DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents

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We have generated mice deficient in 6-methylguanine DNA methyltransferase activity encoded by the murine Mgmt gene using homologous recombination to delete the region encoding the Mgmt active site cysteine. Tissues from Mgmt null mice displayed very low 6-methylguanine DNA methyltransferase activity, suggesting that Mgmt constitutes the major, if not the only, 6-methylguanine DNA methyltransferase. Primary mouse embryo fibroblasts and bone marrow cells from Mgmt –/– mice were significantly more sensitive to the toxic effects of the chemotherapeutic alkylating agents 1,3-bis(2-chloroethyl)-1-nitrosourea, streptozotocin and temozolomide than those from Mgmt wild-type mice. As expected, Mgmt-deficient fibroblasts and bone marrow cells were not sensitive to UV light or to the crosslinking agent mitomycin C. In addition, the 50% lethal doses for Mgmt –/– mice were 2- to 10-fold lower than those for Mgmt +/+ mice for 1,3-bis(2-chloroethyl)-1-nitrosourea, N-methyl-N-nitrosourea and streptozotocin; similar 50% lethal doses were observed for mitomycin C. Necropsies of both wild-type and Mgmt –/– mice following drug treatment revealed histological evidence of significant ablation of hematopoietic tissues, but such ablation occurred at much lower doses for the Mgmt –/– mice. These results demonstrate the critical importance of 6-methylguanine DNA methyltransferase in protecting cells and animals against the toxic effects of alkylating agents used for cancer chemotherapy.

Introduction

DNA repair plays an important role in protecting genomes from insults inflicted by certain endogenous metabolites, by agents in the environment and, for a significant number of individuals, by cancer chemotherapeutic agents. Alkylating compounds are particularly cytotoxic, making them good chemotherapeutic agents, but they are also mutagenic and carcinogenic, detracting from their long-term clinical benefits. It is generally accepted that in cultured mammalian cells the DNA alkyltransferase protein O6-methylguanine (O6-MeG) DNA methyltransferase (MTase) provides protection against such toxic and mutagenic effects of chemotherapeutic alkylating agents (Erickson et al., 1980; Samson et al., 1986; Barrows et al., 1987; Dolan et al., 1989; Dumenico et al., 1989; Tano et al., 1990, 1997; Kaina et al., 1991; Wu et al., 1991; Harris and Margison, 1993). O6-MeG DNA MTases repair alkyltransferase damage via an unusual suicide mechanism involving irreversible transfer of alkyl DNA lesions to an internal cysteine residue (Lindahl et al., 1982, 1988). However, because this particular DNA repair protein is expressed at very different levels in mammalian tissues (Grafstrom et al., 1984; Pegg, 1984; Montesano et al., 1985; Pegg et al., 1985; Gerson et al., 1986; Moritz et al., 1995), varying by up to 100-fold, it has been difficult to predict from such in vitro tissue culture experiments the precise role of O6-MeG DNA repair MTase in protecting whole animals from chemotherapeutic alkylating agents. Furthermore, many of these agents produce DNA damage that are substrates for several different DNA repair pathways, hence, the relative contribution of each repair pathway may differ between alkylating agents and between tissues.

Chloroethyl nitrosourea (CNU) alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) induce numerous DNA lesions including highly cytotoxic DNA interstrand crosslinks. These crosslinks are formed via an intermediary O6-chloroethyl group and are thought to interfere with DNA replication (Ludlum, 1990); the repair of such O6-chloroethyl groups by the O6-MeG DNA MTase protein prevents DNA crosslink formation and thus provides protection against the cytotoxic effects of such agents (Erickson et al., 1980; Robins et al., 1983; Samson et al., 1986; Brent et al., 1987; Brent and Remack, 1988). Indeed, O6-MeG DNA MTase levels in cultured mammalian cells correlate with resistance to CNU alkylating agents (Erickson et al., 1980; Tsujimura et al., 1987; Lindahl et al., 1988; Mineura et al., 1990; Moritz et al., 1995; Wang et al., 1996; Phillips et al., 1997).

BCNU and other CNUs are used to treat a variety of tumors, in particular pediatric and adult gliomas, as well as cancers of the lymph, breast, lung and gastrointestinal (Broder and Rall, 1972; Carter et al., 1972; Schabel, 1976; Walker et al., 1978; Goldin and Schabel, 1981; Colvin, 1993). However, the success of such treatments is limited by severe myelosuppression, as well as lung toxicity (Schabel, 1976). Such myelosuppression is almost certainly due to the fact that bone marrow tissue has very low levels of O6-MeG DNA MTase and 3-methyladenine DNA glycosylase activities relative to other tissues (Gerson et al., 1986; Moritz et al., 1995; Glassner and Samson, unpublished results); indeed, introduction of the human O6-MeG DNA MTase gene, MGMT, into murine bone marrow cells significantly enhances resistance to chemotherapeutic
Materials and methods

Reagents

pBluescript and a 129SV λ-fixII mouse genomic library were from Stratagene (La Jolla, CA). pSL301 was from Invitrogen (San Diego, CA). E14 embryonic stem (ES) cells and H-2k were kindly provided by A.Berns and H.t Rieke (Netherlands Cancer Institute, Amsterdam, The Netherlands). The neo and HSV-1k expression cassettes (Tybulewicz et al., 1991) were subcloned from modifications of plasmids pPKG-NEO and pBS-PKG-TK-Δ, respectively (from D.Huszar, GenPharm International, Mountain View, CA). PCR primers were from Ransom Hill Bioscience (Ramona, CA). BCNU and temozolomide were from J. Johnsons (National Cancer Institute, Bethesda, MD). N-methyl-N-nitro-N-nitrosoguanidine (MNNG), MNU, mitomycin (MMC), STZ, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma (St Louis, MO). Wild-type mice [(129×C57d/J)F1] were from Jackson Laboratories (Bar Harbor, ME). Minimal essential medium alpha (α-MEM) and LIF was from Life Technologies (Grand Island, NY). MethoCol™ PF from Stem Cell Technologies (Vancouver, BC, Canada), and fetal bovine serum from HyClone Laboratories (Logan, UT).

Mgmt gene knockout targeting vector

The 129SV λ-fixII mouse genomic library was screened using a 32P-labeled PCR fragment that spanned Mgmt exon 5 (Shiraiishi et al., 1992). A ~13 kb Ncol insert from a positive clone was subcloned into pBluescript to generate pBSMT42. The Mgmt gene targeting construct pMT42-ΔE5-ΔNeo-TK (Figure 2B) was engineered from pBSMT42 in multiple steps. Briefly, two BamHI sites present in the neo cassette of pPGK-NEO were removed and an XhoI-MluI fragment containing this modified neo cassette was subcloned into pSL-PGK-NORL. The larger Ndel-Norl fragment of pBSMT42 (Figure 2B) was subcloned into a pSL301 plasmid lacking an internal BamHI site to generate pSL-PGK-ΔNeo. The smaller Norl-Norl fragment of pBSMT42 served as a probe source. The positively selectable BamHI-MluI neo marker from pSL-PGK-NEOII was cloned into the BamHI-MluI exon 5 region of pSL-MT42-LABam, generating pSLMT42-ΔΔ5-ΔNeo. An HSV-d-containing Norl fragment from pBS-PKG-TK-Δ Norl (Englard et al., 1996) was cloned into the unique Norl site of pSLMT42-ΔΔ5-ΔNeo, thus allowing for negative counter-selection against random integration events, to generate the final Mgmt knockout targeting construct pMT42-Δ5-ΔNeo-TK.

Generation of Mgmt knockout ES cells and mice

An aliquot of 20 µg of an ~12 kb HindIII fragment (from pMT42-Δ5-ΔNeo-TK) was electroporated into E14 ES cells as described (Engeward et al., 1997). G418- and 1-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil-resistant colonies were expanded and frozen at ~80°C (Engeward et al., 1997). Homologous recombinant ES cell clones were identified by Southern blot analysis (Aubusel et al., 1994) using a 32P-labeled BglII-BamHI probe fragment (Figure 2B) and 10–15 karyotypically normal ES cells were injected into C57Bl/6 mice and implanted into X chromosome deficient female B6C3F1 mice; male chimeras were bred with C57Bl/6 females and germline transmission was determined by transmission of the agouti coat color (data not shown).

Mgmt genotyping

Genomic DNA isolated from mouse liver (Laird et al., 1991) was BamHI digested, then subjected to Southern blot analysis using a 32P-labeled BglII-BamHI probe fragment (Figure 2B). Genomic DNA from ear punches (Chen and Evans, 1993) was subjected to multiplex PCR analysis using primers P1, P2, and P3. Primers P1 (5′-GGATCCCTCCGTCTAGACGTT-3′) and P3 (5′-CCCCAGACCTTGACGCTC-3′) detect the wild-type Mgmt allele (542 bp); primers P2 (5′-GGTGGGATGATAATGCCTGTC-3′) and P3 detect the targeted MgmtΔneo allele (409 bp) (Figure 2B). Determining MTase activity levels

Mouse liver extracts were prepared as described (Moritz et al., 1995). ES cells and mouse embryonic fibroblasts (MEFs) were washed in phosphate-buffered saline (pH 7.3), resuspended in 100-300 µl MTase buffer (50 mM HEPES, pH 7.8, 10 mM DTT, 1 mM EDTA, 5% glycerol), sonicated twice, the lysate cleared by centrifugation and aliquots stored at ~80°C. O6-MeG DNA MTase activity was determined by a rapid assay (Margison et al., 1985), using Micrococcus luteus DNA methylated with [3H]MNU (18 or 0.9 Ci/µmol; Amersham, UK) as described (Demple et al., 1983; Karren et al., 1979). DNA concentration in tissue extracts was determined using Hoesch 33258 fluorescence (Gerson et al., 1986) and protein concentration using the Bio-Rad Bradford (Bradford, 1976) Protein Assay (Richmond, CA).

Growth inhibition of MEFs by chemotherapeutic alkylating agents

Primary MEFs from 13-5 day-old mouse embryos were generated and maintained as described (Freshney, 1994). Cells were washed before and after drug treatment in phosphate-buffered saline. Drug exposure was in serum-free medium for 1 h. Cell exposure to UV was in 0.1 ml phosphate-buffered
Results

Generation of Mgmt null mice

The Mgmt targeting vector pMT42-ΔE5-NEO-TK (Figure 2B) was constructed as described in Materials and methods and used to target the Mgmt locus in E14 ES cells by homologous recombination. Our strategy was to delete, from Mgmt exon 5, a region encoding the active site cysteine of Mgmt thought to be required for alkyl transfer (Olsson and Lindahl, 1980; Demple et al., 1985; Santibanez-Koref et al., 1992; Figure 2A). Mgmt heterozygous ES cells were identified by Southern analysis and karyotypically normal cells were injected into mouse blastocysts, generating chimeric mice and germline transmission of the disrupted Mgmt allele. Mgmt +/− offspring were intercrossed and Mgmt null mice were produced in the normal Mendelian ratio. These Mgmt −/− mice developed normally, displaying no obvious phenotypic or pathological abnormalities (data not shown). The structure of the targeted MgmtΔ:neo allele in +/− and −/− Mgmt mice was examined both by Southern (Figure 2C) and PCR (Figure 2D) analysis; Mgmt +/+ , +/− and −/− mice were identified with the expected genotype.

O6-MeG DNA MTase activity levels vary with Mgmt genotype

We examined the levels of O6-MeG DNA MTase activity in the Mgmt +/− E14 ES cells (used to generate the chimeric mice) and the E14 parental cells. MTase activity in Mgmt +/− ES cell extracts was reduced to ~60% of the wild-type level (Figure 3A), consistent with targeting one of the Mgmt alleles. For the mice, we examined MTase activity in liver cell extracts (Figure 3B), because liver normally has the highest activity relative to other tissues (Gerson et al., 1986; Moritz et al., 1995). Clearly, MTase activity reflected the Mgmt genotype. MTase activity in Mgmt +/− liver tissue was ~60% that of wild-type and virtually no activity was found in Mgmt −/− mice (Figure 3B). A similar genotype-dependent reduction in MTase activity for MEF cells was also observed (Figure 3C). Mgmt thus encodes the major O6-MeG DNA MTase activity in the mouse tissues tested; whether the residual activity in Mgmt −/− liver and MEFs represents a second MTase remains to be determined.
alkylating agents, namely BCNU, MNNG, STZ, and temozolomide, for their sensitivity to growth inhibition by a number of chemotherapeutic alkylating agents. Mgmt –/– MEFs are sensitive to chemotherapeutic alkylating agents, namely bone marrow cells. Bone marrow cells were isolated from Mgmt +/+ and –/– mice and were examined ex vivo for their sensitivity to BCNU, MNU, MMC, STZ, and temozolomide. Given that Mgmt activity levels are so low in bone marrow, one might predict that Mgmt would not play a significant role in protecting this tissue from killing by such agents. However, Mgmt –/– bone marrow cells were extremely sensitive to BCNU, MNU, STZ, and temozolomide (but not to the control agent MMC) compared with cells from wild-type mice (Figure 5A–E). These results illustrate that even low levels of Mgmt can protect cells from the toxic effects of chemotherapeutic alkylating agents.

**Mgmt +/+ mice are sensitive to chemotherapy agents**

Mgmt +/+ and –/– mice were exposed to a single injection of graded doses of the alkylating agents BCNU, MNU, STZ, and MMC in order to determine LD50 values. Table I shows that the Mgmt –/– mice were considerably more sensitive to BCNU, MNU, and STZ than the wild-type animals, with the difference in LD50 values ranging from ~2-fold for STZ to ~10-fold for MNU. No difference in MMC sensitivity was observed. These results demonstrate that Mgmt plays an essential role in protecting mammals against the toxic effects of several agents used in cancer chemotherapy and suggests that inter-individual variations in MTase repair capacity might be an important variable in chemotherapeutic tolerance.

**Chemotherapeutic alkylating agents cause severe ablation of hematopoietic cells in both Mgmt +/+ and –/– mice**

The most consistent pathology observed in Mgmt +/+ and –/– mice treated with BCNU, MNU, MMC, and STZ was ablation in bone marrow of the hematopoietic tissues. Tissue sections of sternebrae revealed hematopoietic compartment spaces devoid of progenitor cells, containing instead only expanded sinuses with mature erythrocytes and small numbers of neutrophils, indicative of myeloablation (Figure 6B and D). In contrast, normal hematopoietic compartments of bone marrow from untreated animals had a tightly packed mass of hematopoietic stem cells of several lineages (Figure 6A and C). The atrophy observed following alklyation treatment occurred at much lower drug doses (with the exception of the control agent MMC) in the Mgmt –/– mice than in the wild-type controls (examples from STZ-treated animals are shown in Figure 6). No other consistent pathology was observed following drug treatment. These results demonstrate that the toxicity of alkyl lesions normally repaired by Mgmt is particularly acute in rapidly dividing hematopoietic tissues.
Table I. Sensitivity of Mgmt +/+ and −/− mice to various alkylating agents

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<tr>
<th>Mgmt genotype</th>
<th>LD50 (mg drug/kg body wt)</th>
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<tr>
<td></td>
<td>BCNU</td>
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<tr>
<td>+/+</td>
<td>39</td>
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<td>−/−</td>
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Six- to eight-week-old mice were given a single i.p. injection. One mouse was treated per dose and each dose concentration was chosen to be 50% greater than the preceding, for a total of six doses per treatment regime. The lowest dose at which a mouse dies within 30 days of each treatment was taken as the estimate of the 50% lethal dose (LD50) (Deichmann and LeBlanc, 1943). Similar results were obtained in duplicate trials.

Discussion

During a screen of randomly synthesized drugs in the late 1950s by the National Cancer Institute, MNNG was identified as displaying a limited but consistent antitumor activity in mice. At the time, this discovery seemed counter-intuitive, given that MNNG was a known carcinogen. Synthesis and testing of chemical analogs of MNNG identified MNU, which in addition to displaying higher antitumor activity than MNNG, also appeared to cross the blood–brain barrier and to be active against intracerebrally implanted tumors, one of the first chemotherapeutic agents to display such a property. Further nitrosourea congeners have been employed as effective agents, including temozolomide and UV light. A naturally occurring methylnitrosourea derivative, STZ, was also identified as having antitumor activity, with less severe associated bone marrow toxicity than the CNUs, and it also entered clinical trials in the 1970s (Weiss, 1982; Ludlum, 1997). More recently, new generations of chemotherapeutic alkylating agents, including temozolomide, have been generated and are being evaluated for their clinical efficacy as antitumor agents (Workman et al., 1992; Abrams et al., 1994; Newlands et al., 1997).

Given the wide use of CNUs in the clinical treatment of a variety of cancers and the correlation observed in cultured cells between CNU resistance and DNA MTase activity, the generation of a readily available whole animal model might seem warranted. We targeted the deletion of a highly conserved amino acid sequence PCHR in the mouse Mgmt gene (Santibanez-Koref et al., 1992), which, judging from other cloned MTases, contains a critical active site cysteine residue (Olsson and Lindahl, 1980; Demple et al., 1985). Mice containing the targeted Mgmt alleles exhibited reduced O6-MeG DNA MTase activity depending upon gene copy number. It is important to note that while MTase activity in Mgmt −/− liver and MEF cells appears to be >0, this may be deceptive, since the activity in Mgmt −/− extracts did not increase with increased protein as it does for authentic MTases (data not shown). However, we cannot exclude the existence of another, separately encoded O6-MeG DNA MTase.

Our results demonstrate that Mgmt plays an important role in the sensitivity of murine hematopoietic tissues to chemotherapeutic alkylating agents, consistent with the results of Sakumi et al. (1997) using MNU, a simple methylating agent that is not used clinically. For both Mgmt +/+ and −/− mice, ablation of bone marrow hematopoietic tissue was the most consistent pathology induced by the alkylating agents tested, albeit at lower doses for the Mgmt −/− mice. Thus, even though the level of O6-MeG DNA MTase in wild-type...
Fig. 5. The clonal survival of bone marrow cells isolated from Mgmt wild-type (+/+; ○) or null (−/−; □) mice. Cells were treated with (A) BCNU, (B) MNU, (C) MMC, (D) STZ, and (E) temozolomide. A representative experiment is shown in each panel.

Fig. 6. Tissue section of sternebrae from (B) Mgmt wild-type (+/+; ) and (D) null (−/−; ) mice displaying atrophy and ablation of hematopoietic cells, respectively, following STZ treatment at the indicated doses. Sections from age-matched untreated (A) Mgmt wild-type (+/+; ) and (C) null (−/−; ) sternebrae are shown for comparison. Note the expanded sinusoids in (B) which reflect cell shrinkage and pyknosis and the nearly complete absence of basophilic staining hematopoietic tissues in (D). These findings should be compared with the filled hematopoietic spaces present in the control panels, (A) and (C). The absent tissue in (B) is replaced by expanded sinusoids and in (D) by expanded sinusoids and virtually empty hematopoietic compartments containing mature erythrocytes and serum proteins.
hematopoietic tissues is extremely low relative to other tissues, it nevertheless plays a crucial role in protecting against chemotherapeutic alkylating agents. It will be interesting to determine how Mgmt−/− mice transplanted with Mgmt+/+ bone marrow cells respond to these alkylating agents, and these experiments are underway.

In addition, we have shown that O6-MeG DNA MTase plays a critical role in determining the sensitivity of the whole animal to chemotherapeutic alkylating agents. These results could have important implications for gauging chemotherapeutic drug regimes, given that O6-MeG DNA MTase activity levels probably vary between patients. Indeed, human lymphocyte extracts revealed up to an ~10-fold inter-individual difference in O6-MeG DNA MTase activity (Waldstein et al., 1982a,b; Sagher et al., 1989), these activity levels may vary in response to the chemotherapy itself (Sagher et al., 1988). Such considerations take on added relevance given the demonstration here of the importance of Mgmt in protecting the organism as a whole against the lethal effects of chemotherapeutic alkylating agents.

In addition to MTase repair, base excision repair is now known to play an important role in protecting cells from the cytotoxic effects of CNU alkylating agents. The Saccharomyces cerevisiae MAG1-encoded 3-methyladenine DNA glycosylase protects both S.cerevisiae and Escherichia coli against the cytotoxic effects of N-(2-chloroethyl)-N-nitrosourea (Matijasevic et al., 1993). Moreover, the mouse 3-methyladenine DNA glycosylase protects murine ES cells against BCNU (Engelward et al., 1996), although it does not appear to be required for alkyllation resistance in MEFs (Elder et al., 1998). Such protection may be achieved by the glycosylases preventing the formation of interstrand DNA crosslinks via removal of the crosslink precursor 1,6-ethanoguanine (formed by an intramolecular condensation of the 6-chloroethylnitrosourea lesion, the MTase substrate; Ludlum, 1997). The relative importance of DNA MTase and 3-methyladenine DNA glycosylase in providing CNU resistance remains to be determined and we are currently generating mice deficient in both activities to investigate this issue.

There now exist a large number of DNA repair-deficient mice generated by targeted homologous recombination and this number is growing rapidly (Friedberg et al., 1997, 1998). Mice deficient in base and nucleotide excision repair, mismatch repair, and recombinational repair already exist and each of these pathways has been implicated in the repair of DNA crosslinks (Siebert and Eisenbrand, 1977; Abril et al., 1996; Aquilina et al., 1998; Chen et al., 1998). Together with the O6-MeG DNA MTase-deficient mouse strain described here, these repair-deficient mouse strains should allow us to determine the relative importance of each repair pathway in protecting cells against chemotherapeutic alkylating agents, information which should prove useful in guiding the clinical use of such agents.

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References


