Polyclonal chemoprotection against temozolomide in a large-animal model of drug resistance gene therapy

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Incorporation of drug resistance genes into gene vectors has 2 important roles in stem cell gene therapy: increasing the proportion of gene-corrected cells in vivo (ie, in vivo selection) and marrow protection to permit higher or more tightly spaced doses of chemotherapy in the treatment of malignant diseases. We studied in a clinically relevant canine model of gene therapy the P140K mutant of the drug resistance gene methylguanine methyltransferase (MGMT), which encodes a DNA-repair enzyme that confers resistance to the combination of the MGMT inhibitor O6-benzylguanine (O6BG) and nitrosourea drugs such as carmustine and methylating agents such as temozolomide. Two dogs received MGMT(P140K)-transduced autologous CD34+ -selected cells. After stable engraftment, gene marking in granulocytes was between 3% and 16% in the 2 animals, respectively. Repeated administration of O6BG and temozolomide resulted in a multilineage increase in gene-modified repopulating cells with marking levels of greater than 98% in granulocytes. MGMT(P140K) overexpression prevented the substantial myelosuppression normally associated with this drug combination. Importantly, hematopoiesis remained polyclonal throughout the course of the study. Extrahematopoietic toxicity was minimal, and no signs of myelodysplasia or leukemia were detected. These large-animal data support the evaluation of MGMT(P140K) in conjunction with O6BG and temozolomide in clinical trials. (Blood. 2005;105:997-1002)

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Introduction

Gene transfer to hematopoietic stem cells holds significant promise for the treatment of genetic diseases, AIDS, and cancer. The use of drug resistance genes in stem cell gene therapy has 2 important clinical applications. First, drug selection of genetically corrected cells in vivo increases the proportion of corrected cells in gene therapy protocols for genetic diseases affecting the hematopoietic system. This strategy could circumvent the relatively low gene transfer levels obtained after nontoxic reduced-intensity conditioning regimens. Second, this strategy could also be applied to protect the bone marrow in the context of chemotherapy for solid tumors. Chemoprotection could permit dose-intensified cancer chemotherapy regimens, averting the dose-limiting myelosuppression normally associated with intensified chemotherapy regimens.1-4 Rescue with unmanipulated autologous stem cells has been widely used to this end, but this strategy allows for only a limited number of high-dose chemotherapy cycles and is still limited by short periods of significant myelosuppression in the period immediately after transplantation. In contrast, a genetically protected bone marrow could make possible tightly spaced dose-intensive chemotherapy regimens over extended time periods in the complete absence of any myelosuppression.

A particularly attractive drug resistance gene for use in gene therapy is the DNA-repair enzyme methylguanine methyltransferase (MGMT).3,4 Overexpression of this enzyme renders primary hematopoietic cells resistant to nitrosoureas such as carmustine (BCNU)3-6 and to methylating agents, such as temozolomide.7 Resistance to nitrosoureas in human cancer has been linked to elevated protein levels of endogenous MGMT.8 To overcome this documented resistance mechanism, inhibitors of endogenous MGMT, such as O6-benzylguanine (O6BG), are being evaluated in clinical trials.9,10 Importantly, survival and time to disease progression in glioma patients are favorably correlated to methylation and subsequent silencing of the endogenous MGMT promoter.11

The clinical use of O6BG and BCNU is limited by myelosuppression.12 Mutants of MGMT insensitive to the inhibitory action of O6BG have been developed for use in gene therapy.12,13 These have been shown to confer resistance to the drug combination of O6BG and BCNU15,6 or O6BG and temozolomide7 in vivo in mouse models of stem cell gene therapy.

Successful in vivo selection of genetically modified hematopoietic cells was first reported in murine models more than 10 years ago using multidrug resistance protein 1 (MDR-1) as a drug resistance gene.14 Later, in vivo selection in the murine model was also reported for other selectable markers, dihydrofolate reductase (DHFR)15 and MGMT.4,7 These murine studies have set the stage for large-animal experiments and clinical trials. For both MDR-1 and DHFR, evaluation in clinically more relevant large animals essentially documented less pronounced in vivo selection with unlikely clinical benefit or excessive toxicity.16-18 MDR-1 has been tested in clinical trials,19-21 and these trials confirm the data from large-animal models, showing moderate and transient in vivo selection at best. In contrast, successful in vivo selection...
and marrow protection, using the P140K mutant of MGMT [MGMT(P140K)] and the drug combination of O6BG and BCNU, has recently been demonstrated in a canine model.3 In this study, gene marking of virtually 100% was observed. Temozolomide is an oral methylating agent that has shown promise for the treatment of several malignancies,22 including gliomas,23-25 malignant melanoma,26 and acute leukemia.27 A particularly attractive feature of temozolomide is its favorable toxicity profile, with myelosuppression as the dose-limiting toxicity in most studies. Here, we used a clinically relevant canine model to determine whether genetic chemoprotection of hematopoietic stem cells could allow for in vivo selection and chemoprotection using the drug combination of O6BG and temozolomide.

Materials and methods

Animals

Dogs were raised and housed at the Fred Hutchinson Cancer Research Center under conditions approved by the American Association for Accreditation of Laboratory Animal Care. Animal experiments were reviewed and approved by the Fred Hutchinson Cancer Research Institutional Animal Care and Use Committee. Marrow draws were performed under general anesthesia. Dogs were treated with canine stem cell factor (SCF; 25 µg/kg body weight subcutaneously, once daily) and canine granulocyte colony-stimulating factor (G-CSF; 5 µg/kg body weight subcutaneously, twice daily) for 5 consecutive days before CD34+ cells were isolated from bone marrow (G179) or by leukapheresis (G179) using biotinylated monoclonal anti–canine CD34 antibody clone 1H6 and streptavidin-labeled magnetic beads (Miltenyi, Auburn, CA) as described.28 As preparation for transplantation, the animals received a single myeloablative dose of 920 cGy total body irradiation (TBI). Posttransplantation immunosuppression consisted of cyclosporine (CSP), 15 mg/kg orally, twice daily.

Viral vectors

The oncoretroviral vector plasmid MIEG3P140K has been described. The vector was used to transiently transfect Phoenix–gibbon ape leukemia virus (GALV) packaging cells. The resulting virus-containing medium (VCM) was used to transduce 293T-based Phoenix-RD114 packaging cells, and an RD114 pseudotype, high-titer packaging clone was identified.29 Viral particles were concentrated by centrifugation at 7277 × g for 6 hours to a dilution of concentrated vector at a multiplicity of infection (MOI) of 3 to 4 in the presence of growth factors. After overnight culture in media containing growth factors, cells were re-exposed to the same MOI of concentrated vector for 4 hours. Immediately after this second exposure to VCM (after about 72 hours of total time in culture), cells were washed and reinfused into the irradiated autologous recipient.

O6BG/temozolomide

Fifty milligrams of O6BG (Sigma Aldrich, St Louis, MO) was dissolved in 30 mL of 40% polyethylene glycol in phosphate-buffered saline (PBS), and the concentration was adjusted to 1 mg/kg with prewarmed (37°C) PBS. The drug was further diluted in normal saline to a final volume of 150 mL and was infused over 15 to 20 minutes. Temozolomide capsules (Schering-Plough, Madison, NJ) were administered orally within 5 minutes after completion of the O6BG infusion.

Flow cytometric analysis

Flow cytometric quantification of at least 20 000 events (gated by forward and right-angle light scatter and excluded for propidium iodide, 1 µg/mL) was performed on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ). Analyses of flow cytometric data were performed with CELLQuest v3.3 software (BD Biosciences, San Jose, CA) with gating to exclude fewer than 0.1% control cells in the relevant region for white blood cells. Control cells from a normal dog, matching the cell type assayed, were used to set the gates. FloJo software (Tree Star, San Carlos, CA) was used to score enhanced green fluorescent protein (EGFP)–expressing erythrocytes and platelets. The Overton subtraction algorithm was used to subtract background.

LAM-PCR

Integration site analysis by linear amplification–mediated polymerase chain reaction (LAM-PCR) was performed on canine DNA isolated from either total bone marrow (BM), peripheral blood leukocytes (PBLs), or flow cytometric–sorted cells. One hundred nanograms of DNA served as template for LAM-PCR that was performed as described previously with modifications.3,30-32 Standard LAM-PCR31,32 was combined with a high-throughput screening method.30 Briefly, TaqI and MspI restriction enzymes were used to digest DNA after initially amplifying and creating double-stranded DNA. Enzyme-specific linker cassettes were ligated onto the TaqI and MspI restriction overhangs, and 2 additional rounds of PCR amplified the virus long terminal repeat (LTR) and genomic flanking regions. PCR products were visualized on spreadex gels (Elcrom Scientific, Cham, Switzerland) and directly cloned into TopoTA vectors (Invitrogen, Carlsbad, CA) and plated overnight on selection plates with X-gal (100 µg/mL). Distinct white colonies were selected and grown overnight at 37°C, and plasmid DNA was prepared with a QIAprep 8 turbo miniprep kit (Qiagen, Valencia, CA) as described by the manufacturer. Clones were PCR amplified to assess if a fragment had been inserted. Positive clones were sequenced using M13 forward and reverse primers. Potential genomic insertion sites were analyzed using Blast33 or BLAT34 genome database searching sites. A sequence was scored as a valid insertion site if the fragment of genomic DNA was flanked by both a linker cassette and an LTR sequence. Alignments of the intervening unknown sequences against the July 2003 Human Genome assembly35 confirmed that the sequence was a valid genomic sequence, but due to the low homology between the canine and human genomes, the placement of the sequence in the genome was not carried out.

Clone tracking

Three genomic primers were designed to sequences identified by LAM-PCR from populations sorted for expression of CD14 (ie, monocytes) or a CD21-like antigen (ie, B cells). To PCR amplify specific genomic insertion sites, a forward primer specific to the murine stem cell virus (MSCV) LTR (5′-TGCTTACACAGATATCCTG-3′) and 1 of the 3 reverse genomic primers specific to sequences isolated from the CD14 subset population (1B: 5′-CCCTCCAGCGAGCCTAAGAT-3′; and 6C: 5′-TGGACTGTAGGCAAAGACG-3′) or 1 specific to a sequence isolated from the CD21 subset (21A: 5′-GAGTAAAGCCTGTGGTGCAGT-3′), were used. Products were visualized on 1% agarose TAE (Tris-acetate–ethylenediaminetetraacetic acid [EDTA]) gels.

Results

O6BG/temozolomide-mediated in vivo selection

Two dogs received autologous CD34+-selected stem cell grafts transduced with RD114 pseudotype retroviral vector MIEG3(P140K). The
vector contains the P140K mutant of MGMT [MGMT(P140K)] and, linked via an internal ribosomal entry site, the reporter GFP. The use of RD114 pseudotype vectors produced by 293T-based packaging cells has allowed for improved gene marking in these 2 dogs compared with historic controls. After stable engraftment had occurred, we evaluated the drug combination of O6BG and temozolomide in dog G179. The dose of O6BG was fixed throughout the course of the study at 5 mg/kg with a maximal dose of 50 mg per administration. At the outset of the study, no data were available regarding the toxicity of temozolomide in dogs. Thus, we gradually escalated temozolomide doses from a relatively low starting point. G179 received 4 cycles of a single intravenous infusion of O6BG followed by a single oral dose of temozolomide. Temozolomide doses for these 4 cycles were 50 mg/m², 100 mg/m², 100 mg/m², and 200 mg/m². The mean time period between drug cycles was approximately 30 days, with a range of 27 to 33 days. Gene marking rose in response to the drug treatment in all peripheral blood lineages and in BM CD34+ cells (Figure 1). Increases in marking were sustained for more than 120 days. We then administered 4 additional cycles of O6BG/temozolomide, further escalating the dose of temozolomide. Temozolomide doses in these additional cycles were 300 mg/m², 400 mg/m², 500 mg/m², and 600 mg/m². The mean time period between these cycles was approximately 43 days, with a range of 28 to 59 days. Again, we observed in vivo selection in all lineages (Figure 1). Gene marking in granulocytes peaked at greater than 98% and was sustained at a level greater than 90% for more than 100 days after the last drug administration. A ninth drug cycle with a temozolomide dose of 700 mg/m² was administered on day 559 after transplantation. Gene marking again rose and has remained greater than 98% in granulocytes for more than 85 days.

To ensure reproducibility of these results, a second dog (G197) received a transplant of autologous CD34+ selected cells transduced with a GFP-only vector. Both animals (E977 and G047) were stably engrafted for 857 days and 524 days, respectively, at the time of drug administration. The animals received O6BG (5 mg/kg, 50 mg maximal dose) and temozolomide at a dose of 500 mg/m² (E977) or 600 mg/m² (G047). Both animals showed pronounced thrombocytopenia with a nadir of less than 20 × 10⁹ platelets/L blood (Figure 3). In contrast, the 2 animals with genetically protected BM showed only a moderate drop in platelet count that was most pronounced at the earlier and lower doses of temozolomide. After successful in vivo selection had ensured a sufficient predrug marking level, the nadir after administration of O6BG and temozolomide was markedly blunted in both animals. Specifically, the nadir at 600 mg/m² temozolomide was 146 × 10⁹ platelets/L blood for G179 and 95 × 10⁹ platelets/L blood for G197. Of note, marrow protection was also observed at even higher doses of 700 mg/m² for G179 and 700 and 800 mg/m² for G197. At none of these doses did platelet counts drop to a value of less than 128 × 10⁹/L blood. These data indicate that MGMT(P140K), expressed from an MSCV backbone, mediates effective chemoprotection to the myelotoxic effects of the drug combination of O6BG and temozolomide.

Low average copy number in gene-modified peripheral blood leukocytes

To evaluate the average vector copy number (acn) in PBLs, we compared values for gene marking as determined by quantitative Taqman-PCR (Figures 1C and 2C) with values determined by flow cytometric analysis. The curves obtained by the 2 methods are virtually superimposable. The average ratio of acn to gene marking was 1.11 for dog G179 and 0.98 for dog G197. These data indicate that most gene-modified cells in both dogs carry a single proviral integration.

Multipotential repopulating cells are chemoprotected

The clonal composition of gene-modified hematopoiesis in dog G197 was analyzed by linear amplification-mediated PCR (LAM-PCR). Initially, we performed LAM-PCR on DNA isolated from myeloid (granulocytes and monocytes) and lymphoid (T and B cell) subsets sorted to purity of 97.5% to 99.7% (322 days after
transplantation, after 5 rounds of drug selection). The product of LAM-PCR was analyzed by gel electrophoresis, and several bands were excised and sequenced. In the gel electrophoresis, after using generic LAM primers, no common sequences were identified among the different subsets. To increase sensitivity, we designed 3 sets of primers specific to the genomic sequence flanking 3 individual insertion sites (2 from CD14+ and 1 from CD21+ populations). Using these sequence-specific primer sets, we were able to amplify all 3 insertions from genomic DNA isolated from fluorescence-activated cell sorter (FACS)–purified subpopulations of T cells, B cells, granulocytes, and monocytes. These data strongly suggest that all 3 integrations were derived from a cell with both lymphoid and myeloid potential. The same 3 integration events documented in multiple lineages 322 days after transplantation could also be demonstrated at both earlier and later time points (95 days and 262 days after transplantation; see Figure 4B, pre and early). The fact that some clones were not detectable by LAM analysis in all samples but can be detected by PCR using sequence-specific primers suggests that the sensitivity of gel electrophoresis after LAM-PCR is limited and may underestimate the true number of clones contributing to hematopoiesis in large animals. Similar results were obtained in a nonhuman primate model of stem cell gene therapy in our laboratory (H.-P.K., unpublished data, 2002–2004).

Clonal composition at different time points before and after selection

We also analyzed the clonal composition of the gene-modified hematopoietic cells by LAM-PCR on different time points. DNA isolated from PBLs before selection (pre), after 4 cycles (G179) or 5 cycles (G197) of selection (early), and after 8 cycles (G179) and 9 cycles (G197) of selection (late) was used as starting material for LAM-PCR, and the samples were separated by gel electrophoresis (Figure 4). Both animals show contribution of multiple clones on all time points analyzed. In both animals we observed a tendency toward a reduction in clone number as assayed by gel electrophoresis. The total number of unique bands from 3 triplicate experiments for dog G179 was 32 before selection (pre), 11 after the early rounds of selection (early), and 7 after the last round of selection (late; Figure 4A). The total number of unique bands from 3 triplicate experiments for dog G197 was 37 before selection (pre), 35 after the early rounds of selection (early), and 17 and 11 on 2 time points after the last round of selection (late-1 and late-2; Figure 4B).

To generate more sequence information, LAM-PCR product obtained from dog G197 on different time points after transplantation was shotgun-cloned without prior gel separation,30 and a total of 25 unique sequences were identified. We found 15 sequences before the first round of selection, 10 of which were unique. Twelve
sequences were identified after the fifth round of selection, all of them unique. Three sequences were identified after the last (ninth) round of drug selection, all 3 being unique. Interestingly, we found no overlap between “pre,” “early,” and “late” sequences as assayed by random sampling of peripheral blood. This allows 2 interpretations: either there are different clones contributing to hematopoiesis before and after in vivo selection, or the total number of clones is bigger than anticipated from the visual analysis of electrophoresis gels and sampling error accounts for this absence of any overlap.

**Multipotential gene-modified clones are present before selection and after early and late drug cycles**

To address the question if individual clones survive all rounds of drug administration, we used the 3 genome-specific primers used to characterize the presence of clones in subset populations as described in the paragraph entitled “Multipotential repopulating cells are chemoprotected,” as it refers to CD14+ populations. All 3 sequences could be demonstrated by PCR in total peripheral leukocyte DNA harvested before selection (262 days after transplantation), after the early rounds of selection (262 days after transplantation), and after the last drug cycle (476 days after transplantation). These data indicate that MGMT(P140K)-transduced hematopoietic stem cells active early after transplantation can survive multiple administrations of O6BG and temozolomide, although their overall proportional contribution to hematopoiesis may have changed. A more detailed analysis of the number of clones contributing to hematopoiesis before and after in vivo selection of MGMT(P140K)-transduced canine repopulating cells is currently ongoing in our laboratory and will be reported elsewhere.

**Extrahematopoietic toxicity**

Dogs were regularly examined by a board-certified veterinarian and were weighed at least once per week in the 4-week period after drug treatment. Repeated liver function tests and kidney function tests were carried out. The only abnormalities noted were intermittent mild increases of liver enzymes (alanine aminotransferase [ALT]) in dog G179. It is unclear if this finding is causally related to the drug administrations. Both dogs appeared clinically healthy from the time of neutrophil engraftment up to now. Both animals maintained their weight within 10% of baseline after drug administrations. Morphologic bone marrow examinations of dog G179 (day 581 after transplantation) and G197 (day 489 after transplantation) showed trilineage maturation and a normal blast count. No dysplastic features were seen.

**Discussion**

Here, we demonstrate in a clinically relevant canine model that transplantation of genetically modified MGMT(P140K)-expressing stem cells allows for efficient in vivo selection and marrow protection in conjunction with the drug combination O6BG and temozolomide. In both animals, gene marking increased after repeated doses of O6BG and temozolomide from a moderate baseline to levels greater than 98% in granulocytes. Durable increases in marking were also observed in lymphocytes, red blood cells, platelets, and BM CD34+ cells. Marking was sustained at levels significantly higher than baseline before the first round of drug selection for more than 80 days after several cycles, indicating that selection occurs at the level of an immature hematopoietic progenitor and possibly at the level of a true stem cell. While dogs that received transplants of gene-modified cells expressing GFP but not MGMT(P140K) showed grades 3 to 4 myelosuppression at temozolomide doses of 500 mg/m² and 600 mg/m², the 2 animals with genetically protected BM were able to tolerate temozolomide doses of 500 to 800 mg/m² with no or only very moderate thrombocytopenia. These data indicate that the use of MGMT mutants could be an attractive strategy for clinical marrow protection. Importantly, in clinical trials, the dose-limiting toxicity of temozolomide used as a single dose after O6BG was found to be hematologic with a maximal tolerated dose of 472 mg/m² in a clinical trial for gliomas.36 Marrow protection should help to circumvent this limitation, and the associated intensified drug schedules of increased dose density and dose intensity could result in improved antitumor efficacy. For clinical applications, it would be desirable to replace the total body irradiation regimen used in the present study with a regimen with antiglioma activity. We are currently in the process of evaluating a combination of BCNU and temozolomide to this end in the canine model.

Toxicity in our study has been very acceptable, with no transfusions necessary and no clinical symptoms attributable to the overall very mild myelosuppression associated with drug treatments. We did observe transient intermittent increases in transaminases in 1 of the 2 dogs (G179). However, no elevated transaminases were observed after the highest dose of temozolomide given in this animal, making less likely a causal relationship of temozolomide and the intermittent mild hepatitis-like laboratory abnormalities. Furthermore, no elevation in transaminases was observed in several other dogs that have received temozolomide in our laboratory (H.-P.K., unpublished data, 2002-2004), and liver toxicity has not been prominent in clinical trials involving temozolomide. However, longer follow-up regarding this potential side effect will be needed.

Recent technical advances have made possible the analysis of the clonal composition of the graft. The LAM-PCR has proven particularly useful to this end both in primary samples of animals undergoing gene therapy and in clinical trials.3,31,32,37 We have used this technology to gain a better understanding of the selection process occurring in the dogs presented in this study. We provide evidence that a long-lived cell with both lymphoid and myeloid potential was able to withstand the drug treatment. The presence of multiple clones before and after 9 drug cycles demonstrates that the MSCV backbone achieves expression levels of MGMT(P140K) sufficiently high to protect against repeated drug exposure in vivo. Furthermore, we demonstrate by direct sequencing that multiple clones contribute to hematopoiesis before and after drug selection. It is reassuring that there was no occurrence of monoclonality in the 2 dogs reported here, at least over the observation period of 18 and 16 months, respectively.

We believe that our data should be rapidly translatable to clinical trials. Safety concerns regarding gene therapy have arisen after 2 cases of leukemia were described in patients treated by gene therapy for X-linked severe combined immunodeficiency (SCID).37 Many experts consider the possibility that the occurrence of these severe adverse events was at least in part linked to the specific transgene used in this study.38,39 In support of this theory, leukemias have not been observed in clinical trials of stem cell gene therapy for diseases other than X-linked SCID. A recent retrospective study of a large series of canine and nonhuman primate recipients of gene modified hematopoietic stem cells also revealed no evidence of monoclonality or leukemia.40 However, for a number of malignant diseases, available treatment options are very limited. We believe that the potential benefits of drug resistance gene therapy outweigh the risks in the case of some highly refractory malignancies, especially in the case of glioblastoma.41 Thus, clinical trials of gene therapy for these diseases seem warranted.
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References

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