Biochem. J. (1989) 258, 421–425 (Printed in Great Britain)

A discoordinate increase in the cellular amount of 3-hydroxy-3-methylglutaryl-CoA reductase results in the loss of rate-limiting control over cholesterogenesis in a tumour cell-free system

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Cholesterol biosynthesis was characterized in cell-free post-mitochondrial supernatant systems prepared from both normal rat liver and Morris hepatoma 3924A. The rate of cholesterol synthesis per cell was 9-fold greater in the tumour system than in that from normal liver, and the tumour systems showed the loss of rate-limiting control at the hydroxymethylglutaryl-CoA reductase (HMGR)-catalysed step. The apparent absence of rate-limiting control over cell-free tumour cholesterogenesis was traced primarily to a discoordinate and dramatic increase in the amount of HMGR in the tumour relative to the liver system. Preliminary evidence for an altered control of the post-lanosterol portion of the pathway was also obtained with the tumour system.

INTRODUCTION

Cholesterol is synthesized *de novo* in the cytoplasmic compartment of mammalian cells via a branched pathway which is mandatory for cell maintenance and proliferation ([1,2]; reviewed in [3]). With normal hepatocytes and most other cells, the rate of sterol synthesis is under negative regulation by cellular cholesterol concentrations. In hepatomas and other tumours propagated *in vivo*, unlike normal cells, the negative-feedback regulatory mechanism for cholesterogenesis *de novo* seems to be defective, and in rapidly proliferating tumours sterol synthesis appears to be a continuous process [4–6]. As a result, a significant cholesterol enrichment has been observed in various membranes of tumour cells. Furthermore, in liver, the deregulation of the cholesterol-synthesis pathway has been shown to occur shortly after the dietary administration of hepatocarcinogens [4]. Thus an alteration in the regulation of this pathway might be an important aspect of the mechanism of carcinogenesis.

In normal cells, regulation of cholesterogenesis occurs via several mechanisms, including feedback inhibition by serum-supplied cholesterol of the pathway's major rate-controlling enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGR), at the level of transcription [7,8]. A second, short-term, mode of regulation relies on the reversible activation of HMGR by a cyclic-AMP-independent phosphorylation–dephosphorylation cascade scheme [9]. Previous studies with cell-free as well as whole-cell systems have implied that the rate-limiting properties of the HMGR-catalysed reaction impose a tight control over the flux of carbon delivered to the remaining post-HMGR segment of the cholesterogenic pathway [9,10]. Since the activity of the set of enzymes responsible for generating HMG-CoA from acetyl-CoA (HMG-CoA synthase and acetoacetyl-CoA thiolase) is modulated in concert with changes in HMGR activity, the rate-limiting control over this pathway in normal tissues always remains at the HMGR-catalysed step [11,12]. Yet the underlying cause of the apparent lack of feedback inhibition of cholesterogenesis in tumours remains uncertain [13], and a controversy continues to exist as to whether the regulation of cholesterogenesis at the level of HMGR is altered in tumours.

In the present studies we utilized a lipid-synthesizing, cell-free post-mitochondrial supernatant system (PMS) to characterize the cholesterogenic pathway in Morris hepatoma 3924A and normal liver. These refined PMS preparations have permitted us to examine some of the controlling elements of this critical pathway in the absence of any direct influences of extracellular cholesterol and affiliated plasma-membrane-related phenomena. Our data conclusively demonstrate that the rate-limiting step of the liver cholesterogenic pathway is absent from the tumour system, probably because each tumour cell possesses at least 10 times the amount of immunoprecipitable HMGR. We also noted a small but significantly altered flux control over the post-lanosterol segment of tumour cholesterogenesis relative to normal liver.

EXPERIMENTAL

Materials

All radioisotopes were obtained from Amersham (Arlington Heights, IL, U.S.A.). Dowex AG1X-8 (formate form) resin and gel-electrophoresis supplies were from Bio-Rad (Richmond, CA, U.S.A.). All reagents and enzymes were obtained commercially and used without modification. Monospecific antiserum against rat HMGR was generously given by Dr. Gene Ness of the University of South Florida School of Medicine.

Animals and tissues

Male ACI rats (200–300 g; Harlan Sprague–Dawley, Indianapolis, IN, U.S.A.) were fed on rat chow (5012;

Abbreviations used: PMS, post-mitochondrial supernatant; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; PAGE, polyacrylamide-gel electrophoresis.

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Purina, St. Louis, MO, U.S.A.) *ad libitum* and were housed for at least 10 days under a 12 h-light/12 h-dark cycle before experiments. Morris hepatoma 3924A was maintained as described in ref. [14]. Rats were killed by decapitation at 6 h of the dark phase: both the microsomal HMGR and cholesterogenic activities of liver tissue are at peak diurnal values at this point (see [9]).

Preparation of cell-free, mitochondria-free lysates

Liver or hepatoma 3924A was homogenized in 4 vol. (ml/g) of Buffer A (110 mM-mannitol, 70 mM-sucrose, 100 mM-sodium phosphate, 5 μg of leupeptin/ml, 5 μg of pepstatin/ml, 60 mM-nicotinamide, 10 mM-$MgCl_2$, pH 7.5) with a tight-fitting glass/Teflon homogenizer. The homogenate was centrifuged twice at 12000 *g* (15 min, 4 °C). Both liver and tumour PMS preparations contained minimal mitochondrial enzyme contamination, as determined by assessing fumarate hydratase activity [15]. In addition, mitochondrial HMG-CoA lyase activity (EC 4.1.3.4) was below detectable values in both tumour and liver PMS preparations, as determined enzymically [16]. PMS systems were supplemented with a cofactor and substrate cocktail (1 μmol each of $NADP^+$ and NAD^+, 3 μmol of glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, 3 μmol of ATP, 0.5 μmol of GSH, 0.25 μmol of CoA) in tightly capped vials, which were flushed with O_2/CO_2 (19:1) and preincubated for 5 min at 37 °C. Then [1,5-^{14}C]citrate, [1-^{14}C]acetate or [2-^{14}C]mevalonate (1.5 μCi/ml) was added at a final concentration of 10 mM. Incubations were carried out for a total of 4 h at 37 °C, and were supplemented at 1.5, 2.5 and 3.5 h with an additional 3 μmol of glucose 6-phosphate/ml and at 2.5 h with an additional 3 μmol of ATP/ml. Liver and tumour PMS protein content was determined as described by Bradford [17].

Lipid extraction and analysis

Reactions were terminated by rapidly cooling the vial contents. Portions (1 mg each) of carrier cholesterol and lanosterol were added, together with 1 nCi of [^{3}H]cholesterol to correct for extraction efficiency. The lipid and aqueous components were separated as described in ref. [18]. Saponification of lipid extracts and digitonin precipitation of sterols, as well as the extraction of fatty acids, were performed as described in ref. [19]. Incorporation of ^{14}C or ^{3}H into sterols was determined by liquid-scintillation counting.

Carboxylic acid analysis

The aqueous fraction derived from the lipid extractions was treated with KOH and then acidified in order to cleave CoA esters and to lactonize mevalonate, respectively. Intermediates were resolved on a Dowex AG1X-8 (formate form) ion-exchange column by elution with a 0–3 M-formate linear gradient. The identity of each resolved peak was determined by reference to relative elution volumes of commercial radioactive carboxylic acid standards. The acetoacetate peak was identified enzymically [20].

Immunoprecipitation and SDS/PAGE analysis

Tumour and liver microsomal fractions were prepared as described in ref. [21]. Microsomal pellets, representing 4×10^9 liver or tumour cells, were resuspended in incubation buffer (0.5% Nonidet P-40/0.15 M-NaCl/20 mM-Tris, pH 8, containing the proteinase inhibitors leupeptin, aprotinin and pepstatin at 5 μg/ml each, and 0.1 mM-phenylmethanesulphonyl fluoride). Immunoprecipitations were performed as described in ref. [22], except that the incubation volume and contents were increased 5-fold. The washed pellets were dissolved in PAGE sample buffer and resolved on SDS/PAGE (10%-acrylamide) gels [23]. The relative intensity of the Coomassie Blue-stained band corresponding to the purified HMGR (97 kDa) was quantified by laser densitometry (LKB Ultroscan XL 2222).

Radioisotope-incorporation data as well as microsomal isolation data were normalized per cell, based on the DNA assay [24] of a unit wet weight of tumour and normal liver tissue, and, separately, of a known number of dispersed cells [25] from each tissue.

RESULTS

Lipogenesis in cell-free PMS systems

The PMS systems from both normal rat liver and Morris hepatoma 3924A were able to catalyse cholesterol synthesis from [^{14}C]citrate at linear rates for at least 4 h [liver PMS 1.07 ± 0.18; tumour PMS 10.98 ± 0.98 (nmol of C_2 incorporated/h per 10^9 cells; $n = 8$)], suggesting the validity of the measurements as indicators of true relative pathway activities. For further verification we tested both PMS systems with [^{14}C]acetate (by-passing the ATP citrate-lyase step), and also by using non-radiolabelled acetate or citrate in buffer containing ^3H_2O. All incubation conditions yielded virtually identical rates of C_2 (or ^{3}H) incorporation into cholesterol with each individual PMS system (results not shown). These results indicated that the tumour PMS promoted a 9-fold greater cholesterogenic activity than the normal liver PMS per cell, and they confirm previous long-standing data illustrating the enhanced rate of cholesterogenesis measured in tumour whole-cell preparations [6].

Comparative PMS incubations with acetate and mevalonate as substrates for cholesterogenesis

To determine whether a rate-limiting control site(s) could be demonstrated within the early stages of the cell-free cholesterogenic pathway with either system, acetate and mevalonate were employed independently as lipogenic substrates. With [2-^{14}C]mevalonate, PMS systems from normal liver accumulated digitonin-precipitable sterols at a 6-fold greater rate than when fuelled by [1-^{14}C]acetate (Table 1*a*). Such data imply that in the cell-free normal liver system a rate-limiting site persists between acetate and mevalonate. In contrast, sterol synthesis in the tumour-driven PMS system was identical whether acetate or mevalonate was the cholesterogenic substrate (Table 1*a*). Furthermore, the rate of sterol synthesis from mevalonate onward was similar in both tumour and normal systems, indicating that the rate-controlling characteristics of cholesterogenesis, between acetyl-CoA and mevalonate, are retained by the normal liver, but are absent from the tumour PMS system, again supporting previous observations derived from whole-tissue studies [13].

Carbon flux through early cholesterogenesis

Aqueous fractions generated from the lipid extractions contained the early intermediates of cholesterogenesis. These intermediates were resolved and quantified via

Table 1. Incorporation of ^{14}C from acetate or mevalonate into (*a*) digitonin-precipitable sterols and (*b*) non-saponifiable isoprenoids with normal liver and tumour PMS systems

Liver and tumour PMS were supplemented with 10 mM-[1-^{14}C]acetate or -[2-^{14}C]mevalonate as indicated in the Experimental section. Calculation of ^{14}C incorporation into digitonin-precipitable sterols (*a*) was normalized according to the number of ^{14}C atoms from each substrate: 12 derive from [1-^{14}C]acetate; 5 arise from [2-^{14}C]mevalonate [35]. T.l.c. on silica gel H preparative plates to resolve non-saponifiable isoprenoids (*b*) was performed as described in ref. [36]. The R_F values were: cholesterol = 0.18; lanosterol = 0.32; squalene = 0.74. All values represent means ± S.E.M. from four PMS preparations from each tissue source. Statistical significance was calculated by Student's *t* test: *not significant; **$P \leqslant 0.05$; ***$P \leqslant 0.01$.

	Incorporation (c.p.m./2 h per 10^9 cells)	
	Liver	Tumour
(*a*) ^{14}C source		
[1-^{14}C]Acetate	613 ± 64	5650 ± 531***
[2-^{14}C]Mevalonate	4091 ± 348	5327 ± 491*
Difference (%)...	570	6
(*b*) ^{14}C-labelled component		
Total non-saponifiable isoprenoid	6706 ± 710	7312 ± 678*
Cholesterol	3748 ± 322	4971 ± 475**
Lanosterol	1690 ± 228	1079 ± 100**
Squalene	67 ± 7	73 ± 8*

anion-exchange chromatography. The incorporation of acetyl units into each intermediate remained constant during the 4 h incubation for each tissue system independently (results not shown). However, the steady-state amounts of these early lipogenic intermediates differed between normal and tumour systems (Fig. 1*a*). The normal liver PMS displayed high steady-state amounts of HMG-CoA and relatively low amounts of mevalonate. The tumour system, in contrast, yielded the reverse pattern. Indeed, the percentage C_2 incorporation into mevalonate was more than 10-fold higher in the tumour than in the liver. Fig. 1(*b*) displays this comparison as a positive cross-over point between HMG-CoA and mevalonate for the tumour relative to the liver PMS. Thus, compared with normal liver, the control of cell-free carbon flux through the cholesterogenesis pathway appears to be intrinsically altered in the hepatoma system.

Accumulation of several other early pathway intermediates was also monitored in the two systems (Fig. 1*a*). High steady-state amounts of acetyl-CoA (measured as acetate) were observed in the liver system; the tumour, in contrast, displayed relatively low amounts. Acetoacetyl-CoA (measured as acetoacetate) was not significantly different in amount in the two systems.

Relative amounts of microsomal HMGR protein in liver and tumour

The basis for the observed discoordinate increase in HMGR activity in the tumour system (Table 1*a* and Fig.

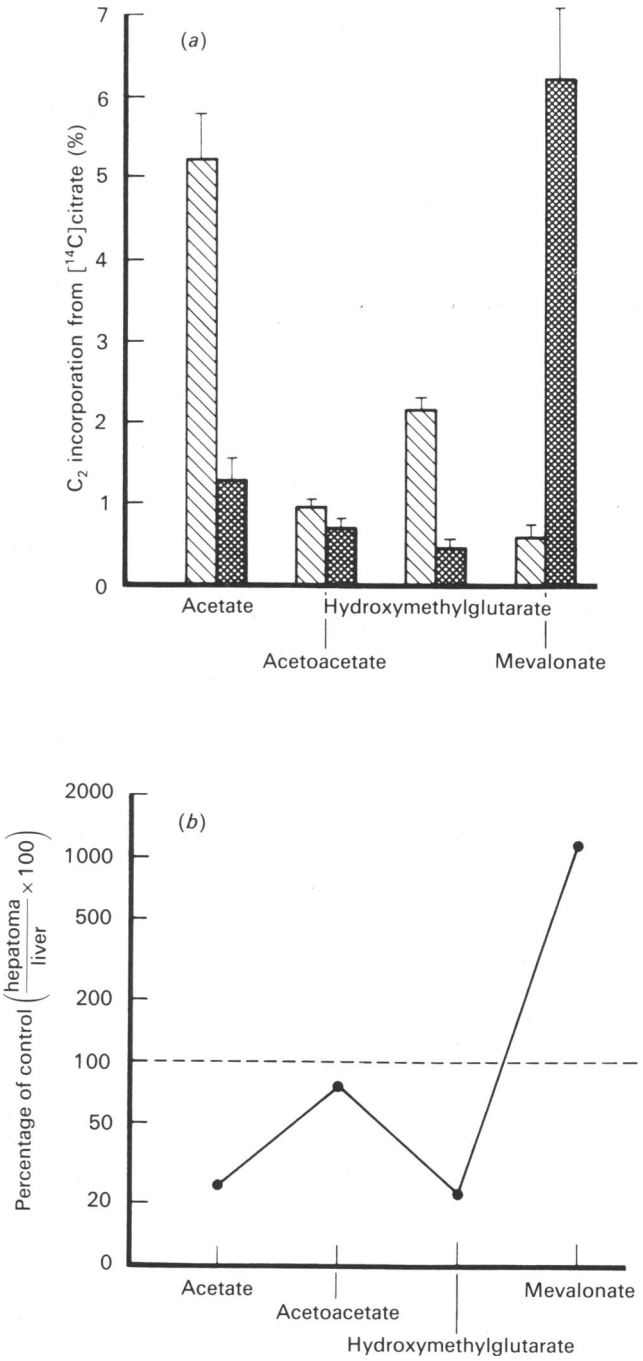

Fig. 1. Steady-state pattern (*a*) and cross-over plot (*b*) of C_2 incorporation into early sterologenic intermediates by liver and tumour PMS systems

Liver and tumour PMS systems were incubated with 10 mM-[1,5-^{14}C]citrate, and C_2 incorporation was determined as in the Experimental section. That steady-state flux had been achieved was ascertained by sampling incubations at 2, 3 and 4 h in separate experiments. Data were normalized according to the number of cells represented by each PMS system. Part (*a*) is expressed as the percentage of total (sterol plus fatty acid) lipid synthesis (C_2 units incorporated from [^{14}C]citrate into the intermediates shown). Part (*b*) expresses the incorporation data of part (*a*) as a cross-over plot, normalized with the normal liver system as 100% (dashed line). Each value represents the mean ± S.E.M. for nine preparations from tumour (▩) and normal liver (▧).

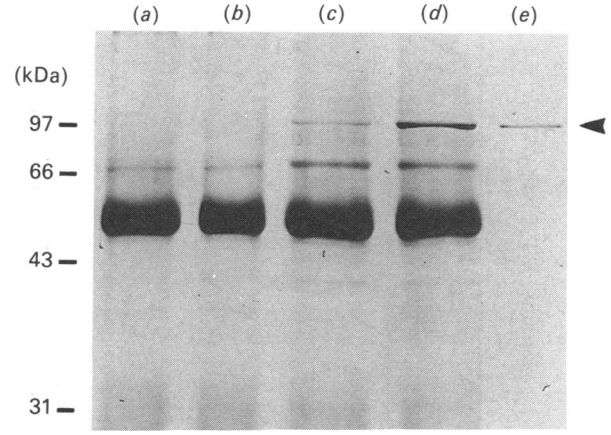

Fig. 2. SDS/PAGE analysis of liver and tumour microsomal HMGR immunoprecipitates

Microsomal fractions were isolated from both normal ACI rat liver and Morris hepatoma 3924A, and immunoprecipitation was performed as described in the Experimental section. Microsomal fractions representing an equivalent number (4×10^9) of liver (lanes *a* and *c*) or hepatoma (lanes *b* and *d*) cells were immunoprecipitated either with normal rat serum (lanes *a* and *b*) or with anti-(rat HMGR) serum (lanes *c* and *d*). Migration of purified detergent-solubilized HMGR (according to [26]) is shown in lane (*e*). The Coomassie Blue stain intensity of the 97 kDa immunoprecipitate (arrow) was quantified by laser densitometry. Relative percentage staining intensities for tumour and liver enzymes were 100 and 9.7 respectively. The bands resolved at lower molecular mass correspond to serum albumin (66 kDa) and the immunoglobulin heavy chain (53 kDa).

1*b*) was clarified by assessing the relative cellular amounts of this enzyme in both systems. Fig. 2 displays the SDS/PAGE pattern of immunoprecipitated normal liver microsomal HMGR and that from the Morris hepatoma. The 97 kDa band corresponding to purified ACI-rat liver HMGR [26] is present in microsomal preparations derived from both tissue sources. Densitometric scans of the gels revealed, however, a 10-fold greater accumulation of this protein in microsomal fractions derived from the tumour, when the same number of cells from each tissue source was used for the immunoprecipitation (see the Experimental section). Consequently, the dramatic enhancement of carbon flux in the cell-free tumour system through the otherwise rate-controlling locus of the cholesterogenic pathway appears to be due to the much greater amount of tumour-cell HMGR.

T.l.c. analysis of non-saponifiable lipids

The non-saponified lipid extracts from the two PMS incubations with [2-^{14}C]mevalonate as substrate were resolved by t.l.c. Table 1(*b*) shows that, per cell, both liver and tumour systems yielded similar degress of ^{14}C incorporation into the total non-saponifiable-lipid fraction. The tumour system, however, displayed a 33% greater accumulation of cholesterol but a 36% lower production of lanosterol than the normal liver system. On the basis of total β-hydroxy sterol synthesis from mevalonate measurable by digitonin precipitation, therefore, both tumour and normal systems generate virtually identical amounts of sterol (Table 1*a*). Nevertheless, resolution of the individual β-hydroxy sterol intermediates shows that the tumour system displays significant differences in the control of post-lanosterol sterologenesis relative to normal liver. This suggests that any rate-regulating elements normally present in this latter segment of the liver pathway [27] are at least somewhat diminished in the tumour. Incorporation of ^{14}C into the pre-cyclic terpene, squalene, was the same for both systems, however.

DISCUSSION

This study provides the first demonstration of the lack of synthetic flux control at the HMGR site of cholesterogenesis in a cell-free tumour-derived system. It is important to note that the retention of rate-limiting flux control in the normal liver PMS occurs despite the preparation having been obtained at the diurnal peak of liver cholesterogenesis, when HMGR activity is highest (results not shown; see [9]). Thus, in normal tissue cell-free systems, under citrate-saturating (10 mM) conditions, activities of other early pathway enzymes are regulated coordinately with that of HMGR, allowing the latter enzyme to maintain a carbon-flux bottleneck manifest by high steady-state concentrations of HMG-CoA (Fig. 1*a*). The tumour system, however, displays no flux-controlling pattern at this locus under the same conditions, and the positive cross-over point at this site (Fig. 1*b*) indicates intrinsic decontrol of the cholesterogenesis pathway in the tumour. It should be emphasized, however, that the regulation of cholesterogenesis in intact cells and tissues is subject to a higher degree of complexity than that manifest in substrate-saturated cell-free systems. Thus Gibbons *et al.* [28] showed that cholesterogenic flux in intact hepatocytes depends also on the cellular concentration of HMG-CoA, the substrate of HMGR, and this, in turn, is a function of the pathway's accessibility to (and sterologenic utilization of) extramitochondrial acetyl-CoA. Interpretation of the results of Figs. 1(*a*) and 1(*b*) therefore can only be cautiously extended to systems *in vivo*.

Nevertheless, the data of Fig. 2 lend strong support for assigning the cause of this pathway decontrol in the cell-free tumour system to a dramatically greater accumulation of cellular HMGR protein in the hepatoma cell. This suggests tht specific synthetic and degradative mechanisms responsible for maintaining the cellular amount of this strategic enzyme are regulated differently in the tumour relative to normal liver. Furthermore, as indicated above, the overall C_2 flux control of cholesterogenesis would depend also on precursor substrate (e.g. citrate) availability in the cytoplasm. Interestingly, the increased capacity for tumour mitochondrial citrate efflux in Morris hepatoma 3924A [29], thereby augmenting cytosolic citrate concentrations [14,30], would contribute to the tumour's capacity for continuously high rates of cholesterogenesis. In this regard, we have noted that, relative to the normal liver PMS, the tumour system exhibited a much higher ATP citrate-lyase activity (results not shown). Consequently, the persistence of such intrinsic characteristics regarding tumour cholesterogenesis would help to explain the apparent loss of feedback inhibition by serum-derived sterols, a well-established hallmark of neoplastic systems *in vivo* [31,13].

There is also evidence for an alteration in flux control in the tumour's post-lanosterol segment of the pathway

(Table 1*b*), suggesting that rate-limiting elements at the later steps of the pathway may also be compromised in the tumour. A relationship *in vivo* between the rate of lanosterol–cholesterol conversion and the activity of HMGR has been recently described [32], which strengthens the belief that the generation of oxysterols, via post-lanosterol metabolism, and the regulation of mevalonate production are integrally linked. Further study is necessary to characterize regulation of these distal reactions in tumours.

Many collateral investigations have implied that an operational cholesterol-biosynthesis pathway is requisite for cell proliferation. Recent evidence has indicated that a non-sterol mevalonate-derived metabolite produced by this pathway is required by the cell before it can enter the S-phase of the cell cycle [33,34]. In view of such findings, the intrinsically decontrolled carbon flux through tumour cholesterogenesis as described here could result in a discoordinate and dramatically elevated synthesis of mevalonate, thereby providing for the production of these non-sterol metabolites in tumours.

This work was supported by grant CA 28677 from the U.S. Public Health Servide to P.S.C.

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Received 7 July 1988/7 December 1988; accepted 21 December 1988