EARLY DETECTION OF BONE MARROW ENGRAFTMENT BY AMPLIFICATION OF HYPERVARIABLE DNA REGIONS

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ABSTRACT

Background and Objective. After allogeneic bone marrow transplantation (BMT) it is important to be able to distinguish between the host and donor origin of cells in order to monitor the engraftment process. However, identifying whether the hematopoietic stem cells are of donor or recipient origin may be a difficult task. DNA studies using Southern blotting techniques or the amplification by PCR of regions in the human genome with high polymorphic neutral sequence variation showing Mendelian inheritance as variable number of tandem repeats (VNTR) can detect the origin of host bone marrow after BMT. We have tried to apply these sensitive systems of detection to the early stages of BMT when small numbers of regenerated cells are available for analysis.

Methods. We used in vitro polymerase chain reaction (PCR) amplification of three single-locus simple repetitive DNA sequences, all of which vary extensively in their repeat number among different individuals (VNTR D1S80, ApoB, and D17S5), to evaluate post transplant engraftment in six patients who showed no signs of peripheral blood engraftment at 2-3 weeks after transplant. We tested 2 patients with chronic myelogenous leukemia (CML), 2 with B-acute lymphoblastic leukemia (B-ALL), 1 with T-acute lymphoblastic leukemia (T-ALL), and 1 with aplastic anemia (SAA), all in prolonged aplasia following allogeneic bone marrow transplantation (BMT).

Results. In a sequential analysis protocol with the different loci, the donor was distinguishable from the recipient in all pairs with at least one of the three markers used. After 16 days (median 16.2; range 15 to 20 days) we found that complete chimerism was present in 5 patients: 4 of donor origin (= engraftment) and one of host origin (= rejection), this last case being one of mixed chimerism. In the 2 cases in which the presence (one complete and one partial) of host DNA was detected, rejection of the donor bone marrow followed, and in 1 patient a second BMT was necessary. The other 4 patients with complete chimerism of donor origin achieved hematological reconstitution and we documented complete engraftment of donor bone marrow a few months later.

Interpretation and Conclusions. Utilizing PCR to document early post-transplant engraftment and chimerism in the first month after BMT has the advantage over Southern blotting of being more sensitive and requiring small amounts of sample. It may also be useful for guiding subsequent therapeutic decisions.

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Key words: bone marrow transplantation, PCR, VNTR
showing Mendelian inheritance as variable number of tandem bone repeats (VNTR) could detect the origin of host bone marrow after BMT.\(^6\)\(^-\)\(^8\)\(^,\)\(^9\)\(^,\)\(^10\)\(^,\)\(^11\) We previously reported on three different VNTRs\(^6\)\(^-\)\(^8\)\(^,\)\(^9\)\(^,\)\(^10\) which were found to be useful in monitoring hematopoietic cell chimerism after BMT.\(^16\)\(^-\)\(^20\) We have now tried to apply these sensitive detection systems to the early stages of BMT when small numbers of regenerating cells are available for analysis. For this purpose, we sequentially amplified three VNTRs to assess hematopoietic cell origin after BMT. Using these techniques, the presence of donor and recipient cells after BMT can be detected at levels approaching 1% by Southern blotting and 0.01% by PCR,\(^6\)\(^-\)\(^11\) respectively.

**Materials and Methods**

**Patients**

Six allogeneic BMT donor-recipient (D/R) pairs were studied. Clinical data, disease status, donor and recipient sex, transplant conditioning regimens, GvHD prophylaxis, and outcome of BMT are shown in Table 1. All D/R pairs were matched at the HLA A, B, C, and DR loci and were non-reactive in mixed lymphocyte cultures (MLC). One of these patients (#4 in Table 1) was transplanted with a serological and molecular HLA-class I and HLA-DRB1, DQA and DQB typed identical unrelated donor;\(^12\)\(^-\)\(^14\) a DP gene mismatch between donor and recipient was present.

**Transplant conditioning regimens**

After informed consent, all patients received a combination of chemotherapy. Conditioning chemotherapy included busulphan (BU in Table 1), cyclophosphamide (Cy in Table 1), etoposide (VP16 in Table 1) and total body irradiation was performed as reported.\(^15\) Unrelated donor bone marrow was not T-cell depleted prior to transplant to prevent graft versus host disease (GvHD). All patients received irradiated leukocyte-depleted blood products throughout the course of their BMT. Post BMT anti-GvHD treatment consisted of cyclosporin A (CsA) in three patients, and CsA plus methotrexate (MTX) in the remaining three patients (Table 1).

**PCR analysis**

The time of bone marrow aspirates and the DNA markers used are indicated in Table 2. After informed consent high molecular weight DNA was extracted from bone marrow samples as previously described.\(^16\) The genomic regions studied were: locus D1S80, with a 16 bp repeat detected by probe MCT 118, located on the short arm of chromosome 1;\(^17\) ApoB, with a 14-16 bp repeat located about 0.5 kb on the 3' side of the last amino acid codon in the apolipoprotein B gene;\(^18\) D17S5, with a 70 bp repeat detected by probe YNZ22, on the short arm of chromosome 17.\(^19\) Analyses on ApoB, D1S80, D17S5, were performed as described.\(^20\) The primers used are reported in the literature.\(^16\)\(^-\)\(^19\)\(^,\)\(^22\)\(^-\)\(^27\) Briefly, the cycling parameters were as follows: 94°C, 20 sec; 55°C, 30 sec, and 72°C, 1 min, for 30 cycles, as previously described.\(^16\)\(^-\)\(^27\) Observed allele number and heterozygosities in the Italian population for these polymorphisms are given elsewhere.\(^26\)\(^,\)\(^27\) SpERMidine 0.25 mM final concentration was added in the PCR reaction mix to increase the specific product yield. After amplification, 1/10 of the sample volume was electrophoresed through standard non-denaturing 8% polyacrylamide gels (D1S80, ApoB) or 2% agarose gels (D17S5). Gels were subsequently stained with ethidium bromide and photographed under UV light. All primers used in this study were synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer (Applied Biosystems, Foster City, CA, USA) and used without further purification. Negative controls were included in all PCRs.\(^20\)\(^-\)\(^23\) In the two CML cases, the presence of bcr-abl neoplastic transcripts was investigated by RT-PCR using a very sensitive procedure.\(^24\) Conditions for preventing and for revealing possible contamination were used as previously described.\(^25\)\(^-\)\(^27\)

**Results**

Assessment of chimerism using hypervariable DNA polymorphisms

Details of chimerism evaluation of the D/R pairs investigated with two or more of the hypervariable DNA markers D1S80, ApoB and D17S5 amplified previously described.\(^26\) The genomic regions studied were: locus D1S80, with a 16 bp repeat detected by probe MCT 118, located on the short arm of chromosome 1;\(^17\) ApoB, with a 14-16 bp repeat located about 0.5 kb on the 3' side of the last amino acid codon in the apolipoprotein B gene;\(^18\) D17S5, with a 70 bp repeat detected by probe YNZ22, on the short arm of chromosome 17.\(^19\) Analyses on ApoB, D1S80, D17S5, were performed as described.\(^20\) The primers used are reported in the literature.\(^16\)\(^-\)\(^19\)\(^,\)\(^22\)\(^-\)\(^27\) Briefly, the cycling parameters were as follows: 94°C, 20 sec; 55°C, 30 sec, and 72°C, 1 min, for 30 cycles, as previously described.\(^16\)\(^-\)\(^27\) Observed allele number and heterozygosities in the Italian population for these polymorphisms are given elsewhere.\(^26\)\(^,\)\(^27\) SpERMidine 0.25 mM final concentration was added in the PCR reaction mix to increase the specific product yield. After amplification, 1/10 of the sample volume was electrophoresed through standard non-denaturing 8% polyacrylamide gels (D1S80, ApoB) or 2% agarose gels (D17S5). Gels were subsequently stained with ethidium bromide and photographed under UV light. All primers used in this study were synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer (Applied Biosystems, Foster City, CA, USA) and used without further purification. Negative controls were included in all PCRs.\(^20\)\(^-\)\(^23\) In the two CML cases, the presence of bcr-abl neoplastic transcripts was investigated by RT-PCR using a very sensitive procedure.\(^24\) Conditions for preventing and for revealing possible contamination were used as previously described.\(^25\)\(^-\)\(^27\) Total RNA extraction, cDNA synthesis and PCR were performed as previously reported.\(^24\)

Table 1. Clinical data from the six transplanted patients.

<table>
<thead>
<tr>
<th>No.</th>
<th>UPN</th>
<th>Name</th>
<th>Diagnosis</th>
<th>Disease status</th>
<th>Donor/recipient</th>
<th>First transplant conditioning</th>
<th>WBC at +20 (x10^9/L)</th>
<th>PLTs at +20 (x10^9/L)</th>
<th>GVHD prophylaxis</th>
<th>GVHD grade</th>
<th>Acute GVHD</th>
<th>Survival</th>
<th>2nd BMT</th>
<th>HLA class I</th>
<th>HLA class II</th>
<th>Donor/recipient</th>
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<tbody>
<tr>
<td>1</td>
<td>159</td>
<td>FA</td>
<td>SAA</td>
<td>Aplastic P</td>
<td>M/F</td>
<td>Cy</td>
<td>0.2</td>
<td>40</td>
<td>CsA</td>
<td>0</td>
<td>neg</td>
<td>44 mo</td>
<td>yes</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>173</td>
<td>NL</td>
<td>B-ALL</td>
<td>Relapse</td>
<td>M/M</td>
<td>Bu+Cy</td>
<td>0.6</td>
<td>40</td>
<td>CsA</td>
<td>0</td>
<td>neg</td>
<td>3 mo</td>
<td>no</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>161</td>
<td>BE</td>
<td>T-ALL</td>
<td>II CR</td>
<td>M/M</td>
<td>TBI+Cy</td>
<td>0.36</td>
<td>28</td>
<td>CsA+MTX</td>
<td>1</td>
<td>neg</td>
<td>23 mo</td>
<td>no</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>170</td>
<td>DM</td>
<td>CML</td>
<td>Blast</td>
<td>M/M</td>
<td>TBI+Cy+VP16</td>
<td>0.1</td>
<td>40</td>
<td>CsA+MTX</td>
<td>2</td>
<td>2</td>
<td>4 mo</td>
<td>no</td>
<td>M</td>
<td>DP mismatch</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>145</td>
<td>MF</td>
<td>CML</td>
<td>CP</td>
<td>F/M</td>
<td>Bu+Cy+VP16</td>
<td>1.4</td>
<td>80</td>
<td>CsA</td>
<td>3</td>
<td>3</td>
<td>4 mo</td>
<td>yes</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>167</td>
<td>TD</td>
<td>B-ALL</td>
<td>Relapse</td>
<td>M/M</td>
<td>TBI+Cy+</td>
<td>0.31</td>
<td>28</td>
<td>CsA+MTX</td>
<td>1</td>
<td>1</td>
<td>36 mo</td>
<td>no</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: No., number of patient; Name, initial of patient’s name; M, male; F, female; Diagnosis, disease classification; SAA, severe aplastic anemia; B-ALL, acute lymphoblastic leukemia of B lineage; CML, chronic myelogenous leukemia; T-ALL, acute lymphoblastic leukemia of T lineage; Disease status, disease phase at BMT; Aplastic P, aplastic phase; II CR, second complete remission; Blast, blast phase; CP, chronic phase; mo, months; Cy, cyclosporin A; MTX, methotrexate; GVHD, graft versus host disease grade; M, method; neg, negative; Bu, busulphan; Cy, cyclophosphamide; VP16, etoposide.
in vitro are reported in Table 2. Figure 1 shows an example of mixed chimerism detected with D17S5; Figure 2 illustrates complete chimerism (T.D. in Tables 1 and 2) detected with ApoB polymorphism. In the D/R couple formed by unrelated individuals (D.M.), as expected, no identical polymorphism was evidenced with either of the 2 markers tested. Polymorphisms which could identify chimerism were found in all 6 (100%) pairs of patients who underwent allogeneic transplantation (Table 2). In 6/6 (100%) pairs, D1S80, the first marker used, was sufficient for identification (so-called informative). The second (ApoB) was informative in 5/6 (83%) cases; and the last polymorphism employed, D17S5, was also able to distinguish between donor and recipient in case #2 (Table 2).

Of the 5 patients who became chimeras, one (M.F. in Tables 1 and 2) displayed a CC of recipient origin (CC* in Table 2) and experienced graft failure (= rejection); bcr-abl mRNA detection by RT-PCR confirmed leukemia relapse at the molecular level. Also, cytogenetic analysis for the Ph+ and Y chromosomes was positive for relapse or non engraftment. After a long period of aplasia (> 40 days) during which only a few cells of host origin were detectable by PCR, this patient underwent a second transplant from his brother. Fifteen days after this second BMT his hematopoiesis was of donor origin (CC), but the patient died of complications due to infection four months after the first BMT. No mixed chimerism, Ph+ metaphases or bcr-abl mRNA (minimal residual disease) was docu-

Table 2. Molecular data from the six transplanted patients. Cytogenetic and molecular evaluation of bone marrow origin by PCR analysis is reported.

<table>
<thead>
<tr>
<th>No. UPN</th>
<th>Name</th>
<th>Diagnosis</th>
<th>Day post-BMT</th>
<th>D1680</th>
<th>ApoB</th>
<th>D17S5 (YNZ222)</th>
<th>Cytogenetic of Y chr.</th>
<th>Clinical status at the date of VNTR analysis</th>
<th>Clinical status after 2 months with the informative VNTR</th>
<th>Courses of, VNTR status at 15-20 days after BMT</th>
<th>PCR of bcr-abl status at 15-20 days after BMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>159</td>
<td>FA</td>
<td>SAA</td>
<td>15 days</td>
<td>CC</td>
<td>ND</td>
<td>ND</td>
<td>Not engrafted</td>
<td>Engrafted</td>
<td>CC</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>173</td>
<td>NL</td>
<td>B-ALL</td>
<td>15 days</td>
<td>MC</td>
<td>MC</td>
<td>ND</td>
<td>Not engrafted</td>
<td>Engrafted</td>
<td>MC/Died</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>161</td>
<td>BE</td>
<td>T-ALL</td>
<td>17 days</td>
<td>CC</td>
<td>Neg</td>
<td>ND</td>
<td>Not engrafted</td>
<td>Engrafted</td>
<td>CC</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>170</td>
<td>DM</td>
<td>CML</td>
<td>20 days</td>
<td>CC</td>
<td>ND</td>
<td>ND</td>
<td>Not engrafted</td>
<td>Engrafted</td>
<td>CC</td>
<td>pos</td>
</tr>
<tr>
<td>5</td>
<td>145</td>
<td>MF</td>
<td>CML</td>
<td>15 days</td>
<td>CC*/CC*</td>
<td>CC*/CC*</td>
<td>ND</td>
<td>CC*</td>
<td>Not engrafted</td>
<td>Not engrafted</td>
<td>Died</td>
</tr>
<tr>
<td>6</td>
<td>167</td>
<td>TD</td>
<td>B-ALL</td>
<td>15 days</td>
<td>CC</td>
<td>ND</td>
<td>ND</td>
<td>Not engrafted</td>
<td>Engrafted</td>
<td>CC</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. CC, complete chimerism; CC*, complete chimerism (results related to the first BMT) means that in case #5 there was failure of the first transplantation after which a second procedure was performed, and this was followed by CC of donor origin; MC, mixed chimerism.
mented for the second BMT in this CML patient.

Patient F.A. (#1 in Tables 1 and 2) received a first BMT from her brother and after 20 days of aplasia was found to possess a few hematopoietic cells of donor origin (CC). After a prolonged aplastic phase without engraftment (no take), an additional late decline in peripheral blood leukocyte count was noted (leukocyte number under \(0.2 \times 10^9/L\)), raising clinical suspicion of graft rejection. She was again conditioned and transplanted from the same donor and achieved an engraftment of donor origin which had lated for 44 months so far.

The other patients had bone marrow donor engraftment confirmed at the molecular level that lasted 3, 23, and 36 months. Patient D.M. died 4 months after BMT of GVHD complications in clinical but not in molecular remission. Patients T.D. and B.E. died of ALL relapse 36 and 23 months after BMT, respectively.

Mixed chimerism was documented 20 days after BMT in patient N.L., who had undergone BMT for B-ALL. After 3 months of molecularly detectable mixed chimerism, together with a complete hematological remission following a rapid increase of host hematopoiesis and clinical relapse of B-ALL, this patient died.

**Discussion**

This study confirms the use of a PCR-based method to monitor engraftment following allogeneic BMT. We describe the use of polymorphic regions to document engraftment or rejection in 6 patients post BMT at time points when peripheral leukocyte counts were low.

Amplified sequence polymorphisms are a useful tool for evaluating hematopoietic chimerism, and they have also been used by others for determining the origin of leukemic cells in patients with recurrent disease after BMT and for the study of marrow engraftment after allogeneic BMT. When limited quantities of DNA are obtainable from an aplastic or still leukopenic patient immediately following BMT, DNA analysis by Southern blot is not always possible. The PCR method overcomes this problem by allowing detection of mixed chimerism at levels below 1%. We did not attempt to quantify our results using the quantitative PCR strategies reported; however, the random distribution of the hypervariable loci on the human genome allows us to assess chimerism even in the presence of chromosomal rearrangements or genetic losses, such as monosomies or deletions, which are frequently reported in leukemias and after hematological disorders.

It has been reported that many different markers need to be employed in order to achieve complete characterization of transplanted tissue origin. In our study, all patients obtained a diagnostic result through the use of only two VNTRs of DNA polymorphism. Our finding that D1S80, a highly polymorphic marker, proved to be the most useful for chimerism detection is in accordance with the high heterozygosity of this locus previously described in a larger sample of Italians. This finding is important because it indicates that 70% or more of the BMT cases in our population may be investigated with only one PCR assay, which reduces the cost and time required for analysis. When, as should always be possible, the polymorphism of the locus is determined prior to BMT, there should be no need to take other polymorphisms into consideration after BMT, thus reducing the analysis time.

Whereas PCR polymorphism gave useful information in all patient pairs, karyotypic analysis was possible in only two patients and on samples collected from 11 to 30 days after BMT. Another advantage of the PCR-based method is that it is applicable to all cases, even when no cytogenetic markers are present (DNA from all nucleated cells is available for analysis by PCR, while cytogenetic analysis is restricted to actively dividing sample cells).

Most microsatellite assays use radioactively labeled PCR products and denaturing acrylamide gels. Our method allows direct analysis of PCR products on ethidium bromide soaked gels. This technique has the advantages of speed, sensitivity, and ease of analysis because it involves only two steps: amplification and electrophoresis.

Detection of engraftment as early as day 7 has been reported with leukocyte counts as low as \(0.2 \times 10^9/L\). Our results suggest late engraftment or failure to engraft. In this study, engraftment was documented in four cases and early graft rejection was documented in one.

In conclusion, our results confirm the previously reported advantages of PCR-based analysis of engraftment following BMT and extend the application of this technique to the early phase of BMT. The method described here provides increased speed, sensitivity and ease of analysis of polymorphic sequences since only two steps (amplification and gel electrophoresis) are required. Finally, it should be noted that this method might prove suitable for other forms of engraftment, including liver transplantation.

**References**