O⁶-Alkylguanine-DNA alkyltransferase and sensitivity to procarbazine in human brain-tumor xenografts

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The level of O⁶-alkylguanine-deoxyribonucleic acid (DNA) alkyltransferase (AT) was determined in 15 human brain-tumor xenografts in athymic mice. This enzyme is a primary intracellular repair mechanism for lesions produced at the O⁶ position of guanine by a wide range of alkylating agents, including nitrosoureas and procarbazine. Its activity ranged from undetectable in five tumor lines to 2338 fmol/mg protein in N-1941, a human glioblastoma xenograft. The sensitivity of 10 of these xenografts to procarbazine was determined and it was found that four of the five tumor lines with AT levels of more than 100 fmol/mg protein had growth delays after procarbazine treatment of less than 20 days, whereas all five lines with undetectable AT levels had growth delays of over 30 days. The primary cytotoxic DNA adduct produced by procarbazine (namely, O⁶-methylguanine) was found to be significantly higher in two sensitive lines with low AT levels than in a highly resistant line with a high AT level. These data suggest that the AT levels of individual brain tumors can be used as predictive indicators of their susceptibility to drugs that exert their antineoplastic effect primarily by O⁶-alkylation of guanine in nuclear DNA.

KEY WORDS • glioma • medulloblastoma • alkylating agent • DNA repair • O⁶-alkylguanine-DNA alkyltransferase • brain neoplasm

Despite aggressive surgery, radiotherapy, and chemotherapy, the most common forms of primary anaplastic neoplasms of the central nervous system (CNS) are lethal diseases. However, there have been objective and clinically significant responses of recurrent CNS tumors to currently available forms of antineoplastic chemotherapy, particularly the alkylating class of drugs. One mechanism by which these drugs exert their effect is modification of the purine or pyrimidine bases of tumor DNA, which may interfere with cell replication. The nitrosoureas and procarbazine, in particular, have been effective in the treatment of gliomas and are known to produce cytotoxicity by alkyl substitution at the O⁶ position of guanine in deoxyribonucleic acid (DNA). A repair enzyme, O⁶-alkylguanine-DNA alkyltransferase (AT), removes the alkyl group from the O⁶ position, leaving intact guanine. This enzyme is present in varying amounts in different human and experimental neoplasms. A panel of human brain-tumor xenografts growing in athymic mice was used to test the hypothesis that the activity of this repair enzyme is related to resistance of the tumors to procarbazine.

Materials and Methods

Tumor Lines

Fifteen human brain-tumor xenografts were used for these experiments. Eight of the tumor lines (N-1941, N-1872, N-1768, N-456, N-519, N-696, N-735, and N-1520) were established in athymic mice as xenografts at Duke University after direct transplantation from resected cerebral glioblastomas. One of these lines (N-696) was simultaneously established in culture and has been described in detail because of its unusual karyotype. Four lines (H-259, U-118 MG, D-54 MG, and U-251 MG) were initially established in culture and later transplanted into mice. Two of these lines were established in Uppsala, Sweden, by J. Pontén, and two were established at Duke University. All four lines were derived from anaplastic gliomas. Three tumor lines (TE-671, D-341 Med, and Daoy) were derived...
from human medulloblastomas and have been described previously.  

Alkyltransferase Assay

Xenografts growing in the subcutaneous flank of athymic mice were excised and rapidly frozen in liquid nitrogen when they reached a volume between 500 and 1000 cu mm. For analysis, the frozen tumor was weighed and suspended in 4 volumes of a buffer that contained 0.05 M Tris-HCl (pH 7.5), 0.002 M ethylenediaminetetra-acetic acid (EDTA), 0.1 M NaCl, 0.001 M diethiothreitol (DTT), 0.02% sodium azide, 0.2 mM phenylmethylsulfonyl fluoride, and 20 trypsin-inhibition units of aprotonin/liter. The tumor was then homogenized* and sonicated for 45 seconds at setting No. 6.† The extract was centrifuged for 60 minutes in an ultracentrifuge at 35,000 rpm.‡ The supernatant was removed in aliquots and frozen at −70°C. Protein was quantified using an assay kit.§

Alkyltransferase activity was determined by quantifying the ability of this protein extract from the tumor to remove tritiated (3H-labeled) methyl groups from the O6-methylguanine in an oligonucleotide substrate.†† Ten- to 130-μl aliquots of tumor extract were added to the assay buffer, which contained 0.01 M Tris-HCl (pH 7.5), 0.002 M EDTA, 0.001 M DTT, 10% glycerol, and 0.02% sodium azide. The addition of 1 μg DNA substrate that contained 1 pmol of 3H-methyl-O6-guanine (specific activity 5.6 Ci/mmol, prepared by reacting calf thymus DNA with 3H-methyl nitrosourea) initiated the reaction. The total volume of the reaction solution was 200 μl. Blank solutions contained buffer and substrate alone. The material was incubated at 37°C for 30 minutes. The addition of 500 μl of 5% (w/v) trichloroacetic acid (TCA) stopped the reaction. The mix was then hydrolyzed for 30 minutes at 80°C and cooled for 10 minutes at 4°C. Bovine serum albumin (100 μg) was added as a carrier protein. Precipitated protein was collected on 4.25-cm GF/F Whatman filters and suspended in 4 volumes of a buffer that contained 0.01 M Tris-HCl (pH 7.5), 0.002 M EDTA, 0.001 M DTT, 10% glycerol, and 0.02% sodium azide. The addition of 1 μg DNA substrate that contained 1 pmol of 3H-methyl-O6-guanine initiated the reaction. The total volume of the reaction solution was 200 μl. Blank solutions contained buffer and substrate alone. The material was incubated at 37°C for 30 minutes. The addition of 500 μl of 5% (w/v) trichloroacetic acid (TCA) stopped the reaction. The mix was then hydrolyzed for 30 minutes at 80°C and cooled for 10 minutes at 4°C. Bovine serum albumin (100 μg) was added as a carrier protein. Precipitated protein was collected on 4.25-cm GF/F Whatman filters and washed with 30 ml 5% TCA and 15 ml of 95% ethanol using a Millipore vacuum filtration apparatus.|| Dry filters were placed in 20-ml scintillation vials. Amersham NCS tissue solubilizer (200 μl) was added to each filter,** followed approximately 5 minutes later by 10 ml scintillation liquid. Radioactivity was determined by scintillation counter.†† The AT activity is expressed as fmols of 3H-methyl transferred/mg protein, as determined by regression analysis.

Xenograft Treatment Methods

Methods of treatment for subcutaneous xenografts in athymic mice have been described elsewhere.7,18 Briefly, congenitally athymic adult BALB/c mice derived from our own breeding colony were used for these experiments. Animals received 50 μl of a tumor suspension in the right flank through a No. 20 needle in a Hamilton syringe. Tumors were serially measured with calipers, and treatment began when the median tumor volume of all animals exceeded 200 cu mm. Procarbazine was administered intraperitoneally at a single dose of 2437 mg/sq m (75% of its lethal dose for 10% of subjects tested in our colony). Control animals received an equal volume of saline. There were 10 control animals and 10 drug-treated animals for each experiment. Serial measurements continued until tumor volumes exceeded 2000 cu mm. Growth delay was calculated by the difference in days between the median of the treated tumors and the median of the control tumors to reach five times their volume on the day of treatment.

Chromatography of DNA Hydrolysates

For adduct analysis, tumors were treated at a volume of 1000 to 1500 cu mm. Nine animals were treated in each group; at 4, 12, and 24 hours after treatment, the tumor was removed from three animals and rapidly frozen in liquid nitrogen. The DNA was subsequently isolated from the tumors by phenol extraction and absorption to hydroxyapatite, as previously described.20 The yield was approximately 1 mg DNA/gm tissue. The DNA was stored at −80°C until further use. For analysis of alkylated purine bases, the DNA was depurinated in 0.1 N HCl for 24 hours at 37°C. High-performance liquid chromatography analysis of adducts was performed using a strong cation exchange column, as previously described.21 Briefly, samples were injected into a Partisil SCX ion-exchange column and the bases were eluted with 50 mM NH4H2PO4 buffer (pH 2, flow rate 1.5 ml/min). Guanine and adenine were quantified by absorbance at 254 nm, and O6-methylguanine was quantified by fluorescence detection (295 nm excitation, 370 nm emission). The mean retention time for O6-methylguanine under these conditions was 8.4 minutes, and the limit of detection was 1 to 2 pmol.

Results

The AT activity was determined in 12 human glioma and three human medulloblastoma xenografts (Table 1). In five lines (D-54 MG, N-1872, U-251 MG, N-735, and N-1520) minimal to no activity was detected and there was no increase in activity with the addition of greater amounts of extract (Fig. 1), suggesting that

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TABLE 1

<table>
<thead>
<tr>
<th>Tumor Line</th>
<th>AT Specific Activity (fmol/mg protein)</th>
<th>Growth Delay† (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-1941</td>
<td>2338</td>
<td>—</td>
</tr>
<tr>
<td>TE-671</td>
<td>2071</td>
<td>1.4</td>
</tr>
<tr>
<td>D-341 Med</td>
<td>1351</td>
<td>—</td>
</tr>
<tr>
<td>N-1768</td>
<td>1200</td>
<td>—</td>
</tr>
<tr>
<td>N-456</td>
<td>1100</td>
<td>6.4</td>
</tr>
<tr>
<td>Daoy</td>
<td>897</td>
<td>3.5</td>
</tr>
<tr>
<td>N-519</td>
<td>741</td>
<td>13.4</td>
</tr>
<tr>
<td>H-259</td>
<td>489</td>
<td>—</td>
</tr>
<tr>
<td>N-696</td>
<td>414</td>
<td>—</td>
</tr>
<tr>
<td>U-118 MG</td>
<td>198</td>
<td>24.0</td>
</tr>
<tr>
<td>D-54 MG</td>
<td>ND</td>
<td>32.0</td>
</tr>
<tr>
<td>N-1872</td>
<td>ND</td>
<td>33.2</td>
</tr>
<tr>
<td>N-735</td>
<td>ND</td>
<td>76.0</td>
</tr>
<tr>
<td>N-1520</td>
<td>ND</td>
<td>74.8</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>ND</td>
<td>64.1</td>
</tr>
</tbody>
</table>

* Tumor lines TE-671, D-341 Med, and Daoy were medulloblastomas. ND = none detected; — = study not performed.
† Growth delay of subcutaneous tumors after treatment with procarbazine.

These lines are methyltransferase (mex)-deficient. The other lines had varying amounts of the repair enzyme, ranging from 198 fmol/mg protein in U-118 MG to 2338 fmol/mg protein in N-1941. All three medulloblastoma lines (TE-671, D-341 Med, and Daoy) had abundant enzyme (2071, 1351, and 897 fmol/mg protein, respectively), whereas the gliomas were more variable (Table 1).

The sensitivity of 10 of these xenograft lines in athymic mice to procarbazine was determined. Of five lines with AT levels exceeding 100 fmol/mg protein (mex-positive lines), four had growth delays of less than 20 days, whereas all five of the mex-deficient lines had growth delays that exceeded 30 days (Fig. 2). Tumor lines N-519 and U-118 MG had relatively long growth delays of 13.4 and 24.0 days, respectively, despite the definite presence of enzyme (Fig. 1); this evidently represents an intermediate level of protection against alkylation. The growth delay of 1.4 days in TE-671 was not statistically significant. The growth delays of 76.0, 74.8, and 64.1 days in N-735, N-1520, and U-251, respectively, are among the longest detected in this system, and there were several cures in these treatment groups.

The level of O6-methylguanine was determined in three xenografts after procarbazine treatment. Two xenografts (D-54 and U-251 MG) were mex-deficient lines that were highly sensitive to the cytotoxic effects of procarbazine, and the third (TE-671) was a mex-positive line that was resistant to procarbazine. The amount of O6-methylguanine formed was six- to tenfold higher at all three time points in the sensitive lines than in the resistant line (Fig. 3).

**Fig. 1.** Varying amounts of a protein extract of three tumor lines, TE-671 (triangles), N-519 (squares), and D-54 MG (circles), were combined with DNA containing [methyl-3H]-O6-methylguanine, and the amount of 3H transferred to the protein was determined (see Materials and Methods section). The protein content of each tumor extract was between 5 and 10 mg/ml.

**Fig. 2.** Relationship of O6-alkylguanine-DNA alkyltransferase (O6-alkG AT) to procarbazine sensitivity in 10 human brain-tumor xenografts. T-C: the growth delay produced by procarbazine in subcutaneous xenografts in athymic mice. Each point represents a single tumor line.

**Fig. 3.** Level of O6-methylguanine (O6-meG, expressed as µmol/mol guanine (G)) in DNA from three human brain-tumor xenografts in athymic mice at 4, 12, and 24 hours after procarbazine treatment. D-54 MG (circles) and U-251 MG (triangles) are methyltransferase(mex)-deficient lines that are highly sensitive to the cytotoxic effects of procarbazine, whereas TE-671 (squares) is a mex-positive line that is resistant to procarbazine.
There was some reduction of adduct concentration between 4 and 12 hours in the two sensitive lines, but no significant level of O⁶-methylguanine was detected even at the earliest time in TE-671. At 24 hours, substantial levels of O⁶-methylguanine were still present in the two mex-deficient lines.

Discussion

Primary anaplastic CNS neoplasms in humans are generally refractory to therapy. The median survival time of adults with anaplastic glioma is less than 18 months, and the 5-year survival rate is less than 10%. Although children with medulloblastoma fare somewhat better, over 50% of these patients have a lethal recurrence within 5 years of diagnosis. Surgery, radiotherapy, and corticosteroids are clearly effective in either prolonging survival or reducing symptoms, but in most cases they cannot eradicate the tumor without producing unacceptable toxicity.

The efficacy of antineoplastic chemotherapy in the treatment of these diseases is uncertain. Several groups of investigators have documented unequivocal responses to chemotherapy in individual patients with recurrent anaplastic glioma or medulloblastoma. However, since these responses occur in a minority of patients, their impact on the median survival time is negligible. Nevertheless, in some cases the response to chemotherapy is profound and prolonged, indicating substantial heterogeneity of these tumors in their sensitivity to conventional chemotherapy. An understanding of the critical factors governing sensitivity to chemotherapy would be a significant advance in our ability to match therapy with individual patients and tumors.

The most active drugs in the treatment of primary CNS tumors are the alkylating agents. These drugs produce their cytotoxic effects by alkylating bases in the DNA of tumors and thereby interfering with cellular DNA replication. The critical cytotoxic lesions produced by nitrosoureas such as BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) or CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) and procarbazine involve O⁶-alkylation of guanine in DNA; procarbazine, decarbazine (DTIC), and methylnitrosourea produce O⁶-methylguanine, whereas BCNU produces DNA cross-links following the initial formation of O⁶-chloroethylguanine. On the other hand, the "classical" alkylators, such as nitrogen mustard, cyclophosphamide, and L-phenylalanine mustard (melphalan), produce cytotoxic alkyl adducts in tumor DNA predominantly at sites other than the O⁶ position of guanine.

The importance of distinguishing between these types of alkylating agents is the existence of an enzyme, O⁶-alkylguanine-DNA alkyltransferase (AT), that repairs O⁶-alkylguanine adducts but has no effect on potentially lethal alkylated lesions at other sites in the DNA. Although AT is present in most normal cells, it is relatively low in normal brain, and it is variably present in animal and human neoplasms. Bodell, et al., have shown that human glioma cell lines that are capable of repairing the O⁶-alkyl lesions are more resistant to the cytotoxic effects of the nitrosoureas in cell culture. Brent, et al., have demonstrated an inverse relationship between AT levels and sensitivity to methylCCNU in human rhabdomyosarcoma xenografts. These observations are extended in the present experiments which demonstrate the range of expression of AT in 15 human brain-tumor xenografts, identify a relationship between the level of this repair enzyme and sensitivity to procarbazine in 10 of these lines, and measure higher levels of the critical cytotoxic adduct (O⁶-methylguanine) in two sensitive tumors with low levels of AT than in a resistant tumor with a high level of AT.

It is very likely that these observations would apply to sensitivity to BCNU as well, and these data suggest that resistance of human gliomas to this class of alkylating drugs is mediated in part by the scavenger repair enzyme (AT) which removes the critical adduct in DNA before cell replication is adversely affected. Furthermore, we believe that this enzyme is absent or present at a very low level in a minority of anaplastic gliomas, and that tumors with the lowest level of the enzyme are likely to be the most sensitive to the nitrosoureas and procarbazine.

It is of some interest that all three medulloblastoma lines and high levels of AT and that the two lines that were tested were highly resistant to procarbazine. Experimentally, medulloblastoma xenografts are resistant to the nitrosourea class of alkylating agents but are highly sensitive to the mustards and cyclophosphamide. Clinically, both cyclophosphamide and melphalan are active, whereas the evidence for sensitivity to the nitrosoureas is less convincing. Results from the present study suggest that the reason for this difference is the presence of large amounts of AT in most medulloblastomas, but firm data to support this assertion are not yet available.

Clearly, there are many factors influencing sensitivity and resistance of brain tumors to antineoplastic chemotherapy. These tumors are notoriously heterogeneous, and a resistant subpopulation of cells will quickly override the effect of an initially active agent. Drug delivery is an important and complex issue that varies with each tumor and with each drug. Human neoplasms are biologically unstable, so a single measurement will not necessarily reflect the permanent state of a tumor. Nevertheless, identification of a phenotypic trait, such as AT, which may predict sensitivity and resistance to therapeutic agents in current use has potentially important clinical implications.

Acknowledgment

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