Lipid Metabolism of Ehrlich Ascites Tumor Cells Grown in Chemically Defined Albumin Medium

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Total lipids of Ehrlich ascites tumor cells grown in nutrient medium supplemented with 1% bovine serum albumin instead of 15% native horse serum and rate of incorporation of [^{3}H]acetate into total lipids are reduced, while the cholesterol content of the cells is higher than of controls grown in normal medium. The lower lipid content of cells cultured in albumin supplemented medium is explained by a lack of exogenous lipids, which are normally taken up from the serum containing nutrient medium. A several fold increase of incorporation of labelled choline and ethanolamine into the phospholipids of the cells grown in the modified medium is observed. We suggest, that the observed stimulation of the incorporation of phospholipid precursors reflects changes in the dynamic state of the membranes produced by serum depletion.

In a previous paper [1] we have presented the results of studies on the incorporation of labelled acetate into the lipids of Ehrlich ascites tumor cells grown in modified medium. These experiments have shown that the metabolism of [^{14}C]pyruvate and [^{14}C]mevalonate are identical in all media tested, while the incorporation of isotopic acetate is higher in medium with dialyzed serum than in medium with delipidized serum. Incorporation of [^{14}C]acetate into the cells is suppressed in the absence of all lipids in the culture medium. Cellular integrity was not impaired in the modified media. These results indicated that the acetyl-CoA-syntethase of Ehrlich ascites tumor cells is not regulated by exogenous lipids as is known to be the case in nonmalignant cells. Meantime we have extended these investigations on the lipid metabolism of these cells grown in chemically defined albumin medium and in the present communication we report the results of these studies.

Materials and Methods

All chemicals used were of analytical grade or for biochemical purposes from Merck, Darmstadt; Serva Heidelberg, Biochemicals were from Boehringer, Mannheim and Sigma, München. Radiochemicals were obtained from Amersham, Braunschweig or Schwarz/Mann, Heidelberg.

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Cell cultures in vivo and in vitro

Hyperdiploid EAT cells grown in the intraperitoneal cavity of female NMRI mice were used for all experiments after a first shortened *in vitro* passage of 14 h in modified Eagle medium supplemented with 15% native horse serum (Behringwerke AG, Marburg) according to Karzel and Schmid [2]. This type of ascites tumor cells is made to grow in suspension culture by using glass vessels which are not coated with silicone. 575 mg/l microcillin Bayer and 30 mg/l streptomycin were present in all cultures. Albumin containing medium was prepared by dissolving 1 g bovine serum albumin (Cohnfraction V, free fatty acids 3–5 µg/g, Serva, Heidelberg) in 100 ml serum free medium. Cell number was determined by turbidity measurements at 578 nm against a blank with the appropriate culture medium [3].

Gravimetric determination of total lipids of cells

Cells were separated from the culture medium by centrifugation, washed twice with Hank's balanced salt solution and precipitated with 0.6 N perchloric acid. The acid insoluble fraction was extracted with chloroform/methanol = 1:1 at 70 °C for 3 min according to ref. [4].

Lipid synthesis

The synthesis of the lipids was determined by incubation of 25 ml cell suspension ($5-7 \times 10^5$ cells/ml) for 20 min at 37 °C with 100 µCi [^{3}H]acetate (spec. act. 0.75 Ci/mmol). Phospholipid synthesis

was measured by incubation with 12 μCi [methyl-^{3}H]choline (spec. act. 10 Ci/mmol), 2 μCi [methyl-^{14}C]choline (spec. act. 52 mCi/mmol) or 2 μCi [2-^{14}C]ethanolamine (spec. act. 50 mCi/mmol). The total concentration of acetate was 200 μM and of choline and ethanolamine 100 μM. The incubation was stopped by chilling the samples in an ice bath. 1 ml of the cell suspension was used for the determination of the radioactivity in the acid insoluble fraction as described in ref. [5]. Cells from 24 ml of the suspension were collected by centrifugation, washed twice with Hank's balanced salt solution, and precipitated with perchloric acid. The radioactivity of the acid soluble fraction was determined by neutralization of 1 ml of the perchloric acid supernatant with potassium phosphate and centrifugation. 0.5 ml of the supernatant were counted in 10 ml Rotiszint 22 in a Packard Tricarb scintillation spectrometer. The pellet was extracted as described before [4]. Lipid extracts were evaporated to dryness and counted in Rotiszint 11 (Roth, Karlsruhe) in a Packard Tricarb scintillation spectrometer.

Uptake of [^{3}H] choline

The uptake of [^{3}H]choline was measured by pulse labelling experiments. 1 ml of the cell culture was shaked with 1 μCi [^{3}H]choline (0.1 mmol [^{3}H]choline, 1:10 diluted with cold choline-chloride) for 10 min at 37 °C; the total concentration of choline was 100 μM. Cells were collected by filtration and washed twice with 10 ml Hank's balanced salt solution containing 100 μM choline chloride. Filters were dried for one hour at 50 °C and radioactivity was measured in Rotiszint in a Tricarb scintillation spectrometer.

Separation of the total lipids by thin layer chromatography

Lipids were separated by thin layer chromatography using 20 × 20 cm silica gel plates without fluorescence indicator from Merck, Darmstadt and Riedel de Haen, Hannover, prewashed with chloroform/benzene = 3:2 and activated at 120 °C for one hour. 1.5 ml of the lipid extract was evaporated in a stream of nitrogen to 0.2 – 0.3 ml and applied to the plates. The plates were developed in chloroform/methanol/H_2O = 75:25:4 (4 cm), chloroform (10 cm) and *n*-hexane (15 cm). The chromatograms were dried and the components were made visible under UV-light after spraying with 0.1% 2.7-dichlorofluoresceine. Thereafter the plates were sprayed with phosphomolybdate and heated for 2 min at 120 °C for additional identification.

Separation of the phospholipids

To separate the phospholipids from nonpolar lipids, the thin layer chromatograms were developed in acetone/petroleum ether = 1:3 (15 cm). Resolution of the phospholipids was accomplished in a second step by running the chromatograms in chloroform/methanol/ammonia = 70:30:5 (10 cm). The main phospholipids were identified by comparison with the following reference substances (purchased from Sigma, Munich) L-phosphatidyl choline, L-phosphatidyl choline dimyristine, phosphatidyl serine, L-phosphatidyl ethanolamine dipalmitine, L-phosphatidyl ethanolamine, sphingomyelin, lysophosphatidyl choline. The exact localization of the labelled compounds was done by autoradiography as described by Randerath [6]. For the quantitative determination of radioactivity the spots were counted in a liquid scintillation spectrometer after scrapping off the labelled areas.

Determination of cholesterol

Cholesterol was determined by gas-liquid chromatography. The sample to be analyzed was mixed with a known quantity of 5-α-cholestan and treated with N-methyl-N-trimethylsilyl-trifluoracetamide. The samples were analyzed in a Varian aerograph Series 1800 gas chromatograph with a glas column of 4% SE 52 on Chromosorb (80 – 100 mesh) at 260 °C with a known quantity of cholesterol as standard.

Results

Total lipids of EAT cells grown in albumin media

In normal medium the total lipids of the cells rose within 24 h from the beginning of the second passage *in vitro* for about 55%, while in serum free medium supplemented with 1% bovine serum albumin only a very small increase was observed. At the end of the fourth *in vitro* passage the total lipids of the cells grown in normal medium were about 80% higher than at the beginning of the second passage; in albumin medium an increase of 35% was found. During the same time in normal medium the

Table I. Total lipids and cholesterol content in $\mu g/10^6$ cells grown in normal and albumin medium.

Medium	At the beginning of the second passage *in vitro*		At the end of the second passage *in vitro*		At the end of the fourth passage *in vitro*	
	Total lipids	Cholesterol	Total lipids	Cholesterol	Total lipids	Cholesterol
normal medium	30.3 ± 2.5 $n = 12$	1.82 *	47.1 ± 15.3 $n = 8$	1.9 *	54.0 ± 14.5 $n = 4$	1.98
albumin medium	32.2 ± 3.5 $n = 12$	1.84 *	33.2 ± 6.9 $n = 8$	2.0	43.5 ± 9.3 $n = 4$	2.46

* Mean values from two different experiments.

cholesterol content was found to be 9%, in albumin medium 34% higher than at the beginning of the second passage (see Table I).

The relative incorporation of [^{3}H]acetate into total lipids and the acid insoluble precipitate and the uptake into the soluble fraction of the cells are summarized in Table II. A reduction of incorporation of label into the total lipids and the perchloric acid soluble fraction of about 45 – 70% of the controls was observed with cells grown in albumin medium.

A comparison of the absolute incorporation rates of radioactivity from [^{3}H]acetate 12 and 24 h after starting the *in vitro* cultures revealed a decrease of about 30 – 40% for the cells in normal as well as albumin medium (details not described).

Synthesis of phospholipids

The uptake of [^{3}H]choline into the cells measured by pulse labelling in the second to fourth passage *in vitro* in normal and albumin supplemented medium is shown in Fig. 1. While the uptake of choline by the cells grown in normal medium is constant, in albumin containing medium a permanent increase of choline incorporation in the course of the experiment is observed. This increased label of the cells

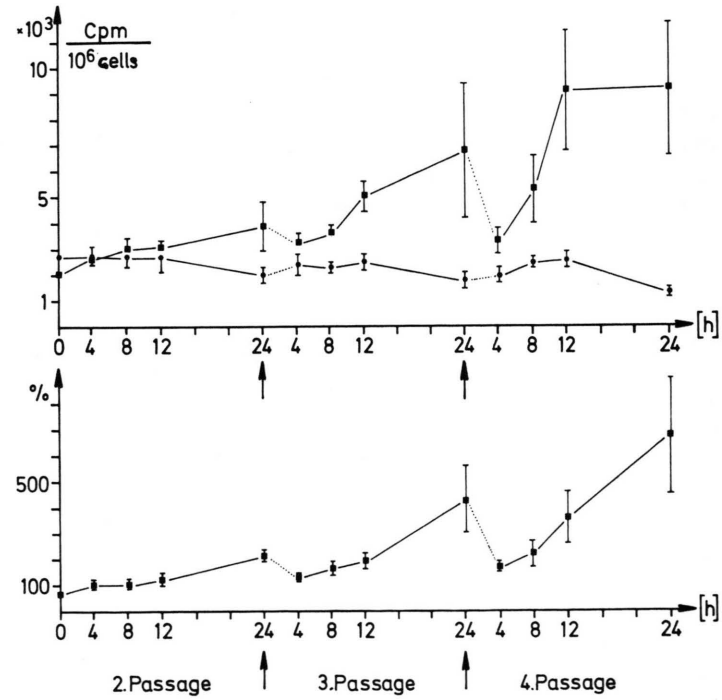

Fig. 1. Uptake of [methyl-^{3}H]choline in Ehrlich ascites tumor cells grown in normal medium — ∗ — and in albumin medium — ■ —. $n = 4$; for further details see text.

Table II. Relative uptake and incorporation of [^{3}H]acetate into EAT-cells in percent of controls. Values for controls in normal medium are normalized to 100%.

Total lipids		Acid insoluble fraction		Acid soluble fraction	
12 h	24 h	12 h	24 h	12 h	24 h
46 ± 3 * $n=2$	52 ± 9 $n=6$	43 ± 4 $n=2$	70 ± 14 $n=6$	60 ± 1 $n=2$	59 ± 21 $n=6$

* Mean ± SE.

grown in albumin medium reflects an enhanced rate of uptake and incorporation into the acid insoluble fraction.

The distribution of the total radioactivity between the acid soluble and insoluble cell fractions at the end of the second and fourth passage *in vitro* is demonstrated in Table III. At the end of the second passage (first passage in albumin medium) the radioactivity in the lipid fraction of the cells was 11 (from [^{3}H]choline) to 13-fold (from [^{14}C]choline) higher than in the cells grown in normal medium; after further 48 h a 23 fold increase of label from [^{3}H]choline was found in the total lipids of cells cultured in albumin medium.

Separation of the lipid extracts by thin layer chromatography revealed three active bands which were made visible by autoradiography. By far the most radioactivity was found in phosphatidyl choline, weak activity in lysolecithine and scarcely detectable activity in a not identified band, which was not observed in every case. These results demonstrate a considerable stimulation of the phospholipid synthesis in cells cultured in medium supplemented with albumin. These observations were confirmed by experiments with [^{14}C]ethanolamine: At the end of the second passage *in vitro*, the relative labelling of the cells grown in albumin medium was 170 ± 12% in the lipids, 173 ± 15% in the acid insoluble fraction and 150 ± 25% in the acid soluble supernatant ($n = 4$; controls = 100%). Thin layer chromatography of the total lipids and analysis of the radioactivity in the phospholipid area showed that 80% of the label was associated with phosphatidyl ethanolamine, further 20% with an unidentified slower moving spot.

Discussion

It is generally known that lipid metabolism of normal and malignant cells grown *in vitro* is decisively influenced by lipids present in the nutrient medium. Since supplementation of culture media with albumin instead of serum makes available only a few fatty acids, considerable alterations in lipogenesis and lipolysis of EAT cells are expected on transfer from normal culture medium to albumin containing medium.

The results of our investigations on total lipid content in cells grown under these culture conditions demonstrate a reduced lipid content and reduced incorporation rates of [^{3}H]acetate into total lipids.

As we have described in an earlier publication, the poor incorporation rates of acetate into lipids of EAT cells cultivated in lipid depleted media are not an expression of a diminished lipogenesis but are brought about by a low level and lack of control of acetyl-CoA synthetase activity in tumor cells as compared to normal cells [1]. The lower lipid content of cells grown in albumin supplemented medium may thus be explained by a simple lack of exogenous lipids which normally are taken up, metabolized and accumulated by cells cultured for several *in vitro* passages [7] in serum containing medium.

Though proliferation of EAT cells in serum free medium is reduced [5], under the same culture conditions, the cholesterol content calculated on a per

Passage	Medium		Total lipids	TCA insoluble fraction	TCA soluble fraction
second passage	normal medium	$n=4$	280 ± 135	435 ± 140	20 100 ± 9 200
	albumin medium	$n=8$	3 050 ± 1 460	3 938 ± 1 960	35 175 ± 11 457
fourth passage	normal medium	$n=4$	278 ± 30	439 ± 53	17 085 ± 1 195
	albumin medium	$n=2$	6 533 ± 3 950	6 230 ± 4 260	26 820 ± 1 370

Table III. Uptake and incorporation of [^{3}H]choline at the end of the second and fourth passage of cells grown in albumin medium (cpm/10^7 cells).

cell basis is higher than in control cells. It seems reasonable that in EAT cells normally meeting most of their demand by uptake of exogenous cholesterol from the medium [8, 9] cholesterol synthesis is stimulated, but the present result is difficult to explain. Although anomalies in the control of cholesterol biosynthesis currently discussed for tumor cells [10, 11] could explain an exceeding synthesis under modified culture conditions, it seems more probable that the ratio of phospholipids to cholesterol has to be corrected due to an enhanced synthesis of phospholipids as revealed by the incorporation rates of isotopic choline and ethanolamine. Changes in the physico-chemical state of cell membranes seem to be possible as a response to the transfer of cells into a culture medium containing albumin instead of the complex serum. Since it is known that phospholipid degradation occurs at a similar rate in growing and non-growing cells [12], the observed several fold stimulation of incorporation of phospholipid precursors could reflect in addition changes in the dynamic state of the membrane (degradation, synthesis, exchange and repair mechanisms) being necessary after serum depletion.

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