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DIFFERENCE IN PLASMA MEMBRANE STRUCTURE BETWEEN TWO SUBLINES OF EHRLICH-LETTRÈ ASCITES TUMOR CELLS

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The plasma membranes of the glycogen-free and the glycogen-containing subline of Ehrlich-Lettrè ascites cells were purified and compared with respect to their enzyme activity, chemical, lipid and protein composition, and membrane fluidity. Both membrane fractions differed in a number of parameters which are discussed as differences in the expression of malignant transformation of the two sublines. 1. The 5′-nucleotidase activity was 3–5-times higher and the sialic acid content 3-times lower in the glycogen-containing than in the glycogen-free subline. 2. Differences were also observed with respect to the phospholipid composition, that is in the relative proportions of mainly phosphatidylcholine, -inositol and -serine. 3. The fatty acid spectrum of the two sublines differed in the C-18 series and in the percentage of polyunsaturated acids, which was about 6% lower in the glycogen-containing line. 4. Measurements of fluorescence polarization (*P*) using 1,6-diphenyl-1,3,5-hextriene as probe generally gave higher *P* values, indicating a decreased membrane fluidity for the plasma membranes of the glycogen-containing subline both below and above the transition temperature at 33°C. 5. Polyacrylamide gel electrophoresis revealed different protein patterns mainly in the molecular weight range of around 90000 and in the range between 31000 and 14000.

Introduction

In a previous paper [1] we have reported on the isolation of two plasma membrane fractions of a glycogen-free cell line of Ehrlich-Lettrè ascites cells. In this article we present data on the composition of the light plasma membranes of the glycogen-free and a glycogen-containing subline [2]. These two cell lines have previously been shown to differ in (a) colchicine resistance [3], (b) lipid synthesis [4], (c) in cell surface properties such as sialic acid content and electrophoretic mobility, and (d) in respiration and anaerobic glycolysis [5]. These cell lines which in the meantime have been adapted to growth as suspension cultures in vitro have recently been shown to differ in the rate of tumor takes in the sense that the glycogen-free subline appears to be the most malignant variant [6]. Since cell surface properties and growth behavior, notably malignant growth, are undoubtedly interrelated, our aim was to isolate and characterize the plasma membranes of these two sublines in the hope that differences between them may give some indication as to the nature in which this interrelationship may be expressed. These investigations may then give some hint as to the direction in which future research should be guided to attack these questions more specifically. Preliminary results of the studies on comparing these two sublines were published some time ago [7].

Materials and Methods

Isolation of ascites cells. Male NMRI mice, about 5 weeks of age, were used in this study for the in vivo propagation of the glycogen-free and the glycogen-containing Ehrlich-Lettrè ascites tumor cells [8]. The cells were harvested 7 days after inoculation. They were centrifuged at 4000 rev./min ($1800 \times g$) for 10 min to remove the ascites fluid, and then washed once with Ringer buffer, pH 7.4.

Reagent grade substrates such as 5'-AMP, ATP, *p*-nitrophenyl phosphate, cytochrome *c*, glucose 6-phosphate, β-glycerophosphate and RNAase-free sucrose were used for the enzyme tests and were purchased from Serva, Heidelberg, F.R.G.

Cells were disrupted after swelling in 2 mM EDTA, pH 7.4, in hypotonic salt solution according to Mamaril et al. [9]. A nuclear-free $12000 \times g$ pellet was prepared from the cell homogenate by differential centrifugation which was then used as starting material for the purification of the plasma membranes by a combination of discontinuous and continuous sucrose-density gradient centrifugations. Details of this purification procedure including the enzymatic assays have been described elsewhere [1]. Protein was determined by the method of Lowry et al. [10], and P_i by the procedure of Eibl and Lands [11]. DNA and RNA were measured according to Schmidt and Thannhäuser [12] and sialic acid according to Warren [13] after dialysis of the membrane suspensions. The lipids were extracted using the procedure of Folch et al. [14], and the phospholipids quantified by phosphorus analysis after two-dimensional thin-layer chromatographic separation using silica gel G-covered plastic sheets from Merck, Darmstadt, F.R.G.

Gas liquid chromatography. The fatty acid composition of the plasma membrane lipid extracts was analyzed as their methyl esters by using a Becker gas chromatograph Model 419. A 1.80 m × 2 mm stainless steel column packed with 15% diethylene glycol succinate on gaschrom P (100–120 mesh) was used. The temperatures of oven and flame ionization detector were 200 and 250°C. Purified nitrogen was used as carrier gas. The percent distribution of the fatty acid methyl esters was obtained by computer-assisted (CDS-111 from Varian GmbH, Darmstadt, F.R.G.) evaluation of the peak areas. The methyl esters were prepared by interesterification of about 100 μg of the Folch lipid extract, dissolved in 1 ml dry 5% (w/v) HCl in methanol under nitrogen atmosphere in a sealed glass tube heated for 30 min in a heating block at 100°C. After evaporation of the reaction solvent the fatty acid esters were taken up in carbon disulfide and injected into the gas chromatograph. Cholesterol was quantified according to Haeffner and Hoffmann [15].

Fluorescence spectroscopy. Fluorescence polarization was measured at 90° relative to the exciting beam, using a Perkin-Elmer MPF 4 fluorescence spectrophotometer. Two commercial polarizers (Zeiss, Oberkochen, F.R.G.) were mounted at the excitation (360 nm) and emission (426 nm) sites of the cuvette. The samples were excited with vertically polarized light and the intensity of the polarized fluorescence emission was measured both at vertical and horizontal orientation of the emission polarizer. The instrument was tuned for optimal intensity of the fluorescence signals. The 1,6-diphenyl-1,3,5-hexatriene-labelled samples were exposed to the excitation light shortly before the measurement to eliminate the possibility of reversible bleaching of diphenylhexatriene [16]. The temperature was controlled with a Haake thermostatic bath and measured directly within the cuvette by means of a digital thermometer. Before each measurement, the membrane suspensions were mixed gently to ensure isotropic distribution. The temperature profiles were measured by scanning from the lowest temperature to the highest and backwards. The plasma membranes suspended in 0.25 M sucrose/2 mM EDTA/20 mM Tris-HCl, pH 7.4, were labelled for 1 h at 37°C with a diphenylhexatriene solution prepared in the same buffer which was practically void of any fluorescence. To correct for straylight of the membrane material which has been shown to be the major source of error [17,18], (7–13% in our experiments), we have also measured the fluorescence polarization of the unlabelled samples and subtracted these values from the labelled specimen. The calculation of the fluorescence polarization was made according to Shinitzky and Inbar [19].

Polyacrylamide gel electrophoresis. Electrophoretic analysis was performed in 10% gels at pH 8.8 and in the presence of 0.1% sodium dodecyl

sulfate (SDS) according to Laemmli [20]. Staining was carried out by using standard technique with Coomassie brilliant blue.

Results

Table I gives the specific activities of both the homogenate and the plasma membrane fractions of the glycogen-free and the glycogen-containing ascites cell line. By comparison of the activities in both preparations we can observe a similar degree of enrichment and thus purification of the two membrane fractions. The major difference between the plasma membrane-bound enzymes was found for the 5'-nucleotidase showing an about 3–5-times greater specific activity in the glycogen-containing cells. Further details of this difference and its possible implication with respect to tumor development are presently being investigated. In Table II data are shown on the chemical composition of the two plasma membranes indicating differences mainly in the sialic acid content. The glycogen-free subline revealed at 3-times greater concentration of sialic acid on its surface than the glycogen-containing cell type which is in accord with the observation on intact cells made by Bohn et al. [5]. The cholesterol content on a protein basis is almost identical with the data reported by Friedberg and Halpert [21], but the phospholipid content was found to be about 21% higher than reported by others [21,22].

The phospholipid composition of the two membrane fractions in comparison to the homogenate is shown in Table III. Sphingomyelin as a major plasma membrane component was in case of the glycogen-free line almost as high as has been found for rat liver plasma membranes [23]. Some differences were observed for the phosphatidylcholine and-inositol content. Whereas the former mainly serves structural functions the latter has been shown to undergo a high turnover rate suggesting that phosphatidylinositol plays an essential

TABLE II

CHEMICAL COMPOSITION OF THE PLASMA MEMBRANE FRACTIONS OF THE GLYCOGEN-FREE AND THE GLYCOGEN-CONTAINING LINE OF ASCITES CELLS

	Glycogen-free	Glycogen-containing
Membrane protein (mg/ml packed cells)	0.20 ± 0.14 [a] (3)	0.40 ± 0.22 (4)
Phospholipid/protein (in mg)	0.71 ± 0.08 (5)	0.74 ± 0.1 (3)
Cholesterol/phospholipid (molar)	0.41 ± 0.06 (7)	0.40 ± 0.07 (4)
Cholesterol/protein (mg/mg)	0.09 ± 0.03 (9)	0.10 ± 0.04 (4)
DNA/protein (μg/mg)	7.3 ± 3.0 (3)	10.9 ± 6.0 (3)
RNA/protein (μg/mg)	65.9 ± 7.3 (4)	61.1 ± 0.6 (3)
Sialic acid (nmol/mg protein)	16.65 ± 2.6 (4)	5.16 ± 1.9 (4)

[a] Values are expressed as means ± S.D. Number of experiments with duplicate analyses in parentheses.

TABLE I

SPECIFIC ACTIVITY OF MEMBRANE MARKER ENZYMES OF THE HOMOGENATE AND THE PLASMA MEMBRANE FRACTIONS OF THE GLYCOGEN-FREE AND GLYCOGEN-CONTAINING CELLS

Values are expressed as means ± S.D. Figures in parentheses refer to the number of experiments with duplicate analyses.

Enzymes	Glycogen-free		Glycogen-containing	
	Homogenate	Plasma membranes	Homogenate	Plasma membranes
5'-Nucleotidase	1.01 ± 0.22 [a] (5)	13.46 ± 2.23(6)	4.89 ± 1.22(6)	44.2 ± 10.3 (3)
(Na + K)-ATPase	12.60 ± 3.70 (9)	130.8 ± 11.1 (7)	27.46 ± 10.0 (5)	196.6 ± 40.1 (3)
Alkaline phosphatase	0.17 ± 0.04 (4)	1.20 ± 0.22(9)	0.20 ± 0.09(3)	1.35 ± 0.15(3)

[a] Activity expressed in nmol/min per mg protein.

TABLE III

PHOSPHOLIPID PERCENT COMPOSITION OF THE HOMOGENATE AND THE PLASMA MEMBRANE FRACTIONS OF THE GLYCOGEN-FREE AND THE GLYCOGEN-CONTAINING ASCITES CELLS

	Glycogen-free		Glycogen-containing	
	homogenate	plasma membranes	homogenate	plasma membranes
Sphingomyelin	15.6 ± 3.6 [a]	30.33 ± 3.52	11.34 ± 2.31	26.0 ± 3.31
Phosphatidylcholine	38.7 ± 3.9	27.20 ± 4.10	39.90 ± 3.41	33.3 ± 2.80
Phosphatidylethanolamine	30.4 ± 4.0	27.90 ± 1.80	33.70 ± 3.0	28.7 ± 2.80
Phosphatidylinositol	4.0 ± 2.3	9.04 ± 2.60	6.28 ± 2.0	6.4 ± 2.0
Phosphatidylserine	4.0 ± 1.7	4.65 ± 1.25	6.10 ± 3.0	2.2 ± 1.7
Cardiolipin	5.2 ± 2.2	0.66 ± 0.22	4.60 ± 0.92	1.4 ± 0.3

[a] Values are expressed as means ± S.D. of four experiments with duplicate analyses.

role in cell differentiation and transformation [24].

Table IV gives the total fatty acid composition of the plasma membranes of the two sublines. In addition to finding trace amounts of saturated odd chain acids with 15 and 17 C-atoms we found differences in the relative proportions of oleic and linoleic acid, and we obtained a greater percentage of polyunsaturated (> 1 double bonds) fatty acids for the glycogen-free ascites cells. The overall composition is similar to the results obtained by others [25] with the exception that we also determined saturated and unsaturated acids with 24 C-atoms. These acids are known to be esterified preferentially to sphingomyelin which is a major plasma membrane constituent [25,26].

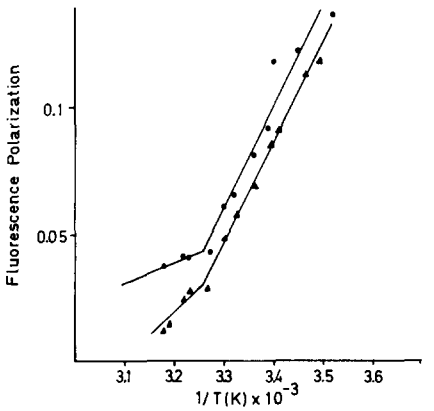
Since fatty acid unsaturation and membrane fluidity are interrelated we have measured the membrane fluidity of isolated plasma membranes of the two cell lines by fluorescence spectroscopy using 1,6 diphenyl-1,3,5-hexatriene as membrane probe. The results demonstrated in Fig. 1 generally show a lower fluorescence polarization for the glycogen-free subline which is to be expected from the greater percentage of polyunsaturated fatty acids compared to the glycogen-containing subline, and which becomes most pronounced above the transition temperature at 33°C.

The protein components of the two membrane fractions were resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate which is shown in Fig. 2. About 23 bands with aparent molecular weights ranging from 174 to approx. 14 K were detected. Major differences between the

TABLE IV

FATTY ACID COMPOSITION OF THE PLASMA MEMBRANE FRACTIONS OF GLYCOGEN-FREE AND GLYCOGEN-CONTAINING ASCITES CELLS

Fatty Acid	Glycogen-free	Glycogen-containing
12:0	0.16 ± 0.06	2.12 ± 0.10
14:0	0.77 ± 0.20	1.93 ± 0.08
15:0	0.11 ± 0.02	2.16 ± 0.85
16:0	12.20 ± 0.53	10.84 ± 0.13
16:1	2.52 ± 0.37	3.81 ± 0.74
17:0	1.18 ± 0.14	1.35 ± 0.12
18:0	19.10 ± 0.60	16.32 ± 0.14
18:1	19.26 ± 0.55	21.66 ± 0.43
18:2	16.86 ± 1.69	13.75 ± 0.49
18:3(14)	2.05 ± 0.38	2.57 ± 0.10
20:0 or 18:3(15)	0.45 ± 0.02	0.69 ± 0.07
20:1	1.37 ± 0.16	1.15 ± 0.09
20:2	1.71 ± 0.31	0.34 ± 0.05
20:3	1.95 ± 0.39	2.49 ± 0.05
20:4	3.72 ± 0.77	3.80 ± 0.11
20:5	0.48 ± 0.08	0.34 ± 0.03
22:0	1.49 ± 0.43	0.98 ± 0.07
22:2	—	0.09 ± 0
22:3	—	0.66 ± 0.01
22:4	0.87 ± 0.19	0.80 ± 0.20
22:5(19)	2.30 ± 0.63	0.95 ± 0.12
22:6	4.10 ± 0.69	2.50 ± 0.13
24:0	0.97 ± 0.06	0.31 ± 0.04
24:1	1.80 ± 0.53	0.53 ± 0.22
> 24	2.43 ± 0.59	3.87 ± 0.29
Unidentified	1.31 ± 0.47	5.06 ± 1.38
% Saturated	39.20 ± 0.66	38.54 ± 1.83
% polyenoic	33.53 ± 0.94	27.63 ± 1.29
C 18:1/C 20:4	5.18	5.70



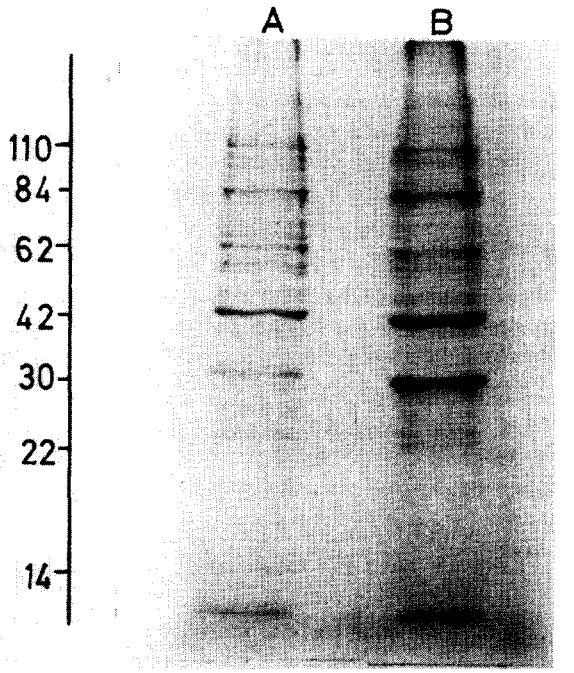


Fig. 1. Temperature dependence of fluorescence polarization. Plasma membranes of the glycogen-containing (●———●) and the glycogen-free cells (▲———▲). About 0.60 mg membrane protein/ml suspended in 0.25 M sucrose/2 mM EDTA/20 mM Tris-HCl, pH 7.4, was labelled with a 7.5 μM 1,6-diphenyl-1,3,5-hexatriene solution prepared in the same buffer. The values are corrected for scattered light imposed by the unlabelled membrane vesicles.

Fig. 2. Polyacrylamide gel electrophoresis of the plasma membranes of the glycogen-storing (A) and the glycogen-free (B) ascites cell subline. Between 100 and 115 μg protein of each membrane preparation were placed on the gel and separated and stained according to standard procedures. The numbers represent the apparent molecular weights ($\times 10^{-3}$).

two sublines were observed around 90 K and in the range between 40 and 14 K molecular weight. The glycogen-free line constains six proteins with apparent weights of 90, 86, 80, 40, 28.5 and 22 K, respectively, which are lacking in the glycogen-containing line, which itself has three proteins banding at positions corresponding to 95, 24.5 and 14 K molecular weight. In addition, one of the heavily stained bands at the postion 42 K was found to be present in minor amounts in the glycogen-containing subline.

Discussion

As has already been stated in the introduction, both ascites cell sublines show different properties related to malignancy and invasive growth behaviour [6]. Recent investigations on the karyotype further revealed that the glycogen-storing subline contains a unique marker chromosome, mar AB, and a reduction in the stemline number from 46 to 41 [27] indicating that both sublines are genetically different species. With respect to differences in malignancy the increased 5′-nucleotidase activity of the glycogen-storing cells seems of interest, since this enzyme may be involved in the regulation of malignant transformation [28], although its significance is far from being resolved. In a separate study we are presently investigating the difference in the membrane-bound 5′-nucleotidase between the two cell types in greater detail in the hope of shedding some further light on the relationship between this enzyme activity and tumor growth (unpublished data).

Early studies on the function of sialic acid as the major carrier of the cell surface charge have

been correlated, in one way or another, with malignant transformation [29,30]. In more recent investigations (for review see Hakomori [31] and Yamakawa and Nakai [32]) it has been found that alterations in glycolipid content may not be correlated in a simple way with the degree of tumorigenicity and malignancy. A higher sialic acid concentration after neuraminidase treatment of intact cells has been obtained for the glycogen-free subline [5] which agrees with our data. Keenan and Morré [33] have shown that both sialic acid and 5′-nucleotidase were increased in mammary carcinomas which is different from our results. Except for the amount of sialic acid the chemical composition of the two membrane fractions is very similar not only with respect to the membrane constituents but also in relation to the impurities.

The differences in phospholipid composition between the two sublines may be of importance concerning the concentration of phosphatidylinositol. This component has been shown to exhibit a high turnover rate in ascites tumor cells [24], and it has also been associated with the growth state and transformation of the cells [34]. Although the cholesterol content of the isolated membranes and of the intact cell [2] is about identical, its synthesis is more than twice as high in the glycogen-free than in the glycogen-containing subline [2]. The importance of this observation with respect to tumor growth remains to be elucidated.

Evidence exists that the fatty acid patterns found in tumors are strongly dependent on the host which has been observed for normal liver and hepatomas [35]. It has further been demonstrated that the fatty acid composition can be altered markedly in vivo by changing the type of fat fed either to normal [36] or to tumor-bearing animals [25,37,38]. However, it has also been observed that the regulation of the de novo fatty acid biosynthesis is defective in minimum deviation hepatomas [39]. This finding leads us to consider that in our ascites cell sublines the regulatory mechanism may be altered since both cell types are being cultivated in the same host. Analysis of the fatty acid composition of the individual phospholipids should give further insight into this problem. The differences in membrane fluidity between the two sublines could possibly cause a change of agglutinability and cell movement through an alteration in microtubular and microfilament activity, both of which are anchored within the plasma membrane [40]. Similar membrane fluidity differences between normal and malignant lympoid cells of mouse have recently been obtained by Koizumi et al. [41].

Although of no major concern in this context we have analyzed the protein patterns of the plasma membranes of both cell types. In accord with other more recent investigations on the plasma membrane protein composition of e.g., thymocytes [42] and Ehrlich ascites tumor cells [43] we have found the most heavily-stained bands in the approximate molecular weight range between 105 and 28.5 K. These data may give some basis for further studies in this direction.

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