Paclitaxel Chemotherapy after Autologous Stem-Cell Transplantation and Engraftment of Hematopoietic Cells Transduced with a Retrovirus Containing the Multidrug Resistance Complementary DNA (MDRI) in Metastatic Breast Cancer Patients

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ABSTRACT

The MDRI multidrug resistance gene confers resistance to natural-product anticancer drugs including paclitaxel. We conducted a clinical gene therapy study to determine whether retroviral-mediated transfer of MDRI in human hematopoietic cells would result in stable engraftment, and possibly expansion, of cells containing this gene after treatment with myelosuppressive doses of paclitaxel. Patients with metastatic breast cancer who achieved a complete or partial remission after standard chemotherapy were eligible for the study. Hematopoietic stem cells (HSCs) were collected by both peripheral blood apheresis and bone marrow harvest after mobilization with interleukin 3, and interleukin 6. The remaining CD34+ cells were stored without further manipulation. All of the CD34+ cells were infused for hematopoietic rescue after conditioning chemotherapy with ifosfamide, carboplatin, and etoposide regimen. After hematopoietic recovery, patients received six cycles of paclitaxel (175 mg/m² every 3 weeks). Bone marrow and serial peripheral blood samples were obtained and tested for the presence of the MDRI transgene using a PCR assay. Six patients were enrolled in the study and four patients received infusion of genetically altered cells. The ex vivo transduction efficiency, estimated by the PCR assay, ranged from 0.1 to 0.5%. Three of the four patients demonstrated engraftment of cells containing the MDRI transgene. The estimated percentage of granulocytes containing the MDRI transgene ranged from a maximum of 9% of circulating nucleated cells down to the limit of detection of 0.01%. One patient remained positive for the MDRI transgene throughout all six cycles of paclitaxel therapy, whereas the other 2 patients showed a decrease in the number of cells containing the transgene to undetectable levels. Despite the low level of engraftment of MDRI-marked cells, a correlation was observed between the relative number of granulocytes containing the MDRI transgene and the granulocyte nadir after paclitaxel therapy. No adverse reactions to the genetic manipulation procedures were detected. Therefore, engraftment of human HSCs transduced with the MDRI gene can be achieved. However, the overall transduction efficiency and stable engraftment of gene-modified HSCs must be improved before MDRI gene therapy and in vivo selection with anticancer drugs can be reliably used to protect cancer patients from drug-related myelosuppression.

INTRODUCTION

The demonstration of stable transfer of therapeutic genes into human cells capable of reconstituting the entire hematopoietic system is an important step in the evolution of gene manipulation from a laboratory technique to a clinical therapy. Although rodent studies have demonstrated that retroviral vectors can transfer genes into HSCs2 stably and efficiently, gene transfer into human HSCs in patients with cancer or leukemia has thus far had limited success. Clinical trials of high-dose

Received 12/14/98; accepted 2/9/99.

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2 The abbreviations used are: HSC, hematopoietic stem cell; ICE, ifosfamide, carboplatin, and etoposide regimen; AGC, absolute granulocyte count; TM, transduction medium; IL, interleukin; SCF, stem-cell factor; nt, nucleotide; DHFR, dihydrofolate reductase.
chemotherapy with HSC support have demonstrated that human HSCs can be transduced ex vivo with retroviral vectors, and that these transduced cells can be detected in peripheral blood and bone marrow for prolonged periods of time after reinfusion (1–4). However, the level of gene marking in patients has been too low (<1%) for most therapeutic purposes.

Previous studies in patients with cancer and leukemia have used retroviral vectors containing the bacterial neomycin phosphotransferase (neo) gene, which was used as a marker of successful transduction and was not otherwise intended to have in vivo biological effects. However, the successful transfer of a gene that could impart a survival advantage to transduced cells and that could, therefore, allow selection and expansion after engraftment has the potential to overcome the low transduction efficiency evident in neo gene marking studies.

Breast cancer therapy offers an ideal context in which to determine the utility of such a gene therapy strategy. High-dose chemotherapy followed by autologous bone marrow and/or peripheral blood progenitor transplantation has been increasingly used in the treatment of various malignancies including breast cancer. Hematopoietic toxicity of high-dose chemotherapy is diminished with the use of HSC support, and clinical studies have demonstrated that the treatment of breast cancer patients with high-dose chemotherapy with HSC support is associated with high clinical response rates (5). In addition, pilot studies have suggested that high-risk stage II breast cancer patients treated with high-dose chemotherapy and HSC support may have increased disease-free survival and overall survival relative to historical controls (6).

Although high-dose chemotherapy regimens used for consolidation therapy are associated with high clinical response rates in metastatic breast cancer, the majority of patients ultimately develop progressive disease (5). The potential of gene therapy to impart the chemotherapy resistance of HSCs would allow the use of high-dose therapy earlier in the overall course of therapy and the safe escalation of antitumor cancer chemotherapy after autologous transplantation. Therefore, a breast cancer treatment regimen that uses high-dose chemotherapy and HSC cell support, followed by further administration of active antitumor cancer drugs, is a rational setting for the study of the transfer of genes that confer resistance to breast cancer drugs into human HSCs. In this setting, the in vivo effect of antitumor cancer therapy on drug-resistance genes transferred into HSCs can be determined in a therapeutic milieu.

Previous studies in transgenic animals have demonstrated that overexpression of the multidrug resistance gene MDRI in murine bone marrow cells results in protection from hematopoietic toxicity from chemotherapy drugs that are substrates for the MDRI drug efflux pump (7–9). Subsequent studies in mice reconstituted with hematopoietic cells transduced with an MDRI retroviral vector demonstrated that MDRI-transduced hematopoietic cells could be expanded in animals after treatment with MDRI substrates (10–13).

Therefore, the transfer of MDRI into HSCs may lessen the subsequent chemotherapy-induced myelotoxicity of breast cancer patients and may ultimately permit treatment with increased dose intensity. This pilot clinical study was initiated to study the reconstitution of breast cancer patients with MDRI gene-transduced HSCs cells after autologous HSC transplantation and to examine the effects of subsequent chemotherapy with the MDRI substrate paclitaxel on the level of hematopoietic cells containing the MDRI transgene.

**PATIENTS AND METHODS**

**MDRI-containing Retroviral Supernatant.** The G1MD vector was produced using the pG1 Moloney murine leukemia retrovirus backbone into which the human MDRI cDNA was inserted as described previously (14, 15). A high-titer, helper virus-free amphotropic producer clone was isolated from the PA317 packaging cell line (G1MD clone 5) and used to produce the clinical grade supernatant that was supplied by Genetic Therapy, Inc (Gaithersburg, MD; Refs. 15, 16).

**Patients.** Patients with histologically confirmed metastatic breast cancer who achieved a complete response or a partial response after three to five cycles of standard induction chemotherapy were eligible for this study. Patients with evidence of metastatic disease in bone (by bone scan or X-ray) at the time of diagnosis or in the central nervous system (by high-dose computed tomography scan of the head) or bone marrow (by histological and cytological evaluation of bone marrow biopsy and/or aspirate) at the time of study entry were excluded from the study. Eligibility criteria included normal renal, cardiac, hepatic, and pulmonary function.

The protocol was approved by the National Cancer Institute Institutional Review Board, the NIH Recombinant DNA Committee, and the United States Food and Drug Administration. All of the patients signed separate informed consents for the chemotherapy to treat their breast cancer and for procedures and risks related to the gene therapy. The schema is shown in Fig. 1. After recovery from induction chemotherapy, patients were treated with cyclophosphamide (4 g/m²) over 4 h ± 1 dose. Mesna (800 mg/m²) was given i.v. admixed with cyclophosphamide and then every 3 h over 30 min starting 3 h after the completion of cyclophosphamide, for a total of 12 doses. Normal saline hydration at 3000 ml/m²/day was started 4 h before, and continued for 24 h after, treatment with cyclophosphamide. Patients received filgrastim 10 μg/kg/day on days 2–15 and underwent apheresis when their WBC increased to greater than 5000 cells/μl after reaching the nadir level. Peripheral blood cells were collected using a Fenwal CS3000 (Baxter Corp., Deerfield, IL) or a Cobe Spectra (COBE, Lakewood, CO). Patients underwent a bone marrow harvest at least 4 weeks after treatment with cyclophosphamide.

After bone marrow harvests, patients were treated with ICE chemotherapy consisting of: (a) ifosfamide (3500 mg/m²) i.v. over 2 h daily × 4 days on days 1, 2, 3, and 4; (b) mesna (700 mg/m²) admixed with ifosfamide, then given as a 3-h infusion starting at the completion of the i.v. ifosfamide in (a) and every 3 h thereafter for six doses; (c) carboplatin (600 mg/m²) over 24 h daily × 3 days on days 1, 2, and 3; and (d) etoposide (250 mg/m²) over 2 h every 12 h on days 1, 2, and 3 for a total of six doses (17). Unmanipulated and G1MD-transduced hematopoietic cells from both the peripheral apheresis collection and the bone marrow harvest were thawed and reinfused on day 7. Patients received filgrastim (10 μg/kg/day) starting on day 7 and continuing until achieving an AGC greater than 2000 cells/μl. Patients were treated with six cycles of paclitaxel (175 mg/m²)
over 3 h every 3 weeks) after hematopoietic recovery. No filgrastim was given after paclitaxel.

**G1MD Retroviral Transduction of CD34+ Cells.** For both the peripheral blood aphereses and the bone marrow harvests, the collected mononuclear cells were CD34+ enriched using a Ceprate stem-cell concentrator column (Cellpro, Bothell, WA) according to the manufacturer’s instructions (18). An aliquot (5 × 10^6) of CD34 selected cells was examined for breast cancer cell contamination by immunohistochemistry using a panel of antikeratin antibodies. All of the CD34+ samples were negative for tumor cell contamination with the limit of detection being 1 tumor cell in 10^5 CD34+ cells.

Two-thirds of the CD34+ cells were placed in freezing medium containing 50% human AB serum, 40% Plasmalyte-A (Baxter, Deerfield, MI), 10% DMSO (Tera Pharmaceuticals, Salt Lake City, UT), 10 μg/ml Darnase (Genentech, Thousand Oaks, CA), and 15 units/ml heparin (Fujisawa, Deerfield, IL). The cells were cryopreserved in a controlled-rate freeze and stored in liquid nitrogen.

One-third of the CD34+ cells were centrifuged at 290 × g for 10 min and resuspended in TM at a concentration of 1–1.5 × 10^6 cells/ml. The TM consisted of three parts DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Biowhittaker, Walkersville, MD) and 1 part supernatant from the amphotropic MDR1 retroviral producer cell line G1MD clone 5 (Genetic Therapy Inc., Gaithersburg, MD). TM was also supplemented with 4 μg/ml protamine sulfate (Fujisawa, Deerfield, MI), 20 ng/ml IL-3 (Novartis, NJ), 50 ng/ml IL-6 (Novartis), and 100 ng/ml SCF (Amgen, Thousand Oaks, CA). Cells were cultured in 650-cm^2 flasks (Costar, Cambridge, MA) for 18–24 h at 37°C in 5% CO. At 24 h and 48 h, the cells were collected by centrifugation at 745 × g for 10 min, resuspended in fresh TM (containing fresh G1MD supernatant), and incubated at 37°C in 5% CO2. Cells were harvested at 72 h by centrifugation at 745 × g for 10 min. Aliquots were taken for PCR analysis of G1MD vector DNA and for testing for bacterial and fungal contamination. The remaining cells were resuspended in freezing medium and stored in liquid nitrogen.

**Detection of Transgenes in Hematopoietic Cells.** PCR analyses were performed to detect G1MD vector DNA in the following samples: (a) in CD34+ cells after the 72-h transduction; (b) in bone marrow cells obtained before the first paclitaxel cycle and after the last paclitaxel cycle; and (c) in peripheral blood samples before each of six cycles of paclitaxel therapy and after the last cycle of paclitaxel.

Cell pellets from bone marrow aspirates were prepared by incubation of the cells in a hypotonic RBC lysis solution (Gentra Systems, Inc. Research Triangle Park, NC) for 10 min at room temperature, followed by centrifugation for 10 min at 2000 × g. Mononuclear and polymorphonuclear cell fractions were isolated from each of the peripheral blood samples using double-gradient centrifugation. Whole blood was layered onto Histopaque-1077 (Sigma, St. Louis, MO) medium which had been carefully layered over an equal volume of Histopaque-1119. After centrifugation for 700 × g for 30 min, the two cell layers at each interphase were carefully aspirated. Mononuclear cells and platelets were found at the plasma/Histopaque1077 interface; granulocytes were found at the 1077/1119 interface. The two cell samples were collected by centrifugation for 10 min at 700 × g and resuspended in HEPES-buffered saline (Biofluids, Rockville, MD). Aliquots of cell preparations were counted with a hemocytometer and stained with Hema 3 stain (Biochemical Sciences, Inc.) to document the purity of the collection. The remaining cells were concentrated by centrifugation and resuspended in a cell lysis solution (Gentra Systems, Inc). All of the samples were stored at −80°C until DNA extraction.

DNA was isolated from the granulocytes, monocytes, and bone marrow cell pellets using the Puregene DNA isolation kit (Gentra Systems, Inc). The frozen samples were thawed and incubated with RNase A for 15 min at 37°C followed by the addition of protein precipitation solution according to the manufacturer’s directions. After centrifugation at 15,000 × g for 3 min, an equal volume of isopropyl alcohol was added to the supernatant. The sample was again centrifuged at 15,000 × g for 1 min; the DNA pellet was washed once with 70% alcohol and resuspended in DNA hydration solution, and the concentration was determined by UV spectrophotometry.

Care was taken to avoid potential contamination of patient specimens with PCR products. DNA extraction and PCR assays were performed in separate rooms. Vector DNA was detected with a PCR assay using MDR1 primers (5′-TGA AAC AAA ACG ACA GAA TAG TAA C-3′ and 5′-AAT ACT AAC AGA ACA TCC TCA AAG C-3′). These primers span nt 3726–4424 on the G1MD (MDR1) cDNA sequences begin at position 1479 in the G1MD vector, and the ATG start site is position 1491; Ref. 15). The size of the amplified product is 699 bp (see Fig. 1.
These primers were chosen to eliminate potential amplification of genomic MDR1 sequences and span more than 10 kb on the MDR1 genomic gene (from exon 18 to exon 23; Ref. 19). Previous studies have demonstrated that, due to cryptic splice sites within the MDR1 cDNA, G1MD producer cell lines generate both full-length and spliced transcripts resulting in full-length and truncated provirus in target cells (15). The shortened provirus produces a truncated MDR1 mRNA that results in a large deletion of coding sequences. The upstream G1MD primer used for viral detection in these studies is located just upstream of the cryptic splice acceptor site. Therefore, the PCR assay recognizes only full-length proviral sequences. The PCR reaction mixtures were incubated at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, at 58°C for 20 s, at 72°C for 50 s, and a final extension of 10 min at 72°C. A nested MDR1 PCR assay was also developed using primers located within the first amplified PCR fragment and which span G1MD nt 3797–4159 and was also developed using primers located within the first amplified PCR fragment and which span G1MD nt 3797–4159 and 5’-GTT CTT TCT TAT CTT TCA GTG CTT G-3’.

The PCR reaction for actin was performed as a control at the same time using primers 5’-CAT TGT GAT GGA CTC and 3’-CAT CTC CTG CTC GAA GTC to amplify an actin cDNA sequence of 763 bp. The PCR reaction conditions for the nested MDR1 reaction were 94°C for 3 min, then 25 cycles at 94°C for 30 s, 58°C for 20 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR reaction for actin was performed as a control at the same time using primers 5’-CAT TGT GAT GGA CTC and 3’-CAT CTC CTG CTC GAA GTC to amplify an actin cDNA sequence of 763 bp. The PCR reaction conditions for the nested MDR1 reaction were 94°C for 3 min, then 25 cycles at 94°C for 30 s, 58°C for 20 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR reaction for actin was performed as a control at the same time using primers 5’-CAT TGT GAT GGA CTC and 3’-CAT CTC CTG CTC GAA GTC to amplify an actin cDNA sequence of 763 bp. The PCR reaction conditions for the nested MDR1 reaction were 94°C for 3 min, then 25 cycles at 94°C for 30 s, 58°C for 20 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR reaction for actin was performed as a control at the same time using primers 5’-CAT TGT GAT GGA CTC and 3’-CAT CTC CTG CTC GAA GTC to amplify an actin cDNA sequence of 763 bp. The PCR reaction conditions for the nested MDR1 reaction were 94°C for 3 min, then 25 cycles at 94°C for 30 s, 58°C for 20 s, 72°C for 30 s, and a final extension at 72°C for 10 min.

The total number of CD34+ cells isolated from peripheral blood mononuclear cellular DNA was screened by PCR for recombinant helper virus genome as described previously (1). Conditions for amplification were 95°C for 2 min, followed by 25 cycles at 95°C for 1 min and 72°C for 1.5 min and a final extension at 72°C for 10 min.

RESULTS

The characteristics of the four patients who received G1MD-transduced CD34+ cells are shown in Table 1. Two additional patients were entered on the trial but were removed from the study before receiving any G1MD-transduced cells. One patient did not mobilize sufficient numbers of CD34+ cells, and the other patient developed progressive disease after stem-cell mobilization and, therefore, was not eligible to receive high-dose chemotherapy with stem-cell support. Four patients were treated with ICE chemotherapy and received the G1MD-transduced CD34+ cells as well as the stored, nonmanipulated cells. All of these four patients recovered from the autologous transplantation and were subsequently treated with six cycles of paclitaxel after hematopoietic reconstitution.

After ex vivo incubation in G1MD retroviral supernatant for 72 h in the presence of SCF, IL-3, and IL-6, a portion of each patient’s CD34+ sample was assayed by PCR for the presence of G1MD vector DNA (Fig. 2). To estimate the relative G1MD vector copy number in the transduced CD34+ cells, the PCR signal obtained from ex vivo transductions was compared with the PCR bands obtained from serial dilutions of genomic DNA from a control cell line containing a single copy of G1MD vector per cell diluted in DNA extracted from nontransduced CD34-selected cells. Also, all of the PCR signals of the MDR1 gene in the G1MD vector were normalized to an actin DNA PCR to control for the equivalence of template DNA. As shown in Fig. 2, based on comparison with the control dilution series, the estimated transduction efficiency for the patient CD34+ samples ranged from 0.1 to 0.5%. In the two patients (patients 1 and 2) who underwent both bone marrow harvest and peripheral stem-cell apheresis, there was no obvious difference in the efficiency of transduction with G1MD vector of either collection.

The total number of CD34+ cells isolated from peripheral blood and bone marrow harvests and the estimated G1MD transduction efficiency of each patient sample is shown in Table 2. Patients 3 and 4 had only transduction of peripheral blood CD34+ cells because of inadequate harvests from their bone marrow BM procedures and limited retroviral supernatant. For all of the patients, the mean total number of CD34+ cells reinfused was 3.25 × 10^6/kg (range, 2.32–4.90 × 10^6/kg). On
the basis of the estimated G1MD transduction efficiency, the estimated mean number of G1MD-transduced CD34+ cells reinfused was $2.1 \times 10^7$/kg.

The time to engraftment for the four patients treated with ICE chemotherapy and rescued with transduced and unmanipulated CD34+ cells is shown in Table 3. The mean time to engraftment to granulocytes $>500$ cells/$\mu l$ was 17.5 days and to a platelet count $>50,000$ cells/$mm^3$ was 22 days from the initiation of chemotherapy. The mean time to engraftment with an absolute granulocyte count $>1000$ cells/$mm^3$ and platelets $>90,000$ cells/$mm^3$ was 25.5 days. No toxicities attributable to the use of gene-transduced cells were evident.

Examination of granulocytes from the patients who received G1MD-transduced CD34+ cells for the presence of G1MD vector DNA by PCR analysis is shown in Fig. 3. As both a positive control and reference, each patient sample is accompanied by a concurrent PCR assay of a cellular DNA containing a single-copy G1MD vector per genome serially diluted in DNA from CD34-selected cells. The top row for each patient sample shows the results obtained after the first PCR reaction for the G1MD vector. This assay, although less sensitive than the nested PCR reaction shown in the middle row, better represents the relative quantitation of G1MD vector DNA (in the range 0.01–10% G1MD copy number per cell) as shown by the results from the dilution series of the control cells. The results of the nested G1MD PCR reaction are shown in the middle row. This assay is capable of detecting up to a single copy of G1MD vector DNA in 30,000 cells (0.003%).

As shown in Fig. 3, one of the four patients (patient 2) had no detectable G1MD marking in granulocytes obtained from peripheral blood after hematopoietic reconstitution or at any time after treatment with six cycles of paclitaxel even using the nested PCR reaction. Each of the other three patients had evidence for G1MD marking in peripheral blood granulocytes at the time of hematopoietic reconstitution. Patient 1 demonstrated detectable G1MD marking in granulocytes until cycle 5 of paclitaxel, but the transgene became undetectable before the last cycle of paclitaxel. In this patient, the highest level of G1MD marking (~1%) was noted at the time of reconstitution, and it decreased gradually over the next 15 weeks of therapy. Patient 3 showed G1MD vector marking in granulocytes at the time of hematopoietic reconstitution and throughout all of the six cycles of paclitaxel. This patient exhibited the highest level of G1MD marking in granulocytes (~9%). G1MD vector DNA was present in granulocytes obtained from patient 4 after each of the first two cycles of paclitaxel. However, after cycle 3 of paclitaxel, G1MD marking in granulocytes fell to undetectable levels in this patient. The highest level of G1MD marking in patient 4 was only 0.01%.

None of the four patients exhibited any evidence of an increase in G1MD vector marking level in granulocytes after the treatment with paclitaxel. Even in patient 3, in whom the high level of G1MD marking was observed, the level of G1MD marking remained unchanged for approximately three cycles of paclitaxel and then decreased during the last two cycles of therapy.

PCR analysis for G1MD vector DNA was also performed on monocytes isolated before each cycle of paclitaxel and at the end of therapy (Fig. 4). The results obtained from analyses of peripheral blood monocytes is similar to that obtained from granulocytes (see Fig. 3). Thus, patient 1 lost G1MD marking in monocytes after cycle 3 (granulocyte marking was lost after cycle 5); patient 2 had undetectable G1MD marking at any time after hematopoietic reconstitution and paclitaxel therapy; patient 3 had G1MD marking in monocytes throughout all of the six cycles of paclitaxel; and patient 4 became negative for G1MD marking before cycle 4 of paclitaxel (same as granulocyte marking).

The relation of relative MDR1 gene marking of granulocytes to AGC nadirs after each cycle of paclitaxel therapy is shown in Fig. 5. The mean AGC nadir during the nine cycles in which the MDR1 transgene could not be detected was 354/$\mu l$. In

### Table 2

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>PB CD34+ posttransduction ($\times 10^6$/kg)</th>
<th>PB CD34+ transduction efficiency (% G1MD+)</th>
<th>PB CD34+ nontransduced ($\times 10^6$/kg)</th>
<th>BM CD34+ posttransduction ($\times 10^6$/kg)</th>
<th>BM CD34+ transduction efficiency (% G1MD+)</th>
<th>BM CD34+ nontransduced ($\times 10^6$/kg)</th>
<th>Total no. of G1MD-transduced cells reinfused ($\times 10^6$/kg)</th>
<th>Estimated no. of G1MD-transduced cells reinfused ($\times 10^6$/kg)</th>
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<td>0.3</td>
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<td>4.84</td>
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<td>ND</td>
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<td>2.34</td>
<td>1.1</td>
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*PB, peripheral blood; BM, bone marrow; ND, not determined.
contrast, the mean AGC nadir during the six paclitaxel cycles associated with the highest levels of the MDR1 gene marking was 3.5-fold higher at 1243/µL. In the seven cycles in which there was an intermediate level (<0.01%) of G1MD marking in granulocytes, the mean AGC nadir was also intermediate at 552/µL. Using Spearman (nonparametric) correlation analysis, a moderate-to-strong correlation (r values ranging from 0.4 to 0.8) was found between G1MD levels and AGC nadir for each cycle of therapy. Although this analysis suggests a possible association between MDR1 marking in granulocytes and subsequent toxicity from paclitaxel treatment, the 22 evaluable chemotherapy cycles reflect the experience of only four patients. Furthermore, four of the six samples with the highest level of G1MD marking (>0.01%) shown in Fig. 5 are from one patient (patient 3). Although patient 3 demonstrated high levels of G1MD gene marking in granulocytes (up to 9% G1MD gene copy number), this apparent association between paclitaxel-induced hematopoietic toxicity and G1MD marking in granulocytes must be regarded as tentative.

**DISCUSSION**

In this study, we have shown that patients with metastatic breast cancer can be reconstituted with MDR1-transduced cells after high-dose chemotherapy with stem-cell support. The transduction efficiency with G1MD vector after a 72-h incubation of CD34+ cells in the presence of SCF, IL-3, and IL-6 was 0.1–0.5%. This transduction efficiency is somewhat lower than our previous clinical trials results using neo retroviral vectors (LNL6 and G1Na) and using the same transduction condition of 72-h incubation of CD34+ cells using the same cytokines (1). In that trial, neo gene transduction efficiency of CD34+ cells ranged from 4 to 77% (mean, 21%; Ref. 1). Although the transduction efficiency of the G1MD vector in the current study is lower, the titer of the clinical grade neo vectors (4.2 × 10^5 to 2.1 × 10^6 biologically active particles/ml) is somewhat higher than the titer of the clinical grade G1MD vector used in the current trial (3.1–6.4 × 10^5 biologically active particles/ml), and the total volume of retroviral supernatant was 4- to 6-fold lower in the current trial because of the limited availability of vector.

Despite the reinfection of a limited number of G1MD-marked CD34+ cells, three of the four patients in this trial had evidence of G1MD marking in both granulocytes and monocytes after engraftment. Indeed, high levels (∼9%) of G1MD marking in granulocytes of patient 3 at engraftment were observed after reinfection of only 3.2 × 10^6 G1MD-marked cells/kg. Because the number of G1MD gene-marked CD34+ cells reinfused in the patient on this trial was < 0.1% of the total number of reinfused cells, the relatively high level of G1MD gene marking in patients suggests a potential benefit of G1MD transduction on progenitor cell repopulating ability. However, it should also be noted that one patient in this trial did not engraft with G1MD-transduced hematopietic cells. Furthermore, in two previous studies of MDR1 gene transfer into hematopietic cells, more than one-half of the patients failed to engraft with MDR1 gene-modified hematopoietic cells (21, 22).

Recently, Bunting et al. (23) compared the repopulating ability of murine bone marrow cells transduced with a Harvey-based retroviral vector containing either MDR1 (HaMDR1) or a mutant DHFR gene in the presence of SCF, IL-3, and IL-6. Competitive repopulation studies indicated that the murine bone marrow cells transduced with MDR1 and then expanded in culture for up to 12 additional days in the presence of cytokines were far more capable of both in vitro progenitor cell expansion and stable engraftment than either DHFR-transduced or mock-transduced cells (23).

The mechanism whereby MDR1 transduction may enhance hematopoietic engraftment is not clear. Recent studies have indicated that MDR1 overexpression can protect cells from undergoing apoptosis (24, 25). Thus, MDR1 overexpression may reduce the susceptibility of ex vivo expanded HSCs to undergo programmed cell death. Conversely, because MDR1 is a marker of early progenitor cells, its expression may play a role in decreasing the primitive cell’s propensity to differentiate into committed progenitor cells (23). Nevertheless, the relatively high level of G1MD marking observed in patients in the current clinical trial (up to 9% G1MD copy number in patient 3), despite the limited number of G1MD-transduced CD34+ cells reinfused, supports the hypothesis that MDR1 gene overexpression may enhance the repopulating ability of HSCs in patients.

Of some concern was the finding by Bunting et al. (23) that mice transplanted with ex vivo expanded, HaMDR1-transduced hematopoietic cells developed a myeloproliferative disorder characterized by high peripheral WBC counts and splenomegaly. However, these workers also noted that animals transplanted with MDR1-transduced cells that were reinfused immediately after transduction did not develop any evidence of a myeloproliferative syndrome. This latter observation is consistent with several previous studies (10, 13, 26) that demonstrated that animals could be safely transplanted with MDR1-transduced hematopoietic cells that were not expanded in culture after transplantation. Furthermore, transgenic animals overexpressing MDR1 in bone marrow cells developed normally and without evidence of any hematopoietic disorder (7). Thus, overexpression of MDR1 in bone marrow cells by itself does not result in myeloproliferation. Conversely, continued passage of HaMDR1-transduced hematopoietic cells ex vivo in the presence of early acting cytokines is apparently a prerequisite for any subsequent risk of myeloproliferation in transplanted animals. It must also be noted that the animal studies by Bunting et al. (23) used a Harvey-based retroviral vector (HaMDR1) and not the Maloney-based G1MD vector used in the clinical trial described in this report. The possible contribution of vector-derived sequences to cellular proliferation will need to be addressed (23).

It is also important to note that none of the patients in the

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<tr>
<th>Patient no.</th>
<th>Days to AGC &gt;500</th>
<th>Days to platelets &gt;50,000</th>
<th>Days to AGC &gt;1000 and platelets &gt;90,000</th>
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<td>Mean</td>
<td>17.5</td>
<td>22.3</td>
<td>25.5</td>
</tr>
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</table>
clinical study described in this report developed elevated WBC counts or any evidence of a myeloproliferative disorder after transplantation with MDR1-transduced cells. This is consistent with other MDR1 gene transfer clinical trials, which indicated that patients can be engrafted safely with MDR1-marked hematopoietic cells (21, 22). Hesdorfer et al. (22) used a 72-h incubation of CD34+ cells with a Harvey-based MDR1 retroviral vector in the presence of cytokines (SCF, IL-3, and IL-6) in fibronectin-coated plates. These investigators found MDR1 retroviral vector marking in bone marrow samples from 2 of 7 patients at the time of hematopoietic reconstitution from high-dose chemotherapy. No data were presented from this trial on long-term MDR1 marking or the results after subsequent treatment of patients with MDR1 chemotherapy. Hanania et al. (21) reported results on 20 patients who received MDR1-transduced cells after high-dose chemotherapy. At hematopoietic recovery, these investigators found detectable MDR1-vector marking in bone marrow samples from 5 of 8 patients who received CD34+ cells transduced ex vivo using a 72-h transduction protocol that used autologous stroma and additional cytokines (IL-3 and IL-6). In contrast, they found no evidence of MDR1-vector marking in bone marrow samples from 5 of 8 patients who received CD34+ cells transduced ex vivo using a 6-h coculture with retroviral supernatant in the absence of cytokines. In addition, Fahman et al. (27) examined the effects of paclitaxel chemotherapy in 10 patients immediately after hematopoietic reconstitution from high-dose chemotherapy and infusion of MDR1-vector transduced CD34 cells. Although three of these patients had evidence of MDR1-vector marking in bone marrow cells at reconstitution and before paclitaxel therapy, none of their patients had evidence by PCR of MDR1 vector DNA in hematopoietic cells after paclitaxel therapy despite repeated testing over time. The authors concluded that only short term reconstituting cells had been modified by MDR1 vector in their study. It should also be emphasized that each of the clinical trials to date, including the one described in this report, used nonexpanded MDR1 transduced cells. No hematopoietic abnormalities have been reported thus far in patients transplanted with MDR1 transduced cells.

Previous murine studies have indicated that MDR1-transduced hematopoietic cells could be selectively expanded in animals treated with MDR1 chemotherapy (10, 13). In the current clinical trial, three of the four patients demonstrated G1MD vector sequences in peripheral blood for 10, 16, and 21 weeks after the infusion of cells. Although this is the first study to detect MDR1 vector-modified hematopoietic cells in patients after chemotherapy, we did not detect any expansion of G1MD-marked granulocytes in peripheral blood during paclitaxel therapy. There are several possible explanations for the progressive loss of hematopoietic cells containing the MDR1 transgene during successive chemotherapy cycles: (a) it is possible that the transduced MDR1 gene in these patients was not expressed at sufficiently high enough levels to protect progenitor cells from paclitaxel toxicity. Because of the low level of transduction, it was not possible to directly measure P-glycoprotein function in...
the MDR1-transduced cells; (b) it is conversely possible that the dose of paclitaxel used, 175–200 mg/m², was not sufficiently toxic to confer a clear survival advantage to the G1MD-transduced HSCs; and (c) because retroviral integration requires cell division, it is possible that the incubation of CD34⁺ cells with SCF, IL-3, and IL-6 resulted not only in increased CD34⁺ cell proliferation and retroviral transduction but also resulted in the differentiation of dividing, gene-transduced cells into cells with finite proliferation and self-renewal potential. These cells would produce MDR1 transgene-containing hematopoietic cells for a limited number of cell divisions until ultimately undergoing apoptosis.

Although the numbers of patients in this study is limited, we did observe an association between higher G1MD marking levels and higher AGC nadirs in patients during the posttransplant paclitaxel therapy (Fig. 5). These results suggest that MDR1 gene therapy may be able both to ameliorate hematopoietic toxicity and to enhance chemotherapy dose intensity. One would not necessarily expect that the relatively small numbers of cells that contain the MDR1 transgene would have a discernible clinical impact. Clearly, these observations must be regarded as tentative at this time. Because many factors contribute to chemotherapy-induced hematopoietic toxicity, additional clinical studies with more patients and improved MDR1 gene transduction of HSCs will be needed to address the potential clinical benefit of this approach.

In order for this strategy to be effective, efforts must be made to improve the efficiency of retroviral gene transfer into HSCs capable of engraftment and self-renewal in patients. In this trial, as well as in previous neo gene marking studies (1–3), the majority of the CD34⁺ cells that patients received were frozen immediately after harvest and were not incubated ex vivo in the retroviral TM. It is possible that the low level of gene marking achieved in these trials may be due to preferential engraftment of the nonmanipulated hematopoietic progenitor cells. Animal studies have indicated that nonmanipulated progenitor cells preferentially engraft in animals compared with hematopoietic cells transduced ex vivo (28). Future trials should explore whether MDR1 gene transfer into hematopoietic progenitor cells can be accomplished without the need for the infusion of nonmanipulated hematopoietic cells using nonmyeloablative conditioning regimens.

MDR1 is one of many genes that confer resistance to antineoplastic agents. Other groups have shown that hematopoietic cells transduced with other drug-resistance genes can protect from hematopoietic toxicity or can be selectively expanded after treatment with appropriate antineoplastic agents including genes encoding O⁶-methylguanyl methyltransferase, aldehyde dehydrogenase, and mutant DHFR (29–39). Although overexpression of MDR1 results in the development of resistance to many of the agents most commonly used to treat breast cancer.

Fig. 4 PCR analysis of the MDR1 transgene in monocytes purified from peripheral blood from patients before each paclitaxel cycle and at the end of therapy. The top panel for each patient, the signal from the nested PCR reaction; the bottom panels, the PCR reactions for actin RNA, which served as a control for equivalent template DNA in each sample; on the left side of each panel, the relative signal strength of genomic DNA from a clonal cell line transduced with a single copy of the G1MD vector diluted to 0.01% in DNA extracted from CD34⁺ cells.

Fig. 5 The relationship between AGC nadir to the relative number of granulocytes containing the MDR1 transgene during paclitaxel therapy. For each cycle of paclitaxel, the AGC nadir is plotted relative to the estimated G1MD gene copy number in granulocytes. ◇, the results for patient 1; ◇, the results for patient 2; □, the results for patient 3; △, the results for patient 4; bar, the mean AGC nadir for each group.
including taxanes, anthracyclines, and Vinca alkaloids, the transfer of other drug-resistance genes alone or in combination may prove useful in protecting HSCs from cytotoxic effects of anticancer therapy.

Approaches to increase the efficiency of gene transfer into a self-renewing stem-cell population also need to be explored. For example, recent studies suggest that HSCs harvested using SCF and G-CSF resulted in increased marking with retroviral vectors in vitro (40). Other studies have indicated that the inclusion of other cytokines including FLT3 ligand (41, 42) or various extracellular matrix molecules (43) during the ex vivo transduction incubation with retroviral vector can increase the efficiency of gene transfer into CD34+ cells in vitro. In addition, retroviral vectors that have an envelope protein derived from the gibbon ape leukemia virus (GALV) may have a higher gene transfer efficiency in hematopoietic cells of baboons (44, 45). Clinical studies will be needed to assess the ability of SCF, FLT3 ligand, or other factors to increase the efficiency of retroviral gene transfer into HSCs capable of long-term engraftment in patients.

REFERENCES


Paclitaxel Chemotherapy after Autologous Stem-Cell Transplantation and Engraftment of Hematopoietic Cells Transduced with a Retrovirus Containing the Multidrug Resistance Complementary DNA (MDR1) in Metastatic Breast Cancer Patients

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