Melanoma Tumors Acquire a New Phospholipid Metabolism Phenotype under Cystemustine As Revealed by High-Resolution Magic Angle Spinning Proton Nuclear Magnetic Resonance Spectroscopy of Intact Tumor Samples

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ABSTRACT

N \textsuperscript{-}[2-chloroethyl]-N\textsuperscript{2}-(methylsulfonyl)]ethyl]-N\textsuperscript{-}nitrosourea (cyste- mustine), a chloroethylnitrosourea antineoplastic drug, provokes cellular proliferation inhibition and redifferentiation, but there was no cell death in B16 melanoma tumors. Because the phospholipid (Plp) metabolism is tightly involved in tumor growth regulation and tumor cell survival, we tested the hypothesis that melanoma tumors undergo adaptive Plp metabolism changes to survive treatment. Measurements of Plp derivatives were performed using a novel proton nuclear magnetic resonance Spectroscopy application using magic angle spinning on intact tumor tissue samples. Phosphatidylcholine levels were obtained from one-dimensional spectra, and relative levels of choline- and ethanolamine-containing compounds were derived from two-dimensional spectra (total correlation spectroscopy sequence). Two major findings emerged from this study: (a) during tumor growth inhibition, there was a transient accumulation of choline, glycerophosphocholine, and glycerophosphoethanolamine and a sustained increase in phosphocholine and phosphoethanolamine, whereas phosphatidylcholine levels remained unchanged; and (b) during tumor growth recovery, only phosphocholine and phosphoethanolamine remained elevated. Therefore, cystemustine-treated B16 melanoma tumors acquire a new Plp metabolism phenotype, a mechanism that could participate in tumor cell redifferentiation and/or survival.

INTRODUCTION

CENU\textsuperscript{3} are anticancer agents that mainly target DNA and create interstrand DNA bonds, thus interfering with tumor cell replication (1). We demonstrated previously that N\textsuperscript{-}[2-chloroethyl]-N\textsuperscript{2}-(methylsulfonyl)]ethyl]-N\textsuperscript{-}nitrosourea (cystemustine), a CENU, provoked growth inhibition of B16 melanoma tumor with a drastic decrease in the number of mitoses, but there was no cell death (2). After a period of growth inhibition, tumor cells recovered a proliferation capability with the restoration of numerous mitoses, but the attained tumor mass remained much lower than that of untreated tumors over long-term follow-up (2). In addition, during the growth-inhibition period, tumor cells exhibited transient arrest in G\textsubscript{2} phase, redifferentiation features like increased tyrosinase activity and pigmentation, large cytoplasm, and loss of aggressiveness (2, 3). During growth recovery, redifferentiation features were still persistent.

Tumor growth regulation and cell survival tightly involves Plp metabolism (4–9). Apoptosis has been shown to be mediated by a drop in PtdCho mass because of an agent-induced blockage of the CDP-Cho pathway or to a mutation in CCT, the key enzyme of the CDP-pathway (5, 6). Cell growth has been shown to be inhibited, and even apoptosis induced, by alkyl-PC (10) or lysoPtdCho analogues (11). Additionally, the overexpression of PEMT, the enzyme converting PtdEth to PtdCho, inhibited hepatoma cell proliferation (7). Apoptosis could be prevented and cell differentiation induced through overexpression of PLC\textgreek{a} (8). Moreover, water-soluble intermediates of Plp metabolism by themselves could play an important role in the regulation of mitogenesis and in cell survival (9). Among these derivatives, PC was shown to promote cell proliferation effects of insulin and insulin-like growth factor-1 (9).

In this study, we tested the hypothesis that cystemustine-TR murine B16 melanoma tumors undergo adaptive changes of Plp metabolism during the sequential phases of tumor evolution: the growth-inhibition period and the growth recovery period during which tumor cells proliferate again and exhibit a persistent redifferentiation pattern.

To this aim, we took advantage of 1\textsuperscript{H}-NMR Spectroscopy, a powerful tool to measure most Plp derivatives in intact cells and tissue or in extracts (4, 12). Because of improved signal/noise ratio techniques, two-dimensional NMR Spectroscopy now permits the identification and quantification of weakly concentrated species (13–15). A novel accessory, HRMAS when upgrading a high-field magnet, improves spectra of cell cultures (16), normal tissue (17, 18), and cancer tissue (19, 20). 1\textsuperscript{H}-HRMAS NMR Spectroscopy allowed us to simultaneously follow the fate of the Plp products and precursors, PtdCho, Cho, PC, CDP-Cho, Eth, PE, CDP-Eth, GPC, and GPE. Water-soluble Plp derivatives by themselves yield important information because their concentration plays a specific role in cell proliferation and survival (9), and they allow some Plp metabolism enzyme activities to be inferred (4).

The data presented in this report demonstrate, for the first time, that cystemustine-TR melanoma tumor cells maintain their PtdCho mass and acquire a new Plp derivative phenotype. During growth inhibition, tumors showed a transient increase in intracellular Cho, GPC, and GPE and a sustained increase in PC and PE, whereas PtdCho levels remained unchanged. During growth recovery, tumors overexpressed intracellular PC and PE, and the CDP-Eth pathway, a mechanism that could participate in B16 melanoma redifferentiation or protection against cell death.

MATERIALS AND METHODS

Chemical

N\textsuperscript{-}[2-chloroethyl]-N\textsuperscript{2}-(methylsulfonyl)]ethyl]-N\textsuperscript{-}nitrosourea (cyste- mustine), a CENU antineoplastic agent (1), has been proposed in the treatment of human malignant melanoma and glioma. Cystemustine was used as a 5 mM solution in 0.9% NaCl. D\textsubscript{2}O and CDC\textsubscript{3}(SDS, Pepsin, France) were used as solvents and as locking media for NMR Spectroscopy.
Cell Cultures
Transplantable B16 melanoma cells originating from C57BL/6J Ico mice were obtained from ICIG (Villejuif, France) and adapted to grow in culture. B16 cells were prepared as described previously (2).

Animal Model. Treatment Protocol
Six- to 8-week-old C57BL/6J male mice were purchased from IFFA CREDO, (L’Arbresle, France). Mice were shaved before s.c. injections into their flank of 5 × 10^5 B16 cells. B16 melanoma tumors became palpable at days 7–9 after cell inoculation. Mice were divided into two groups, a CT group, which received sham injections of saline solution, and a TR group. The TR group received intratumor cyustumiste injections at a dose of 15 μg/gram body weight. Cystemustine was injected at days 11, 14, and 18 from B16 cell inoculation.

At defined times of tumor evolution (days 10, 12, 15, 20, 24, and 29 after B16 cell inoculation for CT and TR tumors and prolonged to days 35, 43, and 54 for TR tumors), three mice of each group were sacrificed according to institutional guidelines for animal welfare and experimental conduct. Tumors were dissected and weighed. The dissection of the s.c. tumor took institutional guidelines for animal welfare and experimental conduct. Tumors were dissected and weighed. The dissection of the s.c. tumor took <2 min. A piece of the tumor <50 mg of the whole tumor was immediately prepared for NMR Spectroscopy as described below or frozen at −80°C in case of delayed examination.

Tumor growth curves were fitted to the Gompertz function and modified for the TR group to include a growth delay period. Ranges for maximum attainable weights and other model parameters obtained in cytostumistine-treated melanoma models have been published previously (2).

Histopathology
Tumors underwent histopathological examination during follow-up. Formaldehyde-fixed tumors were embedded in paraffin, and 4-μm sections were cut for H&E staining analysis. The count of mitoses was performed on 10 HPFs. Cell morphology and pigmentation load were evaluated.

Tissue Extracts
To further investigate the main Plp (PtdCho and PtdEth) content, chloroform/methanol tissue extracts were prepared from days 15–29 in CT and TR groups. Tissue samples were freeze dried and homogenized using a mortar and pestle. Then, 5 ml of chloroform/methanol (2:1, volume for volume) was added to the mixture, and the suspension was vortexed three times for 1 min and centrifuged at 1550 × g for 5 min. The supernatant was collected and evaporated under dry nitrogen for 2 h. Extracts were reconstituted using CDC13 and 50 μl of extract were deposited in the dedicated NMR tube.

NMR Spectroscopy
NMR Spectroscopy was performed on a small bore Bruker DRX 500 magnet (Bruker, Karlsruhe, Germany) equipped with an HRMAS accessory. Samples were set into 4-mm diameter, 80-magnet (Bruker, Karlsruhe, Germany) equipped with an HRMAS accessory.

1H-NMR Spectroscopy of Intact Tumor Material
One-dimensional 1H-NMR Spectroscopy. One-dimensional 1H-NMR Spectroscopy of intact tumor material was used for the quantification of PtdCho. NMR Spectroscopy acquisition and processing were run from a workstation. Aiming for tuning and shimming from a shim table recorded previously, a water signal was obtained, and its frequency was retained for subsequent water signal suppression. An 8-μs radio-frequency pulse was applied, then 32 1H free induction decays were collected with water signal suppression at low power, a 10-ppm spectral width, 8 K complex data points, and a repetition time of 10 s. After Fourier transformation, a baseline correction was applied using a spline algorithm in the spectral domain of interest.

Peak referencing was done on the signal of 3-trimethylsilylpropyl-sulfonate. PtdCho gave rise to a broad (because of restricted mobility) signal at 3.26 ppm (the trimethyl signal of the Cho moiety). However, the PtdCho signal overlapped with other signals at that spectral position and required the use of deconvolution procedures using the XWINNMR software (Bruker) to be quantified. Strong signals that could be separated from PtdCho were those of Taurine (Triplet at 3.27 ppm), PE (3.22 ppm), and GPC (resonating together at 3.32 ppm). Signals of CDP-Eth, GPE, and PtdEth could not be resolved from that of PtdCho. However, they could be considered as negligible in comparison with PtdCho, because PtdCho was the most concentrated derivative among the four and because of its number of spins, the 3.26-ppm PtdCho derivative weighed by 4.5-fold all Eth-containing derivative signals in the 3.2–3.3-ppm spectral area. Internal standardization was performed on the signal of Gly as discussed below in the two-dimensional NMR Spectroscopy section.

Two-dimensional 1H-NMR Spectroscopy. The relative measurement of water-soluble Cho- and Eth-containing derivatives was performed on intact tumor material using a two-dimensional TOCSY sequence (13, 14). The sequence was performed with water signal suppression, using high-resolution sampling (spectral bandwidth of 6 ppm, 256 along the first axis, and 8 K complex data points along the second axis), a number of scans of 8, and a mixing time of 75 ms during which was applied a spin-lock pulse consisting of a cascade of short duration radio-frequency pulses aimed at producing a signal-enhancing polarization transfer effect. The repetition time was 2 s, thus weighting Plp derivatives by a factor of 0.98–0.92 (T1 between 0.5 and 0.8 s) and the longest T1 species (Gly, taurine) by a factor of 0.7 (T1s of −1.7 s). The phased mode was achieved using time proportional phase increment procedure. Raw data were processed with a quadratic sine function, then underwent Fourier transformation.

Proton resonances of the amine groups, Eth and Cho, are characteristic of each Plp derivative (given in ppm) and, because of scalar coupling, give rise to cross-correlation signals between hydrogen nuclei of neighbor C1 groups (the hyphen between chemical shifts indicates the scalar coupling): Cho (3.20 and 3.55–4.07), PC (3.23 and 3.62–4.18), CDP-Chol (3.23 and 3.68–4.39), GPC (3.23, 3.62, and 3.68–4.34), Eth (3.15–3.8), PE (3.22–3.99), CDP-Eth (3.30–4.20), and GPE (3.3–4.12).

The TOCSY sequence was optimized using a cascade of measurements on a mixture of Plp derivative standards, while varying only the mixing time. Optimal signal/noise ratio for most Plp derivatives was obtained for mixing times of 75 and 150 ms over a period of 200 ms (data not shown). We set our choice to 75 ms. The stability of the tissue sample inside the rotor was evaluated by a cascade of TOCSY measurements for ~10 h. We could verify that at room temperature, Plp derivatives varied by <20% after a 3-h stay inside the rotor. Operating with the sample cooled at temperatures <20°C would help improve the biological stability of the sample but at the expense of resolution (17). However, our overall sample processing and acquisition lasted by 105 min after excision, a duration which was short enough to adequately reflect Plp derivative changes that take place in vivo.

For quantification, real-part spectra were phased along both frequency axes simultaneously setting θ at 54.7° (the magic angle) allows the chemical shift spreading to theoretically converge to zero. The HRMAS accessory then allows the use of the same radio-frequency power amplifiers and the same NMR sequences as the spectrometer standard equipment. In contrast, the technique differs from solid-state NMR Spectroscopy, which also uses magic angle spinning but operates at much higher rotation rates with dedicated NMR sequences and stronger radio-frequency power amplifiers.

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and baseline corrected using a low-order spline function. Then, CPVs were measured using the XWINNMR software from a recorded template of regions applied to the cross-peaks of interest (Cho, PC, GPC, CDP-Cho, Eth, PE, GPE, and CPD-Eth). CPVs were measured in the upper half plane of the spectrum where they were exempt from first-axis noise originating from strong signals like that of taurine at 3.27 ppm, also from partial saturation effects because of water signal suppression (21).

Two-dimensional spectra yield relative Plp derivative measurements, because CPVs are proportional to metabolite concentration, although weighted by relaxivity and magnetization transfer effects. T1s of water soluble Plp derivatives were measured using Inversion Recovery-TOCSY (a sequence with TOCSY as the read block). Measured T1s ranged between 500 and 800 ms. For each derivative, T1s were similar in TR and CT groups (data not shown). In addition, Plp derivatives have similar spin systems. Therefore, cross-peak signal variations were mainly because of concentration variations. Internal standardization was performed using the diagonal signal of Gly at 3.56 ppm. The Lys ε-δ cross-peak signal has been proposed as an internal standard in two-dimensional NMR Spectroscopy, because it has been shown to be reasonably constant in a number of cell models and tissue samples (13). Lys ε-δ and Gly signals correlated in our study, irrespective of the sample group (r² = 0.66, n = 42). Normalization to the Lys ε-δ cross-peak signal or the Gly signal yielded similar time course curves (22). We chose to use the Gly signal here because the same standardization could be used in one- and two-dimensional spectra. Finally, standardizing the sample preparation, data acquisition and processing procedures ensured the comparability of spectra.

NMR Spectroscopy of Chloroform/Methanol Extracts

One-dimensional 1H-NMR Spectroscopy. Relative quantification of PtCo and PtOEt was performed using one-dimensional spectra of chloroform/methanol extracts, for which only these two Plp were quantifiable. Using the HRMAS coil did not greatly improve the resolution of extract spectra but gave a signal advantage. Quantification was performed on the signals of PtCo at 3.26 ppm and of PtOEt between 3.05 and 3.15 ppm corrected for their number of spins (nine and two, respectively). After baseline correction, Plp lines were fitted a mixed lorentzian-gaussian line using the XWINNMR software, then integrated, and the proportion of PtCo and PtOEt relative to the sum of both was calculated.

Two-dimensional 1H- NMR Spectroscopy. In extracts, TOCSY spectra were performed for PtOEt assignment. PtOEt gave rise to signals at 3.26 and 3.70 to 3.80–4.30 to 4.40 ppm and PtOEt at 3.05 to 3.15–4.00 to 4.15. The mixing time of the TOCSY sequence was set to 40 ms in accordance with published values for lipids (21).

Statistical Analysis

Data are presented as mean ± SD. Single comparisons between TR and CT groups were performed using the Mann-Whitney test. Time series were analyzed using ANOVA, followed by a post-hoc test with paired comparisons between TR and CT groups.

RESULTS

Cystemustine-TR B16 Melanoma Tumors Exhibit Transient Growth Inhibition and Sustained Redifferentiation. We compared tumor proliferation patterns in CT and cystemustine-TR groups by tumor size measurements, histopathology, and mitosis count. Tumor growth curves are shown in Fig. 1. In the CT group, measurements were only available until day 29 because the animals died. In the TR group, measurements were conducted until day 54. Two phases, accounted for by a Gompertz model, could be distinguished: (a) an exponential phase; and (b) a plateau phase starting from day 29 and characterized by the maximum attainable tumor weight. In the TR group, a growth-inhibition phase characterized by growth delay between days 12 and 24 was observed before a short exponential phase followed by a plateau phase characterized by tumor weights much lower than those of the CT tumors (1.1 ± 0.5 versus 5 ± 0.7 grams, P < 0.001, TR versus CT).

In CT tumors, histopathological examination revealed numerous mitoses, features of aggressiveness, and low-melanin content (Fig. 2A). In TR tumors, cells showed enlarged cytoplasm, features of decreased aggressiveness, and strong increase in melanin content (Fig. 2B). During the plateau phase, these redifferentiation features were still encountered, although they were weaker than during the growth-inhibition phase (Fig. 2C). This observation indicated that cystemustine induced a sustained redifferentiation pattern.

The count of mitoses per 10 HPF in the CT group was 104 ± 19 (days 12–29 average; Fig. 3). The mitosis count dropped at day 12 in the TR group. It averaged 7 ± 5 during the growth-inhibition phase (days 15–24), then increased and reached levels of 77 ± 29/10 HPF during the plateau phase, from days 35–54 (P < 0.05, TR versus CT at day 29).

During Growth Inhibition, Cystemustine-TR B16 Tumors Exhibit Transient Increase in Cho, GPC, and GPE and Sustained Elevation of PC and PE. Cho increased sharply at the beginning of the growth-inhibition phase and reached a maximum (2.2-fold the baseline level) at day 15, remained elevated until day 20, then decreased to CT levels (Fig. 4, A and B and Fig. 5A). PC increased from day 12 and reached a peak by 4.9-fold the pretreatment level at day 20, after the third cystemustine injection, then decreased moderately and was still elevated at day 29 (Fig. 4, A and B and Fig. 5B). After peaking, Cho could have fuelled the CDP-Cho pathway with PC (Fig. 5B) and CDP-Cho (data not shown) both culminating at day 20.

Eth levels did not vary during that period (data not shown). PE increased sharply during the growth-inhibition period and reached a maximum after the third cystemustine injection at day 20 by 3.6-fold, then decreased but remained elevated at day 29 (Fig. 4, A and B and Fig. 5C). The CDP-Eth level increased during the growth-inhibition phase and was still elevated at day 29 (Fig. 5D).

GPC and GPE increased during the growth-inhibition phase (Fig. 4, A and B). GPC and GPE culminated at day 20, after the third injection of cystemustine, by 2.8- and 2.7-fold, respectively, then decreased and reached the baseline at day 29 (Fig. 5, E and F).

During Growth Recovery, Cystemustine-TR B16 Tumors Exhibit Sustained Overexpression of PC and PE. Overall comparison of time course curves showed that PC and PE had parallel evolution, starting shortly after the first cystemustine administration.

During the plateau phase of TR tumors, Cho was low, whereas PC levels remained elevated 3-fold up to day 54 (Fig. 4C and Fig. 5B). CDP-Cho was lower than during growth inhibition.

In parallel, Eth levels did not vary, whereas PE levels remained
DISCUSSION

This study demonstrates the response of Plp metabolism to cystemustine, a DNA-damaging antineoplastic drug in a B16 melanoma murine model, during the sequential phases of TR tumor growth. Two major findings emerged from this study: (a) during growth inhibition, Cho, GPC, and GPE were transiently overexpressed, PE and PC accumulation, and PtdCho levels remained unchanged; and (b) during growth recovery, intracellular content of PC and PE remained elevated, indicating the acquisition of a new PtdCho derivative phenotype by regrowing cystemustine-TR tumors.

In this study, we provide simultaneous follow-up of most Cho- and Eth-containing Plp derivatives, including CDP-Chol and CDP-Eth, in intact tumors by 1H-NMR Spectroscopy. Previous studies of Plp derivative expression have been carried out on cell cultures or tissue extracts (4, 12). The HRMAS technique has been shown to improve the detection of PtdCho in 1H-NMR spectra of intact cells (16). We took advantage of HRMAS to quantify PtdCho levels in our study. We focused on PtdCho derivatives rather than on enzyme activities. However, PtdCho derivatives allow some enzyme activities to be inferred and also have specific consequences when they accumulate (4, 9). PE was the most highly expressed Plp derivative in B16 melanoma. Similar findings were reported in other tumor models (4). Additionally, Ras transformation of NIH 3T3 fibroblast cells was shown to be associated with inhibition of CCT, activation of Cho- and Eth-kinase, and activation of PLC, yielding together an accumulation of PC and PE (23, 24). PC and PE accumulation in cancer cells is considered to play a role in the stimulation of mitogenesis (9).

The first major finding of this study was the profile of PtdCho derivatives during the growth-inhibition phase. Despite oscillations during follow-up, the average PtdCho levels were similar in the TR and CT groups. In the CT group, PtdCho levels were low during the early exponential growth phase possibly because of strong membrane Plp demand that did not allow PtdCho to accumulate. PtdCho homeostasis is critical for cell survival. A recent study demonstrated that a mutant ovary cell line (MT58) exhibiting temperature-sensitive CCT activity underwent a G1 cell cycle arrest, a drop in PtdCho level, and apoptosis at the critical temperature (5). Agents, such as nonmetabolizable analogues of lysoPtdCho, alkyl-PC, and tumor necrosis factor-α, were shown to induce a decrease in PtdCho mass or biosynthesis and provoke apoptosis (6, 10, 11). Alternatively, Plp accumulation inhibited cellular proliferation (25). In this study, the cystemustine-TR B16 tumors may have regulated their PtdCho mass to survive.

**Cystemustine-TR Tumors Maintain PtdMass during Growth Inhibition and Recovery.** PtdCho and PtdEth were the Plp the most highly expressed in melanoma tumors (Fig. 4D). The PtdCho curve displayed some oscillations in both groups (Fig. 6A). In the TR group, after day 12, PtdCho resumed its pretreatment levels for the duration of the growth-inhibition phase (until day 24), after which PtdCho reached a minimum at day 29, coinciding with the restoration of tumor growth. From that time on, the mitosis count increased (Fig. 3). Despite oscillations, the average PtdCho levels were similar in the TR and CT groups during the growth-inhibition phase (PtdCho/Gly time averaged over days 12–29: 2.8 ± 1.1 versus 2.7 ± 0.9, P = NS, TR versus CT) and also over complete evolution. In addition, the relative amounts of PtdCho and PtdEth measured from chloroform/methanol extracts and time averaged over days 15–29 were similar in TR and CT groups (Fig. 6B).

**Fig. 3.** Cystemustine-TR tumors exhibit changes in mitosis count during growth inhibition and recovery. The mitosis count in CT (○) and cystemustine-TR (■) tumors was performed on histopathological slices. Mitosis count dropped during the GI phase and then recovered to large values, although below CT ones, during the GR phase (77 ± 29 versus 104 ± 19 mitoses/10 HPF; P < 0.05, TR versus CT). GI, growth inhibition; GR, growth recovery.

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*Fig. 2. In A–C, cystemustine-TR tumors exhibit histopathological features of decreased aggressiveness and redifferentiation. A, CT tumor at day 29. At the beginning of the plateau phase, the tumor showed important features of aggressiveness with many mitoses and a weak pigment load (×200). B, cystemustine-TR tumor at day 20. During the growth-inhibition phase, the tumor showed features of decreased aggressiveness, few mitoses, and an increased pigment load (×200). C, cystemustine-TR tumor at day 43. During the plateau phase, the count of mitoses was increased, but cells still displayed features of decreased aggressiveness and moderate pigment load (×200).*
It has been shown that in response to Plp overload, e.g., because of an excess in the culture medium, cells protect themselves through PLA2 activation and base-exchange activation (25). Cho may accumulate within the cells because of decreased release from the cells, increased production, or increased extracellular uptake. Because Cho increased at a time when PtdCho was in excess, Cho could originate from phospholipase D activation and/or base-exchange activation between PtdCho and PtdEth or phosphatidylserine (26). The catabolism of GPC into Cho and glycerol-phosphate is also a possible source of Cho through an activation of GPC-phosphodiesterase (27).

It appeared to be a likely involvement of the biosynthetic CDP-Cho pathway during days 20–29. This biosynthetic phase could have

Fig. 4. A–D, typical one-dimensional and two-dimensional 1H-NMR spectra in the 3–4.5-ppm spectral range. A, CT tumor at day 29 (beginning of the plateau phase). PE was the most highly expressed Plp derivative. B, cytosine-TR tumor at day 20 (growth-inhibition phase). Cho, PC, PE, GPC, and GPE levels were increased in comparison with the CT tumor (A). C, TR tumor at day 43 (plateau phase). PE and PC levels were increased in comparison with the CT tumor (A). Cho, GPC, and GPE signals have returned to low levels in comparison with B. D, spectrum of chloroform extract of a CT tumor at day 29. PtdCho and PtdEth signals were easily identified. The number of isocontour lines indicates the higher content of PtdCho in comparison with PtdEth.
prepared the tumor to recover growth. PtdCho is synthesized mainly via the CDP-Cho pathway and methylation of PtdEth involving PEMT (EC 2.1.1.71). In addition, differences between PtdCho profiles (acyl composition) occur according to the involved PtdCho biosynthesis pathway and should play a role in cellular proliferation processes (28, 29).

During growth inhibition, we observed a transient increase in GPC and GPE. These derivatives were released from PtdCho and PtdEth, respectively, through activation of PLA. GPC and GPE could accumulate because of a decrease in their turnover. The most likely hypothesis is that GPC and GPE accumulate because they are produced in excess through an activation of PLA2 (25). The quite elevated PtdCho levels observed during the growth-inhibition phase could be explained by a loss of demand for membrane Plp, thus allowing PtdCho to accumulate.

The involvement of PEMT under cystemustine treatment needs to be addressed. The PEMT pathway is quantitatively significant only in the liver (4). We showed previously in B16 melanoma that the methyl-transferase involved in creatine biosynthesis (EC 2.1.1.2) was activated during the growth-inhibition phase under cystemustine treatment (30). Recently, it has been reported that in Chinese hamster ovary-K1 cell cultures, the overexpression of PEMT did not inhibit

Fig. 5. In A–E, cystemustine-TR tumors show alterations in water-soluble derivatives during growth inhibition and recovery. Time course of Cho (A), PC (B), PE (C), CDP-Eth (D), GPC (E), and GPE (F) tissue relative content (see “Materials and Methods”) in CT (□) and cystemustine-TR (■) tumors. Cystemustine was administered between days 11 and 18. Each data point was the average of three experiments. All variations were statistically significant (Cho: day-12 and day-15 data points, $P < 0.01$, TR versus CT; PC: day-15 data point and from days 24 to 54 data points, $P < 0.05$, day-20 data point, $P < 0.01$, TR versus CT at day 29 for TR data points after day 29); PE: day-12 data point, $P < 0.05$, and from days 15 to 54 data points, $P < 0.01$; CDP-Eth: from day-20 data points, $P < 0.05$; GPC: day-12 and day-24 data points, $P < 0.05$, day-15 and day-20 data points, $P < 0.01$; GPE: day-12 and day-24 data points, $P < 0.05$, day-15 and day-20 data points, $P < 0.01$). GI, growth inhibition; GR, growth recovery.
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the CDP-Cho pathway but paradoxically enhanced it (31). These data are to be compared with the report that the activation of PEMT produces a PtdCho functionally different from that derived from the CDP-Cho pathway (28, 29). Therefore, our data could be consistent with an activation of PEMT under cystemustine, a mechanism that would yield an excess PtdCho production with fatty-acid moieties of unusual types. It was shown that such activation inhibited cellular proliferation in hepatoma cells, and a similar process could account for tumor-growth inhibition in cystemustine-TR B16 melanoma.

The second major finding was that cystemustine-TR B16 tumor cells acquired a new Plp metabolism phenotype developed during the growth-inhibition phase, which became definitively acquired once tumors recovered growth. The new phenotype consisted of a sustained growth-inhibition phase, which became definitively acquired once cells acquired a new Plp metabolism phenotype developed during the growth-inhibition phase, TR tumors transiently overexpressed Cho, GPC, and GPE, whereas they maintained Plp mass at CT values. Over long-term evolution (during growth inhibition and subsequent recovery), TR tumors overexpressed PC and PE, indicating the acquisition of a new phenotype, a mechanism that could be associated with tumor cell redifferentiation and/or survival.

REFERENCES

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