Methotrexate Preconditioning Allows Sufficient Engraftment to Confer Drug Resistance in Mice Transplanted with Marrow Expressing Drug-Resistant Dihydrofolate Reductase Activity

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ABSTRACT

Methotrexate (MTX) is an effective antitumor agent that has been demonstrated to be particularly useful in the treatment of hematopoietic neoplasms but causes substantial hematologic and gastrointestinal toxicity. We previously demonstrated that transplantation with transgenic marrow expressing drug-resistant dihydrofolate reductase (DHFR) into animals preconditioned by irradiation substantially protected recipient mice from the toxic side effects of methotrexate administration. Here we test the use of methotrexate itself as a preconditioning agent for engraftment of drug-resistant transgenic marrow, subsequently conferring drug resistance upon recipient animals. Administration of methotrexate beginning 1 or 2 weeks prior to or on the same day as transplantation with drug-resistant DHFR transgenic marrow did not allow sufficient engraftment to confer drug resistance to most unirradiated recipients. A small number of animals were curiously protected from lethal MTX toxicity but exhibited extremely low hematocrits and were not engrafted with stem cells, as indicated by low engraftment levels assessed in secondary transplant recipients. However, we subsequently found that MTX preconditioning allowed sufficient engraftment of DHFR transgenic marrow to confer drug resistance if MTX administration was withdrawn at the time of bone marrow transplantation (BMT) and withheld until 2 weeks post-transplant. Quantitative molecular analysis of primary and secondary recipients indicated a stem cell engraftment level of approximately 1%, consistent with previous studies demonstrating that a low level of DHFR transgenic cell engraftment was sufficient to confer drug resistance in recipient animals. We conclude that MTX can be used as a preconditioning agent for subsequent engraftment of hematopoietic stem cells, in this case conferring resistance to MTX.

Methotrexate (MTX) is a potent competitive inhibitor of dihydrofolate reductase (DHFR), a key enzyme in the generation of reduced folates crucial for the biosynthesis of purines and thymidylic acid (Blakley and Benkovic, 1984; Blakley, 1995). Due to its substantial antiproliferative activity, MTX has been used effectively as a chemotherapeutic agent in the treatment of both hematopoietic and solid-organ neoplasms, particularly acute lymphocytic leukemia, non-Hodgkin’s lymphoma, choriocarcinoma, Ewing’s sarcoma, and osteosarcoma (Jolivet et al., 1983; Schornagel and McVie, 1983; Bertino, 1993). However, the usefulness of MTX as an antitumor agent is limited by the toxicity for highly proliferative normal cells and tissues of the hematopoietic system and gastrointestinal tract (Margolis et al., 1971; Rivera et al., 1985).

Chemotherapeutic use of MTX and other antifolates might be rendered more effective if gastrointestinal toxicity and myelosuppression could be averted by the expression of a drug-resistant form of DHFR in normal, drug-sensitive tissues. To this end, several investigators have reported experiments in which transplantation with donor bone marrow, expressing drug-resistant DHFR either by retroviral transduction or by germ line transgenesis, rendered recipient animals resistant to MTX (Williams et al., 1987; Corey et al., 1990; Zhao et al., 1994; Morris et al., 1996; James et al., 1997). As a part of these studies, we recently reported that animals subjected to mild preconditioning and subsequently engrafted with as little as 1% DHFR transgenic cells after bone marrow transplantation (BMT) were significantly resistant to doses of methotrexate that are lethal for normal

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ABBREVIATIONS: MTX, methotrexate; DHFR, dihydrofolate reductase; BMT, bone marrow transplantation; APP, amyloid precursor protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; HBV, hepatitis B virus.
animals (James et al., 2000). MTX administration itself can cause severe myelosuppression, potentially resulting in conditions that are sufficiently cytoreduced to allow engraftment of subsequently transplanted DHFR transgenic marrow and resistance of recipient animals to continued methotrexate administration. In this study, we tested this possibility and demonstrated that MTX preconditioning does indeed allow engraftment of subsequently transplanted DHFR transgenic marrow. However, such engraftment did not occur when MTX was administered continuously and immediately post-BMT but rather required the withdrawal of MTX administration during the time immediately following BMT. The results from these experiments demonstrate that the myelosuppressive effect of antifolate administration can be used to create hematopoietic space for subsequent engraftment of hematopoietic stem cells, which may in turn be genetically engineered to express drug-resistant genes or other types of genes to confer therapeutic benefit for the patient.

Materials and Methods

Animals and Bone Marrow Transplant Studies. FVB/N mice were obtained from the National Institutes of Health health facility in Frederick, MD. The line 04 Arg22 DHFR transgenic mice used in this study have been previously described (Morris et al., 1996; James et al., 1997). APP transgenic mice were used to provide a separate, transgenic non-DHFR donor signal and were obtained from Dr. Karen Hsiao [Department of Neurology, University of Minnesota, Minneapolis, MN (Hsiao et al., 1995)]. Animals were provided with food and water ad libitum. Bone marrow transplant experiments were conducted as previously described. Briefly, marrow was flushed from the long bones of the hind limbs of donor DHFR or APP transgenic animals into Dulbecco’s modified Eagle’s medium, and a single cell suspension was prepared by repeated pipetting and passage through a 27-gauge needle. Ten million donor marrow cells were introduced through the lateral tail vein of unirradiated recipient mice. Recipient animals were administered 4 mg/kg methotrexate per day (amethopterin; Sigma-Aldrich, St. Louis, MO) or phosphate-buffered saline (PBS) by intraperitoneal injection before and/or after bone marrow transplantation as described under Results. Control animals were treated similarly to the experimental animals with respect to the timing and volume of materials administered. Peripheral blood was collected weekly from the retro-orbital vein under anesthesia for determination of hematocrit. For secondary transplants, marrow was collected from primary recipients 90 days after drug administration, and 5 × 10⁶ total bone marrow cells were injected intravenously into lethally irradiated (8.5 Gray) FVB/N mice. Statistical analysis was conducted using the Kaplan-Meier product limit method (Kaplan and Meier, 1958), calculating the log-rank statistic (Peto and Peto, 1972). All animal studies were carried out under the guidance of the University of Minnesota Institutional Animal Care and Use Committee.

Southern Hybridization Analysis. Engraftment levels in marrow transplant recipients were determined by Southern hybridization analysis as previously described (Southern, 1975). Briefly, genomic DNA was isolated from marrow and spleen, digested with BglII, electrophoresed through 1% agarose/Tris-acetate, and blotted onto Nytran (Sclerich and Schuell, Keene, NH). Blots were probed with a 1.3-kilobase APP cDNA fragment (kindly provided by Dr. Hsiao), radiolabeled by random priming. Blots were hybridized and washed as previously described (James et al., 1997). Radioactive signals were quantitated using a 445SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For quantitation of DHFR transgene signals (n), the endogenous DHFR gene signal was used as a loading control in the formula n = (a/b)/(c/d), where a and b are the DHFR transgene and endogenous DHFR signals in the sample evaluated and c and d are the DHFR transgene and endogenous signals from a DHFR transgenic (100%) positive control. APP transgene signals were similarly quantitated, where a and c were the test sample APP transgene signal and APP transgenic (100%) positive control, respectively.

Polymerase Chain Reaction. In some cases, engraftment of transgenic cells was determined by polymerase chain reaction (PCR) analysis of genomic DNA extracted from spleen tissues of test animals. Genomic DNA was isolated from liver tissue (for transgene-positive and -negative controls) using the Gentra GENERATION Capture Column Kit (Gentra Systems, Inc., Minneapolis, MN) according to kit instructions. Standard PCR was performed to amplify a DNA sequence specific to the hepatitis B virus (HBV) sequence located within the DHFR transgene. Each reaction contained 10 pmol of HBV-specific oligonucleotide primers (sense, 5′-ACCTCTCTTTACGCGGTGTCCTC-3′; antisense, 5′-AATGTCCATGCGCCCAAGCC-3′) in a 50-μl reaction mixture with 100 mM Tris-HCl (pH 8.5), 50 mM KCl, 3.5 mM MgCl₂, 200 nM of each dNTP, 0.5 U of TaqDNA polymerase, and 300 ng of genomic DNA template for the control reactions or 500 ng of genomic DNA template for the unknown reactions. The cycling parameters were 30 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, followed by a 10-min incubation at 72°C. PCR reaction products were electrophoresed in 1% agarose, and the 381-base pair HBV-specific product was visualized with 0.5 μg/ml ethidium bromide.

Quantitative Real-Time PCR. The ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) was used for these assays. A TaqMan probe and primer set was designed using Primer Express software (Applied Biosystems) and synthesized by Applied Biosystems. The sense primer 5′-CCGTTCCGCGTGCACCTC-3′, antisense primer 5′-AGGATCGTATGGCCTAC-3′, and fluorescent 5-carboxyfluorescein dye-labeled probe 5′-ACCTCTCGACGTG- CATGGAGACCA-3′ were designed to amplify the HBV sequence located within the DHFR transgene (Morris et al., 1996). Calibration standards consisted of mixtures containing both DHFR transgenic and normal liver genomic DNA, corresponding to varying DHFR transgene content (0, 0.01, 0.1, 1, 10, 25, 50, and 100%). Reaction mixtures (25 μl) contained TaqMan Universal PCR Master Mix (Roche Applied Science, Indianapolis, IN), 200 nM each forward and reverse primer, 200 nM probe, and 100 ng of DNA sample (standard or unknown). PCR reaction conditions consisted of 50°C for 2 min and 95°C for 10 min, followed by cycling between a melting temperature of 95°C for 15 s and an anneal-extension temperature of 60°C for 1 min, repeated for 40 cycles. Threshold cycle values obtained for test samples (unin repeat triplicate) were interpolated from the calibration curve to determine DHFR transgene copy number.

Results

We previously reported that FVB/N mice can be protected from methotrexate toxicity by transplantation with marrow obtained from transgenic animals expressing drug-resistant dihydrofolate reductase activity (May et al., 1995; James et al., 1997). Methotrexate causes severe hematologic toxicity in humans and animals, so as a part of our ongoing studies on methotrexate resistance mediated by expression of drug-resistant DHFR activity, we wondered whether MTX itself might be used as a cytoreductive agent to create hematopoietic space for subsequently infused transgenic marrow expressing drug-resistant DHFR activity. We first tested this possibility by transplanting 10⁷ Arg22 DHFR transgenic marrow cells into normal, unirradiated FVB/N mice precoconditioned by the administration of MTX at 4 mg/kg/day. Methotrexate administration was initiated either 2 weeks...
before BMT (Fig. 1A), 1 week before BMT (Fig. 1B), or on the same day as BMT (Fig. 1C), and it continued through day 60 post-BMT. The dose of MTX (4 mg/kg) used in these experiments is a standard dose that we have established in our experimental system and is well tolerated by animals transplanted with Arg22 DHFR marrow, whereas normal animals or animals transplanted with normal marrow succumb to toxicity. It was anticipated that the hematologic toxicity of MTX administered starting at the time of BMT or starting 1 to 2 weeks prior to BMT would provide sufficient cytoreduction to allow engraftment of DHFR transgenic marrow and, subsequently, protection of recipient animals from MTX toxicity, as previously observed for animals preconditioned with reduced doses of irradiation (James et al., 2000). However, we found instead that only three animals survived out of a total of 24 mice transplanted with DHFR drug-resistant marrow in these three study groups (Fig. 1), indicating that continuous methotrexate administration does not allow sufficient engraftment to confer methotrexate resistance of recipient animals.

Interestingly, these three animals survived for several weeks after their hematocrit levels had fallen to around 10 starting at 3 weeks post-transplant, without otherwise showing signs of deteriorating health (Fig. 1, B and C). In contrast, we have previously observed that animals suffering from such a reduced hematocrit level usually succumb to the effects of MTX administration within 1 to 2 weeks after reaching this low level, i.e., less than 15 (May et al., 1995; Morris et al., 1996; James et al., 2000). We hypothesized that these animals had engrafted with cells that were capable of contributing to protection from MTX toxicity but which were either incapable of or somehow prevented from contributing to erythropoiesis. MTX was withdrawn, and within 2 weeks their hematocrit level was normal, indicating that MTX administration was indeed responsible for the maintenance of low hematocrit prior to withdrawal from drug administra-

![Fig. 1. Effect of DHFR marrow transplant on health (hematocrit) and survival of normal animals administered 4 mg/kg MTX per day. A, schematic representation of treatment protocol. Animals were administered MTX intraperitoneally starting at day 0 and continuing until animal death or until drug withdrawal after day 60. 10^7 Arg-22 DHFR transgenic marrow cells (line 04) or normal FVB/N marrow cells were transplanted into recipient animals on day 0 (B), day 7 (C), or day 14 (D) after initiation of MTX administration. B–D, top, Kaplan-Meier plots showing the fraction of animals surviving over the 2-month period of MTX administration; bottom, mean hematocrit values ± S.D. assessed for each group on a weekly basis.](https://jpet.aspetjournals.org/doi/fig/10.1124/jpet.106.110060)
tion. To determine whether these animals had engrafted with hematopoietic stem cells, the animals were sacrificed, harvesting marrow and transplanting it into secondary recipients preconditioned by lethal total-body irradiation. Secondary recipients were allowed to engraft for 4 months before harvesting spleen and marrow and carrying out quantitative Southern hybridization studies to determine the level of engraftment (Fig. 2). The DHFR transgene signal was very low in spleen and marrow (less than 0.1% transgenic material) from all secondary transplant recipients. We conclude from these studies that the small number of animals surviving MTX administration after MTX preconditioning and transplant with DHFR transgenic marrow must have engrafted with drug-resistant hematopoietic progenitors that were capable of mediating protection from methotrexate but which were not primitive enough to mediate long-term regeneration of hematopoietic stem cells in secondary transplant recipients or mediate erythropoiesis.

One possible explanation for the lack of engraftment and drug resistance in animals preconditioned with methotrexate is that methotrexate administration after BMT may have inhibited engraftment of donor, DHFR transgenic stem cells, preventing their contribution to hematopoiesis post-transplant. We have, in fact, observed decreased engraftment associated with methotrexate administration in animals transplanted with transgenic marrow after preconditioning with sublethal doses of irradiation (James et al., 2000). To test this possibility, we preconditioned normal, FVB/N females with 14 days of methotrexate administration at 4 mg/kg/day. The animals were rested for 4 days and then transplanted with $10^7$ Arg22 DHFR transgenic marrow cells. Recipient animals were allowed to recover from BMT for another 14 days and then tested for drug resistance by initiating methotrexate administration at a daily dose of 4 mg/kg. In this experiment, animals preconditioned by MTX administration and transplanted with drug-resistant marrow exhibited long-term resistance to MTX that extended out to 2 months post-transplant (Fig. 3A). Reduced survival was observed for untransplanted animals as well as animals transplanted with normal (APP) marrow. The resistance of DHFR transgenic marrow transplant recipients to the toxic effect of MTX administration was further demonstrated in the maintenance of hematocrit levels observed in these animals; i.e., hematocrit levels did not fall below an average of 30 during the entire period of drug administration (Fig. 3B).

Quantitative Southern hybridization analysis indicated that these animals were engrafted at a level of approximately 1% donor transgenic marrow (Fig. 4). In contrast, in control animals administered PBS rather than MTX both before and after BMT, the engraftment level was nearly undetectable (less than 0.1%). These results using MTX conditioning are similar to our previous studies, in which we have reported protection from MTX toxicity in animals preconditioned with reduced doses of irradiation (1 to 4 Gray) and transplanted with reduced numbers of drug-resistant marrow cells (down to 10), resulting in reduced engraftment levels down to 1% (James et al., 2000).

These results demonstrate that MTX administration, using the schedule described above, allows for engraftment of donor transgenic hematopoietic cells with subsequent drug resistance of recipient animals but that MTX must be withdrawn during the time immediately post-transplant for such engraftment to occur. Finally, we tested whether the engraftment observed in MTX-preconditioned animals was attributable to stem cells by transplanting marrow collected from primary recipients into lethally irradiated secondary transplant recipients. Secondary recipients were sacrificed 4 months post-transplantation, and then DNA was extracted from spleen samples and assayed for engraftment by quantitative PCR (Fig. 5). In Fig. 5, images from standard PCR/agarose gel electrophoresis were shown along with the Arg22 DHFR transgene copy number (per genome equivalents) as determined by real-time quantitative PCR. DHFR transgene engraftment in animals transplanted with Arg22 transgenic DHFR marrow ranged from 0.02 to 3.03 copies per genome equivalent, whereas in control animals that received no bone marrow transplant or were transplanted with APP marrow, DHFR transgene levels were essentially undetectable (Fig. 5). Thus, as we observed for the primary recipients, engraftment levels in secondary transplant recipients were also

![Fig. 2. Southern hybridization analysis of secondary transplant recipients. Bone marrow was harvested from the three surviving animals depicted in Fig. 1, B and C, and each marrow sample was transplanted into three lethally irradiated secondary recipients. After 4 months, the secondary recipients were sacrificed, harvesting marrow and spleen for Southern analysis as described under Materials and Methods. The locations of DHFR-hybridizing BglII fragments corresponding to the DHFR transgene (Tg) and the endogenous DHFR gene (En), used here as a loading control, are shown. Animal numbers correspond to the primary recipient marrow source: 71 was a control animal transplanted with DHFR transgenic marrow and administered PBS; 47 and 48 were the two survivors shown in Fig. 1B; 67 was the single survivor shown in Fig. 1C. Samples from animals transplanted with normal or DHFR transgenic marrow are shown as negative and positive controls, respectively.](image-url)
around the 1% level. These results demonstrate that MTX preconditioning allows for engraftment of hematopoietic stem cells capable of serial reconstitution in secondary irradiated transplant recipients.

Discussion

We conducted experiments to test the effectiveness of methotrexate administration as a preconditioning regimen for engraftment of DHFR transgenic marrow and subsequent MTX resistance of recipient animals. Continuous administration of MTX starting either before or at the same time as transplantation with drug-resistant transgenic marrow did not allow sufficient engraftment to confer drug resistance in recipient animals. However, when MTX was administered for 2 weeks and then withdrawn for 2 weeks immediately following BMT, animals were found to be engrafted at the 1% level with DHFR transgenic marrow and to exhibit substan-
with retrovirally transduced normal hematopoietic cells (Williams et al., 1987; Corey et al., 1990; Zhao et al., 1994) or with DHFR transgenic hematopoietic cells (Morris et al., 1996; James et al., 1997) after preconditioning with total body irradiation. In most cases, lethal traumatic brain injury has been used to maximize the engraftment level of donor hematopoietic cells. This is particularly important for experiments using retrovirally transduced donor material, since the frequency of transduced cells achieved in the test animal would be compromised by a reduced level of overall donor cell engraftment. A key question in these experiments is the level of DHFR transgenic or transduced cell engraftment that is necessary to confer antifolate resistance in recipient animals. We recently reported experiments in which animals were transplanted with DHFR transgenic marrow after preconditioning with sublethal doses of TBI and in which rescue from lethal MTX administration post-BMT was observed in animals engrafted with as little as 1% donor transgenic marrow (James et al., 2000). In the experiments described herein, we similarly found that MTX itself can provide preconditioning to allow engraftment of DHFR transgenic marrow at approximately the 1% level, which is sufficient to protect animals from subsequent MTX administration. These results imply that, in a gene therapy procedure intended to protect the recipient from MTX toxicity by drug-resistant DHFR gene transfer and expression, preconditioning other than that provided by MTX administration itself may not be necessary. The apparent low-level requirement for preconditioning and engraftment in this system is an important consideration, because ex vivo gene therapy protocols have in general avoided using myeloablative conditions.

Although MTX is known to be acutely myelosuppressive and to cause hematopoietic toxicity (Schornagel and McVie, 1983; Bertino, 1993), it has not been previously used as a preparative agent for the purpose of hematopoietic stem cell transplantation. This is perhaps because MTX is not known to be particularly toxic for stem cells on its own. Blau et al. (1996) reported that MTX administered as a single injection caused no toxicity for stem cells in mice. Allay et al. (1997, 1998) reported that the antifolate trimetrexate was not toxic for stem cells when administered on its own but rather required coadministration of a nucleoside transport inhibitor (nitrobenzyl-mercaptopurine riboside-phosphate) to prevent salvage of nucleosides and rescue from antifolate toxicity. However, reduced engraftment has been reported in patients undergoing methotrexate therapy as graft-versus-host disease prophylaxis (Atkinson et al., 1983). In addition, we have recently reported that MTX inhibited engraftment of DHFR transgenic marrow when administered starting immediately post-transplant (James et al., 2000). In this study, we found that MTX administered at a moderate dose (4 mg/kg/day) over a period of 2 weeks, although not extremely toxic for stem cells, created sufficient hematopoietic space to allow subsequent low-level engraftment of drug-resistant hematopoietic stem cells. The ability of drug-resistant marrow to engraft in MTX-preconditioned animals must have resulted either from toxicity of this dose of MTX for stem cells to some extent or from the effect of MTX on the character of the marrow microenvironment, rendering it susceptible to the establishment of newly introduced stem cells in the marrow (Srour et al., 2001).
The results reported herein support the concept that drug resistance conferred by DHFR gene transfer and expression requires only a low level of DHFR gene transfer and cellular engraftment. Results from the experiments described herein also demonstrate that this level of cellular engraftment is achievable without any further cytoablative procedure than that provided by MTX administration itself. Under these conditions, MTX could be administered first for the purpose of providing cytoreductive preparation for engraftment of DHFR-transduced hematopoietic stem cells, and MTX subsequently could be administered as an antitumor chemotherapeutic agent with reduced toxicity for normal tissues resulting from the expression of drug-resistant DHFR activity. Furthermore, under the appropriate pharmacologic conditions (i.e., in combination with nucleoside transport inhibitors), DHFR-expressing hematopoietic stem cells can be expanded in vivo (Allay et al., 1997, 1998; Warlick et al., 2002). Vectors containing a therapeutic gene in addition to a DHFR gene as a selectable marker may thus be initially established at a low level of engraftment following MTX administration and subsequently expanded in vivo to increase the representation of transduced cell numbers in the blood and hematopoietic organs. Such expanded numbers of transduced stem cells may be necessary in the treatment of diseases that would require an increased frequency of transduced cells in the blood to be effective. Drug-resistant DHFR gene transfer and expression thus has potential applications in the treatment of antifolate-sensitive tumors through improved chemotherapy and in the treatment of hematologic disorders through the use of DHFR as a selectable marker (Karllson, 1991; Halene and Kohn, 2000).

References


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