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Sphingolipids, rafts and multidrug resistance

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Chapter 5

Detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) in drug-resistant versus drug-sensitive human ovarian cancer cells differ in sphingolipid content; Enrichment of sphingolipids with specific fatty acids

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ABSTRACT

The majority of multidrug resistant tumor cells show alterations in sphingolipid composition, most notably increased glucosylceramide levels. We have recently shown that glucosylceramide is enriched in detergent-insoluble glycosphingolipid-enriched membrane domains during multidrug resistance acquisition in HT29^{col} human colon cancer cells (Klappe, K., Hinrichs, J.W.J., Kroesen, B.-J., Sietsma, H., and Kok, J.W. (2004) Int. J. Cancer 110, 511-522). Here we show in human ovarian carcinoma cells that 1) detergent-insoluble glycosphingolipid-enriched membrane domains in drug-resistant cells differ specifically in sphingolipid content and not in protein, phospholipid or cholesterol content, as compared to drug-sensitive cells. 2) The cellular pools of glucosylceramide (and other sphingolipids) are too a large extent localized in Triton X-100 X-100-insoluble glycosphingolipid-enriched membrane domains, while Lubrol WX-insoluble glycosphingolipid-enriched membrane domains are less enriched in sphingolipids. 3) In drug-resistant cells, glucosylceramides (and other sphingolipids) with specific fatty acids are enriched in these membrane domains. Most notably, sphingolipids with C24:1 fatty acid were three times more abundant in membrane domains of drug-resistant cells. Together, these data strongly suggests that multidrug resistance-associated changes in sphingolipids play a structural role in detergent-insoluble glycosphingolipid-enriched membrane domains.

INTRODUCTION

Multidrug resistance (MDR) of cancer cells is characterized by cross-resistance towards multiple chemotherapeutic agents (Ling et al., 1983). Different cellular mechanisms underlying this phenomenon have been described, with the overexpression of ATP-binding cassette (ABC) drug transporter proteins, like P-glycoprotein (Pgp), among the most extensively studied (Gros et al., Hipfner et al., 1999, Konig et al., 1999). Cytotoxic drugs are either pumped out of the tumor cell or translocated to the outer leaflet of the plasma membrane by ABC-transporters, thus preventing their intracellular accumulation (Bolhuis et al., 1996, Borst et al., 2000, Gottesman et al., 1993).

In addition to the overexpression of ABC-transporters, virtually all MDR cells exhibit a deviating sphingolipid composition with, most typically, increased levels of glucosylceramide (GlcCer) (Kok et al., 2000, Lavie et al., 1996, Veldman 2002). It has been postulated that enhanced GlcCer levels reflect an increased conversion of ceramide (Cer) into GlcCer by glucosylceramide synthase (GCS), thereby circumventing the unset of Cer-induced apoptosis. In this way increased metabolism of Cer by overexpressed GCS would function as an independent MDR mechanism acting parallel with ABC transporters (Lavie et al., 1997, Liu et al., 1999, Lucci et al., 1999). However, the MDR $HT29^{col}$ cell line displayed increased levels of both GlcCer and Cer, while the expression and activity of GCS were unaltered (Kok et al., 2000, Klappe et al., 2004). Moreover, in these cells multidrug resistance related protein-1 (MRP1) expression and GlcCer levels were shown to develop simultaneously during drug resistance acquisition, suggesting a coupling between both processes (Klappe et al., 2004). Also drug-resistant 2780AD cells displayed similar GCS expression and activity as drug-sensitive A2780 cells (Veldman et al., 2002).

Pgp and MRP1 are known to depend on their lipid environment for optimal functioning (Dudeja et al., 1995, Sinicrope et al., 1992). Interestingly, Pgp was found to have a higher affinity for its substrates when the surrounding lipids are in gel phase rather than in liquid-crystalline phase (Romsicki et al., 1999). This gel phase occurs when lipids have a high degree of saturation, like sphingolipids, which enables them to pack tightly. This is also an important characteristic of membrane microdomains, which are operationally defined as detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs), and include caveolae (Brown et al., 2000, Schroeder et al., 1994). Due to the tight packing of sphingolipids with cholesterol, DIGs are insoluble in cold non-ionic detergents like Triton X-100 and Lubrol (Brown, 2002, Pike, 2003). Indeed, Pgp has been located in caveolae in several cell types (Demeule et al., 2000, Lavie et al., 1998, Yunamae, 2003).

Recently we have shown that overexpressed MRP1 and sphingolipids were co-localized in Lubrol-based DIGs of $HT29^{col}$ cells (Klappe et al., 2004). Furthermore, we have shown that Pgp in 2780AD cells was highly enriched in Lubrol-based and to a lesser extent in Triton X-100-based DIGs (Hinrichs et al., 2004). In the present study we show that the sphingolipid enrichment in drug-resistant 2780AD cells in fact occurs in Triton X-100-based DIGs and selectively involves specific fatty acid-containing sphingolipids, most notably C24:1 in all sphingolipids and C16 in GlcCer and Cer.

EXPERIMENTAL PROCEDURES

Materials

The 2780 cell lines were kindly provided by Dr. E.G.E. de Vries and Dr. H. Timmer-Bosscha (Dept. of Medical Oncology, University Hospital Groningen, The Netherlands). Doxorubicin was obtained from the hospital's pharmacist. Triton X-100 and Sucrose 99+% were purchased from Sigma (St. Louis, MO). All cell culture plastic ware was obtained from Costar (Cambridge, MA). RPMI 1640 medium, Hank's balanced salt solution (HBSS) and antibiotics were from Gibco Laboratories (Paisley, UK). Fetal calf serum was from Bodinco (Alkmaar, the Netherlands). L-[U-^{14}C]serine (specific activity >150 mCi/mmol) and [1-^{14}C]palmitic acid (specific activity 60 mCi/mmol) were purchased from Amersham International (Bercks, UK). Lubrol WX was obtained from Serva (Heidelberg, Germany).

Cell culture and experimental conditions

2780 cells were cultured in RPMI 1640 containing 100 units/ml penicillin, 100 mg/ml streptomycin and 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum. The medium of the 2780AD cells was supplemented with 1 μg/ml doxorubicin. The cultures were passed twice a week. All cells were maintained in a water-saturated atmosphere of 5% CO_2/95% air at 37°C. All experiments took place during the exponential growth phase of the cells.

Isolation of DIGs

DIGs were isolated from cells as described (Lisanti et al., 1995). For each isolation, confluent cells from two 75 cm^2 flasks were washed once with HBSS, harvested by scraping in 2 ml of ice-cold Tris-NaCl-EDTA buffer (TNE) (20 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 1% (w/v) Triton X-100 or 0.5% (w/v) Lubrol and vortexed. After 30 min

incubation on ice, cells were homogenized further by passing the lysate 20 times through a 21-G needle. Of this lysate 2 ml was transferred to a centrifuge tube and mixed with 2 ml of 80% (w/v) sucrose in TNE. On top of this, 4 ml of 35% (w/v) and 4 ml 5% (w/v) sucrose in TNE were successively loaded, resulting in a discontinuous gradient. All solutions contained the following protease inhibitors: 100 µM PMSF, 1 mM EDTA and 1 µM each of aprotinin, leupeptin and pepstatin A. Gradients were centrifuged in a Beckman SW41 swing-out rotor at 36.000 rpm for 18-20 h at 4 °C. Twelve fractions of 1 ml each were collected (from top to bottom), vortexed and stored at -80°C. The protein content of all fractions was measured using bovine serum albumin as standard. For (sphingo)lipid and lipid-phosphate analysis the DIG containing fractions were diluted 3 times with ice-cold TNE and centrifuged in a Beckman TLA 100.3 fixed rotor at 49.000 rpm for 1 h at 4 °C. The pellet was resupended in 1 ml TNE and stored at -80°C.

Equilibrium radiolabeling and analysis of cellular sphingolipids

Sphingolipid pools were metabolically radiolabeled by growing the cells for 48 hours in the presence of L-[U-^{14}C]serine (1 µCi/ml), a precursor molecule for sphingolipid biosynthesis (Babia et al., 1998). Lipids were extracted (Bligh et al., 1959) from the DIG fraction and the cell lysate kept apart after detergent incubation (see Isolation of DIGs). Aliquots of the lipid extracts were taken for determination of the total amount of lipid-incorporated radioactivity. Acylglycerolipids were hydrolyzed during a 1 h incubation at 37°C in $CHCl_3/CH_3OH$ (1:1, v/v) containing NaOH (0.1 M). The remaining lipids were re-extracted and applied on high performance thin layer chromatography (HPTLC) plates. Plates were developed in $CHCl_3/CH_3OH/H_2O$ (14:6:1, v/v/v) in the first dimension and were then sprayed with 2.5% H_3BO_3 (w/v) in CH_3OH and developed in the second dimension, using $CHCl_3/CH_3OH$/25% (w/v) NH_4OH (13:7:1, v/v/v) as the mobile phase. After autoradiography, GlcCer,

lactosylceramide (LacCer) and sphingomyelin (SM) containing spots were identified with the aid of standards and scraped from the plates. Radioactivity was measured by scintillation counting. Lipid levels were expressed as dps incorporated in a specific lipid species per 10^3 dps of total lipid-incorporated radioactivity (Veldman et al., 1998).

Alternatively, lipid pools were metabolically radiolabeled by growing the cells for 48 h in the presence of [1-^{14}C]palmitic acid (1 μCi/ml). Cells were harvested by scraping and centrifuged, followed by lipid extraction from the cell pellet (Bligh et al., 1959). In addition, DIG containing fractions (see "Isolation of DIGs") were pooled, then diluted with 2 volumes of ice-cold TNE and centrifuged in a Beckman TLA 100.3 fixed rotor at 49.000 rpm for 1 h at 4 °C. The pellet was resupended in 1 ml TNE, followed by lipid extraction (Bligh et al., 1959). The lipids were applied on high performance thin layer chromatography (HPTLC) plates. Plates were developed in $CHCl_3/CH_3OH/H_2O$ (14:6:1, v/v/v) in the first dimension and then sprayed with 2.5% H_3BO_3 (w/v) in CH_3OH and developed in the second dimension, this time using $CHCl_3/CH_3OH$/25% (w/v) NH_4OH (13:7:1, v/v/v) as the mobile phase. After autoradiography phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) containing spots were identified with the aid of standards and analyzed using phosphorimaging. Lipid levels were expressed as a percentage of the radioactivity of the total lipid extract applied on the HPTLC plates.

Electrospray tandem mass spectometry

Sphingolipids were extracted and analyzed by liquid chromatography-electrospray tandem mass spectrometry (ESI-MS/MS) on a PE-Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source as described previously (Sullards et al., 2001). Dry N_2 was used as the nebulizing gas at a flow rate of 6 liters/min. The ionspray needle was held at 5500 V, and the orifice and ring voltages were kept low (40 V and 220 V,

respectively) to prevent collisional decomposition of molecular ions prior to entry into the first quadrupole; the orifice temperature was set to 500°C. N_2 was used to collisionally induce dissociations in Q2, which was offset from Q1 by 40-50 V. Q3 was then set to pass molecularly distinctive product ions (N ions) of *m/z* 264.4. Multiple reaction monitoring (MRM) scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. For example, for the ceramides, these transitions occur at *m/z* 538.7/264.4, 566.5/264.4, 622.7/264.4, 648.7/264.4, 650.7/264.4, 676.5/264.4, and 678.5/264.4, which corresponds to ceramides with a d18:1 sphingoid base (sphingosine) and C16:0, C18:0, C22:0, C24:1, C24:0, C26:1, and C26:0 fatty acids, respectively. Quantitation was achieved by spiking the samples prior to extraction with the C12-fatty acid homologs of Cer, SM and GlcCer.

Cholesterol and phosphate determination on cell lysate and DIGs

Triton X-100- and Lubrol-based cell lysates were prepared and part of this lysate was used to isolate DIGs (see "Isolation of DIGs"). The DIG fractions were pooled. After a protein determination (Smith et al., 1985) on both the lysate and the pooled DIG fractions, lipids were extracted (Bligh et al., 1959). In the extract the cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase / peroxidase assay (Gamble et al., 1978). The phosphorus content, as a measure for the phospholipid content in the lysate and the pooled DIG fractions, was determined by a phosphate assay (Bottcher et al., 1961).

RESULTS

Sphingolipid alterations in drug-resistant 2780AD cells occur to a large extent in (especially Triton X-100-based) DIGs

Pgp overexpressing drug resistant 2780AD cells have been shown to display an altered sphingolipid composition compared to drug sensitive A2780 cells (Veldman 2002), while Pgp was found to be localized in Lubrol-based DIGs and to a lesser extent in Triton X-100-based DIGs (Hinrichs et al., 2004). Here we analyzed the sphingolipid compositions of both Triton X-100- and Lubrol-based DIGs in both drug-resistent and drug-sensitive 2780 cells. Total lipid pools were metabolically equilibrium-labeled with L-[U-^{14}C]serine. Lubrol- and Triton X-100-based cell lysates were prepared and fractionated on discontinuous gradients. The low-density membrane fractions were isolated and their sphingolipid compositions analyzed. The sphingolipid compositions of both Lubrol- and Triton X-100-based DIGs of the drug-resistant 2780AD cells were different from those of the drug-sensitive A2780 cells (Table I). While SM levels and especially GlcCer levels had increased in DIGs from drug-resistant cells, LacCer levels had decreased. The amounts of sphingolipids relative to total (radiolabeled) lipids were three times higher in Triton X-100-based DIGs compared to Lubrol-based DIGs. In 2780AD cells, the total measured sphingolipids in Triton X-100-based DIGs represented 62% of total (radiolabeled) lipids in the DIGs, against 23% in Lubrol-based DIGs. The total measured Triton X-100 and Lubrol insoluble sphingolipids represented 9% and 6%, respectively, of the total labeled lipids in the cell lysate.

Table I- *DIG sphingolipids in MDR 2780AD cells versus drug sensitive A2780 cells.*

Triton X-100				
	A2780		2780AD	
	DIGs	*lysate*	DIGs	*lysate*
GlcCer	30.6 ± 4.0	*3.9 ± 0.9*	60.6 ± 4.6*	*8.6 ± 1.5**
LacCer	68.2 ± 12.2	*8.6 ± 2.2*	33.9 ± 1.8*	*5.3 ± 1.7*
SM	444.1 ± 72.7	*54.9 ± 3.5*	528.8 ± 92.2	*79.6 ± 11.2**
Lubrol				
GlcCer	9.5 ± 1.3	*2.4 ± 0.7*	21.7 ± 2.2*	*6.0 ± 1.8**
LacCer	21.4 ± 6.1	*5.4 ± 2.3*	11.9 ± 2.4	*3.1 ± 0.3*
SM	134.8 ± 14.5	*33.2 ± 4.7*	193.4 ± 35.9*	*51.7 ± 8.9**

Total sphingolipid pools of A2780 cells and 2780AD cells were metabolically radiolabeled by culturing the cells in the presence of 1 μCi/ml L-[U-^{14}C]serine for 48h. Triton X-100- and Lubrol-based DIGs were isolated as described in "Experimental procedures". The lipids were extracted from the DIGs and subjected to an alkaline hydrolysis procedure in order to remove glycerol-based lipids. The remaining lipids were subjected to two-dimensional TLC, followed by autoradiography and scintillation counting. Data (mean ± S.D., n=3) are expressed as the percentage of radioactivity incorporated in a given sphingolipid relative to total lipid-incorporated radioactivity of the DIG (**DIGs**) or of the cell-lysate before DIG isolation (***lysate***). *Values are significantly ($P < 0.05$) different, as determined by the two-tailed, unpaired t-test.

Drug-resistant 2780AD cells do not have altered amounts of DIGs

Given the changes in sphingolipid levels in drug-resistant 2780AD cells (10) and the finding that they represent an important share of DIG lipids, we investigated whether 2780AD cells have altered amounts of DIGs compared to A2780 cells. From both cell lines equal amounts of Lubrol- and Triton X-100-treated cell lysates were fractionated on discontinuous gradients. The low-density membrane fractions were isolated and their protein, phospholipid and

cholesterol contents were measured. Between DIGs of A2780 and 2780AD no significant differences were observed in any of the three parameters (Fig. 1). Therefore we conclude that the amounts of DIGs present in drug-resistant 2780AD cells are not different from those in drug-sensitive A2780 cells. There were clear differences between Lubrol- and Triton X-100-based DIGs in both cell lines. Lubrol-based DIGs contained two times more protein and three times more phospholipid, but only slightly elevated cholesterol (not significant), compared to Triton X-100-based DIGs.

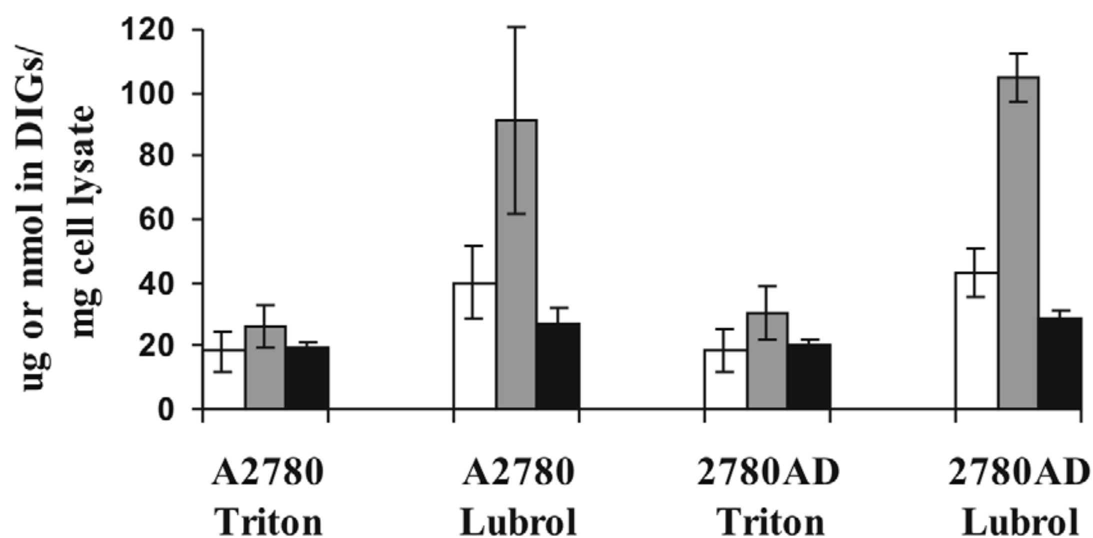

Figure 1 – *Protein, phospholipid and cholesterol content of DIGs from 2780AD versus A2780 cells***.** Triton X-100- and Lubrol-based lysates prepared from A2780 and 2780AD cells were fractionated by flotation in a discontinuous sucrose density gradient. Detergent insoluble floating fractions 4-6 were combined and protein, lipid phosphate and cholesterol contents were determined. Protein content (white bars) was expressed as µg, while lipid phosphate content (grey bars) and cholesterol content (black bars) were expressed as nmol per mg cell lysate. Data represent the mean ± S.D. of three independent experiments. *Values are significantly ($P < 0.05$) different from those of the A2780-derived DIGs, as determined by the two-tailed, unpaired *t*-test.

Phospholipid levels have not altered significantly in DIGs of drug-resistant 2780AD cells

To investigate if drug-resistant versus drug-sensitive cells differed in the levels of individual phospholipids in DIGs, total lipid pools of both A2780 and 2780AD were metabolically

radiolabeled to equilibrium with [1-^{14}C]palmitic acid. Lubrol- and Triton X-100-based cell lysates were prepared and fractionated on discontinuous gradients. The low-density membrane fractions were isolated and the phospholipids were analyzed. Between DIGs of A2780 and 2780AD no significant differences were observed in PC, PE or PS levels (Fig. 2). The phospholipid composition differed significantly between Lubrol- and Triton X-100-based DIGs in both cell lines. While levels of PC were comparable between both types of DIGs, Lubrol-based DIGs contained higher levels of PE and PS.

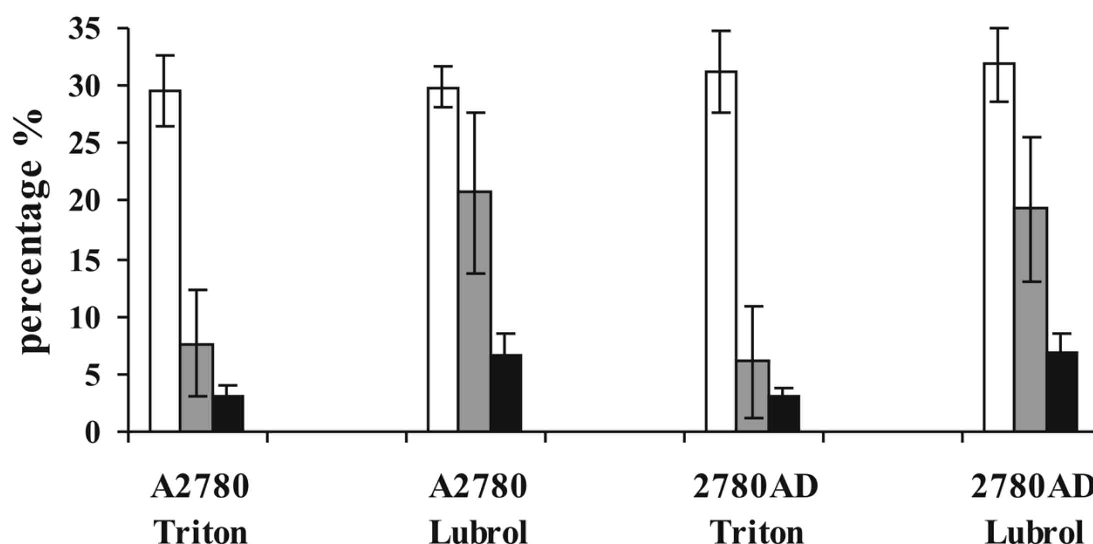

Figure 2 – *Phospholipid composition of DIGs from 2780AD versus A2780 cells***.** Total lipid pools of A2780 and 2780AD cells were metabolically radiolabeled by culturing the cells in the presence of 1 μCi/ml [1-^{14}C]palmitic acid for 48h. Triton X-100- and Lubrol -based DIGs were obtained as described in "Experimental procedures". The lipids were extracted and subjected to two-dimensional TLC, followed by autoradiography and phosphorimaging analysis. White bars: PC, grey bars: PE, black bars: PS. Data (mean ± S.D., n=3) are expressed as the percentage of the radioactivity incorporated in the total DIG lipid fraction. *Values are significantly ($P < 0.05$) different from those of the A2780-derived DIGs, as determined by the two-tailed, unpaired t-test.

C24/1 sphingolipids are highly enriched in Triton X-100-based DIGs of drug-resistant 2780AD cells - DIGs are believed to owe their insolubility, in cold non-ionic detergents like Triton X-100 and Lubrol, to the strong bonds between the long, mainly saturated fatty acid chains of sphingolipids (Brown, 2002, Pike, 2003). If sphingolipid alterations in 2780AD cells play a structural role in DIGs, those with long fatty acids chains and minor unsaturations would be expected to be involved. Since GlcCer and SM were found to be increased in MDR 2780AD cells we compared their fatty acid distribution and that of their common precursor Cer between DIGs of drug-sensitive and those of drug-resistant 2780 cells. Triton X-100-based cell lysates from both cell lines were prepared and fractionated on discontinuous gradients. The low-density membrane fractions were isolated and their sphingolipid composition was analyzed by ESI-MS/MS. The fatty acid distributions were comparable between GlcCer, SM and Cer in one cell type (Table II), but highly different in DIGs from drug-resistant versus drug-sensitive cells (Fig. 3). Most notably, all measured sphingolipids, i.e. Cer, GlcCer and SM, with C24:1 were three times more abundant in DIGs from drug-resistant 2780AD cells compared to those of drug-sensitive cells (Fig. 3). C16 was the major species in all measured sphingolipids in the DIGs. In case of Cer and GlcCer, the C16 species were relatively abundant in DIGs of drug-resistant cells, when compared to the total enrichment factor (Fig. 3). All other fatty acid species were relatively more abundant in DIGs of drug-sensitive cells, when compared to the total enrichment factor. Overall, the levels of GlcCer, SM and Cer were higher in DIGs of 2780AD cells, as indicated by the sum of all fatty acid species (Fig. 3, total). The increases of GlcCer and SM were comparable to those observed using ^{14}C-serine equilibrium labeling.

Table II- *Triton X-100-based DIG sphingolipid species in MDR 2780AD cells versus drug sensitive A2780 cells.*

		C16	C18	C20	C22	C24:1	C24	C26:1	C26
Cer	*A*	*51,3 ± 3,7*	*9,0 ± 1,1*	*2,6 ± 1,1*	*8,5 ± 2,3*	*14,9 ± 1,2*	*13,0 ± 0,8*	*0,4 ± 0,0*	*0,2 ± 0,0*
	AD	60,9 ± 3,1 *	4,8 ± 0,7 *	1,3 ± 0,6	4,1 ± 1,2 *	21,0 ± 1,9 *	7,7 ± 2,3 *	0,3 ± 0,0 *	0,1 ± 0,0 *
GlcCer	*A*	*47,2 ± 5,0*	*7,1 ± 0,3*	*1,8 ± 0,0*	*12,2 ± 3,1*	*14,5 ± 2,0*	*21,3 ± 3,4*	*0,5 ± 0,1*	*0,4 ± 0,1*
	AD	54,3 ± 8,1	3,7 ± 0.0 *	1,1 ± 0,1 *	7,7 ± 0,9 *	22,6 ± 3,8 *	11,9 ± 3,5 *	0,4 ± 0,1	0,2 ± 0,0 *
SM	*A*	*68,6 ± 2,1*	*9,7 ± 1,1*	*1,8 ± 0,6*	*4,2 ± 0,6*	*9,8 ± 1,1*	*5,6 ± 0,6*	*0,4 ± 0,2*	*0,1 ± 0,0*
	AD	71,0 ± 2,5	4,3 ± 0,3 *	0,7 ± 0,1 *	2,1 ± 0,3 *	16,9 ± 2,6 *	4,7 ± 0,3 *	0,2 ± 0,0	0,1 ± 0,0

From A2780 and 2780AD cells Triton X-100-based DIGs were obtained as described in "Experimental procedures". Lipids were extracted from the DIGs and subjected to alkaline hydrolysis to remove glycerol-based lipids. Sphingolipids were analyzed by ESI-MS/MS. Each sphingolipid with a specific fatty acid is expressed as the percentage of the total species of that sphingolipid (mean ± S.D., n=3). *Values are significantly ($P < 0.05$) different, as determined by the two-tailed, unpaired t-test.

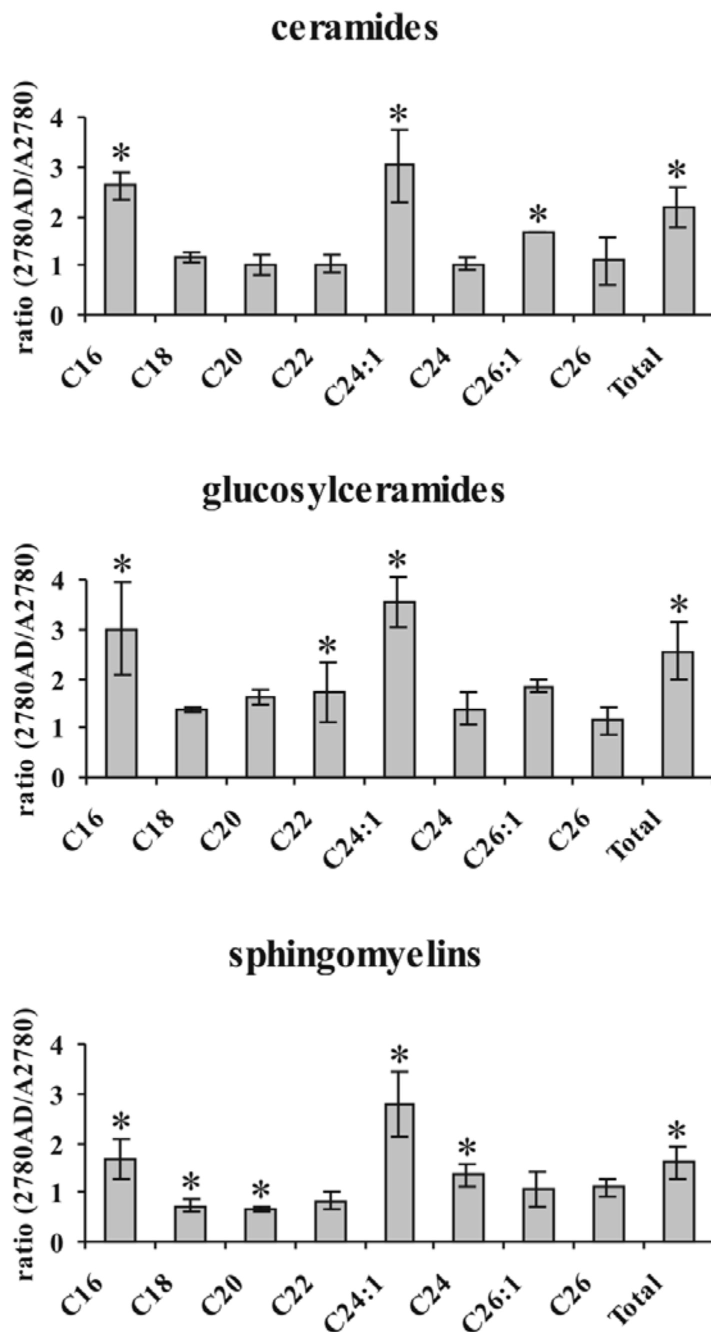

Figure 3 – *Sphingolipid fatty acid distribution of Triton X-100-based DIGs from 2780AD versus A2780 cells***.** From A2780 and 2780AD cells Triton X-100-based detergent insoluble membrane domains were obtained as described in "Experimental procedures". Lipids were extracted from the DIGs and subjected to alkaline hydrolysis to remove glycerol-based lipids. Sphingolipids were analyzed by ESI-MS/MS. The ratio (2780AD/A2780) was calculated by dividing the amount of a given sphingolipid with a a specific fatty acid in 2780AD-derived DIGs by the amount in A2780-derived DIGs (mean ± S.D., *n*=3). *Values are significantly ($P < 0.05$) different, as determined by the two-tailed, unpaired *t*-test.

DISCUSSION

In Pgp overexpressing 2780AD cells, exhibiting typical sphingolipid alterations, Pgp was found to be enriched in Lubrol-based and to a lesser extent in Triton X-100-based DIGs (Hinrichs et al., 2004). In this study, we set out to determine (1) whether MDR-associated sphingolipid alterations occur in DIGs of 2780AD cells, (2) if so, whether they occur in a specific type of DIG, i.e. Triton X-100- or Lubrol-based, (3) if so, whether such alterations in sphingolipids are likely to play a structural role in these DIGs, and (4) whether additional changes occur in other components of DIGs, i.e. phospholipids, cholesterol and protein content, in drug-resistant cells.

In this study the sphingo- and phospholipid compositions as well as cholesterol and protein content of both Triton X-100- and Lubrol-based DIGs between MDR 2780AD cells and their drug sensitive counterpart (A2780 cells) were compared. Both Triton X-100- and Lubrol-based DIGs of 2780AD cells differed in sphingolipid composition from those of A2780 cells in a fashion reminiscent of the differences in total cellular sphingolipid composition as observed earlier (10). Moreover, the differences between both cell lines concerning lipid composition of DIGs appeared to be limited to sphingolipids. The levels of the main phospholipids, PC, PE and PS, as well as the total phospholipid content were similar in DIGs from drug-resistant and drug-sensitive cells. In addition to total phospholipid content, also protein and cholesterol content were similar in DIGs isolated form the two cell lines. Therefore, we conclude that there were no differences in the amounts of DIGs between drug-resistant and drug-sensitive cells. Hence, DIGs from both cell lines differed specifically in sphingolipid composition.

Interestingly, it can be calculated that, based on the data from Table I concerning sphingolipid levels in DIGs in combination with published data on the sphingolipid levels in

whole cells (Veldman et al., 2002), of a given sphingolipid species the pool found in DIGs represents a major fraction of the total cellular pool (c.f. Veldman et al., 2002). Each measured sphingolipid species in DIGs represented at least 69%, in case of Triton X-100, and 44%, in case of Lubrol, of its total cellular pool. This means that the MDR-associated differences in sphingolipid composition are to a large extent DIG-associated.

It appears that the sphingolipid pool in Triton X-100-based DIGs is largely overlapping with that of the Lubrol-based DIGs. First, the differences between drug-resistant and drug-sensitive cells in sphingolipid composition of the DIGs are very similar between Triton X-100- and Lubrol-derived DIGs. Second, while the Lubrol-based DIGs have three-fold higher phospholipid content, this corresponds well with the three-fold lower abundance of sphingolipids relative to total (labeled) lipid in Lubrol-based DIGs. It is therefore likely that at least a considerable fraction of the Lubrol-based DIGs has a Triton X-100-insoluble core and a Triton X-100-soluble/Lubrol-insoluble region. Sphingolipids in Triton X-100-based DIGs would thus be 'diluted' in the Lubrol-based DIGs, due to the addition of a Lubrol-insoluble membrane area with a lower sphingolipid/phospholipid ratio. This notion is consistent with observations by Drobnik et. al. indicating that Lubrol-based DIGs contain at least 75% of Triton X-100-based DIGs (Drobnik et al., 2002). This leads to the conclusion that MDR-associated differences in sphingolipid composition occur to a large extent in Triton X-100-based DIGs.

To investigate if the altered sphingolipid composition of Triton X-100-based DIGs is likely to play a structural role in DIGs we analyzed the fatty acid distribution of DIG sphingolipids using ESI-MS/MS. Most striking, sphingolipids with C24:1 were three times more abundant in DIGs of drug-resistant 2780AD cells (Fig. 3) compared to those of drug-sensitive A2780 cells (Fig. 3). Interestingly, C24:1 was preferentially increased over C24 in DIGs from 2780AD cells. This corresponds with the finding that C24:1 SM was found to be

most typically enriched in rafts compared to total plasma membrane (Pike et al., 2002). C24:1 enrichment in all measured sphingolipids in DIGs from 2780AD cells indicates that the MDR-associated changes in sphingolipid composition of drug-resistant cells at least partly fulfill a structural role in DIGs of these cells.

In case of Cer and GlcCer, also the C16 species were relatively more abundant in DIGs of drug-resistant cells, when comparing to the total enrichment factor (Fig. 3). This is consistent with other observations indicating relative abundance of short-chain fatty acid in raft lipids (Pike et al., 2002). C16-Cer is in contrast to C24-Cer associated with signal transduction in apoptosis (Kroesen et al., 2003). Although beyond the scope of this study, it is conceivable that the C16-GlcCer increase in DIGs of 2780AD cells is correlated with the C16-Cer increase and involved in an apoptosis escape pathway as described earlier (Lavie et al., 1997, Liu et al., 1999, Lucci et al., 1999).

In conclusion, MDR-associated sphingolipid composition changes in drug-resistant 2780AD cells were localized in DIGs and more specifically in Triton X-100-based DIGs. Moreover, sphingolipid composition changes were the only compositional changes observed in the DIGs of these drug-resistant cells. In addition to the changes in sphingolipid species composition, each sphingolipid species measured displayed an altered fatty acid composition relative to drug-sensitive cells. These changes in fatty acid distribution are compatible with a role for sphingolipids in the structural organization of DIGs. This study does not provide evidence for a functional role of DIG-associated sphingolipid changes in MDR. In fact, since Pgp was shown to be especially enriched in Lubrol-based DIGs of 2780AD cells (Hinrichs et al., 2004) it is not likely that its function is directly facilitated by the MDR-related sphingolipid alterations, which occur largely in the Triton X-100-based DIGs. However, as discussed above, sphingolipids and Pgp may be localized in different regions of a large membrane domain, which harbours a Triton X-100-insoluble core and a Triton X-100-

soluble/Lubrol-insoluble perimeter. As such, this large membrane domain may be involved MDR, e.g. in assuring close proximity of Pgp and its amphipathic substrates (cytostatics). Future research will focus on this hypothesis.

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