A Methionine-free Diet Associated with Nitrosourea Treatment Down-regulates Methylguanine-DNA Methyl Transferase Activity in Patients with Metastatic Cancer

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Abstract. *Background: Methionine (MET) depletion used in association with chemotherapy improves the therapeutic index in animal models. This potentiating effect may be due to tumor cell sensitization to chloroethylnitrosoureas through their MET dependency and the down-regulation of O^6- methylguanine-DNA methyltransferase (MGMT). Our purpose was to evaluate the impact of the association of a dietary MET restriction with nitrosourea treatment on MGMT activity in peripheral blood mononuclear cells (PBMCs). Patients and Methods: Six patients with metastatic cancer (melanoma and glioma) received 4 cycles of a MET-free diet with cystemustine (60 mg/m^2). Results: MGMT activity in PBMCs decreased by an average of 13% from 553±90 fmol/mg before the diet to 413±59 fmol/mg after the diet + chemotherapy period (p=0.029). The decrease of MGMT activity was not affected by the duration of the MET-free diet period but seems to be correlated to the plasma MET depletion induced by the MET-free diet.*

Chloroethylating nitrosoureas (CENUs) have documented activity in metastatic malignant melanoma and in recurrent glioma, with a single agent response rate of 10-25% (1-6). The major cytotoxic structural change formed by chloroethylating chemotherapeutic agents is an alkyl adduct at the *O*^{6} position of guanine, which is the direct precursor of a cytotoxic intrastrand DNA crosslink. However, nitrosourea effectiveness is often compromised by resistance mechanisms. The main mechanism of nitrosourea resistance is mediated by the "suicide" DNA-repair protein *O*^{6}-methylguanine-DNA methyltransferase (MGMT). MGMT repairs cytotoxic damage by transfering the alkyl groups from the *O*^{6} position of guanine to an internal cystein residue and in this way inhibits the formation of DNA crosslinks (7). Several studies on animal models and clinical trials have described a close correlation between deficiency in MGMT activity in tumors and sensitivity to the cytotoxic effects of CENU agents and methylating agents (survival and tumor response) (8-11). Thus a possible strategy for increasing the therapeutic index of CENUs is to down-regulate MGMT activity.

In contrast with normal cells, tumor cells are characterized by a high rate of growth with accelerated protein synthesis and transmethylation reaction, entailing a large methionine (MET) requirement in order to proliferate and survive (12-13). Tumors frequently present abnormal MET metabolism, called MET dependency, defined as the inability of tumor cells to proliferate in growth medium when MET is replaced by its immediate precursor homocysteine, whereas normal cells will grow in this medium (13-17). MET dependency of tumors opens interesting avenues for specific targeting of tumor cells. In tumor-bearing animals, it was found that tumor inhibition occurred when animals were fed with a MET-free diet, but interruption of the regimen resulted in tumor regrowth (18). Moreover, during the period of MET restriction, numerous modifications in tumor cells occurred; they mainly concerned tumor cell arrest in S- and G2-phases of the cell cycle, apoptosis, and decreased glutathione content (19) and MGMT activity (18, 20). Thus, these alterations may act synergistically with CENU, since CENU treatment and MET restriction have common targets. The decreased MGMT activity made tumor cells more sensitive to

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carmustine (BCNU) as shown in brain tumor xenografts (18, 20). These data suggest a synergy between MET depletion and alkylating agents in tumor regression. Using a syngenic animal model, some studies demonstrated that a MET-free diet potentiated the effect of CENU on mice bearing B16 melanoma (21).

On the basis of these experimental data, a phase I clinical trial was designed to test the association of a dietary MET restriction with CENU in the treatment of metastatic melanoma or recurrent glioma and to determine the optimal duration of the Met-free diet.

In several clinical trials, an inverse correlation between the MGMT level in tumoral cells and their sensitivity to the effect of alkylnitrosoureas was well established (9-11). Because of the difficulty in accessing to metastases at metastatic stage, numerous clinical trials have attempted to evaluate whether MGMT activity in PBMCs could reflect chemoresistance of tumors. Some authors have shown that MGMT expression in white blood cells mirrors events in the target tumor and might at best serve only as general indicator of the degree of MGMT depletion in the tumor (22, 23). In the present study, we determined the impact of a MET-free diet associated with nitrosourea treatment on MGMT activity in peripheral blood mononuclear cells (PBMC) from blood samples obtained from patients of the phase I clinical trial.

Patients and Methods

Patients and treatment schedule. The study protocol was reviewed and approved by local ethical committees. All the patients included provided their written informed consent for the blood samples. Six patients treated with cystemustine and under a MET-free diet were evaluated for MGMT activity and plasma MET concentration. One patient presented a recurrent glioma (oligodendroglioma) and 5 patients presented a metastatic melanoma, among whom 3 had choroid melanoma. These patients received 4±1 cycles of 2 weeks of chemotherapy associated with a MET-free diet. At each cycle, on the first day patients were allowed to consume a standard diet, and on the following days the MET-free diet. Four periods of 1, 2, 3, or 4 MET-free diet days were randomly tested during the four cycles. Cystemustine treatment was administered *i.v.* at 60 mg/m^2 at 12 a.m. on the last day of the MET-free diet period. The MET-free diet was a mixture of XMET Maxamum (SHS, Liverpool, UK), a MET-free powder as the source of dietary nitrogen, vitamins and minerals, supplemented with soluble DUOCAL (SHS, Liverpool, UK) a medical food providing fat and carbohydrate to meet the energy requirements of a 70 kg man. Patients were advised to spread their consumption of the MET-free diet throughout the day from 8 a.m. to 12 p.m.

MGMT activity. Blood samples were collected at 8 a.m. the day before commencing the MET-free diet and the day following the MET-free diet period and cystemustine administration. PBMCs from whole blood samples were separated using a Ficoll gradient, washed with 0.9% NaCl solution and centrifuged. Cell pellets were stored at –196˚C until used. Cells were suspended in 100 μl/10^6 cells of lysis buffer (70 mM Hepes, pH 7.8; 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 10% glycerol, 1 μg/ml leupeptine-antipain-aprotinine) and sonicated 3 times for 45 s at 1 min intervals on ice. Cell debris we removed by ultracentrifugation at 12000 xg for 10 min at 4˚C. Supernatants were stored at –80˚C after measurement of total protein concentration using the Bradford method.

MGMT activity was determined by measuring the transfer of [^{3}H]-methyl groups from the DNA substrate (obtained by alkylation of calf thymus DNA with [^{3}H]*N*-methyl-*N*-nitrosourea) (24). Aliquots of 25 μg to 175 μg of proteins from PBMC extracts were incubated with [^{3}H]-methylated DNA at 37˚C for 1 h in a total volume of 140 μl of incubated buffer (70 mM Hepes, pH 7.8; 1 mM EDTA, 1 mM DTT). After stopping the reaction with 25 μl of 2 N HCl, samples were heated at 80˚C for 30 min (DNA hydrolysis) and then neutralized with 25 μl of 2 N KOH. *O*^{6}-methylguanine and *N7*-methylguanine (*O*^{6} and *N7*-mG; Sigma, l'Isle-d'Abeau Chesnes, France) (50 mmol/10 μl) were added as internal standards. Different methylating bases (*O*^{6}-mG and *N7*-mG) were separated by reverse-phase high performance liquid chromatography (HPLC) (HP1100 series, Hewlett Packard, Les Ulis, France) using a C18 Kromasil 5 μm x 25 cm column (Interchim, Montluçon, France) eluted at 1.2 ml/min by a 20 mM $NH_4H_2PO_4$ (pH 5.0) mobile phase containing 6% methanol during the 10 first min and 15% during the last 14 min. The methylated DNA adducts were quantified using a Packard Flow scintillation analyzer (500 TR series, Packard Instrument Co., Meriden, CT, USA). MGMT activity was expressed in fmol of methyl groups transferred to protein per mg of total protein in the extract, and measurements were made in triplicate.

Determination of plasma MET. Plasma MET was measured at 12 a.m. the day before the MET-free diet period and then every day of the MET-free diet period. Blood samples were collected in heparinized tube and treated as previously described (25). Two milliliters of plasma were treated for deproteinization with sulfosalicyclic acid (50 mg/ml plasma) for 10 min. After centrifugation (4˚C, 700 xg, 10 min), the supernatant was stored at –80˚C until analysis. Plasma amino acid concentrations were quantified in the supernatant using ion exchange chromatography with ninhydrin detection with an amino acid autoanalyzer (System 6300; Beckman instruments, Palo Alto, CA, USA).

Statistical analysis. Results are expressed as mean±standard error. The evaluations of the cycle effect, during the 2 months of treatment, on MGMT activity and on MET concentration were made using analysis of variance (ANOVA or Kruskal-Wallis *H* test). To evaluate the impact of the association of the MET-free diet with cystemustine or of the MET-free diet respectively on MGMT activity or plasmatic MET concentration, we compared values from before to after treatment using a paired *t*-test. Correlation analysis was performed using the Spearman correlation coefficient.

Results

MGMT activity. MGMT activity in PBMCs of these patients ranged from 84 to 1424 fmol/mg of protein. First, we evaluated the variation of MGMT activity during the 2 months of treatment. At baseline, the mean pre-treatment MGMT

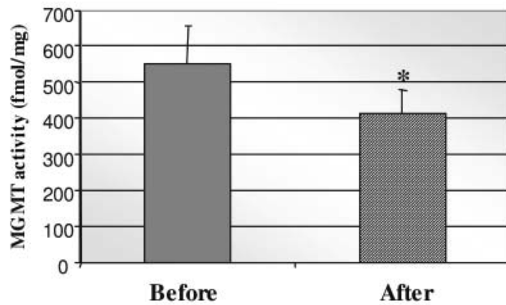

Figure 1. *MGMT activity in PBMC samples before and after MET-free diet plus cystemustine. *p<0.05 with paired t-test after vs. before treatment.*

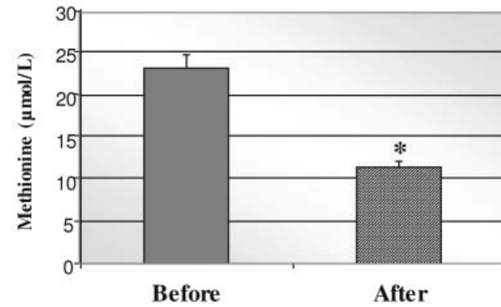

Figure 2. *Plasma MET concentration (12 a.m.) before and after MET-free diet. *p<0.05 with paired t-test after vs. before treatment.*

activity was 294±70 fmol/mg (first cycle) and remained stable for cycles 2, 3 and 4 (p=0.49). Likewise, the MGMT activity after the MET-free diet associated with cystemustine administration did not show any variation (p=0.74).

Second, we evaluated the variation of MGMT activity during cycle. MGMT activity decreased significantly after the MET-free diet associated with cystemustine administration, from 553±90 fmol/mg of protein before treatment to 413±59 fmol/mg after treatment (p=0.029) (Figure 1). This reduction was not observed during the first cycle (p=0.14) but occurred during the subsequent cycles (p=0.01), with a mean decrease of MGMT activity of 36±8% after the MET-free diet associated with cystemustine treatment. Moreover, during the cycle, to determine if the duration of the MET-free diet had an effect on MGMT, we compared the mean MGMT activity after treatment obtained after 1, 2, 3 or 4 days of MET-free diet. This analysis showed that the duration of the MET-free diet had no effect on MGMT activity after treatment.

Plasma MET concentration. At each cycle, as the plasma MET values determined every day of the MET-free diet period (1 to 4 days) were not significantly different (data not shown), we used a mean of these values as plasmatic MET concentration after the MET-free diet. During the 2 months of treatment, no cycle effect was observed on MET concentration, neither on pre-treatment values nor on values determined after the MET-free regimen. During cycles, the plasma MET concentration showed a sharp decline after the MET-free diet administration of an average of 48.5±4% from 23.1±1.6 μg/L before the diet to 11.3±0.7 μg/L after the MET-free diet (p=0.00002) (Figure 2).

MET depletion and MGMT activity. On one hand, we observed an effect on MGMT activity of the MET-free diet associated with cystemustine; and on the other hand, we observed an effect on plasmatic MET concentration of the MET-free regimen. We investigated whether these two observed effects were related. We noticed a tendancy toward a positive correlation between MGMT activity variations and plasmatic MET variations (r=0.517, p=0.15) after the first cycle (Figure 3).

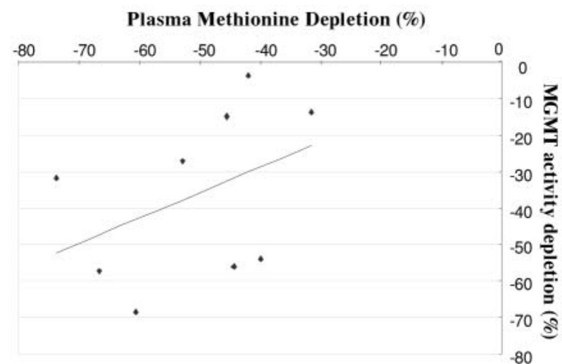

Figure 3. *Correlation between the depletions with treatment of plasma MET and MGMT activity. Spearman correlation coefficient was 0.517 (p=0.15). The line indicates the linear regression analysis (y=0.6904x-1.306).*

Toxicity. Toxicity was evaluated according to the WHO scoring system (26). The major toxicities induced by the MET-free diet and cystemustine treatment were hematological and were mainly responsible for early treatment interruption. Two patients among the six experienced WHO grade 3-4 thrombocytopenia, neutropenia or leucopenia. Non-hematological toxicity was limited, with no WHO grade 3-4 reported.

Discussion

We evaluated the impact of the association of a MET-free diet and CENU treatment on MGMT activity in PBMCs from patients with metastatic melanoma and recurrent glioma. During the treatment period, no change in MGMT

activity was observed. However, close examination during each cycle permitted us to conclude 2 major points. First, we demonstrated that the association of a short period of MET-free diet (1-4 days) with cystemustine treatment led to a reduction of MGMT activity in PBMCs (average of 13%). Second, we showed that the duration of the MET-free diet period of 1, 2, 3 or 4 days did not affect the MGMT activity after treatment. The absence of a cumulative effect of the lengthening of the MET-free diet period on MGMT activity was also reported for plasma MET concentrations.

The down-regulation of MGMT activity after the MET-free diet associated with nitrosourea treatment as obtained in the present study was in agreement with the results expected by MET restriction. In animal models, Kokkinakis *et al.* (20) demonstrated that MET depletion down-regulates MGMT activity in glioma cell lines and in human glial tumor xenografts (18). In our study, the decrease of the MGMT activity in PBMCs observed after treatment with a MET-free diet and cystemustine was not affected by the lengthening of the MET-free diet period but seemed to be influenced by the level of plasmatic MET depletion induced by the diet. In effect, our data noted a tendency towards a positive correlation between the change (from before to after treatment) in MGMT activity and the plasmatic MET depletion (r=0.517, p=0.15, n=9). This hypothesis might be confirmed in a larger cohort of patients. Moreover, as the methylating status of MGMT gene was a major mechanism involved in MGMT regulation (27-29), which could be affected by MET depletion, it would be interesting to evaluate this parameter in order to explain this down-regulation.

Our clinical study was based on experimental data showing that MET restriction may sensitize tumor cells to the antitumoral effect of CENU; one of possible mechanisms identified was the down-regulation of MGMT in tumors (18). The reduction of MGMT activity on PBMCs of patients with metastatic cancer after treatment with a MET-free diet and CENU observed in our clinical study could be considered as a proof of the concept of the association of CENU treatment with MET restriction. Moreover, as expected, toxicity was mainly hematological and seemed to be comparable to that obtained in a previous trial testing cystemustine treatment alone in melanoma and glioma patients (5, 6). Curently, a phase II clinical trial has been initiated in our department to evaluate the therapeutic index of this association, with response rate and toxicity as primary end-points. In addition, an evaluation of MGMT activity in PBMCs and if possible in tumor will be realized. In the futur, the use of methioninase and especially PEGylated methioninase developped by Hoffman and collaborators (30-32) in order to reduce its potential antigenicity and lengthen its effect, could be a new approache to improve the plasmatic MET depletion and could offer the possibility to potentiate more efficacy nitrosourea treatment (18).

Conclusion

These preliminary data show that the association of a MET-free diet and cystemustine treatment down-regulates MGMT activity in PBMCs of patients with melanoma or glioma, and this seemed to be correlated to plasma MET depletion. These results could be considered as a proof of the concept of the association and have motivated us to continue the investigation of this association especially for the therapeutic index in a phase II clinical trial.

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