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Characterization of cellular lipids in doxorubicin-sensitive and -resistant P388 mouse leukemia cells*

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Summary. The purpose of this study was to determine whether changes in cellular lipid composition accompanied the selection of cells that are resistant to the anthracycline doxorubicin. Total cellular lipid extracts from doxorubicin-sensitive and doxorubicin-resistant P388 murine leukemia cells were prepared and separated into neutral glycosphingolipids, gangliosides, phospholipids, and neutral lipid families. No significant quantitative differences in total cholesterol, lipid-bound sialic acid, neutral hexose, and lipid-bound phosphate were found between the two cell lines. Gas-liquid chromatographic analysis of the fatty acids derived from each lipid class demonstrated that sensitive and resistant cells had essentially identical fatty acid compositions. Qualitative evaluation of the four lipid classes by high-performance thin-layer chromatography revealed only minor differences in lipid composition between the resistant and the sensitive cells. Results from this study indicate that although minor differences between the two cell lines are present, no major cellular lipid differences are evident to account for the marked differences in the cellular pharmacokinetics and cytotoxic effects of doxorubicin between doxorubicin-sensitive and doxorubicin-resistant P388 murine leukemia a cells.

Introduction

The anthracycline antibiotics (doxorubicin and daunorubicin) are widely used in the treatment of a variety of neoplastic diseases. Doxorubicin (DOX) is now commonly used in the treatment of patients with acute leukemia and solid tumors, and may be one of the more important antitumor drugs currently available [46]. The success of DOX induction chemotherapy in patients with neoplasms is limited by the emergence of resistant cell populations and by a dose-limiting cardiotoxicity [21, 43, 46].

Classically, the cytotoxic effects of DOX have been thought to act by high-affinity binding and intercalation of DNA [5, 8, 27, 30, 37]. More recent studies examining support-bound DOX complexes indicate that DOX may be actively cytotoxic without entering cells [35, 41, 42]. This suggests that the cell membrane is a target for anthracycline cytotoxicity, which may involve the generation of free radicals at the membrane level [26].

Generalized changes in the membrane composition and structure accompanying the development of anthracycline-resistant cells have been noted [2, 31, 33, 34, 38, 45]. In addition, specific changes in membrane ganglioside composition have been reported in Chinese hamster cells exhibiting pleiotropic resistance to vincristine and daunorubicin [28]. Ramu et al. have recently shown differences in total phospholipid composition and triglyceride levels in P388 cells resistant to DOX [32]. However, it is not clear whether membrane alterations are characteristic of anthracycline resistant cells. In this study we examined the lipid composition of P388 mouse leukemia cells that are DOX-sensitive (P388/S) and DOX-resistant (P388/R) to determine whether a change in any of the four major cellular lipid classes accompanies drug resistance in these cells.

Materials and methods

P388 murine leukemia cells that are DOX-sensitive and DOX-resistant were established in vitro as reported previously [10]. Cells were maintained in suspension at 37°C under a CO_2 concentration of 7.0% in a medium consisting of RPMI-1640 supplement with 10% heat-inactivated fetal bovine serum, 2.0 m*M* *L*-glutamine, 10 μ*M* 2-mercaptoethanol, and 10 units/ml penicillin/streptomycin. Cells were harvested at a concentration of $0.5-1.0 \times 10^6$/ml and washed with phosphate-buffered saline (PBS) three times. Cell pellets were immediately resuspended in chloroform: methanol (2:1, v/v) for lipid extraction and stored at −20°C.

Lipid extraction and separation. The extraction of total lipid was performed in parallel for the sensitive and resistant cells using methods previously reported [22, 24, 25]. Total lipid was obtained by extraction for 30 min at room temperature with 20 cell volumes of each of the following chloroform: methanol (v/v) ratios: (2:1), (1:1), and (1:2). The combined extracts were passed over a sintered glass filter to remove nonsoluble components. The filtrates were then dried in vacuo, resuspended in a small volume of wa-

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ter, and dialyzed overnight with water (mol. wt cutoff 3500, 45 mm diameter tubing, Spectrum Medical Industries, Calif). The dialyzed extracts were lyophylized and resuspended in a small volume of chloroform:methanol:water (30:60:8, v/v) and applied to a DEAE-Sephadex anion exchange column (1 × 25 cm, 40–120 μm mesh, Sigma Chemical Co, St. Louis, Mo.) After 30 min at room temperature, the neutral lipid components were eluted with chloroform:methanol:water (30:60:8, v/v) followed by methanol. The acidic components were eluted with 0.2 *M* sodium acetate in methanol. The neutral and acidic fractions were dried in vacuo, and the acidic fraction was dialyzed against water to remove salt and then lyophylized as described above.

To separate the gangliosides fromt the phospholipids and the neutral glycosphingolipids from the neutral lipids, we used the Florisil column chromatography procedure previously described [36]. To summarize: Each of the neutral and acidic fractions obtained from the DEAE-Sephadex separation were acetylated overnight and applied to a Florisil column (1 × 25 cm, 60–100 mesh, Fisher Scientific, NJ). The four columns (one each for sensitive and resistant, acidic and neutral fractions) were eluted successively with the following solvents: (1) hexane: 1,2-dichloroethane (DCE) (1:4, v/v) followed by DCE alone; (2) DCE: acetone (1:1, v/v; (3) DCE:methanol:water (2:8:1, v/v). This procedure results in separation of the gangliosides, phospholipids, neutral glycosphingolipids, and neutral lipids for both the sensitive and the resistant cell lines. The ganglioside and neutral glycosphingolipid fractions were deacetylated for further analysis as previously described [23].

Analytical methods. To determine whether any specific changes in the lipid fatty acid composition are characteristic of DOX-resistant cells, the fatty acid content of each lipid family was determined. Methyl esters of the free fatty acids were prepared for gas chromatographic analysis using a procedure previously described [14]. Standard fatty acid methyl esters were obtained from Supelco (Bellefonte, Pa). Separation was performed using a Packard gas chromatograph equipped with a 1.8 m packed glass column (1/4 in., 5% Degs-PS on 100/120 Supelcoport, Supelco) using an isocratic temperature of 190°C. Identification of peaks was established by comparison of retention times with standards. The fatty acid content was quantitated by measurement of the percentage area for each peak.

Quantification of the neutral hexose content of the neutral glycosphingolipid was determined using the phenol-sulfuric acid assay previously described [9]. The gangliosides were quantified by determinng the amount of lipid-bound sialic acid using the resorcinol procedure described by Svennerholm [40]. The phospholipid fractions were quantitated by determining the total inorganic phosphate content, by the procedure described by Bartlett [1]. The method used for total protein determination was the Pierce BCA assay (Pierce Chemical Co, Rockford, Ill) [29]. Total cholesterol content of the extracted neutral lipid fraction was determined by the procedure for serum cholesterol that we have modified for use with nonserum samples [47].

Thin-layer chromatography. To identify qualitative differences in each of the families of lipids, the lipid components were compared by HPTLC techniques using standards prepared in our laboratory or obtained through Supelco. The resistant and sensitive cell lines were compared for the presence or absence of compounds visualized with appropriate stains. The following solvent systems, thin-layer chromatography plates, and staining reagents were used: (1) Gangliosides were separated using HPTLC silica gel 60 plates (Merck, Germany) developed in chloroform:methanol: 0.2 *M* $CaCl_2$ (55:40:9, v/v), and visualized with resorcinol reagent (system A); (2) neutral glycosphingolipids were separated using silica gel 60 plates (250 μm, Supelco) developed in chloroform:methanol:water (60:35:8, v/v) and visualized with orcinol reagent (system B); (3) neutral lipid components were separated using silica gel G plates developed in hexane:diethyl ether: acetic acid (70:30:1, v/v) and visualized with a saturated solution of potassium dichromate ($K_2Cr_2O_7$) in 70% sulfuric acid (system C); (4) phospholipids were separated using silica gel G plates developed in chloroform:methanol:acetic acid:water (170:25:40:3, v/v) and visualized with phospholipid stain, allowed to dry, and then sprayed with the saturated potassium dichromate stain to visualize phospholipids which did not stain with the molybdenum blue reagent (system D).

Results

Quantification of lipid components

The quantitative differences in the four lipid families in the P388/S and P388/R cells are summarized in Table 1. Significant quantities of cholesterol, lipid-bound sialic acid, lipid-bound phosphate, and neutral hexose were found for both the sensitive and the resistant cells. In addition, each of these components was quantitatively similar for the two cell lines. Although a slightly higher hexose content was found in the neutral glycosphingolipids and a lesser amount of lipid-bound phosphate was found in the phospholipids of the resistant cells, the difference between sensitive and resistant cells was not significant. These results show no distinct quantitative differences in the total amounts of lipid components between the DOX-sensitive and -resistant cell lines.

Qualitative analysis of lipid components

To determine whether qualitative differences exist between the P388/S and P388/R cells, thin-layer chromatographic analysis was performed. A composite of characteristic chromatographs of the four lipid families is shown in Fig. 1. The HPTLC separation of the gangliosides is shown in Fig. 1A. Both the P388/S and the P388/R cells had gangliosides that migrated with R_f values similar to GM_3, GM_2, and SPG (or GM_1). No evidence was obtained which indicated the presence of more complex gangliosides (including $GD1_a$) in either the P388/S or the P388/R cells. There was also no indication for the synthesis of a new ganglioside species or for the loss of a particular ganglioside in the P388/R cells.

The HPTLC separation of the neutral glycosphingolipids (GSL) is shown in Fig. 1B. The major neutral GSL for both the resistant and the sensitive cells was a compound found to co-chromatograph with the lactosylceramide (LacCer) standard. GL_1 was the only other neutral GSL

Table 1. Summary of quantitative data

Description	Resistant	Sensitive
Total protein	0.11 (0.01) mg	0.13 (0.02) mg
Cholesterol	1.9 (0.1) μg	1.8 (0.1) μg
Phosphate	6.7 (0.2) nmol	7.1 (0.4) nmol
Sialic acid	0.25 (0.03) nmol	0.26 (0.04) nmol
Hexose	0.15 (0.01) μg	0.13 (0.02) μg

Units as presented are per 10^6 cells. Each value is the mean of at least three separate determinations with one standard deviation indicated (in parenthesis)

present in a significant quantity. Overall, a simple neutral GSL pattern was observed; neutral GSLs with longer oligosaccharide chains were not evident in either cell line.

The HPTLC separation of the neutral lipids is shown in Fig. 1C. The major neutral lipid of both cell lines chromatographs with the standard cholesterol. Lesser quantities of compounds that migrated with R_f values similar to cholesteryl palmitate, and cholesteryl acetate or tripalmitin (which co-migrated in this system) were also found. No evidence for the presence of free fatty acid (palmitic and stearic acids) was observed. More polar compounds in approximately equivalent amounts were seen in the neutral lipid preparations from both cell lines. However, these did not migrate with any of the standard neutral lipids used in our analysis.

The HPTLC separation of the phospholipids is shown in Fig. 1D. The major phospholipid found in both the P388/S and P388/R cell lines was identified as phosphatidylcholine (PC). Although phosphatidylserine (PS) had a similar R_f value to PC in solvent D, other solvent systems used resolved PC from PS and clearly demonstrated that PC was the major phospholipid in both cell lines. Compounds migrating with R_f values similar to phosphatidylethanolamine and cardiolipin were also found in significant quantities in both cell lines. Lesser quantities of sphingomyelin (SPM) and lysolecithin (LPC) were found in both cell lines. Phosphatidylinistitol (PI) was not evident in either cell line, although in the system presented in Fig. 1D, PI co-migrates with PS.

Analysis of the fatty acid composition was performed to determine whether a shift in the fatty acid pattern in any of the four lipid families was associated with DOX resistance in these cells (Table 2). Fatty acids that accounted for less than 5% are not included in this table. Fifteen methylated fatty acid standards were used to identify the unknown components. The major fatty acid components of the gangliosides from both the resistant and the sensitive cells were palmitic acid, stearic acid, and lignoceric acid in nearly equivalent quantities. The major fatty acid present in the neutral glycosphingolipids was erucatic acid, with smaller amounts of lignoceric and palmitic acid. The major fatty acid components of the phospholipid fractions were found to be oleic and palmitic acids, with lesser amounts of stearic and linoleic acids. The presence of palmitic acid in the neutral lipid fractions for the sensitive and resistance cells is consistent with HPTLC results which showed compounds migrating with cholesteryl palmitate and tripalmitin. Other fatty acids in the neutral lipid fractions were stearic acid and oleic acid. Therefore, the neutral and phospholipid families for both cell lines contained components with shorter chain fatty acids than did the two glycosphingolipid families. All lipid families contained major fatty acids with zero to one unsaturation.

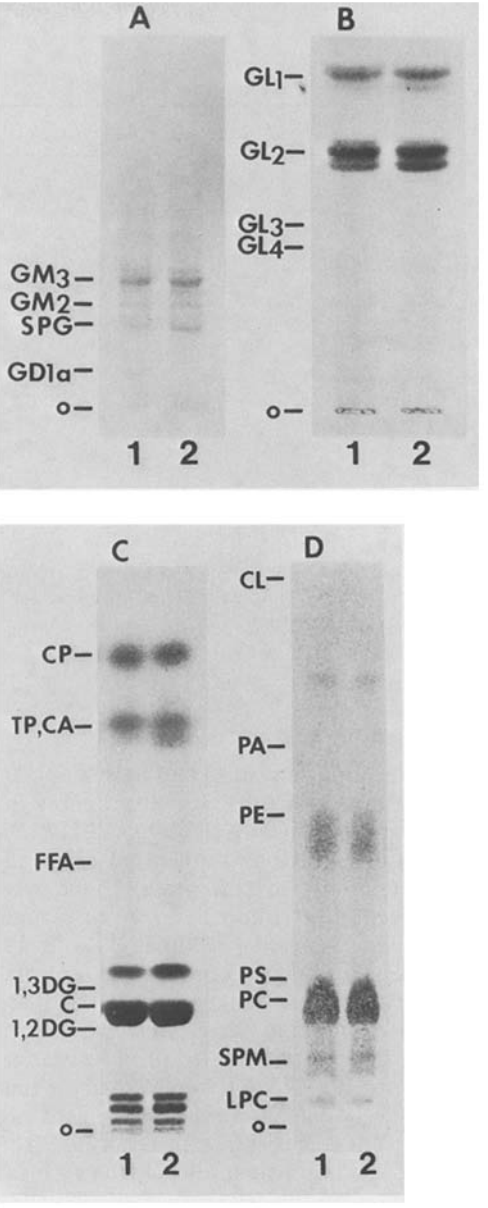

Figure 1. Thin-layer chromatograms of the four lipid families from P388/S and P388/R cell lines. Plates shown are characteristic of those obtained for numerous (>3) cell extracts. Lanes *1* and *2* represent P388/S and P388/R cell extracts, respectively. The origin is indicated by „*o*". Standards as indicated were run concurrently. *A*, Gangliosides separated using system A (see *Methods*). Standards: GM_3, GM_2, SPG, GD_{1a}, *B*, Neutral glycosphingolipids separated using system B. Standards: GL_1, GL_2, GL_3, GL_4, *C*, Neutral lipids separated using system C. Standards: *CP* = cholesteryl palmitate; *TP* = tripalmitin; *CA* = cholesteryl acetate; *FFA* = free fatty acid, *1,3 DG* = 1,3 diglyceride; *C* = cholesterol; *1,2 DG* = 1,2 diglyceride *1D*, Phospholipids separated using system D. Standards: *CL* = cardiolipin (or diphoshatidylglycerol); *PA* = phosphatidic acid; *PE* = phosphatidylethanolamine; *PS* = phosphatidylserine; *PC* = phosphatidylcholine; *SPM* = sphingomyelin; *LPC* = lysophosphatidylcholine

Table 2. Result of gas-chromatographic analysis of four major lipid classes separated

Lipid family	Fatty acid (%)					
	16 : 0	18 : 0	18 : 1	18 : 2	22 : 1	24 : 0
Gangliosides						
R	26	28				30
S	21	25				35
Neutral glycosphingolipids						
R	5				66	11
S	9				68	7
Phospholipids						
R	24	20	35	10		
S	29	17	35	10		
Neutral lipids						
R	15	33	43			
S	10	32	44			

Duplicate determinations were made.
R, DOX-resistant P388 cells; S, DOX-sensitive cells; 16 : 0, palmitic acid; 18 : 0, stearic acid; 18 : 1, oleic acid; 18 : 2, linoleic acid; 22 : 1, erucatic acid; 24 : 0, lignoceric acid

Discussion

Cellular resistance to the anthracyclines had been associated with decreased net uptake of drug, which probably involves an active efflux mechanism [6, 11, 12, 15, 17, 20, 39]. Since both decreased uptake and increased efflux involve membrane interactions, several groups have analyzed membrane lipid and protein compositions of various anthracycline-resistant and -sensitive cell lines [4, 7, 13, 19, 28, 32, 45]. In the present study, we examined the four major lipid classes in P388 mouse leukemia cells that are DOX-sensitive and -resistant to determine whether a change in lipid composition accompanied drug resistance.

Wheeler et al. examined MDAY-K2 cells, to show that progressively higher levels of cell surface sialic acid are found with increasing cellular resistance to DOX [45]. The neuraminidase method used in their study [44] gives a measure of sialic acid residues on the cell surface available for hydrolysis by the enzyme, but does not give a value for the total membrane content of sialic acid. Our measurement of lipid-bound sialic acid did not show any difference between the P388/S and P388/R cells. However, since membrane glycoproteins are also known to contain sialic acid residues, the differences noted by Wheeler et al. [45] may be due to either an increase in sialic acid content of membrane glycoproteins and/or a characteristic of MDAY-K2 cells.

Peterson et al. reported decreased amounts of gangliosides containing higher molecular weight oligosaccharide chains (i.e., gangliosides with more than three sugar residues) in Chinese hamster cells exhibiting resistance to DNR [28]. Our results do show that the major membrane-associated ganglioside produced by both the P388/S and P388/R cells is a ganglioside containing three sugar residues (GM_3). However, both cell lines also contain higher molecular weight gangliosides (apparently GM_2 and SPG). Thus, no differences in ganglioside composition were noted between the sensitive and resistant P388 cell lines studied.

Work by Karczmar and Tritton suggested that the presence of cardiolipin in cell membranes could alter membrane structure and result in increased binding of DOX [18]. Ramu et al. have also shown that P388 cells resistant to DOX contain significantly larger quantities of sphingomyelin and triglycerides than DOX-sensitive P388 cells [32]. This suggests that the loss of cadiolipin from the plasma membrane or increased sphingomyelin or triglyceride levels could result in resistance to DOX. In the present study, the phospholipid and triglycerides were not directly quantitated as described in the previous studies [18, 32]. However, comparison of phospholipid HPTLC patterns showed not distinct differences between the sensitive and resistant cell lines. Furthermore, the total membrane-bound phosphate did not significantly differ for the two cell lines, suggesting that DOX resitance in our P388 cells does not involve a major change in phospholipid composition.

Studies have suggested that the membrane fatty acid composition may be an important factor in the regulation of drug transport and in the sensitivity of cells to antineoplastic agents [3, 16]. Guffy et al. have also shown that the growth of DOX-sensitive L1210 cells in medium supplemented with docasahexaenoic acid (22:6 free fatty acid) resulted in increased sensitivity to DOX [16]. Increased polyunsaturation was also found within the phospholipid components of the more sensitive cells. Since these studies have linked the fatty acid composition of cell membranes with antineoplastic drug activity, we investigated whether changes in cellular fatty acid composition are associated with resistance to DOX in P388 cells. Our results showed no differences in fatty acid composition in any of the four lipid families analyzed from the P388/S and P388/R cells. However, further studies may be necessary to determine whether any changes in the fatty acid composition of individual lipid components occur in association with DOX resistance.

In summary, we have qualitatively and quantitatively characterized four of the major lipid families in P388/S and P388/R cells and have found no distinct differences between the two cell lines. Therefore, a change in total cellular lipid composition is probably not a major factor responsible for expression of DOX resistance in these cells. Whether localized membrane proteins or membrane lipids can effect cellular accumulation or anthracycline sensitivity has not been established. Since a variety of membrane changes have been associated with DOX- and DNR-resistant cells, it seems reasonable to conclude that resistance to these agents can be multifactorial. Although this study has eliminated a number of these possibilities for our P388 murine leukemia cells, further studies on the role of other membrane components, including the role of glycoproteins, in anthracycline resistance are warranted.

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