Lack of adaptation in lipogenesis by hepatoma 9121

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ABSTRACT The "minimal deviation" hepatoma 9121, implanted in rats, was shown to biosynthesize fatty acids from acetate-1-^{14}C at the same rate as normal rat liver but faster than host liver. Feeding the host animals a fat-deficient diet caused fatty acid biosynthesis to be increased 3- to 13-fold in liver, but the dietary regimen did not influence fatty acid biosynthesis in the tumor tissue.

Oxygen consumption and the oxidation of acetate and mevalonate to CO_2 were all affected by the dietary manipulation in liver but not in hepatoma. The fat-deficient diet decreased incorporation of acetate and mevalonate into cholesterol by the liver of control animals, increased it in the liver of host animals, and had no effect on this process in hepatoma. Thus, the transplantable tumor has lost the adaptive power of its parent tissue to respond to the dietary stimulus.

The changes in fatty acid composition in total lipids in response to the fasting and refeeding were also markedly different in hepatoma from those in liver of the host animals. These results support the concept that this tumor is characterized by a loss of some metabolic controls.

KEY WORDS lipogenesis · cholesterol biosynthesis · adaptive · liver · hepatoma · fat-deficient diet · tumor · linoleate accumulation · rat

THE RATIONALE OF STUDYING minimal deviation hepatomas as compared to other types of less differentiated hepatomas is as follows: the fewer the biochemical alterations from the cell of origin, the more likely it becomes that the change(s) necessary for tumor growth will become apparent. With this concept in mind, the hepatoma 9121, a diploid tumor line (1), was chosen for study. This tumor biosynthesizes fatty acids as readily as liver tissue does, and forms cholesterol at a greater rate than liver. Some areas of glycogen are present in the tumor, and it metastasizes to the lung (2, 3).

Various enzymes and enzymatic pathways can be induced or repressed by dietary manipulation (4–8). It has been shown that fasting a rat 48 hr and then feeding it a high carbohydrate, fat-deficient diet (FDD) for a further 48 hr results in increased hepatic biosynthesis of fatty acids (9, 10). A number of reports have shown that cholesterol biosynthesis is depressed in the liver of rats receiving 1% cholesterol in the diet (11, 12). Siperstein, Fagan, and Morris (13) have recently shown that a number of transplantable hepatomas and one human tumor have all lost their ability to regulate cholesterol synthesis by negative feedback control at the β-hydroxy,β-methylglutaryl-CoA reductase step. These workers have suggested that this loss of feedback control may be a property of malignancy per se. Other workers (14–16) have found a lack of adaption in hepatomas from mice and rats fed a fat-deficient diet.

The present investigation was initiated to see if metabolic controls relating to lipogenesis have been lost by the minimal deviation hepatoma 9121. Control and tumor-bearing animals were fasted and then fed an FDD, and the incorporation of acetate-1-^{14}C or mevalonic acid-2^{14}C (MVA-2-^{14}C) by liver and tumor tissue was studied in vitro. Liver tissue from control and host animals was shown to respond to dietary manipulation, but tumor tissue had lost the ability to adapt. Oxygen consumption and carbon dioxide production by the tissues were also studied.

METHODS

The minimal deviation hepatoma 9121 originated at the National Institutes of Health, and was carried in AxC inbred (Irish) rats. This hepatoma is a well-differen-

Abbreviations: FDD, fat-deficient diet; MVA, mevalonic acid.

tiated parenchymal cell tumor induced by *N*-2-fluorenyl diacetamide (17, 18).

The animals were maintained on commercial laboratory chow until the intramuscularly implanted tumors were about 2 cm in diameter. The animals were fasted for 48 hr and both animals from the control groups and tumor-bearing host animals were fed either an FDD (Nutritional Biochemical Corporation, Cleveland, Ohio)[1] or a commercial laboratory chow (Country Best Foods, Agway, Inc., Syracuse, N.Y.) for 48 hr.[2]

The liver and tumors were excised from decapitated animals and placed in cold Krebs' KCl–phosphate buffer (pH 7.4). It is well known that a bicarbonate buffer is a better stimulant of fatty acid synthesis than a phosphate buffer. However, studies in our laboratory (Burns, unpublished observations) have shown that under all conditions tested, i.e. diabetes, starvation, or refeeding, fatty acid biosynthesis undergoes the same qualitative changes (i.e., increase or decrease) regardless of the buffer employed. Fatty acid synthesis in phosphate buffer, which is about 45% that obtained in bicarbonate buffer, can be stimulated 15-fold by fasting and refeeding an FDD.

The necrotic tissue of the tumor was debrided and small fragments were weighed (100 mg per flask) and incubated in Krebs phosphate buffer at 37°C. The liver tissue was sliced (0.5 mm thickness) with a Stadie–Riggs slicer and incubated similarly. Oxygen was the gas phase. Lipogenesis was studied by the addition of acetate-1-^{14}C or MVA-2-^{14}C from the side arm of the Warburg vessel and subsequent analysis described below. Preliminary experiments were conducted by using either substrate levels (12,000 mμmoles/flask) or tracer quantities (40 mμmoles/flask) or acetate-1-^{14}C. Four determinations were made: oxygen consumption, $^{14}CO_2$ evolution, fatty acid labeling, and cholesterol labeling. It was observed previously (19–21) and in these preliminary experiments that the changes elicited by feeding, starvation, and diabetes were seen whether substrate or tracer amounts of acetate were employed, although the order of magnitude of the changes was different. Because of these studies, tracer concentrations of acetate-1-^{14}C and MVA-2-^{14}C (40 mμmoles/flask, containing 2 μc of radioactivity) were used. The usual manometric techniques were employed to obtain oxygen consumption data. A KOH-soaked filter paper in the center well of the Warburg vessel trapped carbon dioxide. The $^{14}CO_2$ was assayed as "infinitely thick" barium carbonate.

The metabolic experiments were terminated by the addition of 0.2 ml of 20% trichloroacetic acid, and each flask was shaken for an additional 30 min to assure complete liberation of carbon dioxide from solution. Contents of the flask were poured over a fine-meshed stainless steel screen, which retained the tissue slices and tissue fragments. The tissues were then rinsed with buffer to remove any adherent acetate or MVA and saponified with 11% ethanolic KOH for 30 min in a steam bath.

The nonsaponifiable fraction and fatty acids were recovered and cholesterol was precipitated from the nonsaponifiable fraction with digitonin overnight. The digitonide was washed twice with acetone, dissolved in methanol, and plated as infinitely thin plates. The cholesterol digitonide was radioassayed on a Nuclear-Chicago D-47 gas-flow counter. The fatty acids were plated as infinitely thin samples and similarly assayed.

An aliquot of the fatty acid fraction was methylated (2% sulfuric acid in methanol), extracted into hexane, and analyzed by gas–liquid chromagraphy on a Perkin–Elmer vapor fractometer No. 154 with a thermistor detector. The column was packed with chromosol P as the stationary phase and coated with diethylene glycol succinate polyester. The area of the peaks was obtained by planimetry.

RESULTS

The oxygen consumption of liver and tumor tissue after dietary manipulation is listed in Table 1. "Control liver" refers to tissue obtained from animals that had no tumor inoculation and were maintained on laboratory chow; "host liver" is the hepatic tissue from animals carrying hepatoma 9121; and FDD refers to tissue from rats fed an FDD for 48 hr after a 48 hr fast. The oxidative capacities of control and host livers were similar; both declined when the animals were fed the FDD. The oxidative ability of tumor tissue, which was lower than that of liver on the control diet, was not altered by the dietary regimen.

The ability of liver and tumor tissue to oxidize acetate-1-^{14}C to $^{14}CO_2$ is represented by the data in Table 2. Fasting and then feeding an FDD decreased significantly the $^{14}CO_2$ production from acetate both by control liver and host liver but did not affect the (lower) rate of oxidation by tumor tissue. $^{14}CO_2$ production from MVA-2-^{14}C (Table 2) was highest in control liver and lowest in the

[1] Fat-deficient diet composition: "vitamin free" casein (21.10%), Alphacel "cellulose" (16.45%), sucrose (58.45%), and salt mixture U.S.P. XIV (4.00%); plus the following vitamin supplements (per kg): choline chloride 5730 mg, nicotinic acid 5730 mg, inositol 292 mg, Vitamin A concentrate (200,000 units per gram) 95.7 mg, Vitamin D concentrate (400,000 units/gram) 66.1 mg, α-tocopherol 227 mg, menadione 2.18 mg, thiamine hydrochloride 22.04 mg, pyridoxine hydrochloride 22.04 mg, riboflavin 22.04 mg, and calcium pantothenate 42.6 mg.

[2] *Big Red Laboratory Chow*. Guaranteed analysis: minimum 24.0% crude protein, 5.0% crude fat, and maximum 5.0% crude fiber. Ingredients: soybean meal, ground oats, wheat midlings, corn meal, ground wheat, dehydrated alfalfa meal, dried skimmed milk, fish meal, ground limestone, vegetable oil, D-activated plant sterol ("Source of Vitamin D-2"), Vitamin A palmitate, salt, manganese oxide, iron carbonate, sodium sulfate, copper oxide, cobalt carbonate, zinc oxide, calcium iodate.

TABLE 1 OXIDATIVE METABOLISM OF LIVER AND TUMOR AFTER DIETARY MANIPULATION

	Oxygen Consumption*	
	μmoles/2 hr	
Control liver	9.1 ± 0.2	$P<0.001$
Control liver, FDD	6.6 ± 0.3	
Host liver	8.2 ± 0.3	$P<0.001$
Host liver, FDD	6.3 ± 0.4	
Tumor	6.4 ± 0.5	$P>0.05$
Tumor, FDD	7.4 ± 0.5	

* At least six animals with duplicate flasks were used for each number presented in all of the tables. Means ± SEM are given. 100 mg of tissue was incubated in 2.0 ml of Krebs phosphate buffer (0.08 M, pH 7.4) at 37°C for 120 min. The gas phase was oxygen. Acetate-1-^{14}C or MVA-2-^{14}C was present in tracer amounts (40 mμmoles/flask containing 2 μc of ^{14}C). FDD: 48 hr fast followed by refeeding with a fat-deficient (high carbohydrate) diet.

host liver. Feeding the animals an FDD caused a decrease in $^{14}CO_2$ production in control livers but an increase in livers from host animals. Dietary alteration did not influence the evolution of $^{14}CO_2$ in the tumor tissue.

Hepatic synthesis of fatty acids from acetate (Table 3) was greatly stimulated both in control and host animals by fasting and refeeding the FDD. The stimulation of the initially low activity in host liver was 16-fold. Tumor tissue was as active in lipogenesis as control or host liver, but feeding an FDD to the host animal did not increase the activity. That is, hepatoma 9121 did not show the adaptive lipogenic response typical of normal liver to the stimulus of fasting and refeeding an FDD.

Table 4 gives the results on cholesterol synthesis. Fasting and feeding an FDD depressed cholesterologenesis in control liver. This finding is similar to that shown by Clarenburg and Chaikoff (4). Although the low level of cholesterologenesis in host liver of rats on laboratory chow was greatly increased on fasting and refeeding the FDD, the final value fell short of that in control liver. This increased cholesterol synthesis under the stimulus of fasting and FDD refeeding is similar to the response of diabetic animals to this treatment (4). On the other hand, tumor tissue, which is an even better cholesterol producer

TABLE 3 FATTY ACID BIOSYNTHESIS FROM ACETATE IN LIVER AND TUMOR TISSUE

	% Incorporation	
Control liver	0.14 ± 0.03	$P<0.001$
Control liver, FDD	0.41 ± 0.09	
Host liver	0.05 ± 0.04	$P<0.001$
Host liver, FDD	0.79 ± 0.21	
Tumor	0.15 ± 0.04	$P<0.1$
Tumor, FDD	0.21 ± 0.07	

Conditions were as in Table 1.

than host or control liver, is unresponsive to alterations in the diet. High cholesterol labeling is an uncommon finding in tumor tissue.

Since the major control of cholesterol biosynthesis is thought to be at the β-hydroxy,β-methyl-glutaryl-CoA reductase step, MVA-2-^{14}C was also investigated as a precursor of cholesterol. Results qualitatively similar to those with acetate were found (Table 4). The lower cholesterol synthesis in host liver implies that biosynthetic enzyme or cofactor activity was depressed, and that this depression was beyond the reductase step, an uncommon finding in cholesterol synthesis.

The fatty acid composition of the total lipids of the tissues under investigation is presented in Table 5. In control and host liver, the most prominent fatty acids were the saturated acids, stearic and palmitic. Feeding an FDD for 48 hr caused major changes in the fatty acid composition of the liver: the percentages of the monounsaturated acids 16:1 and 18:1 doubled at the expense of stearic and the polyunsaturated acids. Similar changes have previously been reported by Allmann, Hubbard, and Gibson (9) for mouse liver.

Tumor tissue showed a fatty acid pattern different from that of its tissue of origin. Oleic acid was by far the most abundant fatty acid present in this tumor, in agreement with the findings of Veerkamp, Mulder, and Van Deenen (22) in hepatomas. However, alteration in the diet of the host animal produced relatively little change (Table 5). Why the percentage of linoleic acid went up

TABLE 2 OXIDATION OF ACETATE AND MEVALONATE BY LIVER AND TUMOR TISSUE

	Acetate-1-^{14}C		MVA-2-^{14}C	
	% incorporation			
Control liver	36.7 ± 2.4	$P<0.001$	5.3 ± 0.4	$P<0.05$
Control liver, FDD	19.8 ± 2.4		2.3 ± 0.4	
Host liver	34.4 ± 3.0	$P<0.001$	0.6 ± 0.2	$P<0.025$
Host liver, FDD	24.4 ± 1.2.		11 ● 0.2	
Tumor	29.0 ± 2.0	$P>0.01$	1.2 ± 0.2	$P>0.05$
Tumor, FDD	26.5 ± 1.8		1.9 ± 0.3	

Incubation conditions were as in Table 1. The carbon dioxide was isolated and plated as barium carbonate ("infinitely thick").

TABLE 4 CHOLESTEROL BIOSYNTHESIS IN LIVER AND TUMOR TISSUE

	Acetate-1-^{14}C		MVA-2^{14}C	
	% incorporation			
Control liver	0.75 ± 0.11	$P<0.05$	8.30 ± 0.80	$P<0.001$
Control liver, FDD	0.43 ± 0.09		3.68 ± 0.49	
Host liver	0.04 ± 0.01	$P<0.05$	1.51 ± 0.25	$P<0.05$
Host liver, FDD	0.20 ± 0.05		2.60 ± 0.39	
Tumor	1.00 ± 0.23	$P<0.1$	3.30 ± 0.84	$P>0.1$
Tumor, FDD	1.35 ± 0.24		3.30 ± 0.54	

Conditions were as in Table 1.

TABLE 5 FATTY ACID COMPOSITION OF TOTAL LIPIDS FROM LIVER AND TUMOR*

	16:0	1 16:	17:0	18:0	18:1	18:2	18:3
	% of total fatty acids						
Control liver	24.6	7.7	2.0	35.3	17.1	11.5	1.8
Control liver, FDD	28.5	12.7	8.7	12.7	32.3	3.8	—
Host liver	27.1	3.4	—	27.9	18.4	18.9	4.4
Host liver, FDD	36.5	13.5	—	12.0	30.0	6.0	—
Tumor	18.8	8.3	—	14.6	58.3	—	—
Tumor, FDD	23.1	7.9	—	16.3	42.1	10.5	—

Fatty acids designated by no. of carbon atoms: no. of double bonds.

* Samples were pooled and analyzed on a Perkin–Elmer vapor fractometer. The variability of different runs on the same pooled samples never exceeded ±3%. There was a small amount of arachidonic (20: 4), but its percentage was not influenced in these experiments.

after the FDD was fed is unknown, but mobilization of lipid from fat depots in the host animal could be an explanation.

DISCUSSION

The first experimental studies suggesting that various control mechanisms in cholesterol metabolism might be lacking in tumor tissue were those of Siperstein and coworkers (11–13), who showed that the negative feedback control of cholesterol biosynthesis had been lost in 1 transplantable human, 1 mouse, and 10 rat hepatomas. They hypothesized that this loss of feedback control might be characteristic of malignancy in the liver. The present investigation was carried out to see if another control mechanism, i.e., enzyme induction, was lacking in this "minimal deviation" hepatoma, as had been previously shown for a mouse and rat hepatoma by Sabine, Abraham, Morris, and Chaikoff (15, 16).

The oxidative capacity of liver tissue from control and host animals was decreased by fasting and refeeding an FDD, an effect that is more probably due to changing from a chow diet to a synthetic diet rather than to the fat deficiency, since unpublished experiments from this laboratory showed that liver from animals receiving an FDD supplemented with corn oil also showed depressed oxygen consumption when compared to liver from chow fed animals (food consumption in the two groups was the same). Oxygen consumption of tumor tissue was not significantly changed by the dietary alterations; when the FDD was fed, the hepatoma utilized as much oxygen per unit weight as the liver from host animals.

The oxidative capacity of hepatoma 9121 (Table 2) was similar to that described previously (23) for the oxidation of palmitate and butyrate in hepatomas 5123C, 5123Tc, and 7787. The oxidation of acetate by hepatoma 9121 was not decreased by feeding the host animal an FDD as it was in host (and control) liver. It seems that the hepatoma has lost this metabolic control. What the control mechanism is, and what the significance of the absence of such control may be, remain unknown at present.

Fatty acid biosynthesis in the liver is sensitive to variations in diet (4–9, 11). Fasting rats and refeeding them an FDD for 48 hr resulted (Table 3) in increased lipogenesis from acetate by normal liver tissue. This metabolic response has been shown to be due to increased synthesis of the enzymes that catalyze lipogenesis (9). Host liver showed a similar, even more dramatic response, but the hepatoma did not. As with cholesterologenesis seen

above, this tumor tissue had lost its ability to respond to signals that control normal tissues. Whether this lack of response is characteristic of malignancy is, of course, unknown, but a survey of other hepatomas with different growth rates and different degrees of differentiation (16) has shown that different (fat-free or high-fat) diets profoundly altered metabolic and enzymatic activities in normal and host liver, but not in the hepatomas.

The explanation of changes in fatty acid composition (Table 5) is not obvious. The increased oleic acid content in liver after the feeding of an FDD could result from mobilization of the oleic acid-rich adipose tissue, but since rat adipose tissue also contains 25% linoleic acid and the percentage of this acid in liver falls, either the mobilization or incorporation into liver lipids would have to be preferential. An increased desaturase activity (stearate → oleate) could also explain some of the results, but palmitic acid does not decline as palmitoleate rises. The strikingly different response of hepatoma fatty acids, in which 18:2 (hitherto absent) becomes a sizable component, can be explained only in terms of mobilization from the host's tissues since tumors, like other mammalian tissues, are incapable of synthesizing this acid.

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References

1. Nowell, P. C., H. P. Morris, and V. R. Potter. 1967. *Cancer Res.* **27:** 1565.
2. Morris, H. P. 1965. *Advan. Cancer Res.* **9:** 227.
3. Morris, H. P. 1966. *In* Gann Monograph. Japanese Cancer Association, Tokyo, Japan, No. I, 1-10.
4. Clarenburg, R., and I. L. Chaikoff. 1966. *Am. J. Physiol.* **210:** 37.
5. Gellhorn, A., and W. Benjamin. 1964. *Biochim. Biophys. Acta.* **84:** 167.
6. Hubbard, D. D., R. E. McCaman, M. R. Smith, and D. M. Gibson. 1961. *Biochem. Biophys. Res. Commun.* **5:** 339.
7. Tepperman, H. M., and J. Tepperman. 1965. *Am. J. Physiol.* **209:** 773.
8. Tepperman, J., and H. M. Tepperman. 1961. *Am. J. Physiol.* **200:** 1069.
9. Allmann, D. W., D. D. Hubbard, and D. M. Gibson. 1965. *J. Lipid Res.* **6:** 63.
10. Benjamin, W., A. Gellhorn, M. Wagner, and H. Kundel. 1961. *Am. J. Physiol.* **201:** 540.
11. Siperstein, M. D., and V. M. Fagan. 1962. *Federation Proc.* **21:** 300.
12. Siperstein, M. D., and V. M. Fagan. 1964. *Advan. Enzyme Regulation.* **2:** 249.
13. Siperstein, M. D., V. M. Fagan, and H. P. Morris. 1966. *Cancer Res.* **26:** 7.
14. Denton, M. D., and M. D. Siperstein. 1964. *Clin. Res.* **12:** 265.
15. Sabine, J. R., S. Abraham, and I. L. Chaikoff. 1966. *Biochim. Biophys. Acta.* **116:** 407.
16. Sabine, J. R., S. Abraham, H. P. Morris, and I. L. Chaikoff. 1967. *Cancer Res.* **27:** 793.
17. Morris, H. P., and B. P. Wagner. 1968. *In* Methods in Cancer Research. H. Busch, editor. Academic Press, Inc., New York. 125–152.
18. Miyaji, H., H. P. Morris, and B. P. Wagner, 1968. *In* Methods in Cancer Research. H. Busch, editor. Academic Press, Inc., New York. 153–157.
19. Emerson, R. J., and J. T. Van Bruggen. 1958. *Arch. Biochem. Biophys.* **77:** 467.
20. Elwood, J. C., and J. T. Van Bruggen. 1960. *J. Biol. Chem.* **235:** 568.
21. Elwood, J. C., A. Marcó, and J. T. Van Bruggen. 1960 *J. Biol. Chem.* **235:** 573.
22. Veerkamp, J. H., I. Mulder, and L. L. M. Van Deenen. 1961. *Z. Krebsforch.* **64:** 137.
23. Bloch-Frankenthal, L., J. Langan, H. P. Morris, and S. Weinhouse. 1965. *Cancer Res.* **25:** 732.