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Characteristic Expression of Lewis-antigenic Glycolipids in Human Ovarian Carcinoma-derived Cells with Anticancer Drug-resistance

Masao Iwamori[1,*], Yuriko Iwamori[1], Kaneyuki Kubushiro[2], Isamu Ishiwata[3] and Kazushige Kiguchi[4]

[1]Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502; [2]Department of Obstetrics and Gynecology, Kawasaki Municipal Hospital, 12-1 Shinkawa-dori, Kawasaki-ku, Kawasaki, Kanagawa 210-0013; [3]Ishiwata Gynecologic Hospital, 1-4-21 Kamimito, Mito, Ibaraki 310-0044; and [4]Department of Obstetrics and Gynecology, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

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By comparing ovarian carcinoma-derived KF28 cells with the corresponding anticancer drug-resistant cells, the taxol- and cisplatin-resistant properties were found to be closely related with MDR1 and BSEP, and MRP2 transporters, respectively. In addition to the transporters expression, the amounts of glycolipids, particularly their longer carbohydrate structures, in the resistant cells increased to 3–4-fold of those in the sensitive cells due to enhanced transcription of the respective glycosyltransferases. The major glycolipids in the sensitive and resistant cells were GlcCer and Gb_3Cer, respectively, and extension of the carbohydrate structure into Lewis antigen characteristically occurred in the resistant cells. Le^b, which was not detected in the cisplatin-resistant cells, was present in the taxol-resistant cells, while Le^x was present in the cisplatin-resistant cells at a higher concentration than in the taxol-resistant cells. 2-Hydroxy fatty acids were significantly abundant in glycolipids of the resistant cells, but they were not detected in free ceramides or sphingomyelin, indicating that the enhanced synthesis of glycolipids in the resistant cells was not linked with the removal pathway for virulent ceramides derived from sphingomyelin. The resistant cells with abundant glycolipids exhibited lower membrane fluidity than the KF28 cells, and this property might be involved in the anticancer drug-resistance.

Key words: ceramides, glycolipids, glycosyltransferase genes, membrane fluidity, transporter proteins.

Abbreviations: The glycolipid nomenclature is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*1*); FABMS, fast atom bombardment mass spectrometry; GC–MC, gas liquid chromatography–mass spectrometry; MDR, multidrug resistance; MPR, multidrug resistance-associated protein; BSEP, bile salt export pump; CMH, ceramide monohexoside; Le, Lewis antigen; CDH, ceramide dihexoside; CTH, ceramide trihexoside; RT-PCR, reverse transcriptase-polymerase chain reaction; BSA, bovine serum albumin.

INTRODUCTION

Amphipathic glycolipids are preferentially distributed in the outer leaflet of a biomembrane and are exposed to the outside of the cell, and their carbohydrate moieties are known to change in association with cellular differentiation and transformation, and to be implicated in histoblood group antigens, receptors for bacterial toxins and viruses, and several carbohydrate-mediated functions (*2*). They are supposed to be abundant in the raft structure of biomembranes, together with sphingomyelin, cholesterol and several membrane proteins such as caveolin, Src, Rac and Rho, and to be involved in the signal transduction cascade through modulation of the activities of proteins in rafts (*3, 4*). However, the mechanism underlying the activation of physiologically important proteins by glycolipids is obscure, because of a lack of information on the three-dimensional structures of proteins before and after modification of their activities. On the other hand, the synthesis of glycolipids has been reported to be coupled with regulation of ceramide-mediated apoptosis, which is initiated by hydrolysis of sphingomyelin by neutral and acidic sphingomyelinases (*5–7*). In particular, ceramide glucosyltransferase has been revealed to attenuate the ceramide-signal from sphingomyelin in response to anticancer drugs (*8*). In relation to these observations, GlcCer is consistently present in multidrug-resistant melanoma cells and is absent or present at very low levels in the corresponding drug-sensitive cells (*9*), and multidrug resistance has been associated with changes in the amounts of glycolipids and caveolin-1 in membrane rafts (*10*). In the case of ovarian carcinoma-derived cells, an increased concentration of GM3 has been

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

demonstrated to be closely related with their resistance to paclitaxel (*11*). We also observed that a change in the glycolipid composition on transfection of the fucosyltransferase gene into human ovarian carcinoma-derived RMG-1 cells resulted in the acquisition of resistance to 5-fluorouracil (*12*). Thus, these findings strongly indicate the possible involvement of the carbohydrate moieties of glycolipids in the anticancer drug-resistance, and provide a clue as to the functional significance of glycolipids in biomembranes. However, it remains uncertain what changes in the composition and amounts of glycolipids are involved in the resistance to anticancer drugs. Accordingly, we analyzed glycolipids in human ovarian carcinoma-derived cells obtained by cloning of cells that survived under cultivation with anticancer drugs and retained their anticancer drug-resistant properties continuously, and found a distinct difference in glycolipids between the sensitive and resistant cells (*13*). Although GlcCer was the major glycolipid in sensitive cells, Gb_3Cer was the major one in resistant cells, irrespective of whether the resistance was to taxol or cisplatin, both of which are versatile anticancer drugs for ovarian carcinomas (*13*). To further characterize the molecular alteration in cells able to survive in the presence of anticancer drugs, we examined the glycolipids with longer carbohydrate chains and their molecular species in comparison with free ceramide and sphingomyelin in the sensitive and resistant cells.

EXPERIMENTAL PROCEDURES

Materials—Sphingolipids from various sources were purified in our laboratory: GlcCer, LacCer, Gb_3Cer, Gb_4Cer, nLc_4Cer and GM3 from human erythrocytes, and GalCer and sphingomyelin from bovine brain, and their N-stearoyl derivatives were prepared by deacylation with sphingolipid ceramide *N*-deacylase (*Psudomonas sp*. TK4), followed by reacylation with stearoyl chloride (*14*). Ceramides, Lc_3Cer, were prepared by treatment of sphingomyelin and nLc_4Cer with *Clostridium perfringens* phospholipase C and *Diplococus pneumoniae* β-galactosidase, both of which were kindly donated by Dr M. Kitamura, National Institute of Infectious Diseases, Tokyo (*15*). Monoclonal anti-GM3 (M2590) and anti-Gb_3Cer antibodies were obtained from Seikagaku Co., Tokyo, and an anti-sphingomyelin antibody (VJ-41) (*16*) and anti-Lc_4Cer antibody (HMST-1) (*17*) were produced in our laboratory. Fatty acids and fatty acid methyl esters, and CMP-[456789-^{14}C]-NeuAc (7 GBq/mmol) and UDP-[6-^{3}H]-Gal (230 GBq/mmol) were purchased from Supelco, Bellefonte, PA, and GE Healthcare Bioscience, Piscatway, NJ, USA, respectively. Cisplatin and taxol were obtained from Bristol-Myers Squibb Co., Tokyo, and Sigma Aldrich, St Louis, MO, USA, respectively.

Cell Lines—The cell lines, KFr13, KFr13TX, KF28 and KF28TX, were kindly donated by Prof. Y. Kikuchi, National Defense Medical College, Saitama, Japan. KF28 was the original human ovarian carcinoma-derived cell line, and KF28TX and KFr13 were taxol- and cisplatin-resistant ones, which were established by repeated exposure of the parent KF28 cells to taxol and cisplatin, respectively. KFr13TX cells were the cell line derived from KFr13 cells through exposure to taxol. Cells were cultured in Dulbecco-modified MEM medium supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

Separation and Quantitation of Lipids—After lyophilization of cells, total lipids were extracted from the lyophilized powder with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, by vol.), and the extracts were combined. Ceramides and glycolipids were developed on TLC plates with chloroform/methanol/acetic acid (94:1:5, by vol.) and chloroform/methanol/water (60:35:8, by vol.), respectively, and their concentrations were determined by TLC-densitometry at the analytical wavelength of 500 nm after visualization with cupric acetate-phosphoric acid and orcinol-H_2SO_4 reagents using *N*-stearoyl sphingosine and *N*-stearoyl derivatives of glycolipids as standards, respectively. Then, the lipid extracts were fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience). The neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis (*16*). The gangliosides and neutral glycolipids thus obtained were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) and chloroform/methanol/water (60:35:8, by vol.), and then visualized with resorcinol-HCl and orcinol-H_2SO_4 reagent, respectively. The density of spots was determined at the analytical wavelengths of 580 nm for resorcinol-HCl-positive spots and 420 nm for orcinol-H_2SO_4-positive spots, respectively, using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard glycolipids: *N*-stearoyl derivatives of GalCer, LacCer, Gb_3Cer and GM3 (0.1 to 1.5 μg) were developed on the same TLC plates for the preparation of standard curves for quantitation.

TLC-immunostaining—The total lipid extracts were applied to plastic-coated TLC plates, which were then developed as earlier. Each plate was incubated with a blocking buffer (1% polyvinylpyrrolidone (PVP) and 1% ovalbumin in PBS) at 4°C overnight and then with anti-carbohydrate antibodies in 3% PVP in PBS at 37°C for 2 h. Afterwards, the plates were washed five times with 0.1% Tween-20 in PBS, and the antibodies bound to the TLC plates were detected using peroxidase-conjugated anti-mouse IgG + M antibodies (Cappel Laboratories, Cochranville, PA), diluted 1: 1000 (by vol.) with 3% PVP in PBS, and with enzyme substrates H_2O_2 and 4-chloro-1-naphthol, as described previously (*15*). The density of spots was determined using 10–100 ng of GM3 and Gb_3Cer as standards for quantitation with a TLC-densitometer as described earlier, the limit of detection being 5 ng.

Structural Analysis of Glycolipids—The ceramides, glycolipids and sphingomyelin were purified on a silica gel (Iatrobeads 6RS8060; Iatron Lab., Tokyo) column by sequential gradient elution with chloroform/methanol (100:0 and 90:10, by vol.), chloroform/isopropyl alcohol/water (85:15:0.2 and 40:60:2, by vol.), and chloroform/methanol (40:60 and 0:100, by vol.), respectively.

The purified ceramides, glycolipids and sphingomyelin were identified by negative and positive ion FABMS (JMS-700TKM; JEOL Ltd, Tokyo), respectively, with triethanolamine as a matrix solvent, and by GC–MS as the trimethylsilyl derivatives of carbohydrates, the methyl esters of fatty acids and the *N*-acetyl *O*-trimethylsilyl derivatives of long chain bases (*16*).

Sugar Transferases—Cells were homogenized in 0.25 M sucrose with a Potter–Elvehjem homogenizer to prepare 10% (w/v) homogenates, which were then centrifuged at $1,000 \times g$ for 10 min at 4°C to remove cell debris, followed by centrifugation at $100,000 \times g$ 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 as the standard (*17*). The activities of LacCer sialyltransferase for the synthesis of GM3 and Lc_3Cer β-galactosyltransferase for the syntheses of Lc_4Cer and nLc_4Cer in the microsomal fraction were determined using CMP-[^{14}C]-NeuAc and UDP-[^{3}H]-Gal as substrates, respectively (*18, 19*). The standard assay mixture for LacCer sialyltransferase comprised 10 nmol of LacCer, 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. 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, Miford, MA, USA), followed by TLC with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.). The radioactivity incorporated into GM3 was determined with a liquid scintillation counter (Tri-Carb 1500; Packard). The mixture for Lc_3Cer β-galactosyltransferase comprised 10 nmol of Lc_3Cer, 5 μg of Triton CF54, 0.2 M KCl, 20 μM $MnCl_2$, 0.45 μM UDP-[^{3}H]-Gal, 50 mM cacodylate-HCl buffer (pH 6.8), and microsomes (0.1 mg protein) in a final volume of 100 μl. After incubation at 37°C for 2 h, the reaction was terminated by the addition of 200 μl of chloroform/methanol (2:1, by vol.), and the lower phase was then applied on a plastic-coated TLC plate. The radioactivity incorporated into Lc_4Cer and nLc_4Cer was determined as earlier. Also, Lc_3Cer β1,3-galactosyltransferase was measured by TLC-immunostaining with anti-Lc_4Cer antibodies after incubation of the mixture with cold UDP-Gal (1.56 mM), instead of radioactive UDP-[^{3}H]-Gal, at 37°C for 2 h. The procedure for TLC-immunostaining was the same as described earlier.

RT-PCR Analysis—Total RNA extracted from the cell lines 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 units of Go Taq DNA polymerase (Promega, Kyoto) under the following conditions: GlcCer β1,4-galactosyltransferase (NM0003360), sense primer, cgtcctgtctgtacttcatc, antisense primer, ggaatgagaactgccac ctt; 35 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s; β1,3-galactosyltransferase (Y15062), sense primer, actggat gtacttctatgag, antisense primer, ttggcattggggcaaaactc; and β1,4-galactosyltransferase (M22921), sense primer, gtttactacctggctgg, antisense primer, ttctgctttgccacgag; 35 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 40 s; LacCer α1,4-galactosyltransferase (NM017436), sense primer, tggaagttcggcggcatcta, antisense primer, caggggg cagggtggtgacg; and α1,2-fucosyltransferase (M35531), sense primer, ggccttcctgctagtctgtcc, antisense primer, caggtgccggagagggaagca; 35 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 40 s. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls. The resulting PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and then examined under a UV transilluminator.

Membrane Fluidity—KF28 and KF28TX cells, at the subconfluence stage, were scraped from plates, centrifuged at $125 \times g$ for 10 min and then resuspended in PBS containing 0.15 mM EDTA. After homogenization with a Potter–Elvehjem homogenizer followed by centrifugation at $100 \times g$ for 10 min, the supernatants were centrifuged at $15,000 \times g$ for 20 min and then at $100,000 \times g$ for 60 min. The resulting membrane fraction was suspended in PBS and 1,6-diphenyl-1,3,5 hexatriene (DPH) in tetrahydrofuran was added to the suspension to a final concentration of 1 μM, followed by incubation at 37°C for 20 min. The fluorescence intensity of the membrane suspension was measured with a fluorescence spectrophotometer (650-40 Hitachi, Tokyo) at an excitation wavelength of 360 nm and an emission wavelength of 426 nm. The vertical (*I*v) and horizontal (*I*h) fluorescence intensities were measured with a polarizing filter, and steady-state emission anisotropy (r) was calculated as $r = Iv - Ih/Iv + 2Ih$. The anisotropy parameter $[(r_0/r)\text{-}1]^{-1}$ was calculated using an r_0 value of 0.365 for DPH (*20, 21*).

RESULTS

Anticancer Drug-sensitive and -resistant Ovarian Carcinoma-derived Cells—KF28 cells were the original human ovarian carcinoma-derived cells, which are sensitive to both taxol and cisplatin, the IC50 concentrations being 4.7 and 0.2 μM, respectively. Through cultivation of KF28 cells with increasing concentrations of taxol and cisplatin, taxol-resistant KF28TX cells and cisplatin-resistant KFr13 ones were obtained, and in the same way, taxol and cisplatin-resistant KFr13TX cells were established by the selection of taxol-resistant cells derived from KFr13 cells (*22*). The cell lines established were confirmed to exhibit anticancer drug-resistance, taxol-resistance for KF28TX and KFr13TX cells, and cisplatin-resistance for KFr13 and KFr13TX cells, respectively, and their drug-resistant properties were revealed to be well correlated with the expression of transporter genes, MDR1 and BSEP for taxol resistant cells, and MRP2 for cisplatin-resistant cells (*13*). In addition, although GlcCer was the major glycolipid in sensitive cells, Gb_3Cer was the major one in both taxol- and cisplatin-resistant cells. Also, GM3 exhibited a two-fold increase in the taxol-resistant cells, but was absent in the cisplatin-resistant cells (*13*). Along with changes in the carbohydrate moieties of glycolipids in resistant cells, 2-hydroxy fatty acids were abundant

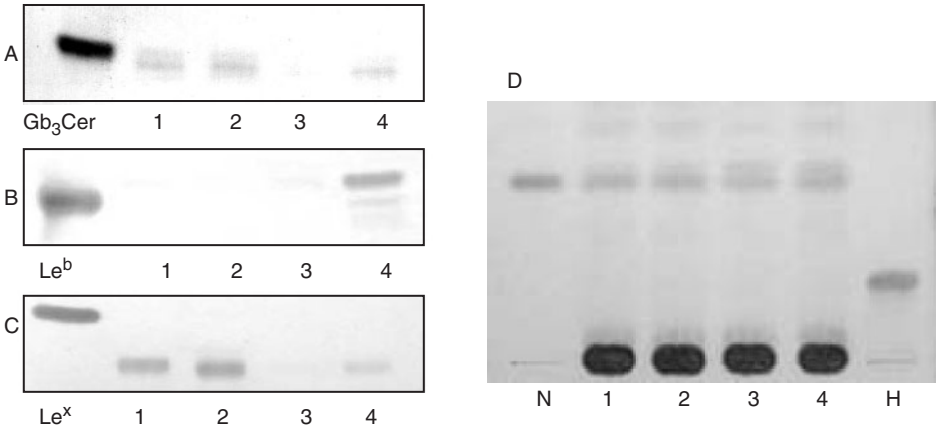

Fig. 1. **TLC and TLC-immunostaining of ceramides and glycolipids from human ovarian carcinoma-derived KF28 cells, and anticancer drug-resistant KF28TX, KFr13 and KFr13TX cells.** The lipids, corresponding to 1 mg (D) and 0.2 mg (A to C) dry weight were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ (55:45:10, by vol.) for A and C, and chloroform/methanol/acetic acid (94:1:5, by vol.) for D, and were detected with anti-Gb_3Cer (A), anti-Le^b (B), and anti-Le^x (C) antibodies, and cupric acetate-phosphoric acid (D). 1, KFr13; 2, KFr13TX; 3, KF28; 4, KF28TX. Le^b, $IV^2Fuc\alpha$,$III^4Fuc\alpha$-Lc_4Cer; Le^x, $III^3Fuc\alpha$-nLc_4Cer; N, N-stearoyl sphingosine; H, 2-hydroxy fatty acid-containing ceramides.

in the glycolipids of resistant cells. Thus, alteration in glycolipid expression, together with transporter proteins, was associated with the anticancer drug-resistance.

Glycolipids with Longer Carbohydrate Chains—Although Gb_3Cer, belonging to the globo-series glycolipids, was the major glycolipid in all anticancer drug-resistant cells (Fig. 1A), Gb_4Cer was not contained in any cells, showing that no further extension of the globo-series carbohydrate from Gb_3Cer occurred in any cells. However, neutral glycolipids with longer carbohydrates were found to be present in resistant cells, and they were characterized as Lewis-antigenic glycolipids (Fig. 1B and C). Glycolipids with Le^x-carbohydrate, which was absent in the original KF28 cells, were abundant in all resistant cells, and their concentrations in cisplatin-resistant KFr13 and KFr13TX cells were higher than that in taxol-resistant KF28TX cells. Judging from its mobility in comparison with that of $III^3Fuc\alpha$-nLc_4Cer as a standard, and the results of negative ion FABMS spectrometry of it, the structure of glycolipids with Le^x-carbohydrate in the resistant cells was thought to be $V^3Fuc\alpha$-nLc_6Cer, as reported for other human ovarian carcinoma-derived RMG-1 cells (*23*). Whereas Le^b ($IV^2Fuc\alpha$,$III^4Fuc\alpha$-Lc_4Cer), which was also present in a trace amount in KF28 cells, was found to be significantly increased in the taxol-resistant KF28TX cells, but was absent in the KFr13 and KFr13TX cells. As a result, the amount of Le^b plus Le^x in KF28TX cells was similar to those of Le^x in KFr13 and KFr13TX cells, and therefore an increase in Lewis antigenic glycolipids was a characteristic of the resistant cells. As shown in Table 1, increases in GalCer, LacCer, Gb_3Cer and Le^x were observed in all anticancer drug-resistant cells, irrespective of whether the resistance was to taxol or cisplatin, and the expression of Le^b and GM3 was characteristically enhanced in the taxol-resistant cells, but not in the cisplatin-resistant ones. As a result, the total amounts of glycolipids in the anticancer drug-resistant cells were 3.4–3.9-fold of those in the sensitive

Table 1. **Concentrations of ceramides and glycolipids in human ovarian carcinoma-derived KF28 cells, and anticancer drug-resistant KF28TX, KFr13 and KFr13TX cells.**

Lipid	KF28	KF28TX	KFr13	KFr13TX
	μg/mg dried cells			
Ceramide	0.15 ± 0.02	0.17 ± 0.01	0.17 ± 0.02	0.18 ± 0.01
GlcCer	0.20 ± 0.01	0.18 ± 0.01	0.08 ± 0.01	0.09 ± 0.02
GalCer	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
LacCer	tr	0.24 ± 0.02	0.24 ± 0.02	0.27 ± 0.02
Gb_3Cer	tr	0.32 ± 0.01	0.44 ± 0.01	0.52 ± 0.02
Le^x	tr	0.02	0.04	0.05
Le^b	tr	0.03	–	–
GM3	0.04 ± 0.01	0.09 ± 0.01	–	–

tr, trace amount.
Values are the means of individual lipids in four different experiments.

KF28 cells. On the other hand, the glycolipid composition in the cisplatin-resistant KFr13 cells was essentially the same as that in the cisplatin and taxol-resistant KFr13TX cells, suggesting that the initial selection of cells with cisplatin, rather than the second selection with taxol, effectively determined the glycolipid profile.

Expression of Glycosyltransferase Genes—To clarify the genetic background of the glycolipid expression in anticancer drug-sensitive and -resistant cells, RT-PCR analysis of glycosyltransferase genes was carried out, as shown in Fig. 2. Although no significant difference in gene-expression between the original and anticancer drug-resistant cells was observed for ceramide glucosyltransferase, GlcCer β-galactosyltransferase (*13*), β1,4-galactosyltransferase or α1,2-fucosyltransferase, the expression of the ceramide β-galactosyltransferase gene for GalCer and the CDH α-galactosyltransferase one for Gb_3Cer in all resistant cells was enhanced compared to that in the original KF28 cells, which is in accord with the higher amounts of GalCer and Gb_3Cer in all resistant cells (*13*). On the other hand, the β1,3-galactosyltransferase and CDH sialyltransferase

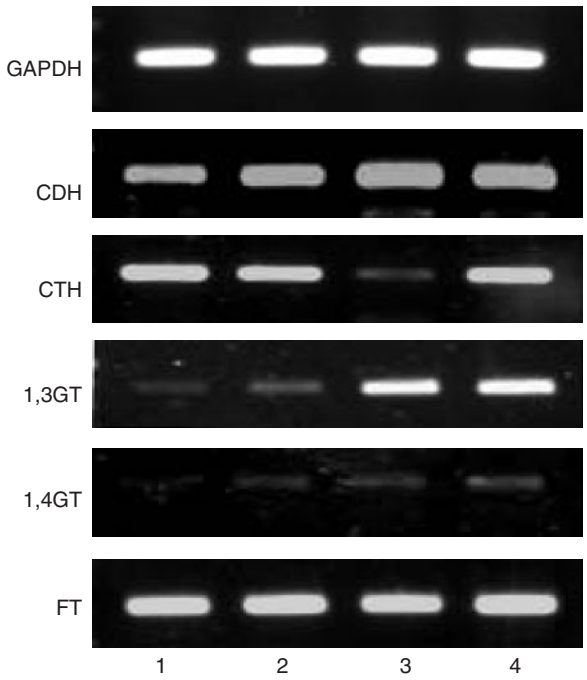

Fig. 2. **RT-PCR analysis of glycosyltransferase genes in human ovarian carcinoma-derived KF28 cells, and anticancer drug-resistant KF28TX, KFr13 and KFr13TX cells.** 1, KFr13; 2, KFr13TX; 3, KF28; 4, KF28TX; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CDH, GlcCer β-galactosyltransferase; CTH, LacCer α-galactosyltransferase; GT, galactosyltransferase; FT, α1,2-fucosyltransferase.

genes for GM3 in KF28 and KF28TX cells were expressed more highly than those in KFr13 and KFr13TX cells, which is consistent with their enzymatic activities (Fig. 3 and Table 2), but not with the amounts of Le^b and GM3. The enhanced syntheses of Le^b and GM3 in KF28TX cells were thought to be regulated through metabolic steps other than those involving β1,3-galactosyltransferase and CDH sialyltransferase, respectively. Thus, the metabolism of glycolipids characteristic of anticancer drug-resistant cells was shown to be regulated through transcription of the respective genes.

Comparison of Ceramide Structures Among Sphingolipids—To determine whether glycolipids that were increased in the anticancer drug-resistant cells were derived through glycosylation of ceramides derived from sphingomyelin or not, the structures of the ceramide moieties of free ceramide, sphingomyelin and glycolipids were determined by FABMS and GC-MS. As shown in Fig. 4, the mobilities of individual glycolipid bands on TLC differed between anticancer drug-sensitive and -resistant cells, probably due to differences in the structures of the ceramide moieties. For instance, the major bands of CMH for all resistant cells were lower than those for the sensitive cells. This was found to be mainly due to their fatty acid compositions, i.e., the proportions of non-hydroxy and 2-hydroxy fatty acids, on FABMS and GC-MS (Fig. 5 and Table 3), the proportions of 2-hydroxy fatty acids in CMH of KF28, KF28TX, KFr13 and KFr13TX cells being 17.2, 74.9, 67.5

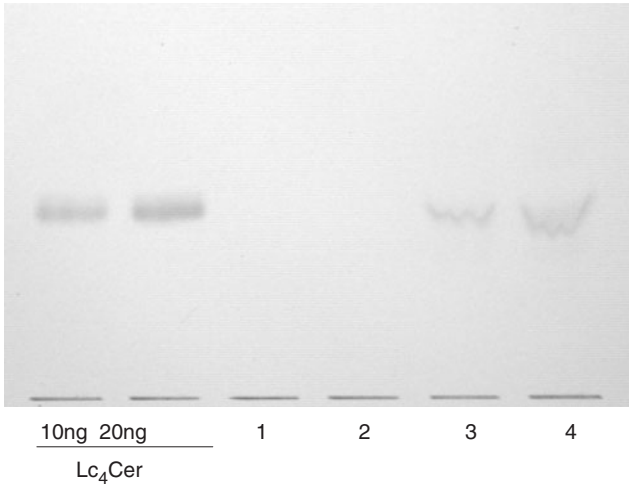

Fig. 3. **TLC-immunostaining with anti-Lc_4Cer antibodies of the products of the reaction with Lc_3Cer β-galactosyltransferase.** The enzyme reaction was carried out with 10 nmol of Lc_3Cer, 5 μg of Triton CF54, 0.2 M KCl, 20 μM $MnCl_2$, 1.56 mM UDP-Gal, 50 mM cacodylate-HCl buffer (pH 6.8), and 100 μg of microsomes at 37°C for 2 h, and the products were developed on a TLC plate with chloroform/methanol/0.5% $CaCl_2$ (55:45:10, by vol.). The heat-denatured microsomal preparation was used as a control. 1, KFr13; 2, KFr13TX; 3, KF28; 4, KF28TX.

Table 2. **The specific acitivities of LacCer sialyltransferase, Lc_3Cer β-galactosyltransferase and Lc_3Cer β-1,3-galactosyltransferase in human ovarian carcinoma-derived KF28 cells, and anticancer drug-resistant KF28TX, KFr13 and KFr13TX cells.**

Enzyme	KF28	KF28TX	KFr13	KFr13TX
	pmol/mg protein/h			
LacCer sialyltransferase	12.1	14.7	0.9	1.6
Lc_3Cer β-galactosyltransferase	52.7	85.2	66.5	70.8
Lc_3Cer β-1, 3-galactosyltransferase	5.3	9.2	ND	ND

ND, not detected.

and 67.8%, respectively. Thus, the higher proportion of 2-hydroxy fatty acids in CMH of the resistant KF28TX, KFr13 and KFr13TX cells caused the lower migration on TLC than in the case of the original KF28 cells. The upper two bands of CMH in KF28 cells were due to different chain lengths of non-hydroxy fatty acids, i.e., lignoceric (24:0) and palmitic (16:0) acid-containing ones for the upper and lower bands, respectively (Table 3). The fatty acid composition of CMH in KF28 and KF28TX cells was essentially maintained in their GM3 and LacCer, there being 62 and 73% of 2-hydroxy fatty acids in the total fatty acids, respectively, while the proportion of 2-hydroxy fatty acids in Gb_3Cer was slightly lower than those in CMH and LacCer, being about 50% for KF28TX, and 41% for KFr13 and KFr13TX cells, respectively. Thus, the anticancer drug-resistant cells, which were established by cloning with cisplatin and taxol, and which retained their resistance, commonly contained glycolipids with higher proportions

of 2-hydroxy fatty acids in their ceramide moieties than in the case of the sensitive cells.

On the other hand, as shown in Fig. 1D, although non-hydroxy fatty acid-containing free ceramides were detected in all cell types, 2-hydroxy fatty acid-containing ones were only detected in trace amounts in both resistant and sensitive cells. Similarly, sphingomyelin from KF28 cells gave molecular ions at *m/z* 703, *m/z* 787, *m/z* 813 and *m/z* 815 corresponding to 16:0, 22:0, 24:1 and 24:0-containing ceramides, respectively, but not the ions of 2-hydroxy fatty acid-containing ones, and the spectrum was essentially identical to those of KF28TX, KFr13 and KFr13TX cells (Fig. 5). Also, when we compared the relative intensities of the molecular ions among sphingomyelin, CMH, LacCer, Gb_3Cer and GM3 from KF28TX cells, a distinct difference in the spectra was observed between sphingomyelin and glycolipids, indicating no metabolic correlation between them, although individual glycolipids carried similar ceramide structures, because they were synthesized via the same metabolic route involving the sequential addition of carbohydrates (Fig. 5). These findings indicate the enhanced expression of glycolipids in anticancer drug-resistant cells is not directly linked with the glycosylation of ceramides produced from sphingomyelin to escape from ceramide-mediated apoptosis.

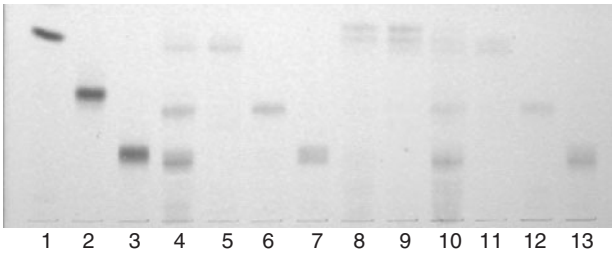

Fig. 4. **TLC of glycolipids from human ovarian carcinoma-derived KF28 cells, and anticancer drug-resistant KF28TX, KFr13 and KFr13TX cells.** The glycolipids were developed on a TLC plate with chloroform/methanol/0.5% $CaCl_2$ (55:45:10, by vol.) and then detected with orcinol-H_2SO_4 reagent. 1, GlcCer; 2, LacCer; 3, Gb_3Cer; 4, neutral glycolipids from KFr13 cells; 5, CMH from KFr13 cells; 6, CDH from KFr23 cells; 7, CTH from KFr13 cells; 8, neutral glycolipids from KF28 cells; 9, CMH from KF28 cells; 10, neutral glycolipids from KF28TX cells; 11, CMH from KF28TX cells; 12, CDH from KF28TX cells; 13, CTH from KF28TX cells.

Membrane Fluidity of KF28 and KF28TX Cells—The anisotropic values for KF28 and KF28TX cells at 37°C were 0.150 ± 0.004 and 0.172 ± 0.005 (mean ± SD), respectively, showing the decreased membrane fluidity of KF28TX cells as compared with that of the original KF28 cells ($P < 0.001$). The change in the membrane fluidity was thought to be due to the concentration of glycolipids with 2-hydroxy fatty acids, and to be also related to the anticancer drug-resistance.

DISCUSSION

As reported in our previous paper (*13*), a distinct correlation of the anticancer drug-resistance to taxol and cisplatin and the expression of transporter genes was observed in human ovarian carcinoma-derived KF28 cells and related taxol- and cisplatin-resistant cells,

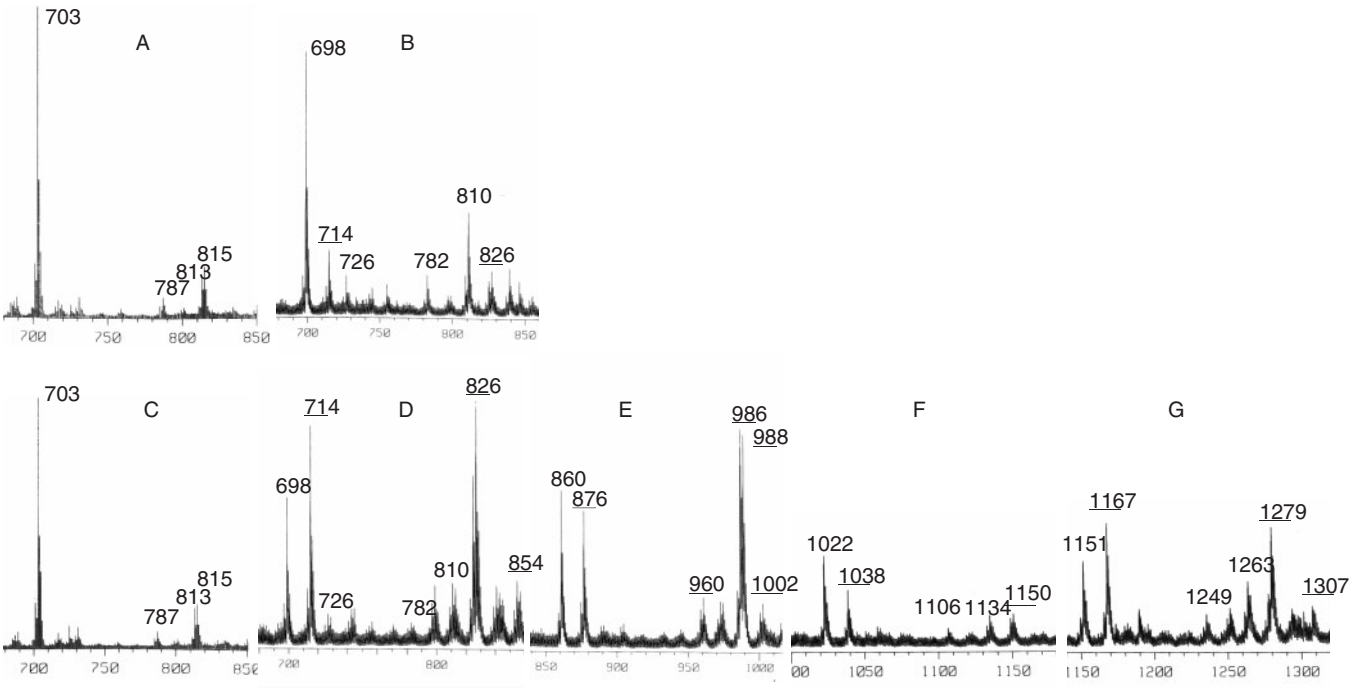

Fig. 5. **FABMS spectra of sphingomyelin and glycolipids.** Sphingomyelin and individual glycolipids were purified by Iatrobeads column chromatography, and then analyzed by positive and negative ion FABMS, respectively, their molecular ion regions being presented. The underlined ions were derived from 2-hydroxy fatty acid-containing molecules. A, sphingomyelin, and B, CMH from KF28 cells, and C, sphingomyelin, D, CMH, E, LacCer, F, Gb_3Cer, and G, GM3 from KF28TX cells.

Table 3. **Fatty acid compositions of sphingolipids in human ovarian carcinoma-derived KF28 cells and taxol-resistant KF28TX ones.**

Fatty acid	KF28				KF28TX					
	ceramide	Sm	CMH	GM3	ceramide	Sm	CMH	CDH	CTH	GM3
16:0	38.4	66.6	38.3	24.4	46.0	62.1	11.7	14.1	25.3	12.6
18:0	3.8	4.6	4.9	3.7	4.8	5.6	1.5	0.5	2.5	4.3
20:0	2.1	1.9	3.6	4.6	–	1.8	0.6	0.2	1.3	1.0
22:0	7.1	4.3	5.1	6.2	2.1	4.4	0.9	0.6	3.5	3.6
23:0	7.1	1.7	2.2	3.4	7.4	2.5	–	2.7	1.5	2.5
24:0	23.5	10.9	14.2	17.4	20.8	11.4	4.6	4.0	8.2	8.0
24:1	14.6	8.8	4.7	6.0	15.5	10.5	2.2	0.7	4.5	3.0
25:0	3.4	1.2	3.8	5.5	3.4	1.7	1.8	2.2	1.8	1.8
26:0	–	–	6.0	7.3	–	–	1.8	2.1	1.8	1.2
16h:0	–	–	8.0	4.5	–	–	18.6	12.9	17.1	18.6
18h:0	–	–	2.7	2.8	–	–	2.5	1.5	3.8	1.3
20h:0	–	–	–	–	–	–	1.0	0.8	0.9	0.7
22h:0	–	–	1.4	1.4	–	–	4.5	3.2	1.9	3.5
23h:0	–	–	–	–	–	–	4.1	4.0	7.9	5.6
24h:0	–	–	5.1	2.8	–	–	20.4	20.6	14.7	17.7
24h:1	–	–	–	–	–	–	14.2	21.1	5.2	6.4
25h:0	–	–	–	–	–	–	4.5	4.0	1.8	3.9
26h:0	–	–	–	–	–	–	5.1	4.5	1.3	4.3

Sm, sphingomyelin; h, 2-hydroxy fatty acids.

which were established by cultivation of KF28 cells with increasing concentrations of taxol and cisplatin, and which retained their taxol- and cisplatin-resistasnce continuously. The anticancer drug-resistant cells were found to exhibit acceleration of the efflux of anticancer drugs through transporters for survival in the presence of xenobiotics (*24, 25*). Also, the mechanism for acquiring the anticancer drug-resistance was thought to involve the virtually ubiquitous inducibility of transporter genes as a response to xenobiotic stimuli. The transcriptional regulation of MDR1 genes is known to be associated with a promotor, i.e., nuclear factor κB, whose activation is generally achieved through the phospholipase C-cascade including protein kinase C, MAP kinase and Ca^{2+} -mobilization, to ensure the rapid emergence of pleiotropic resistance (*26, 27*). Thus, the cells that were able to express the MDR1 and BESP, and MRP2 genes survived in the media containing taxol and cisplatin, respectively.

In addition to the characteristic expression of transporter proteins, we newly found that the anticancer drug-resistant cells contained significantly higher concentrations of glycolipids than those in the original KF28 cells due to enhanced transcription of the respective glycosyltransferase genes. The glycolipids commonly increased in the taxol-, cisplatin-, and taxol- and cisplatin-resistant cells were GalCer, LacCer and Gb_3Cer, respectively, with higher proportions of 2-hydroxy fatty acids in their ceramide moieties as compared with those in the original KF28 cells (*13*). Also, since the glycolipid composition in the cisplatin-resistant KFr13 cells was identical with that in the cisplatin- and taxol-resistant KFr13TX ones, but was different from that in the taxol-resistant KF28TX cells, the initial selection with cisplatin or taxol should have determined the cellular characteristics with the specific expression of glycolipids, i.e., Le^x for the cisplatin-resistant cells, and Le^b and GM3 for the taxol-resistant ones. The enhanced synthesis of glycolipids for the survival of cancer cells in the presence of anticancer drugs has been extensively studied in relation with the detoxification pathway for the removal of free ceramides that trigger apoptosis as second messengers (*28–31*). Since ceramides generated from sphingomyelin by neutral and acidic sphingomyelinases have been proven to initiate apoptotic cellular death (*6, 7, 32*), we compared the molecular species of sphingomyelin, ceramides and glycolipids between the original KF28 and anticancer drug-resistant cells. With the hypothesis that in anticancer drug-resistant cells the pathway for the glycosylation of ceramides from sphingomyelin is activated as compared with in the original sensitive cells, the molecular species of sphingomyelin should be reflected in those of ceramides and glycolipids, even if the pathway is only activated on stimulation with anticancer drugs. As clearly shown in Figs 1 and 5, and Table 2, the molecular species of sphingomyelin and ceramides in the resistant cells were very similar to those in the original cells, but were distinct from those of glycolipids in the same cells, indicating that the enhanced synthesis of glycolipids in the anticancer drug-resistant cells is not linked with cleavage of sphingomyelin. In addition, although the metabolic correlation among CMH, LacCer, Gb_3Cer and GM3 was evident from their similar ceramide structures in all cell lines, 2-hydroxy fatty acids, which were abundant in the glycolipids of the resistant cells, were only present in trace amounts in free ceramides, indicating that free ceramides are not directly utilized for the synthesis of CMH, as observed previously (*33*). In particular, 2-hydroxy fatty acid-containing glycolipids were abundant in the fetal intestine, in which several transporter proteins are highly expressed to sustain the active metabolism in fetal

tissues and cells (*34*). One can suggest that coexpression of transporter proteins and 2-hydroxy fatty acid-containing glycolipids is related to enhancement of the activity of transporter proteins by strengthening the molecular packing through the hydrogen bonds between glycolipids and proteins, or between glycolipids and the other lipids in the raft structures of biomembranes (*3, 4*). It is true that biomembranes with higher concentrations of glycolipids and with higher proportions of 2-hydroxy fatty acids provide rigidity in comparison to those with lower concentrations of glycolipids with non-hydroxy fatty acids. In addition, the 2-hydroxy fatty acids in the ceramide moieties of glycolipids were shown to affect the topology of carbohydrates as to the binding of several bacteria (*35*), suggesting that anticancer drug-resistant cells are covered with reactive carbohydrates.

At present, although the reason why cultivation with taxol and cisplatin led to the selection of KF28TX cells with GM3 and Le^b, and KFr13 cells with Le^x but without GM3 or Le^b, respectively, is obscure, several carbohydrate-mediated properties including cell-to-cell adhesion and negative charges should be involved in the anticancer drug-resistance, as reported previously (*12, 23, 36*). Because transformation-associated alteration of carbohydrates occurs frequently and dramatically in several types of cancers, carbohydrate-specific antibodies have been successfully applied for the clinical diagnosis of cancer patients, such as that against sialyl Le^a for predicting the metastatic potential (*37, 38*), and those against Le^b, Le^y and H antigens for determining the grades of dysplasia and malignancy (*39, 40*). The anticancer drug-resistant cells originating from human ovarian carcinoma-derived cells reported in this paper were useful for characterizing the relationship between the carbohydrate structure and several properties of cancer cells, and an experiment on transfection of the resistant cells with glycosyltransferase genes is now in progress in our laboratory to obtain clues as to the role of carbohydrates in the anticancer drug resistance.

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