Bcr-Abl Expression Levels Determine the Rate of Development of Resistance to Imatinib Mesylate in Chronic Myeloid Leukemia

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Abstract

Chronic myeloid leukemia (CML) starts with the acquisition of a BCR-ABL fusion gene in a single hematopoietic stem cell, but the time to progression is unpredictable. Although the tyrosine kinase inhibitor imatinib mesylate is highly effective in the treatment of CML, its continuous administration is associated with development of resistance, particularly in advanced phase or blast crisis. We investigate here whether a feature of disease progression (i.e., elevated expression of Bcr-Abl in CD34+ progenitor cells from CML patients in blast crisis) has any bearing on the kinetics of resistance to imatinib. By studying cell lines that exogenously express Bcr-Abl over the range found from chronic phase to blast crisis of CML, we show that cells expressing high amounts of Bcr-Abl, as in blast crisis, are much less sensitive to imatinib and, more significantly, take a substantially shorter time for yielding a mutant subclone resistant to the inhibitor than cells with low expression levels, as in chronic phase. Our data suggest that the differential levels of the Bcr-Abl oncoprotein expressed by CD34+ CML cells may reflect the extent and duration of their response to imatinib; the relatively high levels of oncoprotein in advanced-phase disease may underlie the observed rapid development of resistance. (Cancer Res 2005; 65(19): 8912-9)

Introduction

Chronic myeloid leukemia (CML) is initiated in hematopoietic stem cells by the BCR-ABL oncogene generated from the t(9;22)(q34;q11) translocation. The encoded Bcr-Abl oncoprotein is a constitutively activated tyrosine kinase that is necessary and sufficient for the transformation of cells in vitro and induction of a CML-like disease in mice (reviewed in ref. 1).

Imatinib mesylate (Gleevec), an inhibitor of the Bcr-Abl tyrosine kinase, is a well tolerated and highly effective treatment of CML. Imatinib is most efficacious in treatment-naïve CML patients in chronic phase, with 98% achieving complete hematologic and 68% cytogenetic responses (2, 3). Despite its undoubted efficacy, a major problem associated with the administration of imatinib is the development of resistance. In patients with advanced-phase disease or blast crisis, this tends to be particularly rapid, and clinical responses, when achieved, are rarely sustained (4). Resistance develops through selection of subclones containing genetic mutations that enable them to evade the cytotoxic effects of imatinib.

Two major mechanisms of imatinib resistance have been identified: overexpression of Bcr-Abl and mutation of specific amino acids within the Ab1 kinase domain (5). The latter mechanism is believed to be the major cause of secondary resistance to imatinib, but is seldom, if ever, seen in primary refractoriness to the drug (6).

Little is known of the kinetics of resistance development and why it is more frequent in patients with advanced disease. In this report, we studied the response to imatinib in a series of murine cell line clones that exogenously express different levels of the BCR-ABL transgene in a range comparable with that observed in hematopoietic precursors from chronic phase to blast crisis of CML. We show that the amount, rather than just the presence or absence of Bcr-Abl protein, is an important determinant of the degree of imatinib sensitivity and a key factor in dictating the rate by which imatinib-resistant cells emerge and become the dominant leukemic clone.

Materials and Methods

Cell culture. RF10 culture medium consisted of RPMI 1640 supplemented with penicillin, streptomycin, l-glutamine, and 10% fetal bovine serum. Unless stated otherwise, this was further supplemented with 10% conditioned medium from the WEHI-3B cell line, as a source of murine interleukin 3 (IL-3), herein called complete medium.

Vectors. The pMgi210 retroviral vector (7) contains the bicistronic BCR-ABL-ires-eGFP (enhanced green fluorescent protein) cassette and pBabe/Puro contains the puromycin resistance gene (8).

Generation of cell lines. 32D and 32Dp210 (9) cell lines were a gift from Dr. Brian Druker (Oregon Health and Science University, Portland, OR). The latter was originally selected in the absence of IL-3. Generation of the 32D/BCR-ABL/GFP clones has been described elsewhere (10).

Patient samples. Peripheral blood samples were obtained from CML patients at diagnosis of chronic phase and/or blast crisis after receiving informed consent. Mononuclear cells were separated by density-gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway) and cryopreserved. CD34+ cells were isolated by magnetic bead separation (MiniMACS, Miltenyi Biotec, Auburn, CA) and the purity (>90%) of this fraction was confirmed by flow cytometry of cells stained with a FITC-conjugated anti-CD34+ antibody (BD Biosciences).

Immunoblotting. Protein lysates were prepared from CD34+ cells of patients as reported (10). For cell lines, lysates were prepared according to the method of Kabarowski et al. (11). Approximately 20 μg protein were electrophoresed on 8% SDS-PAGE gels, blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA), and immunoscreened with anti-Abl (Ab-3, Calbiochem, San Diego, CA) and antiaktin (Sigma-Aldrich) antibodies. Membranes were stripped in accordance with the instructions of the manufacturer and reprobed, as necessary, with anti-CrkL (C-20), anti-STAT5 (C-17; both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-pSTAT5 (Tyr415), and anti-pCrkL (Tyr207; both from Cell Signaling Technology, Beverly, MA) antibodies. Secondary antibodies were horseradish peroxidase–conjugated rabbit anti-mouse and swine anti-rabbit IgG (DAKO Corporation, Carpinteria, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham, UK).
Kinetics of Imatinib Resistance in CML

**Results**

Bcr-Abl expression is higher in progenitor cells of patients in blast crisis than in those of chronic phase patients. Expression of the Bcr-Abl protein varied over >1 log in the CD34+ cells of the CML samples analyzed, but was significantly higher in patients in blast crisis than in those in chronic phase (P = 0.0079, Mann-Whitney U test; Fig. 1A). Higher BCR-ABL expression in blast crisis was also found at the RNA level by real-time PCR amplification of BCR-ABL and ABL transcripts in CD34+ cells from 40 chronic phase and 11 blast crisis samples (Fig. 1B). The ratio of BCR-ABL/ABL transcripts varied over a 1-log range but was significantly higher in blast crisis than chronic phase (P = 0.015, Mann-Whitney U test). Furthermore, in two patients for whom paired CD34+ samples were available, the BCR-ABL/ABL ratio had increased by 1.5- to 2-fold in the samples taken at blast crisis when compared with those taken at diagnosis of chronic phase. This increase is higher than could be accounted for by the technical variation between duplicate samples in our RT-PCR assays (<1.3-fold variability at the 95% level of confidence at baseline levels of BCR-ABL expression; data not shown).

Phosphorylation of CrkL and Stat5 is higher in progenitor cells of patients in blast crisis than in those of chronic phase patients. Although expression of both CrkL and Stat5 remained constant with disease phase (Fig. 1A), phosphorylation of the two Bcr-Abl substrates was increased in two and three of five blast crisis patients, respectively. This suggests that both Stat5 and CrkL are activated by Bcr-Abl in a dose-dependent fashion, consistent with their role as major tyrosine-phosphorylated substrates of the oncoprotein in CML (14, 15).

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**Figure 1.** Bcr-Abl expression in CD34+ cells and cell lines. A, immunoblot done on protein lysates of CD34+ cells obtained from patients in chronic phase CML and blast crisis. The blot was hybridized with anti-Abl and anti–j-actin antibodies (loading control). After stripping, the blot was reprobed with anti-STAT5, anti-CrkL, anti-pSTAT5, and anti-pCrkL antibodies. The control sample in the first lane is from a patient with non-Hodgkin’s lymphoma (no p210 Bcr-Abl band). The rightmost lane is from a patient with additional copies of the Ph chromosome. Densitometric ratios of p210(Po-Abl)/actin, pSTAT5/actin, and pCrkL/actin are shown below each lane. B, ratio of BCR-ABL/ABL mRNA transcripts present in the CD34+ cells of 25 normal individuals (small black circles), 40 CML chronic phase (light-gray circles) and 11 CML blast crisis (dark-gray circles) patients quantitated by TaqMan RT-PCR. Points, mean of duplicate determinations. Horizontal bars, median values: normal = 6.98 × 10−6, chronic phase = 0.826, blast crisis = 1.04. Paired chronic phase–blast crisis samples from the same patient are linked by lines. C, immunoblot done on protein lysates of the six clones of 32D/BCR-ABL/GFP (x1–x6), 32D, and 32Dp210. The blot was hybridized with anti-Abl and anti–j-actin antibodies (loading control). The six clones express increasing levels of Bcr-Abl (x1, lowest; x6, highest). Densitometric ratios of p210(Po-Abl)/actin, shown below each lane, vary over a 16-fold range (0.04–0.64).
order of magnitude as that seen in the CD34+ cells of patients in bands in clones expressing low levels of Bcr-Abl was of the same with those of CD34+ cells from patients in blast crisis. The median expressing higher levels of Bcr-Abl, these levels were comparable samples was 0.25, compared with a median of 0.17 for the three B, 72 hours incubation with a range of doses of imatinib mesylate.

Figure 2. Sensitivity of 32D, 32D/BCR-ABL/GFP clones, and 32Dp210 to in vitro exposure to imatinib mesylate. A, viability of 32D (C) and imatinib-sensitive cell lines, x1 (V), x2 (A), x3 (+), x4 (-), x5 (●), x6 (▲), and 32Dp210 (●), after 72 hours incubation with a range of doses of imatinib mesylate. B, viability of imatinib-resistant cell lines x1-r, x2-r, x3-r, x4-r, x5-r, x6-r, and 32Dp210-r (same symbols as their sensitive counterparts) after 72 hours incubation with a range of doses of imatinib. Viability was assessed by tetrazolium-based MTS assay. Data have been normalized to 100% (maximum absorbance at 490 nm obtained for each assay) and are representative of three independent experiments. Points, mean of triplicate determinations. Best-fit curves were obtained by nonlinear regression using a sigmoidal log dose-response model (Prism 4.0a, GraphPad).

Newly generated cell lines expressing graded amounts of Bcr-Abl reflect the amounts of oncoprotein in primary chronic myelogenous leukemia progenitors. We have previously transfeceted the 32D cell line and established clones that stably express graded amounts of Bcr-Abl (Fig. 1C). Immunoblotting showed that the ratio of the intensity of the p210BCR-ABL to the actin control bands in clones expressing low levels of Bcr-Abl was of the same order of magnitude as that seen in the CD34+ cells of patients in chronic phase. The median p210/actin ratio for the chronic phase samples was 0.25, compared with a median of 0.17 for the three lowest-expressing clones—x1, x2, and x3. Likewise, in cell lines expressing higher levels of Bcr-Abl, these levels were comparable with those of CD34+ cells from patients in blast crisis. The median ratio for the latter samples [excluding the patient with an additional Philadelphia (Ph) chromosome] was 1.17, whereas for the medium to high expressing clones x4, x5, and x6 it was 0.50.

Cell lines expressing high levels of Bcr-Abl are less sensitive to imatinib mesylate than cell lines with lower expression. Complete inhibition of the growth of all cell lines required a 1 μmol/L dose of imatinib. Four clones, x1, x2, x3, and x4, that expressed low to medium levels of Bcr-Abl had similar mean IC50 values that were not significantly different from each other (0.053, 0.065, 0.048, and 0.061, respectively; P > 0.05, Kruskal-Wallis test; Fig. 2A; Table 1). Cell lines expressing high levels of Bcr-Abl (x5, x6, and 32Dp210), however, were markedly less sensitive to imatinib having mean IC50 values of 0.194, 0.313, and 0.206, respectively (Fig. 2A; Table 1).

The rate at which a cell develops resistance to imatinib is determined by its basal level of Bcr-Abl expression. It has been shown that CML patients in chronic phase respond well to imatinib treatment and achieve lasting hematologic and cytogenetic remissions (2, 3), whereas the responses achieved by patients in blast crisis are rarely sustained and the development of refractoriness to the drug is often rapid (4, 16). Having shown that Bcr-Abl expression is elevated in blast crisis, we asked whether the level of Bcr-Abl in the leukemic clone before the start of imatinib has any bearing on the rate at which resistance develops. Selection of imatinib-resistant subclones able to undergo proliferation in 1 μmol/L imatinib was particularly rapid in cell lines that expressed levels of Bcr-Abl comparable with those of blast crisis. Thus, we obtained imatinib-resistant cultures of these lines after only 32 to 42 days of exposure to the drug (Fig. 3A). In contrast, the emergence of imatinib-resistant subclones in cells expressing levels of Bcr-Abl comparable with those of chronic phase CML was much slower. These lines required a more gradual exposure to imatinib and would only grow in the presence of 1 μmol/L of the drug after 90 to 104 days. A cell line with intermediate expression of Bcr-Abl developed resistance to 1 μmol/L imatinib after an intermediate period of exposure (59 days). These data strongly suggest that the average level at which Bcr-Abl is expressed in a population of clonogenic cells is of primary importance in determining how soon an imatinib-resistant subclone will become prevalent.

Both overexpression of Bcr-Abl and Abl kinase domain mutations are present within imatinib-resistant cell lines. It was possible that the different rates of acquisition of imatinib resistance by the cell lines reflected different underlying mechanisms. One imatinib-resistant cell line (x6-r) was found to overexpress Bcr-Abl in relation to its parental sensitive counterpart due to amplification of the BCR-ABL transgene (Fig. 3B) and had no mutations within the Abl kinase domain (Fig. 3C). In another imatinib-resistant line (32Dp210-r), a point mutation (Phe311Val) within the Abl kinase domain was detected and amplification of BCR-ABL had not occurred. Despite these different mechanisms of resistance, selection of imatinib-resistant subclones was obtained at an almost identical rate in both cell lines (32 days for x6-r and 38 days for 32Dp210-r).

Somewhat surprisingly, we found the same Abl kinase domain mutation, Phe311Val, in five of the seven imatinib-resistant cell

| Table 1. IC50 values for imatinib-sensitive and imatinib-resistant cell lines determined by MTS assay |
|-----------------|-----------------|-----------------|
| Cell line       | Mean IC50 (μmol/L) ± SD | Resistant       |
|                 |                    |                 |
| Sensitive       |                   |                 |
| x1              | 0.053 ± 0.010     | 4.28 ± 0.499   |
| x2              | 0.065 ± 0.011     | 4.97 ± 0.165   |
| x3              | 0.048 ± 0.028     | 4.91 ± 0.248   |
| x4              | 0.061 ± 0.004     | 2.77 ± 0.419   |
| x5              | 0.194 ± 0.018     | 10.3 ± 1.603   |
| x6              | 0.313 ± 0.003     | 3.46 ± 0.056   |
| 32Dp210         | 0.206 ± 0.059     | 4.95 ± 0.366   |
viable cells was sufficient to seed a second 10 mL subculture at a density of increments. The dosage for each clone was increased once the number of A(x1-x6) and 32Dp210 and the imatinib-resistant (BCR-ABL/GFP immunoblots done on protein lysates of the six sensitive (C, bands for resistant clones compared with their sensitive counterparts for x2, that hybridizes to integrations of this plasmid. Note the presence of additional to 1 indicated. above each lane and Abl kinase domain point mutations, if present, are also indicated.

**Figure 3.** Effects of long-term exposure to imatinib mesylate on 32D/BCR-ABL/GFP clones and 32Dp210. A, kinetics of imatinib resistance in x1 (●), x2 (○), x3 (+), x4 (×), x5 (▲), x6 (△), and 32Dp210 (●). Cell lines were exposed to escalating doses of imatinib mesylate from 0.1 to 1 μmol/L in 0.1 μmol/L increments. The dosage for each clone was increased once the number of viable cells was sufficient to seed a second 10 mL subculture at a density of 2 × 10⁵ cells/mL. The day on which the concentration of imatinib was increased to 1 μmol/L is shown for each culture. B, Southern blot of XbaI-digested genomic DNA from each of the imatinib-sensitive (S) and resistant (R) clones of 32D/BCR-ABL/GFP. The filter was probed with a HindIII fragment of pMig210 that hybridizes to integrations of this plasmid. Note the presence of additional bands for resistant clones compared with their sensitive counterparts for x2, x3, and x6. These indicate that genomic rearrangement and amplification underlie the up-regulation of Bcr-Abl expression seen in the resistant clones. C, immunoblots done on protein lysates of the six sensitive (S) clones of 32D/BCR-ABL/GFP (x1-x6) and 32Dp210 and the imatinib-resistant (R) cell lines derived from them. The blots were hybridized with anti-Abl and anti-α-actin antibodies (loading control). Densitometric ratios of p210Bcr-Abl/α-actin are shown above each lane and Abl kinase domain point mutations, if present, are also indicated.

lines (Fig. 3C). The reason for such a high incidence of this particular Abl kinase domain mutation among the imatinib-resistant lines is unclear. Contamination of cultures with a common line containing this mutation may be discounted as Southern blotting revealed unique patterns of hybridization for each resistant clone (Fig. 3B). Cross-contamination of RT-PCR templates was also ruled out because the same sequencing results were reproduced in replicate occasions from newly prepared template RNA from sensitive and resistant clones.

In three of the imatinib-resistant cell lines (x1-r, x2-r, and x3-r), the Phe311Val mutation was accompanied by overexpression of Bcr-Abl (Fig. 3C). Despite containing the same amino acid change, the three lines differed in the rate at which fully resistant subclones became dominant and this was inversely proportional to the increase in Bcr-Abl expression of the resistant compared with its sensitive counterpart (Fig. 3C). A fourth cell line (x5-r) with the Phe311Val mutation did not overexpress Bcr-Abl but had a second mutation in which a change in the nucleotide sequence from TGC to ATC resulted in substitution of Ile for Cys at position 369.

Different mechanisms of imatinib resistance confer different levels of insensitivity to imatinib. To examine the extent of insensitivity of the resistant cell lines to imatinib, we did MTS viability assays using a range of concentrations of the kinase inhibitor and calculated the IC₅₀ values for each of the resistant cell lines (Fig. 2B; Table 1). The least insensitive line was x4-r (IC₅₀ = 2.77 μmol/L), which had a Glu₄⁵⁰Gly substitution. Slightly more insensitive was clone x6-r (IC₅₀ = 3.46 μmol/L), which lacked Abl kinase domain mutations but overexpressed Bcr-Abl. Cell lines x1-r, x2-r, x3-r, and 32Dp210-r were even more resistant to imatinib and had very similar IC₅₀ values (4.28, 4.97, 4.91, and 4.95 μmol/L, respectively). Significantly, these lines all contained the Phe311Val mutation and either overexpressed Bcr-Abl following gene amplification (x1-r, x2-r, and x3-r) or had high basal expression of Bcr-Abl (32Dp210). The most insensitive cell line proved to be x5-r (IC₅₀ = 10.3 μmol/L), which had both the Phe311Val and the Cys369Ile mutations.

Overexpression of Bcr-Abl precedes the appearance of Abl kinase domain mutations in imatinib-resistant cell lines. Because we had obtained imatinib-resistant cell lines in which Bcr-Abl was both overexpressed and its Abl kinase domain was mutated, we hypothesized that one of the resistance mechanisms should precede the other. To determine the order of appearance of these genetic changes, we exposed imatinib-sensitive cell lines to the same incremental doses of imatinib as before, but harvested cells once they were resistant to each dose (Fig. 4A). Immunoblotting done on protein lysates of the harvested cells revealed changes in average Bcr-Abl expression within cultures at each dose of imatinib. In clone x4, which expressed a medium basal level of Bcr-Abl (Fig. 1C), overexpression of this oncoprotein occurred rapidly presumably because this was required to confer resistance to the lowest dose of imatinib, 0.1 μmol/L (Fig. 4B). Further up-regulation of Bcr-Abl expression was observed for this cell line, reaching a peak at 0.5 μmol/L imatinib. In contrast, clones with high basal levels of Bcr-Abl expression, x5 and x6, did not require substantial overexpression of Bcr-Abl to develop resistance to imatinib (Fig. 4B). In all three cases, Abl kinase domain mutations were not detected at low doses of imatinib but these became readily detectable once the cells had become resistant to higher concentrations of the drug. Point mutations were first detected in cells resistant to imatinib doses of 0.4 μmol/L (x5), 0.6 μmol/L (x6), and 0.9 μmol/L (x4; Fig. 4B). It is probable that subclones bearing these mutations were present in the cultures earlier, at lower concentrations of imatinib, but that cell numbers were insufficient for detection of mutations by direct sequencing of PCR products (limit of detection ~20% as also confirmed in our laboratory; ref. 17). However, attempts to increase the sensitivity of detection by using ASO PCR for the three mutations (Glu⁴⁵⁰Lys, Phe³¹¹Val, and Glu³⁶⁹Lys) did not reveal any additional positive data points in the dose-dependent resistance series from each clone (data not shown).

In agreement with the earlier experiments, the time required to induce cultures of these cell lines to grow in the presence of
1 μmol/L imatinib was similar and, more importantly, was also correlated with their basal level of Bcr-Abl expression. Hence, x6, x5, and x4 required 30, 36, and 71 days to become fully resistant to 1 μmol/L imatinib (Fig. 4A) compared with the previously determined rates of 32, 42, and 59 days, respectively (Fig. 3A). In this second set of experiments, clones bearing different Abl kinase domain mutations emerged. Thus, the resistant x6-r clone had a Glu255Lys, x5-r had only a Phe311Val rather than a combination of Phe311Val and Cys369Ile, and x4-r exhibited a Glu261Lys (Figs. 3C and 4B). This finding strongly suggests the presence of multiple mutant subclones within each cell line with potential to be selected for imatinib resistance.

The appearance of Abl kinase domain mutations coincides with a reduction in expression of Bcr-Abl. A downward trend in Bcr-Abl expression was apparent in cell lines x6, x5, and x4 once Abl kinase domain mutations became detectable (Fig. 4B). Overexpression of Bcr-Abl was greatest for cultures of x4 cells growing in 0.5 μmol/L imatinib, but less for cultures growing in 1 μmol/L imatinib. In clones expressing high basal levels of Bcr-Abl, x5 and x6, there was a modest increase in Bcr-Abl expression, reaching the maximum for x5 and x6 at 0.3 μmol/L imatinib. In both cases, expression of Bcr-Abl fell below the levels of the imatinib-sensitive cells once the cultures had been induced to grow in 1 μmol/L imatinib. In all cases, the progressive reduction in Bcr-Abl expression level coincided with the appearance of Abl kinase domain mutations.

**Discussion**

One of the major differences between the chronic phase and blast crisis stages of CML is the responsiveness to antileukemia treatment (18). Accordingly, whereas imatinib can induce hematologic remission in nearly 100% and a major cytogenetic response in 87% of patients in early chronic phase CML (2), only 8% and 16% of patients treated in blast crisis achieve these forms of response (16). Furthermore, the majority of blast crisis patients relapse due to the development of drug resistance within a short period of imatinib administration, which is attributed in part to the appearance of additional cytogenetic/molecular abnormalities in advanced disease. However, an initial reduction in the mass of leukemic cells indicates that these still require Bcr-Abl kinase activity for their survival. Consequently, quantitative, rather than mere qualitative (i.e., presence or absence) differences in Bcr-Abl oncogenic signaling may be responsible for the degree and durability of response of CML cells to imatinib.

In this report, we show for the first time by immunoblotting and real-time PCR that Bcr-Abl expression is elevated in progenitor cells from blast crisis compared with chronic phase CML and that this increased expression of the oncoprotein is associated with marked enhancement of phosphorylation of its key substrates, such as Stat5 and CrkL (14, 15). Notably, unlike previous studies in which this possibility had been raised (19–21), we compared protein and mRNA derived exclusively from CD34+ progenitors, rather than from heterogeneous populations of mononuclear cells. As the CD34+ population includes the mass of immature self-renewing leukemic cells indicates that these still require Bcr-Abl kinase activity for their survival. Consequently, quantitative, rather than mere qualitative (i.e., presence or absence) differences in Bcr-Abl oncogenic signaling may be responsible for the degree and durability of response of CML cells to imatinib.

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due to insufficient biological material. However, whether or not the observed increase in Bcr-Abl expression occurs in all CD34+ cells or is due to the expansion of a subpopulation with preexisting high Bcr-Abl expression, the net result will be an accumulation of cells with high basal expression of Bcr-Abl as the disease evolves.

Although the median difference in BCR-ABL mRNA levels between chronic phase and blast crisis was less marked than that observed for protein levels, up-regulation was found for both parameters. This suggests that the increase in BCR-ABL expression over time is transcriptionally regulated, although we cannot exclude the possibility that disease progression may also cause changes in cell physiology affecting protein translation or stability. Collectively, these data provide evidence that the elevated expression of Bcr-Abl in blastic transformation, even in the absence of Ph chromosome duplication, is closer to being the rule rather than the exception.

To assess whether quantitative differences in Bcr-Abl expression could underlie the diverse response of CML chronic phase and blast crisis cells to imatinib, we studied this phenomenon in BCR-ABL–transduced cell clones expressing variable amounts of the oncoprotein comparable with those observed in primary CML cells. Unlike most of the other BCR ABL–transfected cell lines previously reported (9, 11, 23–31), our clones were isolated and have been maintained continuously in the presence of IL-3. This unique feature removes the selective pressure of growth factor deprivation, which inevitably leads to the emergence and dominance of subclones with either artificially high levels of Bcr-Abl expression or additional mutations that allow them to survive in the absence of growth factor. Our clones maintained a constant basal level of Bcr-Abl expression that was stable over many months in culture, thus allowing the study of the phenotypic effect of oncoprotein levels comparable with those found in chronic phase and blast crisis progenitors. Furthermore, we could investigate the kinetics of imatinib resistance by measuring the time taken for the emergence of a resistant subclone upon in vitro exposure to the kinase inhibitor.

The main conclusion to be drawn from this study is that the rate at which an imatinib-resistant subclone emerges is dependent on the basal level of Bcr-Abl expression by the cell: The higher this level, the faster imatinib resistance will ensue. This is supported by a recent report that provides evidence that BCR-ABL transcription may fluctuate in different CD34+ subpopulations in chronic phase and that there is a correlation between decreased levels of BCR-ABL transcripts and increased sensitivity to imatinib during CML stem cell differentiation in vivo (32). Our results also suggest that this rate may be unaffected by the mechanism of resistance. Significantly, our data are consistent with the clinical findings of Hochhaus et al. (33), who found that the median time to relapse for 23 patients with Abl kinase domain mutations was not significantly different from that of 20 patients without such mutations.

A Phe311Val Abi kinase mutation was particularly prevalent among the imatinib-resistant subclone lines. This mutation is likely to be functionally equivalent to a Phe311Leu substitution reported in a CML patient (34), because leucine only differs from valine in having a single extra alkyl group. In the clones whose sensitive parental cells expressed low levels of Bcr-Abl comparable with those of chronic phase cells (x1-r, x2-r, and x3-r), the Phe311Val mutation was never found as the sole detectable cause of imatinib resistance, being always accompanied by Bcr-Abl up-regulation as well. Thus, only when overexpressed, this mutant form of Bcr-Abl led to a profound reduction in the sensitivity to imatinib, as shown by an average IC50 in those three clones ~70-fold higher than that of their sensitive counterparts (Table 1). In 32Dp210-r, the Phe311Val mutation was detected in the absence of other genetic changes. This is probably because the sensitive cells from which this resistant clone emerged already express a high basal level of Bcr-Abl (Fig. 1C), a level that was maintained in the resistant mutant cells (Fig. 3C). It is of note that the IC50 for imatinib in 32Dp210-r is almost identical to the values for x1-r, x2-r, and x3-r in which the Phe311Val mutation "had to be" accompanied by overexpression of Bcr-Abl to levels similar to those of the sensitive 32Dp210 line for resistance to occur (Table 1; Fig. 3C). Our data suggest that there may be a threshold of Bcr-Abl expression required to confer the full imatinib-resistant phenotype to a relatively "weak" mutation, such as Phe311Val.

To ascertain which imatinib resistance mechanism emerges first, we exposed three of the imatinib-sensitive clones to incremental doses of imatinib in a second round of experiments. In the clone with medium basal expression of Bcr-Abl, subclones overexpressing Bcr-Abl were rapidly selected by low doses of imatinib. Overexpression was not observed in the other two clones because they expressed high basal levels of Bcr-Abl that were presumably sufficient to confer resistance to low doses of imatinib. In all three cases, however, Abl kinase domain mutations could only be detected later at higher doses of imatinib. This suggests that the first response of a leukemic clone to exposure to imatinib is up-regulation of Bcr-Abl expression. Furthermore, the degree of such up-regulation seems to be inversely proportional to the basal level of Bcr-Abl expression. The presence of high oncoprotein levels in the selected subclone may then allow rare cells carrying resistance-endowing mutations to survive and proliferate in increasing concentrations of imatinib. It is known that expression of Bcr-Abl is associated with a "mutator phenotype" (35–38), and up-regulation of this oncoprotein may thus cause an increase in genetic instability and more frequent mutations, including those capable of driving imatinib resistance (39). A similar finding has been reported by Scappini et al. (40), who were able to detect a Thr315Ile mutation in KRM5 cells with high and intermediate, but not low, levels of imatinib resistance. Moreover, recent evidence suggests that the presence of Abi kinase domain mutations is correlated with disease stage and clonal evolution (41). Using a sensitive, fluorescent, allele-specific RT-PCR assay, mutations were frequently detected in pretherapeutic samples from accelerated phase and blast crisis patients but none were found in chronic phase samples (41). The correlation of clonal cytogenetic evolution with the presence of Abi kinase domain mutations implies that genomic instability is responsible for both phenomena (41). Significantly, it appears that as soon as a mutant clone becomes stably established, there is no longer a need for the initial outburst of Bcr-Abl overexpression, as the level of protein tended to decrease substantially once Abi kinase domain mutations were detected. This finding implies that excessive expression of fully active (i.e., refractory to inhibition) Bcr-Abl kinase must have negative consequences that contribute to the extinction of these cells from the culture. This hypothesis is supported by our previous observation (42) that LAMA84-r, an imatinib-resistant CML cell line that overexpresses Bcr-Abl, undergoes apoptosis upon withdrawal of imatinib due to an "acute" accumulation of a vast excess of kinase-active Bcr-Abl protein. Currently, the reasons for this are not understood.

Overall, the results from this study have two major implications. The first refers to the underlying, and largely unknown, mechanism that is responsible for driving expression of the Bcr-Abl
oncoprotein. A single copy of the BCR-ABL oncogene is present on the Ph chromosome generated by a simple t(9;22) reciprocal translocation. If transfection from the Bcr promoter is not significantly altered as a result of the translocation and fusion with Abl sequences, then the Bcr-Abl oncoprotein should be present at a common, basal level in CML progenitors in early chronic phase. Apart from duplication of the Ph chromosome, what triggers and controls progressive increase of BCR-ABL expression during the course of chronic phase through evolution to acute transformation is still unclear.

The second implication of our findings is a practical one. The significant correlation observed between the starting level of BCR-ABL expression and the speed with which resistance to imatinib occurs suggests that this could be a sensitive marker of response to treatment. Although the numbers are too small, it is of note that in our series of 10 patients treated with imatinib, of the 40 tested at diagnosis of chronic phase for BCR-ABL expression in CD34+ cells, the only two patients who did not show a significant (major or complete) cyogenetic response to the inhibitor had pretreatment BCR-ABL/ABL ratios of mRNA transcripts of 1.07 and 0.957, values that are higher than the median for the chronic phase group (0.826). To date, no large clinical study has addressed the issue of a possible association between the pretreatment levels of BCR-ABL RNA and the development of resistance to imatinib. A link between increase in Bcr-Abl and appearance of kinase mutations has been recently reported by Bradford et al. (43), who showed that even a 2-fold increase in the level of BCR-ABL mRNA expression in CML patients responsive to imatinib treatment is predictive of the emergence of a Bcr-Abl mutant clone leading to resistance to the drug. Unfortunately, conventional assays for quantification of BCR-ABL transcripts in patient samples (3,33) are inadequate for testing our specific hypothesis above for two main reasons: (a) they are carried out in RNA extracted from total leukocytes rather than from purified CD34+ progenitor cells, and, (b) the "quantitation" is usually expressed as a ratio of BCR-ABL/control gene transcripts rather than a number of transcripts per cell; thus, a result of, e.g., 10<sup>6</sup> BCR-ABL/control gene transcripts may equally be derived from 10<sup>3</sup> cells expressing on average 10<sup>4</sup> BCR-ABL transcripts each, or 10 cells each expressing 10<sup>5</sup> transcripts. Whereas in the former scenario the bulk of the leukemic cells are at a relatively low-risk of developing resistance, in the latter the probability of a mutant resistant clone emerging soon after the initiation of treatment is considerably higher. It is envisaged that the proper quantification of the BCR-ABL RNA or protein in CD34+ cells before the start of imatinib on a prospective clinical trial will allow the identification of patients who are at risk of poor response or early relapse.

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