

Hepatic Lipids in Tumor-bearing (Glioma) Mice¹

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SUMMARY

Tumor-bearing (glioma) mice have hepatomegaly. Although the neoplasm remains localized, it influences hepatic triglyceride metabolism. In glioma-bearing mice, there is a quantitative decrease in hepatic triglycerides and a decrease in the rate of incorporation of the non-esterified fatty acids into triglycerides. The mechanism of tumor influence on host lipid metabolism is discussed.

Recently, we have described the lipid patterns in experimentally transmissible ependymal glial tumors in mice (8). During this study, hepatomegaly in tumor-bearing animals was observed. Morphologically, the liver was not grossly abnormal, and microscopically there was no cytologic abnormality. Recently, Wilkins and Ketcham (9) have shown that in similar tumor-bearing mice, the number of mitoses of hepatic cells and rate of regeneration following partial hepatectomy were not related to the glioma growth. In an effort to determine the nature of the hepatomegaly in tumor-bearing mice, total lipid analyses were performed on the livers of tumor-bearing and control mice.

MATERIAL AND METHODS

Two transmissible glial ependymomas were obtained for establishment of our colony. Both tumors had been originally induced by intracerebral methylcholanthrene. One, the Perese tumor, was originated in C3H mice at Roswell Park Memorial Institute, Buffalo, New York. The other, the Zimmerman tumor, was originated in C57 black mice at Montefiore Hospital, New York, New York. The colony of tumors was maintained by appropriate s.c. transplantation. Viable portions of tumor were selected for homogenization with sterile isotonic saline in a tubular homogenizer with a glass pestle. The homogenate was prepared on the basis of 1.0 ml saline/mg tumor (wet weight).

In s.c. transplants, the Perese tumor grew to an average of approximately 2 cm in diameter within 2 weeks while the Zimmerman glioma No. 26 grew to an average of approximately 1.5 cm in diameter in 2 weeks.

When the tumors were large, the animals were sacrificed by decapitation and bled. The livers were immediately removed for total lipid analyses. Livers from corresponding control stock animals were similarly obtained.

A. HEPATIC LIPID ANALYSES

Separation of neutral and phospholipids.—The tissue was homogenized in 20 volumes of chloroform-methanol (2:1,

volume for volume) for 3 min in a micro Waring Blender, filtered through Whatman No. 3 paper under slight vacuum, and washed with 50 ml of the above solvent. To the filtrate was added 0.2 volume of 0.73% NaCl; the mixture was stirred, the aqueous layer was aspirated, and 4 successive washes with chloroform-methanol-0.73% NaCl (3:48:47) by volume were performed. Then the total lipid extract was concentrated to dryness and taken up in light petroleum ether. Insoluble material was removed and the extract was concentrated to a small volume (10–15 ml).

Separation into neutral and phospholipids was made on a 10 gm silicic acid column plus 5 gm of Hyflo Super-Cel. The total lipid extract was placed on this column and eluted first with 200 ml of chloroform and then with 200 ml of absolute methanol. Aliquots of the chloroform and methanol fractions were taken to determine the amount of neutral lipids and phospholipids (1), respectively.

Thin layer chromatography of neutral lipids.—Thin layer plates were prepared by mixing a slurry of Silica Gel G and water 1:2 and spreading an even layer 0.25 mm thick on 20- × 20-cm glass plates using a mechanical spreader. The plates were dried at 100°C for 1 hr before use.

Standard solutions of approximately 0.5 mg/ml of cholesterol, cholesterol stearate, triolein, dipalmitin, and palmitic acid in chloroform were prepared. Various amounts of these standards (from 0.2 to 10 µg) were spotted on silicic acid plates and developed in an ascending manner using *n*-hexane, diethyl ether, glacial acetic acid, 80/20/3, v/v/v for 40 min.

Standard cholesterol esters, triglycerides, free fatty acids, and cholesterol for thin layer chromatography were obtained from Applied Science Laboratories, State College, Pennsylvania. Silicic acid and organic chemicals of analytical reagent grade were received from Mallinckrodt Chemical Works. Silica Gel G for thin layer chromatography was obtained from Research Specialties Company.

The plates were sprayed with chromic-sulfuric acid (a saturated solution of K₂Cr₂O₇ in 87% H₂SO₄) and heated at 180°C for 35 min. After cooling, the charred spots were measured on a Photovolt densitometer. As reported by Mangold (3) and Privett *et al.* (5), the areas under the

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densitometer curves are directly proportional to the amount of sample. Privett and Blank (4) recommend the use of chromic-sulfuric acid instead of 50% sulfuric acid as the charring agent. They found that nono-, di-, and triglycerides gave spots of equal intensities after correction for differences in their carbon densities and that unsaturation *per se* had no effect on the yield of carbon. However, a standard curve for the major components was obtained for each plate.

Thin layer chromatography of phospholipids.—The phospholipids were separated on thin layer by the method of Skipski *et al.* (6) with a developing solvent of chloroform-methanol-glacial acetic acid-water, 5:25:7:3 (volume for volume). Sphingomyelin, lecithin, phosphatidyl inositol, phosphatidyl ethanolamine, and polyglycerophosphatides were identified and compared to phospholipids from rat liver.

Fatty acid analyses of individual lipids.—After spraying with 2,7-dichlorofluorescein and localization of spots under ultra-violet light, individual neutral and phospholipids were carefully scraped off the silicee acid plates. Fatty acid esterification was performed by refluxing with 2% H_2SO_4 in methanol for 1 hr, followed by extraction with *n*-hexane (7).

The methyl esters were analyzed on a Barber-Colman model No. 10 gas chromatograph. Separations were carried out by the use of diethylene glycol succinate polyester, 15.4% by weight, on 80-100 mesh, acid-washed, Chromosorb W, supplied by Applied Science Laboratories. The column was maintained at 170°C and the rate of flow of argon was 2.5 ml/sec. The total running time for each prepared sample was 120 min.

The machine was standardized daily with NIH methyl ester standard. Reproducible results within 1.5% of actual composition were obtained. Each fatty acid peak was identified by comparison of retention times with methyl ester standards of myristic, palmitic, stearic, palmitoleic, oleic, linoleic, arachidonic, and docosahexenoic fatty acids which were supplied by Applied Science Laboratories.

The peaks of each chromatograph were designated as follows: C14 (myristic acid), C16 (palmitic), C16:1 (palmitoleic), C18 (stearic), C18:1 (oleic), C18:2 (linoleic), C18:3 (linolenic), C20:4 (arachidonic), C22:6 (docosahexenoic).

The percentile composition of fatty acids was calculated

by measuring the area under each peak by triangulation and equating the sum of the areas to 100%.

B. TURNOVER RATES OF LINOLEATE- ^{14}C IN HOMOGENATES OF LIVER OF TUMOR- AND NON-TUMOR-BEARING ANIMALS

Preparation of linoleate- ^{14}C .—The linoleic acid was dissolved in ethanol, neutralized in NaOH and then taken to dryness. The sodium linoleate was dissolved in 10% human serum albumin so that the final solution contained 21 μ c/ml. The solution was stored in the frozen state.

In vitro incorporation of linoleate- ^{14}C into tissue homogenates.—Individual samples of liver from 3 non-tumor-bearing and 3 tumor-bearing mice were washed in saline and gently blotted dry. They were then placed in a glass tube homogenizer with a motor-driven Teflon pestle. The tissues were homogenized in 15 ml of 0.25 M sucrose solution in the cold. The homogenate was kept in an ice bath for a few minutes until the incubation program could be initiated.

A series of chemicals were added to the homogenate as indicated below. The incubation was allowed (4) to occur for 1 hr at 37°C.

INCUBATION MIXTURE AND ORDER OF ADDITIONS

	Volume (ml)
Tissue homogenate in 0.25 M sucrose	15.0
0.15 M MgCl ₂	0.5
0.5 M Tris ^a	0.5
0.2 M Glycerol	0.5
0.1 M PO ₄ buffer	0.5
1.0 M Sodium succinate	0.5
Linoleic acid- ^{14}C (0.1 ml = 2.1 μ c)	0.2
0.3 M Adenosine monophosphate	0.5

^a Tris buffer from Abbott Laboratories, Chicago, Illinois.

TABLE 1
COMPARATIVE TISSUE WEIGHTS IN TUMOR-BEARING MICE

No. of animals	Type	Mice (gm)	Liver (gm)	Tumor (gm)
6	Control	16.0 ± 1.1 ^a	0.80 ± 0.11	—
6	Subcutaneous ependymoma-bearing	21.9 ± 1.3	0.97 ± 0.04	6.8 ± 1.9

^a Mean ± S.D.

TABLE 2
1-HOUR INCORPORATION OF LINOLEIC ACID- ^{14}C IN HEPATIC LIPIDS OF MICE WITH SUBCUTANEOUS TRANSMISSIBLE GLIOMA

LIPIDS	mg/gm LIVER		LINOLEATE INCORPORATION IN VIVO cpm/gm LIVER	
	3 Control mice	3 Tumor-bearing mice	3 Control mice	3 Tumor-bearing mice
Cholesterol esters	1.50 ± 0.45 ^a	0.93 ± 0.28	6,000 ± 4,000	4,200 ± 2,700
Triglyceride	21.4 ± 5.9	3.5 ± 1.6	158,000 ± 22,000	87,000 ± 34,000
Free fatty acids	0.26 ± 0.20	1.28 ± 0.31	5,000 ± 2,500	5,500 ± 4,600
Diglyceride	0.086 ± 0.081	0.44 ± 0.26	6,300 ± 3,000	4,300 ± 1,900
Cholesterol	1.50 ± 0.3	2.0 ± 0.3	3,300 ± 3,000	400 ± 400
Phospholipid	34.2 ± 6.0	31.2 ± 7.0	149,000 ± 41,000	119,000 ± 50,000

^a Mean ± S.D.

TABLE 3
MOLE PERCENT DISTRIBUTION OF FATTY ACIDS OF HEPATIC NEUTRAL LIPIDS IN TUMOR BEARING MICE

Neutral lipids	No. of animals and type	C14 ^a	C16	C16:1	C18	C18:1	C18:2	C18:3	C20:4	C22:6
Triglyceride	5 Control	0.9 ± 0.4 ^b	23.1 ± 1.4	5.4 ± 0.6	2.1 ± 0.1	34.3 ± 1.3	25.8 ± 1.3	1.7 ± 0.3	1.2 ± 0.3	5.1 ± 1.0
	6 Tumor	0.9 ± 0.02	22.0 ± 1.3	2.3 ± 0.9	3.6 ± 0.9	31.2 ± 1.7	20.5 ± 2.4	1.5 ± 0.1	3.0 ± 1.0	6.0 ± 0.5
Diglyceride	5 Control	1.3 ± 0.5	42.4 ± 0.2	4.1 ± 0.2	7.6 ± 2.2	20.3 ± 1.7	13.3 ± 3.4	Tr	6.9 ± 1.3	6.4 ± 2.0
	5 Tumor	1.5 ± 0.4	35.1 ± 2.7	3.8 ± 0.9	7.3 ± 1.7	25.1 ± 2.4	17.6 ± 1.1	Tr	6.4 ± 2.1	5.0 ± 2.0
Free fatty acids	6 Control	3.0 ± 0.8	69.1 ± 4.1	4.9 ± 0.5	6.5 ± 0.8	10.4 ± 1.7	4.5 ± 1.4	Tr	Tr	Tr
	6 Tumor	3.4 ± 1.3	64.8 ± 7.0	5.1 ± 1.4	8.5 ± 1.9	13.0 ± 3.2	4.7 ± 1.1	Tr	Tr	Tr

^a C14, myristic; C16, palmitic; C16:1, palmitoleic; C18, stearic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic; C20:4, arachidonic; C22:6, decosahexenoic fatty acids; Tr, trace.
^b Mean ± S.D.

TABLE 4
MOLE PERCENT DISTRIBUTION OF FATTY ACIDS OF HEPATIC PHOSPHOLIPIDS IN TUMOR BEARING MICE

Phospholipids	No. of animals and type	C14 ^a	C16	C16:1	C18	C18:1	C18:2	C18:3	C20:4	C22:6
Cardiolipin	6 Control	0.9 ± 0.4 ^b	11.8 ± 3.2	3.4 ± 0.9	5.4 ± 2.6	13.7 ± 1.0	60.6 ± 7.5	Tr	3.8 ± 1.3	Tr
	4 Tumor	Tr	7.4 ± 0.6	2.5 ± 0.1	2.1 ± 0.6	10.3 ± 1.0	75.4 ± 1.0	Tr	1.9 ± 0.2	—
Phosphatidyl ethanolamine	6 Control	31.0 ± 1.3	1.1 ± 0.8	19.3 ± 2.0	12.7 ± 2.5	4.8 ± 1.3	Tr	16.0 ± 1.8	13.4 ± 4.3	Tr
	5 Tumor	Tr	28.4 ± 1.4	Tr	21.1 ± 3.4	7.9 ± 1.5	4.8 ± 1.4	Tr	16.2 ± 3.7	19.6 ± 4.7
Phosphatidyl inositol	5 Control	0.4	9.9 ± 5.0	0.4	31.4 ± 2.9	4.2 ± 2.1	1.3 ± 1.6	Tr	20.0 ± 4.6	Tr
	5 Tumor	Tr	8.4 ± 1.9	Tr	30.1 ± 5.5	2.7 ± 0.5	1.6 ± 0.6	Tr	37.6 ± 6.6	Tr
Phosphatidyl choline	5 Control	Tr	39.7 ± 2.3	1.0 ± 0.1	16.7 ± 2.6	10.6 ± 1.3	11.2 ± 0.8	Tr	13.1 ± 2.3	7.2 ± 2.3
	5 Tumor	Tr	42.9 ± 3.1	0.8 ± 0.1	16.6 ± 2.0	9.6 ± 1.0	13.1 ± 2.3	Tr	12.4 ± 2.3	8.6 ± 3.5

^a C14, myristic; C16, palmitic; C16:1, palmitoleic; C18, stearic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic; C20:4, arachidonic; C22:6, decosahexenoic fatty acids; Tr, trace.
^b Mean ± S.D.

TABLE 5
INCORPORATION OF LINOLEATE-1-¹⁴C INTO LIVER OF TUMOR-BEARING MICE

NO. OF ANIMALS AND TYPE	cpm TOTAL INCORPORATION	cpm/gm LIVER WET WT.	PERCENT INCORPORATION INTO INDIVIDUAL LIPIDS									
			CE ^a	TG	FFA	C	DG	CL	PE	PI	PC	SPH
<i>In vivo</i>												
3 Control	211,000 ±50,000 ^b	333,000 ±27,000	1.8 ±1.2	47.6 ±2.8	1.5 ±0.8	1.0 ±0.8	1.9 ±0.9	1.4 ±1.1	6.1 ±0.8	3.4 ±0.9	32.4 ±9.6	1.4 ±0.6
3 Tumor-bearing	202,000 ±23,000	221,000 ±32,000	1.9 ±0.9	39.4 ±10.0	2.5 ±1.8	0.2 ±0.2	1.9 ±0.6	2.2 ±1.1	5.6 ±0.1	4.0 ±1.3	39.4 ±9.2	2.4 ±1.1
<i>In vitro</i>												
3 Control	3,330,000 ±880,000	4,180,000 ±800,000	1.3 ±0.1	11.9 ±1.7	7.1 ±0.7	0.2 ±0.1	2.6 ±0.6	4.7 ±0.9	5.2 ±0.8	2.0 ±1.0	50.4 ±5.0	5.4 ±2.4
3 Tumor-bearing	3,020,000 ±1,400,000	2,780,000 ±1,800,000	2.0 ±1.8	13.1 ±1.9	27.4 ±5.3	0.3 ±0.1	1.8 ±0.5	5.4 ±0.9	5.0 ±0.7	0.6 ±0.8	39.7 ±8.5	3.9 ±0.9

^a CE, cholesterol esters; TG, triglyceride; FFA, free fatty acids; C, cholesterol; DG, diglyceride; CL, cardiolipin; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PC, phosphatidyl choline; SPH, sphingomyelin.

^b Mean ± S.D.

At the end of the incubation period, the homogenate was placed into a refrigerated preparatory ultracentrifuge (Spineo PR3) and spun at 40,000 rpm on a 40 head ($100,000 \times g$) for 45 min. The sucrose solution was decanted. The specimen was again washed with sucrose solution and the entire sample was then extracted for total lipids by the Folch technic (2).

The method of total lipid extraction, separation of neutral and phospholipids, and subsequent thin layer chromatographic separation of individual lipids has been described. During thin layer chromatography, duplicate plates were utilized, one for the determination of lipid composition and the other for determination of distribution of radioactivity.

Determination of radioactivity.—After separation of neutral or phospholipids by thin layer chromatography, the plates were sprayed with a plastic film. The plate was then placed on a moving thin layer radioactive scanning unit (Nuclear-Chicago Corporation) and the activity in each lipid was recorded graphically. The Geiger tube had linear response, and the relative amounts of activity in each lipid were proportional to the area under each peak. Comparison of graphs from duplicate plates allowed the correlation of radioactive and chemical data.

In vitro incorporation of linoleate-1-¹⁴C into liver of 3 tumor-bearing and 3 non-tumor-bearing mice.—Two-tenths of the final solution of sodium linoleate in 10% human serum albumin was injected into the tail vein of the mouse. This inoculum contained approximately 4.5 µg.

One hr after the i.v. administration of radioactively tagged linoleate-1-¹⁴C, the animals were sacrificed by decapitation and bled. The livers were immediately removed, homogenized, and the total lipid extracted.

RESULTS

Table 1 indicates the weights of tumors, the whole animal, and the livers in both control and tumor-bearing mice.

The liver in the tumor-bearing animals is approximately 10–20% larger by weight than the livers of the non-tumor-bearing animals.

The weights of 14 pairs of livers from tumor- and non-tumor-bearing animals averaged 1.06 ± 0.05 and 0.80 ± 0.1 gm (S.D.), respectively. This hepatomegaly is significant to the 0.001 level ("Student" test).

Table 2 gives the quantitative distribution of hepatic neutral lipids determined by thin layer chromatography and total phospholipid. The most significant finding is the extreme reduction in hepatic triglycerides in the tumor-bearing animal. An accumulation of free fatty acids and diglycerides were also noted. The rate of incorporation of linoleic acid into triglycerides was depressed in the liver of tumor-bearing animals.

Tables 3 and 4 list distribution of individual fatty acids for each neutral lipid and phospholipid. In general, the fatty acids for each hepatic lipid in tumor- and non-tumor-bearing animals were the same.

Table 5 shows the *in vivo* total incorporation of linoleic acid in hepatic lipids of tumor- and non-tumor-bearing animals. The amount of hepatic *in vivo* and *in vitro* linoleic acid incorporation in the tumor-bearing animal is decreased to approximately one-third of the control. In the *in vivo* % distribution of linoleic acid incorporation in individual lipids, there was no significant difference. In the *in vitro* % distribution of linoleic acid incorporation of individual lipids, there was an apparent increase in the free fatty acid fraction in the tumor-bearing animal.

DISCUSSION

During the course of the growth of the tumor over a 20-day period, the host animal (minus tumor) did not show significant weight loss. Previous studies have shown that triglycerides were the major lipids in the glioma (approximately 10 mg/gm of tissue (8)). Although the neoplasm remains localized in the subcutis, it does influence

the hepatic triglyceride metabolism of the host. Since the linoleic acid % distribution of the triglyceride is the same in both tumor and control animals, the amount of linoleic acid incorporation is an indicator of the rate of triglyceride synthesis. In tumor animals, there is a quantitative decrease in triglycerides and a decrease in the rate of triglyceride synthesis in the liver. The mechanism in which the presence of a localized neoplasm influences host lipid metabolism is not clear. If the tumor directly and avidly absorbed triglycerides from the circulation, secondary homeostatic mechanisms would cause increased mobilization from various stores such as subcutaneous fat and the liver. In this way, the reduction of subcutaneous fat and the loss of triglycerides from the liver could be explained. On the other hand, the tendency towards the accumulation of free fatty acids and diglycerides in the liver suggests a specific enzyme block in the biosynthetic pathway of triglycerides in the liver. The role of the neoplasm then may be not simply one of selective lipid absorption, but also may include humoral influence on hepatic metabolic pathway. This pattern of neoplastic influence on hepatic lipids suggests host modification to support the growth of the tumor.

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