Carcinogenesis vol.20 no.4 pp.561–567, 1999

Expression of phosphatidylethanolamine *N*-methyltransferase in Yoshida ascites hepatoma cells and the livers of host rats

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Previous studies have implicated phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2) in the regulation of non-neoplastic liver growth [Tessitore,L., Cui,Z. and Vance,E. (1997) *Biochem. J.*, 322, 151–154]. We have now investigated whether or not PEMT2 is also involved in the control of proliferation of hepatoma cells growing in an animal and cell death by apoptosis in the liver of tumor-bearing rats. PEMT activity was barely detectable and PEMT2 protein was absent in hepatoma cells growing exponentially *in vivo* whereas CTP:phosphocholine cytidylyltransferase (CT) activity and expression were high. The lack of PEMT2 corresponded with the absence of its mRNA. Both PEMT2 protein and mRNA appeared when cells entered the stationary phase of tumor growth and, in parallel, CT expression decreased. The host liver first became hyperplastic and exhibited a slight increase in CT activity and decrease in PEMT2 expression. During the stationary phase of hepatoma growth the host liver regressed and eventually became hypoplastic following induction of apoptosis. The appearance of apoptosis in the host liver was associated with a marked reduction in both CT activity and expression as well as an enhancement of PEMT activity and PEMT2 expression. McArdle RH7777 hepatoma cells underwent apoptosis when transfected with cDNA for PEMT2. The evidence supports the proposal that PEMT2 may have a role in the regulation of '*in vivo*' hepatoma and hepatocyte cell division as well as hepatocyte cell death by apoptosis.

Introduction

Phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the conversion of phosphatidylethanolamine to phosphatidylcholine (PC) in a few bacteria, yeast and in the livers of mammals (1). In spite of the survival of this activity during evolution, phosphatidylethanolamine methylation appears to be redundant since all eukaryotic cells synthesize PC via the cytidine diphosphate (CDP)–choline pathway that is essential for animal cell life (2,3). In fact, hepatoma cell lines have lost PEMT activity and grow very well (4). PEMT activity is mainly located on the endoplasmic reticulum and is catalyzed by an enzyme referred to as PEMT1 (4). Another form of the enzyme, PEMT2, was found associated with the mitochondria-associated membrane (4), an endoplasmic reticulum-like membrane that sediments with mitochondria during fractionation of liver homogenates (5). The gene for PEMT2 was recently cloned and characterized (6). Subsequently, targeted disruption of the PEMT2 gene demonstrated that both PEMT1 and PEMT2 were encoded by a single gene, *Pempt* (7). Expression of PEMT2 in a Chinese hamster ovary (CHO) cell line with a temperature-sensitive mutation in the CDP–choline pathway did not rescue cells from apoptosis at the restricted temperature, suggesting that PC made by the CDP–choline pathway is functionally different from PEMT2-derived PC (8).

We recently showed that PEMT2 was not expressed before birth (9,10) and PEMT2 expression was transiently inactivated during non-neoplastic liver growth after partial hepatectomy (11) and in response to the liver mitogen lead nitrate (12). Under these conditions of liver growth PEMT2 expression was inversely correlated with CTP:phosphocholine cytidylyltransferase (CT) activity and hepatocyte cell division. We also found that PEMT2 permanently disappeared in a hepatocellular carcinoma and its lung metastasis induced by the resistant hepatocyte model of Solt and Farber (13) and the overexpression of PEMT2 by transfection into a rat hepatoma cell line resulted in inhibition of cell growth rates (14). Together, these data suggest that PEMT might have a role in regulation of hepatocyte growth.

Regulation of cell growth and tumorigenesis may be achieved by modulating not only cell replication but also cell death. The *p53* tumor suppressor gene has been implicated in the induction of apoptosis in several cell systems (15–18). To address the question whether or not PEMT is involved *in vivo* in the regulation of both cell division and cell death by apoptosis in the liver, we used hepatoma cells growing in the peritoneal cavity of rats and the liver of tumor-bearing rats. In this model the tumor cells attenuated cell replication and underwent apoptosis when they entered the stationary phase of tumor growth (19). A transient liver hyperplasia occurs in animals bearing an ascites hepatoma, followed by rapid regression, eventually leading to hypoplasia due to cell death by apoptosis (20). Therefore, ascites hepatoma cells and host liver are a suitable model for '*in vivo*' studies on the role of PEMT in both cell division and cell death. In addition, we also investigated whether or not inhibition of the cell growth rate by overexpression of PEMT2 in rat hepatoma cells was related to the capacity of PEMT2 to interfere with cell division or cell death by apoptosis.

We found that both PEMT2 protein and mRNA were absent in tumor cells growing exponentially whereas these cells

Abbreviations: ALT, alanine aminotransferase; CDP, cytidine diphosphate; CHO, Chinese hamster ovary; CT, CTP:phosphocholine cytidylyltransferase; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PKC, protein kinase C; TUNEL, TdT-mediated dUTP nick end labeling.

exhibited high CT activity. PEMT2 expression was eventually induced in stationary cells when the rates of cell division decreased and apoptosis was triggered. Host liver hyperplastic enlargement was associated with a slight decrease in PEMT2 expression, whereas the subsequent involution due to apoptosis was related to an enhancement of PEMT2 expression above the control values. Transfection of McArdle RH-7777 hepatoma cells with cDNA for PEMT2 decreased the rates of cell growth by inducing apoptosis. These data suggest that PEMT2, in addition to a possible role in regulation of liver cell division, may influence the apoptotic process.

Materials and methods

Reagents

[^{3}H]thymidine (20 Ci/mmol) and [^{3}H]putrescine dihydrochloride (26.3 Ci/mmol), the Enhanced Chemiluminescence system (ECL), the Rapid-hyb buffer, Multiprime DNA labeling system kit and [α-^{32}P]desoxyCTP were from Amersham Italia (Milan, Italy). Bovine serum albumin and calf thymus DNA were from Sigma (St Louis, MO).

The *in situ* cell death detection, fluorescein kit was from Boehringer Mannheim (Mannheim, Germany). The agarose gel for electrophoresis was from Bio-Rad (Richmond, CA) and all other common reagents were from Merck (Darmstadt, Germany).

Animals

Male Wistar rats (Charles River, Como, Italy) of 150–200 g body weight had free access to water and a standard AIN-76TM diet (Piccioni, Brescia, Italy) and were maintained on a regular light/dark cycle (08.00–20.00). Rats were inoculated i.p. with 10^8 Yoshida ascites hepatoma cells and killed 4, 7 and 10 days after transplantation by CO_2 asphyxia. Rats were given 500 µCi/kg body wt [^{3}H]thymidine i.p. 1 h before killing.

The tumors were harvested from the peritoneal cavity, the livers were removed, weighed and processed for standard histology. Blood was collected and plasma enzyme analysis for alanine aminotransferase (ALT) (Syncron CX-5 System; Beckman, Milan, Italy) was performed, as an index of hepatocellular necrosis.

Cell culture and treatment

Monolayers of McArdle RH-7777 and PEMT2-transfected hepatoma cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.4 mg/ml G-418 sulfate (Geneticin; Gibco BRL, Basel, Switzerland), 10% fetal bovine serum and 10% human serum. Equal numbers of McArdle RH-7777 and PEMT2-transfected cells were seeded in triplicate in complete DMEM, grown and then trypsinized and counted every 24 h. Average doubling times were calculated starting 24 h after plating. Doubling time was calculated as $0.693/k_g$; $k_g = (\ln t_x - \ln t_o)/t$ (h) = %/h; cells were seeded in triplicate in complete DMEM.

Histology

Smears of tumor cells and slices of liver tissue were stained with haemallume and eosin to evaluate the mitotic index or processed by the TdT-mediated dUTP nick end labeling (TUNEL) technique for determination of the apoptotic index. This is an enzymatic *in situ* labeling of apoptosis-induced DNA strand breaks. The terminal deoxynucleotidyltransferase enzyme labels free 3′-OH DNA with fluorescein-labeled nucleotides. The number of fluorescent cells, i.e. the number of cells with fragmented DNA, were detected and quantitated by fluorescence microscopy (Leitz, Germany), scoring $\geqslant$5000 adjacent cells.

Smears of tumor cells were coated with NTB-2 Kodak emulsion (Kodak, Rochester, NY), dried, immersed in liquid scintillation fluid and sealed in a dark box at –80°C for 2–3 weeks for autoradiography. Slides were then developed and counterstained with haemallume and eosin. Percentages of fragmented nuclei and mitotic and labeling indices were determined scoring $\leqslant$2000 cells.

Biochemical analyses

Homogenates of tumor cells and liver tissues were used to assay transglutaminase (EC 2.3.2.131) activity as incorporation of [^{3}H]putrescine into *N,N′*-dimethylcasein (21). Liver DNA was determined by the method of Burton using calf thymus DNA as standard (22). PEMT activity was assayed as described by Ridgway and Vance (23) and CT activity was measured as reported by Vance *et al*. (24).

To measure DNA fragmentation, DNA was extracted and electrophoresed by standard procedures as described by Tilly and Hsueh (25), with minor modifications. Frozen liver tissues were lysed in homogenization buffer [0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris–HCl (pH 8.0) and 0.2 M sucrose]. SDS (10%) was added to homogenates, then the mixtures were incubated for 30 min at 65°C. After addition of 8 M potassium acetate, samples were kept ice cold for 60 min and centrifuged (5000 *g*) at 4°C for 10 min. Supernatants were collected and sequentially extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). DNA was precipitated overnight with 0.1 vol 3 M Na acetate and 2.5 vol absolute ethanol at ~20°C and sedimented at 5000 *g* for 30 min. The pellet was rinsed with 70% ethanol and air dried. DNA was then dissolved in 10 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA. Subsequently, samples were incubated with 0.5 mg/ml RNase for 1 h at 37°C, then extracted, precipitated and incubated again as described above. The DNA was collected by centrifugation (14 000 *g*) for 30 min at 4°C, washed with 70% ethanol, dried and resuspended in distilled water. DNA concentration and purity were checked spectrophotometrically from the absorbance at 260 and 280 nm. Approximately 10 µg/lane DNA was loaded onto 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and 1 mM EDTA in 40 mM Tris–acetate buffer, pH 8.0, and electrophoresed for 2 h at 50 V in TAE buffer (40 mM Tris–acetate, 1 mM EDTA). Gels were photographed under UV light.

DNA distribution by flow cytometry

Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde for 1 h in ice. After centrifugation, cells were incubated at 37°C in the presence of 0.2% Tween 20 in PBS. After centrifugation, cells were incubated at room temperature in the presence of DNase-free RNase (Type 1-A) and propidium iodide at final concentrations of 0.4 and 0.18 mg/ml PBS, respectively. Flow cytometric analysis of DNA was performed with a FACScan (Becton Dickinson, Sunnyvale, CA). At least 10^4 cells were analyzed for each sample at a flow rate of ~100 cells/s. One filter was used to collect the red fluorescence due to propidium iodide staining of the DNA, transmitting at 585 nm with a band width of 42 nm (FL2).

FL2 was registered on a linear scale. Simultaneously, forward and side light scatter were measured and used to exclude cell debris. Data were recorded and analyzed using Cell-Quest software (Becton Dickinson, San Diego, CA) on a Macintosh computer.

Western blot analysis

Protein samples were separated on 12.5% polyacrylamide gels containing 0.1% SDS (26) and transferred to nitrocellulose membrane by electrophoretic blotting (27). The membranes probed with PEMT2 antibodies were visualized using the ECL system according to the manufacturer's instructions (Amersham).

Northern blot analysis

Northern blot analyses were performed with 10 µg samples of poly(A)$^+$ RNA by electrophoresis in 1% agarose–formaldehyde gels followed by transfer to nitrocellulose filters (28). The membranes were probed with PEMT2 cDNA and CT cDNA.

Hybridizations were done by the Rapid-hyb method obtained from Amersham Life Science. As a control a cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to evaluate the amount of RNA transferred to filters.

Statistical analysis

All values are expressed as means ± SD. Significance of the differences was calculated using Student's *t*-test.

Results

Ascites hepatoma cells

The exponential phase of tumor growth was characterized by high rates of DNA synthesis as reflected in the [^{3}H]thymidine labeling index, percent of cells in S phase and an elevated mitotic index (Table I and Figure 1). The tumor was highly proliferating compared with adult liver. The transition from the exponential to the stationary growth phase was characterized by the appearance of apoptosis, as shown by the significantly higher percent of fragmented nuclei and transglutaminase activity (Table I), the sub G_0/G_1 peak in the DNA distribution (Figure 1) and DNA fragmentation (Figure 3).

Figure 2 shows the inverse relationship between PEMT and CT activities, PEMT activity being barely detectable in the exponentially growing hepatoma cells, but present in the stationary cells, at levels markedly lower than in liver. CT activity decreased from the logarithmic phase to the stationary

Table I. Growth kinetic parameters of exponential AH-130 hepatoma cells

	LI (%)	MI (%)	FN (%)	TA (nmol/mg protein/h)
Adult liver	0.5 ± 0.02	0.1 ± 0.01	0.8 ± 0.2	1.3 ± 0.2
Hepatoma cells				
LOG	52 ± 4[a]	14 ± 2[a]	1.2 ± 0.2	1.7 ± 0.3
STA	11 ± 3[b]	6 ± 1	8.2 ± 0.2[bc]	6.3 ± 0.5[bc]

Rats were inoculated i.p. with 10^8 cells from exponentially growing Yoshida AH-130 ascites hepatomas. Cells were harvested 4 and 10 days after transplantation. Rats were given 500 μCi [^{3}H]thymidine/kg body wt i.p. 1 h before killing. Data are means ± SD; $n = 5$.
LOG, day 4 after transplantation; STA, day 10 after transplantation; LI, labeling index; MI, mitotic index; FN, fragmented nuclei; TA, transglutaminase activity.
[a]$P \leq 0.001$ versus adult liver.
[b]$P \leq 0.05$ versus adult liver.
[c]$P \leq 0.01$ versus 4 days after transplantation (LOG phase).

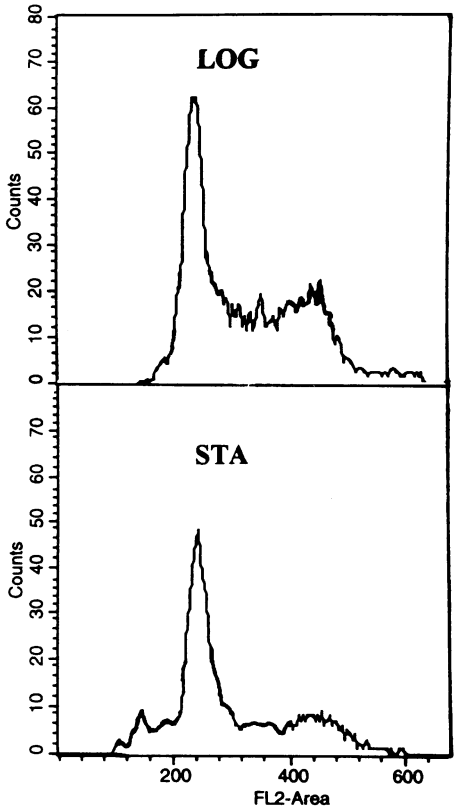

Fig. 1. Flow cytometric distribution of DNA in AH-130 hepatoma cells. Representative profiles for exponentially growing (LOG) and stationary (STA) tumors 4 and 10 days after transplantation, respectively. Distributions were as follows: LOG tumors, 36 ± 2% of cells in G_0/G_1, 49 ± 5% in S and 15 ± 3% in G_2/M; STA tumors, 8 ± 1% of cells in sub G_0, 55 ± 6% in G_0/G_1, 17 ± 3% in S and 20 ± 3% in G_2/M.

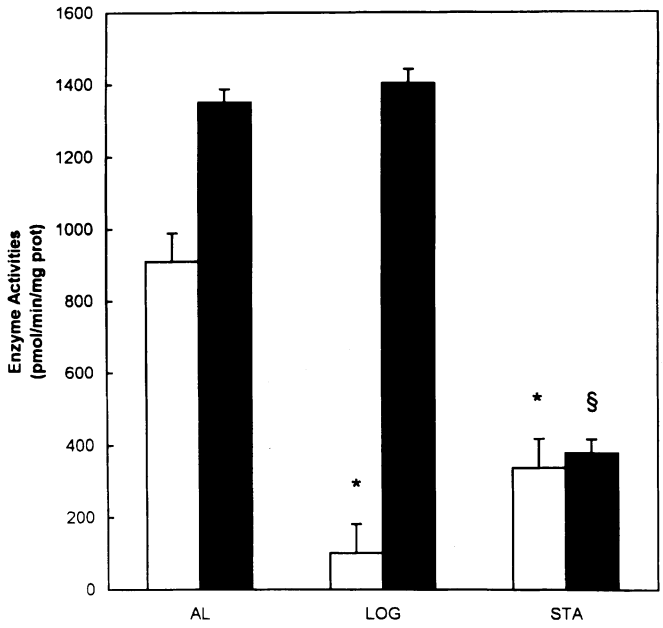

Fig. 2. PEMT and CT activities in AH-130 hepatoma cells. Data are expressed as means ± SD, $n = 5$; *$P \leq 0.001$, §$P \leq 0.0005$ versus AL; #$P \leq 0.005$ versus PEMT 4 days after transplantation (LOG phase). AL, adult liver; LOG, exponential growth; STA, stationary growth; □, PEMT; ■, CT. Similar results were obtained in three other experiments.

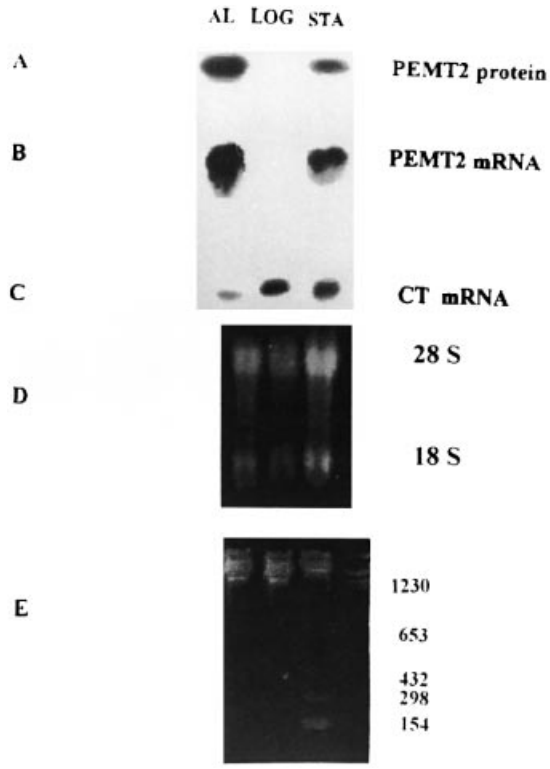

Fig. 3. Expression of PEMT2 and CT in AH-130 hepatoma cells. (**A**) Immunoblot analysis of PEMT2 protein (19 kDa); (**B**) northern blot analysis of PEMT2 RNA (1 kb); (**C**) northern blot analysis of CT RNA (1 kb); (**D**) ethidium bromide stained gel of the same RNA; (**E**) agarose gel electrophoresis is shown and similar results were obtained in three other experiments.

phase of tumor growth. While adult liver displayed a high level of PEMT2 protein, PEMT2 was undetectable in log phase cells and appeared at low levels when hepatoma cells entered the stationary phase of tumor growth (Figure 3).

To determine whether or not the changes in PEMT2 protein mass were due to modulation at the level of expression of the PEMT2 gene, we measured the amounts of PEMT2 mRNA. In the livers of adult rats PEMT2 mRNA was expressed at high levels, whereas it was absent in log hepatoma cells and low in stationary cells. In contrast, CT mRNA was higher in log tumor cells than in the adult liver and markedly decreased in stationary cells (Figure 3). The levels of rRNA were approximately the same in the liver and hepatoma cells.

Table II. Growth kinetic parameters in host liver

Days after transplant	LW%BW	DNA content (mg/liver)	MI (%)	ALT (IU/l)	FN (%)	TA (nmol/mg protein/h)
0	35 ± 0.5	9.5 ± 1.5	0.12 ± 0.01	23 ± 0.9	0.7 ± 0.02	1.3 ± 0.2
4	4.2 ± 1.2[a]	12.2 ± 1.1[a]	1.1 ± 0.03[b]	40 ± 1.5[c]	0.4 ± 0.01	1.4 ± 0.2
7	3.1 ± 0.6	8.9 ± 0.5	0.1 ± 0.01	19 ± 1.2	7.1 ± 0.3[b]	5.1 ± 0.5[c]
10	2.7 ± 0.8[c]	7.8 ± 0.7[b]	0.1 ± 0.02	22 ± 0.5	6.8 ± 0.2[b]	6.7 ± 0.3[c]

Rats were inoculated i.p. with 10^8 cells from exponentially growing Yoshida AH-130 ascites hepatomas and killed 4, 7 and 10 days after transplantation. Data are means ± SD; $n = 5$.
LW%BW, liver weight as a percentage of body weight; MI, mitotic index; ALT, alanine aminotransferase; FN, fragmented nuclei; TA, transglutaminase avtivity.
[a]$P \leqslant 0.05$ versus 0 days after transplantation.
[b]$P \leqslant 0.001$ versus 0 days after transplantation.
[c]$P \leqslant 0.01$ versus 0 days after transplantation.

Host liver

In tumor-bearing rats, liver growth displayed a biphasic pattern. First, the liver enlarged in correspondence with the exponential phase of tumor growth, as shown by the slight increase in liver weight and DNA content. This was probably due to enhanced hepatocyte proliferation as reflected in the mitotic index being higher at 4 days than immediately after tumor transplantion (day 0) (Table II). The plasma levels of ALT activity at 4 days were moderately increased (Table II), consistent with signs of perilobular hepatocyte necrosis far from the central vein (data not shown). The hyperplastic phase was transient, the liver rapidly regressing to a size smaller than at 0 days after transplantion, as shown by a reduction in liver weight and DNA content as well as the disappearance of mitotic figures (Table II). Such involution was characterized by the appearance of apoptosis, the frequency of fragmented nuclei and the activity of transglutaminase being sharply higher than at 0 days after tumor transplantion (Table II) and DNA fragmentation also being present (Figure 5).

The specific activity of PEMT decreased slightly in the hyperplastic liver 4 days after tumor transplantation, then increased during the regression of hyperplasia (day 7) and the following hypoplasia (day 10) (Figure 4). In contrast, CT activity was moderately higher when the liver was enlarged and lower when the liver size decreased to control values (day 7) and eventually to subnormal levels (day 10) (Figure 4). The changes in PEMT activity were due, at least in part, to fluctuations in the amount of PEMT2 protein. In fact, immunoblotting with an anti-peptide antibody specific for PEMT2 revealed that PEMT2 protein was slightly reduced at day 4, while it increased above the control level at days 7 and 10, coincident with a decrease in liver growth (Figure 5). To verify whether or not the changes in PEMT2 protein during liver hyperplasia and the following involution/hypoplasia were controlled at the level of its mRNA, northern blot analysis was performed. The pattern of levels of PEMT2 mRNA in host liver closely paralleled that of PEMT2 protein, being first moderately lower, then markedly higher than in adult liver (Figure 5). The levels of CT mRNA showed an opposite pattern, being first increased then sharply decreased, consistent with the changes in CT activity during host liver growth and regression. A cDNA probe for GAPDH was used as a control to evaluate the amount of RNA transferred to filters.

PEMT2-transfected hepatoma cells

Hepatoma cells transfected with PEMT2 cDNA showed a high level of PEMT activity (712 ± 24 pmol/min/mg protein) compared with hepatoma cells transfected with vector alone (4.1 ± 0.3 pmol/min/mg protein). Exponentially growing monolayers of PEMT2-transfected hepatoma cells exhibited slower cell growth rates, as demonstrated by the higher doubling time (29 h) compared with McArdle hepatoma cells (20 h) (Table III). On analysis of DNA distribution, no significant differences were observed in the percentage of G_0/G_1, S and G_2/M cells between the two cell populations (Figure 6). In contrast, the subpopulation with a DNA content lower than the G_0/G_1 peak, here named 'sub G_0/G_1' cells, was negligible in the McArdle hepatoma cells but significantly increased in the hepatoma cells transfected with PEMT2 (Figure 6). This sub G_0/G_1 cell subpopulation corresponded to apoptotic cells, as indicated by TUNEL analysis and the apoptotic index being ~3% (Table III).

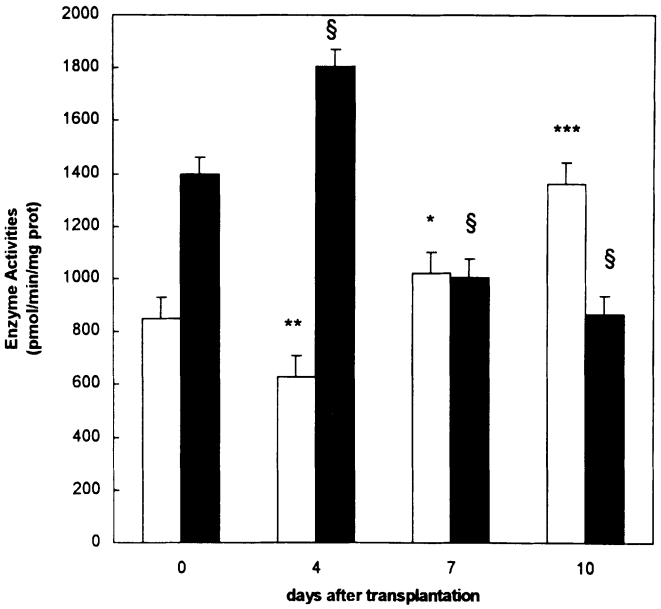

Fig. 4. PEMT and CT activities in the host liver at different times after tumor transplantation. Data are expressed as means ± SD, $n = 5$. *$P \leqslant 0.01$, **$P \leqslant 0.005$, *** and §$P \leqslant 0.0005$ versus 0 days. □, PEMT; ■, CT. Similar results were obtained in three other experiments.

Discussion

The results of the current investigation provide evidence that PEMT2 is inactivated in hepatoma cells growing exponentially in the peritoneal cavity of rats whereas the activity of CT is

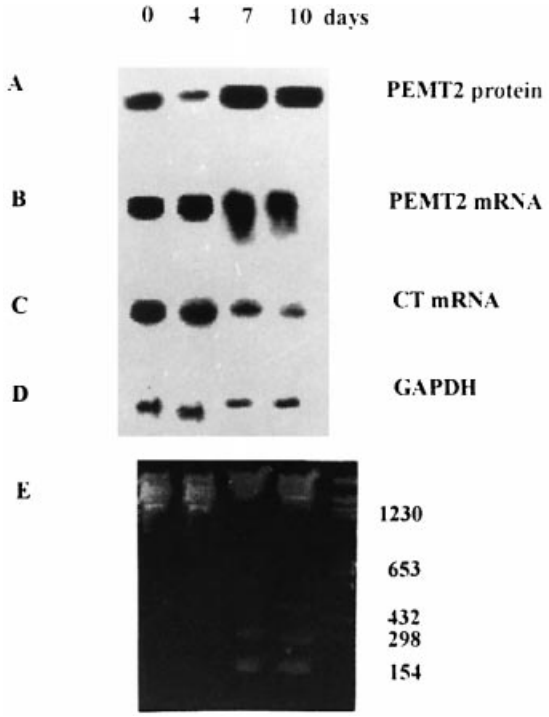

Fig. 5. Expression of PEMT2 and CT in the host liver at different times after tumor transplantation. (**A**) Immunoblot analysis of PEMT2 protein (19 kDa); (**B**) northern blot analysis of PEMT2 RNA (1 kb); (**C**) northern blot analysis of CT RNA (1 kb); (**D**) northern blot analysis of GAPDH, used as a control; (**E**) agarose gel electrophoresis of DNA. A representative immunoblot, northern blot and DNA electrophoresis are shown and similar results were obtained in three other experiments.

Table III. Growth kinetic parameters of McArdle hepatoma cells and PEMT2-transfected hepatoma cells

	Doubling time (h)	S phase (%)	Sub G_0/G_1 (%)	FN (%)
RH-7777	20 ± 0.05	17 ± 0.6	1 ± 0.02	0.1 ± 0.05
PEMT2	29 ± 0.20[a]	16 ± 0.5	8 ± 0.07[b]	2.9 ± 0.1

Data are means ± SD.
RH-7777, McArdle hepatoma cells; H-PEMT2, PEMT2-transfected McArdle hepatoma cells; FN, fragmented nuclei.
[a]$P \leq 0.05$ versus RH-7777.
[b]$P \leq 0.005$ versus RH-7777.

enhanced. In the livers of the host animal PEMT activity and PEMT2 expression is enhanced during regression of the hyperplasia and the subsequent hypoplasia caused by apoptosis. Finally, transfection of McArdle rat hepatoma cells with PEMT caused an apparent decrease in cell growth due to enhanced apoptosis.

PEMT2 and hepatocyte growth

Our finding that PEMT2 expression was undetectable in hepatoma cells growing rapidly *in vivo* supports the possibility that PEMT2 might have an unexpected suppressive role in hepatocyte cell division. These results agree with previous studies in different models of liver proliferation which showed an inverse relationship between PEMT2 expression and hepatocyte cell division (9–12). Moreover, when McArdle hepatoma cell lines were transfected with cDNA for PEMT2, its overexpression resulted in a decrease in the cell growth rates (14). More recently, we found that PEMT2-transfected hepatoma

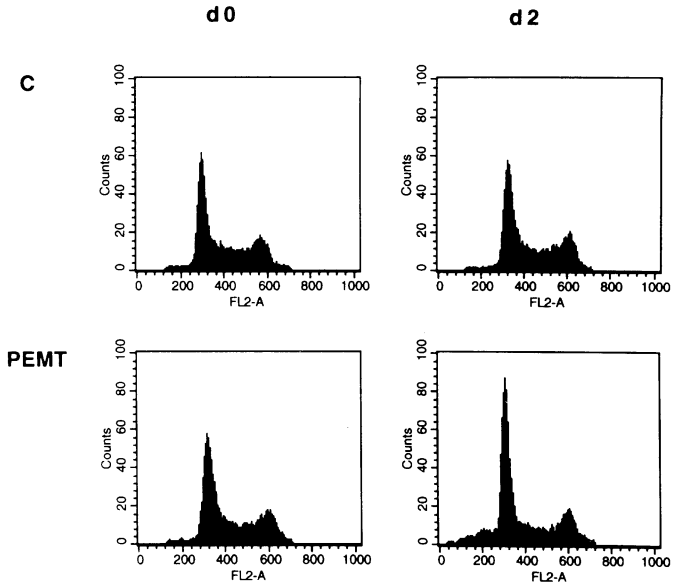

Fig. 6. Flow cytometric distribution of DNA in McArdle hepatoma cells and PEMT2-transfected hepatoma cells. Representative profiles for 0 and 2 days after plating. The propidium iodide fluorescence of PEMT2-transfected cells in the sub G_0/G_1 population was 8% after 2 days plating in comparison with 1% of McArdle hepatoma cells.

cells failed to form anchorage-independent colonies in semi-solid agar and tumors in athymic nude mice, indicating reversion of the neoplastic phenotype (L.Tessitore, E.Sesca and D.E.Vance, unpublished data). This and many previous studies showed a consistent inverse correlation between expression of PEMT2 and hepatocyte growth. Speculation on possible mechanisms by which PEMT2 might inhibit cell proliferation is, therefore, warranted.

PC synthesized by the PEMT2 pathway might be functionally different from the PC derived from the CDP–choline pathway (8). Expression of PEMT2 in CHO cells with a defect in the CDP–choline pathway failed to rescue the growth phenotype even though normal levels of PC were maintained (8). Thus, there appears to be something about PC made via PEMT that does not satisfy the need for CDP–choline-derived PC for cellular growth. The growth suppressive function of PEMT might be related to down-regulation of the CDP–choline pathway. Transfection of hepatoma cells, lacking PEMT activity, with PEMT2 cDNA resulted in a reduction in CT expression, suggesting that PEMT2 down-regulates expression of the CDP–choline pathway (29). Consistently, whenever PEMT2 expression was reduced or suppressed, CT activity increased in non-neoplastic growth of the liver (9–12). The results presented in this paper for the high activity of CT in logarithmically growing hepatoma cells and in the hyperplastic host liver when PEMT2 expression was absent or reduced, respectively, are consistent with the idea that expression of PEMT2 has a negative effect on CT activity. Moreover, enhanced CT activity in *Pemt* 'knockout' mice (7) provides further evidence that PEMT somehow influences expression of the CT gene. Further studies on the transcription factors that modulate CT gene expression may provide a clue as to how PEMT might decrease expression of CT.

How PC made by the CDP–choline, but not the PEMT, pathway promotes growth is not known. One explanation for why PC derived from PEMT might differ functionally from PC derived from the CDP–choline pathway arises from studies

linking the two different sources of PC to the mitogenic pathway mediated by protein kinase C (PKC) (30). PEMT2 expression was absent during liver growth (9,11,12) when the activities of α, β and ζ PKC were enhanced and δ PKC activity was repressed (9,31,32). Thus, possibly diacylglycerol produced from CDP–choline-derived PC is important in activating the α, β and ζ forms of PKC whereas δ PKC might be activated by diacylglycerol derived from PC made via PEMT. Hence, PC produced by the CDP–choline pathway and PC synthesized via the PEMT2 pathway may lead to the activation of different forms of PKC which have opposite functions in the cell cycle (33–35). On the other hand, the correlations between PKC activities and the origin of PC may be unrelated phenomena.

PEMT2 expression is also down-regulated in liver carcinogenesis, PEMT2 being permanently absent in liver cancer and its lung metastasis induced by chemical carcinogens (36). This finding agrees well with the present results of low level expression of PEMT2 in hepatoma cells grown *in vivo* after they entered the stationary phase of tumor growth. Nevertheless, the expression of a small amount of PEMT2 strongly suggests that the PEMT2 gene is intact in transplantable rat liver tumors. Thus, it appears that, at least in the rat, PEMT2 is not a conventional tumor suppressor where loss of function results from mutation or deletion of the gene (37). Work is in progress to define the expression of PEMT2 in human liver cancer.

The choline deficiency model might prove to be useful for studies on the relationships between PEMT, liver growth and hepatocarcinoma. It has been known for many years that rats fed a choline-deficient diet for 1 year or more show an increased incidence of primary liver cancer (38). It would, therefore, be of interest to know what happens to PEMT2 expression in these animals. It is known that PEMT activity increased ~2-fold after 48 h choline deficiency (38). This has been attributed to an increased supply of one of the substrates for PEMT, phosphatidylethanolamine (39). Subsequent studies showed an ~5-fold increase in PEMT2 expression after 3 weeks on the diet (40). The latter experiment was terminated after 3 months, which is before liver cancer is induced by a choline-deficient diet.

PEMT and apoptosis

Our results on the enhancement of PEMT2 expression when the host liver underwent hypoplasia by apoptosis were not unexpected. Recently, it was reported that choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes (41) and increased PC synthesis via the methylation pathway as the intracellular levels of *S*-adenosylmethionine also increased (42). Consistently, supplementation of choline-deficient SV40-immortalized CWSV-1 rat hepatocytes with methyl group donors failed to prevent or correct the choline deficiency (42). However, the present studies are the first evidence linking PEMT2 expression directly with induction of apoptosis in the liver. Moreover, as demonstrated in the current studies, overexpression of PEMT2 in a McArdle hepatoma cell line by transfection with PEMT2 cDNA strongly reduced the cell doubling time by triggering apoptosis without affecting cell division. More recent studies with the MLP29 cell line, derived from mouse embryonic liver, showed that PEMT2 expression was absent when the cells were highly proliferating in culture but was induced when apoptosis was triggered by treatment with retinoic acid (L.Tessitore, E.Medico and P.Comoglio, unpublished data). Further studies are required to understand the relationship between PEMT2 and apoptosis.

Relationship between PEMT1 and PEMT2

Our discussion and experimental approach has necessarily focused on PEMT2 since we only have antibody to PEMT2 and our cDNA may only encode for PEMT2. Although we now know that PEMT1 and PEMT2 are encoded by the same gene (7), we do not know how PEMT1 and PEMT2 differ. Obvious explanations might be alternative splicing to yield different mRNAs or post-translational modifications that modify the antigenicity of the two forms of PEMT so that our C-terminal peptide antibody only recognizes PEMT2. Once we understand the differences between PEMT1 and PEMT2, we will be in a position to determine if expression of PEMT1 has similar effects to PEMT2 on CT expression, hepatocyte growth and apoptosis.

Conclusion

These results provide evidence for a link between PEMT2 expression, hepatic cell division and cell death. Exactly how PEMT2 is a negative factor for hepatocyte growth still needs to be explained.

Acknowledgements

This work was supported by the Associazione Italiana Ricerca sul Cancro, Milano, the Ministero dell'Universita e della Ricerca Scientifica e Tecnologica (40 and 60% funds), Roma, Italy, and the Medical Research Council of Canada. Dr Vance is a Medical Scientist of the Albert Heritage Foundation for Medical Research.

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Received August 10, 1998; revised and accepted November 30, 1998