Transplantation of CCR5Δ32 Homozygous Umbilical Cord Blood in a Child With Acute Lymphoblastic Leukemia and Perinatally Acquired HIV Infection

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Background. Allogeneic hematopoietic cell transplantation (allo-HCT) in a CCR5Δ32 homozygous donor resulted in HIV cure. Understanding how allo-HCT impacts the HIV reservoir will inform cure strategies.

Methods. A 12-year-old with perinatally acquired, CCR5-tropic HIV and acute lymphoblastic leukemia underwent myeloablative conditioning and umbilical cord blood (UCB) transplantation from a CCR5Δ32 homozygous donor. Peripheral blood mononuclear cells (PBMCs) and the rectum were sampled pre- and post-transplant. The brain, lung, lymph node (LN), stomach, duodenum, ileum, and colon were sampled 73 days after transplantation (day +73), when the patient died from graft-vs-host disease. Droplet digital polymerase chain reaction (ddPCR) and in situ hybridization (ISH) were used detect the HIV reservoir in tissues. CCR5 and CD3 expression in the LN was assessed using immunohistochemistry (IHC).

Results. HIV DNA (vDNA) was detected in PBMCs by ddPCR pretransplant but not post-transplant. vDNA was detected by ISH in the rectum at days –8 and +22, and in the LN, colon, lung, and brain day +73. vDNA was also detected in the lung by ddPCR. IHC revealed CCR5+CD3+ cells in the LN postmortem.

Conclusions. HIV was detected in multiple tissues 73 days after CCR5Δ32 homozygous UCB allo-HCT despite myeloablative conditioning and complete donor marrow engraftment. These results highlight the importance of analyzing tissue during HIV cure interventions and inform the choice of assay used to detect HIV in tissue reservoirs.

Keywords. allogeneic bone marrow transplantation; CCR5Δ32; HIV cure; HIV reservoirs.

Despite great advances in HIV treatment, cure remains elusive. The Berlin Patient, who underwent myeloablative, allogeneic hematopoietic cell transplantation (allo-HCT) from an human leukocyte antigen (HLA)-matched, CCR5Δ32 homozygous donor, is the only individual in whom a cure has been achieved [1, 2]. Mechanistically, it is postulated that “cure” was achieved through a combination of myeloablative conditioning and eradication of HIV-infected host cells by graft-vs-host (GVH) immune responses and establishment of donor cells resistant to infection by the patient’s R5 virus. Subsequent allo-HCT transplants in HIV-infected patients using wild-type CCR5 donor cells have not resulted in lasting HIV remission [3].

We describe a case of a child with congenitally acquired HIV who developed acute lymphoblastic leukemia (ALL) with characteristics that were unlikely to be cured with traditional chemotherapy and whose odds for survival was best with an allo-HCT. We chose to use banked 3/6 HLA-matched umbilical cord blood (UCB) because of the lack of a matched sibling donor and because we previously demonstrated that 3/6 matched UCB has equivalent outcomes in children with leukemia, relative to better-matched UCB units [4]. Given the patient’s HIV status and CCR5-tropic virus, we searched for a suitably HLA-matched CCR5Δ32 homozygous donor in the unrelated donor registries [5].

Published reports on CCR5Δ32 homozygous UCB allo-HCT include a 53-year-old male with HIV and myelodysplastic syndrome who died of recurrent disease 2 months after allo-HCT and a 37-year-old male with HIV and aggressive lymphoma who died 3 months after transplant from progressive disease [6, 7]. Although no virologic studies have been reported from the first patient, HIV DNA was cleared from the peripheral blood (PB) of the second patient. However, virologic studies were not performed in secondary lymphatic tissues (LTs), which harbor the
majority of latently infected cells [8–12]. As demonstrated by the Boston Patients, both of whom developed virologic rebound after allo-HCT despite clearing HIV DNA from PB and losing detectable cellular immune responses to HIV, PB analyses cannot reliably predict cure after allo-HCT [3].

Herein, we report our experience using myeloablative conditioning followed by transplantation of a CCR5Δ32 homozygous UCB unit in a child with perinatally acquired HIV and ALL and show the impact of this procedure on the HIV reservoir in multiple tissues using both polymerase chain reaction (PCR) and in situ hybridization (ISH) 73 days after transplantation, when the patient died from complications of graft-vs-host disease (GVHD). Our results describe the timing of viral clearance after transplantation, the management of HIV-infected patients undergoing allo-HCT, and the utility of different methods of detecting HIV when evaluating cure strategies.

CASE REPORT

The patient was diagnosed with perinatally acquired HIV at age 1 and was started on antiretroviral therapy (ART). However, given difficulties tolerating multiple oral regimens and issues with clinic follow-up, the patient had detectable viremia and marked immune suppression with absolute CD4 T cell counts consistently <100 cells/mm³ throughout childhood, with several opportunistic infections including presumed Pneumocystis jirovecii pneumonia at age 7 years. In September 2012, at the age of 11 years, the patient underwent evaluation of pancytopenia and was found to have hypodiploid ALL. After induction using protocol COG AALL1131, the patient had persistent minimal residual disease (MRD; 0.3% using COG reference flow cytometry laboratory). Given the high-risk features of the leukemia and poor response to induction therapy, the patient was referred for consideration of allo-HCT. Because the patient’s HIV was CCR5-tropic, a search for a CCR5Δ32 homozygous UCB unit commenced.

Before transfer to the University of Minnesota (UMN) Bone Marrow Transplant Center, the patient had been treated with raltegravir and tenofovir/emtricitabine but had a difficult time tolerating oral medications due to gastrointestinal (GI) issues. He developed a detectable HIV viral load (VL) and a new M184V mutation. His antiretrovirals (ARVs) were therefore changed to raltegravir and tenofovir and raltegravir were continued, but ritonovir and darunavir were held, and abacavir was substituted given drug/drug interactions. Abacavir was stopped and boosted darunavir was restarted after cyclophosphamide was completed. At day 0, the patient underwent UCB transplant from an HLA 3/6 matched donor. The patient’s HIV PB VL was suppressed (<48 copies/mL) at the time of transplant. ARVs were held for 3 days after transplant given concern that they might interfere with engraftment, but they were then restarted. ARV drug levels were checked weekly, and several dose adjustments were required, although HIV VL remained suppressed. The patient demonstrated neutrophil recovery in the peripheral blood on day 14, and whole-blood chimerism studies demonstrated 100% donor engraftment based on short tandem repeat analysis; the margin of error of this clinical assay is ±5%. On day 21, the bone marrow also demonstrated 100% donor engraftment and 10% cellularity, tri-lineage hematopoiesis, and no evidence of leukemia. Chimerism studies on purified CD3 and CD15 isolated from the peripheral blood on days 35 and 59 also revealed 100% donor engraftment. Thirty-six days after transplant, the patient was transitioned from intravenous to oral CSA. Concurrent with this, there was a drop in the systemic CSA levels, and the patient developed grade IV lower gastrointestinal GVHD at day 38 that was refractory to corticosteroid treatment. He was subsequently treated with antithymocyte globulin, MMF, budesonide, and infliximab. Given drug/drug interactions and concern for toxicity, all ARVs were stopped at day 62. Despite aggressive supportive care, the patient developed multi-organ failure and died 73 days after allo-HCT. The patient’s clinical course is outlined in Figure 1.

METHODS

Clinical and Study Laboratory Procedures
Informed consent from the patient’s guardian was obtained in accordance with the Declaration of Helsinki. The work was approved by the Institutional Review Board of the University of Minnesota, and the clinical treatment protocol was registered with the US Food and Drug Administration (as an amendment to IND 14797) before study initiation. HIV tropism was determined using the Monogram Biosciences phenotypic viral RNA assay. HIV VL was measured twice weekly using the Roche COBAS Ampliprep/COBAS Taqman HIV-1 Test, version 2.0.

Plasma Antiretroviral Drug Levels
Trough plasma concentrations of ARVs were measured weekly from the time of transplant in the Antiviral Pharmacology Laboratory at the University of Nebraska Medical Center using quality-controlled, liquid chromatography/tandem mass spectrometry methods, as previously described [13–15].

Tissue Collection and Processing
Relative to the day of transplant (day 0), PBMCs were obtained at days −61, −20, and −8 and +35 and +49. Rectal biopsies
were obtained via flexible sigmoidoscopy at days –8 and +22. Samples of the brain, lung, lymph node (LN), stomach, duodenum, ileum, and colon were obtained at autopsy. PBMCs and tissues were processed as previously described [12].

Radiolabeled RNA In Situ Hybridization
Radiolabeled RNA ISH (rISH) was performed on tissues as previously described [12]. Five-micrometer-thick sections from the paraffin-embedded tissue were cut through the entire tissue block. Twenty sections from each block were analyzed using every fourth section to measure vRNA+ cells in 20-μm intervals. Photographic images using epifluorescence were taken with a digital camera, and the tif images were analyzed for the area of the sections and the area occupied by silver grains using Photoshop with Fovea Pro. Section weights were estimated from their 5-μm thickness and area and density of fixed tissues. Image analyses were repeated by a blinded expert at an outside institution.

Next-Generation DNA In Situ Hybridization (DNAscope)
After processing as above, next-generation DNA in situ hybridization (DNAscope) was used to detect vDNA as previously described [16]. Image analyses were also repeated by a blinded expert at an outside institution.

Droplet Digital PCR
Droplet digital PCR (ddPCR) was performed at the University of California San Diego as previously described [17, 18]. Two primers, skGag and joPol, were used as described [19, 20].

Figure 1. The patient’s clinical course, including HIV viral load (VL), timing of myeloablative allogeneic umbilical cord blood hematopoietic cell transplant (UCB- allo-HCT), immune suppressant therapy, and tissue sampling time points, is shown from time of transfer to our institution to time of death 73 days after transplantation. Abbreviations: ABC, abacavir; ARVs, antiretrovirals; GVHD, graft-vs-host disease; RAL, raltegravir; r/DRV, ritonavir-boosted darunavir; TBI, total body irradiation; TDF, tenofovir.

Immunohistochemistry
Staining was completed using the Opal 4-color Fluorescent IHC Kit (PerkinElmer). Briefly, slides were dewaxed and hydrated. Antigen retrieval was performed with AR6 buffer in the microwave. After washing, slides were blocked with the PerkinElmer antibody diluent/block for 10 minutes. Slides were drained, and CD3 (clone SP7, Thermo Scientific) was added for 1 hour at room temperature. Following TBST buffer rinses, the Polymer HRP Ms+Rb was added for 10 minutes and washed again. The Opal 520 working solution was added to the slides for 10 minutes. Slides were rinsed. To strip the antibody off, the slides were microwaved again in the AR6 buffer. The staining process was repeated, this time using the CCR5 antibody (Lifespan Biosciences, Inc.) as the primary antibody and the Opal 570 for the fluorophore. Slides were washed, counterstained with DAPI, and mounted in Aqua-Poly/Mount (Polysciences, Inc.).

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RESULTS
Viral load results and ART regimens used throughout the transplant course are detailed in Figure 1. At time of transplant, the trough plasma raltegravir concentration was low at 67.2 ng/mL, so the dose was increased to three 100-mg chewable tablets twice daily. The goal trough (approximately 100 ng/mL) was reached.
the following week and remained stable (median, 89 ng/mL; interquartile range, 85–155 ng/mL) until discontinued. Tenofovir levels were below the limit of detection (10 ng/mL) at the time of transplant; this was felt to be due to adherence and the difficulties of use of oral tenofovir powder. Shortly after transplant, the appropriate strength (250 mg) of tenofovir tablets was obtained, and tenofovir was given as a crushed tablet via a G tube. Levels increased but did not reach the target range until day +42. Darunavir levels (median, 772 ng/mL) were in the target range from time of transplant and remained there until the onset of GI GVHD on day +38, when levels decreased; darunavir remained below target level until it was discontinued on day 57. HIV VL remained suppressed from the time of transplant until death.

HIV DNA was detectable in PBMCs using ddPCR targeting Pol and Gag genes at all time points before transplant (days –61, –20, and –8), with a frequency ranging from 140 to nearly 500 copies of HIV DNA per 10⁶ cells; HIV DNA was also detected at a lower frequency in rectal tissues at day –8 (Table 1). At the time these pretransplant samples were obtained, HIV RNA was detectable in the PB as the patient did not develop a suppressed HIV VL until 5 days before transplant. Following allo-HCT, HIV DNA was not detected by ddPCR in PBMCs at +35 and +49 days, nor was it present in rectal tissues obtained at day +22. Similarly, ddPCR targeting Pol and Gag genes was negative in the brain, LN, stomach, duodenum, ileum, and colon tissues collected at autopsy on day +73. However, ddPCR using a skGag probe was positive in lung tissue at the time of autopsy, with a frequency of 22 copies/10⁶ cells (Table 1).

No HIV RNA (vRNA) was detected by rISH in rectal tissues obtained at days –8 and +22, or in the brain, lung, LN, stomach, duodenum, ileum, and colon at autopsy. However, 2.58 × 10⁵ copies of vDNA/gram of tissue were detected in rectal samples obtained at day –8 and 2.60 × 10⁴ copies/gram at day +22 using DNAscope. At autopsy, 1.06 × 10⁵ copies of vDNA/gram were detected in the LN, 1.57 × 10⁶ copies/gram in the lung, 4.16 × 10⁵ copies/gram in the colon, and 7.35 × 10⁵ copies/gram in the brain (Table 1 and Figure 2). No vDNA was noted in the duodenum, ileum, or stomach.

Analysis of postmortem LN using IHC demonstrated loss of normal lymph node architecture with large areas of necrosis and depleted CD3-positive T cells. Some of the cells stained with CCR5 were consistent with persistence of rare cells of recipient origin, most of which represented CD68-positive macrophages; however, rare CD3-positive cells co-staining with CCR5 were also seen (Figure 3).

**DISCUSSION**

Eradication of the reservoir of vDNA+ cells had not occurred 73 days after allo-HCT, despite myeloablative conditioning, grade IV acute GVHD, and the use of CCR5∆32 homozygous donor cells. This not only informs the clinical management of HIV-infected patients undergoing allo-HCT, but more importantly, highlights the challenges of clearing HIV from tissue reservoirs and provides insight into the utility of ddPCR and ISH in detecting the HIV reservoirs in tissues.

Although the kinetics of donor engraftment in the PB and BM are well characterized after allo-HCT, little is known about

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**Table 1. Results of HIV Reservoir Assessment**

| Time Point* | Tissue          | Assay          | ddPCR Polb | ddPCR Gagc | No. of Cells Assayed by ddPCR | ISH DNAa | ISH RNAe |
|-------------|-----------------|----------------|-------------|-------------|-----------------------------|----------|----------|
| Pretransplant |                 |                |             |             |                             |          |          |
| –60         | PBMCs           | ddPCR Pol     | 211.26      | 146.11      | 74 600                      | –        | –        |
| –20         | PBMCs           | ddPCR Pol     | 496.43      | 140.89      | 112 000                     | –        | –        |
| –8          | PBMCs           | ddPCR Pol     | 260.10      | 152.33      | 193 000                     | –        | –        |
|             | Rectum          | ddPCR Pol     | 71.75       | 35.80       | 53 800                      | 2.58 × 10⁵| 0        |
| Post-transplant |                 |                |             |             |                             |          |          |
| +22         | Rectum          | ddPCR Pol     | 0           | 0           | 16 100                      | 2.60 × 10⁴| 0        |
| +35         | PBMCs           | ddPCR Pol     | 0           | 0           | 135 000                     | –        | –        |
| +49         | PBMCs           | ddPCR Pol     | 0           | 0           | 70 000                      | –        | –        |
| +73 (autopsy)| Brain           | ddPCR Pol     | 0           | 0           | 22 600                      | 7.35 × 10²| 0        |
|             | Lung            | ddPCR Pol     | 0           | 22.57       | 101 000                     | 1.57 × 10⁴| 0        |
|             | Lymph node      | ddPCR Pol     | 0           | 0           | 85 900                      | 1.06 × 10³| 0        |
|             | Duodenum        | ddPCR Pol     | 0           | 0           | 233 000                     | 0        | 0        |
|             | Ileum           | ddPCR Pol     | 0           | 0           | 84 600                      | 0        | 0        |
|             | Colon           | ddPCR Pol     | 0           | 0           | 166 000                     | 4.16 × 10³| 0        |
|             | Stomach         | ddPCR Pol     | ND          | ND          | 0                           | 0        | 0        |

*Abbreviations: ddPCR, droplet digital polymerase chain reaction; ISH, in situ hybridization; ND, not done; PBMC, peripheral blood mononuclear cell.

*Days in relationship to transplant (date of transplant = day 0).

bDigital droplet polymerase chain reaction (PCR) using joPol primer; results reported in DNA copy per 10⁶ cells.

cDigital droplet PCR using skGag primer; results reported in DNA copy per 10⁶ cells.

dCopies of HIV DNA per gram of tissue; assay performed on tissue only.

eCopies of HIV RNA per gram of tissue; assay performed on tissue only.
the turnover of recipient cells and the engraftment of donor cells in secondary LT, including LN and GI-associated LT (GALT). These tissues serve as the primary reservoir of HIV vDNA+ and vRNA+ cells and appear to be the source of viral recrudescence after treatment interruption [21]. In the Berlin Patient, CCR5+-expressing macrophages were detected in rectal tissue more than 5 months after transplant, although no HIV was detected [2]. In our patient, we detected presumed recipient cells (CCR5+CD68+ macrophages and CCR5+CD3+ T cells) in the LN 73 days post-transplant despite full donor engraftment in the PB and BM; we also detected vDNA in the colon and LN at this time point. These findings suggest that (1) the turnover of recipient hematopoietic cells in secondary LT is a protracted process, (2) engraftment in PB and BM may not predict engraftment status in the secondary LTs, and (3) these long-lived, radiation- and chemotherapy-resistant recipient cells can harbor HIV for months after transplantation.

We also found vDNA in our patient’s lung tissue postmortem, which is consistent with evidence that alveolar macrophages (AMs) can harbor HIV DNA [22–24]. There is also evidence that AMs repopulate slowly and remain of recipient origin for at least weeks after allo-HCT, likely explaining the detection of vDNA in this tissue [25].

Although we detected vDNA in multiple tissues, we were unable to assess replication competency. However, we readily detected vDNA despite sampling only a fraction of our patient’s tissues, suggesting that the total number of vDNA+ cells was substantial at the time of death. Given evidence that up to 5% of vDNA+ cells harbor replication competent provirus, it seems likely that withdrawing ART in this individual would have resulted in recrudescence of virus replication [26, 27]. Furthermore, in the Boston Patients, viral rebound after

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**Figure 2.** HIV DNA detected by next-generation in situ hybridization (DNAScope) in rectal tissue (A) obtained 22 days after transplantation and in the rectum (B), lymph node (C), and lung (D) obtained at autopsy 73 days after transplantation. HIV DNA appears red and is highlighted by arrows.

**Figure 3.** Lymph node obtained at autopsy 73 days after transplantation. CD3-positive T cells (green), CCR5-expressing cells (red) and double-positive cells (yellow; denoted by white arrow).
allo-HCT resulted from activation of only 1 or a few latently infected cells, demonstrating that even a very small HIV reservoir post-transplant has the potential to avert cure [3].

There were also differences between measures of HIV DNA by ddPCR and direct visualization by ISH, highlighting the challenge of reservoir measures in tissues. The results of these 2 assays varied, with vDNA being detected in only the rectum and lung post-transplant by ddPCR but in multiple tissues using DNAscope. After processing and disaggregating each tissue, only limited, unsorted cell populations could be assessed by ddPCR. The sensitivity of ddPCR was also likely further impacted by proviral sequence variation as only 2 PCR primers were used. On the other hand, DNAscope utilizes intact tissue, thereby minimizing cellular loss, and has high sensitivity through the use of a combination of ZZ probes in a cocktail probe mixture (40–85 ZZ pairs with approximately 2 to 4.2 kb of genome coverage) and an amplification cascade via sequential hybridization that allows for the visualization of individual virions or vDNA within the nuclei of infected cells [16]. HIV DNA extracted from tissues can be readily quantitated, but the number of CD4 lymphocytes or macrophages being interrogated cannot be measured at present. However, these results highlight the need for multiple measures of vRNA and vDNA to assess the impact of cure interventions, as was done with the Berlin Patient [1].

Finally, our results have implications for the clinical management of HIV+ patients undergoing allo-HCT. First, given our tissue results, we believe that post-transplant ART should be used, even though ART dosing is complex and requires careful attention to drug/drug interactions and an understanding of ARV pharmacokinetics [28]. In our patient, multiple ARV dose adjustments were required due to subtherapeutic plasma drug concentrations, presumably related to impaired GI absorption in the setting of HCT, suggesting that ARV monitoring should be routine for HIV patients undergoing allo-HCT. Furthermore, in this patient, the conversion from intravenous to oral CSA corresponded with the development of GVHD, and drug/drug interactions could have exacerbated this. We were not able to determine the optimal duration of ARVs post-transplant using treatment interruption or additional tissue sampling given our patient’s untimely death, but this will need to be addressed in the future.

Second, in our patient, only the commercially available phenotypic CCR5 trophe assay was used to determine HIV tropism. However, this assay may miss minority CXCR4 variants that can be selected for by CCR5Δ32 allo-HCT, leading to virologic rebound, as has been reported [28, 29]. Although it is unknown if these variants existed in our patient or if they would have emerged post-transplant after stopping ARVs, in the future, consideration should be given to using next-generation sequencing techniques to assess for X4 variants before pursuing CCR5Δ32 allo-HCT.

Third, our case illustrates the risks of a curative approach using allogeneic transplantation in HIV-infected patients. At present, it is unclear whether the use of a biallelic mutated CCR5 donor increases the risk of transplant-related mortality. Individuals with this genetic variation have no overt medical problems [30]. However, cure approaches using transplantation and CCR5Δ32 hematopoietic stem cell grafts in HIV+ individuals have resulted in a higher than expected mortality.

In summary, we present evidence that HIV can persist in tissues months after myeloablative UCB allo-HCT with CCR5Δ32 donor cells despite clearance of measurable HIV DNA from PBMCs. Our experience provides insight into the optimal management of HIV-infected patients with hematologic malignancies undergoing allo-HCT with CCR5Δ32 donor cells and also provides new information about the optimal tools to use when assessing new cure strategies.

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