analysis of glycosyltransferase genes in the mammary glands of mice for nonpregnancy (1), 18th day of pregnancy (2) and 3rd day of lactation (3).** (A) GAPDH; (B) LacCer α2,3-sialyltransferase; (C) β1,4-*N*-acetylgalactosaminyltransferase and (D) GM1b α2,6-sialyltransferase.

Primary Cultured Cells of Murine Mammary Glands—The epithelial cells of mammary glands of mice on 15th day of gestation were cultured in the basal medium, *i.e.* 5% FCS and 5 μg/ml insulin in medium 199, and prolactin- and EGF-containing basal media for 2 days. As shown in Fig. 3, extensive production of fat globules and casein was observed on the cells cultured in prolactin medium, but not in basal and EGF media, indicating that stimulation with prolactin is essential for milk production by the mammary epithelial cells. Whereas, thymidine uptake into cells (5×10^4 per ml) cultured in the basal, prolactin- and EGF media for 24 h amounted to 3,350 ± 240 c.p.m., 3,070 ± 260 c.p.m. and 63,450 ± 870 c.p.m., respectively, revealing that EGF stimulates the proliferation of mammary epithelial cells with down-regulation of milk production.

Ganglioside Metabolism in Primary Cultured Cells of Murine Mammary Glands—As shown in Fig. 4, GD1α was detected in all cells on TLC-immunostaining, but its amount in the cells cultured in prolactin medium was

Table 2. **Specific activities (pmol/mg protein/h) of sialyltransferases in the mammary glands of HR-1 mice.**

Mammary glands		LacCer α2,3-sialyltransferase	Gg_4Cer α2,3-sialyltransferase	GM1b α2,6-sialyltransferase
Nonpregnancy		335.0 ± 27.5	5.6 ± 1.1	tr
Pregnancy	11th day	301.7 ± 15.8	10.2 ± 2.2	tr
	12th day	320.5 ± 35.2	52.5 ± 5.6	66.3 ± 4.3
	15th day	315.8 ± 30.7	108.3 ± 8.8	89.0 ± 7.2
	18th day	312.0 ± 42.1	157.5 ± 12.7	188.2 ± 15.5
Lactation	3rd day	308.5 ± 26.5	183.6 ± 23.0	208.8 ± 18.8

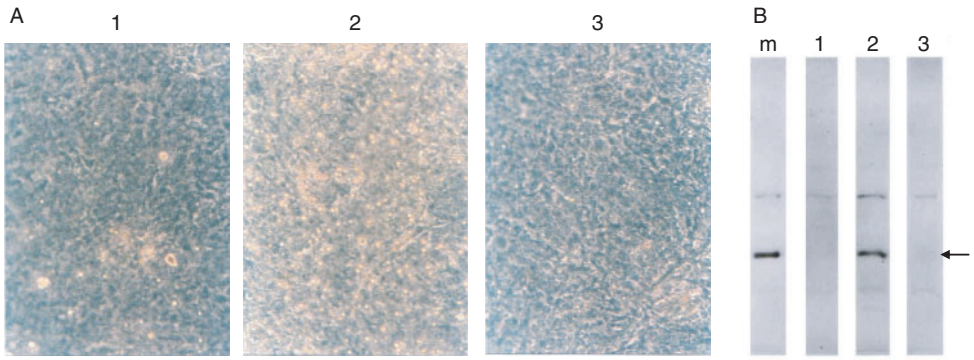

Fig. 3. **Morphology of primary cultured cells from murine mammary glands (A) and western blotting of casein (B).** The epithelial cells of mammary glands of mice at 15th day of gestation were cultured in basal medium (*1*), and prolactin- (*2*) and EGF (*3*)-containing basal media for 2 days. For B, murine milk (m), and media of cells cultured in basal medium (*1*), and prolactin (*2*) and EGF (*3*)-containing basal media were subjected to SDS–PAGE and then transferred to a nitrocellulose membrane, which was stained with anti-casein antibodies. Arrow indicates the position of casein monomer.

123.6 ng/mg of dried cells, which was a twelve times higher concentration than in those cultured in basal (10.3 ng/mg of dried cells) and EGF media (7.0 ng/mg of dried cells), indicating that prolactin stimulates the synthesis of GD1α, together with the production of MFG and casein. In accord with the enhanced synthesis of GD1α, the activities of Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases in the cells in prolactin medium were 3- to 5.6-fold those in basal and EGF media, although LacCer α2,3-sialyltransferase exhibited similar activities in all cells (Table 3). On the other hand, no distinct difference in the expression of the glycosyltransferases gene in Fig. 2 was observed among the cells cultured in basal, prolactin- and EGF media, and the relative intensities were similar to those in the mammary glands of mice at 15th day of gestation, suggesting that prolactin plays a role in regulation of the enzymes for the synthesis of GD1α, not only at the transcriptional level, but also at the translational and enzymatic ones. In fact, when the specific activities in the cells were compared with those in the mammary glands of mice at 15th day of gestation, Gg_4Cer α2,3- and GM1b α2,6-sialyltrasnsferases in the cells cultured in basal and EGF media exhibited only 10–20% of the activities in the glands, but those in the case of prolactin medium exhibited 50–70% of the activities in the glands, indicating that the higher activities of Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases are involved in the prolactin-mediated expression of GD1α.

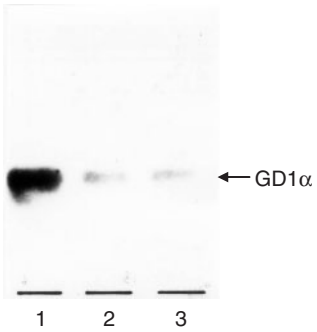

Fig. 4. **TLC-immunostaining with anti-GD1α antibodies of lipids from the primary cultured cells of murine mammary glands.** The epithelial cells (5×10^5) of murine mammary glands were cultured in prolactin (*1*) and EGF (*2*)-containing basal media, and basal medium (*3*). Lipids were extracted from the cells, developed on a plastic TLC plate with chloroform/methanol/0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.), and detected by TLC-immunostaining with anti-GD1α antibodies as described in the text.

DISCUSSION

Cellular proliferation and differentiation of the epithelial cells in mammary glands for the production of milk increase in a rather short period during pregnancy with several hormonal stimuli, *i.e.* insulin, EGF and cortisol in the nonpregnant and pregnant glands, placental lactogen, estrogen and progesterone in the pregnant glands, and prolactin and EGF in the lactating glands (*4*). Since lactation-associated expression of GD1α was revealed to occur in the mid-trimester of gestation, it was supposed to be stimulated by either prolactin or EGF. On 12th day of the mid-trimester of gestation, Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases were found to exhibit increase in their specific activities, resulting in a shift of the metabolism from a-series to α-series gangliosides. In the mammary glands of HR-1 mice on 3rd day of the lactation period, the ratio of GD1a and GD1α, as the terminal products of a- and α-series gangliosides, respectively, reached 1 : 12, although GM3, as a substrate for a-series gangliosides, was supplied sufficiently. A similar metabolic shift due to the increasing activity of α1,2-fucosyltransferase responsible for the modification of the terminal galactose residue was observed previously on transfection of the α1,2-fucosyltransferase gene (*8*), indicating the significance of the terminal modification as to determination of the whole glycolipid composition in tissues and cells. In particular, GM1b α2,6-sialyltransferase, as the terminal modifier of α-series gangliosides in the mammary glands, had the ability to sialylate at the 6th position of *N*-acetylgalactosamine in only GM1b, *i.e.* not in structurally related glycolipids such as Gg_4Cer and GM1a (*9*, *10*), and accordingly active consumption of GM1b by α2,6-sialyltransferase might cause a dramatic increase in GD1α and simultaneous decrease in the concentrations of a-series gangliosides (*4*).

The primary cultured cells from the mammary glands of mice at 15th day of gestation already exhibited the potential to proliferate or differentiate for the production of milk, and consequently, on their cultivation in the presence of EGF or prolactin, the cellular behaviours *in vitro* were readily reproduced, *i.e.* proliferation due to thymidine uptake, and milk production on morphological examination for MFG and western blotting for casein, respectively. Also, the synthesis of GD1α was reproduced in the cells cultured in prolactin medium, but not in those cultured in basal and EGF media, indicating that the synthesis of GD1α is an event occurring on stimulation by prolactin. Although the cells from mammary glands at 15th day of gestation already expressed Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases for GD1α, continuous stimulation after transfer of the cells to cultivation with prolactin was required to retain

Table 3. **Specific activities (pmol/mg protein/h) of sialyltransferases in primary cultured cells from murine mammary glands.**

Cells	LacCer α2,3-sialyltransferase	Gg_4Cer α2,3-sialyltransferase	GM1b α2,6-sialyltransferase
Basal medium	159.7 ± 50.2	17.3 ± 4.7	9.1 ± 3.7
EGF medium	221.2 ± 35.5	25.3 ± 6.5	8.7 ± 2.7
Prolactin medium	160.8 ± 48.0	76.9 ± 15.6	48.5 ± 15.0

their high activities. As shown in Table 3, the activity of LacCer α2,3-sialyltransferase was not significantly different among the cells in basal, EGF- and prolactin media, while those of Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases in prolactin media were more than 3-fold those in basal and EGF media, resulting in a twelve-times higher concentration of GD1α in the cells in prolactin medium than in those in basal and EGF media. On RT-PCR, the mST6GalNAcV gene for GM1b α2,6-sialyltransferase was detected in all cells cultured in basal, prolactin and EGF media at similar intensities, though detailed quantitative analysis by means of real-time PCR is required for determination of the level of mRNA expression. One can suggest that the message level in the cells of mammary glands already stimulated by prolactin *in vivo* does not change on cultivation for 48 h even in the media without prolactin, and additional stimulation with prolactin plays a role in maintaining the higher activity under regulation at the translational and enzymatic levels.

Also, the specific activities of sialyltransferases with different substrates, LacCer, Gg_4Cer and GM1b, could be compared for individual enzymes, but not for different enzymes, because of the differences in the solubility of substrates in aqueous solution, the incubation time for maximal reaction and the requirement of several additives including divalent cations (*9*). In this study, we conveniently used the same conditions for the determination of LacCer α2,3-, Gg_4Cer α2,3- and GM1b α2,6-sialyltransferases for comparison of the altered activities of individual enzymes in the glands during different pregnancy periods and in the cells under different hormonal stimulation, and revealed that Gg_4Cer α2,3- and GM1b α2,6-, but not LacCer α2,3-sialyltransferase, were regulated through stimulation with prolactin to express GD1α as a lactation-associated ganglioside.

The reason why GD1α was newly synthesized in the mammary glands during pregnancy, even though the glands of nonpregnant mice had the ability to synthesize structurally related GD1a, is obscure at present. GD1α was originally discovered in rat ascites hepatoma cells (*18*), and was suggested to be related with the morphology of hepatoma cells, *i.e*. GD1α and fucosyl GM1 being compensatively expressed in free-formed types such as Yoshida sarcoma and rat ascites hepatomas, AH13 and AH66F, and island (aggregated)-formed types such as rat ascites hepatomas, AH109A and AH60C, respectively, suggesting that GD1α prevents the aggregation of cells (*19*). In the same way, GD1α in MFG might play a role by preventing the aggregation of MFG through the repulsion by its carbohydrate structure.

In addition, GD1α has been reported to be selectively distributed in the cerebellar Purkinje cells of mice (*13*), and its synthetic enzyme, mST6GalNAcV, is only expressed in the brain after 12 days gestation, not in other visceral organs (*9*, *10*, *20*). Also, GD3 ganglioside, which was present in significantly high concentrations in the fetal and neonatal brains of vertebrates including man, and is enriched in a microglial lineage (*21–23*), is supplied to human neonates in the colostrum, in which GD3 is a dominant component of the MFG in human milk. Accordingly, the maternal supply of GD1α and GD3 through the milk to neonates seems to be significant for neuronal development and immunological education of infants.

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