Transplantation of Bone Marrow Cells From Transgenic Mice Expressing the Human MDR1 Gene Results in Long-Term Protection Against the Myelosuppressive Effect of Chemotherapy in Mice

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Many human cancers that are initially responsive to chemotherapy eventually fail to respond to treatment. For some drugs, dose escalation that may be required for a cure cannot be achieved because sensitive tissues such as bone marrow (BM) limit cytotoxic therapy. Approaches to prevent or circumvent BM toxicity are therefore a high priority of research on dose escalation protocols. In this study, we have transplanted BM cells from transgenic mice that constitutively express physiologic amounts of a functional human multidrug resistance (MDR1) cDNA to lethally irradiated C57BL/6 × SJL F1 mice (n = 36). From 6 weeks to 10 months after the transplant, all animals contained MDR1 DNA in spleen and BM specimens as indicated by Southern blot analysis, and expressed MDR1 messenger RNA in BM samples as detected by slot blot analysis. In addition, these animals were resistant to the myelosuppressive effect of doxorubicin, daunomycin, taxol, vinblastine, vincristine, etoposide, and actinomycin D, whereas control animals that were reconstituted with normal BM were drug sensitive. Finally, the chemoprotection afforded by the MDR1 gene could readily be reversed by adding chemosensitizers such as cyclosporin A and R-verapamil to chemotherapy. Hence, it appears that BM cells expressing the human MDR1 gene maintain this function after transplantation to host animals for a minimum of 10 months, and confer multidrug resistance to these BM recipients. This selective advantage conferred by expression of the MDR1 cDNA suggests a strategy for the use of MDR1 gene therapy in cancer chemotherapy and for the introduction of otherwise nonsellectable genes into BM. This is a US Government work. There are no restrictions on its use.

CHEMOTHERAPY has proven effective in the treatment of some human cancers that are not amenable to definitive elimination by surgery or radiation. Cures have routinely been produced in testicular cancer, in some leukemias and lymphomas, in Hodgkin’s disease, and in some childhood sarcomas. However, in solid tumors such as breast carcinoma, treatment is at most palliative, remissions are of transient duration, and the overall survival for the patient is sometimes minimal.

Despite initial responses, many human tumors eventually fail to respond to chemotherapy because of the development of drug-resistant cancers or because the chemotherapy is inadequate to deal with a large tumor burden. Dose intensification that might achieve a cure is impossible due to the inherent toxic side effects of chemotherapeutic agents. Until more selective anticancer agents are developed, sensitive normal tissues, such as bone marrow (BM), limit the maximum tolerated doses. Thus, strategies to prevent myelosuppression resulting from chemotherapy is a high priority of research on dose escalation protocols.

The human multidrug resistance (MDR1) gene,1 which encodes a 170-Kd molecular weight (Mr) plasma membrane protein termed P-glycoprotein, is widely expressed in normal human tissues.2,4 It is found on the surfaces of epithelia of the kidney, intestine, liver, and pancreas, in the adrenal cortex, the placenta, and in capillary endothelial cells in testis and brain.5 P-glycoprotein functions as a multidrug transport protein that extrudes hydrophobic compounds from cells.7–9 Sensitive cells become highly chemoresistant against many natural product chemotherapeutic drugs that are recognized and expelled by the multidrug transporter when transfected with the MDR1 gene.7 This accumulated evidence has led to the suggestion that, in normal cells, the multidrug transporter has an important role in removing toxic agents ingested in food or inhaled in air from the body, in transporting steroids in the adrenal, and in protecting vulnerable tissues such as the fetus, the brain, or the testis.7–9

Erythroid and myeloid BM cells do not possess significant amounts of P-glycoprotein as a protective factor, and their resulting sensitivity to chemotherapy is a major limitation for high-dose chemotherapy regimens. To study the effect of expressing the human MDR1 cDNA in BM, we have recently generated transgenic mice whose BM cells constitutively express the human MDR1 cDNA.10–12 These mice express MDR1 RNA in their BM at levels comparable with those found in normal human tissues13 and human cancers,14 and are resistant to the leukopenia induced by many natural product chemotherapeutic agents, including taxol, whose dose can be increased 10-fold before leukopenia results.15 The present study shows that transplantation of these MDR-BM cells to normal recipients results in chemoprotection, thereby providing a model for the use of the MDR1 cDNA to protect BM during dose intensification protocols, and as a selectable marker for the introduction of genes into BM.

MATERIALS AND METHODS

MDR-transgenic animals. The development and characterization of transgenic mice expressing the human MDR1 gene in their BM has been reported elsewhere.10–12 A plasmid carrying the...
full-length MDR1 cDNA under control of a chicken β-actin promoter was injected into fertilized single-cell C57BL/6 × SJL F1 mouse embryos and these transgenic embryos were implanted into foster mice. Inbreeding of these animals made it possible to derive a mouse line (MDR-39) that is homozygous for the MDR1-transgene.12 The amount of MDR1 messenger RNA (mRNA) in BM cells of MDR-heterozygous descendants was comparable with that detected in the MDR-cell line KB-8-5.13 This level of MDR1 expression corresponded to a threefold to 16-fold chemoresistance depending on the drug used.14 Immunocytochemical analysis showed that virtually all of these BM cells contain P-glycoprotein at their membrane surface.15 MDR-homozygous male C57BL/6 × SJL F2 mice of line MDR-39 bred in our specific pathogen-free facility were used as BM donors at the age of 16 weeks.

**BM recipients.** Twelve- to 15-week-old male C57BL/6 × SJL F1 mice (Jackson Laboratories, Bar Harbor, ME) served as recipients. They were maintained in a conventional (short-term experiment) or in a micro-isolator (long-term experiment) environment.

**H-2 typing.** For the long-term experiment, peripheral blood lymphocytes from donors and recipients were collected by eye bleeds and tested against a panel of mouse antimouse antisera (Hazlebot Labs, Rockville, MD). This was performed with a complement-mediated antibody-dependent cytotoxicity assay using trypan blue stain as previously described.10,11

**Transplantation protocols.** C57BL/6 × SJL F1 recipients were lethally irradiated with 1,025 rad over 8.5 minutes from a 137Cs source. Using sterile technique, BM were flushed with Media 199 (GIBCO, Grand Island, NY) containing 50 μg/mL gentamycin from long bones of C57BL/6 × SJL F2 donors homozygous for the MDR1 gene with a 23-gauge needle. The marrows were mechanically resuspended in Media 199 by gentle aspiration through a 19-gauge needle and the suspensions were sterile-filtered through nylon mesh. The cells were then pelleted at 1,200 rpm for 7 minutes, resuspended, and counted. Recipient animals were reconstituted within 4 to 6 hours of lethal irradiation via the lateral tail veins using a 27-gauge needle. Each animal received 10 x 10⁶ BM cells from either the transgenic mice (MDR1) (line MDR-39) or from noyal C57BL/6 mice to show engraftment of non-MDR-expressing BM. Radiation controls were prepared simultaneously to confirm adequacy of the lethal irradiation dose.

Two protocols were applied. In experiment 1 (short-term experiment), BM cells from donors were pooled and transplanted. Sixteen animals received MDR-BM, and three recipients were reconstituted with normal BM. In experiment 2 (long-term experiment), H-2 typing and matched transplantation was performed. Each group of four (time points at 6 weeks, 3 months, and 6 months) or 5 recipients (final time point at 10 months) contained one animal that received normal BM. All others were reconstituted with MDR-BM, each from a different donor.

**Chemotherapy and chemosensitization.** Etoposide was a gift of Bristol-Myers Squibb (Syracuse, NY). All other chemotherapeutic agents were purchased from Sigma (St Louis, MO). Cyclosporin A was provided by courtesy of Sandoz AG (Basel, Switzerland), and R-verapamil by courtesy of BASF Bioresearch Corp (Cambridge, MA). The drugs were administered by a single intraperitoneal injection into the lower right quadrant of the peritoneum. Drug concentrations were adjusted so that a maximum volume of 400 μL was injected per experiment. Peripheral blood was collected by peribordial bleeding with heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and diluted 1:20 (vol/vol) in 3% acetic acid solution for erythrocyte lysis. The refractile viable leukocytes (white blood cells [WBC]) were counted on days 0 (before treatment), 3, 5, and 7 on a ultraplane Neubauer's hemocytometer (Hauser Scientific, Pittsburgh, PA). Drug dosage was selected in accordance with previous studies12 to induce a significant reduction of WBC (≥50%) in unprotected animals within 5 days after administration.

**DNA and RNA analysis.** BM recipients were killed at predetermined times (Table 1) by cervical dislocation. Where applicable (legend to Fig 1), this coincided with day 7 of the chemotherapeutic treatment. Spleens were removed at necropsy and placed on dry ice immediately. BM were flushed from long bones with phosphate-buffered saline (PBS)/1 mmol/L EDTA using a syringe with a 23-gauge needle. Cells were separated from the matrix core by manual pipetting, filtered through gauze, washed twice in PBS, and pelleted at 1,200 rpm for 7 minutes. Spleen and BM specimens were divided into halves for DNA or RNA analysis.

Genomic DNA was isolated from tissue samples using buffered-phenol extraction.26 DNA samples (10 μg each) were digested with EcoRI, electrophoresed on 0.8% or 1% agarose in 1X TBEE-buffer, and transferred to nitrocellulose paper.27 Hybridization was performed with nick-translated probe MDR5A at 42°C as previously described.11

For RNA isolation, solid tissues were first pulverized on a metal surface placed on a bed of dry ice. Specimens were dissolved in guanidinium isothiocyanate and RNA was extracted with a single-step procedure using acid phenol/chloroform.28 Ten micromers of RNA or, if less, all RNA available was transferred to each well of a slot blot apparatus. Hybridization with a nick-translated γ-actin probe29 was performed to assess uniformity of RNA sample loading.

DNA and RNA samples of established cell lines such as KB-3-1 and KB-8-530 were also analyzed for comparison. A DNA or RNA signal of BM specimens was considered to be positive if the intensity was at least as high as that in cell line KB-8-5.

**RESULTS**

**Short-term transplantation experiment.** To address the feasibility of transplanting MDR1 expressing BM cells, BM cells from line MDR-39 were pooled and used as donor marrow for 16 C57BL/6 × SJL F1 recipients (nos. 1–5).

### Table 1. MDR1 DNA and RNA in BM and Spleens of Long-Term Transplant Recipients

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**Abbreviation:** Sp, spleen.
Fig 1. RNA analysis in BM of recipients 43 days after transplantation. BM preparation, RNA extraction, and slot blot analysis using nick-translated probe MDR1 was as detailed in Materials and Methods. Chemotherapy, where applicable, was performed 7 days before the end of the experiment. Animal no. 1 received 5 mg/kg doxorubicin; nos. 2 and 3, 5 mg/kg daunomycin; nos. 4 and 5, 10 mg/kg daunomycin; nos. 6 and 7, 14 mg/kg taxol; nos. 8 and 9, 7 mg/kg taxol; no. 10, 5 mg/kg doxorubicin and 100 mg/kg cyclosporin A; no. 11, 5 mg/kg daunomycin and 100 mg/kg R-verapamil; no. 12, 1 mg/kg actinomycin D; no. 13, 5 mg/kg vinblastine; no. 14, 10 mg/kg etoposide; and no. 16, no chemotherapy. Nos. 17, 18, and 19 (normal BM recipients) received 5 mg/kg daunomycin, 7 mg/kg taxol, or 5 mg/kg vinblastine, respectively.

Through 16; Fig 1). Three controls received normal BM acquired from C57BL/6 × SJL F1 mice (nos. 17 through 19; Fig 1). All animals survived the 6-week experimental phase, whereas radiation controls died between day 10 and day 16. Chemotherapy, where applicable (legend to Fig 1), was administered after 5 weeks. All animals were killed on day 43, and DNA and RNA analysis of the MDR1 gene was performed on spleens and BM cells. All appropriate spleen and BM samples (nos. 1 through 16) contained MDR1 DNA and expressed MDR1 mRNA (data not shown). Neither MDR1 DNA nor its transcripts could be detected in any of the control animals that received normal marrow (nos. 17 through 19) (data not shown). Figure 1 gives an example of the RNA slot blot analysis using extracts of the BM preparations. Varying amounts of MDR1 mRNA (left column) could be detected, which is mainly due to different RNA loading as indicated by the variation in the \(\gamma\)-actin mRNA signal (right column). This reflects the variable recovery of RNA from BM specimens. The results with the characterized cell lines KB-3-1 and KB-8-5, which have undetectable or easily detectable MDR1 RNA levels, are also shown for comparison (Fig 1). Furthermore, our data appear to indicate that drug administration under these test conditions did not significantly affect RNA levels.

To determine the effect of chemotherapy on these BM recipients, seven commonly used MDR-drugs were administered and WBCs performed. Figure 2 shows the results expressed as percent of WBC on day 5 as compared with day 0 (before treatment). The solid bars represent the results found when the same dose of chemotherapeutic agent is administered to normal C57BL/6 × SJL F1 mice. It is evident that the transplantation of MDR1-BM cells afforded chemoresistance against doxorubicin, vinblastine, vincristine, taxol, daunomycin, etoposide, and actinomycin D, because there was no decrease in the peripheral WBC (Fig 2, right side). However, engraftment of normal BM cells did not protect against the myelosuppressive activity of chemotherapy (Fig 2, left side). Figure 3 shows some representative time courses illustrating changes in WBC during chemotherapeutic treatment. The data in Fig 3A show that MDR-BM recipients were refractory to BM marrow toxicity associated with taxol- or vinblastine-chemotherapy (Fig 3A, upper two curves). In animals that were reconstituted with normal BM, the same amount of
the same chemotherapeutic agent led to a significant decrease (Fig 3A, lower two curves), with the WBC decreasing over several days, reaching nadirs on day 5, and then beginning to increase again. These results correspond to those observed with the same regimen in normal C57BL/6 × SJL F1 mice.12 Figure 3B shows that transplanted MDR-BM cells also render protection against doxorubicin and daunomycin (Fig 3B, upper part). This effect can be attributed to the MDR1 gene, because these animals express MDR1 mRNA in their BM (nos. 10 and 11, Fig 1), and this resistance can be overcome by adding chemosensitizers such as R-verapamil or cyclosporin A to chemotherapy (Fig 3B, lower part), previously shown to be potent reversing agents for MDR-BM in vivo.12,14 Hence, it appears that BM cells expressing the human MDR1 gene maintain this function after transplantation to irradiated host animals for a minimum of 6 weeks.

**Long-term expression of the MDR1 cDNA after BM transplantation.** To establish the stability of the MDR1 cDNA expression in a long-term follow-up, we repeated the transplant procedure. The initial protocol was followed as closely as possible, but two changes were introduced. In the initial experiment in which the donor and recipient animals were not matched for histocompatibility antigens, all of the recipient animals lost between 10% and 15% of their total body weight of approximately 25 g in the 6-week experimental course. Roughly half of the animals developed a mild but consistent diarrhea that started 3.5 weeks after irradiation and transplantation and continued in some cases up to the end of the experiment. Because it was not entirely clear whether these symptoms indicated the beginning of a graft-versus-host reaction or were the results of an infectious gastroenteritis or radiation-induced intestinal inflammation, we introduced two modifications. For the long-term experiment (experiment 2), H-2 typing (see Materials and Methods) was performed, and the animals were maintained in micro-isolator cages to provide a pathogen-free environment. Under these conditions, the 10-month experimental phase was completely uneventful.

The results of this long-term experiment are summarized in Table 1. Animal no. 3 received normal BM and did not express the MDR1 gene. All other recipients were reconstituted with MDR-BM cells and exhibited the MDR1 gene over the entire period. Figure 4A gives an example of the Southern blot DNA analysis using the probe MDR5A for hybridization and shows the results at the final time point after 265 days. All appropriate spleen and BM specimens exhibited the 3.1-kb EcoRI band that is diagnostic for the human MDR1-transgene.11

**MDR1 mRNA expression could also be detected in all BM samples from mice that were reconstituted with MDR-BM throughout the experiment (Table 1).** In Fig 4B, examples of the RNA slot blot analysis are shown using the final time point at 265 days. Considering the variation in RNA loading (γ-actin probe), the MDR1 expression in the BM preparations appeared to correspond to the level of expression in the MDR-cell line KB-8-5.17 Spleens contained measurable amounts of MDR1 mRNA at day 43, and in one of three tested specimens at day 90. No transcripts of the MDR1 gene could be detected in spleen samples at later time points.

Chemotherapy of mice at the 265-day time point yielded results that were consistent with findings from the short-term experiment (Fig 3). The control mouse that received
normal BM responded to daunomycin chemotherapy as expected with a decrease in the peripheral WBC (Fig 5), whereas MDR-reconstituted animals were resistant to either daunomycin- or taxol-chemotherapy (Fig 5). Thus, the MDR1 cDNA persists and is expressed constitutively in the BM of irradiated host animals for a minimum of 10 months, conveying chemoresistance to BM recipients.

**DISCUSSION**

For those agents whose maximum tolerated dose is limited primarily by myelosuppression, several strategies are under evaluation to permit dose escalation in the hope of obtaining better clinical results with currently available chemotherapeutic drugs. It is evident that methods to stimulate hematopoiesis in patients undergoing high-dose therapy would, if successful, significantly ameliorate some of the toxicities that prevent large-scale investigation and widespread use of such treatments. Over the past few years, clinical data with major myeloid hematopoietic growth factors, cytokines with hematopoietic activity such as interleukin-3, and erythropoietin have been accumulated. The trials completed so far have generally been small in size, the patients have suffered from a variety of tumor types with various amounts of prior treatment, and different therapeutic regimens have been administered. However, common themes in the clinical effects of these factors have become apparent. These growth factors have reduced the period of dangerous neutropenia by accelerating hematopoietic recovery, but have failed to completely eliminate chemotherapy-associated neutropenia and were much less effective in patients with reduced BM reserves. Hence, there is no reason to think that these factors can replace the requirement for stem cell autografting when severely marrow suppressive or even truly ablative therapy is used.

The autografting of hematopoietic stem cells harvested from BM or peripheral blood to rescue patients from high-dose therapies that initiate severe myelosuppression has been intensively explored in lymphomas, Hodgkin’s disease, breast cancer, and other solid tumors. Response rates in these trials tend to be high, but both the duration of these remissions and overall survival rates as well as the associated morbidity and mortality vary widely with tumor types, amount of prior therapy, and the general performance status of these patients. Nonetheless, these high-dose chemotherapy trials clearly support the concept that many human tumors have a steep dose-response curve, particularly in response to alkylating agents. Although advances in medical technology and improvements in supportive care have made autologous marrow transplantation a relatively safe procedure, many drawbacks still remain. In addition to cost, the major limitation of conventional BM autografting is that patients are left without a functional
BM over the entire chemotherapeutic treatment phase. Furthermore, reconstitution with BM has to take place within a certain time frame to rescue patients from lethal myelosuppression. This necessity, when patients are still in major distress, decreases the chances of engraftment, delays the patient’s recovery, and compromises the application of often lengthy and repeated chemotherapeutic regimens.

The strategy that has been evaluated in our animal studies uses the human MDR1 gene to cause broad spectrum cross-resistance to natural product chemotherapeutic agents. Previous studies had addressed feasibility of transplantation of hematopoietic stem cells mono-resistant to methotrexate and appear to show improved survival and protection from methotrexate-induced marrow toxicity when compared with control animals. The transplantation of MDR-BM cells may be performed at a more favorable time point well ahead of high-dose chemotherapy. Moreover, the patients will benefit from an intact BM over the whole treatment phase because their BM is protected by MDR1 expression. In addition, the functional long-term expression of the MDR1 gene (Figs 4 and 5) permits a flexible chemotherapeutic time schedule so that dose fractioning and individual intermittent recovery phases may prevent or reduce drug toxicities in organs other than BM.

We took advantage in these experiments of a well-characterized MDR-transgenic mouse system, and exploited the relatively easy access to BM cells that constitutively express the human MDR1 gene. The amount of MDR1 mRNA detected after 265 days in recipient animals corresponds to the MDR1 expression of cell line KB-8-5 (Fig 4B), which is similar to expression seen in the marrow of MDR1 donor animals. The present studies confirm that this level of expression is stable for several months in vivo and sufficient to confer resistance against a panel of chemotherapeutic agents currently in clinical use (Figs 2, 3, and 5). In a human situation, MDR1 expression in BM cells will have to be acquired via retroviral transfection, which we have previously shown to be feasible in vitro. However, because efficacy of retroviral gene transfer is less than 100%, more detailed studies are warranted to assess the impact of transplanting BM preparations that contain from 10% to 50% MDR cells, and to develop adequate selection protocols.

The high and persistent degree of chemoresistance (Figs 2 and 5) resulting from MDR1 expression in BM raises safety concerns about applying this approach clinically. For example, if engrafted MDR-BM cells eventually become malignant, it would be difficult to eliminate them by classical chemotherapy for obvious reasons. We, therefore, evaluated the effect of chemosensitizers such as cyclosporin A or R-verapamil (Fig 3B) and showed that MDR1 chemoprotection was readily circumvented with the help of reversing agents. For use in human trials, one may also wish to provide another escape route. A recent approach under discussion is to cotransfect the gene for herpes simplex virus thymidine kinase (HSV-TK) in addition to the MDR1 gene, which would then render these cells selectively susceptible to destruction by antiviral agents such as ganciclovir.

Our present studies addressing the feasibility of transplanting MDR-BM cells have been informative and suggest the possibility of new approaches to gene therapy for cancer and human genetic disorders. We succeeded in establishing stable and long-lasting MDR-BM grafts, and showed a high degree of chemoresistance in animals receiving these transplants. Thus, it appears that this approach merits further attention.

REFERENCES


Transplantation of bone marrow cells from transgenic mice expressing the human MDR1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice

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