Diminished expression of phosphatidylethanolamine *N*-methyltransferase 2 during hepatocarcinogenesis

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Phosphatidylethanolamine *N*-methyltransferase (PEMT) is a liver-specific enzyme that converts phosphatidylethanolamine into phosphatidylcholine. At least two forms of PEMT are present in hepatocytes. However, PEMT activity is negligible in two hepatoma cell lines. Previous studies have indicated an inverse relationship between the expression of one form, PEMT2, and the rate of liver growth, suggesting that this enzyme might be involved in inhibition of hepatocyte proliferation. We have now investigated the expression of PEMT2 at various stages of hepatocarcinogenesis induced by chemical carcinogens. Expression of PEMT2 protein was decreased in liver samples that contained the first detectable proliferative lesions. At later stages of carcinogenesis, PEMT2 expression was obliterated. PEMT activity decreased, the levels of PEMT2 mRNA decreased and there was an increase in the activity of CTP:phosphocholine cytidylyltransferase, a key regulatory enzyme in the CDP-choline pathway of phosphatidylcholine biosynthesis. Southern blot analyses of restriction fragments of DNA showed no changes in the PEMT gene in hepatocarcinoma compared with normal liver. A role for PEMT2 in the control of hepatocyte proliferation remains an intriguing possibility.

Key words: CTP:phosphocholine cytidylyltransferase, diethylnitrosamine, hepatoma, phosphatidylcholine, phosphatidylethanolamine.

INTRODUCTION

Phosphatidylethanolamine *N*-methyltransferase 2 (PEMT2) is one of two isoforms of PEMT in liver that converts phosphatidylethanolamine (PE) into phosphatidylcholine (PC) by addition of three methyl groups. Several studies have demonstrated an inverse relationship between the growth of hepatocytes and the expression of PEMT2 [1–6]. These results suggested the possibility that PEMT2 might have a role in hepatic cell division and might even be a liver-specific tumour suppressor. PEMT2 is an unexpected candidate for a tumour suppressor, since this enzyme has not previously been linked to control of the cell cycle.

PEMT2 is a liver-specific protein that localizes exclusively to mitochondria-associated membranes of hepatocytes [7]. PEMT1 is the major PE methylation activity in liver, and is located on the endoplasmic reticulum [7]. PEMT2 has been purified [8], the cDNA cloned and expressed [8], the gene isolated and characterized [9], and the gene disrupted in mice [10]. Despite the survival of PEMT in liver during evolution, the conversion of PE into PC is not normally required, since all eukaryotic cells make PC via the CDP-choline pathway, which appears to be essential for animal cell survival [11–13].

The CDP-choline pathway for PC synthesis seems to be highly synchronized with hepatocyte proliferation [1–6]. In contrast, the expression of the PEMT2 gene is inversely correlated with the rate of liver proliferation in several experimental systems [1–6]. PEMT activity and PEMT2 protein are absent from rapidly growing embryonic livers, but present in post-natal livers when the growth rates decline [3,4]. Consistent with these observations, PEMT activity and PEMT2 protein are transiently diminished when the growth of adult rat liver is re-initiated after partial hepatectomy [2]. These observations suggest that PE methylation, or PEMT2, might be a negative regulator for cell division of hepatocytes. Moreover, PEMT activity and PEMT2 are absent from two hepatoma cell lines (McArdle RH7777 and HepG2) that divide rapidly [7]. The most compelling evidence that PEMT2 is involved in the suppression of hepatocyte division came from the expression of PEMT2 in McArdle RH7777 cells [1,6]. When PEMT2-transfected cell lines of RH7777 were isolated and characterized, a strong correlation was observed between the expressed PEMT2 level and inhibition of cell division [1].

To explore further a possible link between PEMT2 and hepatocyte growth, we hypothesized that the stepwise process of hepatocarcinogenesis might be accompanied by diminished expression of PEMT2. The present study was designed to characterize PEMT2 gene expression in two carcinogen-induced models of liver carcinogenesis. The results show that PEMT2 gene expression was diminished during liver carcinogenesis. This observation is consistent with a possible role for PEMT2 in the suppression of liver proliferation, and perhaps in hepatic carcinogenesis.

MATERIALS AND METHODS

Materials

[6-^{3}H]Thymidine (20 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). ECL® kits for immunoblots, [^{3}H-*methyl*]choline and [^{3}H-*methyl*]*S*-adenosylmethionine were purchased from Amersham. Diethylnitrosamine, methylnitrosourea and other chemicals were purchased from Sigma unless otherwise noted.

Animal care

Male Fisher 344 rats (2 months old) were purchased from Charles River (Como, Italy). The rats were acclimatized for 1

Abbreviations used: PEMT, phosphatidylethanolamine *N*-methyltransferase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CT, CTP:phosphocholine cytidylyltransferase.

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week to a balanced semi-synthetic diet (Piccioni, Brescia, Italy), with food and water supplied *ad libitum*, and were maintained in a 12/12 h light/dark cycle.

Induction of liver proliferation and hepatocarcinoma by diethylnitrosamine or methylnitrosourea

In the model of diethylnitrosamine induction [14], rats were injected intraperitoneally with diethylnitrosamine (200 mg/kg) disolved in saline. After 1 week, 2-acetylaminofluorene (0.02 %, w/w) was added to the diets for 2 weeks. Treated rats were subsequently fed on regular chow. In the model of methylnitrosourea induction, a 70 % partial hepatectomy was performed 20 h before a single methylnitrosourea injection (60 mg/kg). After 1 week, acetylaminofluorene (0.02 %, w/w) was added to the diets for 2 weeks. Midway through acetylaminofluorene feeding, tetrachloromethane (2 ml/kg) was added to diets. The rats were subsequently fed on regular chow. Controls were normal adult rats fed a chow diet. At various times after initiation (1, 4, 6, 8, 12 and 16 months), visible liver lesions were dissected and homogenized in a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 μM PMSF and 1 mM dithiothreitol.

Immunoblot analysis of PEMT2

Samples of 50 μg of protein from total homogenates were separated on 12.5 % (w/v) polyacrylamide gels containing 0.1 % (w/v) SDS and transferred to nitrocellulose membranes. The membranes were probed with a specific antibody against rat liver PEMT2 [7] and visualised by ECL.

DNA labelling with [^{3}H]thymidine

[^{3}H]Thymidine (500 μCi/kg) was injected into rats intraperitoneally 1 h before they were killed. DNA was extracted from the collected samples of liver and quantified by absorbance at 260 nm.

Northern blot analyses

Total cellular RNA was extracted from liver samples by the guanidinium thiocyanate/phenol/chloroform extraction procedure [15]. The poly(A) mRNA was enriched by passage of the total extract over an oligo(dT)–cellulose column [16]. A sample of 5 μg of poly(A) mRNA was electrophoresed on a 1 % (w/v) agarose/formaldehyde gel and transferred to nitrocellulose membranes by capillary transfer in 10 × SSC (15 mM NaCl/1.5 mM sodium citrate, pH 7.0) overnight. The membrane was baked, pre-hybridized and hybridized by the Rapid-hyb method (Amersham Life Science). A random-primed DNA labelling kit (Pharmacia P-L Biochemicals) was used to label the PEMT2 probe with [^{32}P]dCTP. The membranes were also probed with a glyceraldehyde-3-phosphate dehydrogenase cDNA as an internal control.

Southern blot analyses

Samples of 10 μg of DNA were digested at 37 °C overnight with *Eco*RI, *Bam*HI or *Hin*dIII. DNA was loaded on to a 0.7 % (w/v) agarose gel and electrophoresed overnight. The DNA was transferred to Genescreen Plus (du Pont) according to the manufacturer's instructions. Hybridization was performed with Ready-To-Go (Amersham) using [^{32}P]dCTP-labelled rat cDNA for PEMT2. The filter was washed and exposed to Amersham Hyperfilm for 2 days.

Enzyme assays

Assays for CTP:phosphocholine cytidylyltransferase (CT) activity [17] and PEMT activity [18] were described previously.

RESULTS

Staged samples of liver tumorigenesis were compared with normal livers for the level of PEMT2 protein expression. Liver tumour development can be divided into three major sequential stages [19]: (i) initiation (exposure to carcinogen and DNA damage); (ii) promotion (development of focal lesions and nodules); and (iii) progression (development of hepatoma and metastases). Equal amounts of total cellular protein from staged samples were separated on SDS/polyacrylamide gels and stained with Coomassie Blue (Figure 1, upper panels). This overall profile of cellular proteins served as a control and demonstrated pronounced changes in protein profiles in late stages of carcinogenesis. However, the profile of cellular proteins in foci was

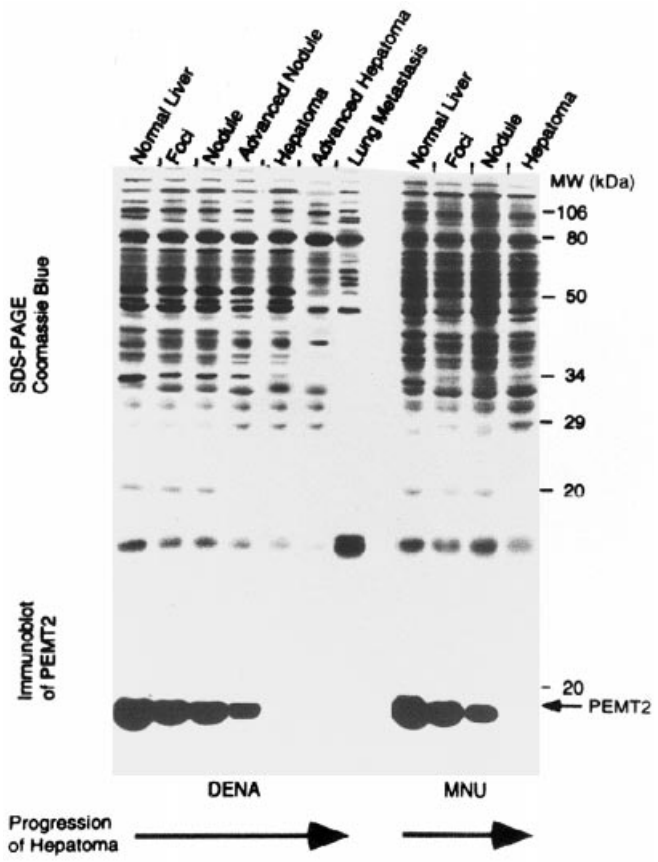

Figure 1 Immunoblot analysis of PEMT2 in proliferative lesions of liver tumorigenesis

Male Fisher 344 rats (2 months old) were acclimatized for 1 week to a balanced semi-synthetic diet, with food and water supplied *ad libitum* and a 12/12 h light/dark cycle. Tumour induction by carcinogens has been described previously [14]. Samples of 50 μg of protein were separated on 12.5 % (w/v) polyacrylamide gels that contained 0.1 % SDS. The gels in the upper panels were stained with Coomassie Blue. The lower panels are immunoblots of similar gels probed with an antibody against rat liver PEMT2. DENA, diethylnitrosamine; MNU, methylnitrosourea. The experiment was repeated three times with similar results. Scanning of the gels with a Storm 840 phosphorimager indicated that the relative intensities of the bands for PEMT in the diethylnitrosamine-treated rats were 1.0 (normal liver), 0.84 (foci), 0.76 (nodule) and 0.40 (advanced nodule). The relative instensities for PEMT in the methylnitrosourea-treated rats were 2.5 (normal liver), 0.84 (foci) and 0.40 (nodule).

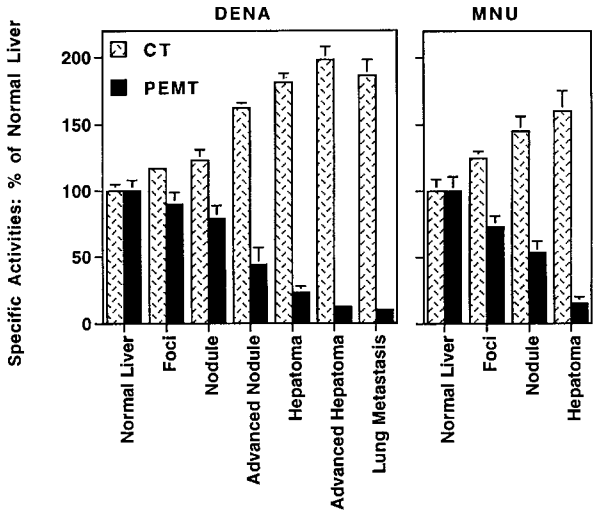

Figure 2 Activities of CT and PEMT at various stages of liver tumorigenesis

The same samples described in the legend to Figure 1 were assayed for enzymic activities of PEMT and CT. Results are means ± S.D. of four samples. The experiment was repeated with similar results. The 100% activity for PEMT2 was 0.87 nmol/min per mg of protein, and that for CT was 2.7 nmol/min per mg of protein. DENA, diethylnitrosamine; MNU, methylnitrosourea. There was no statistical difference between normal liver and foci for animals treated with diethylnitrosamine. The difference in PEMT activity in diethylnitrosamine-treated rats between nodules and normal liver had a P value of < 0.01. All other differences in enzyme activites between normal liver and tumour samples had P values of < 0.005.

Table 1 Incorporation of [^{3}H]thymidine into DNA of normal liver and of proliferative lesions at various stages of hepatocarcinogenesis

At 1 h before they were killed, [^{3}H]thymidine (500 μCi/kg) was injected intraperitoneally into normal rats and rats in which liver tumours had been induced to various stages. Proliferative lesions were dissected, DNA was extracted and quantified by measurement of light absorbance at 260 nm, and radioactivity incorporated was determined by liquid scintillation spectrometry. Data are means ± S.D. from five rats.

Liver sample	$10^{-3} \times {}^3$H incorporation (d.p.m./h per mg of DNA)
Normal liver	35.7 ± 8.2
Nodules	67.3 ± 11.2
Advanced nodules	73.5 ± 12.2
Hepatomas	81.6 ± 10.2

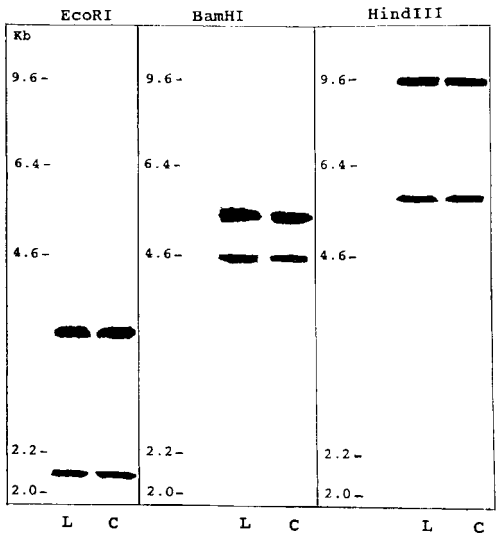

Figure 3 Southern blot analysis of the PEMT2 gene in hepatocellular carcinoma

Restriction fragments were produced from DNA obtained from normal liver (L) or hepatocellular carcinoma (C) using PEMT2 cDNA as a probe. DNA from normal liver samples or hepatocellular carcinomas was digested with the indicated enzymes. The experiment was repeated twice with identical results.

similar to that in normal liver, except in the region of 34 kDa, where some differences were apparent.

Proteins from the samples were subjected to immunoblot analysis with a specific antibody against rat liver PEMT2 [7] (Figure 1). In the diethylnitrosamine model (left panel), we detected a decrease in PEMT2 in liver samples with foci, the first identifiable lesions of hepatic carcinogenesis. The amount of PEMT2 protein was decreased by approx. 60% in advanced nodules, and PEMT had completely disappeared in hepatoma, advanced hepatoma and lung metastases. In the methylnitrosourea model (Figure 1, right panel), the decrease in PEMT2 was similar. Possibly, the gene for PEMT2 or its expression was targeted by both mutagens. If inactivation of a tumour suppressor were to play a role in initiating liver cancer, either by a decrease in the steady-state level of the suppressor or by eliminating its function, this would occur early in tumour development. The decrease in PEMT2 in liver samples with foci, prior to hepatoma development, is consistent with this criterion.

The impact of PEMT2 inactivation on overall PE methylation capacity in the various stages of tumour development was evaluated by assessment of the activity of PEMT (which includes PEMT2 and PEMT1) in homogenates (Figure 2). The decreases in PEMT2 protein shown in Figure 1 were consistent with decreases in total PEMT activity during the various stages of tumour progression. Since PEMT1 has not been purified, nor the cDNA cloned, the role of PEMT1 in tumour development cannot be defined at this time. However, since the activity of PEMT was 50% less in advanced nodules and 85% less in advanced hepatoma and lung metastases than in control liver (Figure 2), it appears that PEMT1 is also inactivated, but at a later stage in tumour development.

Most PC in liver is synthesized via the CDP-choline pathway [20], which is usually regulated by the activity of CT [21–23]. Because of the inverse relationship between PEMT2 and CT activity in various models [1–6], we postulated that the decreased expression of PEMT2 in liver tumours might coincide with the activation of CT. CT activity increased progressively throughout tumour development, with the activity almost doubling in the later stages (Figure 2). The increase in CT activity was nearly a mirror image of the decrease in PEMT activity during carcinogenesis, suggesting a reciprocal relationship between PEMT and CT activities, similar to that observed in other models [1–6]. The results with CT also serve as a control demonstrating that the inactivation of PEMT2 did not result from a general decrease in protein expression during early stages of liver proliferation.

The rate of DNA synthesis in the proliferative lesions of liver carcinogenesis was estimated by measuring the incorporation of [^{3}H]thymidine into DNA. The incorporation of [^{3}H]thymidine was increased by 86% in nodules and by 125% in hepatomas compared with normal liver (Table 1). This progressive increase in DNA synthesis was consistent with the degree of malignancy and the decreased expression of PEMT2.

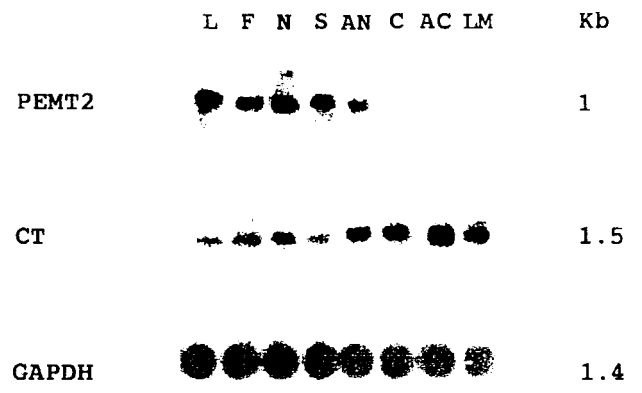

Figure 4 Northern blot analysis of mRNA at various stages of hepatocarcinogenesis

mRNA was prepared as described in the Materials and methods section. Samples were then probed with end-labelled cDNAs for PEMT2, CT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The experiment was repeated twice with similar results. L, normal liver; F, proliferative focal lesions; N, nodules; S, liver samples surrounding the nodules; AN, advanced nodules; C, hepatocellular carcinoma; AC, advanced hepatocellular carcinoma; LM, lung metastases. The figure was scanned with a Storm 840 phosphorimager, and the relative intensities for PEMT/GAPDH were 1.0 (normal liver), 0.89 (proliferative focal lesions), 1.07 (nodules), 0.81 (liver samples from surrounding nodules) and 0.37 (advanced nodules). The relative intensities for CT/GAPDH were 1.0 (normal liver), 1.6 (proliferative focal lestions), 1.5 (nodules), 0.9 (liver samples from surrounding nodules), 6.0 (advanced nodules), 7.1 (hepatocellular carcinoma), 7.2 (advanced hepatocellular carcinoma) and 9.2 (lung metastases).

The above data are consistent with the proposal that PEMT2 might have a tumour-suppressor role in liver. If this were true, we might expect that initiation of hepatocarcinogenesis would involve disruption of the gene that encodes PEMT2 [24,25]. Thus DNA was isolated from normal liver and hepatocarcinoma, digested with restriction enzymes and Southern analyses performed (Figure 3). As the restriction patterns were identical, we could see no evidence for gross disruption of the PEMT2-encoding gene. Obviously, more subtle changes in DNA sequence would not be detected by this technique.

The expression of PEMT2 mRNA was evaluated by PCR and Northern analyses. Reverse transcriptase–PCR indicated the absence of PEMT2 mRNA from hepatocarcinoma samples. Subsequently, the mRNA levels at various stages of hepatocarcinogenesis induced by diethylnitrosamine were studied by Northern analysis. The amount of PEMT2 mRNA was similar in nodules and normal liver, had decreased by approx. 60% in advanced nodules, and was not detected in carcinoma, advanced carcinoma or lung metastases (Figure 4). This decline in PEMT2 mRNA was similar to the decrease in PEMT2 protein in rats treated with diethylnitrosamine (Figure 1). The signal for CT mRNA was enhanced in advanced nodules and in later stages of carcinogenesis compared with that in normal liver and earlier stages of carcinogenesis. However, the apparent enhancement of CT mRNA was much larger than the 2-fold increase in CT activity observed in the later stages of carcinogenesis (Figure 2). The signal for glyceraldehyde-3-phosphate dehydrogenase was similar for all samples, except for diminished expression in lung metastases.

DISCUSSION

Primary liver cancer results in more than 250000 deaths worldwide each year [26]. Chronic infection with hepatitis B virus and exposure to aflatoxin B_1 are primary risk factors. Analysis of liver tumours revealed that certain regions of DNA were deleted [27–29], indicating that specific genes present in normal liver were disrupted in tumours. Therefore, inactivation of a tumour suppressor(s), rather than activation of an oncogene, might be the trigger for liver cancer development. No single gene product has been implicated as a tumour suppressor in liver [27–29]. Retinoblastoma protein and p53 have been linked with many non-hepatic cancers, but mutations in p53 were found in only a small number of liver tumours at late stages [27,30–34]. Inconsistent with p53 being a liver tumour suppressor, many liver tumours contain normal or even elevated levels of p53; moreover, p53 is found in cultured hepatoma cell lines [30].

The results of the present study suggest that a decrease in the expression of PEMT2 might be involved in the development of carcinogen-induced liver cancer in rats. In comparison with the inactivation of tumour suppressors such as p53 and retinoblastoma protein, decreased PEMT2 expression during liver proliferation and carcinogenesis may be unique. For example, p53 is often inactivated by point mutations, and the mutated protein often accumulates in tumour cells [26]. In contrast, PEMT2 is inactivated primarily due to diminished levels of mRNA transcripts and protein. Decreased gene expression of PEMT2 is reversible during partial hepatectomy [2], suggesting that PEMT2 expression can be regulated during proliferation. Although a causal relationship between PEMT2 expression and inhibition of hepatoma cell proliferation has been established for hepatoma cells in culture [1], whether or not inactivation of PEMT2 alone is sufficient to trigger hepatocarcinogenesis remains to be determined. The recent construction of mice that lack PEMT2 [10] should allow us to investigate further the role of PEMT2 in hepatocarcinogenesis. If such PEMT2 knockout mice are more susceptible to chemical induction of liver cancer, a role for decreased PEMT2 in liver carcinogenesis would be strongly implicated. As these PEMT2(−/−) mice have only just become available, it will be some time before we have the answer.

The relationship between PEMT1 and PEMT2 in the carcinogenesis process is not completely defined. However, the present experiments suggest that PEMT1 expression might also be decreased during carcinogenesis. The role of PEMT1 will be better understood when an antibody to PEMT1 becomes available.

The Northern blot analyses of PEMT2 mRNA are consistent with the immunoblot analyses of PEMT2 and with differences in PEMT activity at the various stages of carcinogenesis. Since the Northern blot probe was the cDNA for PEMT2, and since both PEMT1 and PEMT2 are encoded by the same gene [10], it is possible that this probe might detect mRNAs for both forms of PEMT if the enzymes are encoded by different mRNAs. Whether or not the two forms of PEMT result from alternative splicing of a PEMT gene transcript or from post-translational modification is not known. In either case, hybridization of the PEMT2 cDNA to all PEMT mRNA would be likely.

As with any provocative finding, the decreased expression of PEMT2 during liver carcinogenesis raises new questions. Could a decrease in PEMT2 expression permit the growth of liver tumours? Evidence that there is something unique in relation to cell division about PC derived from PEMT2, compared with PC derived from CDP-choline, came from studies with mutant Chinese hamster ovary cells that have a temperature-sensitive defect for CT and the CDP-choline pathway for PC biosynthesis [12,13]. Overexpression of PEMT2 in these mutant cells maintained PC at normal levels, but failed to rescue these cells from death. In contrast, overexpression of CT allowed survival of the cells. Thus it is possible that PC derived from PEMT2 is inhibitory for the biosynthesis of PC derived from the CDP-choline pathway, which is necessary for hepatocyte growth. Evidence in

support of this proposal was obtained from hepatoma cells transfected with PEMT2 cDNA, which resulted in decreased expression of CT and a slower rate of cell growth [1,6]. Direct *in vivo* evidence for a suppressive role of PEMT (or the PC derived from methylation of PE) on the CDP-choline pathway was obtained from PEMT knockout mice, in which there was a 60% increase in the activity of the membrane-associated form of CT, the rate-limiting enzyme in the CDP-choline pathway [10].

Increased cell division is often accompanied by an increase in CT activity [35,36]. CT gene expression is increased during liver regeneration [35] and in growth-factor-stimulated macrophages [36]. However, the data in Figure 2 do not support a role for CT as a liver oncogene, since a significant increase in CT activity is first observed in advanced nodules. Instead, the higher CT activity appears to be one of several mitogenic activities, such as protein kinase C, which are elevated to accommodate the carcinogenic proliferation of liver.

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