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Reciprocal Relationship between *O*⁶-Methylguanine-DNA Methyltransferase P140K Expression Level and Chemoprotection of Hematopoietic Stem Cells

Michael D. Milsom¹, Moran Jerabek-Willemsen^{1,2,3}, Chad E. Harris¹, Axel Schambach⁴, Emily Broun¹, Jeff Bailey¹, Michael Jansen¹, David Schleimer¹, Kalpana Nattamai¹, Jamie Wilhelm¹, Amanda Watson⁵, Hartmut Geiger¹, Geoffrey P. Margison⁵, Thomas Moritz^{1,2}, Christopher Baum^{1,4}, Jürgen Thomale³, and David A. Williams^{1,6}

¹Division of Experimental Hematology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

²Internal Medicine (Cancer Research), University of Duisberg-Essen, Essen, Germany

³Institute of Cell Biology, University of Duisberg-Essen, Essen, Germany

⁴Department of Experimental Hematology, Hannover Medical School, Hannover, Germany

⁵Carcinogenesis Group, Cancer Research UK Paterson Institute for Cancer Research, Manchester, United Kingdom

⁶Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts

Abstract

Retroviral-mediated delivery of the P140K mutant *O*⁶-methylguanine-DNA methyltransferase (MGMT^{P140K}) into hematopoietic stem cells (HSC) has been proposed as a means to protect against dose-limiting myelosuppressive toxicity ensuing from chemotherapy combining *O*⁶-alkylating agents (e.g., temozolomide) with pseudosubstrate inhibitors (such as *O*⁶-benzylguanine) of endogenous MGMT. Because detoxification of *O*⁶-alkylguanine adducts by MGMT is stoichiometric, it has been suggested that higher levels of MGMT will afford better protection to gene-modified HSC. However, accomplishing this goal would potentially be in conflict with current efforts in the gene therapy field, which aim to incorporate weaker enhancer elements to avoid insertional mutagenesis. Using a panel of self-inactivating gamma-retroviral vectors that express a range of MGMT^{P140K} activity, we show that MGMT^{P140K} expression by weaker cellular promoter/enhancers is sufficient for *in vivo* protection/selection following treatment with *O*⁶-benzylguanine/temozolomide. Conversely, the highest level of MGMT^{P140K} activity did not promote efficient *in vivo* protection despite mediating detoxification of *O*⁶-alkylguanine adducts. Moreover, very high expression of MGMT^{P140K} was associated with a competitive repopulation

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Requests for reprints: David A. Williams, Children's Hospital Boston, 300 Longwood Avenue, Karp 07212, Boston, MA 02115. Phone: 617-919-2697; Fax: 617-730-0934; DAWilliams@childrens.harvard.edu.

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defect in HSC. Mechanistically, we show a defect in cellular proliferation associated with elevated expression of MGMT^{P140K}, but not wild-type MGMT. This proliferation defect correlated with increased localization of MGMT^{P140K} to the nucleus/chromatin. These data show that very high expression of MGMT^{P140K} has a deleterious effect on cellular proliferation, engraftment, and chemoprotection. These studies have direct translational relevance to ongoing clinical gene therapy studies using MGMT^{P140K}, whereas the novel mechanistic findings are relevant to the basic understanding of DNA repair by MGMT.

Introduction

Retroviral-mediated transfer of drug resistance genes into hematopoietic stem cells (HSC) has been investigated for its potential to achieve the dual aim of preventing severe dose-limiting toxicity of cancer chemotherapy and enrichment of gene-modified cells *in vivo* (reviewed in ref. 1). Of several drug resistance genes proposed for this application, one of the best characterized is *O*⁶-methylguanine-DNA methyltransferase (*MGMT*; ref. 2). MGMT confers protection against clinically relevant *O*⁶-alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; Carmustine) and temozolomide (Temodar), which are used in the treatment of a variety of tumors, predominantly brain tumors (3, 4).

Hematotoxicity is dose limiting in many treatment protocols using *O*⁶-alkylating agents (5, 6), likely due to low expression of MGMT in the HSC and progenitor (P) compartment (7). In addition, elevated expression of MGMT has been found to be a mechanism of drug resistance in tumor cells, particularly glial tumors, which have poor prognosis (8, 9). To circumvent tumor resistance, pseudosubstrate inhibitors of endogenous MGMT, such as *O*⁶-benzylguanine and *O*⁶-(4-bromophenyl)guanine (lomeguatrib), have been developed. These agents ablate MGMT activity and sensitize tumors to the cytotoxic effects of *O*⁶-alkylating agents (10, 11). However, in human clinical trials the combination of *O*⁶-benzylguanine or lomeguatrib with temozolomide or BCNU has been shown to exacerbate myelosuppression (12–14). This increased toxicity likely relates to the depletion of MGMT activity in bone marrow HSC/P.

Several mutant versions of MGMT are resistant to such pseudosubstrate inhibitors, yet retain alkyltransferase activity. Of these, the P140K mutant (MGMT^{P140K}) seems to be optimal, combining resistance to pseudosubstrate inhibitors with efficient repair of cytotoxic *O*⁶-alkylguanine adducts (15–18). Thus, one goal of current studies is to achieve transgenic expression of MGMT^{P140K} in bone marrow HSC/P. Coupled with *O*⁶-alkylating agent treatment and *O*⁶-benzylguanine depletion, tumor cells will be sensitized while simultaneously protecting the bone marrow compartment to effect a widened therapeutic window. Clinical studies are currently under way using gamma-retrovirus vectors expressing MGMT^{P140K} in patients with poor-prognosis brain tumors (19). In addition, we and others have shown that treatment of mice and dogs that have been transplanted with HSC transduced with *O*⁶-benzylguanine-resistant MGMT vectors allows significant *in vivo* selection of transduced cells (16, 20–23).

The design of safer retroviral vectors has been an area of intense interest in the field of gene therapy since the occurrence of leukemia due to vector insertional activation of the LIM-

only protein 2 (*LMO2*) proto-oncogene in the gene therapy trial for X-linked severe combined immunodeficiency (24). Further observations from clinical trials, as well as numerous *in vitro* and *in vivo* assays, have shown that the powerful enhancer elements present in the long terminal repeats (LTR) of gamma-retroviral vectors can readily alter cell fate by insertional up-regulation of genes that confer a growth advantage (reviewed in ref. 25). One potential solution to this side effect of retroviral insertion is to use internal promoter elements in the context of deletions of the 3' LTR viral enhancer, so-called "self-inactivating" or SIN vectors. These modified vectors have recently been shown to have attenuated ability to activate a variety of growth-promoting genes in an *in vitro* immortalization assay (26). In addition, by coupling the use of a SIN vector backbone with an internal promoter, which has reduced enhancer activity compared with the gamma-retroviral LTR, it is possible to further reduce the risk of transformation via insertional transactivation (27).

The application of this approach to the design of vectors with which to express MGMT^{P140K} creates a conundrum because the detoxification of *O*⁶-alkylguanine adducts by MGMT is a stoichiometric process. Thus, whereas using a weaker enhancer/promoter element to drive expression of MGMT^{P140K} may decrease the risk of insertional mutagenesis, the success of such vectors in chemoprotection and selection may be diminished. In addition, theoretically the risk of cell transformation may be increased due to increased frequency of unresolved *O*⁶-alkyl adducts related to the lower levels of MGMT^{P140K} protein.

In this study, we used a panel of SIN gamma-retroviral vectors that express a range of MGMT^{P140K} activities to establish the level of MGMT expression that is optimal for selection/protection of HSC/P. Importantly, the promoter/enhancer elements used in these vector constructs have differing potential to promote insertional mutagenesis (27). We show that the lower-level expression of MGMT^{P140K} driven by weaker cellular promoters is sufficient for selection of HSC following *O*⁶-benzylguanine/temozolomide treatment and detoxifies *O*⁶-methylguanine lesions to the level of untreated controls. Surprisingly, we also found a previously undescribed deleterious effect of very high overexpression of MGMT^{P140K} on HSC reconstitution *in vivo* and cell proliferation *in vitro*. These studies may further elucidate the function(s) of MGMT protein and have important implications for the use of MGMT^{P140K} vectors in clinical trials.

Materials and Methods

Additional details of methods are provided in Supplementary data 1.

Mice

All animals were maintained in a specific pathogen-free environment and all experiments were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Research Foundation. Both C57BL/6J (referred to as C57B6 hereafter) and B6.SJL-Ptprc^a Pepc^b/BoyJ mice were obtained from The Jackson Laboratory. C57BL/6J pUR288(lacZ)-transgenic mice (referred to as lacZ^{TG}) have previously been described (28).

Generation of viral vectors

SIN gamma-retroviral vectors used in this study have previously been described (29). A cassette consisting of the encephalomyocarditis virus internal ribosome entry site, followed by either enhanced green fluorescent protein (eGFP) or Venus, was inserted directly 3' of the MGM^{P140K} cDNA (Fig. 1A). Viral supernatants were generated as previously described (30). Vector titers were in the range of 8.0×10^5 to 8.4×10^6 IU/mL.

Transduction of 32D cells and primary murine bone marrow cells

Transduction of 5-fluorouracil-pretreated primary murine bone marrow cells was done as described in ref. 30. The volume of viral supernatant used for transduction was adjusted relative to the established titer to achieve a transduction frequency that was similar across all transductions (typically in the range of 19–34%). Thirty hours after the final exposure to viral particles, transduced bone marrow cells were isolated by flow sorting (FACS Vantage, Becton Dickinson). The transduction of 32D cells was done essentially as for bone marrow cells with the exception that cells received a single round of transduction and were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and 10 ng/mL murine interleukin-3 (mIL3; Peprotech).

Transplant and analysis of chimerism

Bone marrow was injected into the tail vein of lethally irradiated recipient mice [11.75 Gy, 56 cGy/min, ¹³⁵Cs source Mark I Model 68A Irradiator (J.L. Shepherd and Associates), split dose]. Peripheral blood cell counts were obtained using a Hemavet 850 FS Hematology Analyzer (Drew Scientific). Following red cell lysis (Pharm Lyse, Becton Dickinson), cells were either directly assessed for fluorescence (FACS Canto, Becton Dickinson) or stained with fluorescently labeled monoclonal antibodies [antimouse CD3ε (145-2C11), B220 (RA3-6B2), TER-119, GR1 (RB6-8C5), MAC1 (M1/70), CD5 (53-7.3), and CD8a (53-6.7); all from E-Bioscience] following the manufacturer's instructions, before flow analysis. Viable cells were identified by exclusion of 7-amino-actinomycin D.

Drug treatment

*O*⁶-Benzylguanine (Sigma) was dissolved at 7.5 mg/mL, using sonication for 1 h at 4°C in polyethylene glycol 400 (Electron Microscopy Sciences). The stock was further diluted to 2.5 mg/mL using ice-cold PBS and then injected i.p. into mice at 30 mg/kg. Temozolomide (Chemodex) was dissolved at 40 mg/mL in DMSO and was diluted to 8 mg/mL in PBS and placed on ice and immediately injected i.p. into mice at 80 mg/kg. Temozolomide was administered 2 h after *O*⁶-benzylguanine, and this schedule was repeated on 2 consecutive days.

Analysis of apoptosis and cell cycle

Cell cycle analysis was done using the Becton Dickinson APC bromodeoxyuridine (BrdUrd) Flow Labeling Kit according to the manufacturer's instructions. 32D cells were starved for 16 h in IMDM, 1% FCS and were then incubated in media with 10% FCS, 10 ng/mL mIL3 at 37°C for the time periods indicated. 32D cells were then labeled with BrdUrd for 20 min at 37°C before fixation.

Quantification of active MGMT

Quantification of MGMT activity was done as described by Watson and Margison (31).

Immunoblots

The lysate from 5×10^5 cells was subjected to immunoblot as described by Gu and colleagues (32). The following primary antibodies were incubated with the membranes at the indicated dilutions: rabbit anti-p21 (C-19; Santa Cruz; 1:1,000); rabbit polyclonal anti-p27 (Cell Signaling Technology; 1:1,000); mouse anti-cyclin D1 (DCS6; Cell Signaling Technology; 1:200); mouse anti-cyclin E (E-4; Santa Cruz; 1:500); mouse anti- β -actin (AC-15; Sigma; 1:5,000); mouse anti-MGMT (MT3.1; Millipore; 1:1,000); and rabbit polyclonal anti-phospho-RB (Ser⁷⁸⁰) (Cell Signalling Technology; 1:1,000). The membranes were then washed thrice and incubated with the appropriate secondary antibody [antirabbit IgG-horseradish peroxidase (HRP) conjugate or antimouse IgG-HRP conjugate, both from Cell Signaling Technologies, diluted 1:2,500]. HRP was visualized using either Lumiglo (Cell Signaling Technologies) or Supersignal West Femto (Pierce). The separation of soluble proteins from chromatin- and nuclear matrix-bound proteins before immunoblot was done as described (33).

Immunocytologic assay for DNA adducts and Comet assay

Lineage-negative (lin^-) bone marrow cells were isolated from mice 2 h after treatment with *O*⁶-benzylguanine and temozolomide as described above. Immunocytologic assay for DNA alkylation products by adduct-specific antibodies was done as described (34) using either mouse anti-(*O*⁶-methylguanine) monoclonal antibody EM2-3 (35) or rabbit anti-imidazole-ring opened (*N*⁷-methylguanine) serum (kindly provided by Dr. A Povey, University of Manchester, Manchester, United Kingdom). The alkaline comet assay was done on sorted lin^- bone marrow cells as described (36). Quantification of fluorescence signals relating to DNA adducts and DNA strand breaks was also as described (36).

Immunofluorescence intracellular staining for MGMT

3T3 cells were grown on glass coverslips in 12-well non-tissue-culture-coated dishes (Becton Dickinson). Fixation and permeabilization were done using the Becton Dickinson Cytofix/Cytoperm Fixation/Permeabilization Solution Kit according to the manufacturer's instructions. The samples were then incubated with mouse anti-MGMT (MT3.1, Millipore) diluted 1:100 in Permeabilization/Wash solution for 1 hour at room temperature. After washing, samples were incubated in Rhodamine-conjugated donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories) at 37°C for 45 min. After washing with Permeabilization/Wash solution, samples were mounted using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI). Images were obtained on a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Inc.) and images were collected with a Hamamatsu camera using Openlab software (Improvision).

Mutation frequency analysis

Mutation frequency was determined in lacZ^{TG} bone marrow as described (37).

Statistical analysis

All quoted errors are SEs of the mean unless otherwise stated. The Student *t* test or Wilcoxon rank-sum test was used to determine statistical significance dependent on whether all data conformed to a normal distribution as defined by the Shapiro-Wilk test.

Results

Bicistronic self-inactivating gamma-retrovirus vectors express a wide range of MGMT activity in addition to a marker fluorochrome

The SIN gamma-retroviral backbones used in this study have previously been shown to express a range of MGMT^{P140K} activity in primary hematopoietic cells and 32D cell lines (29, 38). To facilitate sorting of transduced cells and increase the ease of their detection by flow cytometry, the cDNAs encoding either eGFP or Venus were subcloned into these existing vectors directly 3' of the encephalomyocarditis virus internal ribosome entry site yielding SF-MGMT, EFS-MGMT, and phosphoglycerate kinase (PGK)-MGMT (Fig. 1A). A control vector expressing eGFP, but not MGMT (SF-IG), was also used. Primary hematopoietic cells and cell lines transduced with these modified vectors were readily detectable by flow cytometry and mean fluorescence intensity was directly related to the strength of enhancer present in the vector (Supplementary data 2). Furthermore, transduced primary bone marrow expressed a range of MGMT activity, which spanned ~3 orders of magnitude (MGMT activities of $2,091 \pm 105$, 290 ± 5 , 450 ± 53 , and <3 fmol/ μ g DNA for SF-MGMT–, EFS-MGMT–, PGK-MGMT–, and SF-IG–transduced bone marrow, respectively).

Inverse correlation between MGMT expression and chemoprotection and HSC selection *in vivo*

To evaluate the degree of protection against O⁶-alkylating agents conferred by each SIN gamma-retroviral vector *in vivo*, we conducted competitive engraftment studies using bone marrow that was separately transduced with the panel of vectors shown in Fig. 1A. Transduced and sorted bone marrow was i.v. injected into lethally irradiated recipient mice (4×10^5 per mouse) along with 5×10^5 freshly isolated nontransduced bone marrow. At 7 weeks posttransplant, mice were treated with 30 mg/kg O⁶-benzylguanine and, 2 hours later, 80 mg/kg temozolomide for 3 consecutive days. The percentage of gene-marked cells in the peripheral blood and the peripheral blood leukocyte count were subsequently monitored for 4 months following treatment. In spite of several previous reports of successful selection using MGMT^{P140K} vectors, mice transplanted with cells transduced with the SF-MGMT vector showed no evidence of *in vivo* selection (Fig. 1B; Supplementary data 3). In contrast, robust long-term *in vivo* selection was shown in mice transplanted with both PGK-MGMT– and EFS-MGMT–transduced bone marrow. Surprisingly, these data show that the highest level of MGMT^{P140K} expression was not associated with chemoselection whereas lower levels of expression were sufficient to elicit a survival advantage over nontransduced bone marrow HSC/P cells. As expected, there was no apparent selection of engrafted cells expressing GFP only (SF-IG) compared with the nontreated group.

These results paralleled the protection from peripheral blood leukopenia affected by these same vectors. By 10 weeks posttreatment with *O*⁶-benzylguanine/temozolomide, the peripheral leukocyte counts of animals engrafted with SF-IG- or SF-MGMT-transduced cells had not recovered to the range of nontreated controls (Table 1). In contrast, mice transplanted with EFS-MGMT- or PGK-MGMT-transduced cells showed recovery of peripheral blood leukocyte counts to control levels by a maximum of 5 weeks posttreatment, despite the graft consisting of a mixed chimera of protected and nonprotected cells. In addition, the magnitude of early leukopenia observed at 2 weeks posttreatment was less severe in the PGK-MGMT and EFS-MGMT groups compared with the SF-IG or SF-MGMT group (Table 1). These data show that in spite of very high levels of expression of MGMT^{P140K}, animals transplanted with cells transduced with SF-MGMT showed less protection from chemotherapy-induced pancytopenia than animals transplanted with vectors expressing lower levels of MGMT^{P140K}.

Despite the lesser chemoprotection shown by engraftment of transduced cells and blood cytopenias, mice transplanted with SF-MGMT-transduced cells still showed significantly higher overall survival compared with SF-IG-transduced cells, as did mice receiving cells transduced with either PGK-MGMT or EFS-MGMT (Fig. 1C). Mice receiving cells transduced with PGK-MGMT or EFS-MGMT showed a trend toward higher overall survival compared with SF-MGMT mice, although this difference did not reach statistical differences (Fig. 1C). These data confirm that expression of MGMT^{P140K} provides a survival advantage as previously reported by us and others (16, 20–23).

Bone marrow cells transduced with SF-MGMT detoxify *O*⁶-alkyl adducts *in vivo*

Given the inferior *in vivo* selection and chemoprotection conferred by the SF-MGMT vector, we sought to verify that this construct was able to mediate the detoxification of cytotoxic *O*⁶-alkylguanine adducts. Bone marrow was harvested 2 hours posttreatment with *O*⁶-benzylguanine/temozolomide from transplanted mice as described above. Lin[−] GFP⁺/Venus⁺ bone marrow cells were isolated and *O*⁶-alkylguanine adducts were quantified by immunocytologic assay. Unexpectedly, the SF-MGMT-expressing cells showed equivalent repair of *O*⁶-alkylguanine adducts comparable to cells expressing lower levels of MGMT^{P140K} via PGK-MGMT and EFS-MGMT vectors. Nonprotected SF-IG-transduced cells harbored ~10-fold higher levels of residual lesions (Fig. 2A). As expected, due to the specificity of MGMT for *O*⁶-adducted guanines, the frequency of *N*⁷-alkylguanine lesions and of excision repair-induced DNA single-strand breaks as assessed by alkaline comet assay were equivalent across all groups (data not shown). These data show that all groups received a comparable biological dose of temozolomide and suggest that the defect in chemoprotection and selection of SF-MGMT-transduced cells is not due to defective *O*⁶-alkylguanine adduct repair or to an altered processing of other DNA alkylation products.

To more precisely assess the detoxification of *O*⁶-alkylguanine adducts in long-lived HSC/P, we analyzed the mutation frequency in transduced bone marrow harvested from mice 6 months post-treatment. The mutation frequency in all MGMT^{P140K}-expressing groups, regardless of expression level, was in the range of nontreated transduced control groups (Fig. 2B). In contrast, the mutation frequency of bone marrow in mice transplanted with SF-

IG was in excess of that observed for MGMT^{P140K} vector and control groups, supporting the interpretation that all groups engrafted with bone marrow transduced with MGMT^{P140K}-expressing vectors detoxified the potentially mutagenic *O*⁶-alkylguanine lesions to an equivalent level. These data suggest that the inferior chemoprotection and selection shown in mice transplanted with bone marrow transduced with SF-MGMT are not due to a defective repair of DNA damage but suggest a defect in HSC function.

SF-MGMT–transduced bone marrow shows a competitive engraftment defect in the absence of chemoselection

We next sought to analyze whether very high levels expression of MGMT^{P140K} had a detrimental effect on engraftment of gene-modified HSC in the absence of treatment with temozolomide. To do this, we directly compared engraftment of bone marrow transduced with different vectors expressing MGMT^{P140K} from different promoters (Fig. 1A) within the same recipient mouse. Bone marrow was separately transduced with vectors encoding MGMT^{P140K} from different promoters and coexpressing either eGFP or Venus. Transduced bone marrow cells were isolated by flow sorting and equivalent numbers of eGFP⁺ or Venus⁺ cells were coinjected into lethally irradiated recipient mice along with 5×10^5 freshly isolated nontransduced competitor cells (Fig. 3A). The percentage contribution of each transduced population to the peripheral blood of recipient mice was analyzed at 6 months posttransplant. At the time of sacrifice and analysis, 25% of a femur equivalent of bone marrow from these mice was also transplanted into lethally irradiated secondary recipients to definitively assess HSC activity in each transduced population. The percentage of transduced cells in the peripheral blood of secondary recipients was evaluated at 15 weeks posttransplant.

SF-MGMT–transduced bone marrow showed a significant repopulation disadvantage compared with either EFS-MGMT– or PGK-MGMT–transduced bone marrow (Fig. 3B). The difference in contribution compared with PGK-MGMT was significant in both primary and secondary recipients, whereas the difference in contribution compared with EFS-MGMT reached significance in secondary transplant recipients. There was no significant difference in the contribution of SF-MGMT GFP⁺ versus SF-MGMT Venus⁺ transduced bone marrow in either primary or secondary recipient mice. These data confirm that there was no engraftment bias dependent on the fluorescent protein used to evaluate the peripheral blood marking. Thus, in the absence of chemoselection, very high expression levels of MGMT^{P140K} generated via the SF-MGMT vector are associated with reduced HSC activity as measured in competitive repopulation assays.

32D cells transduced with SF-MGMT show a growth defect

To better identify the potential mechanism(s) by which very high levels of MGMT^{P140K} have a detrimental effect on HSC engraftment, we sought to determine if MGMT^{P140K} expression was detrimental to the growth of the 32D myeloid progenitor cell line. 32D cells were separately transduced with the SF-MGMT, EFS-MGMT, and PGK-MGMT vectors and the percentage of gene-marked cells was followed with time in culture. As seen in primary bone marrow, expression of MGMT was highest in 32D cells transduced with SF-MGMT and considerably lower in cells transduced with PGK-MGMT and EFS-MGMT

(MGMT activities of $1,532 \pm 147$, 371 ± 14 , 185 ± 7 , and <3 fmol/ μ g DNA for SF-MGMT⁻, EFS-MGMT⁻, PGK-MGMT⁻, and SF-IG⁻-transduced 32D cells, respectively). In the absence of drug treatment, SF-MGMT⁻-transduced 32D cells showed a pronounced growth defect resulting in an $\sim 50\%$ reduction in the frequency of transduced cells over 3 weeks of culture (Fig. 4A). In contrast, there was no significant change in the percentage of gene-marked cells in cultures containing SF-IG⁻, EFS-MGMT⁻, or PGK-MGMT⁻-transduced cells, indicating that low levels of MGMT^{P140K} expression did not alter cell growth in culture relative to the nontransduced 32D cells. The growth defect exhibited by SF-MGMT⁻-transduced 32D cells was confirmed at the clonal level, as there was a $\sim 40\%$ reduction in colony formation of these cells compared with 32D cells expressing only eGFP (Supplementary data 4). Finally, there was a $>30\%$ reduction in tritiated thymidine uptake by SF-MGMT⁻-transduced 32D cells compared with control groups (Supplementary data 4).

SF-MGMT growth inhibition in 32D cells is specific to the P140K mutant, which is mislocalized in transduced cells

To determine whether the reduced proliferation of SF-MGMT⁻-transduced 32D cells was specific for the P140K mutation, we transduced 32D cells with an identical vector backbone expressing wild-type (WT) MGMT. Surprisingly, there was no reduction in the growth of 32D cells expressing WT MGMT, suggesting that the growth defect seen in these cells was specific to high-level expression of MGMT^{P140K} and not related to MGMT repair activity per se (Fig. 4A). The lack of growth defect in cells expressing WT MGMT was not due to lower expression levels of MGMT because we also observed normal growth in 32D cells transduced with a 4-fold higher multiplicity of infection of WT MGMT, resulting in ~ 2 -fold increase in levels of MGMT relative to MGMT^{P140K}-transduced cells (MGMT activities of 1,230 and 2,330 fmol/ μ g DNA for P140K- and WT MGMT⁻-transduced cells, respectively).

To determine if the MGMT^{P140K} mutant protein expression mimicked WT protein with respect to intracellular distribution, we next examined the localization of protein in transduced cells. To provide better visualization of protein localization, we used NIH 3T3 fibroblasts and examined fluorescence distribution. As shown in Fig. 4B, WT MGMT was expressed in both the nucleus and the cytoplasm. As a control, the non-DNA-binding mutant MGMT^{Y114E} (39) was examined and seemed to be predominantly cytoplasmic, suggesting that DNA binding is required for nuclear retention. In marked contrast to WT protein, MGMT^{P140K} was almost exclusively localized to the nucleus (Fig. 4B; Supplementary data 5). MGMT^{P140K} expressed at lower levels via the PGK-MGMT and EFS-MGMT vectors was also restricted predominantly to the nucleus, suggesting that the restricted localization was independent of expression level. Additionally, the largely nuclear localization of MGMT^{P140K} was not observed when a range of other MGMT mutants were used (Supplementary data 5).

The lack of cytoplasmic MGMT^{P140K} expression compared with other MGMT mutants was also observed in transduced 32D cell lines, but localization in these cells was more difficult to discern due to the low ratio of cytoplasmic/nuclear volume (data not shown). Therefore, to examine these cells, transduced 32D cells were fractionated by differential lysis of nuclear and cytoplasmic components and analyzed by immunoblot. MGMT^{P140K} expression

was largely restricted to the chromatin-bound fraction (Fig. 4C). In contrast, WT MGMT was abundant in both the chromatin-bound fraction and the cytoplasmic fraction, whereas the MGMT^{Y114E} mutant was expressed primarily in the cytoplasmic fraction. Overall, these data show that the defect in growth of 32D cells overexpressing MGMT is specific to MGMT^{P140K}, and in multiple cell types MGMT^{P140K} shows a more restricted localization to the nucleus, correlating with elevated association with chromatin. This restriction may be associated with DNA binding as the Y114E mutant is predominantly cytoplasmic and does not associate with chromatin.

SF-MGMT expression in 32D cells interferes with cell cycle progression

To determine the basis of the impaired growth of 32D cells transduced with SF-MGMT, cell cycle analysis was done 12 and 24 hours following release from serum starvation. SF-MGMT-transduced 32D cells showed a pronounced delay in the G₀/G₁ to S phase transition compared with SF-IG-, PGK-MGMT-, and EFS-MGMT-transduced cells (Fig. 5A). This delay in cell cycle was associated with a 1.4- to 1.6-fold reduction in SF-MGMT-transduced cells in S phase at 12 hours postinduction, compared with SF-IG-, PGK-MGMT-, and EFS-MGMT-transduced cells, and a 1.2-fold reduction at 24 hours postinduction.

To further explore this cell cycle progression defect at the biochemical level, the expression of cell cycle regulatory proteins was determined. Immunoblot analysis of cell lysates isolated at 6 hours postinduction with serum revealed a demonstrable elevation of p27 in SF-MGMT-transduced 32D cells compared with 32D cells transduced with SF-IG, PGK-MGMT, and EFS-MGMT (Fig. 5B), whereas at this time point postinduction, there was no appreciable difference in the level of p21, cyclin D1, phospho-RB, or cyclin E (data not shown). The presence of elevated p27 confirmed the perturbed G₀/G₁ to S phase transition identified at the level of DNA synthesis and suggests that p27 may be one of the effectors of the MGMT-mediated proliferation defect. We detected no difference in the rate of apoptosis of SF-MGMT-transduced 32D cells relative to control groups, even following prolonged withdrawal of growth factors (Supplementary data 4). Thus, the observed growth defect in 32D cells expressing very high levels of MGMT^{P140K} seems to result from a perturbation of cell cycle progression.

Discussion

The optimal use of MGMT^{P140K} in chemoprotection and HSC selection may require conflicting levels of expression. On the one hand, achieving higher levels of cellular expression should confer more protection against damage induced by the O⁶-alkylating agent because MGMT is not an enzyme and is degraded after acceptance of the alkyl group from the damaged DNA. Conversely, in the context of integrating vectors, high levels of expression are generally reached using strong enhancer elements, which increase the risk of insertional up-regulation of deleterious genes near the proviral insertion site.

In this study, we show that weaker human cellular promoters located internal to the viral LTRs are sufficient to drive the expression of MGMT^{P140K} in HSC/P at levels that efficiently repair O⁶-alkylguanine DNA adducts. This repair leads to protection and *in vivo* selection of gene-modified HSC after combined pharmacologic inhibition of WT MGMT

and temozolomide treatment. Although the EFS and PGK promoters mediate low-level MGMT^{P140K} expression relative to vectors that incorporate the viral SF promoter, the level of exogenous MGMT activity encoded by these vectors is still ~2 orders of magnitude higher than endogenous MGMT activity in hematopoietic cells. Because vectors that incorporate weaker enhancer elements are thought to reduce the risk of insertional mutagenesis, these data suggest that the combination of the EFS or PGK promoter with the MGMT^{P140K} cDNA in a SIN retroviral backbone makes up a superior vector configuration to develop and test in the next generation of clinical trials.

Very high levels of MGMT^{P140K} precluded efficient selection and protection of gene-modified HSC/P, an unexpected finding given the many previous reports of successful use of this cDNA. This finding seems to be unrelated to the efficiency of repair of the O⁶-methylguanine lesion. In support of this finding, we define an engraftment defect resulting from very high levels of MGMT^{P140K} expression. The observed defect in cellular proliferation seems to be specific to the overexpression of the P140K mutant of MGMT. We surmise that very high levels of MGMT^{P140K} may result in defective *in vivo* HSC/P protection and selection due to a proliferation defect analogous to the one we define in 32D cells.

In addition, MGMT^{P140K} protein shows an unusual localization to the nucleus/chromatin relative to WT MGMT. This mislocalization of protein is apparently related to DNA binding. The increased propensity of MGMT^{P140K} protein to bind to chromatin could be responsible for the observed proliferation defect. However, if this is the case, there is a threshold for this effect because vectors using weaker cellular promoters to drive expression of MGMT^{P140K} result in intracellular levels of protein that do not elicit a perceptible proliferation defect but show similar protein distribution abnormalities. An excess of mislocalized protein could be deleterious to several nuclear processes (e.g., by inhibiting the synthesis of DNA and/or RNA or interfering with gene transcription or replication functions). This hypothesis would correlate with the observation that MGMT localizes to the sites of active transcription in a cell cycle-specific manner in the absence of treatment with an O⁶-alkylating agent (40). These data also imply that retroviral vectors containing weak internal promoters in the SIN configuration to express MGMT^{P140K} may be superior in the context of a clinical trial of chemoprotection. This conclusion is in contrast to conventional predications, which would maintain that the achievement of optimal chemoprotection of HSC/P would require higher levels of MGMT expression.

In the setting of numerous previous studies that have documented effective *in vivo* protection and selection of HSC using retroviral overexpression of MGMT^{P140K} (16, 18, 21–23, 41), the data presented here showing that very high levels of this protein result in a deleterious effect on HSC engraftment seem to be unique. The experimental parameters leading to this observation likely include the level of expression of MGMT^{P140K}, which was achieved using the combination of the spleen focus-forming virus (SFFV) promoter and the Pre element within this retroviral construct. Using 32D cells, we observed that the SF-MGMT vector mediated a ~10-fold higher level of MGMT activity compared with a monocistronic MSCV vector encoding MGMT^{P140K} coupled with a Pre (data not shown), which is currently being used in clinical trials (19). Indeed, a 28-fold differential of

transgene expression level has previously been documented between a gamma-retroviral vector incorporating the SFFV enhancer coupled with the Pre, versus the weaker MSCV-based backbone (42). Thus, the level of expression of MGMT^{P140K} mediated by the SF-MGMT vector used in this study likely exceeds that achieved by other vector configurations that have previously been reported.

With respect to the chemotherapeutic regimen used, we have attempted to model a high-dose myelosuppressive schedule administered soon after bone marrow transplant. This model may mimic schedules used in some clinical protocols designed for HSC chemoprotection for the purpose of increasing dose intensity for antitumor therapy. A similar treatment schedule could in the future be used to select for transduced HSC/P in autologous reinfusion and treatment of genetic diseases. This approach places a significant proliferative stress on reconstituting HSC/P, increasing the likelihood that a modest proliferation defect would be noted. In contrast, other published studies have used less severe chemotherapeutic regimens. Examples of these schedules include using a gradual dose escalation of temozolomide, delaying the administration of drug until after bone marrow reconstitution is complete, and/or using an extended schedule that allows more time for hematopoietic recovery between rounds of treatment (21–23, 41). Thus, these less dose-intense or time-compressed treatment schedules may mask any reconstitution defect of gene-modified HSC by reducing the proliferative stress on these cells.

Aside from the direct translational application of our findings to clinical trials using gene transfer of MGMT for HSC chemoprotection, these studies may also have importance for further understanding the basic biology of MGMT. For example, these studies show that MGMT^{P140K} may have a higher binding affinity for nonalkylated DNA. One might interpret this finding as a possible explanation for the slower kinetics of *O*⁶-alkylguanine detoxification by MGMT^{P140K} relative to WT protein (29) in that increased DNA binding affinity may correlate with a reduced rate of processive scanning of DNA. Furthermore, understanding the precise mechanism(s) through which very high levels of MGMT^{P140K} elicit an effect on cell cycle regulatory proteins may allow further insight into the coordination of DNA repair and cellular proliferation in HSC, where a large fraction of cells remain in a quiescent state for prolonged periods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Retrovirus vectors and *in vivo* selection/protection of transduced bone marrow cells. **A**, schematic representation of SIN-MGMT retroviral vectors. Vectors were derived by modification of Sin.SF, Sin.EFS, and Sin.PGK (29). Abbreviations: SF, enhancer/promoter from the SFFV LTR; EFS, truncated form of the promoter/enhancer from the human elongation factor 1 α gene; PGK, promoter/enhancer from the human phosphoglycerate kinase gene; MGMT, the human *O*⁶-methylguanine-DNA methyltransferase cDNA (P140K mutant unless otherwise stated); IRES, the internal ribosome entry site from encephalomyocarditis virus; eGFP, enhanced green fluorescent protein; Venus, modified version of yellow fluorescent protein; Pre, truncated version of the woodchuck hepatitis virus posttranscriptional regulatory element that lacks any X protein coding sequence; U3, deletion in the enhancer/promoter region of the retroviral LTR; Ψ , packaging signal. **B**, change in the percentage chimerism of gene-marked cells following treatment with *O*⁶-benzylguanine/temozolomide. Lethally irradiated recipient mice were injected as described in Materials and Methods. Mice from each cohort were then either treated or not with a *O*⁶-benzylguanine/temozolomide regimen at 7 wk posttransplant. The percentage of GFP⁺ cells in the peripheral blood at 15 wk posttreatment versus 1 wk pretreatment was used to determine the change in frequency of gene-marked cells with chemoselection. $\square$, nontreated

age-matched control mice; ■, *O*⁶-benzylguanine/temozolomide treatment. **, $P < 0.01$ (Wilcoxon's rank sum test); ns, not significant. Data represent the sum of three independent experiments with 11 to 23 mice per experimental group. C, Kaplan-Meier plot of survival of *O*⁶-benzylguanine/temozolomide-treated mice from each experimental group with time posttreatment. *Vertical arrows*, timing of *O*⁶-benzylguanine/temozolomide (6BG/TMZ) treatment. **, $P < 0.01$, versus other groups (log-rank test). Data represent the sum of three independent experiments with 13 to 23 mice per experimental group.

Figure 2.

Repair of temozolomide-induced DNA damage. *A*, residual levels of O^6 -methylguanine in the nuclear DNA of treated transduced cells. Mice were sacrificed 2 h posttreatment with O^6 -benzylguanine/temozolomide as described in Materials and Methods. Bone marrow was pooled from three mice within each experimental group and lin^- transduced cells were isolated by flow sorting. Isolated cells were then subjected to immunostaining for O^6 -methylguanine adducts as described in Materials and Methods. The mean arbitrary nuclear fluorescence units (AFU) of 100 cells are shown minus background. Representative of two experiments. □, SF-IG; ■, SF-MGMT; ▨, EFS-MGMT; ▩, PGK-MGMT. **, $P < 0.01$, versus all other groups (Student's t test). *B*, absolute mutation frequency in transduced bone marrow. At 6 mo posttreatment, the surviving mice were sacrificed. Bone marrow was harvested and LacZ reporter plasmids were subsequently rescued from the resulting genomic DNA as described in Materials and Methods. The mutation frequency in the rescued reporter plasmids was then determined. Each cross represents the mutation frequency determined for bone marrow from a single mouse. Dashed line, the upper range of mutation frequency in nontreated controls (CON).

Figure 3.

Competitive transplantation of MGMT vector-transduced cells. *A*, schematic representation of competitive repopulation assay used to directly compare the engraftment potential of bone marrow populations expressing different MGMT vectors. *B*, competitive repopulation of transduced bone marrow. Lethally irradiated recipient mice were injected with a mixture of sorted bone marrow transduced with the indicated vectors and freshly isolated bone marrow. At 6 mo posttransplant, the contribution of each gene-modified population to the production of peripheral blood leukocytes was analyzed by flow analysis. A portion of the harvested bone marrow was transplanted into lethally irradiated secondary recipient mice as described in Materials and Methods. At 15 wk posttransplant, the frequency of each gene-marked population in the peripheral blood of secondary recipients was determined by flow analysis. Percentage of SF-MGMT- (■), EFS-MGMT- (▨), or PGK-MGMT-transduced (▩) cells in the peripheral blood. *Columns*, mean of eight mice per group; *bars*, SD. **, $P < 0.01$; *, $P < 0.05$, versus the SF-MGMT GFP⁺ graft in the same recipients. SF-MGMT Venus⁺ transduced bone marrow is used to control for bias due to type of fluorochrome used.

Figure 4.

Very high expression of MGMT^{P140K} causes a proliferation defect in 32D cells and correlates with altered subcellular localization. **A**, 32D cells were transduced with the indicated retroviral vectors and the percentage of gene-modified cells in culture was analyzed with time in culture. ■, SF-MGMT; □, SF-IG; ▨, EFS-MGMT; ▩, PGK-MGMT; ▤, SF-MGMT vector containing WT cDNA. *Columns*, mean of three to nine independent experiments; *bars*, SD. **, $P < 0.01$, compared with other groups (Student's t test). **B**, NIH 3T3 fibroblast cells were transduced with the indicated retroviral vectors. Cells were fixed and stained with DAPI and anti-MGMT antibody. Photomicrographs of fixed and stained cells were at $\times 400$ magnification. *Bar*, 9.75 μm . **C**, 32D cells were transduced with SF-MGMT vectors containing the WT MGMT sequence, the P140K mutant, or the Y114E mutant as a non-DNA-binding control. Transduced cells were isolated by flow sorting and cells were lysed and cellular constituents separated as described in Materials and Methods before immunoblot with the indicated antibody. SC, cytoplasmic and soluble cytoplasmic fraction; SN, soluble nuclear fraction; C, chromatin/nuclear matrix-bound fraction. *Lanes 1 to 3*, P140K; *lanes 4 to 6*, WT; *lanes 7 to 9*, Y114E.

Figure 5.

Transduced 32D cells expressing high MGMT^{P140K} show aberrant cell cycle progression. *A*, sorted transduced 32D cells were isolated by flow sorting based on fluorescence and were serum starved for 16 h. Cells were then resuspended in IL3/10% serum-containing growth media. At 12 and 24 h postinduction, cells were pulse labeled with BrdUrd for 20 min and then fixed and stained. The proportion of cells in each phase of the cell cycle was determined by analysis of BrdUrd incorporation versus 7-amino-actinomycin D (7-AAD) staining. ■, SF-MGMT; □, SF-IG; ▨, EFS-MGMT; ▩, PGK-MGMT. *, $P < 0.05$; **, $P < 0.01$, compared with SF-MGMT-transduced group. *B*, lysate from transduced and sorted 32D cells described in *A* was prepared at 6 h postinduction. These lysates were then subject to immunoblot and probed with the indicated antibodies. Lysate from starved (S) nontransduced cells is shown as control to illustrate the level of p27 in noninduced cells.

Table 1
Peripheral blood leukocyte cell count posttreatment with *O*⁶-benzylguanine/temozolomide

Vector	6BG/TMZ	1 wk pretreatment	2 wk posttreatment	5 wk posttreatment	10 wk posttreatment	15 wk posttreatment
SF-IG	No	12.7 ± 0.8	13.8 ± 1.0	14.1 ± 0.8	15.0 ± 0.8	12.0 ± 0.7
SF-IG	Yes	12.4 ± 0.9	4.4 ± 0.5*	5.1 ± 0.3*	11.0 ± 2.7*	16.2 ± 5.4
SF-MGMT	No	11.7 ± 0.8	12.8 ± 0.9	13.4 ± 0.5	13.4 ± 0.8	12.5 ± 1.5
SF-MGMT	Yes	11.7 ± 0.5	6.9 ± 0.8*	7.8 ± 0.9*	9.6 ± 1.0 [†]	15.2 ± 4.8
EFS-MGMT	No	12.3 ± 0.8	12.0 ± 1.0	12.3 ± 0.4	13.9 ± 0.4	11.5 ± 0.5
EFS-MGMT	Yes	13.0 ± 0.7	7.9 ± 0.8*	11.4 ± 1.0	11.7 ± 1.0	14.1 ± 3.3
PGK-MGMT	No	12.4 ± 0.6	11.7 ± 1.1	13.7 ± 1.0	15.7 ± 1.0	10.7 ± 0.8
PGK-MGMT	Yes	12.2 ± 0.8	11.4 ± 0.7	12.5 ± 1.1	13.3 ± 0.8	10.5 ± 1.2

NOTE: The mean peripheral blood leukocyte cell counts ($\times 10^6/\text{mL}$) pretreatment with *O*⁶-benzylguanine/temozolomide and at various time points posttreatment are shown as mean $\pm$ SE. Nontreated control groups (*No 6BG/TMZ*) are also shown.

Abbreviations: 6BG, *O*⁶-benzylguanine; TMZ, temozolomide.

* $P < 0.01$, compared with nontreated control (Wilcoxon's rank sum test).

[†] $P < 0.05$, compared with nontreated control (Wilcoxon's rank sum test).