A Bicistronic Therapeutic Retroviral Vector Enables Sorting of Transduced CD34+ Cells and Corrects the Enzyme Deficiency in Cells From Gaucher Patients

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Corrective gene transfer for therapeutic intervention in metabolic and hematopoietic disorders has been hampered by the relatively inefficient transduction of human hematopoietic stem cells. To overcome this, a bicistronic recombinant retrovirus has been generated that delivers both a therapeutic glucocerebrosidase (GC) cDNA for the treatment of Gaucher disease, and a small murine cell surface antigen (heat-stable antigen [HSA]) as a selectable marker. An amphotropic retroviral-producing cell clone was created, and filtered supernatant was used to transduce NIH 3T3 cells. Sorting of transduced cells by flow cytometry enabled separation into populations based on cell surface fluorescence intensity derived from the expressed HSA. Significant increases in GC enzyme activity were seen for the transduced and especially the transduced and sorted cells. Similarly, increases in GC specific activity were seen in transduced and sorted skin fibroblasts from a patient with Gaucher disease. To streamline future transfer and sorting protocols for hematopoietic cells, transformed B-cell lines from Gaucher patients were created. Type I B cells were transduced and sorted, and large increases in GC specific activity occurred with concomitant increases in integrated retroviral copy numbers. In addition, toward the goal of using this selectable approach for corrective gene transfer to bone marrow stem cells, CD34+ cells were isolated from normal BM donors, transduced, and sorted based on cell surface expression of HSA. Proviral DNA was detected in approximately 40% of clonogenic progenitor colonies derived from unsorted, transduced CD34+ cells, demonstrating the high titer of the vector. However, after sorting, 100% of the colonies had the corrective GC cDNA, demonstrating the efficiency of this selective system for human hematopoietic progenitors. It is expected that strategies based on this approach will allow sorting of transduced cells of many types before implantation of transduced cells to animals or patients. This vector system may also be used to simplify manipulations and studies on retroviral-mediated gene delivery in vitro and for in vivo models.

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RETROVIRAL VECTORS have been used extensively to transfer genes into a variety of cells to investigate the possibility of gene therapy for many disorders. The widespread utility of recombinant retroviruses has been somewhat constrained, though, by the fact that they do not transduce nondividing cells. Transduction of human hematopoietic stem cells has been relatively inefficient, probably because most of these stem cells are quiescent and possibly because specific receptors for current viral vectors are underrepresented on these cells. Approaches to a solution to the problem of infecting early hematopoietic progenitors have included studies of fine-tuning vector delivery techniques to increase the percentage of transduced cells. Additions of growth factors and stromal support to increase cell cycling have shown increased calculated transduction efficiencies of hematopoietic progenitor and stem cells.1–10 Another approach is to select cells that have been transduced by the recombinant retrovirus based on a distinguishing characteristic. One way to achieve this goal is to include a gene in the vector that enables the transduced cell to be resistant to toxic drugs. As an example, the gene encoding the neomycin phosphotransferase protein has been used extensively to track vector integration and expression and as a selective agent based on acquired resistance to added G418.11 The gene for the multidrug resistance (MDR) efflux pump protein has also been used to protect hematopoietic cells from challenge by various drugs.12,14 Further, in a bicistronic plasmid construct, a therapeutic glucocerebrosidase (GC) gene was included with the coding sequence for the MDR efflux pump protein in transfection studies,15 allowing drug selection of transfected cells, although no actual transduction of cells by recombinant retrovirus was demonstrated in that case.

Another strategy to recognize a distinguishing characteristic of transduced cells has been to include a cell surface antigen cDNA in the recombinant retrovirus construct to allow noninvasive separation of cells due to the expressed surface marker. The practicality of this idea was demonstrated as early as 1988,16 although the overall effectiveness of this approach for corrective gene transfer was limited, because cell surface antigens initially chosen as markers were sizeable and addition of a therapeutic gene would have generated a large vector and potentially adversely affected virus titer. Effects of insert size on recombinant retroviral titer have been demonstrated.17 cDNA for the MDR efflux pump has also been used as a cell surface–selectable marker in retroviral constructs,20,21 as has the interleukin-2 (IL-2) receptor22 and the low-affinity nerve growth factor receptor23 in cell-marking studies only. All of these genes are also relatively large, thus possibly limiting their broad utility in therapeutic vectors containing two genes.

An ideal cell surface marker would have low expression on the target cells to keep background reduced, and would react well with characterized antibodies to allow rapid and efficient separation of transduced cells. Further, the cDNA
sequence would be small enough that addition of it to the therapeutic recombinant construct would not significantly alter the titer of the virus produced. Good candidates that meet these criteria are the human hematopoietic cell marker CD24 and its murine homologue, the heat-stable antigen (HSA). These small (~30 amino acids) surface molecules are GPI-linked cell surface glycoproteins and have been isolated based on homology of signal peptide regions, although the mature polypeptides of CD24 and HSA have little amino acid identity. Additionally, antibodies to one do not cross-react with the other. Indeed, a nontherapeutic bicistronic retroviral construct containing the human CD24 antigen has been used to successfully sort transduced and marked primitive murine hematopoietic cells, including those with long-term repopulating ability.

This report describes the generation of bicistronic retroviral vectors that contain the murine HSA cDNA as a selectable marker and a therapeutic human cDNA for the treatment of metabolic deficiency in Gaucher disease. Gaucher disease is a lysosomal storage disorder manifested in hematopoietic cells that is caused by a deficiency in the lysosomal enzyme GC. It is classified as type I, II, and III largely depending on the degree of neurologic involvement. It is demonstrated here that this bicistronic vector system provides for selection and sorting of transduced hematopoietic and nonhematopoietic cells from enzyme-deficient Gaucher patients based on cell surface HSA expression, and further that these cells are corrected for the enzymatic defect. Transfer and selection of transduced human CD34+ cells is also demonstrated, and the presence of the vector in all progenitor colonies examined was observed. This methodology will greatly facilitate identification and isolation of transduced cells for gene transfer and marking experiments in animals and also for gene therapy protocols for humans.

MATERIALS AND METHODS

Plasmid constructions. The human GC cDNA (herein called LGs) was liberated from plasmid p1-GC-BS (kindly supplied by D. Kohn, Division of Research Immunology/BMT, Children's Hospital Los Angeles, Los Angeles, CA) as an XhoI-XhoI fragment and subcloned into plasmid pLGs. A ClaI-ClaI fragment containing the encephalomyocarditis virus internal ribosomal entry site (IRES) sequence (from plasmid pLVSNP; kindly supplied by A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) and the murine HSA cDNA sequence was then added to pLGs to produce vector pLGsEH (Fig 1). All cloning orientations and junctions were confirmed by restriction digests and by DNA sequencing.

Cell lines and virus production. BOSC 23 cells were kindly supplied by W. Pear (Rockefeller University, New York, NY) and were grown as previously described. These cells were transiently transfected with purified pLGsEH using a calcium phosphate protocol, and ectropic virus-containing supernatant was collected. This supernatant was filtered through a 0.45-µm filter and repeatedly administered to amphotropic virus-producing GP+envAM12 cells (referred to as AM12) that were grown in Dulbecco's Modified Eagle Medium (Biofluids Inc, Rockville, MD) with 10% newborn calf serum (GIBCO-BRL, Gaithersburg, MD) and penicillin/streptomycin at 37°C with 5% CO2. The multiply transduced AM12 cells were then diluted to single-cell density and cloned and expanded. GC activity and flow cytometry profiles for numerous candidate clones in comparison to background controls were obtained (Fig 2). Supernatants from high-titer AM12/LGsEH clones were filtered and used in subsequent infections with added 8 µg/mL hexadimethrine bromide (Polybrene; Sigma, St Louis, MO).

NIH 3T3 (tk–) cells were grown under standard conditions. Titers
of supernatants from AM12/LGsEH clones no. 6 and 8 on 3T3 cells were estimated to be at least 10^5 infectious U/mL as measured by comparative Southern blots (see the results).

**Helper virus detection.** Marker rescue assays with multiple serial dilutions were used to attempt to detect replication-competent virus. No replication-competent virus was found at any dilution. Briefly, 3T3/GINa cells (kindly supplied by C. Dunbar) containing a replication-defective provirus harboring the neo^R^ marker were used as hosts and were infected with five serial dilutions of filtered viral supernatants (assayed in duplicate) and passaged for 2 weeks to allow potential virus to spread. Supernatants were then collected and used to infect virgin 3T3 (tk−) cells with GP + AM12HSATKneo cell supernatant¹⁷ serving as a positive control. Selection was then performed with G418 (Sigma), and drug-resistant colonies were scored 2 weeks later (data not shown).

**Gaucher patient cells.** Skin fibroblasts obtained from Gaucher patients under an approved National Institutes of Health protocol were maintained in McCoy’s 5A media (BioWhittaker, Inc, Walkersville, MD) with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were infected six times overnight with AM12/LGsEH pool supernatants in the presence of 4 μg/mL protamine sulfate (Elkins-Sinn, Inc, Cherry Hill, NJ), cultured, and then tested against normal and infection controls for GC expression and cell surface antigen expression, cells were washed once in a solution (HFN) that allows differentiation of the band from the added cDNA sequence.

**Immortalized B-cell lines were made from peripheral blood obtained from Gaucher type I, II, and III patients.** Briefly, 5 × 10⁶ peripheral blood lymphocytes were incubated with 0.5 μL crude supernatant from an EBV-producing marmoset cell line (B95-8; American Type Culture Collection [ATCC], Rockville, MD) in a total of 5.0 mL media containing RPMI 1640 (Biofluids), 15% FBS, penicillin/streptomycin, and a 1:10,000 dilution of OKT3 (ATCC) ascites fluid. The cells were then subjected to fluorescence-activated cell sorter (FACS) scan and sorted (as below).

**CD34⁺ cells.** Bone marrow (BM) from normal donors was collected under a National Institutes of Health protocol, and mononuclear cells were isolated by centrifugation on LSM lymphocyte separation medium (Organon Teknika-Cappel, Durham, NC). CD34⁺ cells were then isolated by the Cellpro LC system (Cell Pro, Inc, Bothell, WA) and transduced four times with viral supernatant in the presence of stromal cells,¹² protamine sulfate (4 μg/μL), IL-3 (20 ng/mL), IL-6 (50 ng/mL), and SCF (100 ng/mL). Cells were plated as previously described²⁻¹² and incubated for about 2 weeks. Colonies were scored and picked into a PCR preparation buffer²⁸ and analyzed by PCR as above.

**Southern blot analysis and polymerase chain reaction.** DNA was purified from cells using a genomic DNA isolation kit (Qiagen, Chatsworth, CA) and the manufacturer’s protocols. Genomic DNA was digested with restriction enzyme NheI (New England Biolabs, Beverly, MA), which cuts in both the 5’ and 3’ retroviral LTRs. After separation by electrophoresis through agarose gels, DNA fragments with copy number controls were transferred to Hybond N membranes (Amersham, Arlington Heights, IL) and probed for the presence of human GC cDNA sequence and/or Moloney murine leukemia virus sequence with appropriate ³²P-labeled random-primer DNA probes.

For analysis of transduced and control skin fibroblasts, a comparative polymerase chain reaction (PCR) technique was used to establish the presence of the cDNA insert in the genomic DNA pool, because the amount of recovered genomic DNA was limiting. Amplification was performed with primers located within the GC coding sequence, allowing differentiation of the band from the added cDNA sequence and of the genomic GC DNA sequence. PCR products, after electrophoretic separation, were transferred to membranes and probed with specific probes (as above).

**In vitro clonogenic progenitor assays.** Sorted and control cells were plated as previously described²³⁻²⁵ and incubated for about 2 weeks. Colonies were scored and picked into a PCR preparation buffer²⁸ and analyzed by PCR as above.

**RESULTS**

**The LGsEH vector.** A recombinant retroviral vector that encodes a bicistronic message containing the therapeutic human GC cDNA and the murine HSA as a selectable marker in a MoMLV backbone is diagramed in Fig 1. This construct uses a "short" form of the human GC cDNA,⁵ which is different from constructs previously used in this laboratory.¹⁶ The IRES sequence from the EMCV was included to enhance translation of the downstream HSA cDNA in the bicistronic construct. The respective orientation of the coding sequences was selected to possibly optimize the therapeutic GC enzyme expression. It follows that if protein expression from the downstream cDNA is reduced compared with that derived from the upstream cDNA due to synthesis of less than full-length mRNA, cells that have been selected
and sorted on the basis of highest HSA levels will likely also have the highest expression of GC enzyme.

High-titer amphotropic virus-producing cell lines from single-cell isolates were identified by the presence of increased GC enzyme expression and HSA surface expression versus levels in untransduced AM12 cells. Figure 2A shows that in clonal isolates no. 6 and 8, GC enzyme specific activity (nanomoles per hour per milligram protein) was found to be 9,504 ± 550 and 8,270 ± 721, respectively, representing a more than 30-fold increase in enzyme specific activity over background levels seen in nontransduced AM12 cells. Similarly, surface expression of the HSA antigen was greatly increased over background (Fig 2B, shown for the no. 8 isolate), and these cells have the same degree of fluorescence shift and narrow peak shape as the positive control GP + AM12HSATKneo viral producer cells that were originally selected on the basis of resistance to G418.

A Southern blot analysis was performed on genomic DNA prepared from AM12/LGsEH isolate no. 8. A single proviral band was seen at the expected size of 4.2 Kbp, which was found to be present in approximately 10 copies per cell (data not shown). This AM12/LGsEH isolate no. 8 was then used as the amphotropic virus producer for subsequent studies. Marker rescue assays demonstrated that this clone contained no helper virus at any dilution.

Sorting of transduced NIH 3T3 cells. NIH 3T3 (tk−) cells were transduced once with the AM12/LGsEH virus supernatant and then subjected to FACS. Figure 3A shows flow cytometric analysis of cell surface HSA expression. Cells were fractionated into categories based on HSA surface-staining intensity, expanded, and then analyzed for GC enzyme activity and for the presence of viral DNA. Figure 3B shows results of the GC enzyme assay. Cells expanded from the top 30% fluorescence intensity pool (M1) showed the highest GC enzyme specific activity (2,918 ± 78 nmol/h/mg protein), representing a 4.6-fold increase over enzyme activity observed in the unsorted pool and a full 11.6-fold increase over background 3T3 GC enzyme activity levels. Cells expanded from the M2 pool (top 31% to 80% fluorescence intensity) also showed increased enzyme activity (two-fold) over the unsorted pool.

A Southern blot analysis was performed on genomic DNA samples isolated from the various FACS fractions (data not shown). The intensity of the band in the transduced unsorted fraction indicated at least one copy of vector per cell, on average. Correll et al.18 found that neo<sup>+</sup> gene vector clones with titers of 2 × 10<sup>6</sup> to 1 × 10<sup>8</sup> CFU/mL produced 0.6 to 1.2 vector copies per cell in transduced 3T3 cells, and that no signal at all was detected by Southern blot if the titer was less than 5 × 10<sup>5</sup> CFU/mL. This would indicate that the clones presently described have high titer (probably >10<sup>6</sup> CFU/mL), although a direct measurement cannot be made, since the vector lacks a drug-selectable marker. An increase in band intensity in the lane loaded with genomic DNA isolated from sorted cells was found to correlate directly with the increased enzyme and cell surface antigen expression observed. It is likely that the portion of cells with the greatest fluorescence shift is derived from cells that took up and integrated multiple copies of the recombinant retrovirus, and not from a large number of cells in which extreme overexpression of the transgene occurred.

Sorting of transduced Gaucher skin fibroblasts. Gaucher skin fibroblasts were cultured and then transduced with the AM12/LGsEH supernatant. Since the fibroblasts are relatively slow-growing, they were transduced multiple times with viral supernatants to ensure that integration of the virus occurred. Figure 4A shows FACS profiles of HSA expression on nontransduced and transduced cells. Transduced cells with the highest fluorescence intensity were then expanded, and analyzed for GC enzyme expression. Normal skin fibroblasts were found to have a 9.4-fold higher enzyme activity level than those derived from this patient (365 ± 3 vs 39 ± 3 nmol/h/mg protein; data not shown). Multiple infections of these Gaucher fibroblasts with the recombinant retroviral supernatant led to greatly increased GC enzyme specific activity levels—in fact, the unsorted population of transduced cells showed a more than 20-fold increase in enzyme levels versus the untransduced patient cell population (data not shown). Indeed, enzyme activity was even 2.4-fold greater than that seen in normal fibroblasts. Transduction of these cells was extremely efficient and enzyme expression from
this construct was greatly elevated, as demonstrated by the observation that essentially the entire population of transduced fibroblasts had a 2-log increase in observed fluorescence over background levels (Fig 4A). Not surprisingly, differences in enzyme activity among transduced unsorted and sorted populations were found to be relatively small (data not shown). To ensure that the increased enzyme expression and HSA surface fluorescence was derived from integrated retroviral copies, a comparative PCR assay was used to examine genomic DNA derived from these cells and from controls. Figure 4B shows that DNA from transduced populations of cells has a band characteristic of the integrated provirus, although, again, differences in band intensity between sorted and unsorted transduced populations are small due to efficient transduction of these cells. Further studies of this bicistronic retroviral construct with respect to the kinetics of integration and protein expression of GC and the HSA marker in different cell types will allow utilization of optimal conditions for infection and sorting.

Sorting of transduced Gaucher type I B cells. To demonstrate the feasibility of this selection-and-sorting approach to correct the Gaucher deficiency in cells of hematopoietic origin, B cell lines from type I, II, and III Gaucher patients were created by EBV transformation of collected patient lymphocytes. Figure 5 demonstrates observed GC enzyme activity for each line. All cell lines from each type showed GC enzyme specific activities that were approximately 30% of normal. Calculated values of GC specific activity were 13.4 ± 0.1 nmol/h/mg protein for a cell line derived from a normal volunteer, and ranged from 3.5 to 4.0 nmol/h/mg protein for Gaucher B cells.

B cells from the Gaucher disease type I line were transduced with the AM12/LGsEH supernatant. A total of four additions of the supernatant to the cultured cells in the presence of protamine sulfate were performed. Figure 6A shows the resulting flow cytometric analysis of untransduced and transduced cells. A large enhancement of the fluorescence signal can be observed for a small number of cells. The portion of cells marked M1 (representing the top 9% in HSA cell surface staining intensity) were then sorted and expanded. Figure 6B shows calculated GC enzyme activity of the transduced and sorted population of Gaucher disease type I B cells in comparison to the unsorted population. An enhancement of GC enzyme activity is seen in the unsorted population versus the untransduced control, although this enhancement still does not reach the levels of enzyme activity observed in the normal B cell line (Fig 6B). In direct contrast, B cells selected for on the basis of HSA surface antigen expression showed greatly enhanced GC enzyme activity levels to more than fivefold over untransduced cell levels, and even to levels more than twofold over those in normal cells.

That the increased GC enzyme expression is a result of increased retroviral copy number is shown by Southern blot analysis in Fig 6C. Genomic DNA was prepared from the
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various transduced and/or sorted cell populations. After restriction enzyme digests and electrophoretic separation, the DNA was probed for the presence of integrated provirus. A reproducible band of faint intensity can be observed in unsorted, transduced Gaucher type I B cells (representing much less than one copy per cell) in comparison to the copy number of controls. Figure 6C shows that sorting of B cells based on cell surface HSA expression and expansion leads to an increased retroviral copy number in the cells, up to one to two copies per cell, which correlated well with the increased enzyme expression seen. These data also demonstrate that for this retroviral construct, even one to two copies per cell of this recombinant retrovirus can lead to greatly increased GC enzyme expression, higher than what is likely necessary for corrective therapy.

FACS profiles and GC enzyme analyses were obtained for sorted B-cell populations after substantial cell expansion. Cell surface expression of the HSA surface marker and increased GC enzyme activity from the added cDNA were maintained in cells in culture for an extended time (data not shown).

Sorting of transduced human CD34+ cells. BM CD34+ cells were isolated from normal donors and transduced with the AM12/LGsEH viral supernatant. Figure 7A demonstrates the observed GC enzyme activity. Transduction with the AM12/LGsEH supernatant led to an increase in GC enzyme specific activity over that seen in normal CD34+ cells, and to an increase even over that in CD34+ cells transduced with a control positive viral supernatant, LG4.28 Cell surface expression of HSA was also demonstrated (Fig 7B), with approximately 10% of the cells showing a fluorescence shift into the M1-gated range. These cells were then sorted on the basis of cell surface HSA expression, and clonogenic colony assays were performed. Similar numbers of G/M and BFU-E colonies were obtained for nontransduced, control LG4 transduced, and AM12/LGsEH transduced CD34+ cells (data not shown), indicating no gross perturbations in the relative proportions of differentiated progeny cells due to expression of the HSA.

Colonies derived from infected sorted and control populations were analyzed by PCR for the presence of proviral sequence. AM12/LGsEH-infected cells had the provirus integrated in 38% to 50% of the colonies (Table 1). This is similar to the infection frequency observed using the control LG4 supernatant (40% to 59%; Table 1) and indicates that the titer of the AM12/LGsEH is relatively high on human
hematopoietic progenitor cells. AM12/LGsEH-transduced CD34+ cells were then sorted by FACS on the basis of cell surface expression of introduced HSA, and positive cells were plated into methylcellulose colony cultures. All 17 colonies picked (Table I and Fig 7C) were positive for the presence of the proviral GC sequence.

**DISCUSSION**

Recombinant retroviral vectors containing the GC gene and the murine HSA expressed in a coordinated fashion have been used successfully to select transduced cells from Gaucher patients by FACS, resulting in metabolic correction of the enzyme deficiency. Infection of CD34+ human BM cells was also demonstrated. Only a single round of immunofluorescence and flow cytometric sorting was necessary in all cases to produce a large increase in GC enzyme specific activity of the cells (derived from the added therapeutic GC enzyme) in the bicistronic retroviral construct. Interestingly, the large increases in GC enzyme specific activity (>30-fold for the AM12 vector-producing cell line, for example) were not accompanied by any changes in growth characteristics or gross morphology of any transduced cells in comparison to untransduced controls. A good correlation was also demonstrated between virus copy number, GC enzyme levels, and HSA surface antigen expression in sorted cells.

Bicistronic retroviral vector constructs containing the GC gene and the MDR1-selectable gene have been generated and used to correct the metabolic deficiency in Gaucher fibroblasts following plasmid transfection, but transduction experiments by virus were not reported. The MDR1 gene can be selected for in vitro and in vivo by cytotoxic drugs and can also be selected by immunoaffinity techniques, since the MDR efflux pump protein is expressed on the cell surface. Experiments in mice have demonstrated that the MDR gene can protect hematopoietic cells from high concentrations of cytotoxic drugs and may therefore be useful in cancer chemotherapy. However, in consideration of constructs designed for broader utility, the MDR1 gene is large (~4,000 bp), and when an additional therapeutic gene is added to the retroviral construct to correct a deficiency, the recombinant vector titer may be low and transduction of target cells, especially hematopoietic stem cells, may be very inefficient. Recently, bicistronic vectors containing the MDR1 gene were reported to have titers in the 10^5 CFU/mL range. In addition, due to the size of the MDR1 protein, higher translational efficiency or increased transcription of the cDNA may be required to generate levels of MDR1 protein on the surface of cells high enough to allow func-

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**Table 1. Results of PCR Analysis forProviral DNA Sequence in CD34+ Infected BM Colonies**

<table>
<thead>
<tr>
<th>Assay 1</th>
<th>Assay 2</th>
<th>HSA-Sorted</th>
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<tbody>
<tr>
<td>LG4</td>
<td>23/39</td>
<td>8/19</td>
</tr>
<tr>
<td>LGsEH</td>
<td>15/40</td>
<td>1/2*</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Only two colonies could be analyzed, due to technical difficulties.
tional sorting with present antibodies.37 In contrast, the vector system described herein, using the small (243-bp) murine HSA, generates high titers that are comparable to those obtained previously for LG4 in this laboratory and used to transduce murine hematopoietic stem cells.38

The efficient identification and separation scheme described here can greatly facilitate studies on murine hematopoiesis and overall gene transfer technology. Using the HSA as an expression marker or as a labeling tag, long-term hematopoietic cell-repopulation and virus-construction studies can be expedited. Among other examples, research on long-term hematopoietic expression from various retroviral LTRs and also on the potential effect of retroviral promoter shutdown38 can be made much easier, since detection of the expressed surface antigen is relatively simple and only a limited number of cells are required for the analysis. Studies on recombinant retrovirus construction, to generate integration site-independent expression and also exploration of regulatable promoters to modulate expression, can also benefit from the ease of detection and tracking of transduced cells and from the limited quantitation of the expressed marker that is possible. Furthermore, studies to examine why certain cell types have much higher degrees of infectivity (for example, compare FACS profiles for patient fibroblasts and patient B-cell lines in this study) than other cells can also benefit from this rapid and convenient approach.

Since now the murine HSA and previously the human CD2425 have both been used to sort retrovirally transduced cells, greater flexibility is provided for studies in murine models and for possible future transduction into human cells. Since no homology exists in the human for the murine peptide, it may be possible that constructs containing HSA can be used to transduce and sort patient hematopoietic stem cells before transplantation. Further, experiments using hematopoietic stem and progenitor cells from mice and humans are needed to demonstrate the feasibility of using this vector system in clinical gene therapy protocols.

It is not clear whether adverse effects from generalized expression of HSA in most hematopoietic lineages can be expected in mice or in humans in vivo. In transgenic mice, significant overexpression of HSA has recently been shown to reduce the overall numbers of thymocytes.39 In preliminary experiments presented here, expression of HSA on primary human hematopoietic cells does not seem to alter the number or type of observed hematopoietic progenitor colonies in vitro. Similar observations have been made in a study by Conneally et al40 using a HSA vector that also contains a neo6 gene. Therefore, HSA expression on human hematopoietic progenitors does not seem to cause abnormal differentiation or maturation of their progeny cells in vitro.

In humans, an immune response could be generated, and animal experiments, particularly in primates or other large outbred animals, are necessary to explore this potential complication. The HSA was originally isolated from a hematopoietic cell line,29 and the cDNA encodes a glycosylated protein with a core of only 30 amino acids that is expressed on developing cells of earlier hematopoietic lineages and on some neural cells. Varied roles for HSA have been proposed, including that of a costimulator for antigen-presenting cells,41,42 a modulator of cell-cell adhesion,43,44 and a factor in the protection of cells from self-complement depletion.45 As inducible and regulatable promoter systems become more characterized and available,46-48 it may be possible to induce HSA expression only during a short time, long enough to allow the efficient sorting the system affords, and then to turn off long-term expression. This would circumvent some of the potential immunologic hazards of using a murine antigen in human systems.

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