ABSTRACT

Normal and malignant cells of the oligodendrocyte lineage show increased sensitivity to alkylating agents compared to astrocytes. One of the most mutagenic DNA lesions formed following exposure to alkylating agents is \( O^6 \)-methylguanine. To determine whether the increased sensitivity to nitrosoureas seen in oligodendrocytes is due to decreased repair capacity for \( O^6 \)-methylguanine, removal of this lesion from DNA was assessed in primary cultures of rat oligodendrocytes, astrocytes, and microglia. Glial cells were exposed to 1 \( \mu \)M 1-methyl-N-nitrosourea for 1 h and allowed 8 or 24 h for repair. Repair was evaluated using an immunoslot blot technique and a monoclonal antibody which recognizes \( O^6 \)-methylguanine (\( O^6 \)MeGua). Astrocytes removed \( O^6 \)MeGua more efficiently (80%) in 24 h) than either oligodendrocytes (20%) or microglia (4%). Determination of \( O^6 \)MeGua-DNA-alkyltransferase (AT) activity revealed that astrocytes contain 0.4 pmol/mg protein, which is average by comparison to the other cell types. Both oligodendrocytes and microglia exhibited very low levels of AT (oligodendrocytes, 0.08; microglia, 0.01 pmol/mg protein). These data are the first to show that within different populations of glial cells, \( O^6 \)MeGua adduct removal is substantially reduced in both oligodendrocytes and microglia. Rapid removal of \( O^6 \)MeGua in astrocytes coupled with persistence of this mutagenic lesion in oligodendrocytes following exposure of the developing central nervous system to nitrosoureas could contribute to the observed formation of oligodendrogliomas. Inefficient removal of \( O^6 \)MeGua in oligodendrogliomas might also account for their response to chemotherapeutic regimens involving alkylating agents such as procabazine, lomustine, and carmustine. The lack of repair of \( O^6 \)MeGua in microglia suggests that primary lymphomas of the central nervous system might be sensitive to treatment with alkylating drugs whose toxicity depends on repair of this adduct.

INTRODUCTION

The brain contains two major types of cells, neurons and glial cells. Although the importance of appropriate neuronal function is obvious, one half of the mass of the vertebrate brain is composed of glia (1). Based on counts of cell nuclei, it is estimated that glial cells outnumber neurons in the CNS by a factor of 10. Historically, glial cells have been regarded as “passive” elements in the nervous system, although recent attention has focused on the importance of active signaling between neurons and glia as a vital form of intercellular communication in the brain. In glia, as in all cells, normal physiology is dependent on genome stability. Yet, cellular genomes are constantly exposed to agents that modify DNA. One of the major types of injury which cells encounter is alkylation damage. Endogenous alkylation may occur at oxygens and nitrogens of purines by S-adenosylmethionine (2, 3) or by the reaction of nitric oxide with secondary and tertiary amines which form nitrosamines that can be metabolized to reactive species and alkylate guanine or adenine (4). Alkylated purines also can result from exposure to environmental pollutants such as \( N \)-nitrosocompounds in air pollution, food products, and tobacco smoke (5). When cells are exposed to alkylating agents, specific lesions are formed in their DNA. The spectrum of DNA adducts depends on the type of alkylating agent. Primarily, alkylation occurs at the ring nitrogens and exocyclic oxygens of DNA bases and the oxygens of the phosphate backbone (6). One of the most mutagenic lesions involves alkylation at the \( O^6 \) position of guanine. The adducted guanine can mispair with thymine, causing a G:C \( \rightarrow \) A:T transition mutation. Although most lesions formed by alkylation events are repaired by base excision, \( O^6 \)MeGua is repaired by a unique protein termed AT which transfers the alkyl group to a cysteine acceptor site in the active site of the protein. Because the cysteine acceptor site is not regenerated, the number of \( O^6 \)MeGua residues removed is equal to the number of active AT molecules within the cell. Repair of \( O^6 \)MeGua is cell and tissue specific. Many studies have correlated the levels of AT with susceptibility to the cytotoxic and tumorigenic effects of alkylating agents (7). However, few studies have measured AT activity in combination with the quantitation of adduct removal.

Gliomas are the most common primary brain tumors (8). Of particular interest is the observation that there is a differential responsiveness of glial cells to alkylating agents. Both normal and neoplastic cells of the oligodendrocyte lineage exhibit increased sensitivity to the cytotoxic and tumorigenic effects of nitrosoureas. An enhanced chemotherapeutic response is seen in oligodendrogliomas (9—11), and oligodendrogliomas are selectively induced following transplacental exposure of animals to EtNU (12). When single-cell suspensions of fetal forebrain of rat pups exposed transplacentally to a single i.v. dose of EtNU on the 14th day of gestation were transplanted into host animals and the host animals subsequently exposed to EtNU at day 8 and at 9 weeks, all rats developed brain tumors which were classified as oligodendrogliomas (13). Possible mechanisms that could cause the increased sensitivity to DNA-damaging agents are an increase in initial DNA damage or a decreased DNA repair capacity in oligodendrocytes. To date, most of the limited number of DNA repair studies exploring the CNS, have focused on the role of DNA repair in the aging brain. Repair has been assessed using homogenized brain tissues from either adult or embryonic brain, with few attempts to distinguish between the repair characteristics of different cell types (14—16). Cell-specific analysis of \( O^6 \)MeGua removal in neural tissue has not been reported. From mixed primary cultures of neonatal rat cerebral hemispheres, it is possible to generate nearly pure cultures of either oligodendrocytes, astrocytes, or microglia (17, 18). The studies described herein are the first to describe the difference in repair of \( O^6 \)MeGua in these cell types.

MATERIALS AND METHODS

Glial Cell Cultures. Cerebral hemispheres from 1- to 4-day-old Sprague Dawley rats were removed aseptically in ice-cold 20 mM HEPES-buffered Earle’s balanced salt solution (120 mM NaCl, 2.5 mM KCl, 10 mM NaHCO₃, 1.8 mM CaCl₂, 1.25 mM MgCl₂, 11 mM dextrose, and 2 mM L-glutamine).
and 1 mM NaH₂PO₄ containing 0.3% (w/v) BSA (fraction V, Sigma A-9647) and 20 mM glucose (HEBSS) as described previously (19). Cells were plated in high-glucose DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS and gentamicin sulfate. After 7 days, macrophages were harvested by rotary shearing (200 rpm/h) according to the methods of Giulian and Baker (17) and plated in a chemically defined medium (20). Subcultured macrophages were typically 95% pure, as determined by morphological criteria and immunofluorescence staining with biotinylated Griffonia simplicifolia isolecitin B₄, a marker for microglia (21). Oligodendrocyte lineage cells (C, phase contrast; D, FITC labeled with GFAP antibody), and microglial culture [E, phase contrast; F, FITC labeled with Griffonia (Bandeiera) simplicifolia lectin I isolectin B₄] assayed for DNA repair.

Oligodendrocyte progenitors (A, phase contrast; B, FITC labeled with A2B5 antibody), astrocytes (C, phase contrast; D, FITC labeled with GFAP antibody), and microglial culture [E, phase contrast; F, FITC labeled with Griffonia (Bandeiera) simplicifolia lectin I isolectin B₄] assayed for DNA repair.

Drug Exposure and DNA Isolation. Astrocytes and oligodendrocytes were grown for 2 days in the presence of 0.2 μCi/ml [³²P]thymidine and 10 μM thymidine and 10 μM methylguanine to uniformly label the DNA. Cells were washed with HBSS and treated for 1 h with MeNU that was dissolved in citrate buffer (pH 4.2). Concentrations ranging from 10⁻² to 10⁻⁴ M were obtained by serial dilution in HBSS. Control cultures were exposed to HBSS with the diluent only. Cells from control cultures and those for 0-h repair were lysed immediately. For repair analysis, the drug was removed and the cultures were replenished with fresh culture media containing 10 μM bromodeoxyxuridine and 1 μM fluoro deoxyxuridine. At the appropriate time points, the cells were lysed, and high molecular weight DNA was isolated using standard phenol-chloroform extractions. The purified DNA was restricted with BamHI, and the completeness of the digest was checked on a 0.8% agarose minigel. Parental DNA was separated from replicated DNA on neutral CsCl gradients. After centrifugation, the gradients were collected, and 10 μl aliquots were trichloroacetic acid precipitated and analyzed by liquid scintillation spectroscopy to identify the positions of replicated and parental DNA. Both parental DNA and replicated DNA were pooled separately, ethanol precipitated, rinsed in 70% ethanol, resuspended in 100 μl TE buffer (10 mM Tris and 1 mM EDTA), and quantitated. Microglia do not replicate in culture. Drug exposure and DNA isolation was identical to that described for astrocytes and oligodendrocytes. However, because there is no replicated DNA to be separated from parental DNA from these cultures, CsCl gradients were unnecessary.

Immunoslot Blot for Detection of O₆-MeGua. Samples containing 3 μg of DNA were heat denatured for 10 min at 95°C, quickly chilled on ice, and mixed with an equal volume of 2 M ammonium acetate. Standards consisted of 3 μg of calf thymus DNA containing serial dilutions of a known amount of O₆-MeGua. Single-stranded DNA was then immobilized on nitrocellulose membranes. Both samples and standards were loaded in duplicate on each membrane. Prior to use, nitrocellulose membranes were presoaked in 1 M ammonium acetate. After application of DNA, slots were rinsed with 200 μl of 1 M ammonium acetate. The nitrocellulose membranes were removed from the filter support, soaked in 5X SSC for 3 min, dried, and the DNA cross-linked to the nitrocellulose using a Bio-Rad GS Gene Linker UV chamber. Before incubation with a high-affinity, low cross-reactivity anti-O₆-alkyl-2'-deoxyguanine Mab, Mab EM21 (24), the membrane was soaked for 2 h at room temperature in PBS containing 0.1% casein and 0.1% deoxycholate to prevent nonspecific binding of the Mab. The membrane was then incubated, either overnight at 4°C or for 2 h at room temperature, in the same solution as above, and then incubated with a high-affinity, low cross-reactivity anti-O₆-alkyl-2'-deoxyguanine Mab, Mab EM21 (24), for 2 h at room temperature in PBS containing 0.1% casein and 0.1% deoxycholate to prevent nonspecific binding of the Mab. The membrane was then incubated, either overnight at 4°C or for 2 h at room temperature, in the same solution as above,

**Fig. 1. Immunocytochemical analysis of glial cells. Phenotypic properties of oligodendrocyte progenitors (A, phase contrast; B, FITC labeled with A2B5 antibody), astrocytes (C, phase contrast; D, FITC labeled with GFAP antibody), and microglial culture [E, phase contrast; F, FITC labeled with Griffonia (Bandeiera) simplicifolia lectin I isolectin B₄] assayed for DNA repair.**
with addition of the primary antibody. Thereafter, the membrane was washed extensively in PBS containing 0.16 M NaCl and 0.1% Triton X-100. The membrane was then incubated for 2 h at room temperature with PBS supplemented with 0.1% casein, 0.1% deoxycholate, and the 125I-labeled secondary antibody. The membrane was washed again extensively in PBS supplemented with 0.16 M NaCl and 0.1% Triton X-100 before being wrapped in cellophane. The resulting bands were quantified using a Bio-Rad Phosphorimager (GS-250 Molecular Imager). Linear regression analysis was performed on the radiographic intensities of the standards (coefficient of correlation was always >0.998). From the regression analysis, fmol of O6MeGua in each of the unknown samples were calculated.

Aldkyltransferase Assay. Cells were assayed for AT activity essentially according to the method of Brent (25). Briefly, cell pellets were resuspended in two volumes of buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM EDTA, 1 mM DTT, 0.02% sodium azide, 0.2 mM phenylmethylsulfonyl fluoride, 20 units/liter aprotonin, 20 μg/ml leupeptin, 156 μg/ml benzamidine, and 87.6 μM pepstatin. Then the cells were disrupted by three rounds of freezing and thawing. After centrifugation at 15,000 × g for 10 min, the protein content of the supernatant (cell extract) was determined with the Bradford method (26) using a Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA). The substrate for the assay was prepared by incubating [3H]DNA with 1 μg of [methyl-3H]DNA substrate at 37°C for 30 min in 200 μl of buffer. The reaction was stopped by adding 500 μl of 5% trichloroacetic acid, thereby precipitating both DNA and protein. The mixtures were heated at 80°C for 30 min to hydrolyze the DNA. The samples were cooled on ice and BSA was added as a carrier. The protein precipitate was collected on glass fiber filters, and 3H content was determined by liquid scintillation spectrometry. For calculations, 1 unit of AT activity was defined as the amount that transfers 1 pmol of [3H]-labeled methyl groups from DNA to protein.

RESULTS

These studies were designed to compare the DNA repair capacity of different populations of glial cells. Therefore, experiments initially were undertaken to verify the purity of glial cultures using well-defined immunocytochemical markers. More than 95% of the cells in undifferentiated oligodendrocyte cultures expressed A2B5 surface immunoreactivity; <3% were GalC⁺ and <2% were GFAP⁺ (Fig. 1, A and B). Astrocytes were >95% GFAP⁺ and essentially devoid of GalC⁺ or A2B5⁺ oligodendrocyte lineage cells (Fig. 1, C and D). Virtually all of the cells in the microglia cultures stained with the biotinylated Griffonia simplicifolia isoelectin B₄ (<2% GFAP⁺ or A2B5⁺ cells). The purity of each of the different populations was >95%, making them suitable for analysis of cell-specific differences in DNA repair capacity.

Astrocytes were exposed to 0.1, 0.5, or 1.0 mM MeNU for 1 h to determine the optimum drug concentration necessary to induce sufficient DNA damage for evaluation of repair kinetics. High molecular weight DNA was isolated, restricted, and subjected to O6MeGua quantitation. As seen in a representative autoradiograph (Fig. 2), O6MeGua lesions were formed in a dose-dependent manner. Based on this data, it was determined that 1 mM MeNU was an appropriate dose for repair studies. Using Student’s t test, statistical analysis of data from at least three separate experiments revealed that O6MeGua formation was not significantly different in the various populations of glial cells following exposure to 1 mM MeNU (astrocytes, 261 ± 93; oligodendrocytes, 230 ± 42; microglia, 148 ± 24 fmol O6MeGua/μg DNA). To ensure that 1 mM MeNU was not overtly toxic under the conditions of study, viability assays using trypan blue exclusion were performed (Fig. 3). Although 1 mM MeNU was not toxic in any of the cell types (viability >80%), trypan blue exclusion data indicated that microglia were more sensitive to higher concentrations of MeNU than either astrocytes or oligodendrocytes.

To assess removal of O6MeGua, the glial cells were exposed to 1 mM MeNU for 1 h and allowed 8 or 24 h for repair. Astrocytes removed O6MeGua very efficiently with 49 ± 6.4 fmol O6MeGua/μg DNA remaining at 24 h. Minimal repair was observed in oligodendrocytes with 181.7 ± 15.9 fmol O6MeGua/μg DNA remaining at 24 h. Virtually no repair was seen in microglia with 141.7 fmol O6MeGua/μg DNA remaining at 24 h. The results of these experiments are shown graphically as percentage of repair in Fig. 4. Next, AT activity was determined in different populations of glial cells. This assay measures 3H-labeled methyl transfer to cellular protein from a substrate prepared by treating calf thymus DNA with [3H]MeNU. The AT activity value was determined from the slope of the line for each cell type (Fig. 5) and expressed as pmol/mg protein. The control for these experiments was a human cell line (CEM) which contains above average levels of AT (0.88 ± 0.078 pmol/mg protein). AT activity of astrocytes was determined to be 0.42 ± 0.035 pmol/mg protein, which by comparison to other cell types puts them in the average range. Oligodendrocytes contained very low AT levels (0.083 ± 0.0037 pmol/mg protein), whereas microglia were essentially devoid of AT (0.01 ± 0.008 pmol/mg protein).

DISCUSSION

The results of these studies are, to our knowledge, the first to show a cell-type-specific difference in repair of O6MeGua adducts in primary cultures of glial cells. This observation is relevant in at least two areas. The first area concerns the increased risk of mutations in
expression of AT are species, tissue, and cell-type specific (12, 27). These analyses were limited to determination of AT expression. Previously, it has been shown that persistence of O6MeGua and the low levels of AT (27). Only recently chemotherapeutic regimens for treatment of primary brain tumors. This work is the first to address sensitivity of microglia to alkylating agents and their ability to remove O6MeGua. Microglia demonstrated the lowest AT levels and were the most sensitive to the cytotoxic effects of MeNU, in agreement with previous studies in which human monocytes have been shown to have lower AT levels compared to liver, small intestine, and colon epithelia (30). Cytotoxicity results from alkylation of DNA and proteins sufficient to critically disrupt cellular metabolism and replication. Microglia which serve as the macrophages of the CNS do not repair this type of DNA damage but rather accumulate it and die. This exquisite sensitivity may explain why transplacental exposure to ethylnitrosourea does not result in the formation of primary lymphomas of the CNS which are thought to be of microglial origin. Currently, the number of patients in clinical trials that utilized chemotherapy with alkylating agents whose cytotoxicity depends on O6MeGua repair or have combined this type of chemotherapy with radiation therapy in the treatment of primary lymphomas of the CNS is small (31—34). However, the observed results of an improved patient survival following use of this type of regimen might be explained by the lack of O6MeGua repair and suggest that development of new protocols based on this strategy could be productive. Despite numerous attempts to improve chemotherapeutic approaches for the treatment of gliomas, successful results have remained limited. Currently, the standard treatment for malignant glioma is maximal tumor resection followed by a combination of radiotherapy and chemotherapy. Although there are some clear examples of effective chemotherapy, the cure rate for the vast majority of patients with primary malignancies of the CNS is deplorable. Resistance to chemotherapy in brain tumors is complex and involves multiple mechanisms (35, 36). Because one of the major mechanisms may involve DNA repair, studies to evaluate different repair pathways in glial cells are crucial.

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Fig. 5. AT activity of glial cells. The assay measures 3H-labeled methyl transfer to cellular protein from a substrate prepared by treating calf thymus DNA with [3H]MeNU. The AT activity, expressed as pmol/mg protein, was determined from the slope of the line for each cell type. The control for these experiments was a human cell line (CEM) which contains medium/high levels of AT. •, astrocytes; O, oligodendrocytes; ▲, microglia; ○, CEM.

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Fig. 4. Repair of O6MeGua in glial cells. Cells were treated with 1 mM MeNU for 1 h. Following drug treatment, cells were either lysed immediately or allowed 8 or 24 h for repair. Control cultures were exposed to the drug diluent only. DNA was extracted and O6MeGua levels were determined using immunoslot blots (37). Data are expressed as percentage of repair and represent the means (n = 3). Bars: S.E.; * P < 0.01. •, astrocytes; O, oligodendrocytes; ▲, microglia.

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