Expression of $O^6$-Methylguanine-DNA Methyltransferase in Six Human Medulloblastoma Cell Lines

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ABSTRACT

Six well characterized human medulloblastoma cell lines (D283 Med, Daoy, D341 Med, D384 Med, D425 Med, and D458 Med) were examined for the expression of $O^6$-methylguanine-DNA methyltransferase (MGMT) by activity and Western and Northern blot analysis. High levels of MGMT activity were present in D283 Med, Daoy, D341 Med, and D384 Med (1.36, 0.80, 1.68, and 1.62 pmol/mg of protein, respectively), but negligible MGMT activity was detected in D425 Med and D458 Med (0.06 and 0.05 pmol/mg of protein, respectively), which were derived separately at different times from the same patient. The presence of MGMT protein and its transcript was demonstrated in D283 Med, Daoy, D341 Med, and D384 Med, but both the protein and the mRNA were undetectable in D425 Med and D458 Med. Nevertheless, all six cell lines contained an apparently unaltered MGMT gene, as determined by Southern blot analysis. The absence of MGMT activity in D425 Med and D458 Med is likely due to the absence of the protein, resulting from a lack of transcription of the MGMT gene. The varying levels of expression of MGMT in medulloblastoma cells found in this study should provide a molecular basis for drug design and selection in chemotherapy of this tumor.

INTRODUCTION

MGMT$^1$ (EC 2.1.1.63) is a ubiquitous DNA repair protein, first discovered in Escherichia coli, that removes simple alkyl groups from $O^6$-alkylguanine in DNA by accepting them irreversibly at a unique cysteine residue, thereby resulting in its deactivation (1–3). The mammalian MGMT plays an important role in carcinogenesis induced by alkylating agents and in antitumor chemotherapy (4, 5). Based on the calculation from its amino acid sequence, MGMT has a molecular weight of 21.7 kDa, but purified human MGMT has been found to migrate at ~25 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6).

The distribution of MGMT activity in normal and neoplastic human cells and tissue has been studied extensively. Normal cells, tissues, and tumor biopsies so far examined contain negligible MGMT activity, although the activity varies among different tissues, with normal brain tissue containing the lowest (4, 7, 8). A significant number of human tumor cells and transplantable xenografts have been determined to lack detectable MGMT activity; these are called Mer$^-$ or Mex$^-$ cells (9–13).

Recently, successful generation of monoclonal and monospecific antibodies against MGMT has allowed immunodetection and quantitation of MGMT protein (12, 14, 15). Moreover, isolation of the human MGMT cDNA clone has provided an opportunity to analyze MGMT gene expression in detail (16–18). Tano et al. (16) found that a Mer$^-$ HeLa and a Mex$^-$ lymphoblastoid cell line had undetectable mRNA, whereas a Mer$^+$ HeLa line clearly expressed a MGMT transcript in Northern blots. Southern analysis indicated that the gene was deleted in the Mer$^-$ HeLa line but apparently was unchanged in the Mex$^+$ line. Rydberg et al. (18) showed essentially the same correlation between mRNA expression and the Mex phenotype in two Mex$^+$ and three Mex$^-$ lymphoblastoid lines; however, there was no evidence for gene deletion, amplification, or rearrangement in any of these cells. Pieper et al. (19) also demonstrated readily detectable expression of an MGMT transcript in two Mer$^+$ cell lines. MGMT mRNA could also be detected in four Mer$^-$ lines, but only after 40 cycles of amplification by polymerase chain reaction. Most recently, Fornace et al. (13) reported 15 human cell lines that included one liver, ovary, bone, bladder, and lung, three colon, and seven brain cell lines. Three of the brain cell lines, however, were derived from a single line, and a fourth had been characterized previously by Pieper et al. (19). MGMT mRNA was undetectable in all the Mer$^-$ lines in this study, whereas there was an approximate correlation between amount of MGMT activity and mRNA level in the Mer$^+$ lines.

The combination of measurement of activity, immunodetection of protein, and analysis of gene expression should generate valuable information to elucidate the molecular basis of MGMT expression and regulation in normal and neoplastic human cells. While providing valuable information, the previous reports did not systematically examine all the parameters of MGMT expression currently amenable to analysis, in a significant number of tumor cell lines representing a single well defined tumor type. In this study, we set out to characterize MGMT expression comprehensively in a group of six well characterized human medulloblastoma cell lines. Two of these cell lines (derived separately at different times from the same patient) contain negligible MGMT activity, MGMT protein, and MGMT mRNA, while the other four lines all have high levels of MGMT.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Six medulloblastoma cell lines, D283 Med, Daoy, D341 Med, D384 Med, D425 Med, and D458 Med, were used in this study. The establishment and characterization of these cell lines were reported previously (20–23). The known MGMT-positive human lymphoblast leukemia cell line CEM-CCRF (24) was used as a control. Daoy grows as an adherent monolayer, and the rest grow in suspension. Medulloblastoma cells were cultured in a zinc-option medium supplemented with 10% fetal calf serum, at 37°C in a 5% CO₂...
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Atmosphere. The absence of contamination with Mycoplasma in those cells was tested as described previously (20). Cells that grow in suspension were harvested by centrifugation and trypsinization. The cells were washed twice with phosphate-buffered saline at 4°C, frozen in liquid nitrogen, and stored at −135°C until use. Medulloblastoma cells were harvested at the same passage number for enzyme activity, Western blot, and Northern blot analysis. Cells for genetic analysis were harvested either at the same passage as described above or at the next passage.

Preparation of Extracts. Frozen cell pellets were suspended with 2 volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 0.02% sodium azide, 0.1 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and aprotonin (20 trypsin inhibitor units/liter; Sigma, St. Louis, MO), sonicated for a total of 90 s, and centrifuged for 45 min in a Beckman type 50 Ti rotor at 40,000 rpm. Supernatants were saved and stored in liquid nitrogen for MGMT activity assay and Western blot analysis.

MGMT Activity Assay. MGMT activity was studied by detecting the appearance of radiolabeled protein after incubation of cell extracts with methylated DNA (24). Briefly, increasing concentrations (0.1 to 0.5 mg of protein) of cell extract were incubated with [methyl-3H]-nitrosourea-treated calf thymus DNA containing 2 pmol of O-alkyl-[3H]-guanine, in a 37°C water bath, for 30 min in 200 µl of buffer (pH 7.5, 10 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 0.02% sodium azide, 10% glycerol). To stop the reaction, 500 µl of 5% trichloroacetic acid were added. Samples were heated to 80°C for 30 min to hydrolyze the DNA and then chilled, by cooling on ice for 5 min. After addition of 100 µl of bovine serum albumin, the protein precipitate was collected on glass fiber filters (Whatman GF/F). The filters were washed twice with 15 ml of 5% trichloroacetic acid and once with 95% ethanol and then were placed in scintillation vials. NCS tissue solubilizer (Amersham, Arlington Heights, IL) was added to solubilize the precipitates, and then scintillation fluid was added. Radioactivity of the methyl-3H-labeled MGMT was measured in a liquid scintillation counter (LKB, Gaithersburg, MD). MGMT activity was calculated from the linear part of the assay.

Western Blot Analysis. Proteins in cell-free extracts were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a Bio-Rad mini-gel apparatus, and the proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA) by PosiBlot pressure blotter (Stratagene, La Jolla, CA), and then chilled, by cooling on ice for 5 min. After addition of 100 µg of bovine serum albumin, the protein precipitate was collected on glass fiber filters (Whatman GF/F). The filters were washed twice with 15 ml of 5% trichloroacetic acid and once with 95% ethanol and then were placed in scintillation vials. NCS tissue solubilizer (Amersham, Arlington Heights, IL) was added to solubilize the precipitates, and then scintillation fluid was added. Radioactivity of the methyl-3H-labeled MGMT was measured in a liquid scintillation counter (LKB, Gaithersburg, MD). MGMT activity was calculated from the linear part of the assay.

RESULTS

MGMT Activity. Medulloblastoma cell lines and a known MGMT-positive line, CEM-CCRF, were assayed for MGMT activity. Table 1 shows a high level of MGMT activity in CEM-CCRF, as expected. MGMT activity in D283 Med, Daoy, D341 Med, and D348 Med ranged from 0.80 to 1.68 pmol/mg of protein. Less than 0.1 pmol of MGMT activity was detected in D425 Med and D458 Med; these two lines were accordingly classified as Mer−.

MGMT Protein. To examine the levels of MGMT protein present in these medulloblastoma cell lines, we performed Western blot analysis of extracted cellular protein with a cocktail of anti-MGMT monoclonal antibodies (4.A1, 21.A8, and 34.B6). These antibodies can detect as little as 20 fmol of MGMT (12). Five dilutions (2, 4, 8, 16, and 32 µg) of protein from CEM-CCRF cells were loaded, and an increasing intensity of immunostained bands was observed at a position corresponding to the transferase. Distinct immunostained bands located at the same position as that of CEM-CCRF were demonstrated in extracted proteins from D283 Med, Daoy, D341 Med, and D348 Med. No anti-MGMT-reactive protein was detected in

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**Table 1 Relative expression of MGMT activity, protein, and mRNA in human medulloblastoma cell lines, compared with CEM-CCRF cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGMT activity (pmol/mg)*</th>
<th>Relative MGMT activity (% of CEM-CCRF)</th>
<th>Relative MGMT protein (% of CEM-CCRF)</th>
<th>Relative MGMT mRNA (% of CEM-CCRF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D283 Med</td>
<td>1.36 ± 0.13 (3)</td>
<td>48</td>
<td>25 ± 3</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>Daoy</td>
<td>0.80 ± 0.20 (3)</td>
<td>28</td>
<td>21 ± 5</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>D341 Med</td>
<td>1.68 ± 0.04 (3)</td>
<td>59</td>
<td>34 ± 6</td>
<td>15 ± 8</td>
</tr>
<tr>
<td>D348 Med</td>
<td>1.62 ± 0.01 (3)</td>
<td>57</td>
<td>40 ± 2</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>D425 Med</td>
<td>0.06 ± 0.05 (3)</td>
<td>2</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>D458 Med</td>
<td>0.05 ± 0.02 (3)</td>
<td>2</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>CEM-CCRF</td>
<td>2.83 ± 0.20 (9)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Activity is expressed as pmol of methyltransfer pmol of extract protein. Values and SD were calculated by linear regression analysis of at least six determinations and averaged for the number of experiments shown in parentheses.

**Table 1** Relative expression of MGMT activity, protein, and mRNA in human medulloblastoma cell lines, compared with CEM-CCRF cells
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Fig. 1. Western blot analysis of protein extract from human medulloblastoma cell lines and CEM-CCRF cells. Lanes 1 to 5, increasing amounts of protein extracts (2, 4, 8, 16, and 32 µg/lane, respectively) from CEM-CCRF cells of known MGMT activity; lane 6, D283 Med (20 µg); lane 7, Daoy (20 µg); lane 8, D341 Med (20 µg); lane 9, D384 Med (20 µg); lane 10, D425 Med (40 µg); lane 11, D458 Med (40 µg). The filter was probed with a cocktail of anti-MGMT monoclonal antibodies 4.A1, 21.AS, and 34.B6. The immunostained bands of increasing intensity at ~25 kDa in CEM-CCRF correspond to the transferase. Immunoreactive protein is also present in D283 Med, Daoy, D341 Med, and D384 Med but not in the Mer− lines D425 Med or D458 Med.

MGMT mRNA Expression. Northern blot analysis was carried out to examine the steady state levels of MGMT mRNA in these medulloblastoma cell lines, using a 32P-labeled human MGMT cDNA (Fig. 2A). The same blot probed with a β-actin cDNA (B). RNA ladder (in kilobases) was used for sizing single-stranded RNA (BRL). Progressively intense hybridization bands of approximately 23, 10.5, and 7.5 kilobase pairs were observed in all samples examined. Therefore, no obvious gene deletion, rearrangement, or amplification of the MGMT gene has occurred in these cell lines.

Relative Expression of MGMT. MGMT activity, as shown in Table 1, and densitometric scans of the MGMT signals in Western and Northern blots, as shown in Figs. 1 and 2, were used to estimate the levels of MGMT activity, protein, and mRNA in medulloblastoma lines, relative to CEM-CCRF cells of known activity. Relative MGMT activity ranged from 28% to 59%, MGMT protein from 21% to 40%, and MGMT mRNA from 13% to 38% in the four MGMT-positive medulloblastoma lines, compared with CEM-CCRF cells (Table 1). The most likely reason that the relative values for MGMT activity in Table 1 are higher than those for the protein or mRNA is the underestimation of activity in CEM cell extracts, due to the presence of MGMT protein inactivated during extraction. Immunostaining of the protein and genetic probing of the mRNA are, therefore, probably the most reliable measures of MGMT expression. Although these numbers were estimates, the presence or absence of MGMT mRNA clearly determines the Mer phenotypes of these cells; among the Mer+ lines, there is a general correlation between all the parameters, exemplified by consistently lowest values for the Daoy line (Table 1).

DISCUSSION

MGMT expression in six medulloblastoma cell lines was examined in this study. MGMT activity in D283 Med, Daoy, D341 Med, and D384 Med ranged from 0.80 to 1.68 pmol/mg of protein; activity in D425 Med and D458 Med was negligible (Table 1). D245 Med and D458 Med were established from D425 Med and D458 Med was established from the...
same patient, the former from the primary tumor and the latter from the metastatic cells in the cerebrospinal fluid, 6 months after diagnosis, following failure of craniospinal radiotherapy and adjuvant cyclophosphamide, vincristine, and cisplatin (23). MGMT protein and mRNA were demonstrated in the four cell lines that contained high levels of MGMT activity, but neither was detectable in D425 Med or D458 Med. An apparently normal MGMT gene was, however, present in all six cell lines. Lack of MGMT activity in D425 Med and D458 Med appears not to be the result of synthesis of a nonfunctional MGMT protein or due to any obvious deletion or rearrangement of the MGMT gene but, rather, due to the absence of MGMT protein and mRNA. A similar phenomenon of lack of transcription of the MGMT gene in Mer− cells containing an apparently unaltered MGMT gene has been reported by several other groups (13, 18, 19). In one instance, deletion of the MGMT gene was reported (16). Absence of mRNA in Mer− cells is most likely due to lack or severe inhibition of MGMT gene transcription; however, the exact mechanism resulting in the Mer− phenotype remains unknown. All data concerning the absence of MGMT mRNA in Mer− cells were obtained from studies with cultured cells. Without a comparable study of tumor biopsy tissues, it is not known whether the deficiency of MGMT in cultured cells occurs before or after their establishment from tissue samples. However, the fact that D425 Med and D458 Med, which were derived at different times from the same patient, are both Mer− is good evidence that this patient’s tumor was really Mer−. Extraction of MGMT protein and mRNA from tumor tissues will not resolve this issue, because of the “contamination” of tumor tissues by normal cells that express MGMT. In situ hybridization or immunohistochemical studies, when an anti-MGMT antibody suitable for such histochemical study is available, may resolve this question. The regulation of MGMT transcription is likely to be complex and will not be fully understood until genomic clones of the MGMT gene are analyzed, likely including promoter and regulatory regions.

Medulloblastoma is the most common malignant brain tumor diagnosed in the pediatric population. Although surgical intervention followed by craniospinal irradiation has resulted in 5-year survival rates of 50–70% for children with standard risk medulloblastoma (29) (i.e., small localized tumors with <1.5 cm² postsurgical residual disease), the majority of children present with advanced disease and demonstrate only a 30% long-term survival. The activity of several chemotherapeutic agents, notably cyclophosphamide, cisplatin, vincristine, and melphalan, has been demonstrated clearly in children with recurrent medulloblastoma, although tumor regressions are universally followed by tumor regrowth and death (30). Two large randomized trials have strongly suggested the benefit, in newly diagnosed patients, of adjuvant chemotherapy consisting of 1-(2-chloroethyl)-3-chlorohexyl-nitrosourea-vincristine (31) or 1-(2-chloroethyl)-3-chlorohexyl-nitrosourea-vincristine-prednisone (32), although inadequate patient staging and fluctuating P values preclude unequivocal confirmation of the benefit of chemotherapy in increasing survival.

The major impediment to chemotherapeutic cure is drug resistance that is present at diagnosis (de novo resistance) or develops in previously chemo-sensitive tumors (acquired resistance). Although the etiology of resistance to chemotherapeutic agents is multifactorial, recent in vitro and in vivo studies have shown that tumor sensitivity to nitrosoureas and procarbazine is inversely related to MGMT levels (11, 33–35). The nitrosoureas and procarbazine have been shown to be inactive in single-agent Phase II trials utilizing radiographic response criteria (30), which may reflect elevated MGMT levels in these tumors (consistent with the current results utilizing medulloblastoma-derived cell lines). The obvious implication of our studies is the potential role for quantitation of MGMT levels in freshly resected tumors (obtained at diagnosis or recurrence), allowing rational selection of chemotherapeutic agents. Avoidance of nitrosoureas or procarbazine in the therapy of tumors with elevated levels of MGMT may spare patients treatment with an ineffective agent. Selection of agents not detoxified by MGMT, such as the nitrogen mustard-based alkylators cyclophosphamide or melphalan, would be reasonable in this setting (36). Alternatively, pretreatment with either methylating agents, such as streptozotocin (37), or O6-alkylguanines (38–40) may be used to deplete MGMT levels, allowing nitrosourea or procarbazine cytotoxicity to remain unaltered by MGMT (although other mechanisms for resistance to these agents may still be operational in the tumors).

The pattern of MGMT expression observed in the human medulloblastoma cell lines is consistent with clinical response of this tumor to nitrosoureas, supports pretreatment measurement of MGMT levels in patients’ tumors, and provides the rationale for use of these lines as models for inactivation of MGMT as a means of reversing/bypassing MGMT-mediated drug resistance in medulloblastoma.

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