RNA-Based Gene Therapy for HIV With Lentiviral Vector–Modified CD34+ Cells in Patients Undergoing Transplantation for AIDS-Related Lymphoma

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AIDS patients who develop lymphoma are often treated with transplanted hematopoietic progenitor cells. As a first step in developing a hematopoietic cell–based gene therapy treatment, four patients undergoing treatment with these transplanted cells were also given gene-modified peripheral blood–derived (CD34+) hematopoietic progenitor cells expressing three RNA-based anti-HIV moieties (tat/rev short hairpin RNA, TAR decoy, and CCR5 ribozyme). In vitro analysis of these gene-modified cells showed no differences in their hematopoietic potential compared with untransduced cells. In vitro estimates of successful expression of the anti-HIV moieties were initially as high as 22% but declined to ~1% over 4 weeks of culture. Ethical study design required that patients be transplanted with both gene-modified and unmanipulated hematopoietic progenitor cells obtained from the patient by apheresis. Transfected cells were successfully engrafted in all four infused patients by day 11, and there were no unexpected infusion-related toxicities. Persistent vector expression in multiple cell lineages was observed at low levels for up to 24 months, as was expression of the introduced small interfering RNA and ribozyme. Therefore, we have demonstrated stable vector expression in human blood cells after transplantation of autologous gene-modified hematopoietic progenitor cells. These results support the development of an RNA-based cell therapy platform for HIV.

INTRODUCTION

Highly active antiretroviral therapy (HAART) has markedly improved the survival of patients with HIV infection, but it is likely never to be curative (1). Although patient compliance has improved with new multiple-drug formulations, virus replication continues during HAART treatment and the risk of development of antiviral resistance remains a concern. Additionally, medications represent up to 84% of AIDS-related healthcare costs (2), and thus other methods that decrease the need for such continuous and lifelong treatment could decrease the need for continuous medication and its attendant cost. We describe here an approach involving a single genetic manipulation to delay or prevent the progression of HIV infection.

Since it was first proposed that gene transfer might “immunize” against intracellular infection (3), investigators have explored the use of genetic medicine to treat HIV. Given the difficulties, risks, and failures associated with human gene therapy, it has remained unclear whether a single gene manipulation could have a lasting impact in this disease setting. Recently, however, the transplantation of allogeneic hematopoietic stem cells (HSCs) with an HIV-resistant genotype [a result of a naturally occurring 32–base pair (bp) deletion in the gene encoding chemokine receptor 5 (Δ32CCR5)], in conjunction with myeloablative therapy for leukemia, resulted in apparent elimination of HIV in the recipient (4). This result supports the idea that replacing a susceptible immune system with a genetically modified, virus-resistant one could result in reduced viral load and perhaps prevent progression to AIDS in HIV-infected individuals. However, the difficulty of finding matched allogeneic homozygous Δ32CCR5 donors precludes widespread use of this strategy. An alternative approach, with ex vivo genetic modification of autologous HSCs to render their progeny HIV-resistant, could potentially be therapeutic.

Multiple antiviral strategies have been proposed for AIDS gene therapy, including those based on protein (5–11) and RNA (12–16). Autologous T cells or CD34+ cells have been transduced with a retroviral vector construct encoding a surface fusion peptide (17), a mutant Rev molecule (18, 19), or a ribozyme targeting the viral vpr/tat (20) or tat/rev (21) encoding RNAs, and then these cells have been infused into HIV-positive individuals. All of these methods were safe and well-tolerated, but they resulted in little long-term genetic marking of peripheral blood cells and variable to no detectable expression of the therapeutic transcript in the peripheral blood.

Our experience in using HSC transplantation to treat AIDS-related lymphoma (ARL) patients (22) suggested that this population offers a unique opportunity to evaluate RNA-based anti-HIV strategies in an ethically acceptable clinical setting that included marrow ablation in the patients. Long-term expression of the RNA transgenes is necessary for success of this procedure, and thus, the transgenes must be integrated into the progeny cells if the therapeutic effect is to be sustained. In this regard, lentiviral vectors have been promoted as an ideal gene de-
livery system because they have been reported to integrate into nondividing cells and do not preferentially insert near gene promoters (23–25). We describe here a clinical trial in which autologous hematopoietic progenitor cells (HPCs) are programmed with an expressed short-hairpin RNA (shRNA) targeting a tat/rev common exon in combination with two HIV-specific RNA-based inhibitors (a nucleolar-localizing TAR RNA decoy and a CCR5 targeting hammerhead ribozyme) (26). We show long-term expression of an ectopically expressed small interfering RNA (siRNA) and ribozyme in multiple peripheral blood cell lineages of patients transplanted with gene-modified progenitor cells.

**RESULTS**

**Participants**

Seven patients consented to participate in the study and were treated as shown in Fig. 1. All patients had non-Hodgkin’s lymphoma; two had relapsed disease, two were induction failures, two were in first partial remission, and one was in high-risk first remission. Secondary exclusion criteria required that the patients could mobilize sufficient HPCs by apheresis (HPC-A cells) for two collections of $2.5 \times 10^6$ CD34+ cells per kilogram of patient weight each: one a therapeutic product that was otherwise unmanipulated (HPC-A-Rx) and the other an experimental research product for our genetic transduction experimental procedure (HPC-A-Exp). Two patients were removed from the study before HPC collection because of either progressive non-Hodgkin’s lymphoma (patient 302) or inadequate HPC-A collections (patient 303). The remaining five patients were able to mobilize an adequate number of cells for the investigational portion of the trial (Table 1). One of these (patient 301), however, received only HPC-A-Rx cells during the transplant because HPC-A-Exp cells failed to pass the 70% viability release requirement. The other four patients who underwent transplantation with both transduced and untransduced cell products had a median age of 42.5 years (range, 25 to 55 years), had CD4 counts at study entry ranging from 18 to 577 cells/μl, and had a viral load ranging from below the limit of detection (LOD) to $\sim 25,000$ copies per milliliter.

After transplantation, HIV RNA in plasma and CD4 counts were followed at monthly intervals (table S1). All patients were maintained on HAART, and their HIV loads were undetectable (<50 gene copies per

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*Table 1. Patient characteristics. UPN, unique patient number; M, male; gc, gene copies; HPC-A-Rx, minimally manipulated HPC-A product; HPC-A-Exp, CD34-enriched, ex vivo transduced HPC-A; ANC, absolute neutrophil count.*

<table>
<thead>
<tr>
<th>UPN</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Cell products infused</th>
<th>HIV load before transplant (gc/ml)</th>
<th>CD4 level before transplant (cells/ml)</th>
<th>Days to engraftment (ANC, &gt;500)</th>
<th>Follow-up (months)</th>
<th>HPC-A-Rx infused</th>
<th>HPC-A-Exp infused</th>
<th>Total infused</th>
<th>% Gene-marked cells infused*</th>
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<td>M</td>
<td>Diffuse large B cell lymphoma</td>
<td>42</td>
<td>Rx only</td>
<td>9830</td>
<td>138</td>
<td>11</td>
<td>NA</td>
<td>2.8 $\times 10^6$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
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<td>Diffuse large cell type (immunoblastic plasmacytoid)</td>
<td>55</td>
<td>Exp/Rx</td>
<td>&lt;400</td>
<td>366</td>
<td>11</td>
<td>24</td>
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<td>7.7 $\times 10^5$</td>
<td>4.7 $\times 10^6$</td>
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<tr>
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<td>Exp/Rx</td>
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<td>206</td>
<td>11</td>
<td>18</td>
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<td>306</td>
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<td>Plasmablastic lymphoma</td>
<td>45</td>
<td>Exp/Rx</td>
<td>2100</td>
<td>18</td>
<td>11</td>
<td>18</td>
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<tr>
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<td>Exp/Rx</td>
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<td>1.6 $\times 10^6$</td>
<td>8.1 $\times 10^6$</td>
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*% Gene-marked cells = % gene marked (estimated from 4-week in vitro culture analysis) $\times$ HPC-A-Exp infused / (HPC-A-Exp infused + HPC-A-Rx infused) $\times$ 100.
CD34 cell selection and lentiviral transduction

HPC-A cells harvested from patients (n = 5) were subjected to CD34 selection, which resulted in a cell population highly enriched (mean enrichment by a factor of 121; range, factor of 63 to 205) for CD34+ cells. Starting with an average of 312 × 10^6 CD34+ cells (range, 122 × 10^6 to 618 × 10^6), 151 × 10^6 CD34+ cells (range, 47 × 10^6 to 317 × 10^6) were recovered (48% average yield) (fig. S1). CD34+ enriched cells were cryopreserved in the vapor phase of liquid nitrogen until 3 days before infusion, at which time they were thawed and transduced.

In vitro analysis of transduced HPC-A products

In vitro analysis was conducted to determine the effects of transduction on the hematopoietic potential and extent of gene marking of the cells. In methylcellulose culture, the frequency and types of colony-forming units were not markedly different for transduced and nontransduced control cells, demonstrating the absence of toxicity of the transduction process (fig. S2). Cells were also placed in liquid culture with a cytokine cocktail designed to promote myelopoiesis or erythropoiesis or plated on a murine stromal cell line previously demonstrated to support the growth of B-lymphoid cells (26, 27). Samples were taken at weekly intervals for phenotypic analysis of myeloid cells (CD14, CD15, CD33), erythroid cells (Gly-A), B lymphocytes (CD10, CD19), and CD34+ stem or progenitor cells. No differences in the kinetics or magnitude of lineage development were observed between the transduced and the nontransduced cell cultures (fig. S3). Together, these results support the overall safety of the cell transduction process with respect to in vitro hematopoietic potential.

Cells from these cultures were also analyzed weekly for the presence of the rHIV7-shI-TAR-CCR5RZ transgene. Quantitative polymerase chain reaction (qPCR) analysis was performed with primers specific for the woodchuck posttranscriptional regulatory element (WPRE) contained within the transgene and for apolipoprotein B (ApoB), a single-copy housekeeping gene, to normalize for cell number. By measuring the ratio of messenger RNA copies of WPRE to ApoB, we observed gene-marking frequencies as high as 23% of the cells (mean, 18%; range, 5 to 23%; n = 5) after 1 week of culture (Fig. 2A). The percentage of gene-marked cells declined rapidly over 4 weeks of culture to ~1% (mean, 0.75%; range, 0.65 to 0.81%; n = 4). Lineage-specific cells were isolated by flow cytometric cell sorting after 2 weeks of culture (erythroid or myeloid cells) or 4 weeks of culture (B cells) and evaluated for gene marking. Although the average percentage of marked cells varied among phenotypically defined populations, gene marking of all subsets occurred at frequencies similar to those of the bulk population (0.7 to 19.5%) (Fig. 2B). Because most of the cells used to initiate culture were negative for mature myeloid progenitors (CD14, CD15, CD33, and Gly-A) and mature cells do not contribute to 4-week cultures, we believe that these results indicate that, at a minimum, multilineage progenitor cells were transduced with the viral vector and that these transduced progenitor cells differentiated into erythroid, monocytic, granulocytic, and lymphoid lineages, each carrying the transgene.

We also analyzed cells derived from limiting dilution assays to verify the gene-marking estimates from bulk PCR analysis and to determine the number of integrated copies of the lentiviral vector per cell. Using cultures of cells plated at 50, 10, or 5 per well and maintained for 4 to 5 weeks, we evaluated ≥ 500 growth-positive wells from four patients and found only six wells positive for transgene from three of the patients (2 of 169 from patient 305, 1 of 125 from patient 306, and 3 of 125 from patient 307). Of interest, the positive wells had one, two, or three copies of the integrated transgene per cell (fig. S4).

Although this low number of transgene-positive wells is consistent with the average frequency of

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**Fig. 2.** Genetic marking of patient samples during in vitro culture. (A) Average gene marking (copies per 100 cells) (horizontal bar) and values from each patient (symbols) in bulk populations of cells over the 4 weeks of bulk liquid culture. (B) For each patient, cells from in vitro culture were also sorted at 2 weeks (CD14, CD15, Gly-A, CD33, and Lin−) or 4 weeks (CD10) for lineage-specific marking analysis as described above. CD34-enriched HPC-A products were cultured in vitro after transduction for 4 weeks in liquid culture or on a murine stromal cell line in the presence of granulocyte-macrophage CSF, interleukin-3 (IL-3), IL-6, Fli-3L, erythropoietin, and thrombopoietin. Cells were harvested weekly, and DNA was extracted for qPCR analysis of the transgene. The average amount of transduction (copies per 100 cells) with the HIV-shI-TAR-CCR5RZ genetic construct is defined as described in Materials and Methods. Copies of WPRE were derived from a standard curve of known quantities of pHIV-shI-TAR-CCR5RZ plasmid spiked into the background of nontransduced PBMCs. ApoB gene copies, used for normalization, were determined from qPCR analysis of a standard curve of DNA ranging from 10 to 1,000,000 PBMCs.
transduced cells estimated from bulk culture, it suggests that the actual percentage of transduced cells in bulk culture is lower than estimated when assuming a single copy per cell.

Northern blot analysis was used to evaluate the expression of the CCR5 ribozyme, TAR decoy, and siRNA sequences in the HPC-A-Exp cell products after in vitro differentiation and expansion. As expected, RNA was present early (day 13) for all three transgene species but was not detectable by Northern blot analyses in cells from 4-week cultures from patient 305, where the frequency of gene marking was only 1% (Fig. 3A). However, using the reverse transcription PCR (RT-PCR) assays described in Materials and Methods, we were able to detect between 10^5 and 10^6 copies of siRNA per 8 ng of total cellular RNA in samples from three time points of in vitro culture of transduced cells from patient 306 (Fig. 3B).

Treatment, gene marking, and expression in patients after transplantation

Four patients received both HPC-A-Exp and HPC-A-Rx cells at the doses listed (Table 1). All patients engrafted at 11 days after transplant [engraftment defined as absolute neutrophil count (ANC) of >500 for 3 consecutive days]. Excluding cytophenias, expected serious transplant-related adverse events during the first 30-day period after transplant included grade 3 hypotension (two patients), grade 3 hypoxia (two patients), grade 3 fever (one patient), or central line infection (one patient). At the 6-month evaluation, patient 307 was found to have a syndrome consistent with Chlamydia and Pneumocystis jirovecii pulmonary infections, which was treated with appropriate antibiotics without sequelae. The only unexpected adverse event was an asymptomatic (grade 2) occurrence of a solitary lytic scapular bone lesion histologically determined to be Langerhans cell histiocytosis, which subsequently resolved without intervention. No vector sequences were detectable by PCR from a biopsy of this lesion.

Median length of follow-up was 18 months (range, 6 to 24 months). All patients have remained in remission from their lymphoma. Gene-marking frequencies of between 0.02 and 0.32% (200 to 3200 copies per 10^6 cells) of peripheral blood mononuclear cells (PBMCs) have been observed for multiple patients and time points (Fig. 4). Patients 304 and 306 had consistently detectable and quantifiable frequencies of gene marking (0.04 to 0.12%) between 2 and 12 months after infusion, whereas patients 305 and 307 showed detectable (≥0.02%) but not quantifiable (≤0.05%) frequencies of marking over the same period. At 18 and 24 months, however, patients 304 and 305 showed increases in the frequency of gene marking from nonquantifiable to 0.18% and 0.11%, respectively, whereas patient 306 dropped to less than half of the 12-month marking level. Thus, our estimates of the frequency of gene-marked, 4-week culture-initiating cells infused (0.11 to 0.15%) correspond well with...
the levels of long-term (>12 months) in vivo gene marking seen in patients 304, 305, and 306 (0.09 to 0.18%).

Expression of the anti-tat/rev siRNA and CCR5 ribozyme in the peripheral blood was also followed for up to 24 months after infusion. Despite the low frequency of gene marking, we detected vector-expressed RNAs in multiple patients’ peripheral blood and bone marrow for up to 24 months after infusion (Table 2). For example, patient 304, whose gene marking varied between 0.05 and 0.1%, had detectable quantities of siRNA and CCR5 ribozyme at 3 and 6 months but siRNA alone at 1, 2, and 12 months and CCR5 ribozyme alone at 4, 8, and 10 months. Patient 305, on the other hand, showed expression of siRNA and CCR5 ribozyme at 1 month and only siRNA sporadically (6 and 8 months) thereafter. However, in patient 306, higher frequencies of gene marking were observed, and we were able to quantify siRNA quantities of siRNA and CCR5 ribozyme at 3 and 6 months but siRNA alone at 1, 2, and 12 months and CCR5 ribozyme alone at 4, 8, and 10 months.

Peripheral blood was isolated from patient 306 at 18 months and separated into mononuclear and granulocytic fractions by density centrifugation. The mononuclear cells were further fractionated into CD3+ (T cells), CD14+ (monocytes), CD19+ (B cells), and cells that are negative for all three markers (Lin−) by fluorescence-activated cell sorting. All subsets showed gene marking at frequencies consistent with the whole-blood marking analysis (fig. S6). These data support the in vitro findings that multilineage progenitor cells (in this case capable of sustaining hematopoiesis in vivo for 18 months) were in fact transduced.

### Table 2. Analysis of RNA expression in peripheral blood and bone marrow of treated patients and monitoring disease outcome.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Cells</th>
<th>1M</th>
<th>2M</th>
<th>3M</th>
<th>4M</th>
<th>6M</th>
<th>8M</th>
<th>10M</th>
<th>12M</th>
<th>18M</th>
<th>24M</th>
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<tr>
<td>304</td>
<td>PBMC</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>PBGC</td>
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<td></td>
<td>BMGC</td>
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<tr>
<td>305</td>
<td>PBMC</td>
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<td>306</td>
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### DISCUSSION

We have previously demonstrated that our standard HSC transplant procedure for high-risk or relapsed ARL resulted in very low mortality and long-term survival (22). We have also suggested RNA-based gene therapy as a potential therapeutic approach to controlling HIV infection (28, 29). On the basis of these results, we designed and conducted a clinical trial to assess the safety and feasibility of HPC-based lentiviral gene therapy for HIV in the context of treatment for ARL. Secondary objectives included monitoring gene marking and RNA expression in the peripheral blood and marrow of treated patients and monitoring disease outcome. Our results offer compelling evidence for the feasibility of the approach.

Unlike the lentivirus-based stem cell transplantation in adrenoleukodystrophy patients (30) in which only transduction-processed HPC-A cells were infused after complete myeloablation, our five-patient study included infusion of an unmanipulated HPC-A graft. This design satisfied the ethical requirements to provide standard-of-care treatment to our relapsed or poorly responsive patients with ARL. Although we were successful in mobilizing enough peripheral blood stem cells in this patient population to prepare both unmanipulated and gene-modified products for infusion in four of five patients, the infusion of the unmanipulated HPC-A product 1 day after the gene-modified product contributed to a reduction in the frequency of gene-modified progenitor cells in vivo by direct competition for engraftment. Additionally, the low level of marking of the more primitive HSCs (as estimated by in vitro analysis) limited the frequency of gene-modified peripheral blood
cells. Thus, we believe that there were insufficient disease-resistant blood cells in these patients to see a clinical (anti-HIV) benefit. Although there is evidence that lentiviral vectors do not need cells to enter the cell cycle to be transduced (31), other studies indicate that cytokines that induce cell proliferation enhance transduction of engrafting cells (32–34). Process development studies are under way in our laboratories to optimize CD34+ cell transduction with minimal loss of hematopoietic potential.

No short-term toxicity was associated with the infusion of the genetically modified HPC-A cells, and observed toxicities were procedure-related events consistent with standard autologous hematopoietic cell transplantation (HCT). It has been noted, however, that siRNA can be toxic to cells in vitro (35), and therefore, a major concern for this study was whether expression of siRNA would be observed in the mature cell compartment of treated individuals. We demonstrated with both in vitro and in vivo studies that there was no overt hematopoietic toxicity associated with the lentiviral transduction process. Specifically, the persistent levels of shRNA expression observed in patients 304 and 306 up to 24 and 18 months, respectively, strongly support the conclusion that constitutive expression of the tat/rev siRNA is not toxic to peripheral blood cells. In addition, given the frequency of genetic marking of T cells, B cells, and granulocytes, there is no evidence for lineage-specific toxicity, consistent with in vitro analysis and preclinical studies with this vector (13, 36).

Patients 306 and 304 showed increased levels of gene marking immediately after viremia (patient 306 at day 0 and patient 304 at 15 months). Although we cannot formally attribute the increase in gene-modified cell frequency to viremia, the selective expansion of disease-resistant cells in the face of viral selective pressure has been observed in a previous HIV gene therapy trial (19). Although it is not possible at this time to predict what percentage of gene-marked cells would be required for clinical benefit in AIDS, a long-term goal of this approach is the selection of genetically modified cells. If HIV could be a selection factor, then treatment interruption of HAART could become a component of gene transfer approaches. Alternatively, the inclusion of a selectable genetic marker (such as O6-methylguanine DNA methyltransferase) in the antiviral construct may allow for prospective chemotherapeutic selection of disease-resistant cells (using O6-benzylguanine and temozolomide).

Support for this strategy comes from studies in large animal models that have demonstrated substantial increases in the percentage of gene-marked cells in peripheral blood after transplant of gene-modified HSCs and drug selection (37–40).

In summary, we have developed methods for the isolation, genetic modification, and infusion of CD34+ cells that support clinical investigation of stem cell gene therapy strategies for HIV. The sustained expression of siRNA and ribozyme for up to 24 months after infusion marks an initial milestone in the development of a stem cell–based genetic therapy for HIV infection. The development of improved transduction processes and revision of transplant procedures to preferentially infuse only transduced cells are likely to lead to higher levels of engrafted genetically modified cells. This would provide a setting for delivery and/or selection of therapeutic levels of HIV-resistant cells.

MATERIALS AND METHODS

Vector production

The lentiviral vector used in this study was manufactured according to current good manufacturing practice requirements in the Center for Biomedicine and Genetics at City of Hope and was fully tested according to Food and Drug Administration guidelines before enrollment of patients. A self-inactivating lentiviral vector was designed to encode three RNAs consisting of an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti–CCR5-specific hammerhead ribozyme as previously described (29). The packaging system used four separate plasmids developed at City of Hope as previously described (41).

HPC collection

HPC-A products were collected after standard salvage chemotherapy or after cyclophosphamide (2 g/m²) plus granulocyte colony-stimulating factor (G-CSF) (10 μg/kg). A minimum of 2.5 × 10⁶ CD34+ cells per kilogram patient weight was collected and cryopreserved without further manipulation (HPC-A-Rx). An additional one or two apheresis collections were performed to collect cells for the genetic modification (HPC-A-Exp). CD34+ cells were enriched from the HPC-A-Exp collection over a Clinimacs device according to the manufacturer’s instructions and cryopreserved with a controlled rate freezer. CD34+ cells were thawed on day −2 before HCT and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2 mM L-glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml/) (CellGenix) at a density of 2 × 10⁶ cells/ml. Prestimulated cells were transduced with lentiviral vector (HIV7-shl-TAR-CCR5RZ) at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm² tissue culture flasks coated with fibronectin (25 μg/cm²) (RetroNectin, Takara Bio Inc.). A sample of CD34+ cells was incubated as described but in the absence of lentiviral vector to serve as a transduction control. Transduced cells were pooled, washed with X-VIVO 15 medium, and resuspended in final formulation buffer [Climacs phosphate-buffered saline–EDTA buffer with 0.5% human serum albumin (HSA)] at a density of 1 × 10⁶ cells/ml for infusion as described below. A sample was taken from each product and tested for total cell count, viability, sterility, and endotoxin.

Transplant eligibility criteria

Patients with HIV and non-Hodgkin’s lymphoma in first complete remission with high or high-intermediate International Prognostic Index scores were eligible for transplant (42). All patients had to be on combination antiretroviral therapy that did not include zidovudine, maintain an HIV viral load of <50,000 gene copies per milliliter, and be willing to suspend antiretroviral therapy during the period of HPC mobilization and collection. The City of Hope’s Institutional Review Board and Institutional Biosafety Committee approved the protocol, and informed written consent was obtained from each patient in the presence of a patient advocate.

Transplantation procedure

About 1 week after completion of aphereses to collect HPCs, patients were admitted to the transplant unit and received chemotherapy exactly as previously described (22). On the day of HCT (day 0), the transduced product (HPC-A-Exp) underwent release testing for endotoxin, sterility, and viability and was then infused. On day +1, the unmanipulated product (HPC-A-Rx) was thawed at the bedside and infused as per guidelines approved by the Foundation for the Accreditation of Cellular Therapy. Safety was assessed for immediate effects of the treatment with the hematologic Common Terminology Criteria for Adverse Events version 3.0 grading system at 24 and 48 hours and 14 days after infusion and at all subsequent visits from 1 to 18 months.
In vitro cell culture

Methylcellulose cultures of CD34+ cells. Samples from each patient were assayed for hematopoietic potential with standard methylcellulose-based colony-forming unit assays and 4-week liquid and stromal cell cultures. The murine stromal cell line used in these studies to support the growth of B-lymphoid lineages from patient CD34+ cells has been previously described (27).

Limiting dilution assay. Limiting dilution analysis (LDA) of cells from each patient was used to isolate the progeny of primitive clonogenic progenitor cells for gene marking and copy number analysis.

Cell phenotype and analysis

Aliquots of cells from bulk liquid and stromal culture were taken weekly for phenotypic and qPCR analysis. Antibodies to lineage-specific cell surface antigens were used to follow hematopoiesis during culture and isolate subpopulations by fluorescence-activated cell sorting. Phenotypic data were collected on an FC500 flow cytometer (Beckman Coulter) and analyzed with FCS Express V3 software (De Novo Software). Samples labeled as described were also sorted on the basis of surface marker expression to >98% purity with a MoFlo cell sorter (Beckman Coulter) for subsequent analysis of DNA marking and gene expression.

DNA analysis of in vitro–derived cells

Samples from in vitro culture were analyzed weekly for the presence of integrated viral vector. qPCR was performed to detect the number of copies of integrated vector per cell. In each case (except for LDA), genomic DNA from ~20,000 cells was used in each reaction. For colonies derived from LDA, DNA from the entire colony was used. The number of copies of WPRE detected was normalized to cell number with qPCR to titrate DNA isolated from patient specimens. A parallel set of qPCR amplifications was performed on all test and reference samples with primers specific for the p21 promoter as an internal control.

DNA marking and gene expression.

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RNA analysis

RNA analysis was performed on gene-modified products from in vitro culture and peripheral blood and bone marrow cells from patients at specific time points after transduction and infusion. Northern blot analysis was used to detect expression of all three RNA moieties from in vitro cultures but was not sensitive enough to detect RNA in peripheral blood samples. More sensitive detection of expression of CCR5RZ was accomplished by real-time RT-PCR, whereas expression of the tat/rev-coding siRNA was analyzed by RT-PCR with the TaqMan MicroRNA Reverse Transcription kit. RT-PCR products were analyzed by gel electrophoresis in a 1% agarose gel, blotting onto nitrocellulose, and hybridizing with radiolabeled sequence-specific probes.

Measurement of in vivo gene marking

The presence of shi-TAR-CCR5RZ–marked cells in peripheral blood was also assessed by qPCR analysis of WPRE sequences with a fixed amount (50 ng) of genomic DNA from peripheral blood. The average percent WPRE+ DNA in samples was determined with a standard curve generated from DNA isolated from a clone with a single-copy integration of the WPRE-containing lentivirus and used in a standard curve to titrate DNA isolated from patient specimens. A parallel set of qPCR amplifications was performed on all test and reference samples with primers specific for the p21 promoter as an internal control.


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