Disorders in methylated species in response to nitrosourea in a melanoma tumor model: demonstration using ^{1}H-^{13}C HRMAS NMR spectroscopy and L-[*methyl*-^{13}C]methionine

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Introduction

Several anticancer drugs exert their cytotoxicity by altering epigenetic methylations (dacarbazine, nitrosoureas) or metabolite syntheses by methylation like 5-fluorouracil (1). However, little is known on changes in the most abundant methylated species of tumor cells, namely creatine and phosphatidylcholine (PtC) (2), although methylation disorders may be prominent features of the response to these anticancer drugs and provide useful NMR spectroscopy biomarkers of the response. Creatine is the product of guanidinoacetate N-methyltransferase and PtC has an accessory biosynthetic pathway by methylation through phosphatidylethanolamine-N-methyltransferase (PEMT). Most methylations use an activated form of L-Methionine (Met) as the methyl donor. Here, we have investigated the impact of nitrosourea on concentrations and L-[*methyl*-^{13}C]Met-induced methyl group enrichments of creatine and PtC in melanoma tumors.

Methods

B16 melanoma tumors were induced in C57BL6/6J mice after inoculation of 5×10^5 cells subcutaneously. Two groups of tumor-bearing mice were performed, an untreated group (UN) and a nitrosourea-treated group (TR). The TR group received fotemustine (18 µg/g) intratumorally at days 11 and 12 after cell inoculation. During follow-up, animals received 6-mg 99%-enriched L-[*methyl*-^{13}C]Met intraperitoneally twice daily from day 12 to day 19. At defined times of tumor evolution (from day 11 to day 33 after cell inoculation, or from day 0 to day 22 of labeling), 3 mice (4 on day 11) were sacrificed. Tumors were dissected and frozen at –80°C. Aqueous and lipid extracts were obtained using a dual extraction procedure. NMR spectroscopy was done at 500 MHz (Bruker, Germany) using a HRMAS coil. Intact tumor samples were set into 4-mm diameter 50-µ rotor tubes, and spun at 4 kHz. They were analyzed using a fully relaxed {^{13}C}-^{1}H SR sequence. Extracts were analyzed using a {^{13}C}-^{1}H SE sequence and a 2D ^{1}H-^{13}C HMQC sequence. The CH_3 signal of total creatine (tCr=creatine+phophocreatine) arose at 3.03 ppm/38 ppm ($^{1}H/^{13}C$) in spectra of water-soluble extracts and the $N(CH_3)_3$ signal of PtC arose at 3.26 ppm/55 ppm in spectra of lipid extracts. The 1D HMQC f_2 axis projection was computed and used to calculate fractional enrichment, f(Y*), according to the formula:

$$f(Y^*) = 0.01\times\left[\frac{S^*_{MQ}(Y^*)}{S^*_{MQ}(X^o)}\times\frac{S^o_{MQ}(X^o)}{S^o_{MQ}(Y^o)}\times\frac{S^*_{SE}(Y^*)}{S^*_{SE}(X^o)}\times\frac{S^o_{SE}(X^o)}{S^o_{SE}(Y^o)} - 1\right]$$

where S held for signal, Y* was a labeled CH_3 group, Y° the same CH_3 group in the HMQC (subscript MQ) spectrum of an unlabeled tumor (natural abundance), X° was an unlabeled signal in the HMQC spectra, and the corresponding signals in SE sequences were given the subscript SE.

Results

The concentration of the methylated species was obtained from {^{13}C}-^{1}H SR spectra of intact samples as described in (2). tCr concentration was 7.1±1.2 *vs.* 4.2±1.3 mM (TR *vs.* UN, p<0.001, Fig 1) and PtC concentration was 15.7±3.3 *vs.* 16.8±2.1 mM (TR *vs.* UN, p=NS).

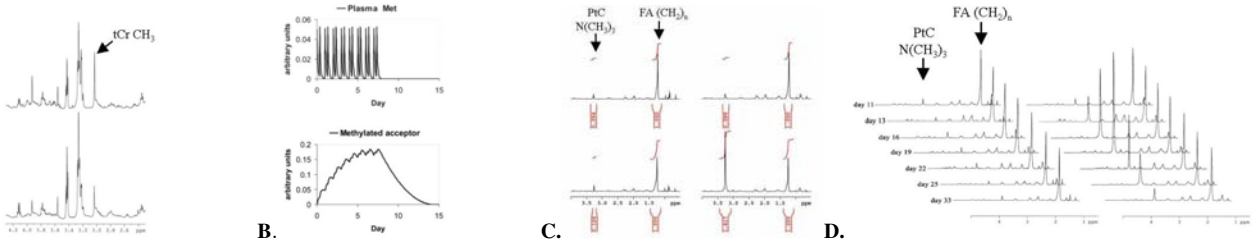

Fig 1 A-D. A-Typical ^{1}H SR spectra of intact tumor samples (bottom, UN; top, TR). **B**-Modeling of the enrichment of plasma Met (top), and tumor tCr or PtC CH_3 groups (bottom). **C**- Set of typical lipid extract spectra used for the calculation of the fractional enrichment of PtC $N(CH_3)_3$. Top left, SE spectrum, unlabeled tumor; top right, f_2 spectrum, unlabeled; bottom left, SE spectrum, labeled tumor; bottom right, f_2 spectrum, labeled. The unlabeled reference was the $(CH_2)_n$ signal of fatty acids (FA) at 1.30 ppm. The calculated enrichment was 12.8%. **D**-UN tumor lipid extracts: stack plot of f_2 spectra (right) and corresponding SE spectra (left) before, during and after L-[*methyl*-^{13}C]Met injection. The start of the experiment was indifferently set to day 11 after cell inoculation or to day 0 of labeling.

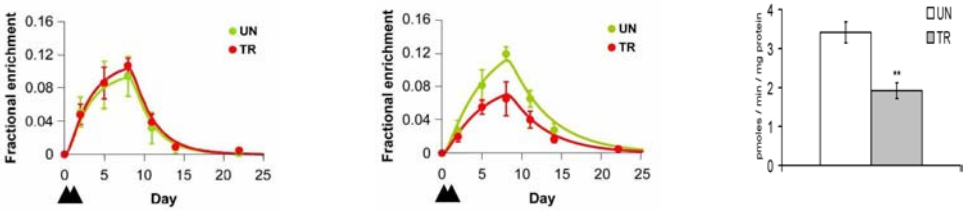

Fig 2. Time course of fractional enrichments of CH_3 residues of tCr (left) and PtC (middle, p<0.01, TR *vs*. UN). Data are mean ± SD of n≥3 tumor samples at each sampling time. Decreased PtC enrichments in response to treatment were confirmed by PEMT activity measurements by an enzymatic method (right, -40%, ** p<0.01, TR *vs*. UN). Arrowheads, fotemustine injections; UN, untreated; TR, fotemustine-treated.

Discussion

Anticancer treatment by nitrosourea increased tCr concentration while it kept tCr CH_3 enrichment unchanged. In contrast, PtC concentration was unchanged but PtC CH_3 enrichment was significantly decreased (Fig 1,2). It was previously reported strong alterations in phospholipid metabolism in response to nitrosourea treatment (3), involving increased PtC turnover. Therefore, reduced PtC labeling and PEMT activity in response to fotemustine were consistent with a regulation of the PtC pool at that time, with increased contribution to PtC synthesis of the unlabeled CDP-choline pathway (3). Phosphocreatine was found to be increased in tumors in response to an alkylating agent (4), consistent with our findings of increased tCr content. Therefore methylations in tumors under nitrosourea treatment may be redirected from PtC to tCr synthesis to cope with methyl group availability. In conclusion, tCr level, not PtC level, is a simple and useful biomarker of the response to nitrosourea. In addition, this experiment demonstrates extensive methylation disorders in nitrosourea-treated tumors that may couple the tCr pool to that of PtC.

References

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