Erythropoietin Promotes Resistance Against the Abl Tyrosine Kinase Inhibitor Imatinib (STI571) in K562 Human Leukemia Cells

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Abstract

Chronic myeloid leukemia is characterized by the Philadelphia chromosome translocation that causes expression of Bcr-Abl, a deregulated tyrosine kinase. Imatinib mesylate (STI571, Gleevec), a therapeutically used inhibitor of Bcr-Abl, causes apoptosis of Bcr-Abl-positive cells. In the leukemia cell line K562, we observed spontaneous resistance to imatinib at very low frequencies when cells were exposed to the drug (1 μM) for more than 4 weeks. Surprisingly, in the presence of erythropoietin (Epo), K562 cells were temporarily able to sustain proliferation in the presence of imatinib, and imatinib-resistant clones could be isolated with high frequencies. From such imatinib-resistant, Epo-dependent clones, sublines could be established that were resistant to imatinib in the absence of Epo. Mitogen-activated protein (MAP) kinase activity was inhibited by imatinib treatment but could be partially restored by Epo. Inhibition of MAP kinase or phosphatidylinositol 3-kinase blocked the protective effect of Epo. The data suggest that K562 cells acquire factor dependency under imatinib/Epo treatment, allowing them to escape from imatinib-induced, immediate cell death. This pool of cells provides the basis for the outgrowth of imatinib-resistant clones of unlimited proliferative capacity. Thus, Epo, an endogenous regulator of hematopoiesis, promotes the development of resistance to imatinib.

Introduction

The fusion protein Bcr-Abl, resulting from the Philadelphia (Ph) chromosome translocation, represents a constitutively active tyrosine kinase, which has been identified as the causative principle for most of the chronic myeloid leukemias (CML) and in Ph+ acute lymphoblastic leukemias (1, 2). Bcr-Abl expression leads to cytokine independence and malignant transformation of cells. Recent advances in the treatment of CML have largely relied on imatinib mesylate (STI571, Gleevec), a rationally designed inhibitor of the Abl tyrosine kinase (3). Imatinib potently suppresses the tyrosine kinase activity of the Bcr-Abl fusion protein and induces apoptosis in Bcr-Abl-positive cells (4–9). The success of this novel drug is based on Bcr-Abl’s central place as the single most important component to maintain factor independence of CML cells. The efficacy of imatinib in the treatment of chronic phase CML is well documented (8). Resistance resulting in failure of treatment at initiation of therapy seems to be rare. However, several recent reports indicate that patients in the accelerated phase and blast crisis that first responded well but relapsed during or after treatment had acquired resistance to imatinib (10, 11). These observations prompted a number of investigations into potential mechanisms enabling leukemic cells to evade the drug regimen.

Recent reports suggest the existence of at least five types of basic mechanisms for the development of imatinib resistance: gene mutations in Bcr-Abl interfering with imatinib binding without abrogating ATP binding (10–13), elevated levels of the Bcr-Abl gene product (10, 14), activation of alternate signaling pathways capable of ensuring cell survival (15), increased levels of the multidrug resistance P-glycoprotein (16), and elevated levels of plasma components that may bind imatinib and therefore lower its effective concentration (17). While most publications focused on the nature of the primary internal resistance mechanisms (18, 19), the potential role of endogenous or therapeutic cytokines as external promoters of resistance development was not investigated. Cytokines play an important role during the development of CML cells, mainly in the chronic phase of the disease. There is evidence for an autocrine interleukin-3 (IL-3) loop in CML (20), and survival of primary CML cells in vitro is stimulated by cytokines (21). Effects of cytokines on imatinib-treated Ph+ cells were investigated in primary CML cells (22) and in cell lines transduced with viral vectors containing Bcr-Abl (4, 23, 24). In both cell systems, increased short-term survival rates of the cells were detected.

For long-term analysis of cytokine effects on imatinib-treated cells, primary cells are not suitable and cell lines derived from CML patients are required. One of the most widely used and well-established Ph+ models is the K562 cell line, which was isolated from peripheral blood of a CML patient in blast crisis (25). K562 cells are highly sensitive to imatinib treatment, which induces partial differentiation followed by apoptosis. Several laboratories reported the generation of imatinib-resistant sublines of K562 cells using a complex drug treatment protocol with gradually increasing drug concentrations over...
several months (14, 15, 26). Development of resistant clones showing molecular changes similar to those found in primary CML cells demonstrates the general potential and suitability of K562 cells for analysis of resistance mechanisms (10, 14, 26).

K562 cells express the p210 form of the Bcr-Abl gene fusion, which is the predominant form found in CML. Imatinib treatment inhibits tyrosine kinase activity of Bcr-Abl and, as a consequence, abrogates phosphorylation of target proteins. Thus, the status of target protein tyrosine phosphorylation has been used as an indicator of Bcr-Abl activity and for the control of drug efficacy in primary as well as in K562 and other leukemia cell lines. Various downstream signaling components of the Bcr-Abl tyrosine kinase that are found in primary cells are also present in K562 cells (2, 27, 28), allowing for the functional analysis of highly relevant signal transducers. In particular, the Raf/mitogen-activated protein (MAP) kinase and phosphatidylinositol 3 (PI 3)-kinase pathways are activated by Bcr-Abl along with signal transducer and activator of transcription 5 (STAT5).

Examining the kinetics of cell death in imatinib-treated cells, we noticed that erythropoietin (Epo) caused a marked delay of apoptosis. We hypothesized that continuous cell cycling in the presence of the Bcr-Abl inhibitor may promote the development of imatinib resistance. Here, we describe that Epo markedly enhanced the frequency of imatinib-resistant K562 cells detected in long-term cultures. Imatinib treatment drives K562 cells into factor dependency that can be met by Epo, thereby ensuring their escape from imatinib-induced cell death. Most of the Epo-dependent cultures ultimately give rise to imatinib-resistant sublines that are able to sustain growth in the absence of Epo. Our data suggest that cytokines may raise the frequency by which leukemic cells develop resistance to imatinib.

Results

Epo Rescues K562 Cells From Imatinib-Associated Cell Death and Supports Their Proliferation

K562 cells are highly sensitive to imatinib (EC50 ~ 0.1 μM; 6). They stop proliferation and undergo apoptosis within 2–3 days of treatment with inhibitory concentrations of the drug (1 μM), resulting in a rapid decline in numbers of viable cells in culture (Fig. 1A). In several cell models, including primary CML cells, cytokines were reported to prevent this apoptotic response. We found that in K562 cells, Epo effectively suppressed immediate imatinib-induced cell death (Fig. 1A), although Epo without imatinib did not enhance proliferation of K562 cells (data not shown). Surprisingly, Epo not only protected from cell death but also allowed cells to proliferate for up to 3 weeks. Proliferation rates were reduced when compared with K562 cells maintained in the absence of imatinib (Fig. 1A). Withdrawal of Epo from imatinib/Epo-treated cultures in this initial phase of treatment led to rapid cell death, resulting in a sharp decline in cell numbers (exemplified in the figure by withdrawal of Epo at day 15). Epo also proved to be effective in supporting cell growth when added after imatinib: Epo exposure 20 h after initiation of imatinib treatment resulted in a 3.8 ± 0.4-fold increase in cell number after 72 h, which was slightly but not significantly lower when compared with a 4.6 ± 0.4-fold increase in cultures that received Epo simultaneously with imatinib (data not shown). These data suggest that in the absence of Bcr-Abl signaling, K562 cells acquire factor dependency. Imatinib itself does not initiate an immediate and irreversible apoptotic response, and the loss of Bcr-Abl signaling may be compensated by alternative signaling through the Epo receptor.

We hypothesized that such conditions may provide an environment for development of imatinib resistance because cell cycling occurs in the presence of imatinib. Therefore, long-term cultures were initiated to assess whether cultures under imatinib and Epo would eventually cease proliferation or whether subpopulations would survive the treatment and form continuous resistant cell cultures. Typically, cultures showed initial proliferation (phase I) when maintained in the presence of imatinib and Epo but stopped proliferating after 2–3 weeks (Fig. 1B). After a lag phase, net cell growth resumed after about 4 weeks of culture (phase II). Under these conditions, cell proliferation was sustained for at least 4 months.

Epo may rescue cells from imatinib-induced cell death by simply inhibiting apoptosis while cells slowly cycle. Alternatively, cell cycling may be normal but cell death results in low net growth rates of the entire culture. To distinguish these two possibilities, proliferation and cell death were monitored in imatinib-treated K562 cells in the presence or absence of Epo. Propidium iodide-labeled cells were analyzed by flow cytometry to assess the amount of cells with fragmented nuclei, indicating dead cells (Fig. 1C, gate 1). A large proportion (45%) of imatinib-treated cells died within the first 3 days. Most cells had stopped proliferating by day 2 (indicated by the lack of the G2 peak in gate 2). The presence of Epo resulted in continued proliferation and much lower proportions of dead cells (5% at day 3). Prolonged exposure to Epo/imatinib resulted in a gradual increase of dead cells (Fig. 1C). This indicates that Epo delayed cell death in a large portion of the culture and prevented cell death of a small subpopulation (indicated by the absence of the G2 peak). This subpopulation is sufficient to sustain long-term cell growth.

In K562 cells, Bcr-Abl causes tyrosine phosphorylation of multiple target proteins, which is reduced by imatinib treatment (26). As expected, the level of overall tyrosine phosphorylation declined and remained low in Epo/imatinib-treated cells during phase I (Fig. 1D). Cells treated with imatinib alone showed only a partial reduction of overall tyrosine phosphorylation at day 2 (Fig. 1D, lane 3) yet stopped proliferating (lack of G2 peak) and underwent apoptosis by day 3. By contrast, more pronounced inhibition of overall tyrosine phosphorylation in the long-term cultures is still compatible with cell growth when Epo is present. CrkL, a major target of Bcr-Abl, is tyrosine phosphorylated in K562 cells but remains predominantly dephosphorylated under imatinib and imatinib/Epo. Therefore, the effect of Epo appears not to be mediated through a restoration of Bcr-Abl-dependent tyrosine phosphorylation of target proteins.

In summary, in phase I, Epo supports limited cell cycling of imatinib-treated K562 cells and delays but does not prevent cell death. Under these conditions, cell cycling appears to be
independent of Bcr-Abl tyrosine kinase activity. In phase II, imatinib-resistant but Epo-dependent cells outgrow from phase I cultures and establish cultures with long-term proliferative capacity.

Epo Promotes the Development of Resistance Against Imatinib in K562 Cells

To examine whether Epo promoted the development of resistant cells from K562 cultures, the frequencies of imatinib-resistant cells could be obtained from K562 cultures in the presence or absence of Epo were analyzed. The ratio of Epo-independent imatinib resistance in K562 cells was investigated by application of inhibitory concentrations (1 μM) of imatinib over a period of 4 weeks. Only 2 of 192 wells showed viable cells, when 6000 cells/well were seeded initially (Table 1). Inclusion of Epo in the assay raised the number of positive wells to 114 of 192 corresponding to an ~100-fold increase in the frequency of imatinib-resistant cultures (estimated by limiting dilution analysis). Withdrawal of Epo in resistant wells is indicated by an arrow. The assay was performed thrice with similar results.

Table 1. Imatinib-Resistant Clones From K562 Cells Cultured in the Presence or Absence of Epo

<table>
<thead>
<tr>
<th>Cells/well seeded</th>
<th>Imatinib</th>
<th>Imatinib/Epo</th>
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<tbody>
<tr>
<td>3000</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>6000</td>
<td>54</td>
<td>1</td>
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<td>Frequency of resistance ($\times 10^{-6}$)</td>
<td>$\sim 1.7$</td>
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FIGURE 1. Survival and resistant growth of imatinib-treated K562 cells in the presence of Epo. A. Growth of untreated K562 cells (squares), imatinib-treated cells (circles), imatinib/Epo-treated cells (closed triangles), or imatinib/Epo-treated cells after withdrawal of Epo (open triangles) was plotted as cumulative changes in cell numbers over 20 days. Epo withdrawal from imatinib/Epo-treated cells at day 15 is indicated by an arrow. The assay was performed thrice with similar results. B. Growth of untreated K562 cells and cells exposed to Epo, imatinib, or imatinib/Epo were collected at the indicated time points, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Frequency histograms of propidium iodide intensities are represented. Nuclei were gated into fragmented (gate 1) and intact (gate 2) populations. Populations with intact nuclei represent cells in all phases of the cell cycle. Peaks representing G1 and G2 phase nuclei are indicated by arrows. The number given for gate 1 indicates the percentage DNA in fragmented nuclei. The experiment was performed six times with similar results. D. Immunoblotting of lysates of untreated cells, Epo-treated cells, and cells treated with imatinib or imatinib/Epo for the indicated times. Total cell lysates were analyzed by Western blot and probed with anti-phosphotyrosine (P-Tyr), anti-CrkL antibody, or anti-α-actin antibodies (actin) to ensure equal protein loading. Tyrosine-phosphorylated CrkL (P-CrkL) migrates slower than the unphosphorylated form (CrkL). The table indicates the percentages of phosphorylated and nonphosphorylated CrkL. Data represent one experiment out of three showing similar results.
clones obtained under Epo/imatinib treatment resulted in cell death over a period of 3 weeks, demonstrating that resistance at this point required the continuous presence of Epo (data not shown). The increase in resistance frequencies by 2 orders of magnitude indicates that reacquired factor dependency facilitates the outgrowth of imatinib-resistant clones in K562 cells with high efficiency.

Characterization of Epo-Dependent and Epo-Independent Imatinib-Resistant K562 Cells

Imatinib-resistant K562 cells that were isolated in the limiting dilution assay (Table 1) were further characterized. The two clones that emerged from cells treated with imatinib alone as well as six randomly selected Epo-dependent, imatinib-resistant clones from the imatinib/Epo-treated cultures (seeded at the lower density of 3000 cells/well) were expanded. Neither of the two clones that were isolated from cultures and exposed to imatinib alone was capable of sustaining unlimited growth in imatinib. The six Epo-dependent, imatinib-resistant clones showed long-term cell proliferation. Clones 1–5 were examined for their ability to sustain growth in imatinib/Epo at concentrations of up to 5 μM (Fig. 2A). Clones in imatinib/ Epo could be maintained in continuous cultures for at least 4 months without any indication of loss of resistance (data not shown). From these clones, resistant sublines (clones 1a–5a) could be isolated that did not longer require Epo to sustain proliferation in the presence of 1 μM imatinib (Fig. 2A). A sixth Epo-dependent clone failed to establish Epo-independent growth and was therefore not included in the figure. Apparently, Epo-dependent, imatinib-resistant cultures from phase II were capable of founding Epo-independent sublines (denoted phase III) with high efficiency (over 80%). In the absence of Epo, four of these sublines (clones 1a–5a) showed resistant growth up to a concentration of 2.5 μM of imatinib and one (clone 2a) even proliferated at 5 μM of the drug. Blocking Bcr-Abl activity by imatinib results in progression of K562 cells into erythroid differentiation, indicated by the production of hemoglobin (4). While Epo- dependent, imatinib-resistant cells (phase II) showed very high ratios of hemoglobin-positive cells as detected by benzidine staining, ratios in imatinib-resistant sublines (phase III) were consistently reduced but still remained above background ratios of K562 cells that were treated with Epo alone (Fig. 2B). Development of resistance appears to shift cells into a less differentiated stage.

Several clinical studies on the efficacy of imatinib in the treatment of CML reported restoration of Bcr-Abl signaling in imatinib-resistant cells on relapse of the disease. Therefore, overall tyrosine phosphorylation was examined in Epo-dependent and Epo-independent resistant clones by Western blotting (Fig. 2, C and D, respectively). While in untreated K562 cells numerous tyrosine-phosphorylated proteins were detected, naïve cells that were exposed to Epo/imatinib for 14 days (phase I) showed only few bands (Fig. 2, C and D). However, Epo-dependent and Epo-independent resistant clones (phases II and III, respectively) showed partial restoration of tyrosine phosphorylation levels with significant differences between the individual clones, including the emergence of new phosphobands in some Epo-independent, imatinib-resistant sublines (Fig. 2, C and D, respectively). As expected, CrkL was mostly phosphorylated in untreated K562 cells (P-CrkL; Fig. 2, C and D) but was dephosphorylated in phase I cells as demonstrated by its increased mobility on the Western blot. In phase III cells, no restoration of CrkL phosphorylation above levels in phase II cells was detectable (Fig. 2D). Bcr-Abl/CrkL signaling therefore appears to be minor compared with wild- type K562 cells, indicating that reactivation of Bcr-Abl is unlikely to be responsible for resistance in these clones. Consistently, phase III cells still showed high ratios of hemoglobin-positive cells, arguing against reactivation of Bcr- Abl (Fig. 2B).

The three phases of imatinib-resistant growth of K562 cells in the presence of Epo are schematically represented in Fig. 3. The K562/Epo/imatinib cell model provides a new paradigm for the generation of imatinib-resistant cells in vitro and for the analysis of their properties in the different phases of resistance development.

Cytokines Other Than Epo Do Not Support Resistance in K562 Cells

Modulatory effects of cytokines on the efficacy of imatinib-dependent cell death have been described for granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3 (4, 6). Therefore, we tested whether imatinib-treated K562 cells would respond to cytokines other than Epo by restoration of cell growth in the presence of imatinib. The influence of the cytokines stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), GM-CSF, or IL-3 on the proliferation of imatinib-treated K562 cells was monitored. None of the four factors was capable of stimulating the growth of imatinib-treated K562 cells (Fig. 4). Combinations of two or of all four factors were equally ineffective (data not shown). These data indicate that proliferation of K562 cells treated with imatinib cannot be experimentally influenced by SCF, G-CSF, GM-CSF, or IL-3, the four major cytokines involved in normal hematopoietic progenitor cell development. As a consequence, further analysis of cytokine-induced effects on imatinib resistance in K562 cells focused on the role of Epo.

Epo-Sustained Growth of K562 Cells in Imatinib Depends on PI 3-Kinase and MAP Kinase Activities

PI 3-kinase and the MAP kinase cascade are essential and sufficient for erythroid differentiation and proliferation (29). To evaluate whether blocking of Epo receptor-dependent cellular signal transduction could influence Epo-induced proliferation of imatinib-treated K562 cells in phase I of resistant growth, inhibitors of PI 3-kinase (LY294002) and the MAP kinase cascade (PD98059) were used. As expected (30), exposure to LY294002 resulted in strongly reduced growth rates of K562 cells (Fig. 5). Doubling times of ~7 days compared with 19 h in untreated cells were estimated. In cultures that also received imatinib or imatinib/Epo, proliferation was completely blocked. This result indicates that proliferation of imatinib/Epo-treated, nonresistant cells is strongly dependent on the activity of PI 3-kinase. Furthermore, PI 3-kinase appears to be required for
FIGURE 2. Analysis of imatinib-resistant clones. A. Growth of imatinib-resistant clones in the presence of 1 μM (squares), 2.5 μM (triangles), or 5 μM (circles) imatinib was plotted as cumulative changes in cell numbers. The measurement was performed by trypan blue exclusion cell counts. Epo-dependent clones (clones 1, 2, 3, 4, and 5) were cultured in the presence of Epo and imatinib (imatinib + Epo). Epo-independent clones (clones 1a, 2a, 3a, 4a, and 5a; imatinib) that originated from the Epo-dependent clones were cultured in 1 μM imatinib but in the absence of Epo for 7 weeks before the presented time course. Points, mean values of three independent measurements. B. Detection of erythroid differentiation by benzidine staining of hemoglobin-producing cells. Cells treated with Epo, imatinib, or imatinib/Epo for 2 days (black bars). Imatinib-resistant, Epo-dependent clones (1, 2, 3, 4, and 5; imatinib + Epo; dark gray bars). Imatinib-resistant, Epo-independent clones (1a, 2a, 3a, 4a, and 5a; imatinib; light gray bars) cultured for 7 weeks in imatinib alone before the experiment. Columns, mean values of six measurements. C and D. Immunoblotting of lysates of untreated cells, cells treated with imatinib/Epo for 14 days, Epo-dependent clones (1–5; C, imatinib + Epo), and Epo-independent clones (1a–5a; D, imatinib). Epo-independent clones (1a–5a) were treated with imatinib alone for at least 7 weeks before the experiment. Samples were probed with anti-phosphotyrosine (P-Tyr), anti-CrkL antibody, or anti-β-actin antibodies (actin) to ensure equal protein loading. Tyrosine-phosphorylated CrkL (P-CrkL) and unphosphorylated form (CrkL) are indicated by arrows. The tables indicate the percentages of phosphorylated and unphosphorylated CrkL. Both experiments were performed thrice with similar results.
Epo-dependent cell proliferation under imatinib treatment. Inactivation of the MAP kinase cascade by the MAP kinase kinase 1 (MEK1) inhibitor PD98059 reduced growth rates of imatinib/Epo-treated K562 cells, and a sharp decline of cell viability was detected after prolonged exposure (>10 days). This indicates that the activation of the MAP kinase cascade seems to be important for phase I survival of imatinib/Epo-treated cells that were rescued from immediate cell death by Epo.

Activation of MAP kinase by Epo treatment of imatinib-exposed cells was confirmed in immunoblotting experiments with an antibody to activation-specific phosphorylation sites of MAP kinase (Fig. 6). Untreated K562 cells showed a high level of phosphorylated MAP kinase (lane 1), which is known to be due to activation by Bcr-Abl (31). In cells treated with imatinib, no phosphorylation of MAP kinase was detectable (lane 2). However, under these experimental conditions, Epo was capable of partially activating MAP kinase (lane 3). In cells treated with Epo alone, phosphorylation levels of MAP kinase were similar as in untreated controls (cf. lanes 1 and 4). The data shown in Fig. 6 represent samples collected after 24 h of treatment. Additional samples were preincubated with imatinib for 1 h and then exposed to Epo for time points ranging from 10 min to 2 h after Epo administration. These samples showed no Epo-dependent MAP kinase phosphorylation, indicating that activation of the MAP kinase signaling cascade by Epo occurs only after prolonged exposure (data not shown). Taken together, these data demonstrate that in Bcr-Abl-inhibited K562 cells sustained Epo signaling partially activates MAP kinase and that survival and proliferation of cells in phase I depends on activation of both MAP kinase and PI 3-kinase.

Activation of STAT5, another direct target of the Epo receptor and a known modulator of expression of antiapoptotic Bcl-xL (32, 33), was examined using an antibody directed against phosphorylated STAT5 (Tyr694). Several reports demonstrated constitutive activation of STAT5 in K562 cells as a result of Bcr-Abl activity (27, 34). On addition of imatinib, STAT5 phosphorylation was reduced, but a significant proportion of STAT5 phosphorylation remained

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FIGURE 3. Schematic representation summarizing the growth phases involved in Epo-promoted development of resistance in K562 cells. In the presence of Epo, K562 cells are capable of surviving imatinib treatment and proliferating for a limited time (phase I: survival). After 2–3 weeks, most cells die and imatinib-resistant clones emerge that are still Epo dependent (phase II: Epo-dependent resistant growth). Epo withdrawal (indicated by arrows) in phase I and early in phase II results in rapid loss of cell viability and collapse of the entire culture. Epo withdrawal later in phase II is again accompanied by massive cell death, but sublines of resistant clones emerge that are capable of sustaining cell proliferation in the absence of Epo (phase III: Epo-independent resistant growth). Overall levels of tyrosine phosphorylation (P-Tyr) during phase I growth gradually decreases and then increases again as imatinib-resistant cultures form (phases II and III). CrkL phosphorylation (P-Crkl), an indicator of Bcr-Abl activity, is reduced under imatinib treatment but does not rise again during development of resistance.

FIGURE 4. SCF, GM-CSF, G-CSF, and IL-3 do not enhance proliferation of imatinib-treated K562 cells. Proliferation rates of untreated control cells or cells exposed to imatinib + Epo, imatinib + SCF (25 ng/ml), imatinib + GM-CSF (5 ng/ml), G-CSF (10 ng/ml), imatinib + IL-3 (3 U/ml), or imatinib alone were measured. The plot shows cumulative changes in cell numbers. Points, mean values of three independent measurements; bars, SE (smaller than symbols).
detectable (Fig. 6, lane 2). Contrary to MAP kinase, the original phosphorylation level of STAT5 was not restored by the addition of Epo for 24 h (lane 3). These results do not exclude a possible role of STAT5 in the survival of imatinib/Epo-treated K562 cells but demonstrate that the rescuing effect of Epo is not mediated by additional Epo-dependent STAT5 phosphorylation.

Physiological Concentrations of Epo Induce Proliferation of Imatinib-Treated K562 Cells

In all experiments described thus far, Epo concentrations of 4 units/ml were used to stimulate cell growth. Epo concentrations in the plasma of healthy subjects are much lower, ranging from 5 to 36 mU/ml (35). To test whether Epo at such low concentrations was still effective, cell growth in the presence of imatinib/Epo-treated K562 cells but demonstrate that the rescuing effect of Epo is not mediated by additional Epo-dependent STAT5 phosphorylation.

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To confirm that proliferation of imatinib-treated K562 cells was due to Epo and not to some other component in the Epo formulation, a neutralizing anti-Epo antibody was used to block binding of Epo to its receptor. As shown in Fig. 7B, the antibody alone had no growth effect on imatinib-treated cells. Exposure of cells to Epo at concentrations of 0.1 and 1 unit/ml, led to proliferation. At Epo concentrations of 0.1 unit/ml, the antibody abolished proliferation of K562 cells completely, which is consistent with Epo being the active principle. At the maximum effective concentration of Epo (1 unit/ml), the antibody in the concentration used (5 μg/ml) was no longer able to block proliferation apparently due to substoichiometric levels with respect to Epo. This latter result demonstrates that the buffer solution of the antibody preparation itself did not interfere with proliferation, because the inhibitory effect could be overcome by higher Epo concentrations. Finally, we determined whether Epo alone acted to stimulate cell proliferation or whether it might require the simultaneous presence of factors in fetal bovine serum that was used as cell culture supplement. Therefore, K562 cells were cultured in a serum-free defined medium for 3 days before the experiment and then incubated with imatinib in the presence or absence of Epo (Fig. 7C). Even under these conditions, Epo was capable of sustaining cell proliferation in the presence of imatinib, excluding the possibility that an unknown serum factor might be essential to support the action of Epo. We conclude that Epo at physiological concentrations can support survival of imatinib-treated K562 cells.

Discussion

Accumulation of mutations in CML from chronic phase to blast crisis due to high genetic instability is well documented (36). Patients that relapse during imatinib treatment frequently...
show specific changes in Bcr-Abl itself, leading to reduced drug binding (37). Less intensely studied is the question whether genetic changes are already present before imatinib treatment or whether resistant clones may develop during treatment. Genetic changes evolving during imatinib treatment are not viewed as a potential reason for imatinib resistance (38). Such changes are expected to happen only in rare cases because Bcr-Abl-positive cells treated with imatinib immediately stop proliferation in vitro (4). With the lack of time for additional cell divisions, it appears unlikely that resistance could occur through de novo genetic changes after initiation of imatinib treatment. However, this assumption may prove invalid in situations that would allow for limited proliferation of Bcr-Abl-positive cells under the selective pressure of an inhibitory agent.

The present study suggests a link between a cytokine-dependent rescue mechanism protecting Bcr-Abl-positive cells from imatinib-associated cell death and the development of imatinib resistance. Antiapoptotic effects of cytokines on Bcr-Abl-positive cell lines during imatinib treatment are well known. Mouse cell lines like 32D, Ba/F3, FDC-P1, and the human cell line MO7e become factor independent when transduced with Bcr-Abl. In the absence of cytokines, imatinib treatment results in cell death that can be prevented by IL-3 (4, 23, 24), demonstrating that factor dependency was reacquired through inhibition of the Bcr-Abl tyrosine kinase. However, these studies did not address the question of whether the combined treatment of cells with imatinib and appropriate cytokines could result in imatinib resistance. Our studies provide the first evidence that cells that are capable of reestablishing cytokine dependency may escape effective drug treatment in the short term and acquire imatinib resistance in the long term. Because the protective effect could even be detected at physiologically low concentrations of Epo, the concept of cytokine-assisted development of resistance may also be of clinical relevance. This conclusion relies on the use of a CML cell line and not on primary CML cells. Therefore, experiments to verify the cytokine effect in primary cells would be necessary. However, long-term cultures of primary leukemia cells that would be required for detecting the outgrowth of resistant clones are not yet feasible.

Signaling pathways activated by the Epo receptor supported survival and substituted for Bcr-Abl signaling for a limited time. MAP kinase and PI 3-kinase, but not STAT5, a transcription factor activated through Janus kinase 2 binding to the Epo receptor (32), appear to be critically involved in phase I. Our findings are consistent with the observation that activation of the PI 3-kinase and the MAP kinase cascades are sufficient for proliferation of Epo-dependent primary cells (29) and suggest that similar mechanisms may regulate proliferation in normal primary cells and Epo-dependent proliferation in the imatinib-treated K562 cell line. We conclude that the mechanism that protects K562 cells from imatinib-associated cell death involves PI 3-kinase and MAP kinase, but is independent of STAT5 activation. The K562 cell line may serve as a model system to further analyze the roles of these signaling cascades in the promotion of resistance development.

Beside Epo, no other factors were effective in K562 cells to protect from imatinib-induced cell death. This does not mean that other cytokines may not be effective in other cell
lines or primary CML cells. Published data on the effects of cytokines on K562 cells are not conclusive. In particular, the presence of functional receptors for IL-3, G-CSF, GM-CSF, and SCF has not been established. However, expression of the Epo receptor in K562 cells is well documented (39). K562 cells may thus present a model system for further studies on the protective effect of Epo as a representative for other cytokines. Investigations whether Epo or other cytokines are able to promote resistance in primary CML cells are limited by the fact that these CML cells only show short-term survival in vitro.

The advantage of the K562/Epo/imatinib model cell for analysis of imatinib resistance lies in the clear distinction and accessibility of the different phases during resistance development. This provides a basis for better understanding the individual steps involved in cytokine-promoted development of resistance. Further analysis of the involvement of the MAP kinase and PI 3-kinase pathways in phase I should provide information on the mechanisms that protect cells from imatinib-induced apoptosis. Analysis of cells in phase II should give insight into the kinetics of resistance development. Finally, analysis of phase III cells is likely to identify in this model system some of the known molecular correlates to clinically observed resistance mechanisms, such as gene amplification or point mutations in Bcr-Abl. In this model, the critical cellular changes allowing for the continuous proliferation under the selective pressure of imatinib may now be examined.

In conclusion, in the K562/Epo/imatinib model system, a new modality of progression toward resistance was identified. Endogenous cytokines may assume an unexpected role in the development of imatinib resistance by supporting factor-dependent growth.

Materials and Methods
Reagents
Cell culture media and supplements were purchased from Life Technologies, Inc. (Basel, Switzerland). Epo, GM-CSF, and IL-3 were generous gifts from Cilag AG (Schaffhausen, Switzerland), Werthenstein-Chemie AG (Schachen, Switzerland), and Novartis Pharma AG (Basel, Switzerland), respectively. SCF and G-CSF were purchased from PeproTech (London, United Kingdom). Epo was used at concentrations of 4 units/ml if not otherwise indicated. Imatinib was kindly provided by Dr. Elisabeth Buchdunger from Novartis Pharma and was prepared as a 10 mM stock solution in DMSO. Imatinib was used at a final concentration of 1 μM. Working solutions were diluted in cell culture media and added directly to cells with no more than 0.1% of final DMSO concentration. LY294002 and PD98059 were purchased from Sigma (Buchs, Switzerland) and Alexis Biochemicals (Lausen, Switzerland) and used as recommended at concentrations of 50 and 20 μM, respectively. Unless otherwise mentioned, all chemicals (analytical grade) were from Sigma, Fluka (Buchs, Switzerland) or Merck AG (Dietikon, Switzerland).

Cell Culture
K562 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640, 10% fetal bovine serum, 2 mM Glutamax I (Life Technologies), 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. Serum-free cultures of K562 cells were maintained in Iscove medium (Iscove’s modification of Dulbecco’s MEM) supplemented with 20% BIT-9500 serum substitute containing bovine serum albumin, insulin, and transferrin (StemCell Technologies, Vancouver, British Columbia, Canada), 2 mM Glutamax I, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of 95% air and 5% CO2. Proliferating cells were passaged every 2–3 days and seeded at a density of 10^5 cells/ml. Slowly proliferating or non-proliferating cultures were kept at a density of 5 × 10^3 cells/ml and medium was partially (50%) replaced every 2–3 days. Hemoglobin-producing cells were detected by benzidine staining of cellular suspensions (40).

Cell Proliferation Assay
Proliferation of cell cultures was monitored by counting viable cells based on trypan blue exclusion. Cumulative cell numbers were calculated by multiplying the number of cells per culture by the splitting factor.

Cell Cycle and Viability Assay
Viability was analyzed by DNA flow cytometry (41). In brief, cells were resuspended in hypotonic fluorochrome solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, and 0.1% (v/v) Triton X-100 and incubated at 4°C for 6 h before analysis.

Antibodies
The following commercially available monoclonal antibodies were used as recommended by the manufacturer: anti-MAP kinase 1/2 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204) E10, anti-phospho-STAT5 (Tyr694), anti-CrkL (32H4), anti-phosphotyrosine (P-Tyr-100; Cell Signaling, Beverly, MA), anti-STAT (sc-835; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibodies to β-actin and neutralizing antibodies to human Epo were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) as well as the horseradish peroxidase-conjugated secondary antibodies to mouse IgG (whole molecule) and rabbit IgG (whole molecule).

Immunoblotting
Cells were washed thrice with Dulbecco’s PBS (Sigma). A sample was removed before the third washing step for protein determination using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). The cell pellet was resuspended in an appropriate amount (100 μl/10^6 cells) of sample buffer (62.5 mM Tris-HCl [pH 7.5], 2% SDS, 10% (v/v) glycerol, 50 mM DTT, and 0.01% bromophenol blue) and lysed by sonication for 10–15 s. Samples were denatured for 5 min at 95°C. Fifteen micrograms of protein/sample were resolved on 12% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose filters. The membranes were blocked with 5% nonfat dry milk in TBS-Tween (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Tween 20), incubated with the
indicated antibodies for immunostaining, and visualized by chemiluminescence and exposure to Hyperfilm (ECL Plus Western Blotting Detection System, Amersham, Buckinghamshire, United Kingdom). As molecular size markers, a prestained SDS-PAGE low range standard (Bio-Rad Laboratories AG, Reinach, Switzerland) was used. Intensities of P-CrkL and CrkL bands on Hyperfilm sheets were determined using a optical digitizing system and the QuantityOne software package (Geldoc, Bio-Rad Laboratories).

**Analysis of Resistance Frequencies and Selection of Resistant Cultures**

Cells were seeded into 96-well dishes (3000 or 6000 cells/well). The culture medium was supplemented with imatinib (1 μM) and Epo (4 units/ml) or imatinib alone. Fifty percent of the medium was changed every third day without removing any cells. Four weeks after initial seeding, wells with proliferating cells were identified using a light microscope. The frequency of resistant clones was calculated using the limiting dilution estimate (42). Briefly, the logarithm of the percentage of wells with no cell growth was plotted against the number of cells seeded per well. A linear regression line was fitted to the two data points (3000 and 6000 cells seeded) originating at the *a priori* value for no cells seeded (100%). The limiting dilution estimate was derived from the abscissa at the ordinate value of 37%, which represents the percentage of empty wells expected at limiting dilution. For further analysis of resistant cultures, cells from individual wells were expanded in imatinib and Epo (1 μM and 4 units/ml, respectively) or imatinib alone after removing Epo by two wash cycles.

**Data Analysis**

Statistical analyses were performed using the software package GraphPad Prism, version 2.0c (GraphPad Software, Inc., San Diego, CA). Error values refer to the SE.

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**References**

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