Imbalance of CDP Pathways and Up-Regulation of Phosphoethanolamine under CENU Treatment in long Term Cultures of malignant Melanoma Cells. A 1H-HRMAS MRS Study.

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Introduction

Phosphatidyl-choline (PtCho), the main plasmatic and cell membrane phospholipid (PL), is a critical compound for cell survival and proliferation. In addition to genotoxicity, anticancer drugs acting through a modification of PtCho level or of its lipid moiety may yield to the arrest of cell proliferation (1). PtCho production involves the Cytidine-diphospho-choline (CDPCho) pathway mainly, also the methylation of Phosphatidyl-Ethanolamine (PtEth). Using 31P-MRS, changes in CDPCho have been shown to be associated with programmed cell death (2). We here investigated PL derivative expression during growth arrest and relapse of B16 melanoma cells exposed to Chloroethyle Nitrosourea (CENU) treatment, using high resolution MRS of a long-term cell culture.

Methods

B16 melanoma cells originating from C57BL6/6J Ico mice were maintained as monolayers in culture flasks using culture medium consisting of Eagle´s MEM-glutaMAX medium, 10 % fetal calf serum and Gentamicin. B16 cells were exposed for 2 hours to 200 mM Cystemustine, a CENU that has been proposed for the treatment of human malignant melanoma. After pulse treatment, melanocytes were rinsed and replaced in fresh culture medium.

MRS measurements were performed several times during long term culture follow-up, each data point being the average of measurements on 2 culture flasks. Cells were harvested by trypsinization, counted, rinsed in saline buffer, centrifugated, then freshly examined.

1H-NMR spectroscopy was operated at 500 MHz (Bruker DRX 500) using an HRMAS accessory. Samples were spinned at 4 kHz in 4 mm diameter Zirconia rotors. At each time, 1D spectra (NS=32, TR=15 sec, spectral width=10 ppm, and 8 Ko complex data points) framing a 2D spectrum (Tocsy sequence optimized for PL derivatives signal-to-noise ratio, mixing time=150 ms) were performed. A baseline correction was applied in all cases. Quantification of PL metabolites was performed using the Bruker Software for spectrum deconvolution (1D) and Cross-Peak Volume measurement (2D). Internal standardization used the ε-CH2 signal of Lysine at 3.02 ppm (1D spectra), and the Lysine ε-δ cross-correlation at 3.02-1.73 ppm (2D spectra).

Results

The cell growth curve showed a marked decrease in cell density between Day 5 and Day 30 after CENU exposure (Fig 1). A Spectrum of the kinetics is presented in Fig 2 with the principal attributions.

Cho and Phosphocholine (PC) decreased to low levels after Day 3 and remained low for the duration of the follow-up. Phosphoethanolamine (PE) increased from Day 3, then showed a second wave from Day 15 to Day 25, then steeply decreased around Day 30. However it still remained elevated at Day 40. CDPEth increased from Day 15 to Day 25 then decreased and returned to baseline at Day 35 (Fig 3).

The comparison between growth curves and PL derivatives showed contemporary and reciprocal changes between cell density and PE level.

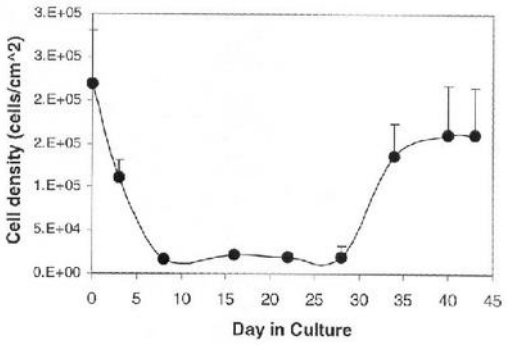

Fig 1: Cell growth curve after CENU exposure.

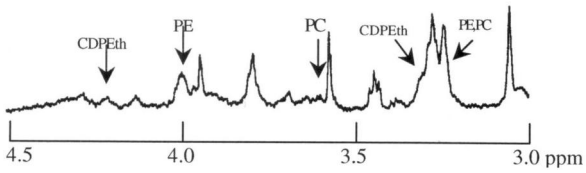

Fig 2: Spectrum of B16 melanoma cell cultures at D15.

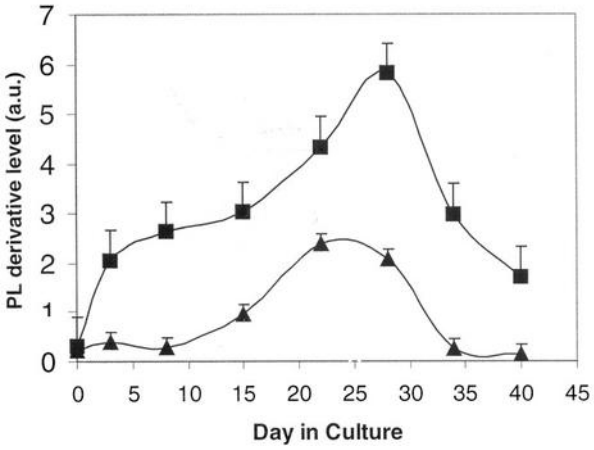

Fig 3: Time course of PE and CDPEth signals.
PE (squares), CDPEth (triangles).

Discussion

Previous studies have shown that Cenu provokes an early arrest in cell cycle G2 phase (days 1-3) (4). Then during growth arrest the number of cells in G2 phase decreases to baseline concomitantly with an increase in cells in G1 phase (4). These modifications are accompanied with an increase in cell size and an accumulation of mature melanosomes, that should require PL synthesis.

From our data, we may conclude that the CDPCho pathway was not activated under CENU. However this may be enhanced by Cho deficiency of the culture medium. To the contrary, the CDPEth pathway is progressively activated during growth arrest, likely aimed at increasing the PtEth level, part of it likely destined to PtCho production. Both PL however participate to membrane synthesis and cell signaling. The early increase in PE might be produced by an activation of Phospholipases-C (5), due to a direct action of CENU or mediated by cell cycle changes (5).

During late follow-up cells relapse although they appear to maintain a modified PL derivative phenotype (elevated PE, low PC) possibly a mutation phenotype acquired under CENU. To our knowledge this is the first MRS study of PL derivative follow-up during a long term culture under anticancer drug.

References

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