O6-Methylguanine-DNA Methyltransferase Activity in Adult Gliomas: Relation to Patient and Tumor Characteristics

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ABSTRACT

The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) confers resistance to therapeutic methylating and chloroethylating agents in human brain tumor-derived cell lines. In this work, we assayed MGMT activity in 152 adult gliomas to establish correlates with patient and tumor characteristics. We also assayed MGMT in histologically normal brain adjacent to 87 tumors to characterize changes in activity accompanying neurocarcinogenesis. MGMT activity was detectable in 76% (115 of 152) of tumors, ranging -300-fold from 0.3 to 28 fmoi/10^6 cells (180-57,000 molecules/cell). Mean activity was 6.6 ± 13 fmoi/10^6 cells and varied 4-fold among diagnostic groups. The mean for oligodendrogliomas was 2-fold lower (P < 0.03), and for mixed oligodendrogloma-astrocytomas, the mean was 4-fold lower (P < 0.006) than for astroglial tumors. Twenty-five % of gliomas had no detectable MGMT activity (Mer phenotype; <0.25 fmoi/10^6 cells or 150 molecules/cell). Glioma MGMT was inversely correlated with age (P < 0.01), consistent with the observed age dependence in the progenitor tissue of brain tumors (J. R. Silber et al., Proc. Natl. Acad. Sci. USA, 93: 6941-6946, 1996). Neither MGMT activity nor proportion of Mer- tumors differed by sex. Glioma MGMT was correlated with degree of aneuploidy (r < 0.006) but not with fraction of S-phase cells. Mean activity in tumors was 5-fold higher than in adjacent histologically normal brain (5.0 ± 7.6 versus 1.1 ± 1.9 fmoi/10^6 cells; P < 0.001). Notably, elevation of tumor activity was observed in 62% of tissue pairs, ranging from 2-fold to >105-fold. Moreover, 64% of Mer- normal tissue was accompanied by Mer+ tumor. These observations indicate that expression of MGMT activity is frequently activated and/or increased during human neurocarcinogenesis, and that the enhancement is not related to proliferation per se. Significantly, enhanced MGMT activity may heighten the resistance of brain tumors to therapeutic alkylating agents.

INTRODUCTION

The DNA repair protein MGMT is a primary defense against cytotoxic O6-alkylguanine adducts (1). MGMT exerts its protective effect by transferring alkyl adducts from the O6 atom of guanine in DNA to an internal cysteine, yielding guanine and S-alkylcysteine. Although the preferred substrate of MGMT is O6-methylguanine in double-stranded DNA, the protein removes larger alkyl groups at progressively slower rates. MGMT also prevents the formation of chloroethylnitrosourea-induced cross-links by reacting with the monoaduct precursors O6-chloroethylguanine and N1,N2,N3,N4-ethanoguanine (2, 3). Importantly, the alkyl receptor site is not regenerated, thereby limiting the number of O6-alkylguanine adducts that can be removed in vivo to the number of MGMT molecules and the rate of synthesis of the protein (1).

Alkylating agents with proven clinical effectiveness against primary brain tumors (4) include nitrosourea and imidazotetrazine derivatives, which produce relatively high yields of O6-methylguanine (5, 6) and O6-chloroethylguanine (7). Work from numerous laboratories has demonstrated a role for MGMT in conferring alkylating agent resistance in cell lines and xenografts derived from a variety of human tumors, including gliomas (reviewed in Refs. 1 and 8). For example, depletion of MGMT activity with the substrate analogue inhibitor O6-benzylguanine (9) increases the rate of killing of human glioma-derived cell lines by clinically used methylating and chloroethylating agents (10-12). Similarly, the response of human glioma xenografts to these agents is markedly improved when MGMT is depleted with O6-benzylguanine (8).

Most human tumors, including those from brain, express MGMT activity, suggesting that MGMT contributes to alkylator resistance in vivo. We reported previously a 200-fold range of detectability activity among 60 pediatric and adult brain tumors (13). Importantly, 27% had no detectable activity (Mer- or methyl repair-deficient status), suggesting that an appreciable fraction of brain tumors may have heightened sensitivity to alkylating agents as a consequence of lacking MGMT. In the present study, we expand our initial findings (13) by assaying activity in 152 adult primary brain tumors and, for 87 cases, in adjacent, histologically normal brain. Our objective is to delineate biological mechanisms underlying brain tumor alkylator resistance by: (a) seeking correlates of glioma MGMT activity with tumor and patient characteristics; and (b) examining the effects of tumorigenesis on activity in normal brain. Our analysis revealed previously unreported associations between glioma MGMT activity and tumor and patient characteristics. It also demonstrated that tumorigenesis in brain is most often accompanied by an increase in MGMT activity, resulting in the majority of cases from loss of Mer- phenotype in normal progenitor tissue. This increase may have significance for clinical response to alkylating agents.

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 87 patients. The specimens included 22 tumors and 63 samples of normal brain whose MGMT activity was reported earlier (13, 14). Diagnosis was obtained from the final neuropathology report, which included flow cytometry analysis of ploidy for 94 tumors and fraction of proliferating cells for 97 tumors. Normal tissue was microscopically free of infiltrating tumor, endothelial proliferation, edema, and gliosis. Demographic information and course of adjuvant therapy was obtained from medical records.

Immediately upon resection, tissue was placed in ice-cold sterile DMEM supplemented with 15% fetal bovine serum and 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 cellular viability and MGMT activity. To determine cell number, a small piece of tissue (0.05–0.1 g) was finely minced with scalp.
blades after removing blood vessels and suspended in PBS. The suspension was serially passed through 18-, 20-, and 22-gauge needles to completely disrupt the tissue. Following 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 resuspending the washed pellet in PBS, cells in a 10-μl aliquot were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 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 transfer of radioactivity from a DNA substrate containing [methyl-3H]O6-methylguanine to protein, as described previously in detail (13, 14). 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-benzylguanine, a potent inhibitor of MGMT (9); and (d) transferred radioactivity migrates on a SDS-polyacrylamide gel at Mr 22,000, identical to that of human MGMT (1).

Additional controls indicate 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 aprotonin, 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 high and low MGMT; and (d) activity migrates on a SDS-polyacrylamide gel at Mr 22,000, identical to that of human MGMT (1). The activity of Mer* samples was calculated to be 0.25 fmol/106 cells or <151 molecules/cell, in agreement with previous results (13, 14).

RESULTS

Patient and Tumor Characteristics. Tumors were obtained from 144 informed patients 18–79 years of age (mean ± SD, 44.3 ± 13.9 years). In accord with the gender bias for gliomas (16), the male:female ratio was 1.6. Individuals treated with alkylating agents were excluded from the study. As indicated in Table 1, individuals treated with adjuvant radiotherapy were similar to unirradiated patients in age, sex ratio, and distribution of tumor diagnoses. Histologically normal brain adjacent to tumor was obtained from 87 patients. These individuals were similar to the entire sample in age (44.3 ± 14.4 years; range, 18–79), sex ratio (1.6), and distribution of diagnoses. Of 152 total tumors, 118 (78%) were astrocytic gliomas, the most frequent human brain tumor diagnosis. These tumors included 26 (17%) astrocytomas, 27 (18%) anaplastic astrocytomas, and 65 (43%) glioblastomas. Other tumors were 22 (14%) oligodendrogliomas and 12 (8%) mixed oligodendroglioma-astrocytomas (Table 1). Eight-five tumors were newly resected, 12 were recurrent after prior surgery, and 55 were recurrent following radiation therapy.

MGMT Activity in Tumors. Measurable tumor MGMT activity ranged ~300-fold from 0.30 to 89 fmol/106 cells (Fig. 1). This inter-individual variability is in accord with our prior findings for 42 adult patients (13) and similar to that observed in a variety of normal and neoplastic human tissues (13, 17–23). Notably, 24% of tumors (37 of 152) had no detectable activity (Mer− phenotype, i.e., <0.25 fmol/106 cells or <151 molecules/cell), in agreement with previous studies (13, 23). For 12 of the 37 Mer− tumors, it was possible to increase the sensitivity of detection by assaying more than the 7.7 X 10^6 cells required to meet our definition of Mer− (see “Methods and Materials”). Increasing the number of cells 2–9-fold did not reveal MGMT activity in these samples.

Mean activity in tumors was 6.6 ± 13 fmol/106 cells and did not differ appreciably between males and females (5.9 ± 11 versus 7.6 ± 15 fmol/106 cells); likewise, there was no significant difference between males and females in the fraction of Mer− tumors (26% versus 22%). Unirradiated and irradiated tumors did not differ in mean level and frequency of Mer− specimens (Table 2), indicating that radiotherapy has no demonstrable effect on MGMT activity in gliomas.

Mean MGMT levels did not differ significantly among the three astroglial tumor groups (astrocytoma, anaplastic astrocytoma, and glioblastoma; Table 2). However, mean activities for oligodendroglioma (4.4 ± 4.2 fmol/106 cells) and mixed oligodendroglioma-astrocytoma (2.1 ± 5.1 fmol/106 cells) were approximately 2–4-fold lower
Table 2 MGMT activity and frequency of Mer− phenotype in adult gliomas as a function of diagnosis and radiotherapy

<table>
<thead>
<tr>
<th>Tumors</th>
<th>n</th>
<th>Mean MGMTa (fmol/10⁶ cells)</th>
<th>Mer− (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All specimens</td>
<td>152</td>
<td>6.6 ± 13 (0.30-89)</td>
<td>24% (37/152)</td>
</tr>
<tr>
<td>By diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>26</td>
<td>7.6 ± 12 (0.80-47)</td>
<td>31% (8/26)</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>27</td>
<td>5.6 ± 7.6 (0.44-28)</td>
<td>30% (8/27)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>65</td>
<td>8.2 ± 17 (0.35-89)</td>
<td>22% (15/65)</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>22</td>
<td>4.4 ± 4.2 (0.30-14)</td>
<td>9% (2/22)</td>
</tr>
<tr>
<td>Oligo-astrob</td>
<td>12</td>
<td>2.1 ± 5.1 (0.29-18)</td>
<td>42% (5/12)</td>
</tr>
<tr>
<td>By treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No radiotherapy</td>
<td>97</td>
<td>6.5 ± 11 (0.40-63)</td>
<td>27% (26/97)</td>
</tr>
<tr>
<td>Adjuvant radiotherapy</td>
<td>55</td>
<td>6.7 ± 16 (0.26-89)</td>
<td>20% (11/55)</td>
</tr>
</tbody>
</table>

a Mean ± SD and range of detectable activity. For purposes of calculation, Mer− specimens were assigned a value of 0.125 fmol/10⁶ cells, one-half the lower limit of detection.

MGMT activity and the fraction of aneuploid cells (Fig. 3A) ranged approximately 30-fold from 0.34 to 9.7 fmol/10⁶ cells; 61% (53 of 87) of specimens were Mer− (Table 4). MGMT activity was inversely correlated with age (r = -0.320, P < 0.01); however, the similar slopes of all of the regression lines suggest that lack of significance for females and other diagnoses is due to smaller sample sizes (data not shown). The inverse correlation of MGMT with age also reflected a modest increase in the fraction of Mer− specimens (Fig. 2B) between patients younger than 35 (16%) and those 35 and older (27%).

Correlation of MGMT with Aneuploidy. Among the 94 tumors analyzed, there was a statistically significant correlation between MGMT activity and the fraction of aneuploid cells (Fig. 3A; r = 0.286, P < 0.006). This correlation reflected a 3-fold higher mean MGMT activity in tumors with an aneuploid content >50% than in tumors with an aneuploid content <50% (4.9 ± 6.4 versus 15 ± 26 fmol/10⁶ cells; t = -1.97; P < 0.06; the t test assumes unequal variances). The association with aneuploidy did not reflect the age dependence of MGMT activity because there was no correlation between fraction of aneuploid cells and age (r = -0.036). Further analysis revealed no sexual dimorphism for the association with aneuploidy.

No correlation (r = 0.061) was found between MGMT activity and the fraction of cells in S-phase in 92 tumors (Fig. 3B), indicating that proliferative state is not predictive of MGMT levels in human brain tumors.

MGMT in Adjacent Histologically Normal Brain. The mean MGMT activity of 87 specimens of histologically normal brain adjacent to tumors was 1.1 ± 1.9 fmol/10⁶ cells. Detectable activity ranged approximately 30-fold from 0.34 to 9.7 fmol/10⁶ cells; 61% (53 of 87) of specimens were Mer− (Table 4). MGMT activity was inversely correlated with age (r = -0.244, P < 0.02), in agreement with an earlier analysis of 117 pediatric and adult patients, which included 63 of the normal brain samples reported here (14). The implications for neurocarcinogenesis of the high frequency of Mer− phenotype and of the age dependence of MGMT in normal brain of brain tumor patients are discussed by Silber et al. (14).

Table 3 MGMT activity and frequency of Mer− phenotype in adult gliomas as a function of age

<table>
<thead>
<tr>
<th>Patient age</th>
<th>All gliomas</th>
<th>Mer− (fraction)</th>
<th>Mer− (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-34</td>
<td>11 ± 21 (0.40-89)</td>
<td>5.3 ± 9.9 (0.26-63)</td>
<td>16% (6/38)</td>
</tr>
<tr>
<td>Male</td>
<td>11 ± 19 (0.40-89)</td>
<td>4.4 ± 6.9 (0.26-36)</td>
<td>14% (3/22)</td>
</tr>
<tr>
<td>Female</td>
<td>12 ± 23 (0.44-81)</td>
<td>5.9 ± 11 (0.70-63)</td>
<td>19% (3/16)</td>
</tr>
<tr>
<td>By diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>11 ± 16 (1.1-47)</td>
<td>3.8 ± 5.6 (0.70-28)</td>
<td>20% (2/10)</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>9.6 ± 1.1 (0.64-28)</td>
<td>4.2 ± 5.7 (0.70-19)</td>
<td>14% (1/7)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>21 ± 34 (0.46-89)</td>
<td>5.7 ± 11 (0.35-63)</td>
<td>10% (1/10)</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>6.9 ± 5.5 (1.2-14)</td>
<td>3.0 ± 2.7 (0.30-11)</td>
<td>13% (4/8)</td>
</tr>
</tbody>
</table>

a Mean ± SD and range of detectable activity. For purposes of calculation, Mer− tumors were assigned a value of 0.125 fmol/10⁶ cells, one-half the lower limit of detection.
Comparison of MGMT in Tumor and Adjacent Normal Brain. For 87 paired tissue specimens, mean activity in tumors was 5-fold higher than in adjacent normal brain (5.0 ± 7.5 versus 1.1 ± 1.9 fmol/10^6 cells; t = 5.1, P ≤ 0.001; t test for paired samples). The trend was consistent across all diagnostic groups (Table 4). Overall, there was a statistically significant correlation between MGMT activity in tumor and normal brain (r = 0.503, P < 0.01). This association reflected a 2-fold lower mean MGMT in tumors adjacent to Mer" compared to Mer" normal brain (3.4 ± 5.3 versus 6.8 ± 9.2 fmol/10^6 cells) and a higher frequency of Mer" tumors paired with Mer" brain (19 of 53; 36%) than with Mer" brain (8 of 34; 24%).

The effects of tumorigenesis on MGMT levels were further characterized by categorizing the paired specimens into three groups. As shown in Fig. 4, tumor activity was higher in a majority (62%) of pairs. The elevation was 1.5-fold to at least 105-fold, assuming the maximum value (0.25 fmol/10^6 cells) for Mer" normal brain. For a minority (30%) of pairs, MGMT did not differ, i.e., both tumor and normal were Mer" in 19 cases. For a relatively few pairs (8%), tumor activity was, at least, 3-30-fold lower than for normal brain; the tumors were all Mer". These three concomitants of tumorigenesis were observed in all diagnostic groups and were independent of age.

The effects of tumorigenesis were also characterized by comparing the Mer phenotype of progenitor tissue and tumor (Fig. 5). In a majority of paired specimens (53 of 87; 61%), the normal brain was Mer", in accord with an earlier study (13). Tumorigenesis was associated with conversion to Mer" phenotype in most of these cases (34 of 53; 64%). Notably, activity in the accompanying Mer" tumors (5.2 ± 6.0 fmol/10^6 cells) was lower than in Mer" tumors that arose from Mer" tissue (9.1 ± 9.6 fmol/10^6 cells). In the remaining pairs with Mer" progenitor tissue (19 of 53; 36%), activity was also undetectable in the tumors, suggesting that the Mer" phenotype was retained during tumorigenesis. In the 34 pairs with Mer" progenitor tissue, 26 (77%) were accompanied by Mer" tumors, suggesting that the Mer" phenotype persisted during tumorigenesis. In the remainder of the 34 pairs, conversion from Mer" to Mer" phenotype was observed.

Several generalizations emerge from analysis of the paired specimens: (a) the progenitor tissue of adult gliomas is predominantly Mer"; (b) the accompanying tumors are predominantly Mer"; and (c) neurocarcinogenesis is associated with loss of Mer" phenotype and elevation of MGMT activity.

DISCUSSION

Repair of cytotoxic DNA damage is a potentially important component of the resistance of brain tumors to chemotherapeutic alkylating agents. Major goals of our laboratory are to systematically document relationships of DNA repair activities in brain to patient and tumor characteristics and to establish the effects of tumorigenesis on these activities. This information is required to achieve our clinical objectives of identifying patients most likely to benefit from alkylating agent therapy and developing strategies to overcome resistance. Moreover, such information will provide insights into the basic biology of DNA repair in human brain and how neurocarcinogenesis alters DNA repair capacity. In the present study, we assayed MGMT, a likely contributor to brain tumor resistance to alkylators, in a large number of adult gliomas and, whenever possible, in adjacent normal brain.

Our analysis revealed previously unreported associations of glioma MGMT activity with tumor and patient characteristics. Mean MGMT level and frequency of Mer" phenotype differed among tumor types; both parameters varied ~4-fold among diagnostic groups (Table 2). Notably, the mean activities of oligodendrogliomas and mixed oligodendroglioma-astrocytomas were 2-4-fold less than that of the combined astrocytic gliomas (Table 2), and these differences were statistically significant. In accord, studies with rat primary glial cell cultures have demonstrated reduced MGMT activity and concomitant...
MGMT activity was inversely correlated with age in adult gliomas (Figs. 1 and 2; Table 3) and has previously been observed in a yet stronger inverse correlation in histologically normal brain adjacent to pediatric and adult gliomas (14). Apparently, neurocarcinogenesis does not abolish the age dependence of MGMT activity in precursor glia. Importantly, we observed no correlation of MGMT activity with age in normal brain of nontumor patients (14), indicating that the association with age in gliomas and their progenitor tissue is characteristic of a subpopulation of individuals with enhanced susceptibility to neurocarcinogenesis. Other studies have reported age-dependent reduction in DNA repair capacity in normal, healthy individuals (29, 30) and in cancer patients (31). However, to our knowledge, age-dependent reduction in a specific DNA repair activity in both tumor and its progenitor tissue has not been reported, nor has the apparent restriction of age-dependence to a subpopulation of individuals.

The incidence of gliomas shows a pronounced gender bias (16), which is apparent in our patient sample (Table 1). In contrast, we found no sex bias in MGMT activity or frequency of Mer⁻ phenotype or in the associations of activity with age and aneuploidy. Preliminary evidence also indicates that activity does not differ by gender in the diagnostic subgroups (data not shown). These findings are consistent with an absence of sexual dimorphism in glioma MGMT activity.

Comparison of MGMT activity in paired tumor and adjacent normal brain emphasizes that elevation of activity is a concomitant of tumorigenesis in the majority of cases. The elevation often encompasses conversion from Mer⁻ to Mer⁺ phenotype and is consistent with the epigenetic regulation of MGMT observed in cultured human cells (32–34). The elevation in tumors suggests that selective pressure for increased MGMT activity may prevail during human neurocarcinogenesis, perhaps exerted by endogenous alkylation (35). The lack of correlation with fraction of S-phase cells indicates that proliferation per se is not a major factor. One significant consequence of this putative selection for enhanced MGMT activity may be greater resistance to therapeutic alkylators.

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