Potential Role of a Mismatched HLA-Specific CTL Clone Developed Pre-Transplant in Graft Rejection following Cord Blood Transplantation

Hiroto Narimatsu, Makoto Murata, Seitaro Terakura, Kyoko Sugimoto, Tomoki Naoe

Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Correspondence and reprint requests: Makoto Murata, MD, PhD, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan (e-mail: mmurata@med.nagoya-u.ac.jp).

Received November 5, 2007; accepted January 1, 2008

ABSTRACT
Graft rejection is a serious complication in cord blood transplantation (CBT), but little is known about the mechanism of rejection. To investigate the potential role of T lymphocytes in graft rejection, we isolated a CD8⁺ cytotoxic T lymphocyte (CTL) clone of recipient origin from blood obtained from a patient with graft rejection after CBT from an HLA-mismatched unrelated donor. The isolated CTL clone specifically recognized an HLA-B*1501 molecule as an alloantigen, which was expressed in donor cells but not in recipient cells. The results of a microchimerism analysis specific for HLA-B*1501 and a polymerase chain reaction assay specific for the T cell receptor on DNA from pretransplant peripheral blood mononuclear cells revealed that the patient was exposed to HLA-B*1501 prior to CBT, and that the CTL clone was in the patient’s blood prior to transplantation. The present study demonstrates a potential role for pretransplant CTL in graft rejection following CBT.

© 2008 American Society for Blood and Marrow Transplantation

KEY WORDS
Graft failure • HLA class I antigen • T lymphocyte • Microchimerism • Reduced-intensity stem cell transplantation

INTRODUCTION
Although cord blood transplantation (CBT) from unrelated donors is an attractive treatment for patients with hematologic disease [1-6], graft rejection is a serious complication and is associated with a high rate of mortality [3,4,7,8]. The mechanisms of graft rejection have been extensively studied in bone marrow transplantation (BMT). One mechanism proposed by the studies in human and animal models is an immunologic response in which the host-derived T lymphocytes recognize donor-specific antigens [9-14]. For CBT, however, the role of the host-derived T lymphocytes in graft rejection has never been demonstrated in humans.

Here, we demonstrate a potential role of the host-derived cytotoxic T lymphocytes (CTLs) for graft rejection after CBT.

MATERIALS AND METHODS

Study Patient
A 59-year-old woman with acute myelomonocytic leukemia received CBT from an unrelated male donor. The details of the CBT procedure and the clinical course of the patient were reported previously [15]. Briefly, the preparative regimen consisted of 125 mg/m² fludarabine and 180 mg/m² melphalan, and 2.9 × 10⁷/kg nuclear cells were infused. White blood cell (WBC) and neutrophil counts increased to 100/µL and 60/µL, respectively, with 65% of donor chimerism on day 16, but both counts subsequently decreased to less than the detection limit of the autohemocytometer on day 26. Graft rejection was diagnosed based on severe marrow hypoplasia and a complete loss of donor chimerism in bone marrow cells on day 28. A second
CBT from an unrelated female donor was conducted on day 35. The infused nuclear cell dose was 2.9 × 10^7/kg. DNA typing of the HLA-A, -B, and -DRB1 loci demonstrated that the recipient was A*1101, B*1501, B*5603, and DRB1*1201/DRB1*1302, the first CBT donor was A*1101/A*2402, B*1501/B*5603, and DRB1*0901/DRB1*1201, and the second CBT donor was A*2402/A*3303, B*4403/B*5101, and DRB1*1201/DRB1*1302. The patient had HLA antibodies, including those against HLA-A33, and broad HLA-DR antigens prior to transplantation, whereas HLA antibody against HLA-B*1501 was not detected. The patient has one 19-year-old daughter. DNA typing of the HLA-A, -B, and -DRB1 loci demonstrated that her daughter was A*1101/A*2601, B*1501/B*5603, and DRB1*0405/DRB1*1201.

**Cell Culture**

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the patient on day 20 were cultured in interleukin-2-containing media without stimulator cells for 14 days, and T lymphocyte clones were isolated by limiting dilution.

**Transfection and CTL Stimulation Assays**

The patient’s B-LCL were transfected by electroporation with the pEAK10 plasmid (Edge BioSystems, Gaithersburg, MD) encoding HLA-B*1501 cDNA, selected for 3 days with 0.8 μg/mL of puromycin, and assayed as a target for N19D8 CTL. Cytotoxicity was determined using a chromium release assay [16]. Interferon-γ release assays were conducted as previously described [16]. Briefly, COS cells were transfected with a plasmid encoding HLA-B*1501, B*5603 (negative control), or B*4403 (negative control) cDNA using the FuGENE transfection reagent (Roche, Indianapolis, IN). COS transfectants were cocultured with N19D8 CTL, and interferon-γ production was measured in the supernatant after 24 hours using an enzyme-linked immunosorbent assay (Endogen, Pierce, Rockford, IL).

**Polymerase Chain Reaction (PCR) Specific for the T Cell Receptor**

The T cell receptor Vβ repertoire was determined by flow cytometry using an IOTest Beta Mark Kit (Beckman Coulter, Fullerton, CA). The nucleotide sequences of the CTL clone’s uniquely rearranged T cell receptor Vβ chain gene were determined by direct DNA sequencing of the amplified PCR product [17]. To determine the presence of the N19D8 clone-specific T cell receptor rearrangement, nested PCR was performed on genomic DNA extracted from a CTL clone N19D8 and PBMCs using a T cell receptor Vβ17-specific primer set for the first PCR: 5'-TTTCAGAAAGGAGATATGAGCT-3' (sense) and 5'-TTCTGATGGCTCAACAC-3' (antisense) followed by a second primer set specific for the N19D8 clone T cell receptor: 5'-GGAGATATAGCTGAAGGGGA-3' (sense) and 5'-CCCCGCAAAGCTACTCA-3' (antisense). PCR products were sequenced and confirmed to be identical in sequence to the N19D8-specific T cell receptor rearrangement. The PCR was performed with thermalcycler (Model 9600; Perkin-Elmer, Boston, MA) for 35 cycles under the following conditions: denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 1 minute in the first step, and denaturation at 95°C for 1 minute, primer annealing at 67°C for 15 seconds, and primer extension at 72°C for 1 minute in the second step. Each reaction contained 0.4 mL of Advantage 2 Polymerase Mix (Clontech Laboratories Inc., Palo Alto, CA).

**Microchimerism Analysis**

The presence of the microchimerism was determined using a nested PCR approach on genomic DNA extracted from a fingernail sample and PBMCs as previously described [18]. Briefly, nested PCR was performed on genomic DNA using an HLA-B-specific primer set for the first PCR: 5'-GCGGCGGGCGCGAGCAGCACCTGA-3' and 5'-GCCGGGGGCGCCAGGACCCGG-3' (1:1 mixture; sense) and 5'-GAGGCCCATCCCCGGGCACTTAT-3' (antisense) followed by a second primer set specific for HLA-B*1501: 5'-ACCCTGACACACAGATCTCC-3' (sense) and 5'-CTTGCGCTCGTAGGGGCGG-3' (antisense). The touchdown procedure [19] was performed as first-step PCR under the following conditions: (1) denaturation at 96°C for 20 seconds and primer annealing at 72°C for 2 minutes for 5 cycles, (2) denaturation at 96°C for 20 seconds and primer annealing at 70°C for 2 minutes for 5 cycles, (3) denaturation at 96°C for 20 seconds and primer annealing at 68°C for 2 minutes for 4 cycles, and (4) denaturation at 96°C for 20 seconds and primer annealing at 72°C for 2.15 minutes for 15 cycles. The second-step PCR was performed for 28 cycles under the following conditions: denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute, and primer extension at 72°C for 1 minute. FastStart Taq DNA Polymerase (Roche) and AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA) were used in the first- and the second-step PCR, respectively.

**RESULTS**

**Isolation of CTL Clone N19D8**

Two CTL clones were isolated from the peripheral blood of the patient just after the onset of graft rejection. One clone lysed B-LCL from the patient but failed to lyse B-LCL from the donor of the first CBT. The other clone, designated N19D8, lysed...
B-LCL from the donor but not from the patient (Figure 1A). Thus, we further investigated the N19D8 clone because this clone had the possibility of being involved in immunologic graft rejection. Flow cytometry analysis and sex chromosome fluorescein in situ hybridization revealed that the N19D8 clone was a CD3⁺CD4⁻CD8⁺ T cell and originated from the patient (data not shown).

**CTL Clone N19D8 Recognizes the HLA-B*1501 Molecule**

In a cytotoxicity assay using a panel of B-LCL derived from unrelated individuals, N19D8 CTL lysed all of 4 B-LCL lines from unrelated individuals that shared HLA-B*1501 but failed to lyse B-LCL from the donor of the second CBT and 7 unrelated individuals without B*1501 (Table 1). These results indicated that N19D8 CTL recognized the HLA-B*1501 molecule as an alloantigen, which was only expressed in donor cells, or a minor histocompatibility antigen presented by HLA-B*1501 molecule.

To determine if the lack of recognition by N19D8 CTL was solely because of the absence of HLA-B*1501 gene expression, the patient’s B-LCL were transfected with HLA-B*1501 cDNA and used as a target in cytotoxicity assays. The B*1501-transfected patient B-LCL was lysed almost as well as the first CBT donor’s B-LCL (Figure 1A). Furthermore, COS cells transfected with HLA-B*1501 cDNA alone stimulated interferon-γ production by N19D8 CTL (Figure 1B). Thus, we concluded that the N19D8 CTL clone recognizes mismatched HLA-B*1501 as an alloantigen.

**The Presence of the N19D8 CTL Clone and HLA-B*1501 Microchimerism Prior to Transplantation**

We next determined whether the N19D8 clone developed prior to transplantation using nested PCR assays specific for the CTL clone’s uniquely rearranged T cell receptor Vβ17 chain gene. PCR products were detected by amplification of DNA from pretransplant as well as posttransplant PBMCs (Figure 2), demonstrating that the N19D8 CTL clone developed in the patient prior to the first CBT. We further tested pretransplant PBMCs for chimeric cells with the HLA-B*1501 gene using PCR. Indeed, the HLA-B*1501 microchimerism was detected by amplification of DNA from the pretransplant patient PBMCs using standard PCR as well as nested PCR (Figure 3), indicating that the patient was exposed to HLA-B*1501 prior to CBT. The HLA-A*2601 microchimerism was also detected from the pretransplant patient PBMCs. From the patient PBMCs 3 months after transplantation, HLA-A*2601 and HLA-B*1501 microchimerisms were detected (data not shown).

**DISCUSSION**

Although direct verification would be difficult, it is reasonable to conclude that the N19D8 clone was involved in the graft rejection based on the following clinical and laboratory findings. First, the patient rejected the first cord blood graft with HLA-B*1501, whereas the second graft without B*1501 was successfully engrafted. This clinical course is consistent with allo-activity of the N19D8 clone. Second, CD3⁺CD4⁻CD8⁺ T lymphocytes accounted for the majority of the lymphocytes in the patient’s blood just after the onset of graft rejection (84.8%, data not shown). Third, no clones that recognize the other mismatched HLA antigens or minor histocompatibility antigens...
were isolated by the limiting dilution cloning from the patient just after the onset of graft rejection; N19D8 was the only clone with cytotoxic activity against the first donor cells. Last, the patient developed graft loss with a transient lymphocyte increase after reaching transient mix chimerism. It is difficult to differentiate immunologic graft rejection from other causes of graft failure in CBT [7]; however, this clinical course is consistent with those of patients who developed immunologic graft rejection following myeloablative or nonmyeloablative BMT [14,20]. Taken together, the results described above support the involvement of immunologic mechanisms such as cytotoxicity of T lymphocyte in the graft rejection.

The following routes of exposure of the patient to HLA-B*1501 can be considered. One possibility is that the patient was exposed to HLA-B*1501 during her pregnancy; her 19-year-old daughter has HLA-B*1501, presumably as an inherited paternal allele.

### Table 1. Cytotoxicity Assays for N19D8 CTL against B-LCL

<table>
<thead>
<tr>
<th>B-LCL</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>Sex</th>
<th>Lysis by CTL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient B-LCL</td>
<td>A*1101</td>
<td>A*2402</td>
<td>B*4403</td>
<td>B*5603</td>
</tr>
<tr>
<td>First-donor B-LCL</td>
<td>A*1101</td>
<td>A*2402</td>
<td>B*1501</td>
<td>B*5603</td>
</tr>
<tr>
<td>Second-donor B-LCL</td>
<td>A*2402</td>
<td>A*3303</td>
<td>B*4403</td>
<td>B*5101</td>
</tr>
<tr>
<td>L17</td>
<td>A*2402</td>
<td>A*3303</td>
<td>B*1501</td>
<td>B*4403</td>
</tr>
<tr>
<td>L79</td>
<td>A*2402</td>
<td>A*3303</td>
<td>B*1501</td>
<td>B*4403</td>
</tr>
<tr>
<td>JMD</td>
<td>A*0201</td>
<td>A*2901</td>
<td>B*1501</td>
<td>B*4403</td>
</tr>
<tr>
<td>L16</td>
<td>A*1101</td>
<td>A*2402</td>
<td>B*5401</td>
<td>B*5101</td>
</tr>
<tr>
<td>L19</td>
<td>A*2402</td>
<td>A*3303</td>
<td>B*0702</td>
<td>B*4403</td>
</tr>
<tr>
<td>L25</td>
<td>A*1101</td>
<td>A*2402</td>
<td>B*5401</td>
<td>B*5201</td>
</tr>
<tr>
<td>L59</td>
<td>A*2603</td>
<td>A*3303</td>
<td>B*3501</td>
<td>B*4403</td>
</tr>
<tr>
<td>L73</td>
<td>A*0201</td>
<td>A*2402</td>
<td>B*0702</td>
<td>B*6701</td>
</tr>
<tr>
<td>L74</td>
<td>A*1101</td>
<td>A*3303</td>
<td>B*4002</td>
<td>B*5101</td>
</tr>
<tr>
<td>RAR</td>
<td>A*2901</td>
<td>A*3101</td>
<td>B*2705</td>
<td>B*4403</td>
</tr>
</tbody>
</table>

NA indicates not available.

*E:T ratio was 10:1.

Figure 2. Presence of the N19D8 clone in pretransplant peripheral blood from the patient. Detection of lymphocytes with the N19D8 clone-specific T cell receptor in pretransplant PBMCs. Nested PCR was performed on genomic DNA using a T cell receptor Vβ17-specific primer set for the first PCR. Genomic DNA was prepared from PBMCs obtained from the patient 1 month before the first CBT (pre PBMCs) and 3 or 9 months after the second CBT (post PBMCs). Genomic DNA from the N19D8 clone was used as a positive control. Genomic DNA prepared from PBMCs obtained from unrelated volunteers were used as negative controls. PCR to detect β-globin was used as an internal control in each assay.

Figure 3. Presence of the N19D8 clone in pretransplant peripheral blood from the patient. The presence of HLA-B*1501 microchimerism in pretransplant PBMCs. Lanes 1 and 6 are standard PCRs specific for HLA-B*1501 on genomic DNA from pretransplant PBMCs from the patient and B*1501-positive PBMCs from the patient’s daughter (positive control), respectively. Lanes 2-3, 4-5, and 7-8 are nested PCR specific for HLA-B*1501 on diluted (in parentheses) first PCR products from a posttransplant fingernail sample from the patient (negative control), pretransplant PBMCs from the patient, and PBMCs from the patient’s daughter (positive control), respectively. PCR to detect complement factor 4 was used as an internal control in each assay.
is known that fetal hematopoietic chimerism can persist for decades, maintaining a paternal-specific CTL developed during pregnancy over a long period of time [21,22]. Another possibility is exposure via blood transfusions. The patient had previously received blood transfusions from random donors, and development of HLA-B*1501-specific CTL as a consequence of contamination by HLA-B*1501-positive WBCs in the transfusion products cannot be completely ruled out. Future studies are needed to determine which of these events was related to graft rejection. It is to be noted that the presence of an HLA-B*1501 microchimerism was demonstrated even after the onset of the graft rejection. Although the mechanism is uncertain, the N19D8 CTL clone might not be able to completely eliminate B*1501-positive cells. Further studies focusing on this basic mechanism are warranted.

Interestingly, the patient had the HLA antibodies including those against the second donor HLA, HLA-A*3303, whereas the patient did not have the HLA antibody against HLA-B*1501. This suggested that HLA-antibodies did not have clinical impact on the graft rejection in this patient. However, it is inconsistent with the previous studies in solid organ transplantations or bone marrow transplantations [23,24]. This inconsistency indicates the clinical impact of HLA antibodies is controversial in CBT. Future studies would allow a proper interpretation.

In conclusion, the results of the present study demonstrate a potential role for pretransplant CTL in graft rejection following CBT. Further studies focusing on mismatched HLA-specific CTLs should help to establish an optimal strategy for overcoming graft rejection in CBT.

ACKNOWLEDGMENTS

We are grateful to Chika Wakamatsu, Yuka Nomura, and Ryohei Tanizaki for technical assistance. We are also grateful to Dr. Etsuko Maruya and Dr. Hiroh Saji (HLA Laboratory, NPO, Kyoto, Japan) for detecting microchimerism in this study. This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan (to M.M.) and the National Institute of Biomedical Innovation (to T.N.).

REFERENCES


