Transfer and expression of the human multiple drug resistance gene into live mice

(bone marrow transplantation/gene therapy)

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Communicated by Richard Axel, June 26, 1992

ABSTRACT The human multiple drug resistance (MDR) gene has been used as a selectable marker to increase the proportion of bone marrow cells that contain and express this gene by drug selection. By constructing retroviral vectors containing and expressing the MDR gene and a nonselectable gene such as the β -globin gene, enrichment for cells containing both of these genes can be achieved. A retroviral construct containing MDR cDNA in a Harvey virus-based vector has been used to transfect our ecotropic 3T3 retroviral packaging line GP+E86. Clones have been isolated by exposure of the retrovirally transduced cells (MDR producer cells) to colchicine (60 ng/ml), a selective agent that kills MDR-negative cells. Flow cytometry analysis (fluorescence-activated cell sorting) with an antibody to MDR demonstrates expression of human MDR protein on the surface of these colchicine-resistant producer clones. Untransfected GP+E86 cells are negative. Colchicine-resistant clones were titrated using clone supernatants and the highest titer clone (4×10^4 viral particles per ml) was cocultured with 10^6 donor mouse bone marrow cells for 24–48 hr. The donor cells were then injected into congenic irradiated mice, and the presence of the MDR gene was assayed by the polymerase chain reaction (PCR) analysis using MDR-specific primers. In one experiment eight of nine transduced mice were positive for MDR by PCR of peripheral blood 14 and 50 days posttransplantation; after 240 days three of nine transduced mice were positive. Bone marrow obtained from one of these positive animals was stained with the MDR monoclonal antibody and the granulocyte population was analyzed by FACS. Approximately 14% of the total granulocyte pool contain increased levels of MDR protein. In addition, the bone marrow cells of several mice initially positive for MDR gene by PCR, and subsequently negative, were exposed to taxol, a drug whose detoxification depends on MDR gene expression; a positive signal was obtained in all of these mice, indicating drug selection of MDR-positive marrow cells. Cell sorting studies of these mice also show an increased number of high-MDR-expressing marrow cells, selected after exposure to taxol. Thus, in this live animal model MDR transduction is effective in selecting a human MDR-expressing population of marrow cells resistant to taxol chemotherapy. This strategy may, thus, be useful in humans to prevent the marrow toxicity induced by anticancer agents such as taxol and as a selectable marker to enrich for cells simultaneously transduced with a nonselectable gene.

Gene therapy in animals, including humans, requires safe and efficient gene transfer and high-level gene expression. Retroviral vectors have been used extensively in gene transfer because of their efficient entry into cells and integration into

the host genome (1–3). Previous studies have indicated that high-titer retroviruses, primarily those containing the neomycin-resistance gene (*neoR*), can be used to transduce mouse bone marrow cells efficiently, resulting in stable, long-term integration of the transferred gene in the majority of mouse bone marrow cells (1–9). Similar experiments using the human β -globin gene in irradiated mice have been less successful (10, 11). In an attempt to increase the number of transduced cells and to provide a potential selection *in vivo* and *in vitro* for the continued selection of transduced cells, we have used the human multiple drug resistance gene (*MDR1*, also called *PGY1*; subsequently referred to as MDR). In studies in transgenic mice it has been shown that the insertion of a human MDR gene leads to the resistance of mouse bone marrow cells to drugs normally toxic to these cells (MDR-responsive drugs) (12, 13). These MDR-responsive drugs include colchicine *in vitro* and such important chemotherapeutic agents *in vivo* as the anthracyclines (daunomycin and Adriamycin), vinca alkaloids (vincristine and vinblastine), the etoposides (including VP-16), and taxol.

With safe and efficient transfer and expression of the MDR gene in bone marrow cells, two types of experiments could eventually be done in humans. First, in patients with cancer not involving the bone marrow, in which routinely high-dose chemotherapy is combined with autologous bone marrow transplantation, the insertion of the MDR gene into bone marrow cells might provide resistance to otherwise toxic MDR-responsive chemotherapeutic agents. It has been shown that in MDR transgenic mice the administration of daunomycin results in no change in their leukocyte (WBC) counts, whereas control animals have a marked decrease in WBC counts (12, 13). Normal bone marrow cells usually have low levels of MDR and, thus, are particularly sensitive to MDR-responsive drugs; MDR gene insertion may be a way of providing normal bone marrow cells with high-level MDR expression, which could lead to (i) the resistance of these cells to the toxic effects of subsequent chemotherapy and (ii) the generation of an enriched population of MDR-expressing cells, which eventually might be further increased in number by exposure to MDR-responsive chemotherapeutic agents.

If a nonselectable gene such as the human β -globin gene is placed on the same retroviral vector as the MDR gene, then selection of cells using MDR-responsive drugs may allow the *in vivo* and *in vitro* selection of cells also containing the nonselectable gene; this could provide a unique *in vivo* method of selection in animals and humans, not available

Abbreviations: MDR, multiple drug resistance; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; WBC, leukocyte.

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using other selectable markers such as neoR or dihydrofolate reductase due to their toxicity. Amplification of the MDR gene by drug selection of cells may also contribute to increased MDR expression after drug selection (14, 15).

In this paper we show (i) the isolation of high-titer retrovirus MDR-containing producer cell lines by their resistance to colchicine, (ii) the high-level expression of MDR on the surface of these cells, (iii) the use of these high-titer MDR producer cell lines to transduce mouse bone marrow cells with the MDR gene, (iv) high-level and long-term expression of MDR in these mouse marrow cells, and (v) the exposure of mice, whose marrow is transduced with MDR, to taxol, a drug that requires MDR expression for its detoxification, results in an enrichment of marrow cells expressing high levels of MDR. The long-term expression of MDR indicates that bone marrow stem cells have been transduced. This experimental model should be useful in humans to prevent marrow toxicity and to coselect transduced cells containing a nonselectable gene on the same MDR retroviral vector DNA.

MATERIALS AND METHODS

Preparation and Analyses of Viral Producer Lines. Fifty thousand GP+E86 ecotropic packaging cells (16) were transfected with 10 μ g of the retroviral vector pHaMDR/A (Fig. 1) (17) by the calcium phosphate coprecipitation method (18). Successfully transfected cells were selected in medium containing 60 ng of colchicine per ml (12). Forty-eight colchicine-resistant clones were isolated from MDR transfected cells, whereas none was seen in untransfected cells. The resistant clones were grown and titered for viral production on uninfected NIH 3T3 cells, as described (16, 17). Genomic DNA was made from the 10 clones with the highest titers. DNA was digested with *Eco*RI, separated on a 1% agarose gel, and transferred by the method of Southern. Blots were probed with a 3.4-kb *Eco*RI fragment of the human MDR cDNA labeled with ^{32} P by nick-translation. GP+E86 and GP+E86-pHaMDR/A cells were trypsinized and transferred to Petri dishes for 18 hr with HXM medium (hypoxanthine/xanthine/mycophenolic acid). To quantitate the amount of MDR expressed on the surface of these clones, the cells were analyzed by fluorescence-activated cell sorting (FACS); the cells were stained with an MDR monoclonal antibody, 17F9 (1 μ g per 10^6 cells), for 20 min on ice. The 17F9 antibody was a gift of David Ring (Cetus). Cells were then washed with 1 $\times$ phosphate-buffered saline (PBS)/3% fetal calf serum (FCS) and stained with an IgG2b secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1 μ g per 10^6 cells) for 20 min on ice. Cells were again washed with 1 $\times$ PBS/3% FCS and resuspended in 300 μ l of 1 $\times$ PBS/3% FCS. Analysis was performed on a FACS 4 (Becton Dickinson).

Bone Marrow Transplantation. Marrow was harvested from the hind legs of 12-week-old C57BL/6J mice 48 hr after they had received one dose of 5-fluorouracil (500 mg/kg) (9). Aliquots of

harvested bone marrow (3×10^6 cells) were cocultured by layering them onto 100-ml plates containing semiconfluent GP+E86 MDR producer cells (3). Cocultures were incubated for 24–48 hr in 10 ml of α minimum essential medium (α MEM) containing 15% FCS, 15% WEHI conditioned medium, 1% penicillin/streptomycin solution (GIBCO), Polybrene (2 μ g/ml), and interleukin 6 (200 international units/ml). In some experiments, stem cell factor (a gift of K. Zsebo, Amgen) was used (10–100 ng/ml). The unattached bone marrow cells were aspirated, concentrated by centrifugation at $800 \times g$ for 10 min, and then counted in a hemacytometer using acetic acid and trypan blue to determine the number of viable nucleated cells (9). Finally, aliquots of $1-2 \times 10^6$ viable nucleated cells in 0.5 ml or less of α MEM were collected in 1-ml tuberculin syringes and kept at room temperature in preparation for injection into irradiated recipient mice (usually <6 hr after removal). Recipient C57BL/6J (6–12 weeks old) mice were irradiated with 975 rads (1 rad = 0.01 Gy) using a γ source; donor marrow was then infused slowly through the central tail vein of the recipients (9). Recipient mice were maintained in sterile cages and continued on a regimen with tetracycline started 3 days prior to irradiation and transplantation. These conditions were used for a minimum of 2 weeks posttransplantation.

Analysis of Transplanted Mice. *Polymerase chain reaction (PCR).* For the detection of the MDR gene in the blood of live recipient mice, PCR analysis was used with appropriate MDR cDNA probes (19). Seventy-five microliters of peripheral blood was obtained from recipient mice tail veins and collected in microhematocrit capillary tubes; the tubes were then spun for 4 min in the microhematocrit centrifuge to obtain the "buffy coat" containing nucleated WBCs. The buffy coat cells were treated with a lysis buffer and then incubated with proteinase K (0.06 mg/ml) at 55°C for 1 hr, followed by 95°C for 10 min to inactivate proteinase K. Salt/ethanol-precipitated DNA or DNA lysate was used directly in the MDR PCR reaction.

PCR was carried out with 25 μ l of DNA solution with 1 unit of AmpliTaq polymerase and appropriate reaction kits (Perkin-Elmer/Cetus) in a final volume of 50 μ l. We used 35 PCR cycles; each cycle included 15 sec of denaturation at 94°C, 15 sec of annealing at 55°C, and 1 min of extension/synthesis at 72°C. MDR-specific sequences were amplified using the sense-strand primer CCCATCATTGCAATAGCAGC (residues 2596–2615) and the antisense-strand primer GT-TCAAACCTCTGCTCCTGA (residues 2733–2752), which

FIG. 1. Retroviral vector pHaMDR/A containing MDR cDNA. pHaMDR/A is a Harvey virus-based retroviral vector containing full-length MDR cDNA [4.1 kilobases (kb)] with *Sac* II and *Xho* I sites at its 5' and 3' ends, respectively. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Stu* I; X, *Xho* I. bp, Base pairs; LTR, long terminal repeat; MSV, murine sarcoma virus.

FIG. 2. Southern blot analysis of GP+E86 MDR producer clones. DNA was digested with *Eco*RI and probed with a 3.4-kb *Eco*RI MDR cDNA probe. The arrow indicates the appropriate MDR DNA band. Lanes 1–9, GP+E86 MDR clones; lane GP+E86, negative control; lane pHaMDR/A, positive control.

FIG. 3. FACS analysis of untransfected (A) and MDR transfected (B) cell lines. Cells were stained with MDR antibody 17F9 and then a secondary IgG2b antibody conjugated to FITC. y Axis, relative number of cells; x axis, amount of MDR protein.

yield a 167-bp product (19). Ten microliters of the PCR product was examined on a 4% agarose/NuSieve gel for the presence of the expected band.

Cell sorting. Bone marrow cells from sacrificed recipients were obtained by flushing the femurs and tibias of mice with α MEM. Erythrocytes were lysed for 30 sec with cold H_2O and isotonicity was restored with 3.5% NaCl. The remaining

FIG. 4. PCR from peripheral blood with MDR-specific primer. Lanes 1–9, signal obtained from 75 μ l of mouse peripheral blood DNA 50 days posttransplantation with bone marrow exposed to producer lines expressing MDR (arrow at left indicates expected size band); lanes PC and CI, same amount of peripheral blood from untreated mice; lane G+, DNA from 8×10^5 MDR producer cells; PB, a PBR marker; lane G, a ϕ X marker.

population of nucleated cells was stained with MDR monoclonal antibody 17F9 (1 μ g per 10^6 cells) as described earlier.

Taxol experiments. Four mice transduced with the MDR gene 14 months earlier were initially positive by MDR PCR; however, after several months, all of these mice lost their MDR PCR signal presumably because of dilution with non-transduced cells. These four mice were exposed once to taxol (7 mg/kg) injected intraperitoneally. Seven days following taxol administration, the MDR PCR was repeated. MDR FACS analysis as described earlier was also performed on peripheral blood samples of these mice before and after taxol.

RESULTS

The High-Level Producer Cell Line. The high-level producer ecotropic cell line was characterized by Southern blot (Fig. 2) showing that there was integration of the human MDR gene into the genome of these cells. The high level of expression of MDR P glycoprotein on the surface of these cells was demonstrated by FACS analysis as described in *Materials and Methods* (Fig. 3). Other antibodies, recogniz-

FIG. 5. FACS analysis of MDR-expressing macrophage/granulocyte population in MDR-transplanted mouse bone marrow cells. Bone marrow cells were obtained by flushing femurs and tibias of an MDR-transplanted mouse with α MEM. The erythrocytes were lysed and the remaining cell population was stained with MDR monoclonal antibody 17F9 and a goat anti-mouse IgG2b FITC-conjugated secondary antibody. The macrophage/granulocyte population was determined by a gate based on forward and side scatter, and only this population is represented. (A) Bone marrow from untransplanted mouse. (B) Bone marrow from transplanted mouse; the distinct population to the right of the gate shows (vertical line on graphs) a logarithm greater MDR fluorescence, indicating significantly increased expression of MDR over endogenous levels. This population represents >13% of the gated macrophage/granulocyte cells.

ing internal epitopes of the MDR P glycoprotein that were used to quantitate MDR protein expression, gave high background staining levels with cells that had not been transduced with the human MDR gene (data not shown).

MDR Gene Transfer and Expression in Live Mice. Several different batches of irradiated recipient mice transduced with bone marrow containing the human MDR gene were analyzed for the content and expression of the MDR gene over time. Over 90% of the mice analyzed between 14 and 50 days posttransplantation contained the MDR gene by MDR PCR analysis (Fig. 4) of their tail vein blood. MDR analysis by PCR was continued in some of these animals for up to 8 months. In the surviving animals at 8 months $\approx 33\%$ contained the MDR gene by PCR. PCR analysis of some of these mice is shown in Fig. 4.

At 8 months some of the animals were sacrificed, and the marrow was analyzed for the content and expression of the human MDR gene by Southern blot and FACS analysis. Southern blot analysis of the bone marrow of one of these animals (data not shown) demonstrates a clearly positive signal of the MDR gene. FACS analysis of the bone marrow cells of this mouse was performed using gating conditions that excluded long-lived lymphocytes from the sort procedure by size and morphology. The cells analyzed were predominantly granulocytes. In this mouse a distinct population of cells, representing $\approx 14\%$ of the total granulocyte pool, contained significantly increased levels of MDR protein (Fig. 5). This result indicates that, in this animal, bone marrow stem cells were clearly transduced initially since mature granulocytes containing high levels of MDR protein were present as long as 8 months posttransplantation. This occurred even without further selection by exposure to MDR-responsive drugs, such as taxol or daunomycin, which has been shown in transgenic animals to increase MDR expression (12, 13).

Enrichment for MDR Expression Following Taxol Administration. Following a single dose of taxol, four mice initially positive for MDR by PCR, who had subsequently become negative by this assay, were shown to have again a positive MDR PCR signal (Fig. 6). In one of these four mice, FACS cell sorting of peripheral blood cells indicated that 7% of the total WBC population was positive for an increased level of MDR expression as compared to $<1\%$ of marrow cells in this mouse prior to taxol administration (Fig. 7).

DISCUSSION

There has been some concern about the efficiency of gene transfer into bone marrow stem cells, especially in experi-

FIG. 6. PCR analysis of peripheral blood of mice following taxol. Mice 2 and 8 were negative for MDR by PCR prior to exposure. Following taxol both mice show a positive PCR signal (arrow at right).

ments involving the human β -globin gene (10, 11). However, in the experiments in this paper we show that a human gene (MDR) cDNA, contained in a retroviral vector, in an appropriately high-titer producer cell line clearly leads to relatively efficient and long-term transduction of mouse bone marrow cells. These results do not quite match those obtained using a *neoR*-containing retrovirus (3–9). However, this may be due to the relative differences in retroviral titer between MDR producer cells (10^5) and *neoR* producer cells (10^6 , 10^7). In addition, the present experiments indicate that the β -globin gene *per se*, as part of a retroviral construct, is particularly difficult to transduce into bone marrow cells since packaging lines and producer lines of $\approx 10^5$ virus per ml, similar to those titers in the MDR experiments reported here, do not transduce marrow in our experience (data not shown). This is in sharp contrast to the results in this paper with the MDR gene and perhaps other genes as well—i.e., adenosine deaminase gene (*ADA*) (19). The differences between the ability of different retroviral vectors containing different

FIG. 7. FACS analysis of peripheral blood of mice following taxol. Peripheral blood WBCs were obtained from untransduced and MDR transduced mice after taxol administration. Dead cells were excluded from analysis by addition of propidium iodide (y axis). MDR expression (x axis) is quantitated by the staining of cells with the monoclonal antibody 17F9 and an IgG2b FITC-conjugated secondary antibody. (A) FACS analysis of untransduced mice who received taxol shows that between 0.19% and 0.65% of the cells lie beyond the gated line indicated. One of these mice is represented here. (B) One of the MDR transduced mice treated with taxol shows high-level MDR expression of 6.9% of the WBC population. These cells are indicated as a distinct population beyond the gated vertical line.

cellular genes and cDNAs to be transduced when incorporated into a retrovirus remain to be explored in greater detail.

The data presented in this paper provide strong evidence that bone marrow stem cell infection is possible with appropriate retroviral vectors and producer lines containing human genes. These experiments represent only a start of the potential optimization of this system. The use of (i) long-term bone marrow culture to provide repeated infection by retrovirus, (ii) isolated stem cells to provide higher virus to cell ratios, and (iii) growth factors leading to stem cell proliferation in these populations provides additional steps that can be used to increase retroviral gene transfer (20, 21). In the case of isolated bone marrow populations, containing the majority of hematopoietic stem cells, it has already been shown that the presence of growth factors, primarily stem cell factor and interleukin 3 and interleukin 6, causes an increase in the number of proliferating stem cells available for retroviral gene transfer (22, 23). The use of *in vitro* or *in vivo* selection of transduced bone marrow cells expressing high levels of MDR is possible by exposure of the cells to MDR-responsive chemotherapeutic agents. The efficacy of this procedure in enriching the bone marrow of live animals for cells expressing high levels of MDR is demonstrated in the preliminary results of others, using murine bone marrow transduced with MDR (24) as well as the data in this paper by MDR PCR analysis. However, the increased MDR PCR data do not distinguish between (i) an increase in MDR gene due to gene amplification in already transduced cells or (ii) an increase in the relative number of MDR transduced cells in the bone marrow. The FACS results presented in this paper showing an increase in high MDR-expressing cells (Fig. 7) demonstrate that selection for MDR-expressing cells has occurred.

Our results suggest that use of MDR in patients with advanced cancer undergoing autologous bone marrow transplantation in association with high-dose chemotherapy may be a feasible first clinical trial of this therapeutic modality, if it can be shown that human bone marrow stem cells can be appropriately transduced and express high levels of MDR in an animal model such as immunocompromised mice. Subsequent experiments using the MDR gene as a selectable marker on a retroviral vector, also containing a nonselectable gene such as the human β -globin gene, may enrich the population of marrow cells for cells containing both genes. If this strategy is successful, then this methodology could be applied to other genetic diseases in which the gene of interest is not selectable.

This work was supported by National Institutes of Health Grants DK-07373 and HL-28381 and the Cooley's Anemia Foundation.

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