Cholesterol biosynthesis in transplantable hepatomas: evidence for impairment of uptake and storage of dietary cholesterol

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ABSTRACT Cholesterol feeding inhibits cholesterol biosynthesis in normal but not in malignant liver tissue. It has been postulated that hepatomas have suffered a specific intracellular deletion of the cholesterol feedback control mechanism, but there is little direct evidence to support this hypothesis.

Rats bearing Morris transplantable hepatomas were fed high cholesterol diets for periods of up to 21 days. Cholesterol biosynthesis, as expected, was suppressed in the normal liver but not in hepatomas. The livers accumulated large amounts of cholesteryl ester but the hepatomas showed little or no increase in ester content.

Cholesterol-1α-^{3}H was administered intragastrically to other tumor-bearing rats. Uptake of radioactivity by the tumors was much slower than by normal liver. Comparison of the specific activities of liver and tumor cholesterol with that of the plasma suggested that the liver took up dietary cholesterol selectively from the blood, while the appearance of radioactivity in the tumors could be explained by slow equilibration with plasma cholesterol.

Our results suggest that the insensitivity of cholesterol biosynthesis to dietary cholesterol in hepatomas could be explained by an impairment in the uptake and storage of dietary cholesterol and that the concept of an intracellular deletion of the feedback mechanism requires further evidence.

SUPPLEMENTARY KEY WORDS cholesteryl ester

CHOLESTEROL biosynthesis in the normal liver of higher animals is markedly inhibited by cholesterol feeding (1). Although the precise mechanism is unknown, this control is effected by a sensitive feedback regulation of the microsomal enzyme HMGCoA reductase, which catalyzes the reduction of HMGCoA to mevalonic acid. As the major metabolic fate of mevalonic acid is its conversion to sterols, this reaction is an appropriate regulatory step in cholesterol biosynthesis.

It has been reported by Siperstein and his colleagues that many hepatomas, including the Morris minimal deviation rat transplantable hepatomas, do not show feedback inhibition of cholesterol biosynthesis (2, 3); these observations have been confirmed by Sabine, Abraham, and Chaikoff (4). There are at least two possible explanations for the insensitivity of these tumors to dietary cholesterol. The tumors might not remove dietary cholesterol from the blood in the same way as normal liver and the inhibitor would not then gain access to its site of action. If, however, hepatomas take up dietary cholesterol in a normal manner one would have to postulate an intracellular defect of the feedback mechanism.

We have tested the hypothesis that rat transplantable hepatomas do not handle dietary cholesterol in the same way as normal liver tissue. We have studied both the accumulation of cholesterol with prolonged cholesterol feeding and the rate of uptake of radioactive cholesterol following intestinal absorption in both liver and tumor tissue. Our results suggest that the ability of rat hepatomas to take up dietary cholesterol is markedly impaired.

MATERIALS AND METHODS

Buffalo strain rats bearing Morris minimal deviation transplantable hepatomas (Nos. 7793, 7794A, and 7787), transplanted intramuscularly in both thighs, were flown

Abbreviations: HMGCoA, 3-OH-3-methylglutaryl coenzyme A; DPS, digitonin-precipitable sterols.

from the United States. The rats were allowed free access to rat chow (Oxoid Ltd., London, England) and water until the combined weight of the tumors was approximately 7–15 g. At this time the animals weighed 300–360 g, and their livers weighed 8–13 g.

For studies of hepatic accumulation of cholesterol, a diet containing 2% cholesterol (Calbiochem, Los Angeles, Calif.) and 5% oleic acid (British Drug Houses, Ltd., Poole, Dorset, England) was prepared by mixing these lipids with normal chow. Control rats were fed a diet containing 5% oleic acid without added cholesterol. Both diets were fed for periods of up to 21 days.

In studies of hepatic uptake of labeled cholesterol, animals were given either a tracer amount of cholesterol-1α-^{3}H (250 μCi, specific activity 10.4 Ci/mmole; Radiochemical Centre, Amersham, England) or the same amount of radioactivity diluted with 100 mg of unlabeled cholesterol. The doses were dissolved in 3 ml of olive oil and administered by gastric intubation. The syringe and intubation tube were flushed with 0.5 ml of olive oil; the radioactivity remaining was measured and found to be approximately 10% of the initial amount. Animals were killed 6–96 hr after the tracer dose, and 3–30 hr after the larger dose. Blood was collected by cardiac puncture following ether anesthesia and the livers and tumors were then removed. The tumors contained areas of necrotic tissue; these areas were avoided in the selection of material for study.

Liver, tumor, and plasma lipids were extracted by the method of Folch, Lees, and Sloane Stanley (5). The free and esterified cholesterol were separated by column chromatography using silicic acid prepared by the method of Barron and Hanahan (6). Esterified cholesterol was eluted with 3% ether in hexane, and free cholesterol was eluted with ether. The cholesterol content in both fractions was measured, after prior saponification of the ester fraction, by the method of Zlatkis, Zak, and Boyle (7). Aliquots were also taken for measurement of radioactivity using as the scintillation fluid a solution of 50 mg of 1:4-di-2-(5-phenyl-oxazolyl)-benzene (POPOP) and 4 g of 2,5-diphenyloxazole (DPO) per liter of toluene.

Values of both free and esterified cholesterol were corrected for loss by the use of trace amounts of ^{14}C-labeled cholesterol and cholesteryl ester as internal recovery standards. Radioactivity was measured in a Philips Liquid Scintillation Analyzer; the degree of quenching was estimated by the external standard ratio method and appropriate corrections were made.

Liver slices (approximately 0.5 mm thick) were prepared using a McIlwain tissue chopper. Tumor tissue was too friable to permit the use of the tissue chopper and was sliced by hand. After weighing, the tissues were transferred to the outer compartment of a 50-ml Erlenmeyer center-well flask containing 7 ml of Krebs bicarbonate buffer (pH 7.4) in which 56 μmoles of acetate-2-^{14}C was dissolved (specific activity 0.1 μCi/μmole). The flasks were flushed with 95% O_2–5% CO_2, sealed with rubber self-sealing caps, and shaken in a Mickle metabolic shaker at 130 double oscillations per minute for 2 hr.

At the end of the incubation, incorporation of the ^{14}C-labeled acetate into CO_2, DPS, and fatty acids was measured. 0.5 ml of Hydroxide of Hyamine 10X (Packard Instrument Co., Downers Grove, Ill.) was introduced into the center well, and 0.5 ml of 5 N H_2SO_4 was added to the incubation fluid. The flasks were allowed to stand in ice water for 1 hr with intermittent shaking. The Hyamine containing the evolved CO_2 was then transferred to a counting vial for measurement of radioactivity. The tissue in the flasks was dissolved by heating at 70°C for 1 hr, after the addition of 1 ml of 90% KOH (w/v). The mixture was acidified and extracted (5), and the lipids were taken to dryness. The dry residue was saponified by heating at 70°C for 1 hr in a stoppered tube with 3 ml of ethanol and 0.3 ml of 50% KOH (w/v). After the addition of 3 ml of water the nonsaponifiable lipids were extracted twice with 10 ml of hexane. The pooled extracts were evaporated to dryness and dissolved in 4 ml of ethanol–acetone 1:1; 2 ml of 0.5% digitonin in 50% ethanol was then added, and the mixture was allowed to stand overnight. The precipitate of sterol digitonide was collected by centrifugation, washed with acetone then ether, and dissolved in methanol. An aliquot was taken for counting. The aqueous layer after saponification was acidified by the addition of 1 ml of 4 N HCl and the fatty acids were extracted twice with 10 ml of hexane. These extracts were pooled, backwashed with water to remove any remaining labeled acetate, taken to dryness, and dissolved in the scintillation fluid. In this fraction the radioactivity was presumed to be present only in fatty acids.

RESULTS

Accumulation of Dietary Cholesterol

The results of feeding cholesterol for up to 21 days are presented in Table 1. Incorporation of acetate into cholesterol by slices of liver was inhibited by cholesterol feeding, but acetate incorporation by the tumor slices was unaffected. This confirms the findings of Siperstein and Fagan (2) and of Sabine et al. (4). Incorporation of acetate into CO_2 and fatty acids was unaffected by cholesterol feeding in both liver and tumor.

The esterified cholesterol content of the liver was far greater in cholesterol-fed rats than in control animals, but the free cholesterol of the liver showed little or no increase with cholesterol feeding. These results are in

TABLE 1 EFFECT OF FEEDING CHOLESTEROL ON THE INCORPORATION OF ^{14}C-LABELED ACETATE INTO DPS AND ON THE CONTENT OF FREE AND ESTERIFIED CHOLESTEROL IN LIVER AND TUMOR TISSUE IN HEPATOMA-BEARING RATS

Diet*		Acetate-^{14}C Incorporation		Free Cholesterol		Esterified Cholesterol	
Days	Cholesterol	Liver	Tumor	Liver	Tumor	Liver	Tumor
Tumor 7787		*nmoles/g/2 hr*		*μ moles/g of tissue*			
7	−	130.0	290.7	5.1	5.8	0.8	3.8
7	+	3.2	276.5	5.2	5.2	15.5	3.9
14	−	67.9	149.8	6.0	5.1	1.5	5.7
14	+	3.1	246.3	6.4	7.6	27.6	3.8
21	−	147.5	355.4	3.9	3.8	0.8	4.2
21	+	4.2	233.6	7.8	4.4	36.2	7.8
Tumor 7793							
7	−	70.5	110.0	6.2	3.6	3.4	1.1
7	+	4.5	65.0	6.2	3.7	16.6	0.4
14	−	140.3	142.3	6.9	9.9	3.4	2.1
14	+	2.0	158.0	6.3	5.0	47.6	2.4
21	−	131.0	130.9	7.4	7.6	0.8	2.7
21	+	1.0	121.7	8.3	5.2	38.8	6.3
Tumor 7794A							
3	−	130.4	143.9	5.1	4.0	0.7	2.4
3	+	3.0	120.8	8.1	4.7	16.5	5.0
7	−	81.1	154.3	5.1	3.8	0.8	4.0
7	+	3.5	128.6	5.5	3.7	14.2	3.9
14	−	235.9	75.6	5.5	5.9	1.4	9.3
14	+	4.8	114.5	6.9	5.3	23.3	3.0
21	−	107.6	143.4	5.7	5.7	2.0	9.4
21	+	4.7	106.5	6.4	5.9	36.5	4.3

* The diet was normal rat chow plus oleic acid (5%). Plus sign (+) indicates that cholesterol (2%) was added to the diet.

agreement with those found in the normal rat by Gould and Swyryd (8). The cholesterol content of the tumors showed no such tendency to increase, either in free or esterified form. The level of esterified cholesterol in tumors of the control animals was often, but not always, higher than that found in the corresponding liver. A high level of cholesteryl ester has been described in other malignant tumors, but the distribution of the cholesteryl ester within the cell, and its physiological significance, are unknown (9).

The mean plasma cholesterol level was higher in cholesterol-fed animals (96 mg/100 ml ± 7.4 [SE]) than in oleic acid-fed controls (81 ± 4.7). The control animals, however, had increased plasma cholesterol levels when compared with tumor-bearing animals fed only a diet of normal chow (56 ± 3.6).

Uptake of ^{3}H-labeled Cholesterol

(*A*). The radioactivity in dpm/g in liver and tumor (7787) was measured at different time intervals following a tracer dose of ^{3}H-labeled cholesterol administered intragastrically. The results are presented in Table 2. In the early period after cholesterol-^{3}H administration, the amount of administered cholesterol-^{3}H in tumor tissue was much less than that in the liver of the same animal. This relationship was true of both free and esterified fractions. At 96 hr similar amounts of radioactivity were present in both liver and tumor, but this relationship was due, primarily, to a fall in the cholesterol-^{3}H content of the liver rather than a marked rise in the cholesterol-^{3}H content of the tumor.

In Fig. 1 we have plotted the specific activity of both free and esterified cholesterol in tumor and liver in relation to the specific activity of free and esterified cholesterol in plasma. The specific activities of both fractions of liver cholesterol were initially higher than those of the plasma; they then fell, and at 96 hr were about equal to those of plasma. The cholesterol specific activity in the tumor, however, was much lower than that of the plasma for the first 24 hr, but the specific activity of the tumor cholesterol increased slowly until it was similar to that of plasma cholesterol.

In vitro acetate incorporation into DPS in both liver and tumor was unaffected by the tracer dose of cholesterol that was administered.

(*B*). Animals in another group (bearing hepatoma number 7794A) were given a single dose of 100 mg of cholesterol-^{3}H by gastric intubation. Animals were killed 3–30 hr after administration of the dose and the same measurements were made as in the tracer experiments.

The results are presented in Table 2. The tumor contained much less of the administered radioactivity than the liver, and this was true for both free and esterified fractions. Again the specific activity of the liver cholesterol exceeded that of the plasma, while tumor cholesterol specific activity was far lower than that of the plasma. This study was not continued beyond 30 hr, and the

TABLE 2 RADIOACTIVITY OF LIVER, TUMOR, AND PLASMA AFTER ADMINISTRATION OF ^{3}H-LABELED CHOLESTEROL TO ANIMALS BEARING MORRIS TRANSPLANTABLE HEPATOMAS*

Hr after Administration	Free Cholesterol			Esterified Cholesterol		
	Tumor	Liver	Plasma	Tumor	Liver	Plasma
	dpm × 10^{-3} per g of tissue or per ml of plasma					
A. Tracer dose, tumor 7787						
6	23.6	439.6	55.7	3.3	45.7	36.7
12	124.1	1,488.8	233.6	23.1	273.9	135.4
18	179.3	1,356.2	137.8	26.5	229.1	143.2
24	358.8	1,600.1	170.1	113.1	364.6	213.3
48	280.2	643.8	97.6	71.3	79.1	138.6
96	359.3	390.5	44.2	126.9	52.3	77.5
B. 100-mg dose, tumor 7794A						
3	11.9	494.2	29.7	9.0	54.3	22.3
6	188.7	919.7	113.4	8.6	554.0	88.5
12	150.1	1,598.7	186.9	38.5	769.2	141.2
18	291.6	1,394.6	159.1	86.4	682.5	177.3
24	145.5	1,212.3	129.4	82.3	433.5	138.3
30	262.0	1,820.2	179.7	208.9	938.0	276.7

* The results at each time point were obtained from one tumor-bearing animal.

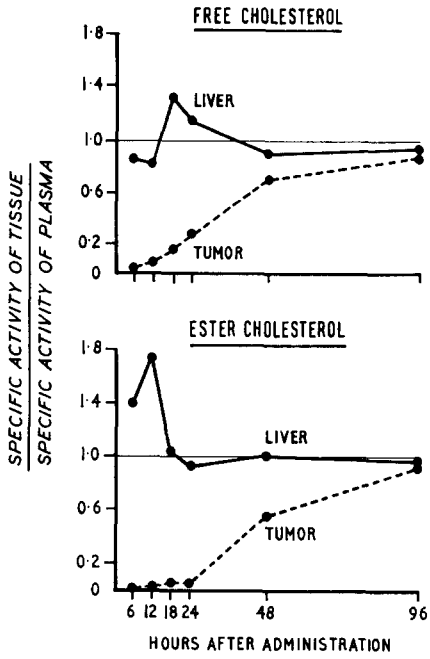

FIG. 1. The ratios of the specific activities of liver and tumor cholesterol to the specific activity of plasma cholesterol at various time intervals after the administration of an intragastric tracer dose of ^{3}H-labeled cholesterol. The results at each time point were obtained from one tumor-bearing animal.

marked rise in the tumor/plasma specific activity ratio seen at 48 and 96 hr after a tracer dose was not observed.

In vitro acetate incorporation into DPS in the normal liver seemed slightly inhibited at 18 hr after this large single dose of cholesterol, but levels were far higher than those found in animals fed cholesterol for prolonged periods.

DISCUSSION

In these experiments we have confirmed the results of Siperstein and Fagan (1, 2). Cholesterol biosynthesis by normal liver was markedly inhibited by cholesterol feeding, but synthesis in malignant liver was unaffected. However, measurement of the cholesterol content of liver and tumor revealed another difference in the response of these two tissues to the oral administration of cholesterol. In normal liver there was a striking increase in esterified cholesterol but the free cholesterol content rose by only a small amount; in tumor tissue no tendency was found for either free or esterified cholesterol to accumulate. Both free and esterified cholesterol have been invoked as inhibitors of hepatic cholesterol synthesis (8, 10, 11), and it is clearly a possibility that the failure of dietary cholesterol to suppress cholesterol biosynthesis in hepatomas may be related to the inability of the tumor tissue to accumulate cholesterol in one or both of these forms.

Siperstein and Fagan studied mice bearing transplantable hepatomas (2). After they were fed a diet containing large amounts of radioactive cholesterol for 2, 4, or 10 days, the cholesterol specific activity of the liver was only twice that of the tumor. It was argued that differing rates of cholesterol synthesis would have led to a greater dilution of labeled dietary cholesterol in the uninhibited tumor than in the inhibited liver. On this basis the specific activity data were interpreted as evidence that the rates of penetration of dietary cholesterol into tumor and liver were approximately the same.

We studied tumor-bearing rats hours after the administration of a single dose of radioactive cholesterol and our results are not compatible with the conclusions of Siperstein and his colleagues (1–3). Following intragastric administration of tritiated cholesterol the rate of appearance of radioactivity was far slower in tumor tissue than in the liver. The specific activities of hepatic free and esterified cholesterol rapidly exceeded those of the plasma, suggesting an efficient extraction of cholesterol-containing chylomicrons and very low density lipoproteins of intestinal origin. The specific activities then fell to approximate those of the plasma during the subsequent period of equilibration. This pattern of response of the liver to cholesterol of intestinal origin is well recognized (12, 13). By contrast, the specific activity of tumor cholesterol was initially very low following the administration of tritiated cholesterol but appeared to increase gradually so that at 96 hr it was close to that of plasma. This was true of both free and esterified fractions. This slow rise in tumor specific activity suggests that there was little selective uptake of cholesterol of intestinal origin and that the increase in radioactivity of tumor cholesterol could best be explained by slow equilibration with the cholesterol of the plasma (14). It would seem likely, therefore, that the failure of hepatomas to accumulate dietary cholesterol is related to their relative inability to remove it from the blood.

To explain the lack of inhibition of cholesterol synthesis in hepatomas, Siperstein and Fagan (1, 2) postulated an intracellular disturbance of the mechanism regulating cholesterol synthesis. This was a logical consequence of their belief that hepatoma cells take up dietary cholesterol as readily as normal hepatocytes. Our failure with rat hepatoma to demonstrate uptake and storage of dietary cholesterol makes it unnecessary to postulate that the abnormality is inside the cell.

Our findings do not, of course, provide evidence that the intracellular mechanism is intact. Such evidence is difficult to obtain, as many problems beset the study of the regulation of cholesterol biosynthesis in cell-free systems. Sabine and Chaikoff, however, studied an analogous situation in the mouse hepatoma BW 7756 (15). The feeding of fat leads to inhibition of fatty acid production in normal mouse liver, and it has been suggested that long-chain fatty acids and their coenzyme A deriva-

tives are the specific intracellular inhibitors. Fat feeding failed to suppress fatty acid synthesis in the mouse hepatoma, but addition of both palmitic acid and palmityl CoA led to inhibition of synthesis in a cell sap–microsomal preparation from the tumor. Sabine and Chaikoff (15) postulated that the intact tumor failed to respond because the inhibitors, which were active in a cell-free system, were unable to penetrate the malignant cells. They suggested that a similar explanation could be true for the failure of feedback inhibition of cholesterol biosynthesis; our results clearly support their hypothesis.

Lack of cholesterol feedback inhibition is found in the apparently normal livers of mice from strains in which there is a high incidence of spontaneous hepatoma; a similar abnormality is found in livers of rainbow trout and rats which have been fed the potent carcinogen aflatoxin (10). The finding that premalignant liver tissues fail to suppress cholesterol synthesis in response to cholesterol feeding led Siperstein to suggest that an intracellular deletion of cholesterol feedback control might be an important etiological factor in carcinogenesis. Such a concept is clearly of great importance; however, no data has yet been presented that premalignant liver tissue can take up and store dietary cholesterol normally. Our finding that these functions are impaired in malignant liver tissue removes the need to postulate that the loss of cholesterol feedback inhibition is due to an intracellular abnormality. The same may be found to be true for the premalignant liver. Clearly, further evidence is needed before we can accept with confidence either that hepatomas have suffered an intracellular deletion in cholesterol feedback control or that such a lesion is of importance in the development of the malignant state.

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