BCR-ABL Gene Rearrangement and Expression of Primitive Hematopoietic Progenitors in Chronic Myeloid Leukemia

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Chronic myeloid leukemia (CML) is characterized by an initial chronic phase of expanded yet orderly clonal hematopoiesis that is distinguished by the BCR-ABL gene rearrangement. We found that although the mature myeloid compartment in patients with CML was expanded and entirely derived from the dominant leukemic clone, the primitive hematopoietic progenitor compartment did not show a corresponding expansion and was substantially enriched for cells without the BCR-ABL gene rearrangement. More importantly, primitive progenitors exhibiting the BCR-ABL gene rearrangement did not express either the BCR-ABL hybrid mRNA or fusion protein (P210). Expression of P210 protein increased with myeloid commitment in vivo as well as with growth factor--induced proliferation and differentiation of the primitive CML progenitors in vitro. This differential expression of BCR-ABL between primitive and mature CML progenitors may explain the expansion of the leukemic clone at the level of mature myeloid progenitors and granulocytes without a concomitant expansion of primitive CML progenitors. Because BCR-ABL mRNA is minimally expressed or may be absent in primitive CML progenitors, these cells may escape detection by reverse transcriptase-polymerase chain reaction and eradication by antisense oligonucleotides targeted against BCR-ABL mRNA.

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MATERIALS AND METHODS

Identification and isolation of hematopoietic progenitors. BM aspirates were obtained from six patients with chronic-phase CML and five normal volunteers after informed consent approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions. BM mononuclear cells were obtained by Ficoll-Hypaque density centrifugation (density < 1.077) and fractionated by CCE using a Beckman J-6M centrifuge with a JE-6B elutriator rotor and a standard chamber (Beckman Instruments, Palo Alto, CA). A masterflex peristaltic pump (Cole Palmer, Chicago, IL) equipped with a potentiometer provided precisely metered flow of medium. About 10^6 cells were loaded into the chamber at a flow rate of 15 mL min^-1 and a rotor speed of 1,230g. Cerebrospinal fluid cells were eluted while the cells were being loaded into the chamber. With the rotor speed held constant, the cells were eluted by changing flow rates, collecting 200 mL each at flow rates of 25 mL min^-1 (FR25), 29 mL min^-1 (FR29), and 33 mL min^-1 (FR33). The cells remaining in the chamber at the end of CCE were collected as the "rotor-off" fraction (RO).

Cells from each CCE fraction were separately labeled with phycocyanin (PE)–conjugated anti-CD34 antibody and fluorescein isothiocyanate (FITC)–conjugated anti-CD33, anti-CD19, and anti-CD5 monoclonal antibodies (MoAbs) (Becton Dickinson, Mountain View, CA). Cells were sorted on an Epics 753 Dual Laser Flow Cytometer (Coulter Electronics, Hialeah, FL). Windows were chosen on the basis of the fluorescence pattern of control samples labeled with FITC–conjugated mouse IgG1 and PE–conjugated mouse IgG1 (Becton Dickinson).

Determination of the BCR-ABL gene rearrangement. High molecular weight DNA was prepared from 10^6 nucleated cells from each subpopulation studied, digested with either BamHI, BglII, or EcoRI restriction endonuclease (determined for each patient with CML by screening for rearrangements in DNA isolated from whole marrow).
according to manufacturer's recommended conditions (New England Biolabs, Beverly, MA), separated by agarose electrophoresis, and conditions described by Church and Gilbert with a 600-bp BCR fragment subcloned from the commercially available BCR probe, Pr-1 (Oncogene Science, Mineola, NY), which was radiolabeled by the random primer method. Repeated sequences were removed by preannealing the labeled probe DNA with placenta DNA. Autoradiography was performed as previously described. A Hoefer (San Francisco, CA) GS300 scanning densitometer and GS360 data system were used to quantitate autoradiographic signals. Densitometric analysis of the relative intensity of the rearranged band compared with the BCR germline band was used to estimate the proportion of cells with no BCR-ABL rearrangement because of the densitometric signal from the rearranged band was subtracted from the percentage of the signal from the germline band to determine the proportion of cells with no BCR-ABL rearrangement.

Polymerase chain reaction (PCR) amplification of BCR-ABL mRNA. Total RNA was prepared from 10^6 cells of each sorted subpopulation by the method of Belyavsky et al. First-strand DNA was synthesized with MMLV reverse transcriptase, buffers, and conditions described by BRL (Bethesda Research Laboratories, Gaithersburg, MD) using 2-ng random hexanucleotides (Pharmacia Fine Chemicals, Uppsala, Sweden) as primers in the presence of four units RNasin (Promega, Madison, WI). Random hexanucleotide primers ensured efficient cDNA synthesis necessary for PCR amplification to be sensitive and reproducible. This cDNA product was divided for two PCRs: one with CML-specific primers and one with β-actin specific primers. The following oligonucleotide primers were used: bcr 5' sense primers: CML A, 5'GGAAGTCGAGATCGCTGACC 3'; abl 3' antisense primers: CML B, 5'TCAAGACCTGAGCTCTAAGTC 3'; and β-actin primers: 5' primer (exon I): 5'AGGCTGCAGACCCTGAAGGGCTTTTGGAACTCTGCTTA 3' and 3' primer (exon III): 5'GCCACGGCCCTGGTGGAGCTGACT 3'. The β-actin primers were designed to span introns to avoid contamination of DNA that could contaminate the RNA preparations. PCR buffer has been described and 2 U AmpliTaq (Perkin Elmer–Cetus, Norwalk, CT) was added.

A Coy tempcycler (Model 50, Coy Laboratory Products Inc, Grass Lake, MI) was used with the following program: 5 minutes 95°C; 40 cycles of 1 minute 95°C, 30 seconds 65°C, 1 minute 72°C; one final 72°C extension for 5 minutes. BCR-ABL PCR products were analyzed by electrophoresis, transfer, and hybridization to end-labeled (5'End Labelling System, Dupont NEN, Boston, MA) BCR-ABL junction–specific oligonucleotide probes complementary to the b2/a2 splice (CML C) (5'SGCTGAAGGGCTTCTGGTGAACCTGCTGA 3') or b2/a3 splice (CML D) (5'GCCTGAAGGGCTTCTCCATTGATG3') to assure specificity of PCR for BCR-ABL beyond the mobility of the PCR product on the gel.22 Each analysis included a positive control (K562) and RNA-free reagent control. All cell fractions yielded a strong β-actin–specific PCR DNA fragment of 527 bp that provided a positive control for interpretation of negative RT-PCR results.

Immunoperoxidase assay for detection of P210. Cytospin preparations were made (Shandon Cytospin III, Pittsburgh, PA) of the various cell subpopulations. Cells were fixed with methanol at 4°C and incubated with an affinity-purified P210 rabbit polyclonal antibody (bcr Ab-1; Oncogene Science, Mineola, NY). Immunoperoxidase staining was performed by the avidin–biotin–peroxidase complex method per manufacturer's instructions (Vector, Burlingame, CA). Phosphate-buffered saline with 0.2% bovine serum albumin (Sigma, St Louis, MO) and 0.05% Tween 20 (Sigma) was used as the sample and wash buffer. 3-Amino-9-ethylcarbazole (AEC) (Lipshaw, Detroit, MI) was used as the peroxidase substrate and the cells were counterstained with Giemsa. Each experiment included the BCR-ABL–positive K562 cell line and the patient's own CML granulocytes as positive controls. Positively stained cells showed cytoplasmic localization of P210. There was no detectable staining of either normal granulocytes or HL-60 cells, which served as negative controls. The percentage of cells staining positive was measured by analysis of at least 100 cells in each subpopulation. P210 expression was also graded as 0, 1+, or 2+ based on the intensity of the immunoperoxidase staining reaction.

Cell cultures. Primitive hematopoietic progenitors (5 × 10^3 cells) were placed in liquid cultures containing 250 μL McCoy's medium, 20% fetal calf serum, c-kit ligand (recombinant human stem cell factor, Amgen, Thousand Oaks, CA) at 100 ng mL^-1, recombinant human interleukin-3 (IL-3) at 10 ng mL^-1, and recombinant human interleukin-6 (IL-6) at 100 U mL^-1 (Genzyme, Boston, MA). The cells harvested from liquid cultures at 7 to 14 days were analyzed for P210 expression, BCR-ABL mRNA and BCR-ABL gene rearrangement.

Methylcellulose assays for granulocyte-macrophage colony-forming units (CFU-GM) using 10% phytomengin-stimulated leukocyte-conditioned medium were performed as previously described. CFU-GM were scored after 14 days of incubation at 37°C and 5% CO2.

RESULTS

Primitive hematopoietic progenitors were characterized as CCE FR25 cells that expressed CD34 and lacked lineage-specific antigens (lin−: myeloid–CD33+, B cell–CD19−, T cell–CD5−). These FR25 CD34 lin− cells were a uniform population that displayed a high nuclear/cytoplasmic ratio and were previously described, the relative size of the primitive hematopoietic progenitor compartment in patients with CML was reduced compared with normal individuals despite expansion of more mature myeloid progenitors and granulocytes. The FR25 CD34 lin− subpopulation represented an average of only 0.06% (range 0.01% to 0.5%) of the total marrow mononuclear cells in the six patients with CML compared with 0.2% (range 0.1% to 1.0%) in normal individuals (P = .04, Wilcoxon rank test).

Because of the small number of FR25 CD34 lin− cells recovered from the patients with CML (median of 2 × 10^6, range 10^6 to 10^9), we used a sensitive indirect immunoperoxidase assay for the BCR-ABL fusion protein P210 to identify the Ph-positive primitive hematopoietic progenitors. P210 was not detected in the FR25 CD34 lin− cells isolated from

| Table 1. Analysis of Primitive Hematopoietic Progenitors (FR25 CD34lin−) for P210 Expression, BCR-ABL mRNA, and BCR-ABL Gene Rearrangement |
|-----------------|-----------------|-----------------|-----------------|
| Patient No.     | % Cells Expressing P210 | BCR-ABL mRNA Expression | % Cells With BCR-ABL Gene Rearrangement* |
| 1               | 0                | Negative         | 0               |
| 2               | 0                | Negative         | 30              |
| 3               | 0                | Negative         | 50              |
| 4               | 0                | Trace            | 50              |
| 5               | 0                | Negative         | 30              |
| 6               | 0                | Trace            | 70              |

* Estimated by densitometric quantification of autoradiograph signals.
any of the six patients studied (Table 1). P210 was readily detected in the larger and more differentiated (RO CD34+lin-, RO CD34+CD33+ progenitors from all patients (Table 2). Granulocytes from all six patients and K562 cells were uniformly positive for P210 expression, with the most intense cytoplasmic staining.

To further characterize the primitive hematopoietic progenitors, we used RT-PCR for BCR-ABL mRNA to identify Ph-positive progenitors. RT-PCR for BCR-ABL mRNA confirmed the protein results. BCR-ABL fusion transcripts were either undetectable or detected in trace amounts (two patients) in RNA isolated from 10⁷ FR25 CD34+lin- cells (Table 1: Fig 1). Strong β-actin-specific PCR signals were detected in the 10⁷ FR25 CD34+lin- cells from six all patients. In contrast, BCR-ABL mRNA was easily detected by RT-PCR in 10⁴ RO CD34+CD33+ cells and in 10⁴ unseparated mononuclear cells from all patients. BCR-ABL mRNA was also easily detected by RT-PCR in 10⁶ normal marrow cells mixed with one CML BM mononuclear cell and in a single K562 cell (alone or mixed with 10⁶ normal cells). K562 cells have previously been shown to express fourfold to eightfold more BCR-ABL mRNA than CML marrow cells. The trace RT-PCR signal for BCR-ABL mRNA seen in 10⁷ FR25 CD34+lin- cells from two patients was substantially weaker than the signal obtained from a single K562 cell (<0.01 as intense by densitometric scanning).

To study the proliferative capacity of the FR25 CD34+lin- cells, they were placed in liquid culture with a combination of growth factors (c-kit ligand, IL-3, and IL-6). No increase in cell number occurred in the absence of growth factors. However, in the presence of growth factors, there was an 86 ± 20-fold expansion in the number of cells after 14 days in culture; Wright-Giemsa staining of the cultured cells showed differentiated myeloid cells, mostly granulocytes and macrophages. Although there were no CFU-GM in the FR25 CD34+lin- cells at day 0, the 7-day cultured FR25 CD34+lin- cells from the six patients yielded a mean of 307 ± 33 CFU-GM colonies per 10⁶ cells. The cells were harvested from liquid cultures after 14 days and analyzed for BCR-ABL mRNA and P210 expression. Surprisingly, P210 and BCR-ABL mRNA were readily detected in the differentiated cultured cells from all patients, with the majority of cells showing P210 expression (Table 2).

The emergence of cells expressing BCR-ABL after in vitro culture could result from preferential expansion of CML progenitors whose numbers before in vitro culture were too small to detect; we thought that the high sensitivity of RT-PCR for detecting BCR-ABL mRNA made this unlikely. Another possible explanation was that primitive CML progenitors were present in the primitive progenitor compartment before culture, but BCR-ABL was either not expressed in these cells or was expressed at very low levels that could not be detected. To address the latter possibility, the FR25 CD34+lin- cells were analyzed for BCR-ABL gene rearrangement by recombinant DNA analysis. FR25 CD34+lin- cells from all six patients were enriched for primitive hematopoietic progenitors without the BCR-ABL gene rearrangement (Table 1: Fig 2); there was no detectable BCR-ABL gene rearrangement in the FR25 CD34+lin cells from one patient. However, at least 30% of the FR25 CD34+lin- cells from five of the patients harbored the BCR-ABL gene rearrangement. All of the larger and more differentiated progenitors (RO CD34+lin-, RO CD34+CD33+) from all patients displayed the BCR-ABL gene rearrangement (Table 2), as did 100% of the marrow mononuclear cells and granulocytes from all six patients. Furthermore, about two thirds of the cells from the 14-day liquid cultures of FR25 CD34+lin- cells displayed the BCR-ABL gene rearrangement; because the cell number increased nearly 100-fold in culture, there was marked proliferation of both CML and normal FR25 CD34+lin- cells.

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**Table 2. Analysis of P210 Expression, BCR-ABL mRNA, and BCR-ABL Gene Rearrangement in Subpopulations of CML BM**

<table>
<thead>
<tr>
<th>Population</th>
<th>% Cells With P210</th>
<th>Intensity of P210</th>
<th>BCR-ABL mRNA</th>
<th>% Cells With BCR-ABL Gene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR25 CD34+lin-</td>
<td>0</td>
<td>–</td>
<td>–/Trace</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>Cultured FR25 CD34+lin-</td>
<td>67 ± 4</td>
<td>+</td>
<td>+</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>Rotor off CD34+lin-</td>
<td>83 ± 7</td>
<td>++</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Rotor off CD34+CD33+</td>
<td>100</td>
<td>++</td>
<td>+</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percentages represent the mean ± SEM of six patients.
† Undetectable BCR-ABL mRNA expression (+), detectable BCR-ABL mRNA expression (++)

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Fig 1. Expression of BCR-ABL fusion gene mRNA by RT-PCR. Total RNA was prepared from 10⁷ cells from each designated cell subpopulation. RO, mature hematopoietic progenitors (RO CD34+lin-, CD33+ cells); PHP, primitive hematopoietic progenitors (FR 25 CD34+lin- cells) from patients 1 and 2. All cell fractions yielded a strong β-actin specific band (not shown). K562 represents one K562 cell as a positive control and C is a negative reagent control.
PRIMITIVE PROGENITORS IN CML

Although the mature myeloid compartment is expanded in patients with CML and entirely derived from the Ph-positive clone, the primitive hematopoietic progenitor compartment phenotyped as FR25 CD34^+lin^- cells is not correspondingly expanded and continues to harbor a substantial fraction of normal cells. Moreover, the Ph-positive cells that were present in the primitive hematopoietic progenitor compartment did not express BCR-ABL or expressed it at low levels that were not detectable by RT-PCR. This differential expression of BCR-ABL between primitive and more mature CML progenitors may explain the expansion of the Ph-positive clone at the level of relatively mature myeloid progenitors and granulocytes without an analogous expansion of primitive CML progenitors.

However, there is some limited expansion of primitive CML progenitors, with about 40% of the FR25 CD34^+lin^- cells being Ph positive (Table 2). This limited expansion of primitive CML progenitors could result from these cells expressing low levels of BCR-ABL that are difficult to detect. Alternatively, like normal stem cells, primitive CML progenitors may usually be quiescent in vivo; primitive CML progenitors may be transcriptionally silent when in G0, expressing BCR-ABL only when they enter the cell cycle.

Both Ph-positive and normal FR25 CD34^+lin^- cells proliferated extensively in liquid culture. Moreover, the FR25 CD34^+lin^- cells produced over 300 GFU-GM per 10^5 cells after 1 week in culture, although they generated no CFU-GM before expansion in liquid culture. The marked myeloid proliferation and differentiation of both the CML and normal FR25 CD34^+lin^- cells in culture demonstrates that they represent early hematopoietic progenitors.

Our findings should have important clinical implications. RT-PCR is currently used as the most sensitive technique for detecting residual CML cells after allogeneic BM transplantation (BMT) or treatment with interferon. Because our data suggest BCR-ABL mRNA is either absent or minimally expressed in primitive CML progenitors, these cells may escape detection by RT-PCR. Selective inhibition of Ph-positive progenitors by BCR-ABL antisense oligodeoxynucleotides in vitro has recently been demonstrated; however, antileukemic therapy using antisense oligonucleotides targeted against BCR-ABL mRNA may not be effective in eradicating primitive Ph-positive cells that do not express BCR-ABL mRNA. The persistence of normal stem cells in patients with CML should enable the clinical isolation of normal stem cells from these patients for autologous BMT. It is imperative to confirm the purity of putative normal cells from patients with CML by testing for the presence of the BCR-ABL gene rearrangement (by recombinant DNA analysis or fluorescent in situ hybridization) to exclude transcriptionally silent stem cells harboring the BCR-ABL translocation.

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REFERENCES


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