

REVIEW

Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia

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Chronic myeloid leukemia (CML) has been studied intensively for many years; yet its treatment remains problematic and its biology remains elusive. In chronic phase, the leukemic clone appears to be maintained by a small number of BCR-ABL-positive hematopoietic stem cells that differentiate normally and amplify slowly. In contrast, as these cells enter the intermediate stages of lineage restriction, their progeny are selectively expanded and generate an enlarged pool of neoplastic progenitors. Recent analyses of purified subsets of primitive CML cells have provided a coherent explanation for this dichotomous behavior of BCR-ABL-positive stem and progenitor cells based on the discovery of an unusual autocrine IL-3/G-CSF mechanism activated in them. This only partially counteracts *in vivo* signals that maintain normal stem cells in a quiescent state but, when active in CML stem cells, promotes their differentiation in favor of their self-renewal. In more differentiated CML progenitors, the same mechanism has a more potent mitogenic effect which is then extinguished when the cells enter the terminal stages of differentiation. Thus, further expansion of the clone is limited until inevitably additional mutations are acquired that further distort or override the regulatory mechanisms still operative in the chronic phase.

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Introduction

The chronic phase of chronic myeloid leukemia (CML) is characterized by the presence of an abnormally amplified multilineage clone that produces functionally normal mature blood cells of all types except for those of the T lineage.¹ However, disease symptoms usually develop as a result of the markedly elevated output of granulocytes that is the biologic hallmark of this disease. The expanded clone responsible is believed to be initiated by the chance occurrence in a hematopoietic stem cell of a rare mutational event in which the majority of the 3' end of the *c-abl* gene from chromosome 9 replaces the 3' end of the *BCR-1* gene on chromosome 22.^{2,3} A characteristically shortened derivative chromosome 22 (known as the Philadelphia chromosome or 'Ph'), thus created by this reciprocal exchange of chromosome ends, has been recognized for four decades as diagnostic of >90% of cases with features associated with CML.^{4,5} The BCR-ABL fusion gene harbored on the Ph is transcriptionally active and encodes a 210 kDa oncoprotein (p210^{BCR-ABL}) that has transforming activity in both primary mouse bone marrow cells and established cell lines.^{6–10} P210^{BCR-ABL} differs from the normal p145^{c-ABL} in its preferential location in the cytoplasm and its constitutively elevated tyrosine kinase activity. Mutational

analyses suggest that both of these properties of p210^{BCR-ABL} are critical to its transforming activity.^{11,12} Recent findings indicate that the formation of the BCR-ABL gene may not, alone, be sufficient to produce disease. This is suggested by the finding that cells containing detectable levels of BCR-ABL transcripts exist in the blood of a high proportion of normal adults.^{13,14} In addition, even when a BCR-ABL⁺ clone does expand to the point where a disease entity is recognized (ie to an extent that allows >10¹² cells to be produced daily), this occurs only after a prolonged latent period (on average, 6–7 years, as calculated from the follow-up of survivors of the atomic bomb explosions in Japan at the end of World War II¹⁵). Thus the net growth advantage that expression of BCR-ABL confers on a CML stem cell must be relatively subtle. In light of these considerations, it is perhaps not surprising that the identification of therapies able to effectively target the CML stem cell compartment have remained elusive. In this review, we will summarize some of our recent studies of these cells that provide new insights into their deregulated growth and suggest new avenues for exploration in a clinical setting.

The Ph⁺ BCR-ABL⁺ stem cell: basic concepts

Cell of origin of the disease

Although the granulocytic lineage is the one that is typically the most affected in CML, an accompanying hypercellular marrow containing an increased number of erythroblasts and megakaryocytes was recognized more than 50 years ago as a typical feature of CML. Indeed, this latter observation formed the original basis of the concept that CML arises in a pluripotent stem cell,¹⁶ later confirmed by a variety of elegant studies using cytogenetic, FISH and X-linked markers of clonal expansion.^{4,17–22} These showed that erythroid cells, megakaryocytes, and B and T cell precursors, are all commonly produced within the neoplastic clone. More recently, Gunsilius *et al*²³ reported that the BCR-ABL gene could be found in endothelial cells produced from a CML patient's cells. If confirmed, these data might place the level of the BCR-ABL mutation in an even earlier cell type, ie one with endothelial as well as hematopoietic differentiation potential.

Morphology and phenotype of the neoplastic cells

CML results in the production of granulocytes, red cells and platelets that, although derived from Ph⁺/BCR-ABL⁺ precursors, are morphologically indistinguishable from their normal counterparts. A definitive diagnosis of CML therefore relies on the cytogenetic or molecular demonstration in blood or marrow of either the Ph chromosome or a BCR-ABL gene

rearrangement. Similarly, since the cells in the Ph⁺/BCR-ABL⁺ clone undergo normal differentiation, analysis of changes in cell markers that accompany this process in normal cells do not allow individual Ph⁺ and Ph⁻ elements to be distinguished from one another at any stage of differentiation. This also applies to functionally defined progenitors, including those classified as various types of colony-forming cells (CFCs), as well as even more primitive cells detected by their ability to generate progeny CFCs after 5–8 weeks in co-cultures containing stromal cells and referred to as long-term culture-initiating cells (LTC-ICs).^{24,25} However, some differences are seen at the progenitor population level due to the fact that a much higher proportion of primitive Ph⁺ cells are actively cycling relative to their normal (Ph⁻) counterparts. For example, most primitive CML progenitors are characterized by an upregulated expression of HLA-DR^{26–28} which is known to represent an early response to growth factor activation by their predominantly quiescent, primitive normal counterparts.²⁹

Transplantability

The ability of Ph⁺ CML cells to engraft humans was first discovered by accident following the transfusion of leukopenic patients with freshly leukapheresed blood cells from donors with CML at a time when leukemic stem cells were not known to be present at high levels in these collections.^{30,31} More recently, further evidence that CML cells can engraft has been provided by analysis of cells from CML recipients of retrovirally marked autografts at the time of subsequent disease relapse.³² However, none of these studies provided any quantitative indication of the relative numbers of transplantable CML stem cells in a given patient nor whether their engraftment properties are affected by the disease process. Interestingly, in other studies with similar types of autotransplants, prolonged disease-free outcomes have been encountered,^{33,34} suggesting that the number of CML 'stem' cells in many patients may actually be quite small and/or that many of these cells are not readily transplantable. Attempts have also been made to produce the disease in xenogeneic hosts by transplantation of immunodeficient mice with CML patients' cells. Thus far, engraftment of irradiated SCID or NOD/SCID mice with Ph⁺/BCR-ABL⁺ cells from chronic phase CML patients has been found to require the transplantation of very large inocula, consistent with experience in patients.^{35–37} Moreover, in most cases, a selective engraftment of normal (Ph⁻) cells has been seen, particularly when the more primitive human cells detectable in these mice are analyzed.^{35–39} Thus, although it is clear that some CML cells can produce leukemic progeny following their intravenous injection, none of the xenotransplant models used, to date, have lived up to initial hopes to consistently transfer the human disease to animal hosts at frequencies or levels adequate for their further study on a routine basis.

More recently, it has been found that the transplantability of normal hematopoietic stem cells can fluctuate according to their cell cycle status.^{40,41} This may explain, at least in part, the relatively poor engraftment obtained with transplants of primitive CML cells. Only one-twentieth of the potentially transplantable stem cells in normal human cord blood or fetal liver actually seed to the marrow of NOD/SCID mice,^{42,43} and the ability of even this fraction to engraft markedly decreases both as they enter G₁ from G₀ and then again as they transit S/G₂/M.^{41,44} The proportion of many primitive types of CML progenitors that are proliferating is known to be much higher

than is the case for their normal counterparts.²⁵ It would thus be anticipated that this would also be true for the CML stem cell population. Accordingly, these cells would be expected, on this basis alone, to appear selectively compromised in their ability to engraft NOD/SCID mice. Conversely, the detection of any Ph⁺ progeny in NOD/SCID mice might then indirectly infer the presence, in chronic phase CML patients, of rare G₀/G₁ stem cell populations. As discussed below, we have recently shown that such G₀/G₁ CML cells can be found in all patients examined to date.

Deregulated hematopoietic cell functions in CML

The BCR-ABL fusion protein has a wide range of substrates, and it is likely that many of these contribute to producing the altered behavior of Ph⁺/BCR-ABL⁺ stem cells. Many theories have been formulated to explain the myeloid expansion that is typical of chronic phase CML. These emphasize to varying degrees the abnormalities in control of proliferation, apoptosis, cellular adhesion and the organization of the cytoskeleton that have been noted in various studies of BCR-ABL expressing cells. As noted above, marked numerical perturbations occur in the subpopulations that make up the CML clone as the neoplastic stem cells begin to differentiate. Of interest, the leukemic stem cell compartment appears to remain quite small but entry into the intermediate compartments of progenitors (of all lineages) is accompanied by a rapid expansion of these cells. However, ultimately an increased output of mature cells is largely restricted to the granulocyte lineages. It is therefore likely that the cell context in which BCR-ABL is expressed has a significant impact on how cellular behavior is altered. In this review, we have focused on mechanisms relevant to the deregulated turnover that is characteristic of the most primitive elements in the CML clone and their ability to generate an expanded population of intermediate progenitor types.

Proliferative activity and stem cell turnover

Evidence of a perturbation of the cell cycle regulation of Ph⁺/BCR-ABL⁺ stem cells is inferred from changes that affect neoplastic cells detected by surrogate assays for these and closely related populations of primitive Ph⁺/BCR-ABL⁺ cells. These include assays for LTC-ICs and CFCs defined either by their high proliferative potential (HPP)^{25,45} and/or their multipotent differentiation ability (referred to as colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte, or CFU-GEMMs.^{25,45,46} However, it has been a challenge to acquire data even on these cell types, both because of their relatively low frequency in the blood and marrow of CML patients and because of their lack of a unique phenotype, which precludes their direct distinction or separation. Also, because of the frequent prevalence of residual normal cells in the most primitive compartments and the wide variation in proportions of different types of normal and leukemic cells in individual CML patients, the progenitors investigated in any given experiment (or their clonal progeny) must be genotyped. This, in turn, limits the number of samples that can be analyzed and, hence the ultimate precision of the data obtained.

Nevertheless, the results of several approaches have provided strong support for the view that the turnover of BCR-ABL⁺ stem cells, like that of their immediate progeny, is deregulated. The first approach involves exposing the cells to

an agent that is specifically toxic to S-phase cells (eg high specific activity of ^3H -thymidine or hydroxyurea) for 15–30 min. This methodology was first used to demonstrate that the HPP-CFCs in the marrow of normal adults are largely quiescent, as are all of the CFCs normally found in the blood. In contrast, analogous measurements applied to CFCs from CML patients showed that the majority of the leukemic CFCs, regardless of their location (in bone marrow or blood), are at any given moment in cycle.⁴⁵ Later, a more prolonged ^3H -thymidine suicide procedure was used to show that in normal adults, most (>90%) of the LTC-ICs are quiescent, whereas in CML, most (>90%) of their leukemic counterparts are proliferating.^{47,48}

A second approach to assessing the cell cycle status of primitive hematopoietic cells has exploited FACS technology for isolating viable cells in G_0 , G_1 and $S/G_2/M$ based on differences in their DNA and RNA content as visualized by co-staining with Hoechst 33342 and Pyronin Y (Figure 1). Such experiments have confirmed that there is an increased proportion of primitive leukemic cells (CFCs and LTC-ICs) in chronic phase CML patients that are cycling (in $S/G_2/M$).⁴⁸ Unexpectedly, these analyses also provided the first direct evidence that there is, in addition, a consistently detectable fraction of G_0 CML cells within these same primitive leukemic cell populations. Furthermore, using a high resolution procedure for tracking cell division, it was possible to isolate a subset of $\text{Ph}^+/\text{BCR-ABL}^+$ CFCs that remained viable and functionally unaltered, but did not enter mitosis for 5 days in the presence of a growth factor cocktail that is potently mitogenic for primitive normal cells (Figure 2). These latter studies thus demonstrated the depth of quiescence that a proportion of primitive CML progenitors reach *in vivo* that, nevertheless, can be reversed after several days of growth factor stimulation *in vitro*. Interestingly, single cell RT-PCR analyses showed that the primitive quiescent CML cells (isolated by either strategy) expressed BCR-ABL transcripts. This latter observation suggests that the BCR-ABL oncoprotein alone may not be sufficient in these cells to counteract all of the inhibitory cues emanating from the environment *in vivo*. Indeed, such a possi-

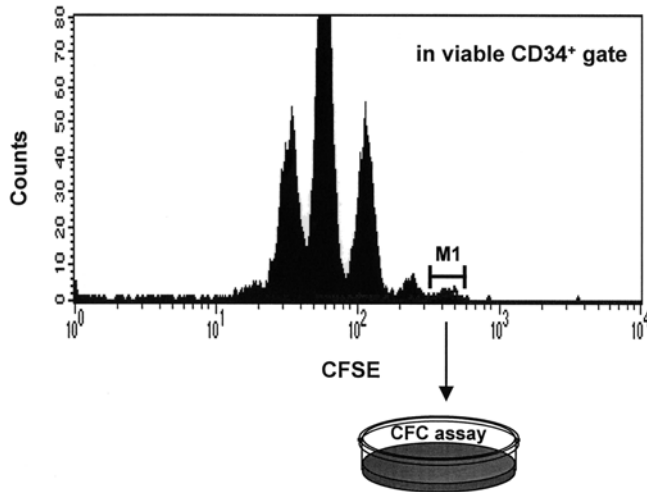


Figure 2 FACS strategy for the isolation of deeply quiescent CD34^+ cells. Lineage-depleted, CD34^+ cells were labeled with CFSE and a homogeneous population of viable CD34^+ , CFSE^+ cells was isolated by FACS. These cells were then cultured for 3 days in the presence of five stimulatory growth factors (Flt3-ligand, Steel factor, IL-3, IL-6 and G-CSF) selected to drive primitive cells into cell division. At the end of the third day the cells were analyzed in terms of residual CFSE fluorescence and those cells which had not yet undergone cell division were identified in M1. The quiescent cells present in M1 were then isolated by FACS and cultured in standard CFC assays. (Reproduced with permission by the American Society of Hematology, Washington DC, from Ref. 48.)

bility was raised a decade ago from studies indicating that exposure of primitive cycling CML CFCs to non-toxic concentrations of $\text{TGF-}\beta$ *in vitro* can transiently arrest their entry into S-phase.⁴⁹

We also found that relatively low numbers (10^5) of primitive CD34^+ G_0 CML cells were able to engraft irradiated NOD/SCID mice and produce detectable numbers of leukemic progeny 6–8 weeks later.⁴⁸ This indicates that the quiescence of these transplantable leukemic cells is also reversible *in vivo*, thus pointing to the likely importance of these cells in sustaining and spreading the disease in patients. The consistently demonstrable presence of this previously unrecognized compartment of primitive, transiently quiescent CML cells in chronic phase patients provides a possible explanation for both the historic failure of intensive (but non-myeloablative) chemotherapy regimens to eradicate chronic phase disease,^{50,51} and the occasional observation of late relapse many years post transplant.^{52,53} On the other hand, these clinical findings could also have other explanations and neither permits any direct quantitative inferences to be made about the frequency of primitive neoplastic cells that are quiescent in an individual patient. The availability of methods that allow the reproducible isolation of these cells and their further characterization should now facilitate investigation of the role of these primitive quiescent leukemic cells in disease progression and response to therapy (see below).

A third approach to evaluating normal and leukemic stem cell dynamics in CML patients has involved a comparison of telomere length measurements performed on their granulocytes (which are typically >95% leukemic) and on those from age-matched normal individuals. Telomere length in normal somatic cells decreases with each cell division and changes in telomere length can thus serve as an indicator of their mitotic history, with shortening to a critical level acting as a checkpoint to signal senescence.⁵⁴ The main factor that elongates

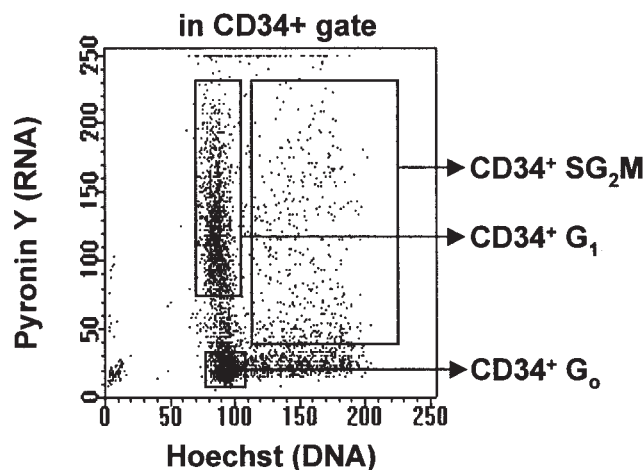


Figure 1 FACS strategy for the isolation of cells in different phases of the cell cycle. Lineage depleted, CD34 -enriched cells were labeled with propidium iodide (PI), CD34-FITC , Hoechst 33342 and Pyronin Y. An initial gate was set on viable PI^- cells and a second on the CD34^+ fraction. Thereafter, cells were identified and sorted in $G_0/G_1/S/G_2/M$ of the cell cycle according to their level of Hoechst and Pyronin Y staining. (Reproduced with permission by the American Society of Hematology, Washington DC, from Ref. 48.)

SPOTLIGHT

telomeres is the enzyme telomerase (TERT), whose expression is normally restricted to primitive cell types, particularly cells of the germ line.^{55,56} However, low levels of TERT have also been documented in later types of primitive hematopoietic cells⁵⁷ and in certain lymphocytes.⁵⁸ In tissue culture, when human cell lines are immortalized, telomerase expression is frequently seen to be upregulated and high levels of telomerase are characteristic of most human cancers.⁵⁷ Conversely, forced overexpression of telomerase can extend the number of cell divisions possible and contribute to full malignant transformation, suggesting a causal relationship between telomere shortening, senescence and the circumvention of this response by malignant cells.^{59–62} Interestingly, telomere length comparisons between normal and CML cells have only recently become informative. This is largely due to the very wide variation in telomere length seen between normal individuals, even when those of the same age are compared,^{63,64} compounded by the imprecise methodology available until recently for making telomere length measurements. Techniques that utilize flow fluorescence *in situ* hybridization (flow-FISH) or quantitative FISH⁶⁵ have provided a major advance in this regard as has been their application to assessments of either very large numbers of individuals or, even better, of non-clonal (eg T-lineage) cells from the same patient.

Telomere length measurements performed on granulocytes from large numbers of normal individuals of different ages now suggest that, in the adult, all of the stem cells from which they originate will divide, on average, only once every 1–2 years.⁶³ Several studies have now reported that the leukemic cells (which are predominantly mature myeloid cells in chronic phase CML patients) have shorter telomeres than normal granulocytes or bone marrow cells.^{66–68} In the study that we undertook, telomere length measurements were obtained on peripheral blood leukocyte cells from >150 CML samples and from >300 normal individuals. In some of the CML samples, purified endogenous normal (T) cells were also analyzed. These normal vs CML comparisons revealed an average overall difference in telomere length between the normal and leukemic cells of ~1 kb, indicative of a somewhat faster turnover of the leukemic stem cells (Figure 3). Assuming no change in other kinetic parameters, this difference can be estimated to represent ~10 more self-renewal divisions than

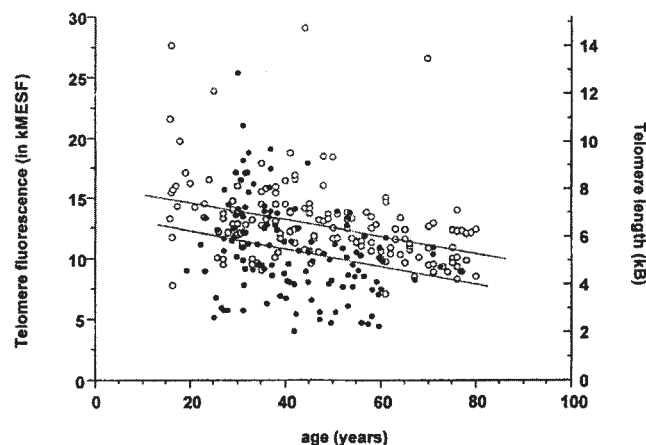


Figure 3 Telomere length in blood or marrow cells from CML patients (solid symbols) is shorter than in blood cells from age-matched normal individuals (open symbols). Telomere length was determined by flow-FISH. (Reproduced with permission by the American Society of hematology, Washington DC, from Ref. 67.)

would be executed by normal stem cells between the time the clone was initiated and the time of diagnosis (ie ~1–2 extra divisions per year over an estimated average 6 to 7 year latent period).

An even greater loss of telomere length is seen when blast phase CML cells are analyzed by quantitative methods.⁶⁹ In fact, our finding of a significant correlation between reduced telomere length and the proportion of patients entering blast phase disease within 2 years of the time when telomere length measurements were performed,⁶⁷ provides strong evidence that telomere shortening may be a rate-limiting step contributing to the onset of blast crisis. More recently, a significant correlation between the degree of telomere shortening at diagnosis and the prognostic index, as measured by the Hasford score, has been demonstrated (MW Drummond, T Holyoake, unpublished findings). Furthermore, in these latter studies, the degree of telomere shortening could be correlated with disease progression from early chronic phase, to accelerated phase and finally into blast crisis (Figure 4).

Taken together, these data not only support previous evidence that CML stem cells cycle *in vivo* more actively than their Ph⁻ counterparts, they strongly indicate that telomere length may serve as a useful surrogate marker of disease progression. They also support the concept that reduction of telomere length to a critical point may drive the selection of a clone of blast phase cells in which expression of telomerase has been upregulated. It is thus interesting to speculate that strategies to inhibit the telomerase activity in these cells might have therapeutic potential by inducing their senescence.⁷⁰

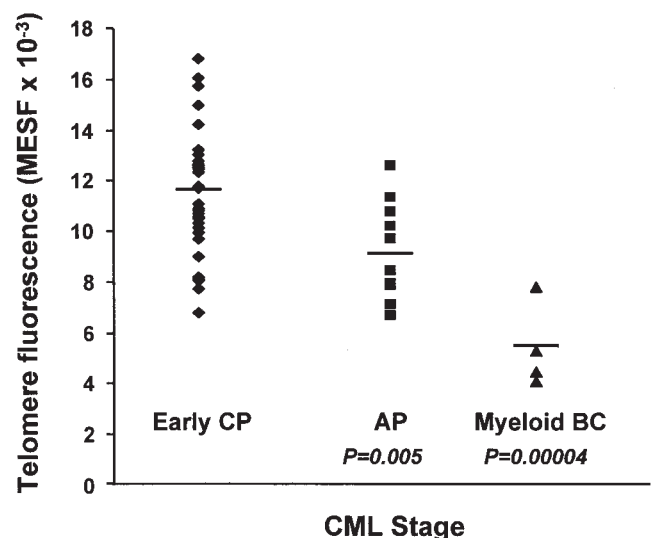


Figure 4 Loss of telomere fluorescence in CML (as measured by flow-FISH), in the myeloid compartment of peripheral blood. Fluorescence intensity is expressed as molecules of equivalent soluble fluorochrome units (MESF), with 10^{-3} MESF approximating to 0.5 kb of telomeric repeat sequence. Granulocytes (or blasts in blast crisis CML patients) were gated using their known forward and side light scattering characteristics. Total peripheral blood leukocytes were used when the granulocyte population was not clearly defined. The bars represent mean values. Significant loss of telomere signal is seen in both accelerated phase disease (AP, $n=11$) and myeloid blast crisis (BC, $n=4$) as compared to chronic phase (CP, $n=32$) within 1 year of diagnosis (P values as shown). Median ages for all three series were similar (56, 61 and 58 years respectively), thereby excluding age-related telomere shortening as a major cause for the observed differences (unpublished observations). The range of telomere signals at all stages is wide, a phenomenon also observed for normal individuals of the same age (see Figure 3).

In vitro response to STI571

STI571 is a small molecule inhibitor, with great specificity for the p210^{BCR-ABL} tyrosine kinase. *In vitro*, this agent selectively suppresses the growth of primary CML CFCs and of BCR-ABL⁺ cell lines. In phase I–II clinical trials, initial results with single agent STI571 therapy have appeared far superior to those achievable using any other non-transplantation treatment modality, to date, leading to the reappearance of Ph⁻/BCR-ABL⁻ hematopoiesis in >50% of chronic phase patients within 1 year of continuous treatment.⁷¹ These clinical results, although preliminary, have raised much interest in the possibility that STI571 will prove to be an important advance in the long-term treatment of patients with CML. However, given previous knowledge of the slow but eventual ability of a small Ph⁺/BCR-ABL⁺ stem cell compartment to re-initiate disease even after several years of apparent normal hematopoiesis, it could be particularly important to understand the relative sensitivity of the quiescent CML progenitor cells to STI571.

As a first step towards this goal, we have recently begun to compare the ability of STI571 to initiate an apoptotic response in cycling vs quiescent CML cells. Initial results show that a 6-day exposure in serum-free culture to 10 μ M STI571 is, as expected,^{10,72} sufficient to kill >90% of the CD34⁺lin⁻ CML population, the majority of which are proliferating cells. In contrast, when the small quiescent Ph⁺/BCR-ABL⁺ subset was selectively isolated (by their resistance to cell cycle entry for 4 days *in vitro*), these cells were found to be fully resistant to the same STI571 concentration (Figure 5).⁷³ We therefore anticipate that STI571, as a therapeutic single agent, will be unlikely to achieve long-term cures unless administered over very long periods of time, and that the use of additional agents that target the primitive quiescent leukemic cells more effectively will prove necessary to fully eradicate the disease. The recent reports of WT-1 as a relevant antigen in CML⁷⁴ and the proposed role of WT-1 to induce cellular quiescence in primitive precursors⁷⁵ suggest that immunotherapy against this antigen may be an interesting candidate.⁷⁶

Another mechanism proposed for the resistance of some CML cells to STI571 *in vivo* is through binding to plasma alpha-1-acid glycoprotein (AGP). However, we have also now

shown that, although plasma levels of this glycoprotein are significantly higher in CML patients than in normal controls, purified AGP from CML patients' plasma does not block the effect of STI571 on either Ph⁺ cell lines or primary CD34⁺lin⁻ cells from chronic phase patients.⁷⁷ These results cast doubt on the likelihood that AGP will prove a useful target for enhancing the anti-leukemic activity of STI571 *in vivo*.

BCR-ABL-induced growth factor independence

As discussed previously, the increased turnover of primitive CML cells has been recognized for many years. Nevertheless, consensus has not been reached as to the precise molecular mechanism responsible. In part, this may be due to the relative paucity of data from studies of primitive CML cells from patients with chronic phase disease in spite of an abundance of information from studies of BCR-ABL-transduced cell lines or various Ph⁺/BCR-ABL⁺ cell lines established from samples obtained from CML patients in blast crisis, or from mature CML cells that are actually incapable of further division. Interestingly, one of the earliest findings from studies of BCR-ABL-transduced hematopoietic cell lines was the activation of an autocrine mechanism which could confer partial or complete growth factor autonomy in cells that were previously factor-dependent.^{78,79} However, until recently, investigators have struggled to extrapolate these findings to primary CML cells, at least for patients in the chronic phase of the disease. Moreover, leukemic CFCs were well known to exhibit a normal dose response to exogenous growth factor stimulation when assayed under standard *in vitro* conditions. Many groups have therefore concluded that the growth requirements of CML progenitors are unaltered and a role for autocrine mechanisms has been largely and generally discounted as irrelevant to the pathogenesis of naturally arising CML. For the same reasons, the acquired factor independence of many of the BCR-ABL⁺ cell lines available has focused attention on the anti-apoptotic activity of the BCR-ABL oncoprotein. Although the results of experiments to examine this potential role of p210^{BCR-ABL} have been conflicting, at least five studies comparing the effect of growth factor withdrawal on normal vs leukemic CFC survival have shown the response of the leukemic cells to be superior.^{80–84} In the three studies from our own group, the number of CFCs was found not only to be maintained upon factor withdrawal but also to have increased several-fold within 3 weeks of culture in serum-free medium. Importantly, this growth factor-independent proliferation was shown to be a property exclusive to the primitive Ph⁺/BCR-ABL⁺ cells and is lost when they reach the stage where they lose expression of CD34. Conversely, this capacity for autonomous growth is most pronounced in the most primitive subsets of Ph⁺/BCR-ABL⁺ CD34⁺ cells, as identified by their lack of expression of CD45RA, CD71⁻, or CD38⁻ (Figure 6). The unexpected but convincing picture of primitive CML cell factor independence that emerged from these experiments prompted us to undertake a renewed search for evidence of an autocrine mechanism. The combined results of subsequent RT-PCR analyses, high sensitivity ELISAs, intracellular flow cytometry and bioactivity assays provided definitive evidence of the consistently activated production of IL-3 and G-CSF (but not several other growth factors) in CD34⁺ cells isolated directly from chronic phase patients. Moreover, the activation of IL-3 and G-CSF in these cells was shown to mirror precisely their differentiation stage-specific growth factor independence (Figure 7). Antibody neutralization experiments confirmed the dependence

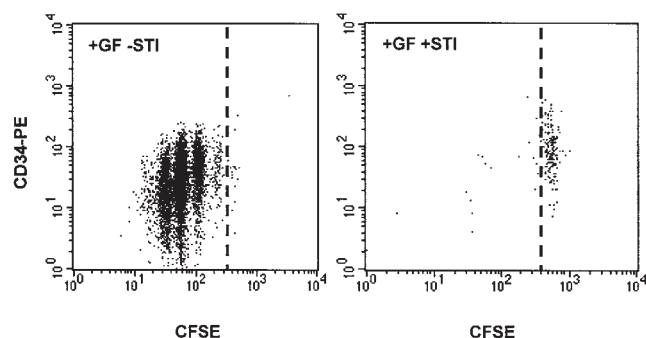


Figure 5 Demonstration that quiescent CD34⁺ CML cells are insensitive to STI571. Using the FACS strategy outlined in the legend to Figure 2, purified CD34⁺, CFSE⁺ CML cells were cultured in the presence of the same five growth factor combination as described in Figure 2 and in the presence (right dot plot) or absence (left dot plot) of 10 μ M STI571 (provided by Novartis, Basel, Switzerland). As shown, although all the dividing cells (identified to the left hand side of the dotted line) were killed by STI571, the quiescent cells (right hand side of dotted line) remained viable (PI⁻) and retained a high level of CD34 expression. (Reproduced with permission by the American Society of Hematology, Washington DC, from Ref. 73.)

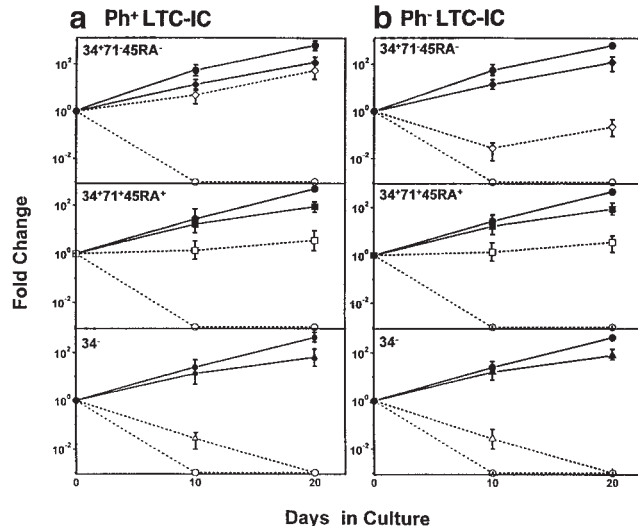


Figure 6 Factor-independent growth of CML cells in culture decreases with their differentiated state. Highly purified $CD34^+CD71^-CD45RA^-$ (diamonds), $CD34^+CD71^+CD45RA^+$ (squares), and $CD34^-$ (triangle) CML cells ($n = 5$) or normal marrow cells (circles, $n = 3$) were cultured at 10^5 cells per ml in SFM with (solid symbols) or without (open symbols) the same five growth factors described in Figure 2. Viable cell numbers were determined by hemacytometer counts of trypan blue-excluding cells. Results are expressed as the mean fold-change compared with input values \pm s.e.m. (a) CML patients with predominantly Ph^+ LTC-IC ($n = 3$). (b) CML patients with predominantly Ph^- LTC-IC ($n = 2$). (Reproduced with permission by the National Academy of Science of the United States of America, Washington, DC, from Ref. 83.)

of the primitive leukemic cell proliferation on this autocrine loop although intracellular binding of these two growth factors to receptors on the endoplasmic reticulum likely contributes to the general inability of exogenous anti-IL-3 and anti-G-CSF antibodies to completely abrogate the autonomous growth behavior.

The constitutive production of IL-3 and G-CSF by primitive CML cells fits remarkably with previous Ph^+ cell line work,

with the clinical picture of CML and with the known stage-specific responses of normal cells to high levels of these cytokines. IL-3, as a single factor, has been shown to support the survival of primitive normal bone marrow cells and to activate proliferation of quiescent LTC-ICs and CFCs.⁸⁵ However, in the absence of adequate co-stimulation by FL and SF, excess IL-3 delivers a potent differentiation-inducing stimulus to $CD34^+CD38^-$ cells including the LTC-ICs. Transplantation of murine bone marrow cells, transgenically engineered or retrovirally transduced to produce IL-3 induces a myeloproliferative syndrome that shares many similarities with human CML.⁸⁶⁻⁸⁸ Likewise, administration of pharmacological doses of IL-3 enhances the production of basophils and mast cells,^{89,90} and G-CSF strongly promotes terminal granulopoiesis *in vivo*,^{90,91} both of which are features of CML. Enhanced proliferation of the Ph^+ clone due to autocrine growth factor production has thus emerged as a potentially important mechanism underlying the 'apparent' growth factor independence of primitive CML cells and the accelerated expansion of the clone as cells transit the intermediate stages of differentiation.

These key observations have also allowed previous, apparently discrepant, findings regarding the factor dependence of CML CFCs to be explained. CFC assays rely on the ability of $CD34^+$ cells to initiate cell division as single cells and then produce colonies of terminally differentiating $CD34^-$ cells by executing their final amplifying divisions. Thus, initiation of colony formation by CML progenitors plated in the absence of growth factors would have to occur in the absence of any paracrine stimulation and the majority of the CML cells subsequently generated in each colony would be relatively late types of factor-dependent cells. Thus, in standard semi-solid assays of purified progenitors, only a few abortive colony starts would be expected in the absence of added growth factors, as was originally observed by Strife *et al*.⁸⁰

Signaling changes induced by BCR-ABL

BCR-ABL-expressing cells have also been found to show many similarities to those induced by IL-3 stimulation⁹² or forced

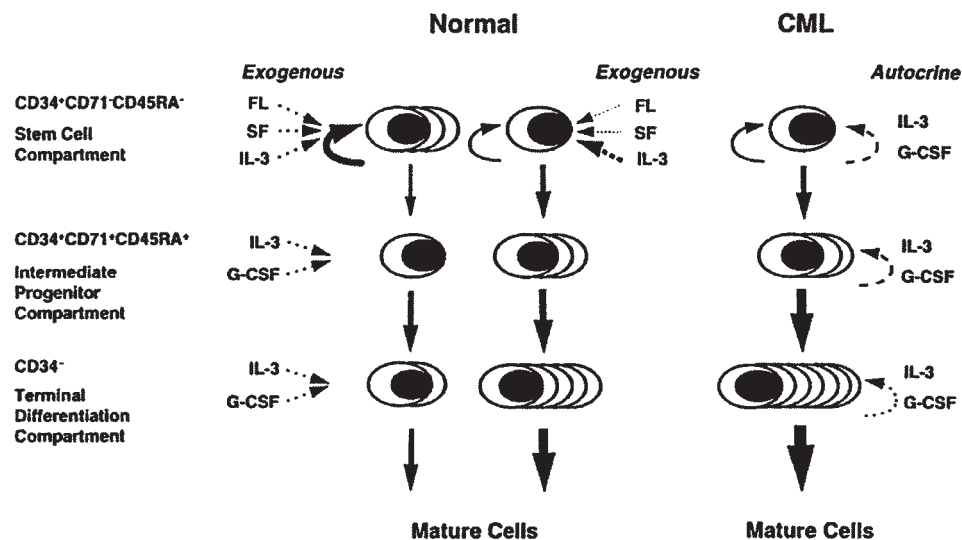


Figure 7 Schematic representation of the differential effects of different levels of IL-3 and G-CSF stimulation on primitive normal cells by comparison to the observed alterations in CML patients where an autocrine IL-3/G-CSF loop in the earliest leukemic compartments has been demonstrated. (Reproduced with permission by the National Academy of Science of the United States of America, Washington, DC, from Ref. 83.)

IL-3 overexpression.^{86–88} In particular, the increased phosphorylation of STAT5, previously thought to be an immediate function of the BCR-ABL oncoprotein, has now been shown in primary CML CD34⁺ cells to occur largely as a consequence of BCR-ABL-induced activation of IL-3 auto-stimulation, since STAT5 activation in these cells could be blocked by neutralization of IL-3 activity.⁸³ We have also shown that the primitive, quiescent Ph⁺/BCR-ABL⁺ cells, that are present in most CML patients, are silent with respect to detectable levels of IL-3 and G-CSF mRNA, although BCR-ABL transcripts are present and both IL-3 and G-CSF transcripts reappear when the cells spontaneously re-enter the cell cycle *in vitro*.²⁸ The fact that the primitive, quiescent CML cells remain viable in the absence of exogenous IL-3 or G-CSF makes two strong inferences. The first is that BCR-ABL alone may provide a sufficient anti-apoptotic signal to keep these cells alive under conditions of factor deprivation that their normal counterparts may not withstand. The second is that the autocrine IL-3 (and G-CSF) mechanism may be required for their factor-independent proliferation, as indicated by our antibody neutralization studies.

Subsequent studies have demonstrated that the production of IL-3 is consistently activated in murine bone marrow cells transduced with a BCR-ABL retrovirus.^{93,94} We have confirmed these findings and have further shown that this occurs in very primitive cells.⁹⁵ Furthermore, activation of expression of both IL-3 and G-CSF in this model occurs a few days after transduction but following the onset of BCR-ABL expression (unpublished findings), again consistent with a causal linkage of factor-independent growth and the autocrine IL-3/G-CSF mechanism secondary to BCR-ABL expression. At present, very little else is known about the mechanism by which the BCR-ABL oncoprotein elicits this autocrine activity, except that its SH2 domain appears to be important for this to occur *in vitro*.⁹⁶

Recently, it has been shown that BCR-ABL-transduced murine myeloid cells that are unable to produce IL-3 can still display leukemic activity.⁹⁷ Although initially this may seem to contravene the conclusions reached above, in fact this is not surprising at all. There are a number of signaling pathways which BCR-ABL is known to activate that may be candidates for promoting primitive CML cell viability independently (as well as downstream) of IL-3 or G-CSF, eg the ras, PI3K/Akt and Jak/Stat pathways. In addition, we have found BCR-ABL-activated GM-CSF as well as G-CSF to exert potent paracrine effects in BCR-ABL-transduced normal and IL-3^{-/-} mouse bone marrow cells.⁹⁵ Furthermore, in the murine models employed, the levels of BCR-ABL transcription and translation are likely to be greater than in primary chronic phase CML cells due to the use in transduction models of a powerful promoter in the retroviral LTR. In addition, in murine models, leukemogenic activity is measured by the generation of a rapidly fatal, polyclonal, 'CML-like' syndrome within a few weeks after the transplantation of millions of transduced cells into myeloablated recipients. This contrasts markedly with the long latent period of many years for the clonal disorder that produces CML in people. The occurrence, in this model, of acute B cell leukemias in addition to 'CML-like' disease, may also suggest a number of different target cells, some of which may not be as early in the stem cell hierarchy as the pluripotent cell mutated in primary disease. Our finding of a discrepancy between the presence of BCR-ABL transcripts and IL-3/G-CSF transcripts in the quiescent stem cell fraction²⁸ suggests that IL-3 and G-CSF activation may depend on a threshold level of BCR-ABL transcripts within the cells. For example, in the

presence of low levels of BCR-ABL transcripts (and p210^{bcr-abl}), IL-3 and G-CSF are not activated and the cells remain viable but do not divide. As BCR-ABL transcripts rise above a certain threshold, transcription of the IL-3 and G-CSF genes is activated and this is accompanied by the entry of these cells into cycle. Under conditions where BCR-ABL transcripts are more elevated (eg in retrovirally transduced or actively proliferating CD34⁺ CML cells), transcription of both IL-3 and G-CSF (and even GM-CSF) is never shut off and a state of more sustained proliferation may be achieved. Thus the autocrine IL-3/G-CSF mechanism that is characteristic of primitive chronic phase CML cells may be key to their slow but inexorable expansion that results in a disease with a course quite distinct from the human Ph⁺ acute leukemias. Future investigation of both human and murine models of CML using various transduction and transplant strategies should allow further examination of these possibilities as well as providing more refined models for evaluating new therapeutics.

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