Chronic Myelogenous Leukemia: Amplification of a Rearranged c-abl Oncogene in Both Chronic Phase and Blast Crisis

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The specific genetic events that distinguish the blast crisis from the chronic phase cells of chronic myelogenous leukemia (CML) are unknown. The most common karyotypic change that occurs as CML evolves from chronic phase to blast crisis is the development of multiple Philadelphia (Ph') chromosomes, each of which is presumably harboring a translocated c-abl oncogene. We describe here a patient with CML who presented in lymphoid blast crisis with three Ph' chromosomes/metaphase associated with an amplified, rearranged c-abl oncogene fragment and high levels of the aberrant 8-kilobase bcr-abl transcript. This rearranged c-abl fragment was amplified to a similar degree in both the patient's blast crisis cells and in his terminally differentiated granulocytes, but the level of the aberrant CML-specific bcr-abl transcript was some eight- to 16-fold higher in the blast crisis cells than in the granulocytes. This analysis indicates that genomic amplification of a translocated c-abl oncogene, although perhaps important in the evolution of CML, nevertheless cannot, by itself, be the sole genetic event giving rise to blast crisis.

CHRONIC MYELOGENOUS LEUKEMIA (CML) is a clonal disorder involving a pluripotent stem cell from which granulocytes, erythrocytes, platelets, monocytes, macrophages, and B lymphocytes arise. Over 90% of cases of CML exhibit the Philadelphia chromosome (Ph') (22q-), which results from a reciprocal translocation involving the distal portions of the q arms of chromosomes 9 and 22 and which is the most specific chromosome abnormality associated with any human malignancy. A prominent feature of CML is the distinct clinical phases of the illness. The relatively benign chronic phase, characterized by hyperplasia of terminally differentiated granulocytes, inevitably progresses to the blast crisis phase in which immature blasts predominate and that usually proves lethal.

Recent observations have provided considerable insight into the molecular genetics of the Ph' chromosome formation in CML. The c-abl oncogene is defined by virtue of its homology to v-abl, the transforming sequences of the Abelson murine leukemia virus. In CML, this oncogene is translocated from its usual position on the distal end of the q arm of chromosome 9 to a relatively restricted region on the Ph' termed the breakpoint cluster region (bcr). As a result of this translocation, a hybrid bcr-abl transcript is generated. This abnormally large transcript (8.2 kilobase [kb]) probably codes for the abnormally large abl-related protein product (P210) that has been demonstrated in CML cells. Although the physiological function of the c-abl oncogene is unknown, these findings indicate that abnormalities in the structure and expression of this oncogene may be critical to the pathogenesis of CML.

Several lines of evidence indicate that enhanced expression of the altered c-abl oncogene may be important in the progression of CML from chronic phase to blast crisis. We have previously demonstrated that this oncogene is amplified some four- to eightfold in the K-562 CML blast crisis cell line. The development of multiple Ph' chromosomes, each of which is presumably harboring a translocated c-abl oncogene, is the most frequent karyotypic abnormality that occurs as CML evolves from the chronic phase to blast crisis. In addition, enhanced expression of the abnormal 8.2-kb bcr-abl hybrid transcript is noted in CML blast crisis cells but not in chronic phase cells. Thus, one genetic event leading to enhanced expression of the translocated c-abl oncogene during progression of CML from the chronic phase to blast crisis may be an increase in the copy number of the abnormal bcr-abl locus.

Is the enhanced expression of the aberrant 8.2-kb bcr-abl transcript due solely to the presence of the multiple Ph' chromosomes in certain CML cells? Is the presence of multiple Ph' chromosomes the main genetic change distinguishing CML chronic phase from blast crisis? We have been able to approach these questions by using a molecular genetic analysis of blast crisis and chronic phase cells from a patient with CML who presented in lymphoid blast crisis with multiple Ph' chromosomes/metaphase associated with amplification of a rearranged c-abl oncogene. Although there was a marked difference in the level of expression of the aberrant bcr-abl transcript in the blast crisis cells, both of these cell types in this patient exhibited similar amplification of the rearranged c-abl fragment, which suggests that both the blast crisis and chronic phase cells harbored multiple Ph' chromosomes.

MATERIALS AND METHODS

Case Summary

The patient was a 42-year-old man who presented with a 1-month history of weight loss, malaise, fatigue, and headaches. His physical exam revealed pallor and splenomegaly without adenopathy. A complete blood cell count (CBC) revealed a hematocrit value (Hct) of 29, platelet count of 32,000, and a WBC count of 430,000 with...
54% blasts, 3% lymphocytes, 4% monocytes, 3% nucleated RBCs, 10% myelocytes, 3% metamyelocytes (metas), 7% bands, and 16% segmented neutrophils (seggs). A bone marrow aspirate and biopsy sample revealed a marrow packed with over 90% blasts that were terminal transferase-positive. Karyotypic examination revealed 52 XXXY, +5, +11, +20, +3 Ph in virtually all unstimulated metaphases. In contrast, phytohemagglutinin (PHA)-stimulated metaphases displayed a 46 XY karyotype. Therapy was initiated with oral hydroxyurea followed by two 3-week courses of vincristine, prednisone, and Adriamycin. Six weeks following initiation of therapy, the patient was noted to be in partial remission with an Hct of 28, WBC of 110,000 consisting of 8% lymphocytes, 8% monocytes, 5% bands, and 38% seggs. The patient refused further chemotherapy and was discharged. Two months later he returned with a peripheral WBC count of 110,000 consisting of greater than 90% lymphoblasts. Therapy with oral hydroxyurea was re instituted.

**Cell Separation**

At initial presentation the peripheral blood was diluted 1:1 with plain RPMI, layered over isolymph (Gallard/Schlesinger, Carle Place, NY), and spun at 1,800 rpm for 20 minutes. The interface cells were washed several times in RPMI and upon Wright staining were found to consist of over 85% blasts, with the remaining cells being predominantly myelocytes and metamyelocytes. The RBC-granulocyte isolymph pellet was brought to 25 mL with plain RPMI and mixed with 25 mL 3% dextran (T-500) in phosphate-buffered saline. The RBCs were allowed to sediment for 1 to 1.5 hours, and the supernate was harvested and the cells pelleted. The contaminating RBCs were lysed several times in 0.2% NaCl. The pelleted cells were then washed several times in plain RPMI and consisted of greater than 95% mature granulocytes.

After the chemotherapy-induced partial remission, peripheral blood was subjected to 3% dextran sedimentation followed by lysis of RBCs as described above. These cells consisted of approximately 7% myelocytes, bands, and seggs, with the remaining cells consisting of mature lymphocytes and monocytes. There were fewer than 5% blasts in this cell population.

At the time of relapse, mononuclear cells were isolated following isomyth gradient centrifugation of peripheral blood. This fraction consisted of greater than 95% blasts.

**DNA and RNA Extractions**

DNA extractions from the various leukocyte populations were performed by digesting nuclei with protease K followed by phenol/chloroform extraction and ethanol precipitation as previously described. RNA was extracted by homogenizing the cells in guanidine thiocyanate followed by ultracentrifugation through a cesium chloride cushion as described by Chirgwin et al. Poly A + RNA was selected by using oligo-dT cellulose.

**Southern, Northern, and Dot Blot Hybridizations**

Restriction endonuclease digestion of genomic DNA was performed following manufacturer specifications. Southern and Northern blots were made following standard procedures. DNA dot blot analysis on the total cellular RNA was performed as previously described. All blots were hybridized to nick-translated probes and subsequently washed under exactly the same conditions described previously.

**Molecular Probes**

5' abl. A molecular probe from the 5' end of the c-abl oncogene was cloned from a genomic library constructed from the K-562 CML blast crisis cell line. DNA from this cell line was partially digested with Sau 3A and fractionated by sucrose density gradient centrifugation, and the 15- to 23-kb DNA fragments were isolated. This DNA was ligated with Bam-digested EMBL 3 phage vector DNA, and the ligation mixture was packaged in vitro. Approximately 500,000 recombinant clones were screened with a nick-translation 550-bp HindII-Smal fragment derived from the pAB1 sub9 plasmid, which contains a v-abl insert. This 550-bp fragment represents the most 5' sequences of v-abl. Following screening, ten positive clones were isolated and purified. One of these clones contained a 17-kb genomic insert that exhibited a restriction map similar to that of the previously published 5' end of c-abl and from this clone a 0.3-kb HindIII-EcoRI, fragment free of human repetitive sequences was isolated and used as a molecular probe.

**Genomic bcr probe.** A 1.4-kb BglII-Sst1 human genomic fragment representing sequences within the bcr gene that are immediately 5' to the bcr itself was isolated from a normal genomic library. Details of the cloning procedure will be published elsewhere.

**bcr cDNA probes.** A 2-kb bcr cDNA clone was isolated from an HL-60 cDNA library screened with the aforementioned 1.4-kb BglII-Sst1 human genomic bcr fragment. A 700-bp Pst fragment near the 3' end of this clone that contains sequences that are represented in the normal bcr transcript but not in the aberrant bcr/abl transcript was utilized as a probe designated 3' bcr.

**v-abl.** This probe is a 1.5-kb BglII fragment isolated from the v-abl insert of the pAB1 sub9 plasmid.

**Actin.** The actin probe used is a 2.0-kb chick pA 1 β actin cDNA Pst fragment.

In all cases the probes were purified from their respective vectors using the glass bead technique of Vogelstein and Gillespie prior to nick translation and hybridization to the appropriate blots.

**RESULTS**

**Cell Harvesting and Separation**

We obtained peripheral blood cells from this patient at three different times during the course of his illness including (1) at initial presentation, at which time he had a WBC count of 430,000 with 54% blasts; (2) during a brief chemotherapy-induced partial "remission" when his peripheral count was 7,000 with >70% terminally differentiating granulocytes and <5% blasts; and (3) during relapse of blast crisis when his peripheral count rose to greater than 100,000 with over 90% blasts. At initial presentation, we separated the immature blast cells from the mature terminally differentiated granulocytes by isolymph gradient centrifugation as described in Materials and Methods. DNA and RNA were extracted from these cells as well as from the mature granulocytes isolated at the time of partial remission and from the immature blasts isolated at the time of relapse.

**Lymphoid Nature of the Blast Crisis Cells**

Many cases of CML blast crisis are B lymphoid in origin and exhibit immunoglobulin gene rearrangements. This patient's blast cells were positive for terminal transferase, thus suggesting their lymphoid origin. This was confirmed by Southern blot analysis of the blast cell DNA, which revealed several extra nongermline bands hybridizing to a heavy-chain immunoglobulin joining region probe (JH) (Fig 1). The
AMPLIFIED \( \text{bcr-abl} \) GENES IN CML

Fig 2. Enhanced expression of the aberrant 8.2-kb \( \text{bcr-abl} \) transcript in the CML blasts. Poly A+ RNA (5 \( \mu \)g/line) was electrophoresed in a formaldehyde gel, transferred to nitrocellulose, and hybridized to a \( \nu\)-\( \text{abl} \) probe. The normal 7.4- and 6.6-kb transcripts as well as the aberrant 8.2-kb transcripts are noted. RNA samples are from K-562 (lane 1), HL-60 cells (lane 2), and the patient CML blasts (lane 3).

identical restriction pattern was seen with this probe in DNA from both the blasts at initial presentation and at relapse (Fig 1). In contrast, predominantly the germline pattern was seen in the granulocyte populations isolated at initial presentation and during the chemotherapy-induced partial remission (Fig 1). Therefore these blots not only reveal the B lymphoid nature of the patient’s blasts but also indicate that the separated granulocyte fraction is not contaminated with any significant number of these blasts.

RNA Analysis

RNA from the patient’s blast and initial presentation granulocyte populations were subjected to Northern and dot blot analysis. The patient’s blast cells exhibited the abnormal 8.2-kb \( \text{bcr-abl} \) hybrid transcript that has been previously described in \( \Phi^\nu \) chromosome-positive CML cells (Fig 2). The relative amount of this transcript is quite high and is comparable with that found in the K-562 cell line, which exhibits a four- to eightfold amplification of the \( \text{c-abl} \) oncogene (Fig 2). Although we did not obtain enough granulocyte RNA to perform Northern blots, we did note by RNA dot blot analysis a marked decrease of at least eightfold in the level of \( \text{abl} \)-related transcripts in the granulocyte population compared with the blasts (Fig 3). In addition, a similar eightfold decrease in the level of \( \text{bcr} \)-related transcripts was noted in the granulocyte population \( \nu \) the blasts. These results indicate that the level of \( \text{bcr-abl} \) hybrid transcripts is significantly higher in the blasts \( \nu \) the granulocyte population in this patient. It is of interest that hybridization of this dot blot to a 3’ \( \text{bcr} \) probe, which measures levels of \( \text{bcr} \) transcripts from normal chromosome 22 but does not detect \( \text{bcr-abl} \) hybrid transcripts, reveals a similar decrease in levels of normal \( \text{bcr} \) transcripts in the granulocyte populations \( \nu \) the blasts (Fig 3). As a control, we hybridized these same samples to an actin probe that revealed similar levels of actin-related transcripts in both (Fig 3).

c-\( \text{abl} \) Rearrangement and Amplification in Both Blasts and Granulocytes

We hybridized Southern blots of restriction digests of DNA from patient blasts and initial presentation granulocyte fractions with a 300-bp R\(_7\)-HindIII \( \text{c-abl} \) probe (probe...
B) located within a 12-kb BglII fragment on chromosome 9 at the 5' end of c-abl (Fig 4). This probe detected an extra fragment in both the blast and granulocyte DNA digests, thus indicating that the break on chromosome 9 in this patient lies within this 12-kb BglII fragment and that both the blast and granulocyte subpopulations harbor a similar chromosome 9 breakpoint (Fig 4). In both the blast and granulocyte fractions, this probe hybridizes more intensely to the rearranged BglII fragment than to the germline fragment, which suggests that the rearranged c-abl fragment is amplified in this patient. We also noted a rearranged and amplified KpnI fragment in genomic digests of blast and granulocyte DNA hybridized to a chromosome 22 probe (probe A) located just 5' to the breakpoint cluster region on chromosome 22 (A). genetic maps of the bcr region on chromosome 22 (A), the 5' c-abl region on chromosome 9 (B) and the 9:22 Ph' breakpoint region on the Ph' chromosome in this particular patient (C) are shown. These maps were derived from data from references 4, 5, and 17 and from our own mapping data. The approximate breakpoints on chromosomes 22 and 9 in this patient have been previously mapped and are indicated by arrows. Probe A is a 1.4-kb BglII-SstI chromosome 22 fragment located immediately 5' to the breakpoint cluster region (BCR) and hybridizes to a 19-kb KpnI germline fragment as indicated in A. Probe B is a 0.3-kb PstI-HindIII fragment located in the 5' c-abl region of chromosome 22 that hybridizes to a 12-kb BglII germline fragment as indicated in B. c-abl exons are denoted by A. These probes were hybridized to Southern blots of genomic digests of CML DNA from patient blasts (lanes 1 and 3) and from initial presentation patient granulocytes (lanes 2 and 4). Lanes 1 and 2 are BglII digests; lanes 3 and 4 are KpnI digests. In both blasts and granulocytes probe B identifies the 12-kb germline BglII fragment as well as a rearranged, amplified 7.5-kb fragment as indicated (lanes 1 and 2). Probe A identifies the 19-kb KpnI germline fragment as well as a rearranged, amplified 7.8-kb fragment as indicated (lanes 3 and 4). These rearranged fragments are 9:22 junction fragments as indicated in C. Densitometric scanning reveals a ratio of rearranged:germline fragment of approximately 6:1 using probe B (lanes 1 and 2) and 10:1 using probe A (lanes 3 and 4) in both the blast and granulocyte DNA digests.
granulocytes exhibited a germline immunoglobulin heavy-chain pattern and were therefore representative of chronic phase cells. If one of the major genetic events that distinguishes these lymphoid blast crisis cells from the terminally differentiated chronic phase granulocytes is the development of multiple Ph' chromosomes with concomitant amplification of the rearranged c-abl fragment, then one would predict that the terminally differentiated granulocytes would not display amplification of the rearranged c-abl fragment (ie, precursors of these granulocytes display a single Ph'). However, our Southern blots show that this is clearly not the case, since this rearranged c-abl fragment is amplified to a similar degree in both the blast and granulocyte cell populations. Indeed, these Southern blots indicate that the terminally differentiated granulocytes also harbor multiple Ph' chromosomes. Therefore, the development of multiple Ph' chromosomes per se cannot be the sole genetic event that leads to the blast crisis phenotype, and one would predict that there are other genetic factors besides multiple Ph' chromosomes that distinguish the blast crisis from the chronic phase cells.

One potential problem in interpreting our Southern blots is that the enhanced hybridization of the bcr and abl probes to the rearranged fragments may be secondary to preferential transfer from the agarose gel to nitrocellulose of the smaller, rearranged fragments compared with the larger, germline fragments. Indeed since the rearranged bcr and abl fragments most likely are present on each individual Ph' chromosome, one would predict a ratio of intensity of hybridization of rearranged:germline fragment of 3:1 with these probes (ie, three Ph' chromosomes/one normal chromosome 9 or 22/metaphase). However, our densitometric scanning indicates a hybridization intensity ratio of 6:1 with the S' c-abl probe and 10:1 with the bcr genomic probe (Fig 4), which suggests that there indeed has been preferential transfer of the smaller rearranged fragments in these blots. However, the important observation is that the hybridization intensity ratios of rearranged-germline fragments using both abl and bcr probes are virtually identical in the blasts (which by karyotypic analysis harbor three Ph' chromosomes/metaphase) and the chronic phase granulocytes. Thus even though preferential transfer of the smaller rearranged fragments is probably present in these blasts, this does not interfere with our conclusion that the chronic phase granulocytes are similar to the blast crisis cells in harboring multiple Ph' chromosomes.

Although we noted amplified, rearranged c-abl sequences in both the lymphoid blast crisis cells and the chronic phase granulocytes in this patient, the blast crisis cells had an eight- to 16-fold greater level of bcr-abl-related transcripts than the chronic phase granulocytes (Fig 3). Thus, the presence of amplified c-abl sequences per se within the genome of certain CML cells does not necessarily lead to enhanced expression of these sequences. What controls the expression of the aberrant bcr-abl transcript in CML? The c-abl oncogene in CML is translocated into the bcr gene on the Ph', and transcription of this oncogene may then come under control of those factors that normally control transcription of the intact bcr gene on the normal chromosome 22. Consistent with this hypothesis is our finding that there is a decrease in the normal chromosome 22 bcr transcripts in the CML granulocytes v blast crisis cells that is almost
of chronic myelocytic leukemia: Origin of some lymphocytes from scripts of abl clustered within a limited region, bcr, some CML cell populations (for instance, the granulocytes) are found to express high levels of the bcr-abl transcript even though they harbor multiple Ph' chromosomes. An alternative hypothesis to explain the evolution of CML is that a further genetic event or mutation, perhaps involving another oncogene in addition to the Ph' translocation, gives rise to blast crisis.

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