O$^6$-Methylguanine-DNA Methyltransferase-deficient Phenotype in Human Gliomas: Frequency and Time to Tumor Progression after Alkylating Agent-based Chemotherapy

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ABSTRACT

The DNA repair protein $O^6$-methylguanine-DNA methyltransferase (MGMT) contributes to the resistance of human brain tumor cell lines and xenografts to methylating and chloroethylnating agents. We assayed MGMT in 174 newly diagnosed or recurrent gliomas to (a) quantitate changes in MGMT activity associated with alkylating agent-based chemotherapy; and (b) assess the contribution of MGMT to clinical outcome. Glioma MGMT activity ranged 300-fold, averaging 3,800 molecules/cell or $<0.25$ fmol/10$^6$ cells. Tumors treated with surgery alone and tumors recurring after surgery and radiation did not differ significantly in frequency of the Mer$^-$ phenotype (29% versus 24%). However, the frequency of the Mer$^-$ phenotype among tumors recurring after surgery, radiation, and alkylating agent-based chemotherapy was 7-fold lower than in tumors treated with surgery alone (4.3% versus 29%; $P \leq 0.02$) and 6-fold lower than in tumors recurring after surgery and radiation (4.3% versus 24%; $P \leq 0.05$). In contrast to gliomas, there was no relationship of alkylating agent-based therapy with the frequency of the Mer$^-$ phenotype in paired histologically normal brain. These data suggest that alkylating agents, either alone or synergistically with radiotherapy, selectively kill Mer$^-$ glioma cells in situ. Importantly, Mer$^-$ and Mer$^+$ tumors did not differ in time to tumor progression following treatment with alkylating agents, indicating that although Mer$^-$ glioma cells may be differentially killed by alkylators, factors other than Mer phenotype were the principal determinants of time to clinical progression. Nonetheless, our results support the possibility that complete ablation of glioma MGMT with substrate analogue inhibitors could improve the efficacy of alkylating agent-based chemotherapy.

INTRODUCTION

Despite advances in surgical technique and radiation therapy, the prognosis for malignant gliomas remains poor, with 2-year survival rates $<20\%$ (1, 2). The chloroethylnating agents carmustine (BCNU)$^4$ and lomustine (CCNU) and the methylating agents procarbazine and temozolomide, in single agent or combination chemotherapy, have been shown to modestly increase response rates and survival times when used as an adjuvant to surgery and radiation (1, 2). The clinical effectiveness of these agents is attributed, in part, to their relatively high yields of cytotoxic $O^6$-alkylguanine adducts in DNA (3, 4).

Intrinsic and acquired resistance to alkylating agents limits the efficacy of these drugs in the therapy of gliomas. A large body of evidence demonstrates that the DNA repair protein MGMT contributes to alkylating agent resistance in human brain tumor cell lines and xenografts (5–10). MGMT mediates resistance by removing cytotoxic alkyl adducts from the $O^6$ atom of guanine in DNA to an internal cysteine, yielding guanine and $S$-alkylcysteine. Because the alkyl receptor site in MGMT is not regenerated, the number of adducts that can be removed from DNA in vivo is limited by the number of MGMT molecules and the rate of synthesis of the protein (5).

Most human neoplasms (5), including those from brain (11–18), express MGMT activity, suggesting that MGMT contributes to the alkylating agent resistance of tumors in vivo. We have observed a 300-fold range of detectable activity among 152 adult brain tumors (18). Importantly, 25% of specimens had no detectable activity (Mer$^-$), implying that an appreciable fraction of brain tumors may have heightened sensitivity to alkylating agent-based therapy

$^4$ The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MGMT, $O^6$-methylguanine-DNA methyltransferase; TTP, time to tumor progression.

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alkylating agents as a consequence of lacking MGMT. Evidence of preferential killing of Mer− glioma cells in situ would support this idea and would strengthen the rationale for antiresistance strategies using inhibitors of MGMT, such as O6-alkylguanine (19).

In the present study, we analyzed the relationship of prior alkylating agent-based therapy to MGMT activity in recurrent gliomas, and the relationship of MGMT activity to response to alkylator therapy. We observed a 6- to 7-fold reduction in the frequency of the Mer− phenotype among tumors that recurred after treatment with alkylators, whereas the frequency of the Mer− phenotype in adjacent, microscopically normal brain was unchanged. We interpret these findings to suggest that Mer− glioma cells are selectively killed in situ by alkylating agent-based chemotherapy. However, despite the putative selective killing of Mer− cells, we found that the Mer− phenotype was not associated with a longer time to tumor progression after alkylating agent-based therapy. This result indicates that variables other than Mer phenotype can be overriding determinants of clinical response to current treatment regimens that include alkylators. Nonetheless, our observations support the idea that complete ablation of glioma MGMT activity with substrate analogue inhibitors might improve clinical response.

MATERIALS AND METHODS

Tissue. Tumors were resected at the University of Washington Medical Center from 1991 to 1996. Subcortical normal brain adjacent to tumor was obtained from 104 patients. The specimens included 152 tumors and 70 normal brain samples for which MGMT activity had been reported in earlier studies of the relationship of activity to patient and tumor characteristics (15, 18, 20). Diagnosis was obtained from the final neuropathology report. Normal tissue was microscopically free of hypercellularity, infiltrating tumor, endothelial proliferation, edema, and gliosis. Demographic information together with course and response to therapy was obtained from medical records.

Immediately upon resection, tissue was placed in ice-cold DMEM supplemented with 15% fetal bovine serum and was transported to the laboratory within minutes. We have successfully established cell lines from tumors held overnight on ice in supplemented DMEM. We have also found identical MGMT activities in aliquots of brain tumor and normal brain processed either immediately upon arrival in the laboratory or after being held overnight on ice in supplemented medium, demonstrating that our protocol for transporting specimens preserves cell viability and MGMT activity. To determine cell number, a small piece of tissue (0.05–0.1 g) was finely minced with scalpels, after blood vessels were removed, and suspended in PBS. The suspension was serially passed through 18, 20, and 22 gauge needles to completely disrupt the tissue. After filtration of the suspension through a 60 μm mesh, cells were pelleted by centrifugation. Contaminating erythrocytes were eliminated by hypotonic lysis, and debris was removed by repeated washes with PBS. After the washed pellet was resuspended in PBS, cells in a 10-μl aliquot were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and the total cell number was determined by counting with a hemacytometer. The remaining tissue (0.5–5 g) was divided into two or more aliquots, frozen in liquid nitrogen, and stored at −80°C.

MGMT Assay. The MGMT content of extracts (i.e., high speed supernatants of whole tissue sonicates) was assayed by quantitating the transfer of radioactivity from a DNA substrate containing [methyl-3H]O6-methylguanine to protein, as described previously in detail (18, 20). MGMT content is the mean of at least five determinations that generally differed by no more than 20%. We have validated this assay for extracts prepared from tumor and normal brain by demonstrating that (a) the increase in radioactivity transferred to protein is linear over a 3- to 5-fold range of added extract; (b) the transfer of radioactivity is sensitive to protease digestion; (c) transfer of radioactivity is prevented by O6-alkylguanine, a potent inhibitor of MGMT (19); and (d) transferred radioactivity migrates on a SDS-polyacrylamide gel at M, 22,000, identical to that of human MGMT (5).

Additional controls indicated that the wide range of MGMT activity observed is unlikely to be due to degradation of MGMT and/or its [3H]DNA substrate during extraction and assay, or to a diffusible inhibitor in extracts. (a) The protease inhibitors aprotinin, leupeptin, and pepstatin did not increase activity when present together during extraction and assay. (b) All Mer− samples displayed linearity of activity with added extract. (c) Additive amounts of activity were found for mixed extracts of Mer− specimens with high and low MGMT activities. (d) Assay of an 8:1 (v/v) mixture of every Mer− extract with a Mer− extract yielded activity expected for the Mer− sample. (e) Extracts prepared from mixtures of Mer− brain tissue and Mer− cultured medulloblastoma cells yielded activity expected of the cultured cells.

We define the Mer− phenotype here as MGMT activity <0.25 fmol/10^6 cells or 151 molecules/cell. Mer− phenotype is a functional term that refers to the limit of detection in the particular assay used. In our biochemical assay, the definition of Mer− phenotype was established by (a) the specific activity of the [methyl-3H]-O6-methylguanine moiety in the DNA substrate (20 Ci/mmol); (b) counting efficiency equal to 14%; (c) the requirement that extract from at least 7.7 × 10^6 cells be included in the assay; and (d) the requirement that extract from 7.7 × 10^6 cells yield less than 12 cpm (86 dpm) above an unincubated control that displays 25–40 cpm when counted for 10 min. On average, the Mer− tumor and normal brain samples reported here yielded 2.4 ± 1.6 cpm (mean ± SD) above the unincubated control; these counts were sporadic and were not proportional to added extract. Notably, the limit of detection in our assay is lower than that of histocytochemical procedures (~4000–30,000 molecules/cell (17, 21)) and comparable to that of oligonucleotide-based assays (~200 molecules/cell (22)) and Western assay (~80 molecules/cell if 7.7 × 10^6 cell-equivalents could be analyzed (23)).

Alkylating Agent-based Chemotherapy. Sixty-two patients received alkylating agent-based chemotherapy. Eight of these individuals were treated with BCNU. Of the eight, five received 200 mg/m^2 BCNU i.v. every 6–8 weeks; three received 80 mg/m^2 BCNU and 33 mg/m^2 cisplatin i.v. daily for 3 days every 6 weeks for three cycles and then 80 mg/m^2 BCNU i.v. daily for 3 days every 6 weeks for two cycles. Fifty-four patients were treated with procarbazine and CCNU in combi-
nation with other drugs. Of the 54, 41 (PCV group) received 110 mg/m² CCNU p.o. on day 1, 60 mg/m² procarbazine p.o. on days 8 through 21 and 1.4 mg/m² vincristine i.v. on days 8 and 29. This cycle was repeated every 6 to 8 weeks for up to 8 cycles. Nine patients in the PCV group also received 300 mg/m² hydroxyurea p.o. every 6 h during radiotherapy. Thirteen of the 54 patients treated with procarbazine and CCNU (6-drug group) received 30 mg/m² 6-thioguanine every 6 h for 12 doses, 50 mg/m² procarbazine every 6 h for 4 doses, 1 dose of 400 mg/m² dibromodulcitol, and 1 dose of 100 mg/m² CCNU (all p.o.) in 3 days, followed by a continuous i.v. infusion of 1000 mg/m² of 5-fluorouracil on days 14 and 15 and 1000 mg/m² hydroxyurea every 4 h for 4 doses on day 14. This cycle was repeated every 6–8 weeks. Chemotherapy was terminated upon unacceptable levels of toxicity or evidence of tumor progression.

All 62 patients treated with alkylators also received prior radiotherapy (52–62 Gy) in 4 to 6 weeks. Of the 62, 28 individuals had newly operated tumors that were treated with alkylators within 2 to 4 weeks of completing radiotherapy. Another six individuals had tumors recurring after initial surgery only and were treated with radiation and alkylators after a second surgery. The remaining 28 individuals had tumors recurring after initial surgery and radiation and were given alkylating agents after a second surgery.

Statistical Analysis. Standard statistical procedures (24) were applied as specified by using Microsoft Excel (Microsoft, Redmond, WA). For purposes of calculation, Mer samples were assigned a value of 0.125 fmol/10⁶ cells, i.e., one-half the lower limit of detection.

RESULTS

Patient and Tumor Characteristics. One hundred seventy-four glial tumors were obtained from 166 informed patients (Table 1). Patient age ranged from 18 to 79 years (mean ± SD, 45 ± 13 years), and the ratio of males to females was 1.9; in accord with the known gender bias for gliomas (25). One hundred twenty-two tumors (70%) were astrocytic gliomas, the most frequent glial tumor diagnosis; they included 16 astrocytommas, 23 anaplastic astrocytommas, and 83 glioblastomas. Other tumors included 30 oligodendrogliomas and 22 mixed oligodendroglioma-astrocytommas.

As indicated in Table 1, tumors were divided among three treatment groups. One hundred tumors were treated with surgery only, including 23 that recurred after prior resection without adjuvant therapy. The 77 newly operated and 23 recurrent tumors did not differ significantly in MGMT activity (6.8 ± 11 versus 3.7 ± 5.5 fmol/10⁶ cells) or in fraction of Mer− specimens (27% versus 35%). Fifty-one tumors were recurrent following surgery and radiotherapy, and 23 were recurrent following surgery, radiotherapy, and alkylating agent-based chemotherapy.

MGMT in Tumors. The MGMT activity of tumor extracts was quantitated in our standard biochemical assay, which yields the average activity of many millions of cells. Tumors with activity <0.25 fmol/10⁶ cells or 151 molecules/cell, the limit of detection of the assay, were assigned the Mer+ phenotype ("Materials and Methods" and Refs. 18 and 20). The MGMT activity of all 174 tumors covered a 300-fold range, from 0.30 to 89 fmol/10⁶ cells (~180 to 54,000 molecules/cell), the mean being 6.3 ± 12 fmol/10⁶ cells (~3,800 ± 7,200 molecules/cell). Twenty-four percent of tumors had no detectable activity (Mer− phenotype). Males and females did not differ significantly in mean activity (5.8 ± 11 versus 7.2 ± 15 fmol/10⁶ cells) or fraction of Mer− tumors (24% versus 25%). The wide variation in activity is similar to previous observations in brain tumors and other neoplastic human tissues (11–18). A detailed analysis of the relationships of MGMT activity to patient and tumor characteristics (e.g., age, diagnosis, ploidy, and proliferative rate) for 152 of the specimens has been presented elsewhere (18).

The MGMT activity in 174 tumors grouped by treatment regimen and diagnosis is summarized in Table 2. There was no statistically significant difference in mean MGMT (6.1 ± 10 versus 5.9 ± 13 fmol/10⁶ cells) or fraction of Mer− specimens (29% versus 24%) between tumors treated with surgery alone and tumors recurrent after surgery and adjuvant radiotherapy. In contrast, the frequency of the Mer− phenotype among tumors recurring after surgery, radiation, and alkylating agent-based chemotherapy was 7-fold lower than for tumors treated with surgery alone (4.3% versus 29%; P ≤ 0.02; χ² = 6.14) and 6-fold lower than for tumors recurring after surgery and radiotherapy (4.3% versus 24%; P ≤ 0.05; χ² = 3.94). Comparable findings applied to the large astrocytic glioma subgroup where the frequency of the Mer− phenotype among tumors recurring...
portion of adjuvant radiotherapy was not associated with an altered proportion of Mer allele in recurrent tumors. The distribution of MGMT activity among tumors recurring after surgery and radiotherapy (4.5% versus 24%) was comparable to that of tumors recurring after surgery, radiation, and alkylating agents. Moreover, ANOVA revealed no significant difference in the distribution of activity among Mer allele tumors in the three treatment groups.

**MGMT in Paired Normal Brain.** Histologically normal brain adjacent to 104 tumors was analyzed as summarized in Table 3. The patient subgroup was similar in age and gender bias to all patients (data not shown), and mean MGMT activity of the 104 tumors (4.4 ± 7.0 fmol/10⁶ cells) was comparable to the mean of all 174 tumors (6.3 ± 12 fmol/10⁶ cells).

Measurable MGMT activity of the 104 normal brain specimens ranged 68-fold from 0.34 to 23 fmol/10⁶ cells. Mean activity was 1.4 ± 3.3 fmol/10⁶ cells, 3.1-fold lower than the mean of the adjacent tumors (4.4 ± 7.0 fmol/10⁶ cells). Sixty-two percent of the normal tissue specimens was Mer allele. The high frequency of the Mer allele phenotype in normal brain adjacent to tumors and the overall elevation of tumor activity are in accord with previous reports (15, 18, 20).

As summarized in Table 3, treatment with alkylating agents had no statistically significant relationship with mean MGMT activity or frequency of the Mer allele phenotype in normal tissue. In particular, the frequency of the Mer allele phenotype in normal brain from individuals treated with surgery alone, with surgery and radiation, and with surgery, radiation, and alkylating agents was 60, 63, and 63%, respectively. In contrast, the frequency of the Mer allele phenotype among the paired tumors recurring after surgery, radiation, and alkylating agents (6.3%) was 5-fold lower than the frequency in tumors treated with surgery alone (34%; P ≤ 0.05; χ² = 5.43) and 5-fold lower than in tumors recurring after surgery and radiotherapy (30%; P ≤ 0.05; χ² = 3.92). These latter data confirm that the subgroup of 104 tumors paired with normal brain (Table 3) showed a correlation of alkylating agent-based therapy with Mer allele frequency comparable to that observed in the entire tumor population (Table 2).

**Tumor MGMT and Clinical Response to Alkylating Agents.** The foregoing results suggest that the Mer allele phenotype could be a determinant of the alkylating agent survival of glioma cells in situ. To assess the role of the Mer allele phenotype in clinical response to alkylators, we examined the relationship of tumor MGMT activity to TTP, i.e., the interval from initiation of alkylating agent-based chemotherapy to the appearance of radi-

### Table 2: Tumor MGMT activity and treatment regimen

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>n</th>
<th>MGMT³</th>
<th>Fraction Mer²</th>
<th>n</th>
<th>MGMT³</th>
<th>Fraction Mer²</th>
<th>n</th>
<th>MGMT³</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Surgery only</td>
<td>100</td>
<td>6.1 ± 10 (0.40–63)</td>
<td>29%</td>
<td>51</td>
<td>5.9 ± 13 (0.30–89)</td>
<td>24%</td>
<td>23</td>
<td>7.9 ± 18 (0.35–81)</td>
<td>4.3%</td>
</tr>
<tr>
<td>Surgery + RT</td>
<td>63</td>
<td>7.0 ± 12 (0.49–63)</td>
<td>27%</td>
<td>37</td>
<td>6.8 ± 15 (0.30–89)</td>
<td>24%</td>
<td>22</td>
<td>6.5 ± 18 (0.35–81)</td>
<td>4.5%</td>
</tr>
<tr>
<td>Surgery + RT + AA</td>
<td>22</td>
<td>5.3 ± 7.7 (0.40–32)</td>
<td>23%</td>
<td>8</td>
<td>4.6 ± 3.8 (0.70–11)</td>
<td>23%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mixed oligo-astro³</td>
<td>15</td>
<td>3.7 ± 7.4 (0.55–28)</td>
<td>47%</td>
<td>6</td>
<td>2.4 ± 5.1 (0.30–13)</td>
<td>50%</td>
<td>1</td>
<td>39</td>
<td>0</td>
</tr>
</tbody>
</table>

* a: Tumors recurrent after surgery and radiotherapy.
  b: Tumors recurrent after surgery, radiotherapy, and alkylating agent-based chemotherapy.
  c: Mean ± SD, in fmol/10⁶ cells, calculated by using a value of 0.125 fmol/10⁶ cells (one-half the limit of detection) for Mer− tumors, and range of detectable activity.
  d: Mixed oligodendroglioma-astrocytoma.

![Distribution of MGMT activity in Mer+ tumors grouped by treatment regimen.](Image)

**Fig. 1** Distribution of MGMT activity in Mer+ tumors grouped by treatment regimen. □, tumors treated with surgery alone; ■, tumors recurring after surgery and radiotherapy; ▶, tumors recurring after surgery, radiotherapy, and alkylating agent therapy. ANOVA revealed no significant difference in the distribution of activity in the three groups.

after surgery, radiation, and alkylating agent-based chemotherapy was 6-fold lower than for tumors treated with surgery alone (4.5% versus 27%; P ≤ 0.05; χ² = 4.43) and 5-fold lower than for tumors recurring after surgery and radiotherapy (4.5% versus 24%; P ≤ 0.05; χ² = 3.86). Thus, we infer that, whereas adjuvant radiotherapy was not associated with an altered proportion of Mer− tumors, treatment with radiation and alkylating agents was associated with reduced frequency of the Mer− phenotype in recurrent tumors. We believe the likeliest explanation for the observed frequencies is that Mer− glioma cells are preferentially killed by alkylating agents, acting alone or synergistically with radiotherapy (see “Discussion”).

Of importance is the concomitant observation that, although the proportion of Mer− tumors differed following treatment with alkylators, the distribution of MGMT activity among recurrent Mer− tumors showed no statistically significant alteration (Fig. 1). A shift to higher values might be expected if higher MGMT activity conferred greater resistance to alkylating agent killing. In fact, the mean activities of Mer− tumors in the three treatment groups in Table 1 were not statistically different, i.e., 8.6 ± 11 fmol/10⁶ cells for the tumors treated with surgery alone, 7.7 ± 15 fmol/10⁶ cells for tumors recurring after surgery and radiation, and 8.2 ± 19 fmol/10⁶ cells for tumors recurring after surgery, radiation, and alkylating agents. Moreover, ANOVA revealed no significant difference in the distribution of activity among Mer− tumors in the three treatment groups.

### Table 3: Tumor MGMT activity and clinical response to alkylating agents

<table>
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<th>Treatment Regimen</th>
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<td>24%</td>
<td>23</td>
<td>7.9 ± 18 (0.35–81)</td>
<td>4.3%</td>
</tr>
<tr>
<td>Surgery + RT</td>
<td>63</td>
<td>7.0 ± 12 (0.49–63)</td>
<td>27%</td>
<td>37</td>
<td>6.8 ± 15 (0.30–89)</td>
<td>24%</td>
<td>22</td>
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</tr>
<tr>
<td>Surgery + RT + AA</td>
<td>22</td>
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* a: Tumors recurrent after surgery and radiotherapy.
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**Fig. 1** Distribution of MGMT activity in Mer+ tumors grouped by treatment regimen. □, tumors treated with surgery alone; ■, tumors recurring after surgery and radiotherapy; ▶, tumors recurring after surgery, radiotherapy, and alkylating agent therapy. ANOVA revealed no significant difference in the distribution of activity in the three groups.
for 14 Mer... more favorable outcome. Thus, as indicated in Table 4, the mean relationship between MGMT activity and TTP (6 tumours (11 droxyurea, and 5-fluorouracil (6-drug protocol). The remaining 12 patients treated with procarbazine/CCNU were also given 6-thioguanine, dibromodulcitol, hydroxyurea, and 5-fluorouracil.

Of the 62 patients who received alkylating agent-based chemotherapy, 45 had progressed. For the 45 patients with documented tumor progression, linear regression analysis revealed no statistically significant relationship between MGMT activity and TTP (r = 0.061; P = 0.65). In accord, the Mer− phenotype was not associated with a more favorable outcome. Thus, as indicated in Table 4, the mean TTP for 14 Mer− tumors (7.1 ± 6.9 months) and 31 Mer+ tumors (11 ± 10 months) did not differ significantly (t = −1.28; P = 0.20). This finding was consistent within all treatment protocols, i.e., it applied to patients receiving BCNU alone, PCV, or the 6-drug protocol. However, TTP was negatively correlated with patient age (r = −0.342; P = 0.02), as observed in other studies (1, 17). Limiting analysis to the 32 anaplastic astrocytomas and glioblastomas likewise revealed no statistically significant relationship between MGMT and TTP (r = 0.017) or a difference in TTP between 9 Mer− tumors (5.1 ± 4.9 months) and 23 Mer+ tumors (8.8 ± 9.6 months).

We also analyzed separately the 22 newly operated tumors treated with alkylators and the 23 tumors treated with alkylating agents after surgery for recurrence (Table 4). Linear regression analysis revealed no correlation between MGMT and TTP in either group. In accord, mean TTP did not differ significantly for Mer− and Mer+ tumors from newly operated patients (6.8 ± 5.7 versus 9.0 ± 9.1 months; t = −0.738; P = 0.45) or from patients operated for recurrence (7.7 ± 8.8 versus 12 ± 11 months; t = −1.08; P = 0.30).

Lastly, we analyzed the relationship of the Mer phenotype to the proportion of progression-free patients by using the Kaplan-Meier method, which allowed inclusion of the 17 individuals who were progression free at the end of the study. Log-rank analysis revealed no significant difference between 20 Mer− and 42 Mer+ tumors in the proportion of progression-free patients (Fig. 2A). Grouping of the 62 tumors on the basis of other MGMT activities (i.e., the mean, one-half the mean, and 1.5 times the mean) likewise revealed no significant difference. Comparable results were obtained if analysis was limited to anaplastic astrocytomas and glioblastomas (Fig. 2B) or to malignant oligodendrogliomas and mixed oligodendroglioma-astrocytomas (data not shown).

**DISCUSSION**

Greater efficacy of alkylating agent therapy for brain tumors may be achievable if tumor resistance mechanisms can be identified and disabled. A large body of data strongly suggests that MGMT, a DNA repair activity that removes potentially lethal alkyl adducts from the O6 position of guanine, plays an important role in human brain tumor alkylating agent resistance.
Cultured mammalian cells lacking MGMT, referred to as Mer$^-$ or Mex$^-$ (5), as well as yeast (26) and bacteria (27) genetically deficient in MGMT are hypersensitive to killing by alkylators. Moreover, inhibition of MGMT activity with the substrate analogue $O^6$-benzylguanine potentiates the cytotoxicity of clinically relevant chloroethylylating and methylating agents in human brain tumor-derived cell lines and xenografts (6–10). By analogy with the enhanced cytotoxicity observed in numerous model systems, our present findings for 174 newly diagnosed or recurrent gliomas suggest that glioma cells deficient in MGMT are selectively killed in situ by alkylating agent-based chemotherapy.

Ionizing radiation is a mainstay of therapy for malignant brain tumors and, as an adjuvant to surgery, substantially improves survival of adult and pediatric patients (1, 2). We found no difference in mean MGMT activity or in frequency of the Mer$^-$ phenotype between gliomas treated with surgery alone and gliomas recurring after surgery and radiation therapy (Table 2), nor did we find any difference in the normal tissue adjacent to tumors (Table 3). Notably, it is possible that radiation reduces the cytotoxicity of subsequent alkylator therapy, as well as the differential susceptibility of Mer$^+$ and Mer$^-$ cells, by selecting for variants that tolerate increased levels of DNA damage without lethality (28, 29).

In contrast to radiotherapy, we found a strong association between alkylating agent-based chemotherapy, acting either alone or synergistically with radiation, and MGMT activity in tumors (Tables 2 and 3). Whereas 29% of tumors from patients treated with surgery alone and 24% of tumors recurring after surgery and radiotherapy were Mer$^-$, only 4.3% of tumors recurring after surgery, radiotherapy, and alkylating agents had the Mer$^-$ phenotype. Importantly, there was no association of alkylator therapy with the frequency of the Mer$^-$ phenotype in adjacent, histologically normal brains (Table 3).

The reduction in frequency of the Mer$^-$ phenotype among tumors exposed to alkylators was statistically significant for all gliomas and for the astrocytic glioma subgroup, and is based on the assumption of equal Mer$^-$ frequencies in the three treatment groups at the start of therapy. This assumption is supported by consideration of the indications for the three therapeutic regimens and by MGMT measurements of relevant tumor populations. With respect to the surgery versus surgery plus radiotherapy groups, tumor grade is the primary indication for radiation, with high-grade, but not all low-grade, gliomas receiving radiotherapy. This criterion would not be expected to bias Mer$^-$ frequencies, because we have observed that the proportion of Mer$^-$ tumors among astrocytomas, anaplastic astrocytomas, and glioblastomas did not differ statistically (18). The primary indications for alkylating agent therapy are recurrence and high grade. Recurrence per se would not be expected to bias Mer$^-$ frequencies because, as documented above, tumors recurrent after resection without adjuvant therapy did not differ from newly operated tumors in the Mer$^-$ fraction. And as mentioned, tumor grade would not be expected to be a source of bias because the three astroglial diagnoses were found to be associated with similar Mer$^-$ frequencies (18). Although advanced age is a contraindication for alkylator therapy, the three treatment groups had very similar mean ages and standard deviations (Table 1). Finally, with respect to possible bias in assignment of Mer$^-$ tumors to alkylator regimens, the Mer phenotype was not known by the attending physicians.

We believe the likeliest explanation for the reduced frequency of the Mer$^-$ phenotype in recurrent gliomas from patients treated with alkylators is selective killing of Mer$^-$ cells and differential regrowth by surviving Mer$^+$ cells. This explanation is consonant with the large body of data documenting the greater susceptibility of MGMT-deficient prokaryotic and eukaryotic cells to alkylating agent-induced killing (5, 26, 27). Importantly, intercellular heterogeneity of MGMT content has been observed in gliomas, including the occurrence of a minority of Mer$^+$ cells within Mer$^-$ tumors (30). Such heterogeneity is consistent with the epigenetic regulation of MGMT activity.

![Fig. 2](image-url) Proportion of progression-free patients as a function of time after initiation of alkylating agent therapy. A, results for 62 gliomas, 20 Mer$^-$, and 42 Mer$^+$. B, results for 19 anaplastic astrocytomas and 24 glioblastomas, 15 Mer$^-$, and 28 Mer$^+$. 

**Fig. 2**
observed in cultured human cells (31) and the frequent alterations of epigenetic control in tumors (32). The foregoing explanation is also in accord with the results of Phillips et al. (33) who observed selective expansion of Mer− cells following BCNU treatment of heterogeneous xenografts composed of Mer− and Mer+ cells. It also accounts for the unchanged frequency of the Mer− phenotype we observed in normal brain adjacent to tumors because selection would be absent or relatively weak in tissue where proliferation is rare or nonexistent. A significant point is that acquisition of the Mer− phenotype by a formerly Mer− tumor could reflect increased MGMT activity in only a fraction of the cell population. This is possible because our assay measures a cellular average, and increased activity in even a small minority of cells could elevate the mean into Mer− range.

Our data are also consistent with an epigenetically controlled, alkylation-induced increase in MGMT activity that converts tumors from the Mer− to the Mer+ phenotype. However, we do not favor this interpretation for three reasons. First, there was no alkylation-associated difference in the frequency of the Mer− phenotype in normal brain tissue (Table 3). Second, the 1.3-fold greater mean MGMT activity among alkylation agent-treated tumors (Table 2) was fully accounted for by the decreased frequency of Mer− tumors and, as shown in Fig. 1, was not accompanied by a shift toward higher MGMT activity among Mer− tumors. Third, in contrast to the well-documented, selective killing of MGMT-deficient prokaryotic and eukaryotic cells, we are unaware of any clear precedent for a long-term, epigenetically regulated increase in MGMT activity in response to alkylators. Although these reasons do not exclude an epigenetic mechanism, they reduce its likelihood in our view.

Importantly, Mer+ tumors that were recurrent after alkylator therapy did not exhibit elevated levels of MGMT (Fig. 1). We interpret this observation to suggest that widely different MGMT levels in Mer+ tumors prior to alkylation (e.g., 180–54,000 molecules/cell; Table 2) were associated with comparable cell survival. In other words, an MGMT level of at least 151 molecules/cell (our definition here of the Mer+ phenotype) enhanced survival, but beyond that, increased activity did not further protect cells from killing. Failure of higher MGMT levels to promote greater survival would occur when factors other than O6-alkylguanine intervene to become the limiting determinants of cell killing. Examples of such factors include other potentially lethal DNA lesions caused by alkylators, e.g., 3-methyladenine (34). Our results for gliomas in situ are consistent with the finding that resistance to alkylating agents in brain tumor cell lines is determined by factors in addition to MGMT (6–8) and provide strong rationale for the identification of such factors.

Despite our inference that alkylating agents selectively kill Mer− cells, we observed that the Mer− phenotype was not associated with longer TTP (Table 4) or higher probability of remaining progression free after alkylating agent therapy (Fig. 2). One would expect a longer TTP for Mer− tumors if (1) the Mer phenotype was the primary determinant of alkylating agent survival, and hence of residual tumor burden, and (2) if residual tumor burden was the primary determinant of TTP. Our findings are consistent with at least two possibilities. The first is that the putative difference in the killing of Mer− versus Mer+ cells was not large enough to confer a clinically manifest difference between Mer− and Mer+ tumors in the number of cells surviving alkylating agent therapy. This possibility is consistent with the conclusion that resistance to chloroethylnitrosourea and methylating agents in brain tumor cell lines is determined by factors in addition to MGMT (6–8). The second possibility is that other variables limit TTP, e.g., vascularization in areas of surviving cells.

Belanich et al. (17), in a retrospective study of malignant astrocytic gliomas treated with radiation and BCNU, found that MGMT levels ≤60,000 molecules/nucleus, quantitated in individual cells by immunocytochemistry, were associated with a longer TTP. There are major differences in clinical and analytic protocols between our work and that of Belanich et al. One difference is the alkylating agent therapy, i.e., the majority of patients in our study received the methylating agent procarbazine in addition to the chloroethylnitrosourea CCNU. A second difference is the method of MGMT quantitation, i.e., biochemical assay of tissue extracts that measures the average of millions of cells versus quantitative immunofluorescence microscopy of individual cells in fixed tumor sections. Direct comparison of the two studies is precluded by these differences.

Finally, our evidence suggesting that Mer− glioma cells in situ are more susceptible to killing by alkylators supports the ongoing clinical trials of MGMT inhibitors such as O6-benzylguanine (19). If chemical inhibitors can be used to convert all tumors cells to the Mer− phenotype, the number of surviving cells may be reduced to well below the levels achievable at present, and this reduction may lead to improved response in at least some cases.

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