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Alterations in biosynthesis and homeostatis of cholesterol and in lipoprotein patterns in mice bearing a transplanted lymphoid tumor

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The membrane fluidity of murine lymphoid GRSL tumor cells has been shown to depend on their site of growth in the host. Tumor cells located in ascites, in contrast to those in the enlarged spleen, show a very high plasma membrane fluidity, mainly due to a decreased level of cellular (membrane) cholesterol. Yet, the rate of cholesterol biosynthesis in the tumor cells as estimated by the activity of HMG-CoA reductase and the incorporation of [^{14}C]acetate into cholesterol was extremely high when compared to various lymphoid cells in normal control mice. Also the rate of biosynthesis and the cholesterol content in liver and spleen of tumor-bearing mice were substantially higher than in the organs of control mice. Blood plasma cholesterol, however, was decreased in tumor-bearing mice as a result of altered lipoprotein patterns. Outgrowth of the tumor was accompanied by a strong reduction in plasma high-density lipoproteins. Low-density lipoproteins became transiently increased, but eventually all lipoproteins, and consequently the plasma cholesterol content decreased to very low levels, especially so in the ascites plasma. The low transfer of [^{14}C]cholesteryl ester-labeled lipoproteins between blood and ascites plasma after either intravenous or intraperitoneal injection suggested a hampered flow between the two compartments. Also apparent differences in cholesteryl ester fatty acid composition between lipoproteins of the blood and ascites plasma indicated the lack of a rapid equilibration between the two compartments. The results suggest that the limited availability of lipoproteins as an additional source of cholesterol to the rapidly growing ascites cells promotes their high membrane fluidity.

Introduction

The lipid fluidity of cell membranes is an important parameter which may determine membrane-associated enzymatic activities [1–3], carrier-mediated transport activities [4,5] and the expression of cell surface antigens and receptors [6–9]. In tumor cells, membrane fluidity may also determine the sensitivity of the cells to immune attack [10–13], and to chemotherapeutic drugs [14–16].

Previously [17–19] we have demonstrated that tumor cells of a transplanted murine leukemia (GRSL) show an abnormal plasma membrane fluidity, depending on their anatomic site of growth: GRSL cells growing in the ascites fluid show an extremely high membrane fluidity, while those in the enlarged spleen exhibit a somewhat lower membrane fluidity than do normal lymphoid cells.

Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; DPH, 1,6-diphenyl-1,3,5-hexatriene; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

It is important to get more insight into the mechanisms which regulate membrane fluidity in vivo. Then, one might possibly be able to modulate a suboptimal degree of fluidity, e.g. by dietary means. In previous work GRSL ascites cells have been shown to shed significant amounts of rigid (cholesterol-rich) vesicles in their bathing fluid, which may fluidize their surface membrane [17,18,21,22]. In the present paper we have studied three major physiological factors which also may determine cell membrane fluidity in GRSL leukemia: First, the increased rate of cholesterol biosynthesis in the lymphoid tumor cells and in the liver of the tumor bearers. Second, the significant alterations in the amount of cholesterol and in the lipoprotein content and distribution in blood and ascites plasma during tumor growth. Third, the availability of these plasma lipoproteins to the various subsets of tumor cells. The results described below may help to understand the differences in membrane fluidity observed among distinct subpopulations of GRSL tumor cells growing at different sites in the mouse.

Materials and Methods

Chemicals

The following radiolabeled compounds were obtained from New England Nuclear: DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl-coenzyme A (50 mCi/mmol), DL-[5-^{3}H]mevalonic acid (dibenzyl ethylene diamine salt; 5.2 Ci/mmol), [2-^{14}C]acetic acid (sodium salt; 50 mCi/mmol), and [4-^{14}C]cholesterol (60 mCi/mmol). Unlabeled HMG-CoA, glucose 6-phosphate (disodium salt), NADP, glucose-6-phosphate dehydrogenase, dithiothreitol and soybean trypsin inhibitor (Type I-S) were obtained from Sigma. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, U.K.)

Buffered solutions

KES buffer: 10 mM potassium phosphate (pH 6.8)/2 mM EDTA/250 mM sucrose. DENP medium: 10 mM dithiothreitol/10 mM EDTA/100 mM NaCl/50 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (pH 6.5). Medium A: 0.04 M NaCl/0.1 M KCl/5 mM $MgSO_4$/0.02 M Tris-HCl (pH 7.6). EBSS medium: Earle's balanced salt solution, without Ca^{2+}, Mg^{2+}, obtained from Flow Laboratories. Hanks' solution, obtained from Oxoid (London, U.K.). PBS: 0.02 M phosphate-buffered saline (0.85% NaCl) (pH 7.3).

Animals and cells

GR mice of 8–12 weeks old, and weighing 20–24 g were used throughout the experiments. GRSL 18 leukemia cells, originating from a spontaneous thymus-derived lymphoid leukemia in the GR mouse strain, were maintained by weekly intraperitoneal transplantation of $1 \cdot 10^7$ ascites tumor cells (transplant generations 80–110). The tumor grew out in ascites (up to $(2–5) \cdot 10^8$ tumor cells) and infiltrated the lymphoid organs. The spleen, for instance, became thereby 3–4-times enlarged, most of the lymphoid cells being tumor cells [19]. Under these conditions the mean survival time of the mice was 8 days and all animals died within 9 days. The mice were given ad libitum access to a standard pelleted diet (Hope Farms) and water, but were fasted overnight before they were killed for the experiments, routinely 7 days (unless otherwise stated) after inoculation of the GRSL tumor cells.

Blood was collected in heparinized tubes and processed as described before [20]. The ascites fluid varying from 0.5 to 0.7 ml, was collected by eluting the peritoneal cavity with 2–3 ml EBSS medium. Ascites GRSL cells and extracellular membrane vesicles [21,22] were separated by centrifugation at $250 \times g$ for 10 min and at $105\,000 \times g$ for 60 min, respectively. The latter supernatant contained the diluted ascites plasma. Haematocrit levels of samples of the peripheral blood and the ascites fluid were determined in special haematocrit tubes. They amounted to $45 \pm 1\%$ for normal blood, $18 \pm 2\%$ for leukemic blood, and $33 \pm 6\%$ for leukemic ascites fluid (means of four animals). No erythrocytes were present in the ascites fluid.

Splenic lymphoid cells were separated from erythrocytes by centrifugation (20 min, $400 \times g$ at room temperature) of single-cell suspensions in Hanks' solution over a $17\frac{1}{2}\%$ metrizamide solution. Thymocytes were prepared as single-cell suspensions from normal thymuses from 3–5-week-old GR mice [22].

Preparation of subcellular fractions

The various types of lymphoid cells were disrupted by pumping single-cell suspensions in Hanks' solution at 0°–4°C through an air-driven cell disruptor (Stansted Fluid Power Limited, Stansted, Essex, U.K.; model AO 612, disrupting valve 516), using an air pressure of 45 lb/inch^2 [19]. Optimal cell disruption occurred at cell densities of $5 \cdot 10^7$ cells/ml for GRSL ascites cells, $1 \cdot 10^8$ cells/ml for normal or leukemic splenocytes, and $2 \cdot 10^8$ cells/ml for normal thymocytes [19]. The plasma membranes were purified from $(1.95–945) \cdot 10^4$ g · min pellets of the cell homogenates, utilizing a discontinuous sucrose gradient, in Medium A, as described in detail before [21]. The purity of the plasma membrane preparations was ascertained routinely by electron microscopy and by marker assays, as described previously [21].

For the comparative HMG-CoA reductase assays, animals were killed at a fixed time, 6.00 h a.m., in view of the diurnal variation in the activity of this enzyme. The lymphoid cells were disrupted as described above, using KES buffer. The livers were excised, rinsed in KES buffer and minced with scissors. Then, a 10% cell homogenate was made in a Potter-Elvehjem homogeniser by three stokes at moderate speed. Crude microsomal pellets of all cell homogenates were prepared by differential centrifugation between $12\,000 \times g$ for 15 min and $105\,000 \times g$ for 90 min. Pellets of the latter centrifugation were suspended at a concentration of 5 mg protein/ml in DENP medium. The suspended microsomes were quick-frozen and stored overnight at −70°C, prior to use.

Assay of HMG-CoA reductase

The activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase was basically determined according to Philipp and Shapiro [23]. Microsomes at a concentration of 2.4 mg protein per ml DENP were preincubated for 10 min at 37°C. To 50 µl of this suspension was added 25 µl cofactors mix (5.2 µmol glucose 6-phosphate, 530 nmol NADP and 0.5 µmol EDTA) and 5 µl (1 unit) glucose-6-phosphate dehydrogenase. After another 5 min of preincubation 20 µl [^{14}C]HMG-CoA (50 nmol; 60 000 dpm) was added. The reaction was terminated after 30 min incubation at 37°C by adding 30 µl 12 M HCl. [^{3}H]Mevalonic acid lactone (35 000 dpm) was used as an internal standard. The samples were shaken vigorously and incubated for 30 min at 37°C to allow complete lactonization of the mevalonic acid and then were deproteinized by centrifugation. The reaction product was separated by thin-layer chromatography using acetone/toluene (1 : 1, v/v) in the presence of anhydrous Na_2SO_4, and counted in a liquid scintillation counter. Preliminary studies showed that excess of substrate was present and that the assays were linear with respect to the quantity of microsomal protein added and to time.

Incorporation of [^{14}C]acetate

[^{14}C]Acetate incorporation into sterols and fatty acids was determined in vitro in liver, spleen, thymus and GRSL ascites cells. Livers were cut into thin slices (maximally 1 mm). Spleen and thymuses were minced with scissors. Ascites cells were washed free from their bathing plasma and surrounding membrane vesicles [22]. Per assay 30–50 mg wet tissue or cells $((2.5–4) \cdot 10^7$ GRSL ascites cells) was used, to which 300 µl [^{14}C]acetate (1.5 mM; 5 µCi) in EBSS was added. This concentration of acetate gave rise to an optimum rate of incorporation into cholesterol for at least 4 h. The reaction was allowed to proceed for 2 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2, and was stopped by addition of cold methanol, immediately followed by extraction of the lipids in chloroform/methanol (2 : 1, v/v). After Folch's partition [24], the organic phase was evaporated and lipids were transesterified in 0.2 M NaOH/methanol for 1 h at 50°C. Thin-layer chromatography was performed on Merck silica-plates using *n*-hexane/diethyl ether/acetic acid (85 : 15 : 2, v/v), the spots of cholesterol and fatty acyl methyl esters were extracted and their radioactivity was counted.

In vivo incorporation of acetate into cholesterol and fatty acyl moieties was estimated by injection of 25 µCi [^{14}C]acetate in 300 µl EBSS in the tail vein. 2 h later the mice were bled and their organs were excised. Lipids were extracted, processed and counted for radioactivity as described above.

Estimation of membrane lipid fluidity

The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for

measuring the degree of lipid fluidity in the various plasma membrane preparations by steady-state fluorescence polarization (P_{DPH}). The measurements were performed at 25°C with an Elscint apparatus, model MV-1A (Elscint Ltd., Haifa, Israël) as described previously [21].

P_{DPH} values mainly reflect the orientational constraint of the motions of the probe, and these values, or rather the r_s values (steady-state fluorescence anisotropy) can be quantitatively converted into lipid order parameters, S_{DPH}, using a semi-empirical relationship [25]. Membrane lipid fluidity may be defined as the reciprocal of the lipid order parameter [25]. In the present paper we used for calculation of S_{DPH} the equations: $r_\infty = (4\ r_s/3) - 0.10$ (valid for the region $0.13 < r_s < 0.28$ or $0.18 < P_{DPH} < 0.37$) and $(S_{DPH})^2 = r_\infty/r_o$, in which r_∞ represents the limiting hindered fluorescence anisotropy, and $r_o = 0.4$ is the theoretically maximal fluorescence anisotropy [25].

Plasma lipoproteins

Lipoproteins from the peripheral blood and the ascites plasma were fractionated by density gradients of NaBr/NaCl/EDTA solutions, essentially according to Redgrave et al. [26], with some modifications [20].

Lipoproteins from both normal and tumor-bearing mice (4 days after transplantation) were radiolabeled in vivo in their cholesteryl ester moieties by intravenous (i.v.) injection of [^{14}C]cholesterol (50 μCi) complexed to bovine serum albumin [27], and isolation of the blood plasma 6 h later. During that period the plasma enzyme lecithin : cholesterol acyltransferase esterified part of this cholesterol. Remaining unesterified [^{14}C]cholesterol in the plasma was reduced by exchange with the cholesterol of unlabeled erythrocytes. 1.4 ml plasma was three times incubated with 1.0 ml suspensions of 50–70% erythrocytes from normal and leukemic mice, respectively, in phosphate-buffered saline, for 1 h at 37°C. This resulted in a decrease of the unesterified [^{14}C]cholesterol in both plasma samples from 40% to 10% of the total labeled cholesterol. After density gradient centrifugation [^{14}C]cholesteryl ester-labeled HDL from normal mice was recovered from fractions 7 and 8, [^{14}C]cholesteryl ester-labeled LDL from tumor-bearing mice from fractions 4–6 [20]. Labeled HDL and LDL ($(5–10) \cdot 10^4$ dpm) in 0.25 ml saline were injected i.v. or i.p. in normal mice and tumour bearers to study their distribution in the various organs and body fluids. (Samples of) organs, blood plasma, etc. were weighed, solubilized in Soluene-350 (Packard Instrument Co.), decolourized in isopropanol with H_2O_2, and the radioactivity was counted in Dimilume-30 (Packard). For calculation of the ^{14}C recovery, the total blood plasma volume was assumed to be 5 ml per 100 g body weight [28].

Chemical analysis

Protein was determined according to Lowry et al. [29]. Membrane and lipoprotein lipids were analyzed as described in detail before [20,22]. Briefly, lipids were extracted with chloroform/methanol (2 : 1, v/v), followed by partition according to Folch et al. [24]. The phospholipids were quantitated by phosphate analysis [30]. Free cholesterol and cholesteryl esters were determined enzymatically, using the Merckotest cholesterol kit (Merck A.G., Darmstadt, F.R.G.). In plasma samples and lipoprotein fractions this was done directly, i.e. without lipid extraction. In membrane preparations, a sample of the lipid extract, after Folch's partition, was dried and taken up in 100 μl ethanol, followed by addition of 2 ml of the enzyme reagent. Cholesteryl esters of the lipoproteins were isolated by thin-layer chromatography of the lipid extracts, according to Kunz [31], and transesterified with boron trifluoride/methanol [32]. The fatty acid profiles were determined by gas-liquid chromatography on a wall-coated-open-tubular glass scolumn, 25 m × 0.21 mm, coated with Silar 5CP, using a temperature program [22].

Results

Plasma membranes were isolated from splenic and ascitic GRSL cells and analyzed in comparison with those from normal splenocytes and thymocytes. Table I shows the fluorescence polarization data (P_{DPH}) and the structural order parameters (S_{DPH}) of these membranes measured at 25°C. The values of the plasma membranes isolated from the leukemic spleen cells are higher, while those of the ascites cell membranes are lower than those of the plasma membranes isolated from

TABLE I

LIPID STRUCTURAL ORDER AND CHOLESTEROL CONTENT IN ISOLATED PLASMA MEMBRANES FROM NORMAL AND LEUKEMIC (GRSL) LYMPHOID CELLS AT DIFFERENT LOCATIONS

Mean data are presented ± S.D. (number of experiments in parentheses), partly derived from Van Blitterswijk et al. [19]. n.d., not determined.

Plasma membranes isolated from	P_{DPH}	S_{DPH}	Cholesterol/protein (nmol/mg)	Cholesterol/phospholipid (molar ratio)
Normal cells				
Thymocytes	0.303 ± 0.005 (11)	0.71	780 ± 95 (3)	0.74 ± 0.05 (3)
Spleen cells	0.302 ± 0.005 (3)	0.70	440 ± 43 (3)	0.68 ± 0.07 (3)
Leukemic cells				
Spleen cells, day 7	0.316 ± 0.008 (3)	0.73	534 ± 22 (3)	0.70 ± 0.02 (3)
Ascites cells, day 4	0.278 ± 0.007 (4)	0.66	n.d.	0.39 ± 0.02 (2)
Ascites cells, day 7	0.261 ± 0.014 (13)	0.63	336 ± 54 (9)	0.32 ± 0.08 (7)

the normal cells. The P_{DPH} values of ascites cell plasma membranes isolated on day 4 after transplantation of the tumor were slightly higher than of those on day 7 (significance: $p < 0.04$, according to Student's t-test). Table I furthermore shows that the cholesterol content of the splenic GRSL tumor cell membranes is in the range of the cholesterol content of the normal cell membranes, whereas that of the GRSL ascites cell membranes is much lower.

Cholesterol biosynthesis was measured in the various lymphoid cells and in the livers of tumor-bearers and normal control mice. This was done by determination of the activity of HMG-CoA reductase, i.e., the rate-determining enzyme of the cellular cholesterol biosynthesis [33,34], and by measuring the rate of [^{14}C]acetate incorporation into cholesterol. The results of both assays (Table II) indicate a very high cholesterol biosynthesis in the GRSL ascites cells. The specific activity of HMG-CoA reductase in microsomes of GRSL ascites cells is 12-fold and 29-fold higher than that in the microsomes of normal thymocytes and spleen cells, respectively. In GRSL ascites tumor cells, the incorporation of [^{14}C]acetate into cholesterol is 4- and 8-times higher than in normal thymocytes and spleen cells, respectively, on a cell weight basis. Both assays indicate that the cholesterol biosynthesis in the GRSL tumor cells located in the spleen is 2- to 3-fold enhanced, as compared to normal spleen cells. Table II also shows that the HMG-CoA reductase activity in leukemic liver microsomes is about 3-fold higher than in the normal liver microsomes. However, the [^{14}C]acetate incorporation assay (Table II) suggests much larger (13-fold) difference.

[^{14}C]Acetate incorporation into cholesterol was also measured in vivo, 2 h after i.v. injection (Table III). Incorporation per gram tissue in the leukemic spleen is two times higher than in the normal spleen. Incorporation in the liver of leukemic animals is about three times higher than in that of control animals. The apparent difference in the cholesterol biosynthesis in these organs be-

TABLE II

RELATIVE RATES OF CHOLESTEROL BIOSYNTHESIS AS DETERMINED IN VITRO BY TWO ASSAYS IN CELLS/TISSUES OF LEUKEMIC (GRSL-BEARING) MICE AND OF NORMAL CONTROL MICE

Figures presented are mean values ± S.D. of three experiments.

Cells or tissue	Leukemic or normal	HMG-CoA reductase activity [a]	[^{14}C]Acetate incorporation in cholesterol [b]
Liver	leukemic	30.7 ± 5.3	460 ± 45
	normal	11.7 ± 2.4	35 ± 8
Spleen	leukemic	43 ± 15	243 ± 60
	normal	12 ± 2	127 ± 21
Thymus	normal	28 ± 4	217 ± 15
Ascites	leukemic	350 ± 16	950 ± 65

[a] Expressed in pmol mevalonic acid/mg microsomal protein/min.

[b] Incorporation in dpm/mg (wet weight) organ or cells per 2 h in liver slices, minced spleens, minced thymuses or ascites cells.

TABLE III

IN VIVO CONVERSION OF [^{14}C]ACETATE TO STEROLS AND FATTY ACYL MOIETIES IN LEUKEMIC MICE AND NORMAL CONTROL MICE, AS RECOVERED FROM LIVER, SPLEEN AND BLOOD PLASMA[a]

	Condition of mice	Liver (dpm/g tissue)		Spleen (dpm/g tissue)		Blood plasma (dpm/ml)	
		expt. 1	expt. 2	expt. 1	expt. 2	expt. 1	expt. 2
Cholesterol	Normal	1653	2036	1180	1540	400	330
	Leukemic[b]	4807	5930	2300	3220	760	1160
Fatty acyls	Normal	3842	3770	2010	1850	600	600
	Leukemic	8891	11941	990	1260	610	690

[a] 2 h after i.v. injection of 25 μCi [^{14}C]acetate.
[b] 7 days after transplantation of $1 \cdot 10^7$ GRSL tumor cells.

tween leukemic and normal mice is also reflected in the radiolabeled cholesterol recovered in the blood plasma. Table III furthermore shows that the in vivo incorporation of [^{14}C]acetate into fatty acyl residues recovered in the liver, spleen and blood plasma of tumor bearers, were increased (2–3-fold), decreased (about half) and similar, respectively, when compared with normal mice. The ratio leukemic/normal of ^{14}C incorporation into fatty acyl groups of liver and spleen in vivo is the same as that measured in vitro (data not shown).

Since the cholesterol biosynthesis in the liver and the spleen of the tumor bearers was significantly increased (Table II) we have also determined the absolute cholesterol contents in these organs (Table IV). These were 1.6-fold and 2.2-fold increased, respectively, relative to normal liver and spleen, per weight unit. In contrast, the cholesterol content in the blood plasma, 7 days after inoculation of the tumor, was dramatically decreased (by a factor 0.37). At that time the cholesterol concentration in the ascites plasma was even lower, i.e. half of that in the blood plasma (Table IV).

Fig. 1 shows the lipoprotein profiles in blood and ascites plasma of leukemic mice, 4 and 7 days after transplantation of the tumor, and in normal control blood plasma, as characterized by the concentrations of free and esterified cholesterol in density gradient fractions. In control mice the major proportion of the plasma cholesterol was contained in the HDL (fraction 7–9). Only very small amounts were found in the VLDL (fraction 1) and LDL (fractions 3–6). 4 days after tumor transplantation the major lipoprotein peak in the blood plasma had shifted into the LDL region, as demonstrated before [20,35]. On day 7, all blood plasma lipoproteins, except VLDL, had reached extremely low levels (see also Table IV). The lipoprotein profiles of the ascites plasma showed a similar trend during outgrowth of the tumor (Fig. 1), although the shift of the lipoproteins from the HDL to the LDL region did not occur simultaneously with the shift in the blood plasma [20]. It was observed that the ratios free/esterified cholesterol in blood and ascites plasma had changed from 0.45 (control) to 1.0–1.2 in tumor bearers (day 7).

To find out whether lipoproteins are a supplementary source of cholesterol for the ascites tumor, lipoproteins of normal and tumor-bearing mice were biosynthetically labeled with [^{14}C]cholesteryl

TABLE IV

CHOLESTEROL CONTENT OF SPLEEN, LIVER, BLOOD PLASMA AND ASCITES PLASMA IN LEUKEMIC MICE AND NORMAL CONTROL MICE

Figures presented are mean values ± S.D. (number of experiments in parentheses).

	Total cholesterol			
	mg/g organ		mg/dl	
	Spleen	Liver	Blood plasma	Ascites plasma
Normal	2.6 ± 0.6 (5)	3.1 ± 0.3 (5)	154 ± 17 (45)	–
Leukemic[a]	5.8 ± 0.9 (5)	4.9 ± 0.5 (5)	57 ± 17 (20)	28 ± 7 (9)

[a] Leukemic mice, 7 days after transplantation of $1 \cdot 10^7$ GRSL tumor cells.

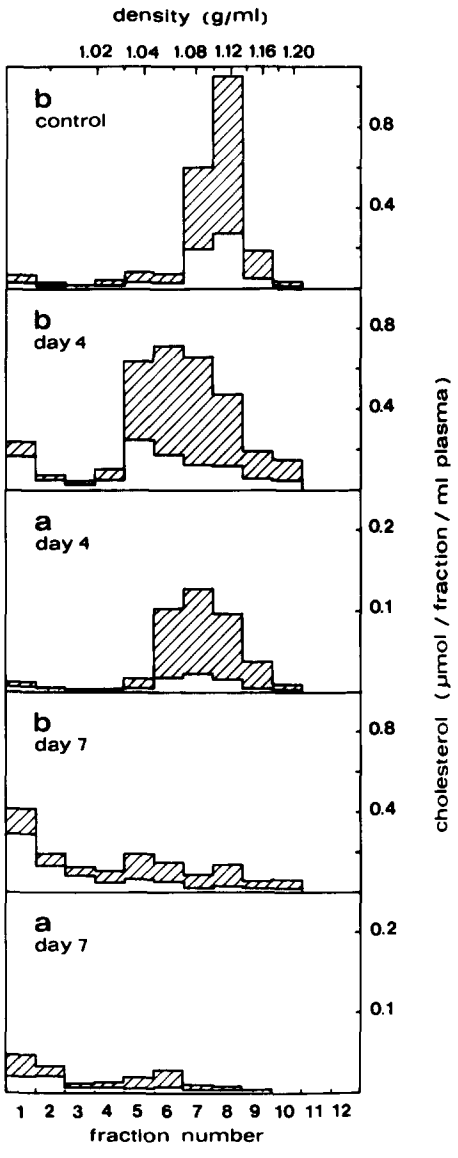

Fig. 1. Lipoproteins of blood (b) and ascites plasma (a) of control and tumor-bearing mice at 4 or 7 days after transplantation, as characterized by the distribution of free and esterified cholesterol in density gradients. Ascites plasma was collected from each mouse in 1 ml PBS. Hatched and blank areas represent esterified and free cholesterol, respectively.

ester in vivo (see Methods), isolated and used for tracing experiments. As reasoned previously [35], radiolabeled cholesteryl esters are a suitable marker of lipoproteins for metabolic studies, since plasma cholesteryl ester transfer activity in the mouse may be low or absent. Results are summarized in Table V. [^{14}C]HDL injected i.v. into normal control mice steadily declines in the blood plasma, down to 71% in 4 h, and to 7.5% in 23 h. When injected i.v. into tumor bearers (day 4), [^{14}C]HDL could be recovered from the peritoneal cavity (ascites fluid) in only very small amounts (1.8% after 23 h). This is also the case for i.v. injected [^{14}C]LDL (1% after 4 h). In normal control mice i.p. injected [^{14}C]HDL rapidly appears in the blood plasma with a maximum (45% of the injected dose) 4 h after injection. However, in tumor-bearing mice i.p. injected [^{14}C]HDL or [^{14}C]LDL are much longer arrested in the ascites (45% and 33%, respectively, after 4 h) and radioactivity only slowly appears in the blood plasma (12% and 7%, respectively, after 4 h). It can be concluded that in leukemic mice the flux of lipoproteins from the peripheral blood into the ascites compartment and vice versa is limited.

The above conclusion is supported by the finding that the composition of fatty acyl residues of the lipoproteins in the blood and ascites compartments is very different, most clearly so in the cholesteryl esters (Table VI), but also in the phospholipids (data not shown). Table VI shows that the cholesteryl esters of HDL and LDL in the ascites plasma contain more saturated (16 : 0, 18 : 0) and monounsaturated (mainly 18 : 1) fatty acids, and less polyunsaturated (18 : 2, 20 : 4, 22 : 6) fatty acids than those in the blood plasma. Ascites plasma VLDL is characterized by an increased 16 : 0, a decreased 18 : 1, but virtually a similar percentage of polyunsaturated fatty acids, compared with the blood plasma VLDL.

Discussion

The present paper is an extension of a previous study on various subsets of lymphoid GRSL tumor cells, which exhibit different membrane lipid fluidities, depending on their site of growth in the mouse [19]. For instance, GRSL ascites cells show a high membrane fluidity, whereas splenic GRSL cells show a low membrane fluidity, mainly due to a low and a high cholesterol/phospholipid molar ratio, respectively, in these membranes (Table I). The cholesterol requirement of a cell for building its membranes is fulfilled by a dual supply: (1) by the cell's own biosynthesis and (2) by the extracel-

TABLE V

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF [^{14}C]CHOLESTERYL ESTER-LABELED LIPOPROTEINS

	Mode of injection	Condition of mice [a]	Circulation time (h)	Recovery of radiolabel (%)			
				Blood plasma	Liver	Spleen	Total ascites
[^{14}C]HDL	i.v.	control	4	71	21	0.7	–
			23	7.5	12	n.d. [b]	–
		day 4	23	2.9	26	n.d.	1.8
	i.p.	control	4	45	n.d.	n.d.	–
		day 4	4	12	13	1.0	45 [c]
[^{14}C]LDL	i.v.	control	4	27	22	1.0	–
		day 4	4	53	24	1.2	1.0
	i.p.	day 4	4	7	4	0.3	33 [d]

[a] Condition of mice: Normal controls or 4 days after transplantation of $1 \cdot 10^7$ GRSL tumor cells. Data are means of two mice.
[b] n.d., not determined.
[c] 38% of radioactivity in ascites plasma, 7% associated with ascites tumor cells.
[d] 30% of radioactivity in ascites plasma, 3% associated with ascites tumor cells.

lular plasma lipoproteins, which are presumably taken up largely by receptor-mediated endocytosis [36,37]. The present paper elaborates some aspects of these two routes, to understand better how membrane fluidity of tumor cells is determined in vivo.

A measure for the relative amount of cellular cholesterol biosynthesis was obtained by two in vitro assays: (1) the activity of the rate-limiting enzyme HMG-CoA reductase, measured in microsomes, and (2) the incorporation of [^{14}C]acetate into cholesterol, measured in whole cells or tissue slices (liver). Although the latter method may possibly underestimate sterol biosynthesis [38], it may be a useful complementary method in comparative studies. The results of both assays have indicated that cholesterol biosynthesis in the GRSL ascites cells is extremely high, 4–8 times that in the splenic GRSL cells, which in turn show a 2–3 times higher rate of biosynthesis than the spleen cells of normal control animals. An increased cholesterol biosynthesis in animal and human leukemic cells has more generally been found (reviewed in Refs. 17, 18), human chronic lymphocytic (B-cell) leukemia being an exception [36,39].

The cholesterol biosynthesis in the livers of the leukemic mice is clearly higher than normal, which is in agreement with literature data [33]. However, the [^{14}C]acetate incorporation in cholesterol meaured in liver slices indicates a much larger difference between normal and leukemic mice than the HMG-CoA reductase acitvity in the microsomes. No clear explanation can be offered for this apparent discrepancy. It could be possible that microsomes as assay system, rather than tissue slices, show a different sensitivity to feed-back regulatory products, including those of the alternate pathways of mevalonate metabolism [40]. Further, it cannot be excluded that the differences are partly due to differences in acetyl-CoA pool-

TABLE VI

FATTY ACID COMPOSITION (WEIGHT %) OF THE CHOLESTERYL ESTERS IN THE LIPOPROTEINS OF BLOOD PLASMA (B) AND ASCITES PLASMA (A) OF MICE BEARING GRSL LEUKEMIA

Values are the means of two consistent experiments. Only major components are listed.

Fatty acid	HDL		LDL		VLDL	
	B	A	B	A	B	A
16:0	6.1	17.9	9.8	17.8	19.9	26.9
16:1	0.9	3.1	1.4	1.4	5.2	5.2
18:0	1.6	4.5	3.3	5.9	5.1	6.1
18:1	8.2	16.9	13.8	22.4	39.6	30.0
18:2	45.0	31.9	39.2	31.1	23.6	22.6
20:4	28.6	11.7	23.6	9.4	3.8	2.1
22:6	8.2	4.0	7.5	3.7	1.9	1.8

size and/or that the acetyl-CoA pool in the livers of tumor-bearing mice supplies the varous metabolic pathways in different proportions.

Enhanced incorporation of [^{14}C]acetate into cholesterol in leukemic mice was also found in vivo (Table III). Of course, in this case we are dealing with a continuous transfer of synthesized cholesterol between the organs and the plasma lipoproteins, and vice versa [41]. Therefore, it is conceivable that the enhanced uptake of the radiolabel in cholesterol molecules in the leukemic mice showed up to a similar extent (2–3-fold) in liver, spleen and blood plasma (Table III).

The absolute cholesterol contents per organ weight unit in the livers and the spleens of leukemic animals were also enhanced, 1.6- and 2.2-fold, respectively (Table IV). In the spleen, this enhancement factor can only to a small extent be accounted for by the lymphoid tumor cells, of which the free cholesterol/phospholipid ratio is not significantly different from normal, whereas their cholesteryl ester content has been found to be only 6% enhanced [19]. Rather, this enhancement must be due to a larger proportion of erythrocytes, as we have actually encountered in the enlarged spleen, and which in addition show some 25% increased membrane cholesterol/phospholipid molar ratio [19].

In contrast to the organs (liver and spleen), the cholesterol content in body fluids (blood and ascites plasma) of tumor-bearing mice decreased to very low levels (Table IV, Fig. 1). It was shown that outgrowth of the GRSL tumor was accompanied by dramatic alterations in the plasma lipoproteins profiles (Fig. 1). HDL, the major lipoprotein in mice, became strongly reduced, while LDL and VLDL were several-fold increased. Finally, on the seventh day after tumor transplantation all lipoproteins except VLDL were decreased to very low levels, even more extremely so in the ascites plasma. As reported previously, the altered lipoprotein density profiles are probably a direct consequence of decreased lipoprotein lipase and hepatic lipase activities [20,35]. Also in mice bearing the Ehrlich ascites tumor a decrased HDL and a (much) increased VLDL level have been found in the blood plasma [42]. However, total blood plasma cholesterol levels in this model were higher than normal [42,43], while the cholesterol levels in the ascites plasma stayed rather constant during outgrowth of the tumor [42].

Despite the very high endogenous cholesterol biosynthesis in the GRSL ascites cells, the cholesterol content in these cells and their plasma membranes (Table I) is very low, as compared with normal lymphoid cells and GRSL tumor cells isolated from the spleen [19]. The direct availability of blood plasma lipoprotein cholesterol to the spleen cells may allow then to maintain a 'normal' cholesterol/phospholipid ratio in their plasma membranes. In contrast, lipoprotein cholesterol may be withheld from the ascites cells as a result of a hampered flow through the peritoneal cavity. This supposition was verified by the following experimental evidence. Firstly, influx of lipoproteins into the ascites compartment appeared to be limited, as demonstrated by the fate of intravenously injected [^{14}C]cholesteryl ester-labeled lipoproteins (Table V). Secondly, due to the absence of plasma cholesteryl ester transfer activity [35], the cholesteryl esters in blood and ascites plasma showed a characteristic distribution of their fatty acids among the lipoprotein subclasses (Table VI), as has also been found in Ehrlich ascites plasma [44,45]. However, the fatty acid composition of the cholesteryl esters in the various lipoprotein subclasses in the ascites plasma was strikingly different from that in the blood plasma (Table VI), indicating that equilibration of lipoproteins of the ascites plasma with those of the blood plasma is strongly hindered. This is furthermore corroborated by the finding that the total lipoprotein and cholesterol concentrations in the ascites plasma were roughly half of those in the blood plasma (Table IV, Fig. 1), more or less similar as in the Ehrlich ascites tumor model [42,45].

We have got preliminary results (unpublished) showing that the cholesterol content of GRSL ascites cell membranes and ascites plasma can be substantially elevated (but not to a 'normal' level) by feeding the mice a cholesterol-rich diet This also supports the proposition that the availability of lipoprotein-cholesterol to the ascites cells is a limiting factor for them to obtain a higher membrane cholesterol/phospholipid molar ratio.

Apart from the high proliferation of the GRSL ascites cells (outgrowth up to 50-times the injected amount in 7 days) and the restrained supply of

lipoprotein in the ascites fluid, there is a third factor which may contribute to the low cholesterol content of the ascites cells and their plasma membranes. That is the shedding of special cell surface domains, in the form of cholesterol-enriched membrane vesicles in vivo. These rigid vesicles, about which we have published extensively before [17,18,21,22], could be isolated from the extracellular ascites fluid in yields as high as half of the yield of plasma membranes isolated from the ascites cells. Their cholesterol/phospholipid molar ratio amounted to 1.10, i.e. 3-times higher than of the purified plasma membranes. The mechanism of this shedding of vesicles is still unknown, and has been the subject of some speculations elsewhere [17,18,21,22]. It may be possible that there is some inherent change in the plasma membrane structure of GRSL ascites cells that prevents it from holding a normal amount of cholesterol, and that the shedding of rigid vesicles is a direct consequence of this change.

In conclusion, we have demonstrated that outgrowth of the transplanted GRSL tumor leads to alterations in the cholesterol levels in organs, cells and body fluids, as well as to increased biosynthesis of cholesterol in the liver and in the lymphoid tumor cells. Differences in these respects were found in tumor cells located in ascites as compared with those in the spleen. The very high lipid fluidity in the GRSL ascites cell membranes may be caused or promoted by the limited availability of plasma lipoproteins to these cells and by the shedding of rigid membrane vesicles from their surface. Studies on this altered cholesterol homeostasis in tumor-bearing mice are currently being pursued further, as to see whether various dietary lipids may affect these cholesterol levels and cell membrane fluidities.

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