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O⁶-Methylguanine-DNA methyltransferase deficiency in developing brain: Implications for brain tumorigenesis

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Abstract

The DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT) is a cardinal defense against the mutagenic and carcinogenic effects of alkylating agents. We have reported evidence that absence of detectable MGMT activity (MGMT[−] phenotype) in human brain is a predisposing factor for primary brain tumors that affects *ca.* 12% of individuals [J.R. Silber, et. al. Proc. Natl. Acad. Sci. USA 93 (1996) 6941–6946]. We report here that MGMT[−] phenotype in the brain of children and adults, and the apparent increase in risk of neurocarcinogenesis, may arise during gestation. We found that MGMT activity in 71 brain specimens at 6 to 19 weeks post-conception was positively correlated with gestational age ($P \leq 0.0015$). Moreover, the proportion of specimens exhibiting MGMT[−] phenotype (MGMT content < 0.42 fmol/10⁶ cells or 255 molecules/cell) declined progressively from 76% (16/21) at 6 to 8 weeks to 13% (1/8) at 15 to 19 weeks. All liver specimens that accompanied MGMT[−] brain (15/15) had measurable MGMT activity, demonstrating that the phenotype was not systemic in these cases. In contrast to MGMT, apurinic endonuclease, DNA polymerase β and lactate dehydrogenase activities were found in every brain extract assayed, and showed no significant relationship with gestational age. The observed gestational pattern has at least two implications for neurocarcinogenesis. (1) Early in development, brain tissue that has MGMT[−] phenotype and is rapidly proliferating may be especially vulnerable to alkylation-induced mutations, including mutations that lead to brain tumors. (2) Persistence of prenatal MGMT deficiency into postnatal life in a sub-population of individuals may increase brain tumor risk. Our findings provide possible mechanistic insight into epidemiologic data associating maternal alkylating agent exposure with brain tumor incidence.

1. Introduction

Alkylating agents, particularly N-nitroso compounds, are potent neurocarcinogens in certain rodent strains and primate species [1–3]. Transplacental exposure can be especially damaging. For example, a single dose of ethylnitrosourea to pregnant rats, representing *ca.* 25% of the

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LD₅₀ for adults, resulted in a 98–100% incidence of neurogenic tumors in offspring [reviewed in 1,2]. Central nervous system tumors were also frequent in the offspring of patas and rhesus monkeys exposed to ethylnitrosourea early in gestation [3]. Methyl- and ethylnitrosourea-induced tumors of rodent brain are associated with persistent O⁶-alkylguanine adducts in DNA [4–6], and administration of N-nitrosodimethylamine to pregnant patas monkeys generates O⁶-methylguanine in fetal brain DNA [7]. O⁶-alkylguanine adducts are highly genotoxic. These lesions are mutagenic, producing predominantly GC to AT transitions [8,9]. They are also clastogenic and recombinogenic, promoting sister chromatid exchange, chromosome deletions and rearrangements [reviewed in 9]. Moreover, specific O⁶-alkylguanine adducts in particular genes are believed to be causally related to induction of cancer in animal models [10,11].

The brain of rodents [12] has low levels of O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein that removes alkyl adducts from the O⁶ atom of guanine [8]. MGMT repairs DNA alkylation damage by transferring a single alkyl group from O⁶-methylguanine to an internal cysteine in a “suicide” reaction that irreversibly inactivates the protein. Methyl groups are the preferred substrate, though larger alkyl groups are removed at slower rates. MGMT is a crucial defense against mutagenesis, as evidenced by bacteria, yeast, and mammalian cells that are deficient in MGMT and display increased rates of spontaneous and alkylating agent-induced mutation [8–11,13]. MGMT also protects against alkylation-induced carcinogenesis, as demonstrated in transgenic mice. Expression of exogenous MGMT in mouse tissues decreases tumor formation induced by alkylators [10,11], and conversely, deletion of the MGMT gene is accompanied by increased methylnitrosourea-induced tumor formation [10,11,14,15].

Emerging evidence indicates that suppression of MGMT expression is associated with human carcinogenesis [14,15]. In the case of brain, MGMT content is relatively low [15], and we have observed that a substantial fraction of specimens contains no measurable activity [15]. We have also observed that MGMT[−] phenotype in brain is associated with gliomas [16], the most common primary brain tumor. We found that histologically normal brain adjacent to tumors displayed MGMT[−] phenotype in over half of cases (64/117 or 55%), whereas brain tissue from control individuals without brain tumors exhibited MGMT[−] phenotype in a minority of cases (5/43 or 12%). This 4.6-fold difference in frequency was highly significant ($P \leq 0.001$), indicating that MGMT[−] phenotype may be a risk factor for human neurocarcinogenesis. The incidence of MGMT[−] phenotype in normal brain was independent of tumor grade and histology [17], suggesting that inability to remove O⁶-alkylguanine adducts promotes the genesis of glial tumors of all major diagnostic types. Few exogenous risk factors for gliomas have been identified [18,19], suggesting that endogenous factors such as MGMT[−] phenotype may be especially important.

We report here that 50% of human embryonic (<9 weeks post-fertilization [20]) and early fetal brain specimens we examined exhibited MGMT[−] phenotype. We also report that the proportion of brain specimens with MGMT[−] phenotype declined progressively during the initial half of gestation, reaching the fraction observed in postnatal brain [16]. This developmental pattern suggests that MGMT[−] phenotype in the brain of children and adults, together with a concomitant increase in brain tumor risk [16], may originate *in utero*. The deficiency of MGMT activity during gestation has potential significance for prevention via reduction of maternal alkylating agent exposure [19], and for the time of occurrence of mutations that culminate in brain tumors.

2. Materials and Methods

2.1 Tissue

Fresh tissue obtained in accord with an IRB-approved protocol was provided by the Central Laboratory for Human Embryology, Department of Pediatrics, University of Washington. Brain and liver were transported to our laboratory in cold DMEM/F12 supplemented with 10% fetal bovine serum. Specimens were washed with PBS, and flash-frozen in multiple aliquots. To determine cell number, a small piece of fresh tissue was suspended in PBS and completely disrupted by serial passage through 18, 20 and 22 gauge needles. The cell suspension was stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and total cell number determined by counting using a hemacytometer. Preparation of extracts (i.e., high speed supernatants of whole tissue sonicates) was carried out as previously described [16].

2.2 MGMT

MGMT activity was measured in a standard biochemical assay that quantitates transfer of radioactivity from a DNA substrate containing [*methyl*³H]O⁶-methylguanine to protein, as we have previously described in detail [16,17,21]. We have used this procedure to quantitate MGMT in extracts of human brain tumors [17,21,22] and normal brain [16,17,21], and to document the association between brain tumors and MGMT[−] phenotype in adjacent, histologically normal brain [17,21,22]. All activities reported here represent the mean of at least 5 determinations that generally differed by no more than 20%, and are validated by numerous controls [17,21]. As in previous reports [16,17,21], every MGMT⁺ specimen displayed linearity of activity with added extract.

MGMT[−] phenotype is a functional term that refers to the limit of detection in the particular procedure employed, and varies among procedures and laboratories. We define MGMT[−] phenotype in this study as MGMT activity < 0.42 fmol/10⁶ cells (< *ca.* 0.16 fmol/mg protein or < 255 molecules/cell). The limit of detection in our assay is comparable to that of oligonucleotide-based assays (*ca.* 200 molecules/cell [23]) and Western assay (*ca.* 135 molecules/cell when 4.6×10^6 cell equivalents are analyzed [24]) and is lower than that of histochemical procedures (7000–30,000 molecules/cell [25,26]). Every MGMT[−] extract, when mixed 8:1 (v/v) with MGMT⁺ extracts, yielded activity expected for the MGMT⁺ extracts, indicating that MGMT[−] extracts did not contain a diffusible inhibitor of MGMT activity.

2.3 Apurinic endonuclease

Activity was assayed as described previously [27,28]. Briefly, acid depurinated, supercoiled plasmid DNA was incubated with extracts, after which nicked, relaxed and supercoiled DNA was resolved by agarose gel electrophoresis. Photographs of ethidium stained gels were scanned by using Adobe Workshop 2.5, and DNA band density was quantitated by using NIH Image version 1.55. Activity (fmol abasic sites incised/min/cell) was calculated from the fraction of nicked molecules, and was a linear function of added extract in all cases. *E. coli* endonuclease, III a reference enzyme, also yielded linearity with added enzyme.

2.4 DNA polymerase β

DNA polymerase β (Pol β) activity was quantitated, as in earlier work [16], by using an activity gel assay [29] with recombinant rat pol β as a reference. Briefly, extracts were subjected to electrophoresis in an SDS-polyacrylamide gel containing gapped DNA in the matrix; following renaturation, Pol β was visualized by incorporation of radioactivity from α -³²P-TTP into a 39 kDa band. Activity, determined by phosphor imaging analysis of band intensity, was a linear function of the amount of Pol β standard (0.01–1.0 pg), and of added extract, in all cases.

2.5 Lactate dehydrogenase

Activity was quantitated by the Cabaud-Wroblewski procedure [30] with a kit from Sigma, and was a linear function of added extract.

2.6 Statistical analysis

Linear regression analysis was carried out with Microsoft Excel (Microsoft Corp., Redmond, WA). For purposes of calculation, MGMT⁻ extracts were assigned an MGMT activity of 0.21 fmol/10⁶ cells, i.e., one-half of the lower limit of detection.

3. Results

The purpose of this work was to assess the idea that the frequent MGMT⁻ phenotype in human brain, and an accompanying risk of neurocarcinogenesis, may arise during gestation. The hypothesis we investigated derived from an earlier study [16] in which we observed an age dependence of MGMT⁻ phenotype in the histologically normal brain of brain tumor patients. In that study, we found that the frequency of MGMT⁻ phenotype in brain adjacent to primary brain tumors was correlated with age, increasing from 22% in children to fully 75% in adults over 50. In contrast, the frequency of MGMT⁻ phenotype in the brain of individuals without brain tumors (12%) was unrelated to age. These observations suggested the following hypothesis: MGMT⁻ phenotype in brain is a prenatal characteristic that persists into postnatal life in a subpopulation of individuals -- 12% in our sample -- who are thereby predisposed to the age-dependent occurrence of brain tumors.

To investigate this hypothesis, we investigated the developmental pattern of MGMT activity in brain. We quantitated MGMT activity in extracts of 71 brains ranging in age from approximately 42 days (6 weeks) to 130 days (19 weeks) post-fertilization. We found that half of extracts (36/71) contained no detectable MGMT activity (MGMT⁻ phenotype, < 0.42 fmol/10⁶ cells (< ca. 0.16 fmol/mg protein or < 255 molecules/cell). MGMT activity in the remaining 35 extracts varied from 0.49 ± 0.08 to 11 ± 1.1 fmol/10⁶ cells (295 to 6,625 molecules/cell), a range consistent with that previously observed in adult [16] and pediatric [22] brain (240 to 18,700 molecules/cell). As shown in Fig. 1A, MGMT activity in developing brain was strongly correlated with gestational age ($r = 0.371$, $P \leq 0.0015$). As illustrated in Fig. 1B, this correlation reflected a decline in the proportion of MGMT⁻ extracts from 76% (16/21) at 6 to 8 weeks of age to 13% (1/8) at 15 weeks and older. Liver, in addition to brain, was obtained in 36 cases. All liver extracts were MGMT⁺, including 15 paired with MGMT⁻ brain, and had activity that varied from 0.43 ± 0.10 to 255 ± 30 fmol/10⁶ cells (260 to 154,000 molecules/cell).

To determine whether the gestational pattern of MGMT activity in brain extended to other activities that repair alkylated DNA, we assayed apurinic endonuclease (Ap endo) activity and Pol β , key base excision repair activities [31]. Ap endo activity was found in all 36 extracts examined, 18 of which had no measurable MGMT (Fig. 2A). The mean activity (0.10 ± 0.091 fmol abasic sites incised/cell/min) was similar to that previously observed in adult [27] and pediatric [28] brain (0.13 ± 0.053 fmol abasic sites incised/cell/min). Pol β activity was likewise found in all 14 extracts examined, 6 of which had no measurable MGMT (Fig. 2B). The mean activity (0.24 ± 0.22 pg/10⁶ cells) was also similar to that observed [16] in the brain of adults (0.16 ± 0.15 pg/10⁶ cells). Importantly, neither Ap endo nor Pol β activities showed a statistically significant relationship with age, as determined by linear regression analysis ($r = 0.16$; $P > 0.40$ and $r = -0.25$; $P > 0.35$ respectively) and illustrated by the mean activities during development (Fig. 3B,C). These observations are consistent with the essentiality of Ap endo activity [32] and pol β for early mouse development [33]. We also assayed lactate dehydrogenase, an enzyme with no known DNA repair function. All 30 extracts examined had activity, and, again, the activity had no statistically significant relationship with age, as

determined by linear regression analysis ($r = -0.16$; $P \leq 0.35$) (Fig. 2C) and illustrated by the mean activities during development (Fig. 3D). In striking contrast, the age dependence of mean MGMT activity for the entire sample population (Fig. 3A), was manifest in each of the three subsets of extracts assayed for Ap endo ($r = 0.64$; $P \leq 0.0001$), Pol β ($r = 0.62$; $P \leq 0.020$) and lactate dehydrogenase ($r = 0.39$; $P \leq 0.04$) activities.

To summarize, the data we present here show that: (1) Half of embryonic and early fetal brain specimens we examined contained no detectable MGMT activity (MGMT⁻ phenotype, <255 molecules/cell); (2) the proportion of brain specimens that exhibited MGMT⁻ phenotype decreased from 76% to 13% between 6 to 19 weeks post-fertilization; (3) the proportion of specimens with MGMT⁻ phenotype in brain at 19 weeks of gestation reached the proportion observed [16] in adult brain (12%)¹. Taken together, the results provide evidence of developmental regulation of MGMT activity in human brain, and indicate that MGMT⁻ phenotype in the brain of children and adults may arise as a “developmental holdover”.

Discussion

Primary malignant brain tumors are among the most rapidly lethal human cancers, with two year survival rates for adult gliomas being less than 20% [34]. The incidence is strongly age dependent, peaking in the sixth and seventh decades of life [18]. However, 10% of new cases are children under the age of fifteen, and brain tumors are the leading cause of death from childhood cancer [35]. Despite intensive investigation, the etiology of primary brain tumors remains largely unknown. Heritable syndromes that predispose to neurocarcinogenesis, and high dose ionizing radiation, a strongly associated exogenous risk factor, account for relatively few cases [18,19]. Our evidence [16] indicates that lack of detectable MGMT activity (MGMT⁻ phenotype) in brain is a susceptibility factor that affects a sizable minority of individuals (12% in our sample population). Apparently, failure to express this important DNA repair activity may be among the earliest events in progenitor tissue that predispose to tumor formation.

Based on earlier findings [16], we postulated that MGMT⁻ phenotype in brain is a gestational trait that is carried into postnatal life by a subgroup of individuals who thereby incur increased risk for brain tumors. In support of this premise, we found that MGMT activity was strongly correlated with gestational age, and the proportion of specimens displaying MGMT⁻ phenotype gradually fell to that found in adults. The data are consistent with a transition from MGMT⁻ to MGMT⁺ phenotype that may involve uniform up-regulation of MGMT activity in all cells, or an increase in the proportion of cells that express MGMT activity. The putative transition is not highly stage-specific, and would occur during a period when major morphological features of the brain are still forming [20]. As noted above, the frequency of MGMT⁻ phenotype that we observed at a gestational age of 15 to 19 weeks (13%) was essentially the same as we found in adult brain (12%). Overall, the results indicate that MGMT activity in human brain is developmentally regulated and suggest that the MGMT⁻ phenotype in the brain of children and adults may arise *in utero*. Our data do not address the possibility that MGMT⁻ phenotype in the brain of children and adults might also arise by loss of MGMT expression in MGMT⁺ tissue, either late in gestation or post-gestationally.

The observed pattern of MGMT activity in developing brain has potential implications for the occurrence of alkylation-induced genomic damage that contributes to brain tumors. The mammalian blood-brain barrier, which is an important defense against exposure to endogenous

¹In the work on adult brain [16], MGMT⁻ phenotype was defined as <0.34 fmol MGMT/10⁶ cells or < 204 molecules/cell. The 20% difference relative to the definition used here (204 vs. 255 molecules/cell) reflects the small amounts of embryonic tissue available and does not alter the conclusions or implications of the present study.

and exogenous alkylators, is not completely formed until after birth [36]. One implication is that brain may be especially susceptible to the mutagenic and clastogenic effects of O⁶-alkylguanine adducts early in gestation when brain cells have MGMT[−] phenotype and are dividing rapidly. Even if such cells eventually become MGMT⁺, previously suffered point mutations and chromosomal aberrations would be fixed. In accord with this idea, gliomas frequently exhibit genetic alterations that may be attributable to unrepaired O⁶-alkylguanine. For example, GC to AT transitions (the characteristic point mutation caused by O⁶-methylguanine [8,9]) in *p53* [37], *RB* [38] and *PTEN* [39], together with loss of the second allele, underlie the abrogation of programmed cell death and cell cycle regulation found in human gliomas [40]. Human embryonic brain may thus resemble embryonic rodent brain, which is deficient in MGMT and particularly vulnerable to nitrosourea-induced tumors [1–3]. These considerations suggest that alkylation damage incurred during embryogenesis may contribute disproportionately (relative to damage incurred at other periods of life) to the overall incidence of human brain tumors, and raise the issue of maternal exposure (19). Another implication of the observed gestational pattern is that the MGMT[−] phenotype that prevails in embryonic brain may persist into postnatal life in a subpopulation of individuals, thus increasing risk for alkylation-related neurocarcinogenesis.

Human exposure to alkylating agents is believed to be continuous and life-long, and to originate from diverse, endogenous and exogenous sources. Possible endogenous alkylators include S-adenosylmethionine, lipid peroxidation products and nitrosated bile acids, peptides and amino acids [41]. Notably, azaserine and nitrosated glycine react with DNA *in vitro* to produce O⁶-methylguanine [41,42]. Based on the neurocarcinogenicity of *N*-nitroso compounds and certain precursors in experimental animals [1–3], it has been hypothesized that exogenous agents of particular relevance may include *N*-nitrosamides and nitrosamide precursors, e.g. sodium nitrite [19]. Although epidemiologic evaluation has been complicated by manifold problems, and the data have not been consistent [reviewed in reference 19], several recent studies offer some support for this hypothesis. Notably, the majority of studies to date of maternal diet and childhood brain tumors suggests that exposure during gestation to endogenously formed nitroso compounds may be associated with tumor occurrence [19]. Our present observations imply that maternal exposure during the first trimester could have particular causal relevance. Other recent studies suggest that exposure to dietary sources of nitroso compounds is associated with increased risk of adult gliomas, and, conversely, that ingestion of agents that inhibit endogenous formation of these compounds is associated with reduced risk [e.g., 43,44]. Stronger epidemiologic data might be obtainable for adult tumors if it were possible to identify individuals with MGMT[−] phenotype in brain, since this subgroup may have heightened susceptibility to neurocarcinogenesis by nitroso compounds. Unfortunately, there is no readily assayable biomarker for this subpopulation, such as MGMT in lymphocytes [16].

The mechanisms controlling MGMT expression in normal mammalian tissue are not yet completely defined, although epigenetic regulation is mediated by CpG methylation in MGMT[−] cell lines and human tumors [45]. DNA methylation is involved in early development [46] and could be especially important in determining tissue-specific MGMT expression in human brain. Polymorphisms have been documented at the human MGMT locus [47,48], some of which might be relevant as well. Thus, individuals with MGMT[−] phenotype in brain could carry an MGMT allele(s) that is particularly subject to down-regulation. Alternatively, MGMT[−] phenotype in brain may be independent of genotype at the MGMT locus and represent an ontologic variation determined entirely by other factors.

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Fig. 1. MGMT activity in developing brain. (A) MGMT activity in high speed supernatants of tissue extracts is shown as a function of days post-fertilization. The overlapping crosses at 53–60 days on the X-axis represent 18 specimens without detectable activity (MGMT⁻ phenotype). The line was derived by linear regression analysis. X, MGMT⁻ phenotype; O, MGMT⁺ phenotype. (B) The percent of specimens with MGMT⁻ phenotype is shown as a function of weeks post-fertilization. The number of specimens in each age group was 21, 29, 13 and 8, respectively, in order of increasing age.

Fig. 2. Apurinic endonuclease (A), DNA polymerase β (B), and lactate dehydrogenase (C) in developing brain. Activities in high-speed supernatants of tissue extracts are shown as a function of days post-fertilization, together with the lines derived by linear regression analysis. X, MGMT⁻; O, MGMT⁺.

Fig. 3.

Dependence of MGMT (A), apurinic endonuclease (B), DNA polymerase β (C), and lactate dehydrogenase (D) activities on gestational age in developing brain. Mean activities and standard errors of the mean are shown as a function of weeks post-fertilization. The number of extracts in each age group is indicated. For purposes of calculation, a value of 0.21 fmol/10⁶ cells (half the limit of detection) was assigned to MGMT⁻ extracts.