High-Dose Multidrug Resistance in Primary Human Hematopoietic Progenitor Cells Transduced With Optimized Retroviral Vectors

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Retroviral transfer of the multidrug-resistance 1 (*mdr1*) cDNA into primary human hematopoietic progenitor cells (HPC) of cancer patients undergoing high-dose chemotherapy has been proposed to protect the bone marrow from the dose-limiting cytotoxicity of cytostatic agents. Preclinical studies performed with vectors derived from the Moloney murine leukemia virus (MoMuLV) or the related Harvey murine sarcoma virus have established that chemoprotection of HPC is feasible. The efficacy of vector-mediated multidrug-resistance under high doses of cytostatic agents, however, remained unclear. We report here that this goal can only be achieved with improved vector design. Novel vectors termed SF-MDR and MP-MDR, which are based on the spleen focus-forming virus or the myeloproliferative sarcoma virus for the enhancer and the murine embryonic stem cell virus for the leader, significantly elevate survival of transduced primary human HPC under moderate doses of colchicine and paclitaxel in vitro when compared with a conventional MoMuLV-based vector. Importantly, SF-MDR and also MP-MDR confer an absolute advantage at high doses of paclitaxel in vitro corresponding to peak plasma levels achieved in patients during chemotherapy. This observation has important consequences for a variety of ongoing and planned gene therapy trials.

THE OUTCOME of pilot gene therapy trials has emphasized the need for careful development and extensive preclinical testing of basic gene transfer technologies.[1,2] Obviously, each single approach requires optimization of vector design and transduction protocols to increase the efficiency and to reduce potential risks of the gene transfer. We have focused on the improvement of retroviral vector design to obtain high and stable gene expression levels in early hematopoietic cells and their progeny. Such vectors are needed for a variety of clinical applications. Among these are bone marrow (BM) stem cell protection to reduce the myelotoxicity of anticancer chemotherapy, correction of metabolic disorders involving hematopoietic cells, suppression of pathogenic viruses like human immunodeficiency virus (HIV) homing to a subset of hematopoietic cells, and finally, correction of oncogene expression in leukemic cells.[3]

Relying on genetic observations made in the murine system, we have recently succeeded in cloning novel retroviral vectors that enable highly efficient gene expression in early hematopoietic cells.[4] These vectors were termed Friend mink cell focus forming (FMCF)/murine embryonic stem cell virus (MESV) hybrid vectors FMEV, and myeloproliferative sarcoma virus MPSV /MESV hybrid vectors (MPEV). Studied on a panel of murine and human myelo-erythroid cell lines, these vectors clearly exceed the activity of conventional retroviral vectors based on the Moloney murine leukemia virus (MoMuLV) and related viruses, eg, the Harvey murine sarcoma virus (HaMuSV).[4,5] The efficiency of FMEV and MPEV results from the use of potent retroviral enhancers combined with exclusion of a potential transcriptional repressor element colocalizing with the retroviral primer binding site (PBS). Both FMEV and MPEV are arranged in a classical backbone based on murine type C-retroviruses, currently the best vector architecture for gene transfer into hematopoietic cells.[6,7] The enhancer/promoter-sequences of MPEV and FMEV were derived from the U3-regions of MPSV and FMCF-related viruses, respectively, which permit expression in early hematopoietic cells as well as in most primitive embryonic stem cells.[4] The FMCF-related polycythemic strain of the spleen focus-forming virus (SFFVp) contains the most potent retroviral enhancer assembly known so far for gene expression in these cells. At the molecular level, FMEV activity is governed by the gain of a binding site for the ubiquitous transcription factor, Sp1, and by an altered enhancer core that is a high-affinity target for the polyoma enhancer binding protein/core binding factor (PEBP/Cbf) family of transcription factors which possibly interact with *ets*-transcription factors binding to the neighbouring LVb-element[5] (Baum et al, manuscript submitted). The cis-regulatory elements of MPSV are closely related to those of the lymphotropic MoMuLV. Again, the gain of a binding site for Sp1 is partly responsible for the increased activity of the MPSV enhancer in more primitive hematopoietic and embryonic cells.[8,9] When compared with the SFFVp enhancer, the MPSV enhancer has fivefold reduced activity in myelo-erythroid cells, whereas the MoMuLV-enhancer is up to one order of magnitude less active in this compartment.[4] To overcome transcriptional repression of U3-mediated transactivation directed by a repressor binding to the PBS, we equipped the vectors with a leader derived from MESV.[10]

Here we report that MPEV and, even more, FMEV greatly

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elevate the efficiency of selectable retroviral gene transfer in primary human CD34$^+$ hematopoietic progenitor cells (HPC). This is exemplified by transfer and expression of the human multidrug resistance 1 (*mdr1*) cDNA, which encodes the membrane-located drug efflux pump P-glycoprotein (Pgp). Pgp confers resistance to a variety of cytotoxic agents, many of which are of outstanding importance in anticancer chemotherapy.[11] Retroviral transfer and expression of *mdr1* in primary murine and human HPC by vectors based on cis-regulatory elements of either MoMuLV or HaMuSV origin is known to result in enhanced expression of Pgp, with no significant differences observed between these two backbones. Both vector types confer a dominant selectable phenotype in the presence of cytotoxic agents like colchicine or paclitaxel in vitro, and, in mouse models, also in vivo.[12-21] The efficiency of protection against high doses of chemotherapeutic agents, however, was previously not systematically addressed.

We show that expression of *mdr1* by both FMEV and, somewhat less effectively, MPEV significantly increases the survival of transduced primary human HPC under moderate drug doses when compared with cells transduced with a conventional MoMuLV vector. Furthermore, in these cells only FMEV and MPEV mediate high-dose resistance, and thus background-free selection. As a representative for more primitive progenitor cells, we chose the murine cell line, FDCP-mix A4. This factor-dependent, diploid, and nontumorigenic cell line still represents one of the best characterized model systems for multilineage HPC available.[22] FDCP-mix A4 cells share flow-cytometric characteristics of early progenitor cells (Thy-1^{lo}/Sca-1$^+$); they can be differentiated into all nonlymphoid hematopoietic lineages, with 5% to 20% spontaneous myeloid differentiation occuring under standard culture conditions,[22-24] in contrast to the FDCP-mix 15S cells which are blocked in differentiation and were analyzed in our previous study.[4] Retroviral gene transfer into FDCP-mix cells is restricted by a block occuring at the preintegration level that is not related to the cell cycle, but depends on the state of differentiation, being more pronounced in multipotent clonogenic blast cells.[23] A similar block has recently been observed in human primary CD34$^+$/CD38$^-$ early HPC.[25] We show that background-free selection and long-term survival of *mdr1*-transduced FDCP-mix cells in the presence of myelosuppressive drug doses can only be achieved with the use of improved vectors such as FMEV or MPEV.

MATERIALS AND METHODS

Cell lines and primary cells. Establishment of amphotropic producer clones based on GP&envAM12 cells releasing the high-titer replication-incompetent vectors SF-MDR, MP-MDR, and V-MDR has been described.[4] As a control, we used high-titer producer cells GP&envAM12 releasing retroviral vector MP1N, expressing the neomycin resistance gene (neoR) in an MPEV backbone.[5] The vectors are depicted in Fig 1. Cell-free supernatants were titrated on ecotropic packaging cells GP&*env*86 as described. Maintenance of FDCP-mix A4 cells was as described.[23,24] CD34$^+$-selected human HPC were obtained from BM aspirates donated by healthy adult volunteers. Mononuclear cells were separated by centrifugation on a Ficoll-gradient (Biochrom KG, Berlin, Germany) according to the manufacturer's instructions, and enriched for CD34$^+$ cells using the magnetic beads based MACS CD34 progenitor cell isolation kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's instructions.

Transduction protocol and methylcellulose assays. Mononuclear CD34$^+$ cells were transduced by cocultivation of 2 to 4 $\times$ 10^5 cells on a confluent layer of irradiated (2,000 rad) virus producers grown in a gelatinized 25 cm^2 flask. Titers of cell-free supernatants obtained from the producers during the experiments ranged from 3 to 5 $\times$ 10^5/mL for SF-MDR and MP-MDR and 1 to 5 $\times$ 10^6/mL for V-MDR. During transduction, cells were kept in α-MEM (GIBCO-BRL, Paisley, Scotland), supplemented with 10% horse serum and 10% fetal calf serum (FCS; Stem Cell Technologies, Remagen, Germany), 10 U/mL interleukin-3 (IL-3), 200 U/mL IL-6, 50 ng/mL stem cell factor (SCF) (all growth factors were obtained from Boehringer Mannheim, Mannheim, Germany) and 4 mg/mL polybrene (Sigma, Deisenhofen, Germany). BM cells were procured after 48 hours and assayed for colony formation using a standard methylcellulose-assay basically performed according to Sutherland et al.[26] Complete methylcellulose medium contained 0.67% MethoCult H4100, 5% PHA-LCM (both Stem Cell Technologies), 30% FCS (PAA, Linz, Austria), 1% bovine serum albumin and 5 U/mL Epo (both Boehringer Mannheim). One thousand or 3,000 cells were plated per 1.2 mL complete methylcellulose medium in Petri-dishes 3 cm in diameter and incubated at 37°C in 5% CO_2 in a humidified incubator. When cytostatic drugs were added to the cultures, cells were plated at 1 $\times$ 10^4 per 1.2 mL and dish. The cytostatic drugs colchicine (Sigma) or paclitaxel (Taxol^R, Bristol GmbH, München, Germany) were present at increasing doses, as indicated in Results.

Colonies were scored morphologically after 18 to 20 days and counted as granulocyte and/or macrophage colony-forming units (CFU-G, CFU-M or CFU-GM, in an additive manner just CFU-GM) or erythroid burst forming units (BFU-E). Individual colonies were randomly picked for polymerase chain reaction (PCR) (see below). The same procedure was used to transduce FDCP-mix A4 cells, which were maintained as described above. Colony forming ability of FDCP-mix cells in the presence of colchicine was scored by plating in soft-agar medium at a density of 1 $\times$ 10^4/mL and counting living colonies after 14 to 17 days. Liquid culture selections of FDCP-mix cells were performed at 30 ng colchicine/mL. Cells were removed every 24 to 48 hours from these cultures to monitor the percentage of living and Pgp$^+$ cells by flow cytometry.

Flow cytometry. Pgp activity in FDCP-mix cells was determined using the rhodamine 123 (Rh123) efflux assay[4] with minor modifications: staining medium contained 1 mg/mL Rh123. Efflux time was 1 hour to allow quantitative efflux of Rh123 and sensitive detection of Pgp-activity. Propidiumiodide staining was used to monitor dead cells.[27] CD34$^+$ cells were detected by staining with the phycoerythrin-conjugated monoclonal antibody (MoAb), CD34/anti HPCA2 (Becton Dickinson, Heidelberg, Germany), was used according to the manufacturer's instructions. Detection of Pgp with the human-specific MoAb 4E3 (Signet, Dedham, MA) was performed as described.[4] Flow cytometric measurements were performed with a FACSCalibur flow cytometer (Becton Dickinson) at an excitation wavelength of 488 nm, using 530/30-nm (green fluorescence), 585/40-nm (red fluorescence) bandpass filters, and 650-nm (red fluorescence) longpass filters. Statistics were analyzed using CellQuest software (Becton Dickinson).

Single colony PCR. Colonies randomly picked from 14 to 17 days old methylcellulose cultures were washed and lysed. Half of the lysate was subjected to a nested PCR for detection of amplificable genomic DNA using primers specific for the human hematopoietic cell kinase (HCK) gene[28] (5′-TTCCCACTCGCCTCTAAGCC-TCTGATG-3′, 5′-TCGTCCTGCACTGTCTGAGTCCCTTC-3′, 5′-TCCACTGGGCAGATTAGAATCTTTG-3′, 5′-GCGTATTGG-

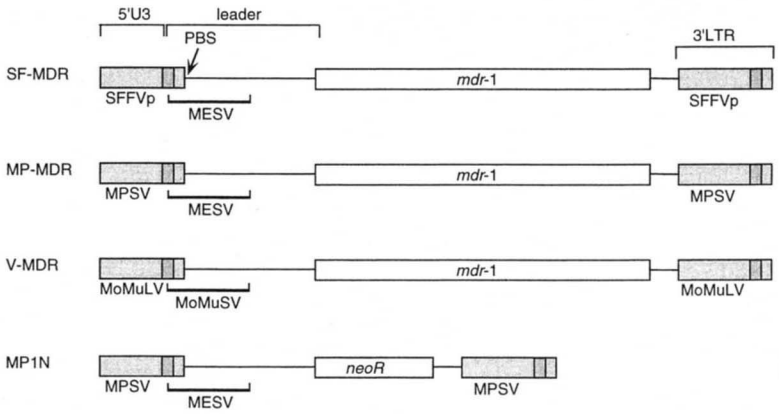

Fig 1. Retroviral vectors used in this study. Shown are proviral forms after genomic integration. SF-MDR, an FMEV-type vector, containing the LTR of SFFVp cl 5.7 and the leader of MESV. MP-MDR (MPEV-type) containing the LTR of MPSV and the leader of MESV. V-MDR (MoMuLV-type) containing the LTR of MoMuLV and the leader of MoMuSV. The arrow points to the position of the PBS.

AACCCTGGGGTTTGTG-3′). HCK$^+$ colonies were then subjected to a second PCR for detection of the presence of retroviral vector U3-sequences with a pair of primers (5′-CGCCATTTTGCAAGGCATGG-3′, 5′-CGCGCGAACAGAAGCGAGAAG-3′) derived from highly conserved parts of the U3-region that detects sequences of SFFVp, MPSV, and MoMuLV present in SF-MDR, MP-MDR, and V-MDR, respectively. The size of the amplification product is 311 bp for SFFVp, 342 bp for MPSV, and 344 bp for MoMuLV. The PCR-program was run 1 minute at 94°C, 1 minute at 62°C, 1 minute at 72°C for 30 cycles and the product was analyzed after electrophoresis in a 1.2% agarose-gel by Southern blotting and hybridization with an internal U3-fragment.

RESULTS

High-dose multidrug resistance in selected human CD34$^+$ progenitor cells transduced with SF-MDR and MP-MDR. We have previously reported that improved *mdr1* expression obtained with FMEV and MPEV results in high-dose chemotherapy resistance in a panel of murine and human hematopoietic cell lines.[4] To address the question whether this would also hold true when targeting primary human HPC, we performed a series of experiments with affinity-purified CD34$^+$ primary human BM cells. Purity of CD34$^+$ human HPC after MACS-separation was in the range of 92% to 98% as determined by flow cytometry (not shown). We first analyzed the sensitivity of untransduced CD34$^+$ human HPC to colchicine and paclitaxel (Taxol^R), two cytotoxic agents transported by Pgp. Paclitaxel had an ID50 close to 10 ng/mL, while the ID50 for colchicine was approximately 5 ng/mL (Fig 2). For both drugs, more than 95% of colonies were suppressed at doses equivalent to twice the ID50, with BFU-E being slightly more resistant than CFU-GM (scored as CFU-G+CFU-M+CFU-GM). Three times the ID50 completely eliminated growth of untransduced cells, a situation equivalent to high-dose treatment of patients with paclitaxel, where peak plasma levels during three-hour infusions range between 20 and 36 ng/mL, followed by severe and dose-limiting myelosuppression[29] (Fig 2).

Because we wanted to compare drug resistance levels achieved with different *mdr1*-transducing retroviral vectors under nonlimiting infectivity, we used a coculture protocol to maximize the transduction efficiency of CD34$^+$ human HPC. The titers of cell-free supernatants of these producers used for coculture ranged from 3 to 5 $\times$ 10^5/mL for SF-MDR and MP-MDR and 1 to 5 $\times$ 10^6 for V-MDR. In the absence of cytotoxic agents, cloning efficiency of human HPC after transduction varied from 8% to 11% when plated at 1,000 cells/1.2 mL and 4% to 6% when plated at 3,000 cells/1.2 mL in colony assays (Table 1). To determine the actual transduction rate on human HPC, integrated viral DNA was measured by PCR in genomic DNA prepared from unselected clonal colonies randomly picked from methylcellulose cultures. Amplifiable DNA was prepared from 80% to 90% of the picked colonies, 50% to 66% of which contained vector sequences (Table 1). Thus, despite the variations in retroviral titer, during coculture equivalent fractions of CFC were transduced in our protocol, indicating that the limiting event was imposed by the target cells. Compared with cells transduced by a vector transferring control cDNA

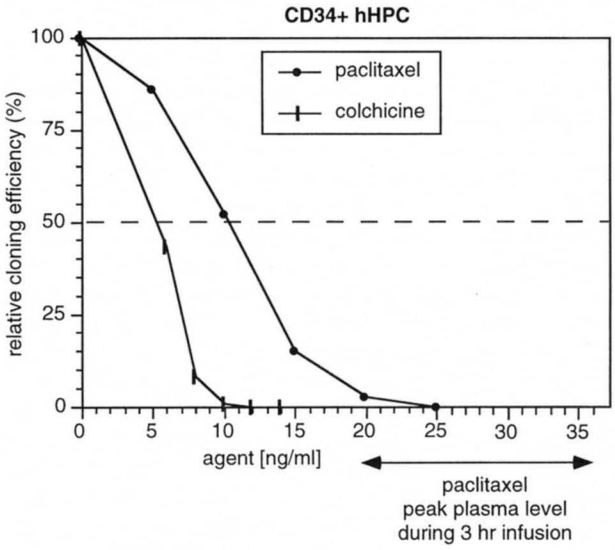

Fig 2. Toxicity of colchicine and paclitaxel on primary CD34$^+$ human HPC assayed in methylcellulose colony assays. The dashed line indicates 50% inhibition of colony formation ability, which was used to determine the ID50 of the drugs. The double-headed arrow delineates the peak plasma level of paclitaxel achieved during a 3-hour infusion of 135 to 175 mg drug/m^2 body surface.[29]

Table 1. Cloning Efficiency Without Selection, But Following Transduction: Fraction of Transduced Clones of CD34⁺ hHPC

Experiment No.	%CD34⁺ Cells*	Vector	Titer†	Cloning Efficiency (%)			PCR⁺ Clones‡
				CFU-GM	BFU-E	Total CFU	
1	96.9	SF-MDR	4×10^5	2.5	2.5	5.0	ND
		MP-MDR	3×10^5	2.1	2.3	4.4	ND
		V-MDR	5×10^6	3.0	1.5	4.5	ND
		MP1N	1×10^6	1.8	1.4	3.3	ND
2	99.1	SF-MDR	5×10^5	2.9	2.5	5.4	14/24 (58%)
		MP-MDR	3×10^5	3.7	2.8	6.5	11/21 (50%)
		V-MDR	5×10^6	3.8	2.8	6.6	10/20 (50%)
		MP1N	1×10^6	3.2	1.4	4.6	4/7 (54%)
3	96.9	SF-MDR	ND	2.9	2.0	4.9	7/14 (50%)
		MP-MDR	ND	4.4	2.8	7.2	6/12 (50%)
		V-MDR	ND	2.9	1.5	4.4	6/9 (66%)
		MP1N	ND	2.5	1.2	3.7	5/7 (71%)

* Purity of CD34⁺ hHPC obtained after separation on MACS-column as determined by flow cytometry using CD34⁺ specific MoAb.

† Retroviral titer of cell-free supernatants obtained from packaging lines used for coculture experiments was determined by limiting dilution on GP&env 86 fibroblasts and subsequent colchicine selection. Colonies were counted after 14 days.

‡ PCR for detection of integrated vector-sequences was performed on genomic DNA prepared from randomly picked colonies.

(neoR), cloning efficiency and colony distribution in the absence of cytotoxic agents was not altered, indicating that *mdr1*-transduction had no effect on the cloning efficiency.

The most sensitive assay to monitor the strength of the multidrug-resistant phenotype obtained after transduction is to plate cells at increasing doses of cytotoxic agents transported by Pgp. The dose-response of the plating efficiency significantly correlates with the expression levels and the activity of Pgp.[4] When we exposed transduced cells to 16 ng colchicine/mL, controls died, whereas V-MDR transduced cells were reduced in their plating efficiency to 2% of unselected cells corresponding to roughly 4% of infected cells (Fig 3A). Transduction with SF-MDR or MP-MDR elevated the cloning efficiency in comparison to V-MDR by a factor of 6 and 5, respectively. At 24 ng colchicine/mL, the cloning efficiency following selection of cells transduced with SF-MDR was more than 10 times higher than that of V-MDR transduced cells. MP-MDR protected sixfold better than V-MDR (Fig 3A). Three consecutive experiments were performed with varying preparations of CD34⁺ cells, but under the same transduction conditions. Cells were plated at doses of 12, 18, and 24 ng/mL colchicine. The ratios observed in the initial experiment remained constant, leading to a significantly elevated cloning efficiency of cells infected with SF-MDR, irrespective of the drug dose. The biggest advantage over V-MDR transduced cells was seen at the highest dose (24 ng/mL) with a 20-fold increased plating efficiency of SF-MDR infected cells. The absolute numbers of clonogenic cells obtained with SF-MDR at high doses were always higher than those seen with V-MDR at the lowest dose. The differences between SF-MDR and V-MDR transduced cells were highly significant, irrespective of the drug dose ($P < .05$ according to t-test). MP-MDR again was less active than SF-MDR, resulting in leakiness of chemoprotection especially at higher doses, but it still clearly exceeded selection efficiency of V-MDR infected cells, with a highly significant difference at selection with 24 ng colchicine/mL. In addition, the size of colchicine resistant clones was generally larger with cells infected with SF-MDR and MP-MDR than with V-MDR.

Colony morphology was somewhat altered in the presence of colchicine resulting in less intense hemoglobinization in cells arising from BFU-E and reduced spread and altered size of cells arising from CFU-GM (CFU-G+CFU-M+CFU-GM). Colony morphology was best preserved upon transduction with SF-MDR, irrespective of the drug dose, and at medium doses also with MP-MDR. Morphological screening at 12 ng/mL of colchicine revealed similar distribution of CFU-GM and BFU-E as in the absence of cytostatic drugs. Thus, no gross preference for protection of individual lineages was apparent. However, because at higher doses morphological discrimination became difficult especially with V-MDR transduced cells, data given in Fig 3 only reflect the total numbers of CFUs (BFU-E + CFU-GM).

When cells were plated in the presence of paclitaxel, residual control colonies could be detected with up to 30 ng/mL, but these were only small clusters of mainly dead cells at the time of observation (Fig 3B). Again, SF-MDR and MP-MDR protected clearly better than V-MDR, resulting in excellent detoxification even under moderately increased (MP-MDR) or high doses of taxol (SF-MDR). The ratios between the different classes of vector infected cells were almost identical to those found with colchicine treatment.

An excellent internal control for measuring indirectly the transcriptional strength of the vectors is the ratio of the plating efficiencies at high doses (30 ng, ie, 3 times the ID50) in comparison to low doses (15 ng, ie, 1.5 times the ID50). This ratio was 29% for SF-MDR, 12% for MP-MDR, and only 4% for V-MDR. In comparison with the neoR-control, V-MDR did not significantly increase the plating efficiency of infected cells at high doses of paclitaxel. This value corresponded to peak plasma levels obtained during a 3-hours infusion in patients undergoing cancer chemotherapy (see Fig 2). V-MDR mediated significant protection against paclitaxel only at 15 ng/mL, which is not strongly myelosuppres-

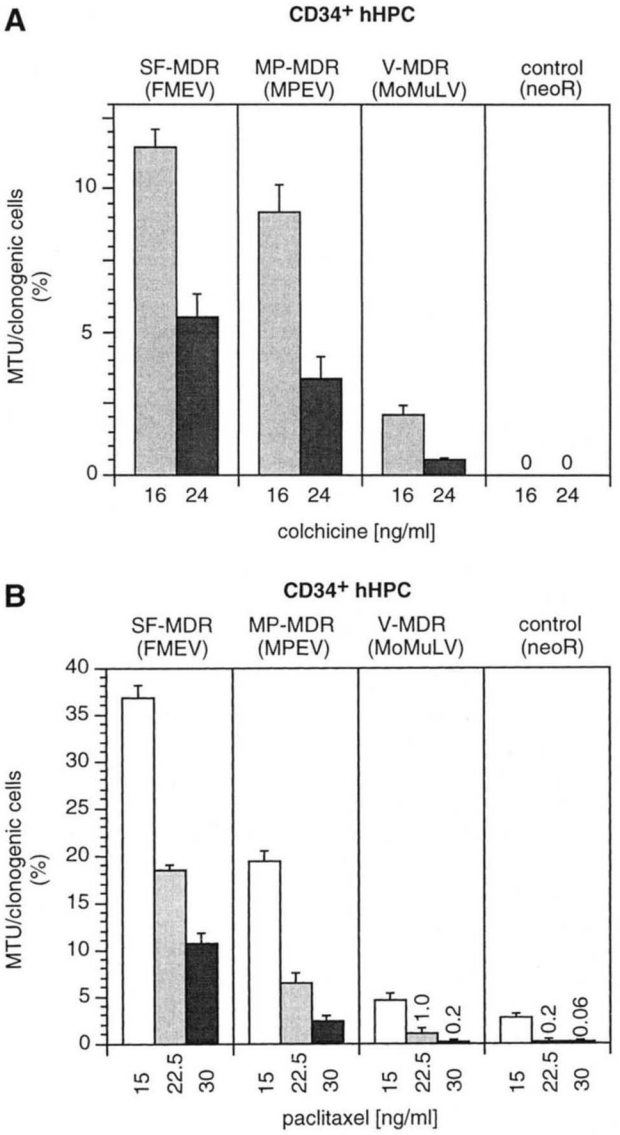

Fig 3. Optimized retroviral FMEV and MPEV vectors transfer high-dose multidrug resistance to primary CD34⁺ human HPC. (A) The fraction of multidrug-resistant units (MTU) obtained at increasing drug doses per clonogenic cells obtained in absence of cytostatic agents. Error bars indicate standard deviations of triplicate platings. Consecutive experiments with varying preparations of CD34⁺ cells gave essentially equal results. (B) Only SF-MDR (FMEV) and MP-MDR (MPEV) mediate resistance against high doses of paclitaxel corresponding to chemotherapy peak plasma levels. Assay conditions were basically identical to those described in (A).

sive and might therefore correspond to a subtherapeutic dose in vivo.

Colony morphology under paclitaxel was better than under colchicine. Especially in cells transduced with SF-MDR and MP-MDR, the majority of resistant colonies could be reliably differentiated at the morphological level. Again, no obvious difference was observed in the lineage distribution when compared with unselected cells; V-MDR protected colonies were again significantly smaller in size and more compromised in morphology (see above).

When cell-free supernatants were used for transduction, the frequency of transduced CFU was in the range of 3% to 14% as determined by single colony PCR. Although the highest infectivity was observed with the high-titer supernatant of V-MDR, the ratios of drug-resistant colonies and the responses to increasing doses of drug were equivalent to those observed after coculture infection (not shown).

Only SF-MDR and MP-MDR mediate background-free selectability against cytotoxic agents in the early multipotent hematopoietic cell line, FDCP-mix A4. Methylcellulose assays of human HPC reflect primarily late lineage-committed progenitor cells that have lost self-renewal capacity. To study the expression efficiencies of different *mdr1*-vectors on more early, multipotent myeloid cells, we analyzed chemoprotection levels achievable on early passages of the murine FDCP-mix A4 stem cell line.

As a consequence of the primitive differentiation state, FDCP-mix A4 cells have a rather high endogenous expression of murine Pgp, resulting in survival of clones even under doses higher than 15 ng/mL colchicine. We cocultured FDCP-mix A4 on the same producer lines as those used for transduction of human HPC and plated cells in soft-agar without drug or with increasing doses of colchicine. Twenty-four hours after release from coculture and before starting drug-selection, cells were analyzed by flow cytometry for Pgp-function using the Rh123 efflux assay under highly sensitive conditions. The number of *mdr1*-transduced cells determined by flow cytometry was in the range of 3% to 4% after coculture and before selection, with no significant differences between the vectors detected in this assay (Table 2). When cells were plated in colony assays at increasing doses of colchicine, even at 20 ng/mL more than 1% of control (neoR-vector infected) clonogenic cells survived when scored microscopically 14 to 17 days after plating. However, between 20 and 22 ng of drug, colony formation was heavily suppressed (Table 2). At first glance, *mdr1*-transduction by the different vectors did not lead to dramatic differences in clonability except when cells were plated at 24 ng/mL colchicine. Then an order of magnitude increase of multidrug-resistant units were observed with SF-MDR and MP-MDR in comparison with V-MDR. Despite the presence of 3% *mdr1*-transduced cells according to flow-cytometric criteria, the number of selectable cells infected with V-MDR did not significantly exceed those infected by the neoR-control vector (Table 2). The expression level of *mdr1* achieved with V-MDR in clonogenic cells therefore was not sufficient to withstand colchicine-cytotoxicity. In contrast, 1% of the calculated clonogenic cells survived at 24 ng/mL when infected with SF-MDR and MP-MDR, here referred to as multidrug-resistance–transduced units (MTU) (Table 2). With respect to the rate of Pgp⁺ cells detected by flow cytometry before exposing cells to colchicine, this corresponds to roughly 25% of transduced and vector-expressing cells.

Retroviral transduction efficiency in FDCP-mix A4 cells could also reflect the probability of preferential infection of differentiating colony-forming cells, unable to self-renew during prolonged passage.[23] Therefore, we randomly picked 10 clones surviving soft-agar selection at 20 ng/mL colchi-

Table 2. SF-MDR and MP-MDR Mediate Long-Term Survival of Clonogenic Early Myelo-Erythroid Progenitor Cells FDCP-Mix A4 in the Presence of High Doses of Colchicine

Vector (type)	Rh123-Efflux$^+$ (unselected)*	MRU (%)† With Colchicine at:			MTU (%)‡ With Colchicine at:			Long-Term Survival§
		20 ng/mL	22 ng/mL	24 ng/mL	20 ng/mL	22 ng/mL	24 ng/mL	
SF-MDR (FMEV)	4.3 ± 0.64	4.9 ± 1.17	2.5 ± 0.87	1.3 ± 0.6	3.2	2.3	1.23	7/10
MP-MDR (MPEV)	3.6 ± 0.45	3.9 ± 0.4	1.7 ± 0.89	1.0 ± 0.32	2.2	1.5	0.93	8/10
V-MDR (MoMuLV)	3.9 ± 1.78	2.9 ± 0.55	0.7 ± 0.29	0.13 ± 0.16	1.2	0.5	0.06	0/10
MP1N (control)	0.6 ± 0.78	1.7 ± 0.91	0.2 ± 0.21	0.07 ± 0.03	0	0	0	0/10

* *mdr1*$^+$ cells as determined by flow cytometry using the Rh123-efflux assay (see Materials and Methods). Mean values ± SD of three experiments performed 24 hours after coculture and before subjecting cells to colchicine.

† MRU, multidrug-resistant colonies after retroviral transduction with the different vectors in percent of clonogenic cells obtained in the absence of colchicine. Only viable colonies were counted 2 weeks after plating in soft-agar. Mean values ± SD of three assays.

‡ MTU, multidrug-resistant transduced units, were determined as MRU obtained with mdr1-transducing vectors SF-MDR, MP-MDR, or V-MDR minus MRU of control infected cells (MP1N).

§ Long-term survival in the presence of colchicine was assessed by transfering 10 randomly picked MRU for each vector into liquid culture medium containing 20 ng/mL colchicine and propagating clones for 3 weeks under these conditions. Proliferating clones surviving this procedure are named long-term survivors.

cine and propagated these in liquid culture medium containing the same dose of colchicine. Continued survival and proliferation during this procedure gives us an indication of colonies that are derived from early blast cells which have self-renewal capacity. Importantly, 70% to 80% of SF-MDR and MP-MDR transduced colonies showed sustained proliferation in liquid culture, whereas none of the V-MDR or control-vector transduced colonies survived. When unselected cells were plated after transduction in liquid culture containing 30 ng colchicine/mL, the resulting survival curve as assessed by flow-cytometric detection of living cells reflected an absolute advantage for chemoprotection of FDCP-mix cells with SF-MDR or MP-MDR as compared with those transduced by V-MDR (Fig 4).

The decrease of the number of viable cells obtained between days 2 and 9 could be explained by the generally low transduction frequency in clonogenic FDCP-mix cells (see also Table 2). SF-MDR as well as MP-MDR transduced cells grew out by day 10 to produce stable and normal proliferating cultures under selection, whereas neoR (MP1N) or V-MDR transduced cells died off. The surviving SF-MDR/MP-MDR infected cells were Pgp$^+$ as determined by the Rh123 efflux assay (not shown). The Pgp activity intimately correlated with vector-derived expression of human Pgp, as was shown by flow cytometric analysis after staining MoAb 4E3, which is specific for human Pgp (Fig 4). Thus, only SF-MDR and MP-MDR, the two vectors optimized for high gene expression in early hematopoietic cells, mediated background-free selection and long-term survival in the presence of truely cytotoxic drug doses in this system.

DISCUSSION

We have reported on the development of novel retroviral vectors termed FMEV and MPEV designed to achieve potent gene expression in early hematopoietic cells. When tested on a panel of murine and human hematopoietic progenitor cells, *mdr1*-transduction with these vectors impressively improved chemoprotection and this difference was most pronounced at high doses of cytotoxic agents.[4,5] We report here that primary, lineage-committed human HPC transduced by FMEV and MPEV are similarly protected at very high levels. We further provided evidence that MPEV, and even more so, FMEV could confer an absolute advantage for selection of *mdr1* vector infected immature, multipotent hematopoietic progenitor cells. Chemoprotection mediated by SF-MDR (FMEV-type vectors) and MP-MDR (MPEV) resulted in a four to sixfold increased plating efficiency at moderate doses of cytostatic agents colchicine and paclitaxel, compared with that provided by a conventional MoMuLV-based vector currently used in clinical trials.[12,15] The advantage in chemoprotection with SF-MDR, and somewhat less effectively, also with MP-MDR was most pronounced when drug doses were increased in vitro to obtain peak plasma levels which are achieved during a conventional three-hour infusion of paclitaxel in patients. Likewise, when targeting FDCP-mix cells representing immortalized, CFU-S equivalent myelo-erythroid progenitor cells, only SF-MDR and MP-MDR allowed background-free selection with increased doses of cytotoxic agents. Thus, this study shows that the experimental system used for vector development was predictive for primary human HPC. Simultaneously, it represents the first systematic and comparative study of vector-mediated cancer drug resistance in primary human HPC, and, as such, provides important preclinical data needed toward successful establishment of the stem cell protection approach in patients.

Previous studies with *mdr1*-transducing vectors with cis-regulatory elements of MoMuLV or HaMuSV have clearly established that retroviral transduction with *mdr1* resulted in a dominant selectable phenotype in vitro and, in the murine system, also in vivo.[12-21] No significant differences in *mdr1*-expression were observed in this system between cells transduced with HaMuSV-based or MoMuLV-based vectors.[12,15,20] Both vectors were accepted for clinical trials.[30,31] Why should there be need for further improved vector-mediated chemoprotection?

The present study clearly shows that chemoprotection with a nonoptimized vector is leaky. Residual toxicity of cytotoxic agents resulted in markedly reduced cloning efficiency and reduced proliferation capacity of transduced cells, especially with high doses. In contrast, the overall number of protected

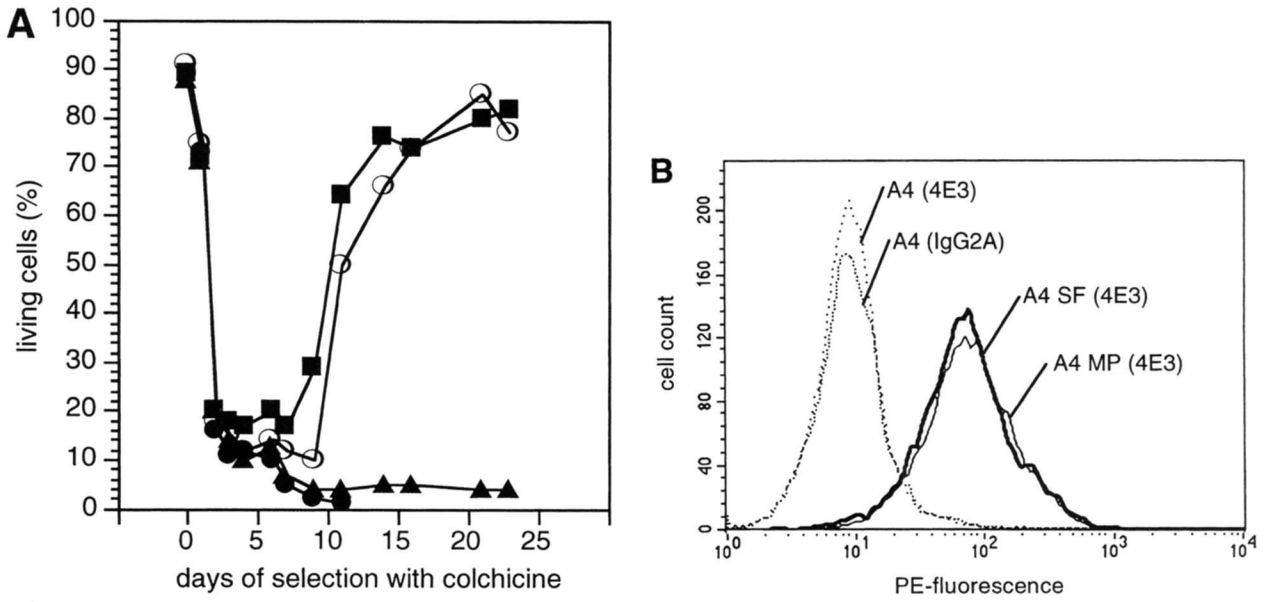

Fig 4. Background-free selectability of early multipotent myeloid progenitor cells FDCP-mix A4 requires transduction with optimized retroviral vectors (■) SF-MDR or (○) MP-MDR. (A) Long-term survival of transduced FDCP-mix cells in liquid culture containing 30 ng colchicine/mL. (●) MP1N and (▲) V-MDR transduced cells do not show self-renewal. The percentage of living cells in the culture was determined by flow cytometry using propidiumiodide staining. The percentages of *mdr1*-transduced cells as determined by flow cytometry (Rh 123 efflux$^+$ cells) are shown in Table 2. (B) Multidrug resistance in FDCP-mix A4 cells correlates with vector-mediated expression of the human Pgp detected by MoAb 4E3. Staining with isotype control MoAb IgG2A is shown as control. Untransduced cells are negative for human Pgp. A4 SF and A4 MP: descriptions for mass cultures of FDCP-mix A4 transduced with SF-MDR and MP-MDR, respectively. The assay was performed at day 14 of the selection experiments shown in (A).

progenitor cells as well as the quality of chemoprotection was significantly elevated if optimized vectors were used. In fact, only the optimized vectors mediate true high-dose multidrug-resistance in the in vitro model. This suggests that the primary goal of the chemoprotection approach can only be achieved upon careful elaboration of the vector design. In stem cell protection, highly efficient detoxification is needed in both early and late hematopoietic progenitor cells to guarantee benefit from the gene transfer.[32] Enhancing the protection level in late progenitor cells like those monitored in the methycellulose-assays of human HPC in the present study is important to counteract the dose-limiting short-term myelotoxicity of cytostatic agents used in anticancer-chemotherapy. Depending on the number of protected progenitors, this will mitigate or even abrogate the myelopoietic decline after chemotherapy, hopefully leading to a definite palliative advantage for the patient. Equally important is efficient transduction of even more primitive, multipotent progenitor cells with self-renewal properties. Provided that this compartment will become drug resistant even in the high-dose situation, the gene therapy approach offers striking advantages in comparison with autologous support using untransduced human HPC. First, a well protected stem cell pool will guarantee intact hematopoiesis even with repeated application of chemotherapy. Under these circumstances, intensification of drug regimens might be possible for optimization of tumor-cytotoxicity as long as the nonhematopoietic toxicity is not dose-limiting. Second, only gene transfer mediated chemoprotection of early progenitors can result in a reduced incidence of long-term myelotoxic side effects like myelodysplasia or induction of secondary hematopoietic malignancies by chemotherapy.

Even if the transfer frequency to early human HPC can be improved, in vivo selection of transduced HPC might still be needed in a number of gene transfer protocols intending to correct inherited metabolic disorders involving hematopoietic cells, eg, lysosomal storage diseases or hemoglobinopathies, or to counteract HIV-infection. The results provided in the present study indicate that background-free selection with *mdr1* as a marker can be performed with the use of FMEV and MPEV in vitro. Selection has been possible even with vectors based on MoMuLV or HaMuSV in vivo, but the putative therapeutic window might be very small in the murine model systems.[15,17,19,20] The relatively high Pgp-level in undifferentiated, untransduced stem cells raises doubts whether safe and background-free selection is possible with vectors not being optimized for gene expression in these cells. Improving expression of, eg, the *mdr1* gene in human HPC with vectors like FMEV and MPEV should significantly aid not only with respect to the in vivo selectability, but also in the expression of a second therapeutic gene. Cotransfer of a second gene along with *mdr1* can be effectively performed with the FMEV and MPEV backbones, as is it was described for conventional vectors[33] (Baum, unpublished data, February 1996).

Although not formally shown in this study, for the following reasons we anticipate that FMEV and MPEV will also prove to be superior to conventional retroviral backbones

for gene expression in early, long-term repopulating HPC: (1) In our in vitro model, the advantageous properties of the cis-acting elements included in FMEV and MPEV became most evident with an increasing developmental primitivity of the cells under investigation.[4,5] (2) Expression of the human alkyltransferase gene from MPEV efficiently protects long-term culture-initiating cells from repetitive nitrosourea toxicity.[34] (3) It has recently been reported that SFFVp-based vectors allow strikingly sustained multilineage gene expression within the murine hematopoietic system in vivo.[35] Because FMEV use the same potent enhancer, and combine it with a fully permissive leader sequence including a nonrestrictive PBS, they should further exceed the transcriptional potency of SFFVp-based vectors. Alterations of the PBS are indeed associated with improved gene expression from retroviral vectors in HPC in vivo.[7] (4) In contrast to conventional MoMuLV-related vectors, both MPEV and FMEV efficiently express genes in undifferentiated totipotent embryonic stem cells, which share transcriptional characteristics with hematopoietic stem cells[4,9] (Baum et al, manuscript in preparation).

To definitely address the chemoprotection quality in immature long-term repopulating HPC, we have recently initiated a series of comparative in vivo experiments in a murine model system. Preliminary data indicate that chemoprotection with the improved vectors enables dose-escalation of paclitaxel without dose-limiting myelotoxicity in mice. However, serial transplantations similar to those performed with V-MDR transduced cells[15] have to be awaited to ensure that the enhanced expression levels of Pgp after transduction with SF-MDR and MP-MDR are compatible with the biological function of the hematopoietic target cells in vivo.

A consistent observation in our earlier studies was that the retroviral titers obtained with FMEV and MPEV were generally up to one order of magnitude reduced when compared with MoMuLV-based vectors. The most likely explanation for this phenomenon is a sequence variation affecting the packaging signal derived from the MESV-based leader.[36] This sequence was preserved in MPEV and FMEV backbones, because we obtained evidence for a second putative repressor element colocalizing with the packaging sequences of MoMuLV, but not MESV.[36] Further dissection of these elements will hopefully allow generation of vectors that avoid transcriptional repression while maintaining all cis-regulatory elements needed to achieve high-titer supernatants. Already, the titers obtained with the current generation of FMEV and MPEV should be sufficient to achieve clinically useful transduction rates with cell-free supernatants under optimized transduction conditions, eg, with the use of autologous stroma,[37] or flow-through transduction.[38]

On condition of encouraging results obtained with the murine in vivo model, FMEV and MPEV will presumably soon enter the phase of clinical investigation.

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