Detection of Breakpoint Cluster Region-Negative and Nonclonal Hematopoiesis In Vitro and In Vivo After Transplantation of Cells Selected in Cultures of Chronic Myeloid Leukemia Marrow


Philadelphia (Ph1) chromosome-positive clonogenic progenitors usually disappear within 4 to 6 weeks in long-term cultures established from the marrow of patients with chronic myeloid leukemia (CML). In contrast, coexisting chromosomally normal hematopoietic cells are relatively well maintained. Thus, even though normal cells are initially undetectable, they may become the predominant population. Recently, we have begun to explore the potential of such cultures as a strategy for preparing CML marrow for autografting, and based on cytogenetic studies of the differential kinetics of Ph1-positive and Ph1-negative clonogenic cells, have chosen a 10-day period in culture to obtain maximal numbers of selectively enriched normal stem cells. Here we present the results of molecular analyses of the cells regenerated in vivo for the initial three CML patients to be treated using this approach by comparison with the differentiated cells generated by continued maintenance of an aliquot of the autograft in vitro (using a slightly modified culture feeding procedure to enhance the production and release of cells into the nonadherent fraction after 4 weeks) for the one patient whose genotype made molecular analysis of clonality status also possible. These analyses showed that cells with a rearranged breakpoint cluster region (BCR) gene were not detectable by Southern blotting in either in vitro or in vivo populations of mature cells that might be assumed to represent the progeny of primitive cells present at the end of the initial 10 days in culture. Production of BCR-negative cells was also shown to be temporally correlated with the appearance of nonclonal hematopoietic cells both in culture and in vivo. These findings provide support for the view that prolonged maintenance of CML marrow cells in long-term culture may allow molecular characterization of both the BCR-genotype and clonality status of cells with in vivo regenerative potential.

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The Philadelphia (Ph1) chromosome is the hallmark of chronic myeloid leukemia (CML), a clonal myeloproliferative malignancy believed to originate in a pluripotent hematopoietic cell. Molecular studies have identified a small region on chromosome 22, called the breakpoint cluster region (BCR), which is broken and rejoined to the c-ABL protooncogene, normally located at the distal end of the long arm of chromosome 9. This rejoining results in the formation of the hybrid BCR-ABL gene and the synthesis of an abnormal gene product with tyrosine kinase activity analogous to that exhibited by the oncogenic murine v-ABL gene product. Using appropriate DNA probes, it is now routinely possible to detect the presence of nondividing Ph1-positive cells using Southern or polymerase chain reaction (PCR) techniques, thereby increasing accessibility of the neoplastic clone to studies beyond those dependent on cytogenetic analysis (which are restricted to proliferating cell populations). Molecular studies have thus facilitated assessment of treatment response in CML in a variety of settings, in particular to demonstrate persistence of residual disease after suppression of the neoplastic clone by interferon or intensive therapy supported by syngeneic or allogeneic bone marrow transplantation. Such studies have also made it possible to analyze various purified subpopulations of hematopoietic cells. These have helped to establish a lack of correlation between the particular site of the breakpoint within the BCR-1 gene and the involvement of lymphoid and/or myeloid cells in the neoplastic clone.

In this report we show how molecular studies may be useful to assess the hematopoietic stem cell population present in 10-day culture-purged CML marrow autografts based on the demonstration of the disappearance of clonal and BCR-positive cells, and the reappearance of nonclonal and BCR-negative cells after further maintenance of the cells in vitro, or in vivo following transplantation of the cultured autograft.

MATERIALS AND METHODS

PATIENT SAMPLES. All marrow and blood samples in this study were obtained with informed consent from the first three CML patients entered into a clinical trial developed in Vancouver. This trial was designed to evaluate the potential of cultured marrow autografts to allow hematologic recovery following myeloablative therapy and has been described previously. A blood sample was obtained from each patient before harvesting the marrow used for autografting, and then periodically after transplantation as part of the routine follow-up procedure. Light density (<1.077 g/cm³) blood mononuclear cells were separated from the red blood cells (RBCs) and granulocytes by density gradient centrifugation on Ficoll-Hypaque, and a greater than 95% pure granulocyte fraction...
then recovered from the pellet by lysis of the RBCs with NH4Cl-Tris-HCl. Bone marrow samples were collected in preservative-free sterile heparin and then divided in aliquots as required for initiation of colony assays or long-term cultures, for cryopreservation in dimethyl sulfoxide (DMSO), and for DNA extraction following washing in phosphate-buffered saline (PBS) and lysis of contaminating RBCs.

Cultures. The procedure for initiating, maintaining, and harvesting cells for autografting from long-term marrow cultures was based on that used in our laboratory for many years, with the minor modification that all handling of the cells or medium for the first 10 days was eliminated. Most of the cultured cells were then harvested and used for autografting as previously described, although a small number of sample cultures were maintained for up to 8 weeks and periodically analyzed for clonogenic cell content.

In the case of unique patient no. (UPN) 248, long-term marrow cultures were also set up with a thawed aliquot of cryopreserved marrow from the same harvest used to initiate cultures for autografting. The recovery of viable cells (based on 1% nigrosin dye exclusion) after thawing using DNAase was 40%. These viable cells, 10^7, were then inoculated into each of eight 75-cm^2 flasks containing 30 mL of standard long-term culture medium. These flasks were maintained for the first 4 weeks with weekly half medium changes. All of the medium containing the nonadherent cells was then removed from each flask and the cultures refed with the same medium (30 mL/flask), but containing 30% fetal calf serum (FCS) rather than horse serum, and to which 10% medium from confluent 5637 cell cultures (obtained from the American Type Tissue Collection, Rockville, MD) was also added. This modified feeding procedure was repeated at weekly intervals until termination of the experiment at week 8.

Molecular studies. DNA extractions, digestions, and Southern analyses were as previously described. Dilution of pretransplant CML marrow DNA with normal human DNA showed a linear relationship between the proportion of CML DNA run in each lane and the intensity of the rearranged BCR-gene band measured densitometrically over a range of 2% to 90% (data not shown), thus validating the use of Southern analysis to provide a quantitative measure of the proportion of BCR-positive cells in test samples. Operational criteria used to infer monoclonality, oligoclonality, and polyclonality from methylation-sensitive changes in phosphoglycerate kinase (PGK) band intensities were as defined by Vogelstein et al.

RESULTS

Patients. Table 1 summarizes the clinical information on the three CML patients studied. A more detailed presentation of the treatment, and clinical and laboratory findings on these patients has been published previously. All had Ph'-positive CML and were in a first, second, or third chronic phase at the time of autografting. Southern analyses of BamHI-digested DNA from pretransplant samples of marrow and blood cells showed unique rearrangements of the BCR-1 gene in each patient (Fig 1 through 3 and Table 2). It should be noted that direct metaphase preparations of pretreatment marrow samples showed exclusively (Fig 1) or predominantly (Figs 2 and 3B) Ph'-positive metaphases consistent with clonal dominance of the neoplastic clone in the most terminal compartments of all three patients studied, despite the penetration into the clonogenic compartments of significant numbers of Ph'-negative cells in UPN 248 (Fig 3A). The BCR data for pretransplant granulocyte and marrow samples are also consistent with this finding.

In one patient (UPN 248), the presence of a polymorphic Bgl I site in one of her X-linked PGK genes also made it possible to undertake clonality studies. Southern analysis of DNA from pretransplant blood and marrow samples from this patient after sequential digestion with EcoRI, Bgl II, Bgl I, and then Hpa II, showed a pattern typical of a monoclonal cell population (Fig 4). Neither of the other two patients was suitable for clonality studies because of homozgyosity at both the hypoxanthine phosphoribosyl transferase (HPRT) and PGK loci (data not shown).

Analyses of cultured marrow cells. Cytogenetic studies of erythroid and granulopoietic colonies produced by progenitors harvested from the long-term marrow cultures established from all three patients indicated that after 4 to 6 weeks Ph'-positive clonogenic cells had declined to very low levels, but that Ph'-negative clonogenic cells were readily detectable. Because cytogenetic analyses tend to be biased towards the selection of larger colonies containing at least 500 immature cells (to ensure the presence of some metaphase cells), it was of interest to investigate the pattern of population changes demonstrated by a less selective, but nevertheless quantitative technique, that could be applied to all hematopoietic cells in the culture, eg, by Southern analysis of extracted DNA. Because one, but only one, of the patients (UPN 248) offered the opportunity to assess changes in BCR-positivity and clonality simultaneously, it was of greatest interest to analyze the cells in cultures established with marrow from this individual. However, to undertake such studies, it was important to identify a practical procedure that would allow us to obtain appropriate samples of cultured cells.

In established (>4 weeks old) long-term cultures initiated with either normal or CML marrow, usually less than 20% of all the cells present are in the nonadherent fraction. Consequently, the adherent layer, even if incomplete as is often the case in cultures initiated with CML marrow, usually contains more than 80% of all the cells present. However, a significant proportion of these are stromal cells of nonhematopoietic origin. On the other hand, it has been shown.

Table 1. Patient Characteristics

| Patient (UPN) | Age* (y) | Sex | Pre-IMT Status % Ph'-positive cells† | Outcome
|---------------|---------|-----|-------------------------------------|--------
| 208           | 44      | F   | CML in 1st CP (100%)                | Alive on a-interferon, clinically normal 26 mo after autografting |
| 232           | 53      | F   | CML in 3rd CP (90%)                 | Died of recurrent blast phase disease on day + 125 |
| 248           | 41      | F   | CML in 2nd CP (84%)                 | Died of therapy-related toxicity on day + 28 |

Abbreviation: UPN, unique patient number.

*At the time of autografting.
†As determined from direct marrow preparations.
that the nonadherent fraction, although smaller, consists almost exclusively of terminally differentiating granulopoietic cells that are continuously released as a result of the proliferative activity of more primitive hematopoietic cells that remain in the adherent layer.\textsuperscript{15,19,22} Thus, assessment of the nonadherent cell fraction might offer a strategy for assessing the genotype of hematopoietic cells active in the adherent layer 1 to 2 weeks previously. In preliminary experiments we found that the production and release of hematopoietic cells into the nonadherent fraction appeared to be enhanced when we switched the feeding procedure after 4 weeks to a complete, rather than a half, medium change and altered its composition to resemble more closely that used to stimulate terminal granulopoiesis in methylcellulose assays containing a source of granulopoietic stimulating factors (see Materials and Methods for details). Other experiments have since shown that addition of such factors to long-term marrow cultures enhances the number of both clonogenic granulopoietic cells and terminally differentiated granulocytes and macrophages found in the nonadherent fraction.\textsuperscript{23,24} To ensure that the nonadherent cells obtained after initiation of this modified feeding procedure could be used to provide sequential samples of the preceding activity of the hematopoietic population remaining in the adherent layer, all nonadherent cells were removed each week for analysis. Using this method, 5 to 10 \mu g of DNA were routinely obtained per 30 mL culture (each initiated with 10\textsuperscript{6} cryopreserved cells).

Figures 3 and 4 show the results of both BCR and clonality (PGK) analyses of marrow cells from UPN 248 before and
NORMAL STEM CELLS IN CML MARROW CULTURES

Fig 3. (A) Analysis of BCR rearrangement in BamH1-digested DNA from nonadherent cells collected weekly from long-term marrow cultures, compared with pretransplant granulocyte DNA from UPN 248. The BCR rearrangement is no longer detectable after 5 weeks. Adherent cells collected by trypsinization after 5 weeks (1 flask) and 8 weeks (7 flasks) also did not show detectable BCR rearrangement (data not shown). Cytogenetic data are from analysis of in vitro colony assays. (B) BCR analysis of BamH1-digested DNA from a granulocyte sample from UPN 248 taken 28 days after autografting. Cytogenetic data are from analysis of bone marrow metaphases.

after varying periods of time in culture. At the end of the first week in culture, cells showing the BCR rearrangement typical of this patient’s clone were still prevalent (Fig 3A). DNA extracted from these cells also showed a monoclonal pattern of methylated fragments (data not shown). The same results were also obtained on the original cultures that had been initiated with fresh marrow cells and harvested and infused after a continuous period of 10 days in culture (89% nonadherent cells in this case). These findings are consistent with the continued presence of Ph1-positive clonogenic progenitors in the cells infused (16% Ph1-positive). Analysis of the nonadherent population obtained after longer periods of culture showed a decline in BCR-positive cells, which fell below the limit of detectability by 5 weeks and remained undetectable thereafter (Fig 3A). BCR-positive cells were also not detectable in the adherent layer after 5 to 8 weeks (data not shown). Clonality studies showed a concomitant emergence of a second PGK band in Southern blots of HpaII-digested samples of her nonadherent cells, indicating the emergence of new hematopoietic clones in her cultures within 5 weeks (Fig 4). This pattern continued for all subsequent samples (data not shown).

Analyses of posttransplant samples. Results for each patient are shown respectively in Figs 1, 2, 3B, and 4, and are summarized in Table 2. Twenty-eight days after the autograft when patient UPN 208 had largely regenerated her hematopoietic system and the peripheral granulocyte count had already recovered to $2.2 \times 10^9/L$, these cells did not...
include detectable (ie, >2%) BCR-positive cells (Fig 1). However, a subsequent sample taken approximately 3 months after autografting showed the reappearance of a minor (~5%) BCR-positive population, which contained the same rearrangement seen before autografting and which remained detectable at about the same low level for another 8 months. Cytogenetic data obtained during the first year from either direct marrow preparations or colonies derived from marrow or peripheral blood progenitors gave the same picture, although given the size of the BCR-positive population that transiently reappeared 3 months after transplantation, it was not always possible to obtain sufficient metaphases to detect its presence. Because of the persistence of Ph'-positive/BCR-positive cells in this patient for another 9 months, she was started on treatment with α-interferon (α-IFN). The BCR-positive population then disappeared and all subsequently analyzed samples up to 2 years after autografting remained BCR-negative (Table 2). Cytogenetic data during this period also showed the disappearance of Ph'-positive cells (Kalousek DK, Barnett MJ, Eaves CJ, Eaves AC: unpublished findings, 1990).

In UPN 232, hematopoietic recovery was slower; neverth-

![Table 2. Summary of BCR Rearrangement in Three Patients](image)

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Table 2. Summary of BCR Rearrangement in Three Patients

Presence (+) or absence (−) of DNA showing a rearranged BCR gene as determined by Southern blot analysis.

Abbreviations: BM, bone marrow; G, granulocytes; LDC, peripheral blood light density (<1.077 g/cm³) cells; T, T lymphocytes; non-T, T lymphocyte-depleted LDC; BM autograft, cells harvested from 10-day-old BM cultures and used as autograft.

![Fig 4. Clonality analysis using the BglI polymorphism of the PGK gene. DNA samples obtained from UPN 248. DNA was digested with EcoRI, BglII, and BglI without (unmarked lanes) or with HpaII (lanes marked H). Pretransplant granulocyte (G) and bone marrow (BM) DNA show a monoclonal pattern, whereas granulocytes obtained 28 days after autografting (G, d + 28) and nonadherent cells obtained from 5-week-old long-term marrow cultures (NA CELLS, wk + 5) showed emergence of nonclonal cells as indicated by the appearance of the 1.3-kb allele.](image)
less, by day 26 after autografting the granulocyte count was at $0.6 \times 10^9/L$. As shown in Fig 2, Southern analysis of granulocytes obtained at this time showed that the original BCR-positive clone had been largely, but not completely, replaced by BCR-negative cells. This replacement was confirmed by cytogenetic studies of direct metaphases in a marrow sample obtained 3 weeks later (day +46) and by repeat Southern analysis of the peripheral blood granulocytes sampled on day +57. By day +77, a marked increase in the abnormal BCR band had become apparent in a Southern blot of DNA from the granulocytes in her circulation. A marrow sample obtained 5 days later was heavily infiltrated with lymphoblasts and a high proportion of proliferating cells with a duplicated Ph chromosome. No further follow-up on this patient was possible as she succumbed to this third and final recurrence of blast phase disease.

The last patient (UPN 248) also showed early evidence of hematologic recovery after autografting, but could only be studied for a limited period because she developed overwhelming therapy-related toxicity. Only two samples of circulating granulocytes were available for DNA studies and these were obtained 24 and 27 days after autografting, at which time her peripheral granulocyte count was $1.2 \times 10^9/L$. In neither of these preparations were BCR-positive cells detected (Fig 3B and Table 2). Because clonality studies (by analysis of the methylation status of the PGK gene) were also possible in this patient, both posttransplant granulocyte DNA preparations were also evaluated in this way. As illustrated in Fig 4, the typical monoclonal pattern exhibited by this patient's pretransplant marrow and granulocytes was replaced in the posttransplant samples by a pattern indicative of the presence of new clones of hematopoietic cells.

**DISCUSSION**

The primary purpose of this study was to use molecular techniques to evaluate and compare the clonality status of cells generated both in vitro and in vivo from precursors present in 10-day-old cultures of CML marrow. A major advantage of molecular techniques is their applicability to nondividing as well as dividing cell populations. Moreover, using methylation-sensitive analysis of certain X-linked genes, it is possible (in informative subjects) to examine the clonality of cell populations by a method that is independent of BCR gene rearrangement measurements. In this study, both of these approaches were applied to the assessment of relatively pure populations of mature hematopoietic cells (primarily granulocytes) separated from posttransplant peripheral blood samples or harvested as the nonadherent fraction of 5- to 8-week-old long-term marrow cultures. Information obtained from analyses of these cells was then used to assign genotypes to their precursors present in the suspension of cells harvested from the 10-day-old cultures used for autografting. In every case studied, both in vitro and in vivo, BCR data confirmed and/or extended results obtained by cytogenetic analyses (and more recent follow-up data on patient UPN 208). More interestingly, in the one patient where clonality studies could also be undertaken, reappearance of BCR-negative hematopoiesis both in vitro and in vivo was paralleled by the emergence of new clones of differentiating granulocytes.

This result brings the number of patients whose Ph-negative (BCR-negative) hematopoietic cells appearing after several weeks in culture could be subjected to an independent analysis of clonality status to a total of three. Interestingly, in each the marker used to determine clonality has been different. The first such patient studied was a cytogenetic (Turner's syndrome) mosaic, the second a G6PD heterozygote, and the third, described here, heterozygous for a $B^2$ polymorphism in the X-linked PGK gene. Only in the case described here was it possible to document complete disappearance of Ph-positive (and BCR-positive) cells concurrent with the appearance of new clones of normal cells. The present studies also now provide evidence of the production of nonclonal, Ph-negative (BCR-negative) cells over a similar time frame in vivo (ie, within the first month after transplanting 10-day cultured cells). These findings are important because they highlight the possibility that placing human marrow under conditions used conventionally to initiate long-term cultures may allow preservation for at least 10 days of cells with in vivo reconstituting potential. Such knowledge may be critical to optimizing the design of future therapies involving transplantation of marrow that is to be manipulated or activated in vitro.

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**REFERENCES**

8. Popenoe DW, Schaefer-Reg K, Mears JG, Bank A, Leibowitz
D: Frequent and extensive deletion during the 9,22 translocation in CML. Blood 68:1123, 1986


Detection of breakpoint cluster region-negative and nonclonal hematopoiesis in vitro and in vivo after transplantation of cells selected in cultures of chronic myeloid leukemia marrow


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