Resistance to Imatinib of Bcr/Abl P190 Lymphoblastic Leukemia Cells

Suparna Mishra, Bin Zhang, Jess M. Cunnick, Nora Heisterkamp, and John Groffen
Division of Hematology/Oncology, MS#54, Section of Molecular Carcinogenesis, Childrens Hospital of Los Angeles Research Institute, Los Angeles, California

Abstract
Around 20% of patients with acute lymphoblastic leukemia are Philadelphia chromosome positive (Ph-positive acute lymphoblastic leukemia) and express the Bcr/Abl tyrosine kinase. Treatment with the tyrosine kinase inhibitor Imatinib is currently standard for chronic myelogenous leukemia, which is also caused by Bcr/Abl. However, Imatinib has shown limited efficacy for treating Ph-positive acute lymphoblastic leukemia. In our study, we have investigated the effect of Imatinib therapy on murine P190 Bcr/Abl lymphoblastic leukemia cells. Three of four cultures were very sensitive to treatment with 5 μmol/L Imatinib. Significant cell death also initially occurred when the same cultures were treated in the presence of stromal support. However, after 6 days, remaining cells started to proliferate vigorously. The Bcr/Abl tyrosine kinase present in the cells that were now able to multiply in the presence of 5 μmol/L Imatinib was still inhibited by the drug. In concordance with this, the Abl ATP-binding pocket domain of Bcr/Abl in the resistant cells did not contain point mutations which would make the protein Imatinib resistant. The effect of stroma in selecting Imatinib-resistant lymphoblasts did not require direct cell-cell contact. SDF-1α could substitute for the presence of stromal cells. Our results show that stroma selects Imatinib-resistant Bcr/Abl P190 lymphoblasts that are less dependent on Bcr/Abl tyrosine kinase activity. Therefore, therapy for Ph-positive acute lymphoblastic leukemia, aimed at interfering with the protective effect of stroma in combination with Imatinib, could be of benefit for the eradication of the leukemic cells. (Cancer Res 2006; 66(10): 5387-93)

Introduction
The fusion of the BCR gene to the ABL proto-oncogene is the primary cause of Philadelphia chromosome-positive (Ph-positive) leukemias. The P210 form of Bcr/Abl is predominantly found in chronic myelogenous leukemia (CML) whereas many Ph-positive acute lymphoblastic leukemia patients have a smaller fusion protein, P190, which shares the same Abl moiety with P210. Ph-positive acute lymphoblastic leukemia constitutes ~20% of all cases of acute lymphoblastic leukemia and has a very poor prognosis (1, 2).

Imatinib (STI 571, Gleevec) is a potent inhibitor of the deregulated tyrosine kinase activity of Bcr/Abl (3). This inhibitor is used in the treatment of both CML (4) and Ph-positive acute lymphoblastic leukemia. The response rate, however, for Ph-positive acute lymphoblastic leukemia is lower than that of CML patients when used as a single agent (5, 6) and relapse is frequent.

The subject of Imatinib resistance has been intensively studied although many of the mechanisms identified to date come from studies involving CML patients and/or P210 Bcr/Abl-positive primary leukemic cells/cell lines. Mechanisms of resistance that involve the oncoprotein itself include BCR/ABL gene amplification, Bcr/Abl protein overexpression (7–9), and point mutations of the Bcr/Abl kinase in the ATP-binding pocket which prejudice binding of Imatinib (9–13). Overexpression of multidrug resistance (14), other protein tyrosine kinases (15–17), and Bcl-2 (18) has also been reported to contribute to Imatinib resistance. In addition, gene expression profiling done on primary cells taken from Ph-positive patients who responded to Imatinib treatment versus cells taken from non-responders showed up-regulation or down-regulation of many other genes, of which the significance remains to be determined (15).

Few studies have examined the question of why P190 Bcr/Abl lymphoblastic leukemia responds poorly to Imatinib therapy. We have previously established a transgenic mouse model for lymphoblastic leukemia caused by Bcr/Abl P190 (19). Malignant lymphoblasts from these transgenics can be cultured and used to test the effect of drug treatment (20, 21). In the current study, we have used such leukemic cells to investigate whether the low response to Imatinib as a single agent, seen in patients with Ph-positive acute lymphoblastic leukemia, is reproduced in vitro. We conclude that stroma exerts a protective effect that contributes to the poor response to this drug.

Materials and Methods
Lymphoblastic leukemia cells. The PLC1 pro-B lymphoblastic leukemia cell line has previously been described (20, 21). Fluorescence-activated cell sorting (FACS) analysis done on these cells 17 days after isolation and when cultured on mouse embryonic fibroblasts (MEF) showed that all of the cells were Gr-1, Mac1 negative; 29% of the cells were CD45R positive; and 61% were Gr-1, Mac1 negative; 7 of 13 contained CD45R single-positive and CD45R/Thy1.2 double-positive cells. This is a fairly typical immunophenotype seen on approximately half of the independent lymphoblastic leukemia cell populations isolated from different individual P190 Bcr/Abl transgenic mice: whereas most are negative for both Gr-1 and Mac-1, 6 of 13 were exclusively CD45R positive and 7 of 13 contained CD45R single-positive and CD45R/Thy1.2 double-positive populations. CD45R, Thy1w double-positive cells are early murine B-cell progenitors (22). This pro-B cell is the target of v-Ab1 transformation (22, 23).

Fifteen nude mice (female, 6 weeks old; Harlan Sprague-Dawley, Indianapolis, IN) were transplanted with 1 × 10³ PLC1 cells. At the first signs of visible lymphomas, mice were treated orally with 50 mg Imatinib/kg body weight (eight mice) or vehicle (seven mice) once daily. Every day, the compound was weighed and mixed in peanut butter/oil at 10 mg/mL before oral administration. Imatinib was obtained from Novartis (Basel, Switzerland). Mice were monitored daily for general health. When mice appeared moribund or the diameter of any lymphomas reached 1 cm, mice were sacrificed. Lymphoblasts were isolated from lymphomas of S-6 and S-11
Stroma affects treatment with Imatinib. We then grew lymphoma cells isolated from two randomly chosen Imatinib-treated (S-6 and S-11) and two vehicle-treated mice (V-7 and V-10) using a coculture system in which the Bcr/Abl P190 lymphoblasts are cultured in the presence of stroma, consisting of a layer of mitotically inactivated (irradiated) MEFs (20). After culture for 1 week, we treated the cells with 5 μM Imatinib. One set of S-6, S-11, V-7, and V-10 cells was cultured in wells containing the irradiated murine fibroblast stromal layer (Fig. 1A). The other set was cultured in wells of Transwell inserts (0.4-μm pore size; Corning, Acton, MA) placed in the wells of a 24-well plate or were seeded directly into the wells without any Transwell inserts. All wells contained irradiated MEFs.

Treatment of lymphoma cells with SDF-1α. PLC1 lymphoblastic leukemia/lymphoma cells or original V-7 cells were grown for several days in vitro. Cells (1 × 10^6) were plated directly in tissue culture wells of a 24-well plate or on an irradiated MEF feeder layer on tissue culture wells. Cells were then treated with 5 μM Imatinib with or without supplementation with SDF-1α (Peprotech, Rocky Hill, NJ) at a final concentration of 0.1 μg/mL in complete lymphoblast medium. An equal volume of 0.1% bovine serum albumin (BSA)/PBS was added to control wells. AMD3100 (Sigma-Aldrich, St. Louis, MO) was added at a concentration of 100 μM/L. Fresh medium was added every other day including fresh Imatinib, SDF-1α, or AMD3100.

Sequencing for ABL mutations. The doubly transgenic mouse line from which the PLC1 cells were isolated carried ~2 × two copies of a human BCR/ABL. P190 transgenic construct. DNA was isolated from V-7 and S-6 when these cells were able to proliferate in 5 μM/L Imatinib. A 417-bp fragment was amplified and sequenced using forward primer 5′-agagataccacctgaaggagatggtcttg-3′ and reverse primer 5′-ggatcggatttttcagatgcttgagtg-3′, including nucleotides 876 to 1,293 (residues 293-432) of c-Abl (NM_005157). This region encompasses point mutations detected in human patients at amino acid residues T315, F317, M351, and H396 (i.e., refs. 24, 25). We also sequenced a larger 675-bp region including both the ATP binding pocket and the activation loop using the primers and as described by Sacha et al. (25).

Western blot analysis. Cells were treated for 6 hours with water or Imatinib. SDS-BS lysates (25 μg) were run on 7.5% (for antiphosphotyrosine or Bcr/Abl) or 15% SDS-PAA (29:1 acrylamide/bisacrylamide, for Crkl) gels. Blots were first reacted with PY20-horseradish peroxidase (1:2500, BD Transduction, San Jose, CA), then stripped (Chemicon, 30 minutes) and re-reacted with anti-Bcr N-20 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Anti-Crkl antibodies (H62) were from Santa Cruz Biotechnology.

FACS analysis. At the end of the 14-day treatment period with vehicle or 5 μM/L Imatinib, lymphoma cells were assessed for cell-surface marker expression by staining with anti-CD45R, anti-Thy1.2, and anti-Sca-1 (BD PharMingen, San Jose, CA). We also analyzed the V-7 cells grown without Imatinib and V-7 cells grown in 5 μM/L Imatinib using anti-CD43-FITC, anti-CD24-R-PE, anti-CD45R-APC, and anti-IgM-PerCP-Cy5.5 (BD PharMingen, San Jose, CA) antibodies. Anti-Crkl antibodies (H62) were from Santa Cruz Biotechnology. Intracellular ABL antibodies were stained with anti-CD43-FITC, anti-CD24-R-PE, and anti-CD45R-APC (BD PharMingen) according to the instructions of the supplier to avoid the nonspecific binding to Fc receptors. The cells were analyzed using dual flow cytometry on a FACScan (Becton Dickinson, San Jose, CA).

Statistical analysis. Significance testing was done using Student’s t test, where P < 0.05 was considered statistically significant.

Results

Isolation of lymphoblasts from nude mice transplanted with leukemic cells and treated with Imatinib. The PLC1 lymphoblastic leukemia cell line was previously established from the lymphoma of a transgenic P190 BCR/ABL mouse (19, 20). Nude mice were each transplanted with 1 × 10^6 of these cells and treated with a dose of 50 mg/kg Imatinib or vehicle as described (21). This treatment was chosen based on previous in vivo treatment protocols with murine cells (28, 29). However, this dose of drug had no clinical benefit in our model: after 23 days of treatment, the average lymphoma burden in the Imatinib-treated group was not statistically different from that of the vehicle-treated group (not shown). At sacrifice, we isolated lymphoblasts from the lymphomas of a number of the vehicle-treated and drug-treated mice. Because amplification of the P210 BCR/ABL gene (9, 30, 31) or stabilization of P210 Bcr/Abl protein (8) was reported to generate Imatinib resistance in some patients, we extracted DNA and protein and evaluated these for possible differences with respect to P190 Bcr/Ab protein levels or increase in copy number of P190 BCR/ABL. However, no differences were found (not shown).
respectively) pCrkl. and tyrosine-phosphorylated and non-tyrosine-phosphorylated (p-Crkl and Crkl, at the bottom. Antibodies used for Western blotting are indicated dramatically to V

cultures had a viability of 91%, but within 24 hours, this dropped dramatically to ≤34%, and as low as 18% for the S-6 culture (Fig. 1A and B). The result of this short-term treatment showed that Imatinib was efficient in the initial eradication of a large number of leukemia cells regardless of whether they came from drug or vehicle-treated mice or whether they had stromal support or not. The longer-term treatment yielded a very different outcome that depended on how the cells were cultured. The viability of three of four cultures growing on tissue culture plates continued to decline, after the initial drop in viability at 24 hours, to the extent that almost no viable cells remained from day 6 onwards (Fig. 1B). The S-11 culture, which had an intrinsically higher proliferation rate than all other cultures, was an exception and started to proliferate vigorously. Interestingly, cells growing with stromal support showed a different outcome (Fig. 1A). In the course of continued treatment with Imatinib, the viability of all cultures, both from vehicle-treated and drug-treated mice, improved to ≥45% by day 10. Because the experiment was done with a fixed number of initial cells, this indicates that after initial cell death had occurred, remaining cells started to actively proliferate. We considered the possibility that Imatinib may not work effectively in the presence of stroma. To investigate this, PLC1 cells were grown in the presence and in the absence of stroma and were treated with 1 or 5 μmol/L Imatinib. As shown in Fig. 2A, treatment with both 1 and 5 μmol/L Imatinib had a very prominent effect on the Bcr/Abl P190 tyrosine kinase activity. This was visible as an overall decrease in the levels of phosphotyrosine. In addition, tyrosine-phosphorylated P190 Bcr/ Abl was eliminated (see Fig. 2A, lanes 2-3 and S-6, both anti-p-Tyr and shift in P190 Bcr/Abl on anti-Bcr Western blot). Tyrosine phosphorylation of the adapter protein Crkl, a substrate of Bcr/ Abl, has been used as a readout of inhibition of the Bcr/Abl tyrosine kinase activity in human patients treated with Imatinib (4). In PLC1 cells not treated with Imatinib, a major portion of Crkl was tyrosine phosphorylated as shown by its retarded mobility (Fig. 2A, lanes 1 and 4, anti-Crkl Western blot). Treatment with 1 μmol/L Imatinib caused increased appearance of the non-tyrosine-phosphorylated Crkl band and this was enhanced by 5 μmol/L Imatinib (Fig. 2A, lanes 3 and 6). Notably, Imatinib was equally effective in blocking Bcr/Abl tyrosine kinase activity when the cells were grown in the absence or in the presence of stroma was grown in wells in which the stromal layer was absent (Fig. 1B). Parallel cultures treated with vehicle only maintained good viability under both types of culture conditions (not shown). At the start of Imatinib treatment on day 0, all lymphoma cell cultures had a viability of ≥91%, but within 24 hours, this dropped dramatically to ≤34%, and as low as 18% for the S-6 culture (Fig. 1A and B). The result of this short-term treatment showed that Imatinib was efficient in the initial eradication of a large number of leukemia cells regardless of whether they came from drug or vehicle-treated mice or whether they had stromal support or not. The longer-term treatment yielded a very different outcome that depended on how the cells were cultured. The viability of three of four cultures growing on tissue culture plates continued to decline, after the initial drop in viability at 24 hours, to the extent that almost no viable cells remained from day 6 onwards (Fig. 1B). The S-11 culture, which had an intrinsically higher proliferation rate than all other cultures, was an exception and started to proliferate vigorously. Interestingly, cells growing with stromal support showed a different outcome (Fig. 1A). In the course of continued treatment with Imatinib, the viability of all cultures, both from vehicle-treated and drug-treated mice, improved to ≥45% by day 10. Because the experiment was done with a fixed number of initial cells, this indicates that after initial cell death had occurred, remaining cells started to actively proliferate. With prolonged treatment, the viability increased to ≥86%. At the end of the period of observation, the cells were able to grow in 5 μmol/L Imatinib. These results showed that the cells isolated from the vehicle-treated and the drug-treated mice behaved very similarly in culture, suggesting that the in vivo drug treatment had not selected for increased Imatinib resistance. However, the presence of stroma clearly provided protection against Imatinib-induced cell death.
(Fig. 2A, compare lanes 1-3 with 4-6). These results showed that the activity of P190 Bcr/Abl in PLC1 cells is effectively blocked by treatment with 5 μmol/L Imatinib and that stroma did not influence the effect of Imatinib on Bcr/Abl activity.

Do drug-resistant cells contain point mutations in the Abl kinase domain? To investigate whether the long-term culture of the V-7 or S-6 cells on stroma in the presence of 5 μmol/L Imatinib had promoted outgrowth of Imatinib-resistant variants of P190 Bcr/Abl, we did Western blot analysis on V-7 and S-6 cells while they were actively growing in 5 μmol/L Imatinib. We compared these to V-7 cells that had been isolated from the nude mice (original V-7 cells) treated either with water or with 5 μmol/L Imatinib. As shown in Fig. 2B, lysates from the original V-7 cells contained abundant phosphorylase, tyrosine-phosphorylated Bcr/Abl P190, and tyrosine-phosphorylated Crkl (Fig. 2B, lanes 1). Treatment of these cells with 5 μmol/L Imatinib caused a dramatic reduction in the phosphorylase levels of all these proteins (Fig. 2B, lanes 2). Surprisingly, when we compared this to the V-7 and S-6 cells that were 5 μmol/L Imatinib resistant and were growing in this dose of drug, the reduction of phosphorylase in general, in Bcr/Abl P190, and in Crkl was very similar to the levels found in the original PLC1 or V-7 cells when they were exposed de novo to 5 μmol/L Imatinib. We conclude that in the 5 μmol/L Imatinib–resistant cells, the P190 Bcr/Abl tyrosine kinase activity was still being effectively blocked by drug treatment.

We also grew the V-7 and S-6 cells that were able to proliferate in 5 μmol/L Imatinib for 1 week without Imatinib and then reexposed them to the dose of Imatinib (5 μmol/L) in which they had been able to grow. As shown in Fig. 3A and B, both V-7 and S-6 showed a reaction very similar to that originally exhibited by the cells: a steep decline in viable cell number between days 0 and 6 was followed by recovery and regrowth to the original cell number at around day 14. When we again cultured these cells for 1 week in the absence of Imatinib and then reexposed them to 5 μmol/L Imatinib, again a percentage of cells was killed during the first 24 hours, but both cultures rapidly recovered and were proliferating at the next time point. We also treated the V-7 cells that were able to grow in 5 μmol/L Imatinib (V-7 1st) with 10 μmol/L drug over a period of 14 days. As shown in Fig. 3C, treatment with a higher concentration of drug caused a decline in viable cell numbers in the first 6 days of treatment, followed by regrowth.

Resistance against Imatinib in human patients is frequently associated with the outgrowth of a clone expressing a Bcr/Abl protein with point mutations in the ATP binding site, and T315 is the most frequently mutated residue in patients (25). To conclusively exclude the possibility that these cultures represented outgrowth of cells containing point mutations in the Abl ATP binding domain, we isolated DNA from V-7 and S-6, which were resistant to 5 μmol/L Imatinib. However, sequence analysis of the regions of Abl encoding the ATP binding pocket as well as the activation loop showed that there were no point mutations present in these samples (not shown). These combined results conclusively show that the ability of a cell to survive Imatinib treatment is not caused by the emergence of clones with Imatinib-resistant Bcr/Abl proteins.

FACS analysis of drug-sensitive and drug-resistant cells. We next compared V-7 cells that were able to proliferate in 5 μmol/L Imatinib to those that had been cultured under identical conditions but in the absence of Imatinib using FACS and CD43, CD24/ HSA, CD45R/B220, and anti-IgM cell-surface markers. These were used by Hardy et al. (26) and Li et al. (27) to subclassify early B-lineage cells in the bone marrow. As shown in Fig. 4, resistant and sensitive cells were positive for both CD24 and CD43 markers for pro-B/pre-B cells. In agreement with these cells being progenitor B-cells, no surface IgM expressed on more mature B cells was detected. We also used Sca-1, a stem cell marker (32). There were significantly fewer brightly Sca-1+ staining cells in the V-7 population growing in 5 μmol/L Imatinib than in the population grown in its absence. CD45R, Thy1low double-positive cells are early B-cell progenitors (22). FACS analysis using B220/CD45R and Thy1.2 showed that there were fewer CD45R, Thy1.2 double-positive cells and more CD45R single-positive cells in the population growing in 5 μmol/L Imatinib than in the population growing in its absence. In addition, staining for CD45R was decreased. Additional analysis of S-6 grown in the absence or presence of Imatinib using Sca-1 and B220/Thy1.2 confirmed the decreased numbers of Sca-1-positive and B220/Thy1.2–positive

Figure 4. FACS analysis of V-7 cells able to grow in 5 μmol/L Imatinib or grown in the presence of vehicle. V-7 cells were stained with the antibodies indicated. The percentage of the total number of gated cells of selected quadrants is indicated below the plots.
cells in the Imatinib-resistant population (not shown). These results indicate that the ability to proliferate in 5 \( \mu \)mol/L Imatinib is associated with a selection within the population as measured by a shift in cell-surface markers.

**Treatment of lymphoma cells with 5 \( \mu \)mol/L Imatinib ex vivo in the presence or absence of Transwell inserts.** To investigate whether direct contact with the stromal layer was necessary for the lymphoma cells to survive in the presence of long-term treatment with Imatinib, the same four original lymphoma cell lines (S-6, S-11, V-7, and V-10) were cultured in the presence of stroma but with Transwell inserts placed in the wells, physically separating the lymphoma cells from the stroma (Fig. 5A, V-7 and V-10), or without the Transwell inserts, allowing direct contact of lymphoma cells with stroma (Fig. 5B, V-7 and V-10). As shown in Fig. 5 for V-7 and V-10, all lymphoma cells were initially severely affected by Imatinib, with a drop in viability from 90% to 5-10% (Fig. 5B) or even lower (Fig. 5A) within the first 2 days. The cells in direct contact with the stroma (Fig. 5B) started to rapidly recover. After day 13, the maximum cell density of the culture had in fact been reached and the cells started to die. The recovery of cells that were not in direct contact with the MEFs was slower and they proliferated less, but they nonetheless reached 80% viability at day 20 of treatment (Fig. 5A). Similar results were obtained with S-6 and S-11 (not shown). These data indicate that direct contact with stroma is not necessary for lymphoma cells to recover from the effect of long-term treatment with Imatinib and suggest that some soluble factor(s) produced by the stroma was responsible for the protective effect.

**Effect of SDF-1\( \alpha \) on the viability of lymphoma cells treated with 5 \( \mu \)mol/L Imatinib.** MEFs have been used as a crucial support for the culture of embryonic stem cells and they are known to secrete numerous growth factors (33, 34). SDF-1\( \alpha \) is an important cytokine that stimulates proliferation and chemotaxis of B-lineage cells (35, 36). To test this cytokine for a protective effect against Imatinib, we plated the parental lymphoblastic leukemia PLC1 cells on tissue culture plates in the absence of stroma. Cells were treated with 5 \( \mu \)mol/L Imatinib plus 0.1 \( \mu \)g/mL SDF-1\( \alpha \) or with 5 \( \mu \)mol/L Imatinib plus 0.1% BSA. As is shown in Fig. 6A, the SDF-1\( \alpha \)-supplemented cells...
maintained a significantly higher viability \((P < 0.01\) and \(P < 0.001\) at 11 and 22 hours, respectively) than the control cells. In a similar experiment, we also tested thrombopoietin but detected no protective effect (not shown).

We then investigated if SDF-1x was able to provide long-term protection. We repeated the experiment by treating V-7 with Imatinib for 14 days. As shown in Fig. 6B, when the cells were cultured without stroma but in the presence of SDF-1x, SDF-1x provided protection against Imatinib to an extent similar to that provided by MEFs.

SDF-1x signals through the CXCR4 receptor. The nonpeptide bicyclam AMD3100 was developed as an anti-HIV drug to block CXCR4 and exhibited low toxicity (37). We tested the ability of this drug to counteract the protective effect of the stroma. V-7 cells were treated with 5 \(\mu\)mol/L Imatinib in the presence of stroma and in the presence or absence of 100 \(\mu\)mol/L AMD3100. As shown in Fig. 6C, AMD3100 treatment was partly able to abrogate the protection against Imatinib treatment provided by the stroma.

**Discussion**

In the current study, we treated nude mice transplanted with PLC1 P190 Bcr/Abl pro-B lymphoblastic leukemia cells with a once-daily dose of 50 mg/kg Imatinib. The treatment regimen chosen for these experiments was based on the results of Buchdunger et al. (28) and Druker et al. (29) who found that this dose provided statistically significant prolongation of life when mice were transplanted with transformed murine cells. This dose achieves maximum concentrations of >3 \(\mu\)mol/L in vivo (38). We found that even 1 \(\mu\)mol/L Imatinib can effectively block the tyrosine kinase activity of Bcr/Abl P190 in cultured PLC1 cells. However, we found that this treatment provided no positive benefit in our nude mouse model. Our results agree with those of le Coutre et al. (38), who also found, using human CML cell lines injected s.c. into nude mice, that a dose of 50 mg/kg, given once daily via i.p. injection, provided no therapeutic benefit compared with mice treated with solvent. They showed that in their model, >80% of the Bcr/Abl kinase activity was restored in vivo after 8 hours (38). Therefore, it is possible that the lack of effect of Imatinib in our mouse model was partly caused by failure to achieve continuous blocking of the Bcr/Abl tyrosine kinase activity. Indeed, Western blot analysis of samples of the lymphomas taken from the drug-treated mice >14 hours after the last treatment showed that the Bcr/Abl tyrosine kinase activity was not blocked (results not shown).

We isolated lymphoma cells from the mice that had been treated with 50 mg/kg of drug and from controls after 23 days of treatment. Mechanisms of Imatinib resistance identified previously include BCR/ABL gene amplification and Bcr/Abl protein overexpression but we found no evidence that these had occurred in cells taken directly from the mice at sacrifice. In addition, there was also no overall difference in sensitivity towards Imatinib ex vivo between two cultures established from lymphomas taken from Imatinib treated versus two cultures taken from vehicle-treated mice, making it unlikely that the 23-day in vivo drug treatment had caused any selective outgrowth of drug-resistant cells.

By growing the cells ex vivo on tissue culture dishes and treating them with 5 \(\mu\)mol/L Imatinib, we showed that three of four different lymphoblastic leukemia populations taken from these mice were not inherently drug resistant: under the culture conditions used, which included supplementation with IL-3, these cells were eradicated. When we provided the same four cultures with stromal support, the initial reduction in viability was less pronounced and all four cultures were actively growing in 5 \(\mu\)mol/L Imatinib by day 14.

We examined two of these 5 \(\mu\)mol/L Imatinib–resistant lines in detail. The presence of 5 \(\mu\)mol/L Imatinib was still highly effective in blocking the Bcr/Abl tyrosine kinase activity. When cells were reexposed to the same dose of Imatinib after having not been treated for a week, the viability of the culture again declined dramatically by 80%. These results do not support the hypothesis that exposure to 5 \(\mu\)mol/L Imatinib had allowed the outgrowth of a drug-resistant subclone. Similarly, a higher dose of Imatinib was still able to eradicate a significant number of cells. We investigated directly by sequencing if the Bcr/Abl protein contained point mutations surrounding the Abl ATP-binding site, but we did not detect such mutations in the cells able to grow in 5 \(\mu\)mol/L Imatinib. We conclude that a part of each leukemic cell population is largely dependent on Bcr/Abl kinase activity for survival and will die when that activity is abruptly blocked by administration of Imatinib. A minority population is less dependent on Bcr/Abl and, if additional prosurvival support in the form of a fibroblast feeder layer is provided, can grow under selective pressure. That minority population does not contain mutations in the Bcr/Abl kinase domain that would render Imatinib ineffective but differs from the original population in that it contains less cells positive for Sca-1 and double positive for B220/Thy-1. Although we tentatively conclude that this population is more mature, further experiments will be needed to substantiate this.

What is the nature of the protection provided by the fibroblasts to the Bcr/Abl leukemic cells? It clearly is not related to metabolizing Imatinib because the Bcr/Abl kinase activity was effectively blocked by Imatinib when the cells were cocultured with the MEFs. The effect of the tumor microenvironment on drug resistance, as it specifically relates to leukemia caused by Bcr/Abl, has not been studied. However, the positive role of stroma on the survival of other leukemias has been well documented (i.e., refs. 36, 39–45). Some acute lymphoblastic leukemia cells may not require direct contact with stroma for survival but instead can rely on growth factors and other cytokines produced by it (46, 47). Because SDF-1 is an important cytokine for normal and leukemic B-lineage cells, affecting survival as well as migration (48–50), we tested its effect. We provide evidence that SDF-1x is one of the soluble factors produced by MEFs that is responsible for rescuing and/or ensuring the survival of the small population of P190 Bcr/Abl lymphoblasts not killed by the treatment with Imatinib. Our results using AMD3100, a specific CXCR4 inhibitor, suggest that SDF-1x is not the only factor produced by the fibroblasts that provides protection. Our results with AMD3100 are consistent with those of Burger et al. (50), who also found that CXCR4 inhibitors could not completely block the protective effect of stroma during treatment of chronic lymphocytic leukemia cells with fludarabine.

Our study shows that the culture of leukemia cells in the presence of stroma provides a valuable model to test the efficacy of drugs ex vivo and may point to mechanisms of drug resistance that are overlooked when the cells are cultured in the absence of stromal support. We conclude that for Imatinib to be...
effective in the long term, strategies other than continued and prolonged treatment with the same drug may be needed. Our study also suggests that the P190 Bcr/Ab1 leukemia population may consist of a mixture of cells that are acutely dependent on the Bcr/Ab1 P190 tyrosine kinase activity for their survival and cells that are not. Fully characterizing these cells will eliminate ways to will be a future challenge.

References


Resistance to Imatinib of Bcr/Abl P190 Lymphoblastic Leukemia Cells

Suparna Mishra, Bin Zhang, Jess M. Cunnick, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/10/5387

Cited articles

This article cites 48 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/10/5387.full#ref-list-1

Citing articles

This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/10/5387.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link:
http://cancerres.aacrjournals.org/content/66/10/5387.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.