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Methylator Resistance Mediated by Mismatch Repair Deficiency in a Glioblastoma Multiforme Xenograft[1]

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ABSTRACT

A methylator-resistant human glioblastoma multiforme xenograft, D-245 MG (PR), in athymic nude mice was established by serially treating the parent xenograft D-245 MG with procarbazine. D-245 MG xenografts were sensitive to procarbazine, temozolomide, *N*-methyl-*N*-nitrosourea, 1,3-bis(2-chloroethyl)-1-nitrosourea, 9-aminocamptothecin, topotecan, CPT-11, cyclophosphamide, and busulfan. D-245 MG (PR) xenografts were resistant to procarbazine, temozolomide, *N*-methyl-*N*-nitrosourea, and busulfan, but they were sensitive to the other agents. Both D-245 MG and D-245 MG (PR) xenografts displayed no O^6-alkylguanine-DNA alkyltransferase activity, and their levels of glutathione and glutathione-*S*-transferase were similar. D-245 MG xenografts expressed the human mismatch repair proteins hMSH2 and hMLH1, whereas D-245 MG (PR) expressed hMLH1 but not hMSH2.

INTRODUCTION

Procarbazine is a methylhydrazine derivative with demonstrated activity against CNS[3] tumors (1–5). Unfortunately, most patients ultimately develop resistance to this methylator, with a consequent dismal outcome. Definition of the mechanisms of resistance to procarbazine has been limited to identification of the role of AGAT, which removes the methyl adduct at the O^6 position of guanine (6).

Here, we report the *in vivo* generation of a human glioma multiforme xenograft resistant to procarbazine and studies that confirmed our hypothesis that this resistance is secondary to an *in vivo*-acquired mismatch repair deficiency.

MATERIALS AND METHODS

Xenografts. D-245 MG is a xenograft derived from a human malignant glioma, as described previously (6). D-245 MG (PR) was established by treating D-245 MG-bearing mice with procarbazine at a dose of 700 mg/m^2 daily on days 1–5 and, after the xenografts had regressed and regrown, transplanting them into the next generation of mice. The resulting xenografts were again treated with procarbazine, and this cycle was repeated until the tumors were procarbazine resistant. D-245 MG (PR) xenografts were treated each generation with procarbazine; a subline was serially transplanted without further procarbazine treatment.

Animals. Athymic BALB/c (*nu/nu*) mice were bred, handled, and used for all experiments as described previously (7).

s.c. Xenograft Transplantation. s.c. tumor transplantation into the right flank of the animals was performed as described previously, with inoculation volumes of 50 μl (8).

Tumor Measurements. Tumors were measured twice weekly with hand-held vernier calipers (Scientific Products, McGraw, IL). Tumor volume was calculated according to the following formula: [(width)^2 × (length)]/2.

Drugs. The following drugs were used: procarbazine, MNU, BCNU, and cyclophosphamide from the Pharmaceutical Research Branch of the National Cancer Institute (Bethesda, MD); 9-aminocamptothecin and CPT-11 from Pharmacia and Upjohn (Kalamazoo, MI); topotecan from SmithKline Beecham (King of Prussia, PA); busulfan from Glaxo-Wellcome (Research Triangle Park, NC); and temozolomide from Schering-Plough (Kenilworth, NJ).

Drug Regimens. All chemotherapeutic agents were injected i.p. at doses representing fractions of 1.0 or 0.38 of the dose lethal to 10% of nontumor-bearing animals (LD_{10}). Procarbazine was administered at a dosage of 2436 mg/m^2 in 0.9% saline on day 1 or 700 mg/m^2 in 0.9% saline daily on days 1–5. Cyclophosphamide was dispensed at 1391 mg/m^2 in 0.9% saline on day 1, and temozolomide was dispensed at 411 mg/m^2 in 10% DMSO, both in 0.9% saline, on a daily basis on days 1–5. Topotecan was administered at a dose of 5.7 mg/m^2 in normal saline on days 1–5 and 8–12. 9-Aminocaptothecin was administered at a dose of 1.5 mg/m^2 in 10% DMSO, both in 0.9% saline, on days 1–5 and 8–12. BCNU was administered at a dose of 100 mg/m^2 in 10% ethanol, both in 0.9% saline, on day 1. Busulfan was administered at a dose of 60.3 mg/m^2 in 10% DMSO, both in 0.9% saline, on day 1. CPT-11 was given daily at 120 mg/m^2 in 10% DMSO, both in 0.9% saline, on days 1–5 and 8–12.

Tumor Therapy. Groups of 8–10 randomly assigned mice bearing D-245 MG or D-245 MG (PR) tumors were treated i.p. by injection of chemotherapeutic compounds, according to the doses described above, when the median tumor volume exceeded 200 mm^3.

Assessment of Response. Xenograft response was assessed by growth delay [T − C being the difference in days between the median time for the tumors of treated (T) and control (C) animals to reach a volume of five times greater than the volume at the time of treatment] and by treated *versus* control tumor regressions. Statistical analyses were performed as described previously (9).

Measurement of AGAT Activity. D-245 MG and D-245 MG (PR) tumors were obtained from untreated mice. AGAT activity was measured as described previously (10, 11).

GSH Assay. Total GSH was determined by the methods of Tietze (12) and Griffith (13), with minor modifications as described previously (14, 15). Groups of untreated animals bearing s.c. D-245 MG and D-245 MG (PR) xenografts were used for GSH measurements. Animal sacrifice, tumor preparation, and GSH analysis were performed as described previously.

GST Measurements. GST measurements were performed using the assay of Habig *et al.* (16), with minor modifications. Groups of untreated animals bearing s.c. D-245 MG and D-245 MG (PR) xenografts were used for GST measurements. Xenografts were homogenized in a Brinkman polytron in 0.1 M potassium phosphate buffer-1 mM EDTA, pH 7.4, and handled as described previously (17).

Microsatellite Studies. Randomly chosen microsatellite loci (*D1S102, D2S123, D2S390, D4S174, D6S253, D7S460, D10S212, D11S935, D14S48, D14S49, D15S118, D16S422, D18S53,* and *D19S246*) were amplified by PCR from genomic DNA isolated from D-245 MG and D-245 MG (PR). The polymerase reaction mixture contained 1× PCR buffer (Life Technologies, Inc., Grand Island, NY), 200 μM each dGTP, dATP, and dTTP, 2 μM dCTP,

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[3] The abbreviations used are: CNS, central nervous system; AGAT, O^6-alkylguanine-DNA alkyltransferase; MNU, *N*-methyl-*N*-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GSH, glutathione; GST, GSH *S*-transferase.

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0.4 μCi of [^{32}P]dCTP, 0.25 mM spermidine, 0.15 mM each primer, 20 ng of DNA and 0.25 units of Taq DNA polymerase in a volume of 5 μl. The samples were subjected to an initial denaturation at 95°C for 4 min, followed by 27 cycles of amplification (20 s at 94°C, 50 s at 55°C, and 50 s at 72°C). We then electrophoresed the samples, added an equal volume of formamide stop buffer, and heated the samples at 95°C for 5 min. From 2 to 2.5 μl of product were size-fractionated in adjacent lanes on 6% polyacrylamide-7.7 M urea gels (Sequal gel; National Diagnostics, Atlanta, GA) for 2.5 h at 1500 V. The gels were dried and exposed overnight to Hyperfilm-MP (Amersham, Arlington Heights, IL). The PCR amplifications were repeated at least three times to rule out PCR artifacts.

Determination of hMLH2 and hMSH2 Protein Levels in Nuclear Extracts. Cell suspensions were obtained from xenografts by removing necrotic and connective tissue from the tumors and then pressing the tissue through a cytosieve in 20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.2 M sucrose (0.1% from a saturated solution in isopropanol), phenylmethylsulfonyl fluoride (0.1% from a saturated solution in isopropanol), 0.5 mM DTT, and 1 μg/ml leupeptin. Nuclei were prepared and extracted as described in Holmes *et al.* (18). Nuclear extracts (50 μg total protein) were separated by electrophoresis through a 7.5% SDS-polyacrylamide gel followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA). hMLH1 and hMSH2 were detected with the mouse monoclonal antibodies [anti-MLH1, human (Ab-1); anti-MSH2, human (Ab-1); Calbiochem, San Diego, CA]. Immune complexes were assayed using horseradish peroxidase-conjugated antimouse antibody (Amersham), then visualized with an enhanced chemiluminescent reagent (Amersham).

Characterization of *hMSH2* Mutation. RNA was prepared from D-245 MG parent and procarbazine-resistant tumors by the method of Chomczynski and Sacchi (19) and was used to generate cDNA with random hexamer primers and Superscript II reverse transcriptase (Life Technologies, Inc.). The cDNA was amplified using the two sets of *hMSH2* primers and the PCR conditions outlined in Liu *et al.* (20). The resulting reverse transcriptase-PCR products were then used in a linked T7 transcription-translation system (Amersham), the [^{35}S]methionine-labeled protein products of which were analyzed by SDS-PAGE. The presence of a mutation that resulted in truncation of the encoded polypeptide could be determined by comparing the polypeptides produced by D-245 MG with D-245 MG (PR) RNAs.

Cell Line Establishment. D-245 MG and D-245 MG (PR) xenografts were washed in Hanks' buffer containing gentamicin, amphotericin B, streptomycin, and penicillin and were disaggregated by dissection and/or a tissue press with a 1.5-mm mesh opening. Selective growth of human cells was accomplished by periodic growth in serum-free medium and growth on mitomycin-C-treated 3T3 Swiss fibroblasts. To evaluate for presence of contaminating murine fibroblasts, we periodically quantitated mouse *versus* human lactate dehydrogenase isoenzyme profiles.

Cell Line Response to Streptozocin. Cells from D-245 MG (passage 41) and D-245 MG (PR; passage 56) shown to be free of murine fibroblast contamination were seeded at a concentration of 1×10^5 cells/well in 12-well Linbro cluster plates. Streptozocin was added in 20% fetal bovine serum plus DMEM/F12 at concentrations of 0.01–10 mM. After a 7-day incubation, the nuclei were released (21) and counted using a Coulter Counter.

RESULTS

Xenograft Response to Chemotherapy. D-245 MG xenografts were sensitive to all agents tested, with the largest growth delays seen with the methylating agents procarbazine, MNU, and temozolomide (Table 1). D-245 MG (PR) developed resistance to procarbazine after 10 passages, with virtually no response to any of the three methylating agents tested (Table 1). D-245 MG (PR) retained sensitivity to the alkylating agents BCNU and cyclophosphamide and to the topoisomerase 1 inhibitors 9-aminocamptothecin and CPT-11, but it was resistant (compared with D-245 MG) to busulfan (Table 1). D-245 MG (PR) xenografts, which were not maintained with continued treatment with procarbazine for seven passages, displayed continued resistance to procarbazine, with growth delays of 4.5 and 0.2 days and regressions in 0 of 10 and 0 of 10 animals, respectively, in duplicate experiments.

Establishment of Cell Lines and Sensitivity to Streptozocin. Cell lines free of murine fibroblast contamination were successfully established, with current passage levels for D-245 MG and D-245 MG (PR) of 41 and 56, respectively. D-245 MG was more sensitive to streptozocin than was D-245 MG (PR), a cell kill of 50% being produced by doses of 0.32 and 1.92 mM, respectively (Fig. 1).

AGAT Activity. No AGAT activity in either D-245 MG or D-245 MG (PR) xenografts was detected (assay sensitivity, 9 fmol/mg protein).

GSH Levels. GSH levels of D-245 MG and D-245 MG (PR) were 1.84 ± 0.09 and 1.76 ± 0.20 mmol/g wet weight (mean ± SD), respectively.

GST Activity. The GST activity of D-245 MG and D-245 MG (PR) xenografts was 77.4 ± 12.4 and 41.9 ± 4.6 mmol/min/mg (mean ± SD) protein, respectively.

Microsatellite Studies. Microsatellite patterns at 15 different loci mapped to various chromosomes were analyzed (Table 2). Differences in the microsatellite patterns of D-245 MG and D-245 MG (PR)

Table 1 *Chemotherapy of D-245 MG and D-245 MG (PR) xenografts growing s.c. in athymic nude mice*[a]

Agent	Fraction of LD_{10}	D-245 MG		D-245 MG (PR)	
		T − C[b] (days)	Regressions	T − C[b] (days)	Regressions
Procarbazine	1.0	74.7	9/9	2.1 (NS)	0/9 (NS)
	1.0	66.2	9/9	1.2 (NS)	0/10 (NS)
	1.0	70.5	10/10	3.3 (NS)	0/9 (NS)
MNU	1.0	81.1	10/10	5.3 (NS)	0/10 (NS)
Temozolomide	1.0	111.9	9/9	5.6 (NS)	0/10 (NS)
	1.0	108.3	8/8	0.6 (NS)	2/10 (NS)
BCNU	1.0	56.7	10/10	74.7	9/9
	1.0	63.8	10/10	65.5	8/8
	0.38	19.1	8/10	30.7	8/9
	0.38	19.7	10/10	30.8	6/7
9-Aminocamptothecin	1.0	19.0	9/10	24.2	9/9
	1.0	22.0	7/8	29.3	9/10
Topotecan	1.0	16.5	5/8	17.7	8/9
	1.0	13.1	8/9	23.5	8/10
CPT-11	1.0	25.9	10/10	33.4	10/10
	1.0	27.9	7/7	33.7	9/9
Cyclophosphamide	1.0	23.0	7/10	33.7	7/7
Busulfan	1.0	21.3	5/9	7.1	0/9 (NS)
	1.0	21.6	8/10	5.8	0/10 (NS)

[a] All values are statistically significant ($P < 0.01$) unless otherwise indicated (NS).
[b] T − C, growth delay in days, is defined as the difference between the median time required for tumors in treated (T) and control (C) animals to reach 5 times the volume measured at the initiation of treatment.

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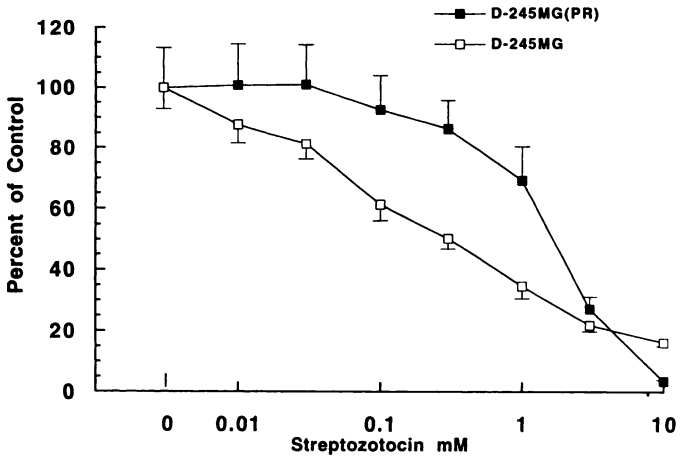

Fig. 1. Dose-response curve for cell lines derived from D-245 MG and D-245 MG (PR) after streptozocin treatment.

Table 2 *Alterations in microsatellite patterns in D-245 MG (PR)*

Locus	Alleles	Change in D-245 MG (PR)
D1S102	1	No change
D2S123	2	Lost one allele and had additional bands at other
D2S390	1	Additional bands
D4S403	2	Small decrease in size (1 bp) of one allele
D4S174	2	Size increase in one allele
D6S253	1	No change
D7S460	2	Lost one allele and instead had smaller less intense bands
D10S212	1	Lower size band
D11S935	2	No change
D14S48	2	Lost one allele and had additional less intense smaller size allele
D14S49	2	Additional bands at one allele
D15S118	1	No change
D16S422	2	Additional bands at both alleles
D18S53	2	Decrease in size of both alleles
D19S246	2	No change

DNA were seen at 10 microsatellite loci (Table 2). The two main types of microsatellite aberrations observed were allelic shift due to increase or decrease in number of repeats at one or both loci and decrease in intensity or loss of one allele accompanied with additional bands at the other allele (examples shown in Fig. 2).

hMLH1 and hMSH2 Protein Levels. D-245 MG xenografts synthesized the hMLH1 and hMSH2 polypeptides in amounts similar to those found in HeLa S3 cells that were grown either in spinner culture or as xenografts (Fig. 3). D-245 MG (PR) also produced a similar level of hMLH1. However, the nuclear extract of cells from this tumor does not contain any detectable hMSH2 polypeptide (Fig. 3), which was shown by *in vitro* synthesized protein assay to be a truncated protein (data not shown). The prominent band present in all of the xenograft extracts, but not the HeLa spinner culture extract, is most likely the heavy chain of mouse IgG, as it was observed when the immunoblots were incubated with the secondary antimouse IgG antibody alone.

DISCUSSION

Procarbazine is a methylhydrazine derivative that has been used alone and in combination for treatment of CNS malignancies (22). Five studies have evaluated the activity of single-agent chemotherapy with procarbazine against CNS tumors, observing results in patients with recurrent (1, 2, 4) or newly diagnosed (3, 5) malignant CNS tumors. Kumar *et al.* (1) reported a 48% response rate in 43 patients with progressive primary or metastatic brain tumors, whereas the European Organization for Research on Treatment of Cancer Brain Tumor Group (2) observed no responses in 17 patients with recurrent CNS tumors. Rodriguez *et al.* (4) reported response rates of 14% in 37 patients with recurrent glioblastoma multiforme and 15% in 46 patients with recurrent anaplastic astrocytoma. Newton *et al.* (5) observed a 35% response rate in 31 patients with recurrent glioblastoma multiforme and anaplastic astrocytoma. Finally, Green *et al.* (3) demonstrated that patients with newly diagnosed malignant glioma treated with radiation and procarbazine had a superior survival to those receiving radiotherapy alone. These results, while supporting a modest benefit for the use of procarbazine in patients with malignant glioma, also suggest that most such patients demonstrate either *de novo* or acquired resistance to this methylating agent, with subsequent tumor progression and death.

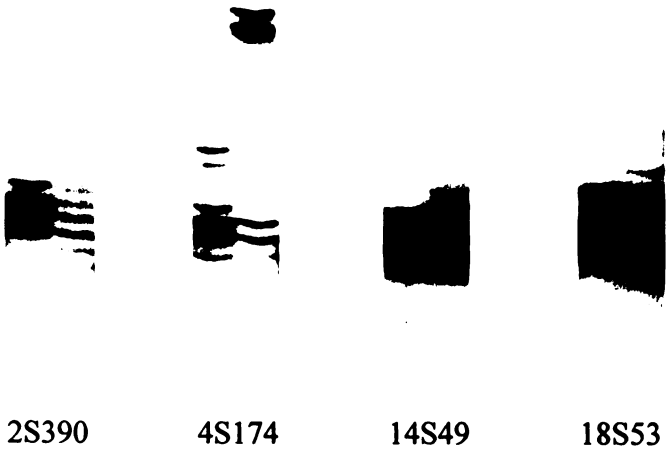

Fig. 2. Examples of microsatellite aberrations in D-245 MG. For each locus, the microsatellite pattern from the D-245 MG xenograft line is shown in the left lane and that from the D-245 MG (PR) line in the adjacent right lane. All four loci shown exhibited extra alleles.

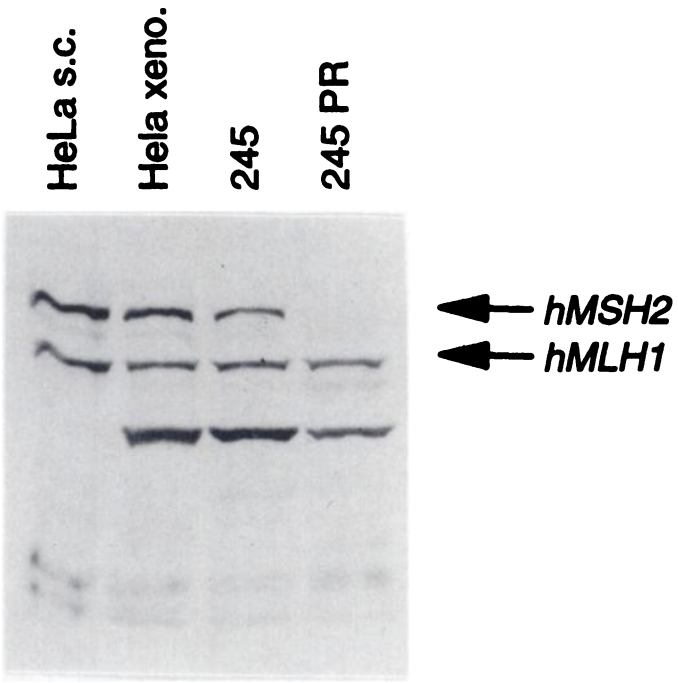

Fig. 3. Nuclear extracts from HeLa S3 cells (grown as spinner culture or as a xenograft) and from D-245 MG parent and D-245 (PR) xenografts. The extracts were subjected to electrophoresis through a SDS-polyacrylamide gel, transferred to a polyvinyl difluoride membrane, and visualized as immune complexes with monoclonal antibodies to both hMSH2 and hMLH1 polypeptides.

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The current laboratory studies were designed first to generate an *in vivo*-acquired resistance to procarbazine in a human glioblastoma multiforme xenograft growing s.c. in athymic nude mice and then to define the mechanisms of this resistance. Profound resistance to procarbazine was noted after nine serial treatments and subsequent passages of the xenograft line D-245 MG (PR), providing the opportunity for phenotypic and genotypic exploration of this resistance. Schold *et al.* (6) reported the only known mechanism of resistance to procarbazine in finding an inverse relationship between procarbazine induced growth delay and AGAT activity in a panel of CNS tumor xenografts in athymic nude mice. However, both D-245 MG and D-245 MG (PR) were Mer^-, displaying no measurable AGAT activity. Additional studies addressing xenograft GSH content, GST activity, and isozyme composition were not helpful in defining the mechanism of procarbazine resistance.

Treatment of the parent and resistant xenografts with a spectrum of antineoplastic agents revealed striking resistance to not only procarbazine but also to two other methylators, temozolomide and MNU. Cross-resistance to BCNU, cyclophosphamide, topotecan, CPT-11, and 9-aminocamptothecin was not observed. Finally, cell lines derived from D-245 MG and D-245 MG (PR) displayed disparate cytotoxicity to the methylator streptozocin, with marked resistance in D-245 MG (PR). This pattern of methylator resistance was striking and suggested similarities to methylation tolerance in bacterial and mammalian cells (23), the tolerance of which results from failure of the cells to conduct mismatch repair (24–26). The microsatellite instability observed in D-245 MG (PR) suggested that an *in vivo*-acquired failure to conduct mismatch repair in D-245 MG (PR) was responsible for the methylator resistance in this xenograft. Western blot analysis confirmed the absence of detectable hMSH2 polypeptide in D-245 MG (PR), supporting this conclusion. D-245 MG (PR) does express full-length *hMSH2* message, which produces a truncated protein product *in vitro*.

The current results broaden the therapeutic implications and consequences of abnormal mismatch repair in human tumor cells. Mismatch repair deficiency has been associated with human tumor cell resistance to methylators (25, 26), although we are not aware of any previous reports detailing serial *in vivo* methylator treatment resulting in acquired mismatch repair deficiency and development of resistance. More recent studies suggest that alkylator and intercalator resistance as well as resistance to other DNA-modifying agents may also be associated with a mismatch repair deficiency (27–29). The cross-resistance of D-245 MG (PR) to busulfan suggests that mismatch repair deficiency may be associated with resistance to this alkylator as well. The implications for patients with malignancies treated with these agents include absolute resistance to these antineoplastics, with subsequent tumor progression. When methylator or alkylator resistance is observed, a deficiency in mismatch repair will need to be considered.

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