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The elusive CML stem cell: does it matter and how do we eliminate it?

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Abstract

CML is a clonal multistep myeloproliferative disease originating from and ultimately sustained by a rare population of BCR-ABL⁺ cells with multilineage stem cell properties. Imatinib, the most successful of molecular targeted therapies, has revolutionized treatment of patients with CML. Despite this achievement, CML is often not curable, largely due to the innate insensitivity of CML stem cells, particularly when in a quiescent state. This failure of not only imatinib but also the second-generation tyrosine kinase inhibitors frequently leads to relapse upon drug discontinuation. Thus, any curative therapy must eliminate CML stem cells. A comprehensive understanding of the biological properties of CML stem cells and an elucidation of the molecular mechanisms and signaling pathways enabling these CML stem cells to self-renew, combined with insight into the regulation of apoptosis signaling and the mechanisms governing the interaction of CML stem cells with their bone marrow microenvironment, will facilitate the development of therapies for targeting these cells. In this seminar, we will discuss the biological properties of CML stem cells and potential strategies to eliminate them.

Introduction

CML is a myeloproliferative disorder characterized by the formation of the *BCR-ABL* fusion gene, which codes for a constitutively active tyrosine kinase that is necessary and sufficient for malignant transformation. The advent of imatinib, a *Bcr-Abl* tyrosine kinase inhibitor (TKI), has revolutionized the molecular targeted therapies. This is the most successful example of molecularly targeted therapies changing the natural course of a malignant disease. Imatinib has drastically improved the prognosis of patients with CML and become the standard-of-care for newly diagnosed CML. However, imatinib-based therapy faces three major challenges.

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The first is the development of resistance in approximately 40% of patients due to mutations in the *BCR-ABL* gene.^{1;2} With the development of more potent TKIs, such as nilotinib and dasatinib, most of the imatinib resistance caused by *BCR-ABL* gene mutations (except the T315I mutation) can be overcome,^{3;4} although other mutations often develop later.

The second is the limited response in patients with blast crisis (BC) CML.⁵ This is not surprising, as the evolution of CML from chronic phase (CP) to BC is accompanied by additional chromosomal and molecular changes; these cells may not depend solely on *Bcr-Abl* for survival.⁶ Therefore, targeting multiple pathways may be required for treating advanced CML.⁷

Finally, the third, and the toughest of them all, is the insensitivity of CML stem cells to both imatinib and second-generation TKIs.^{8;9}

CML is a stem cell disease sustained by a rare population of primitive CD34⁺/*BCR-ABL*⁺ progenitor cells with stem cell characteristics. This cell population makes up approximately 0.5% of the total CD34⁺ compartment and has the ability to self-renew, engraft in *NOD/SCID* mice, and initiate leukemia; therefore, these cells are candidates for CML stem cells.^{10;11} One characteristic of CML stem cells is that they are quiescent. Therapeutic agents currently used to treat CML, such as imatinib and other TKIs, and conventional chemotherapeutic drugs, work by inhibiting cell proliferation and inducing subsequent apoptosis. Thus, they are not effective against nonproliferating progenitor/stem cells.^{9;12;13} This ineffectiveness is supported by the clinical observation that complete cures are rare and relapse is nearly inevitable upon withdrawal of imatinib, even in patients whose levels of *BCR-ABL* transcripts are undetectable during imatinib therapy. These quiescent CML stem cells persist even when complete clinical and cytogenetic responses are achieved with imatinib¹¹. More than 90% of CML patients who are treated with standard-dose (400 mg) and more than 60% who are treated with 800-mg doses of imatinib daily retain residual *BCR-ABL* transcripts detectable by RT-PCR. These *BCR-ABL*⁺ cells not only can cause relapse but also provide a sanctuary for *BCR-ABL* mutations and thus might be behind the emergence of drug-resistant clones. Clearly, the cure for CML depends on the elimination of quiescent CD34⁺ CML stem cells. The curative approach that eliminates stem cells—stem cell transplantation—can be performed in only a few patients, and is associated with a high risk of morbidity and mortality. Therefore, to reach an ultimate cure, development of new and more effective drugs and treatment strategies involving the elimination of CML stem cells is needed.

Biology of CML Stem Cells

In order to identify relevant selective targets to eliminate CML stem cells, a deep understanding the biology of normal hematopoietic stem cells (HSCs) as well as CML stem cells and their difference is needed. Stem cell research is a fascinating field but the rarity of the stem cell populations, their genetic instability, and the lack of easy assays for identifying them make this a daunting task. With great effort, several of the critical biological properties of CML stem cells have been elucidated over the past decade, and many similarities have been found between CML stem cells and normal HSCs.

Definition and properties of CML stem cells

As with HSCs, stemness is the fundamental property of CML stem cells (Figure 1). CML stem cells originate from HSCs by the acquisition of the *BCR-ABL* mutation, which can self-renew and maintains the indolent CP disease. At this stage, CML stem cells and “differentiated” CML cells are functionally, morphologically, and phenotypically almost indistinguishable from their normal counterparts¹⁴, although the number of differentiated cells produced from *BCR-ABL*⁺ stem cells is greatly altered. BC CML evolves from CP CML with additional genetic

and epigenetic changes that are not fully understood. In advanced CML, committed granulocyte-macrophage progenitors (GMP) have gained an abnormal self-renewal capacity by the activation of β -catenin (Figure 1)¹⁵. The coexistence of these mutated progenitors, which acquire stem cell characteristics, is responsible for the rapid expansion of the advanced disease.

HSCs are functionally defined as long-term culture-initiating cells capable to engraft immunocompromised mice. Once transferred into *NOD/SCID* mice, HSCs are able to recapitulate the organ system and generate functional blood cells in recipient organism. In parallel, CML stem cells, once transferred to sublethally irradiated *NOD/SCID* mice, are able to engraft and initiate leukemia. CML stem cells are also long-term culture-initiating cells and are kinetically quiescent. The quiescent state is temporary and reversible. Under specific circumstances, the quiescent CML stem cells can give rise leukemic cells and therefore serve as a reservoir for the activated stem cells that show slightly reduced self-renewal activity. This quiescent compartment represents the most resistant and hardest to eliminate subpopulation. Like normal HSCs, CML stem cells reside in the bone marrow niche *in vivo*, which provides a shelter to protect them from various insults-including therapies.

Biomarkers of CML stem cells

Phenotypic biomarkers—CML stem cells in the CP are phenotypically similar to normal HSCs. They both are characterized by flow cytometry as having low forward- and side-light scatters, representing a small size and agranular cytoplasm. Although not precisely defined phenotypically, CML stem cells are believed to share a cell surface phenotype with normal HSCs, including the expression of CD34, Thy1/CD90, and CD133, and the lack of expression of CD38, CD45RA, CD71, and several lineage markers of mature blood cells.

CD123 (the α chain of the IL-3 receptor) and CD33 have been described as stem cell markers for myeloid leukemias. They were found to coexpress with CD34⁺CD38[−] CML stem cells in most samples analyzed¹⁶. A recent study identified a novel Lin[−]BCR-ABL⁺CD34[−] stem cell population from CML patients at diagnosis¹⁷. These Lin[−]BCR-ABL⁺CD34[−] cells are quiescent, able to engraft immunodeficient mice, and insensitive to imatinib. This study not only supports the emerging theory of the heterogeneity of leukemia stem cell populations, but also suggests that it is not sufficient to characterize leukemia stem cells solely by relying on phenotypic markers.

Molecular biomarkers

BCR-ABL: Due to the rarity of CML stem cells, *BCR-ABL* expression in lin[−]CD34⁺CD38[−] CML cells has been evaluated primarily by RT-PCR, which has shown elevated levels of the *BCR-ABL* mRNA compared to more mature cells. Using p-CrKL as a surrogate marker, Copland *et al.* were able to determine the level of *Bcr-Abl* signaling in CD34⁺CD38[−] cells taken from CML patients by flow cytometry and found it to be higher than that of the total CD34⁺ population⁹. Similarly, our group has found *Bcr-Abl* tyrosine kinase activity to be higher in quiescent primitive CD34⁺ CML progenitor cells than in proliferating cells by measuring p-CrKL levels using laser scanning cytometry (unpublished results).

Signaling pathways for self-renewal: Although the ability to self-renew is believed to be vital to the maintenance of CML stem cells, the underlying mechanisms and signaling pathways involved remain poorly defined. Recent studies from Zhao *et al.* have provided strong evidence that Wnt/ β -catenin signaling is indispensable for the maintenance of normal and CML stem cells¹⁸ and this has been further supported by the studies showing that leukemic GMP cells from BC-phase patients are capable of serially transplanting leukemia into immunocompromised mice (via an improper activation of the β -catenin pathway) with an efficiency even higher than that seen in leukemic HSCs *in vivo*¹⁹. Hedgehog (Hh) signaling

has been shown to be essential for the expansion and maintenance of CML stem cells;^{20,21} and a critical role of the promyelocytic leukemia protein (PML) in HSC maintenance has been demonstrated.²²

Apoptosis regulators: Apoptosis, programmed cell death governed by the death receptor mediated extrinsic pathway and the mitochondrial mediated intrinsic pathway, plays an important role in many facets of normal physiology in animal species. The proteins of Bcl-2 family are the key regulators of the intrinsic pathway, while inhibitors of apoptosis family of proteins (IAPs) inhibit caspases and suppress both the intrinsic and extrinsic pathways (Figure 2). Impaired apoptosis, characterized by the imbalanced expression of anti- and proapoptotic proteins, is a hallmark of many malignant cells. Many antiapoptotic proteins are known to be highly expressed in CML blasts and contribute to chemoresistance.

To assess the expression levels of antiapoptotic proteins in quiescent primitive CD34⁺ CML progenitor cells, our group labeled mononuclear cells from BC CML patients with 5-(and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE), a fluorescent cell permeable dye that halves with each cell division as described previously.²³ After coculturing with stromal cells as a source of growth factors for 4 to 6 days, CML cells were stained with CD34 antibody and then FACS-sorted into proliferating (CD34⁺CFSE^{dim}) and quiescent (CD34⁺CFSE^{bright}) CML progenitor cells. mRNA levels of antiapoptotic proteins were determined by real-time RT-PCR. We found that quiescent CD34⁺ cells expressed as much Bcl-2, Bcl-xL, Mcl-1, and XIAP as their proliferating counterparts (n=11, unpublished results).

ABC transporters: HSC is characterized by the surface expression of ABC transporters, in particular ABCB1 (p-glycoprotein) and ABCG2 (BCR-P), which efflux specific drugs. Both of these transporters were found to be overexpressed in primitive CML cells obtained from CP CML patients compared to their normal counterparts.²⁴ Interestingly, reduced expression of OCT-1, a transporter responsible for the uptake of specific drugs, including imatinib, was found in primitive CML cells obtained from CP CML patients compared to their normal counterparts. The combination of increased expression of ABCB1 and ABCG2 and decreased expression of OCT-1 may contribute to the poor response to therapy of CML progenitors.

Others: Recent studies have shown that the tumor suppressor PTEN is downregulated by *Bcr-Abl* in CML stem cells and that deletion of PTEN accelerates-whereas overexpression of PTEN delays-the development of CML in a mouse model, demonstrating the critical role of PTEN in regulating stem cells in leukemia.²⁵ A study by Naka and colleagues demonstrated an essential role of TGF- β -FOXO in maintaining CML leukemia-initiating cells.²⁶ The arachidonate 5-lipoxygenase gene was recently identified as a regulator inducible by *Bcr-Abl* and unresponsive to imatinib that is crucial for CML stem cell function.²⁷ Systematic gene profiling of CD34⁺-primitive CML cells demonstrated the activation of multiple signaling pathways, along with a reduction of genes for DNA repair, abnormal adhesion, and homing, and activation of the proteasome-ubiquitin protein pathways.

The altered phenotypic markers and deregulated molecular markers that are essential for biological functions, self-renewal, and survival of CML stem cells are the bases for finding targets of choice to selectively eliminate CML stem cells.

Elimination of CML Stem Cells

The ability to therapeutically attack CML stem cells hinges upon identifying unique targets. The only marker indisputably distinguishing CML stem cells from normal HSCs is the presence of the *BCR-ABL* fusion gene, which is the foundation for imatinib-based therapy. Unfortunately, it has been established both *in vivo* and *in vitro* that CML stem cells are

insensitive to this therapy. We and others have demonstrated that the *Bcr-Abl* tyrosine kinase signaling pathway is constitutively activated, as measured by p-CrKL in quiescent CD34⁺ CML progenitor cells. Copland *et al.*⁹ showed that dasatinib does inhibit *Bcr-Abl* kinase activity, as reflected by decreased p-CrKL levels. However, it does not decrease the number of nondividing cells among primitive CD34⁺38[−] CML cells, suggesting that the quiescent self-renewable and transplantable CML stem cells do not depend on *Bcr-Abl* for survival, which explains the historical failure of TKIs and other cell cycle-dependent therapies. Clearly, alternative strategies are urgently needed to eliminate quiescent primitive CD34⁺ CML progenitor cells. Cell surface markers, signaling pathways central to self-renewal, and apoptosis regulators are among several promising targets that have emerged for the elimination of CML stem cells.

Elimination of CML stem cells by antibody-targeting cell surface antigens or ligand-based delivery of immunotoxins

The expressions of cell surface antigens, such as CD123 and CD33, in CD34⁺38[−] CML cells represent attractive targets for antibody-based curative treatment approaches. Recombinant IL-3-diphtheria toxin conjugate (DT-IL3) has been shown to be active against AML blasts and AML stem cells both *in vitro* and in animal models, while exerting minimal effects on normal bone marrow cells^{28;29}. A multicenter phase I/II trial of the drug in advanced AML and MDS is ongoing. A preclinical study of DT-IL3 therapy for CML showed promising results in that the agent induced apoptosis in CD34⁺38[−]123⁺ cells obtained from CML patients, sensitized imatinib-induced cell death in CML cell lines, and prolonged survival of CML-burdened mice.³⁰ Gemtuzumab ozogamicin (GO, Mylotarg) is a humanized monoclonal antibody, linked to a cytotoxic calicheamicin derivative, targeting the CD33 antigen that is expressed on the surface of the majority of AML blasts and leukemia stem cells but not on normal stem cells. GO alone or in combination with other therapeutic agents is a treatment option for CD33⁺ AML³¹. However, this treatment option has not been explored in CML, partly because TKI-based therapy is highly effective for patients with this disease. The recognition that CML stem cell resistance to TKIs leads to relapse and the discovery of CD33 expression on these cells suggest merit for the evaluation of GO against CML stem cells.

Since different neoplastic clones and different patients present heterogeneous profiles of antigen expression, combinations of various antibody-based targeted drugs and combinations of these drugs with conventional therapy are likely required to treat individual patients.

Elimination of CML stem cells by targeting self-renewal signaling pathways

The ability to self-renew, a key characteristic of stem cells, is absolutely critical for maintaining and expanding CML stem cells. Although pathways that regulate self-renewal are tightly controlled in normal HSC, they are often constitutively activated or aberrantly regulated in CML stem cells. This over-active self-renewal might make them more susceptible than normal HSCs to agents that inhibit self-renewal pathways and thus open a therapeutic window. Clearly, selectively targeting the self-renewal machinery in CML stem cells constitutes one of the top choices for eliminating them. Regrettably, the mechanisms governing the regulation of self-renewal in normal HSCs and their deregulation in CML stem cells are mostly unknown. However, as mentioned earlier, evidence has accumulated suggesting that Wnt/β-catenin and Hh signaling drive the quality of stemness. Other mechanisms may also play vital roles.

Using a conditional β-catenin knockout model, Zhao and colleagues reported that loss of β-catenin impairs the self-renewal of normal and CML cells *in vivo*, implying that Wnt/β-catenin is a major player in the maintenance of normal and CML stem cells¹⁸. This finding, together with a previous report demonstrating that the acquisition of self-renewal by β-catenin activation in myeloid progenitors is crucial in the evolution of CP CML to BC CML¹⁵, implies that the

Wnt/ β -catenin signaling pathway is a potential therapeutic target for eradication of this and possibly other stem cell diseases. However, caution needs to be taken, since the dependence on this pathway is shared by normal and CML stem cells. A therapeutic window most likely exists for the use of Wnt/ β -catenin pathway inhibitors in patients with aberrantly activated Wnt/ β -catenin signaling, particularly in BC CML patients whose myeloid progenitor cells gain self-renewal capacity via the activation of β -catenin.

The Hh signaling pathway is a developmentally conserved regulator of stem cell function and it has been shown to be active in several hematological malignancies. Smoothened (SMO) is a transmembrane protein that relays Hh signaling. Using mice lacking SMO or those treated with SMO inhibitor cyclopamine, Zhao and colleagues have shown a reduction in CML stem cell population and prolonged survival in irradiated mice transplanted with *BCR-ABL*-expressing HSCs. Conversely, they found that transgenic expression of an activated form of SMO increased the number of CML stem cells and accelerated cancer progression²¹, implying that perturbation of Hh signaling blocks CML progression by disturbing CML stem cells. However, normal HSC renewal was also impaired in this study, hindering the clinical application of this approach. Interestingly, three other studies have provided evidence that Hh signaling is not required for adult hematopoietic homeostasis^{20;32;33}. Two groups used conditional mice in which *SMO* was deleted after induction in HSCs and found that loss of SMO in adult mice had no significant effect on hematopoiesis, even under stressed conditions and after prolonged treatment with Hh antagonists. They speculated that the systemic administration of Hh antagonists is unlikely to cause significant hematological toxicity and that Hh signaling is a promising target for CML stem cells^{32;33}. A third group had similar results and also showed that Hh signaling is aberrantly activated in CML stem cells through upregulation of SMO²⁰. Cyclopamine can block tumor progression in a variety of mouse models, and its effect on CML stem cells is evident. Examination of SMO inhibitor LDE225 showed that it targets CML stem cells and, when combined with nilotinib, LDE225 eliminates CML stem and progenitor cells³⁴.

A role of tumor suppressor PML in stem cell biology has only recently been proposed. A study by Pandolfi and colleagues illustrated that PML is highly expressed in CD34⁺ CML cells and that patients with higher PML expression displayed lower complete molecular and cytogenetic responses compared with patients with low PML expression²². Furthermore, they demonstrated that *PML*^{-/-} leukemia initiation cells showed remarkably decreased colony numbers in long-term culture. In a series of bone marrow transplantation experiments, *PML*^{-/-} CML stem cells gradually lost the ability to generate CML-like disease in recipient mice. They further demonstrated that inhibition of PML by As₂O₃ greatly reduced the number of quiescent CML stem cells *in vitro* and *in vivo*. Significantly, when As₂O₃ and Ara-C were combined, the antileukemic effect was dramatically improved, showing no CML-like disease in recipient mice after long-term follow-up. Loss of PML had less effect on normal HSC functions. Since As₂O₃ is in clinical use for APL with a known safety profile, the combination of As₂O₃ with clinically effective TKIs aimed at eliminating CML stem cells may be worthwhile.

Elimination of CML stem cells by apoptosis-inducing therapies

Apoptosis deregulation likely plays an important role in protecting CML stem cells from death. Indeed, cells expressing the CML-inductive *BCR-ABL* gene were found to have high levels of antiapoptotic Bcl-X_L and Mcl-1 and low levels of proapoptotic Bim. Inhibition of *Bcr-Abl* tyrosine kinase in these cells decreased the levels of Mcl-1 and increased the levels of Bim^{35;36}. ABT-737, a selective inhibitor of Bcl-2, Bcl-X_L, and Bcl-w, has shown potent antileukemic activity, including effects against AML progenitor/stem cells³⁷. Inhibition of Bcl-2/Bcl-X_L by ABT-737 (Figure 2) was recently shown to augment the killing of imatinib

in CML cells³⁸ and the second-generation *Bcr-Abl* TKI INNO-406, in combination with ABT-737, was reported to greatly enhance apoptosis, even in INNO-resistant cells with *BCR-ABL* point mutations, except in cell with T315I mutation³⁹. Also, the Bcl-2 antisense oligonucleotide Genasense was found to be active against imatinib-resistant *Bcr-Abl*⁺ cells⁴⁰. We have reported that survivin, an IAP protein, is regulated by *Bcr-Abl* tyrosine kinase and is highly expressed in CML, and that targeting survivin overcomes imatinib resistance⁴¹. We also found that XIAP, the most potent caspase inhibitor of the IAP family, is highly expressed in CML cells and that triptolide, an antitumor agent isolated from a Chinese herb, decreases XIAP, Mcl-1 (Figure 2), and *Bcr-Abl* levels and promotes *Bcr-Abl*-independent apoptosis in CML cells⁴². Interestingly, we found that quiescent primitive CD34⁺ CML progenitor cells expressed similar levels of Bcl-2, Bcl-xL, Mcl-1, and XIAP. Collectively, these results suggest that Bcl-2 and IAP proteins are attractive targets for not only killing bulk blasts and proliferating CD34⁺ cells, but also for having the potential to eliminate quiescent primitive CD34⁺ CML progenitors.

In order to investigate the effect of activating the apoptotic machinery on viability of quiescent primitive CD34⁺ CML progenitors and their responses to TKIs, we treated peripheral blood or bone marrow samples obtained from patients with BC CML with imatinib, ABT-737, and both *in vitro*. All of these patients had been treated with and failed imatinib and dasatinib/nilotinib clinically. As expected, imatinib had little effect on the viability of either proliferating or quiescent CD34⁺ cells. ABT-737 alone at nanomolar concentrations was sufficient to induce apoptosis not only in proliferating but also in quiescent primitive CML CD34⁺ progenitor cells. Interestingly, when imatinib and ABT-737 were combined, the effects were more pronounced, with combination index of less than 1, suggesting synergism between the two agents. Importantly, we found that the combination of ABT-737 and imatinib synergistically induced apoptosis not only in proliferating but also in quiescent cell populations⁴³. The mechanism of this synergy is under investigation; a representative result is shown in Figure 3. TKIs have been clinically proven to treat CML, and ABT-263, an orally available derivative of ABT-737, is being tested in clinical trials for hematological malignancies. The combination strategy holds promise for expedited translation into the clinic.

XIAP acts downstream of apoptotic cascades (Figure 2). Mcl-1 is not inhibited by ABT-737 and is therefore a major resistance factor of ABT-737 (Figure 2). Taking advantage of triptolide's ability to decrease both XIAP and Mcl-1, we tested the effectiveness of triptolide in CML and reported recently that triptolide induces cell death independent of cellular responses to imatinib in BC CML cells, including quiescent CD34⁺ primitive progenitor cells⁴². A water-soluble derivative of triptolide is under clinical investigation.

Induction of apoptosis to selectively kill CML stem/progenitor cells was also reported using farnesyltransferase inhibitor BMS-214662. BMS-214662 was found to potentially induce apoptosis of CML stem/progenitor cells in part through Bax activation⁴⁴.

Interferon α therapy

The fact that highly successful imatinib-based therapy fails to eliminate CML stem cells, leading to disease relapse upon discontinuation of treatment, whereas Interferon (IFN)- α , although less effective overall, sometimes cures the disease, supports the idea of revisiting IFN- α therapy. A number of clinical observations over the years have provided evidence that treatment with IFN- α offers long-lasting remissions⁴⁵, which may be attributed to its potential of reducing or eliminating malignant quiescent CML stem cells⁴⁶. Also, studies have shown that combined treatment with imatinib and IFN- α resulted in higher remission rates for CML^{47,48}. The results of the SPIRIT trial were updated at the recent ASH meeting, confirming that the pegylated form of IFN- α 2a in combination with imatinib showed significantly higher rates of molecular response and undetectable molecular residual disease⁴⁹. The requirement

of a longer treatment period for response, the long-lasting effect, and the high molecular remission rate provide strong but indirect evidence that IFN- α may preferentially target the CML stem cell compartment. This is supported by an *in vitro* study demonstrating that IFN- α was more toxic to primitive CML progenitors responsible for the maintenance of long-term cultures.⁵⁰

IFN- α exerts antitumor effects via multiple mechanisms that are not fully understood. In addition to indirectly modulating immune and antiangiogenic responses, IFN- α directly induces the expression of number of genes, including TRAIL, Fas/FasL, caspase-8, XIAP associated factor-1 (XAF-1), all of which promote apoptotic cell death⁵¹. In particular, it was reported that in melanoma cells, the IFN-dependent protein induction of XAF-1 determines the proapoptotic effect of IFN- α ⁵². XAF-1 is known to antagonize XIAP and promote caspase-dependent apoptosis. We found that IFN- α increases XAF-1 protein levels in both imatinib-sensitive KBM5 and imatinib-resistant KBM5STI571 cells (unpublished results), supporting a proapoptotic effect for IFN- α in CML cells. Interestingly, Essers M *et al.*⁵³ recently reported that IFN- α activates dormant HSCs and promotes their proliferation by increasing phosphorylation of STAT1 and Akt and the expression of target genes and by upregulating stem cell antigen-1 in an *in vivo* murine model, suggesting that IFN- α may target CML stem cells by recruiting quiescent primitive CD34⁺ CML progenitor cells into the cell cycle. Therefore, it is likely that IFN- α drives quiescent primitive CD34⁺ CML progenitor cells to exit the dormant stage and enter the active cycle, and that these cells then become sensitive to imatinib. The same concept was used to promote the cell cycle reentry of stem cells by pulsing them with growth factors in combination with imatinib to improve stem cell killing *in vitro*⁵⁴. Furthermore, because IFN- α promotes apoptosis, we speculate that the combination of IFN- α and imatinib may synergize cell death and enhance the elimination of quiescent CD34⁺ CML progenitor cells.

Other therapies

The PI3K/AKT signaling pathway plays a central role in cell survival. The downregulation of PTEN by *Bcr-Abl* and the activation of PI3K/AKT signaling in CML stem cells²⁵ also provide the rationale for a potential therapeutic strategy. A vaccine made from irradiated CML cells showed promising results in reducing/eliminating residual CML cells in patients with measurable cancer cells after imatinib treatment⁵⁵. In addition, the selective requirement of the arachidonate 5-lipoxygenase gene for CML stem cell function warrants further study.

Future Perspectives

Evidence has accumulated in recent years demonstrating that normal hematopoiesis requires complex bidirectional interaction between HSCs and the bone marrow microenvironment (niche). These interactions are critical for the maintenance of normal HSC quiescence and their proper functioning and for protecting them from environmental insults. Since CML stem cells share many characteristics with normal HSCs, it is not surprising that these mechanisms are used by CML stem cells to help them maintain their functioning and protect them from therapies, thereby contributing to disease relapse. The self-renewal, proliferation, differentiation, and apoptosis of CML stem cells are not only determined intrinsically, but also affected extrinsically by the niche. Strategies to selectively block homing and engraftment of CML stem cells will release these cells from their sanctuary niche. However, very little is known about the interaction between CML stem cells and their niche. How to interrupt this interaction to eliminate CML stem cells is an open question. Interestingly, CD44 was found to be required for homing and engraftment of CML stem cells⁵⁶. We reported that CXCR4 was downregulated by *Bcr-Abl*, and that imatinib upregulates CXCR4, induces CML cell migration, and promotes survival of quiescent CML cells⁵⁷. CD44 expression is upregulated by oncogenic pathways such as β -catenin-TCF4 and Ras-Raf-ERK, and downregulated by the tumor

suppressor p53⁵⁸. The combination of *Bcr-Abl* and CXCR4 inhibition and blocking CD44 may eliminate CML stem cells by disrupting leukemic cell-stromal interactions and impeding the trafficking of CML stem cells to the supportive microenvironment.

Benefits of combination therapy over monotherapy are emerging. We and others have observed that combining TKIs and various targeting agents, including those targeting cell surface markers, blocking self-renewal, and inducing apoptosis, enhances the effect of TKIs on CML cells in various cell compartments, even in cells resistant to TKIs. The mechanisms of this synergy are largely unknown. Nevertheless, it is clear that to efficiently eliminate CML cells, which have multiple characteristics, an appropriate combination strategy is a must.

Conclusion

The eradication of leukemia by targeting malignant stem cell populations is a promising new treatment strategy. CML is sustained by a rare population of leukemic cells with stem characteristics. These cells are unresponsive to imatinib-based therapy and are responsible for disease recurrence. Therefore, elimination of these cells is necessary to prevent relapse and to cure patients with CML. Based on what we have learned about CML stem cells, we suggest herein that targeting cell surface markers and essential self-renewal mechanisms, and inhibiting survival proteins to activate apoptotic pathways are plausible options for eliminating CML stem cells. Although these strategies are promising, targeting stem cells for cancer therapy is still a field in its infancy. The experiments thus far have been carried out primarily *in vitro* or in mouse model systems and may not accurately reflect human CML. Clinical evaluation is needed to validate the practical usefulness of these strategies. Targeting the interaction between CML stem cells and their bone marrow microenvironment is another avenue to explore for eliminating CML-initiating cells and reaching an ultimate cure for CML. Combination therapies aimed at CML stem cells, proliferating leukemic cells, and their niche, simultaneously or in sequential therapies, will bring new hope for rapid elimination of the entire leukemic cell population, avoiding the repopulation of leukemic cells upon cessation of therapy and the evolution of resistant clones, and may yield long-lasting benefits for patients with CML.

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References List

1. Branford S, Rudzki Z, Walsh S, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 2003;102:276–283. [PubMed: 12623848]
2. Nardi V, Azam M, Daley GQ. Mechanisms and implications of imatinib resistance mutations in BCR-ABL. *Curr.Opin.Hematol* 2004;11:35–43. [PubMed: 14676625]
3. Shah NP, Tran C, Lee FY, et al. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 2004;305:399–401. [PubMed: 15256671]
4. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* 2005;7:129–141. [PubMed: 15710326]
5. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002;99:3530–3539. [PubMed: 11986204]

6. Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood* 2004;103:4010–4022. [PubMed: 14982876]
7. Walz C, Sattler M. Novel targeted therapies to overcome imatinib mesylate resistance in chronic myeloid leukemia (CML). *Crit Rev Oncol Hematol* 2006;57:145–164. [PubMed: 16213151]
8. Graham SM, Jorgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 2002;99:319–325. [PubMed: 11756187]
9. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML, but does not eliminate the quiescent fraction. *Blood* 2006;107:4532–4539. [PubMed: 16469872]
10. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood* 1999;94:2056–2064. [PubMed: 10477735]
11. Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL. Punish the parent not the progeny. *Blood* 2005;105:1862–1866. [PubMed: 15528314]
12. Holtz MS, Forman SJ, Bhatia R. Nonproliferating CML CD34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli. *Leukemia* 2005;19:1034–1041. [PubMed: 15815728]
13. Heaney NB, Holyoake TL. Therapeutic targets in chronic myeloid leukaemia. *Hematol. Oncol* 2007;25:66–75. [PubMed: 17441215]
14. Eaves, CJ.; Eaves, AC. Progenitor cell dynamics. In: Carella, AM.; Daley, GQ.; Eaves, CJ.; Goldman, JM.; Hehlmann, R., editors. *Chronic myeloid leukaemia: biology and treatment*. London: Martin Dunitz; 2001. p. 73-100.
15. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N.Engl.J.Med* 2004;351:657–667. [PubMed: 15306667]
16. Florian S, Sonneck K, Hauswirth AW, et al. Detection of molecular targets on the surface of CD34+/CD38-- stem cells in various myeloid malignancies. *Leuk Lymphoma* 2006;47:207–222. [PubMed: 16321850]
17. Lemoli RM, Salvestrini V, Bianchi E, et al. Molecular and functional analysis of the stem cell compartment of chronic myelogenous leukemia reveals the presence of a CD34- cell population with intrinsic resistance to imatinib. *Blood* 2009;114:5191–5200. [PubMed: 19855080]
18. Zhao C, Blum J, Chen A, et al. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell* 2007;12:528–541. [PubMed: 18068630]
19. Abrahamsson AE, Geron I, Gotlib J, et al. Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. *Proc.Natl.Acad.Sci.U.S.A* 2009;106:3925–3929. [PubMed: 19237556]
20. Dierks C, Beigi R, Guo GR, et al. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell* 2008;14:238–249. [PubMed: 18772113]
21. Zhao C, Chen A, Jamieson CH, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 2009;458:776–779. [PubMed: 19169242]
22. Ito K, Bernardi R, Morotti A, et al. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature* 2008;19(453):1072–1078. [PubMed: 18469801]
23. Holtz MS, Slovak ML, Zhang F, et al. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. *Blood* 2002;99:3792–3800. [PubMed: 11986238]
24. Jiang X, Zhao Y, Smith C, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. *Leukemia* 2007;21:926–935. [PubMed: 17330101]
25. Peng C, Chen Y, Yang Z, et al. PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice. *Blood* 2010;115:626–635. [PubMed: 19965668]
26. Naka K, Hoshii T, Muraguchi T, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 2010;463:676–680. [PubMed: 20130650]
27. Chen Y, Li D, Li S. The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. *Cell Cycle* 2009;8:3488–3492. [PubMed: 19823023]

28. Feuring-Buske M, Frankel AE, Alexander RL, Gerhard B, Hogge DE. A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors. *Cancer Res* 2002;62:1730–1736. [PubMed: 11912147]
29. Black JH, McCubrey JA, Willingham MC, et al. Diphtheria toxin-interleukin-3 fusion protein (DT (388)IL3) prolongs disease-free survival of leukemic immunocompromised mice. *Leukemia* 2003;17:155–159. [PubMed: 12529673]
30. Frolova O, Wang R-Y, Korchin B, et al. Targeting IL3 Receptor in Chronic Myeloid Leukemia [abstract]. *ASH*. 2009
31. Sievers EL, Larson RA, Stadtmauer EA, et al. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J.Clin.Oncol* 2001;19:3244–3254. [PubMed: 11432892]
32. Gao J, Graves S, Koch U, et al. Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell* 2009;4:548–558. [PubMed: 19497283]
33. Hofmann I, Stover EH, Cullen DE, et al. Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. *Cell Stem Cell* 2009;4:559–567. [PubMed: 19497284]
34. Irvine DA, Zhang B, allan ek, et al. Combination of the Hedgehog Pathway Inhibitor LDE225 and Nilotinib Eliminates Chronic Myeloid Leukemia Stem and Progenitor Cells [abstract]. *ASH*. 2009
35. Aichberger KJ, Mayerhofer M, Krauth MT, et al. Identification of mcl-1 as a BCR/ABL-dependent target in chronic myeloid leukemia (CML): evidence for cooperative antileukemic effects of imatinib and mcl-1 antisense oligonucleotides. *Blood* 2005;105:3303–3311. [PubMed: 15626746]
36. Aichberger KJ, Mayerhofer M, Krauth MT, et al. Low-level expression of proapoptotic Bcl-2-interacting mediator in leukemic cells in patients with chronic myeloid leukemia: role of BCR/ABL, characterization of underlying signaling pathways, and reexpression by novel pharmacologic compounds. *Cancer Res* 2005;65:9436–9444. [PubMed: 16230407]
37. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*. 2006
38. Kuroda J, Kimura S, Andreeff M, et al. ABT-737 is a useful component of combinatory chemotherapies for chronic myeloid leukaemias with diverse drug-resistance mechanisms. *Br J Haematol* 2007;140:181–190. [PubMed: 18028486]
39. Kuroda J, Kimura S, Strasser A, et al. Apoptosis-based dual molecular targeting by INNO-406, a second-generation Bcr-Abl inhibitor, and ABT-737, an inhibitor of antiapoptotic Bcl-2 proteins, against Bcr-Abl-positive leukemia. *Cell Death Differ* 2007;14:1667–1677. [PubMed: 17510658]
40. Tauchi T, Sumi M, Nakajima A, et al. BCL-2 antisense oligonucleotide genasense is active against imatinib-resistant BCR-ABL-positive cells. *Clin Cancer Res* 2003;9:4267–4273. [PubMed: 14519654]
41. Carter BZ, Mak D, Schober WD, et al. Regulation of survivin expression through bcr-abl/ MAPK cascade: targeting survivin overcomes Imatinib resistance and increases Imatinib sensitivity in Imatinib responsive CML cells. *Blood* 2006;107:1555–1563. [PubMed: 16254145]
42. Mak DH, Schober WD, Chen W, et al. Triptolide induces cell death independent of cellular responses to imatinib in blast crisis chronic myelogenous leukemia cells including quiescent CD34+ primitive progenitor cells. *Mol.Cancer Ther* 2009;8:2509–2516. [PubMed: 19723894]
43. Mak DH, Schober WD, Konopleva M, et al. Inhibition of Bcl-2/Bcl-XL Promotes Apoptosis in Blast Crisis CML Including Quiescent Primitive Progenitor Cells Regardless of Cellular Responses to Tyrosine Kinase Inhibitors [abstract]. *ASH* 2009. 2009
44. Copland M, Pellicano F, Richmond L, et al. BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors. *Blood* 2008;111:2843–2853. [PubMed: 18156496]
45. Kantarjian HM, O'Brien S, Cortes JE, et al. Complete cytogenetic and molecular responses to interferon-alpha-based therapy for chronic myelogenous leukemia are associated with excellent long-term prognosis. *Cancer* 2003;97:1033–1041. [PubMed: 12569603]
46. Krause DS, Van Etten RA. Bedside to bench: interfering with leukemic stem cells. *Nat.Med* 2008;14:494–495. [PubMed: 18463658]

47. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* 2007;109:58–60. [PubMed: 16973963]
48. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N.Engl.J.Med* 2006;355:2408–2417. [PubMed: 17151364]
49. Guilhot F, Preudhomme C, Guilhot J, et al. Significant higher rates of undetectable molecular residual disease and molecular responses with pegylated form of interferon $\alpha 2a$ in combination with Imatinib (IM) for the treatment of newly diagnosed chronic phase (CP) chronic myeloid leukaemia (CML) patients (pts): confirmatory results at 18 months of part 1 of the spirit phase III randomized trial of the french CML group (FI LMC) [abstract]. *ASH* 2009. 2009
50. Angstreich GR, Matsui W, Huff CA, et al. Effects of imatinib and interferon on primitive chronic myeloid leukaemia progenitors. *Br.J.Haematol* 2005;130:373–381. [PubMed: 16042686]
51. Chawla-Sarkar M, Lindner DJ, Liu YF, et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 2003;8:237–249. [PubMed: 12766484]
52. Leaman DW, Chawla-Sarkar M, Vyas K, et al. Identification of X-linked inhibitor of apoptosis-associated factor-1 as an interferon-stimulated gene that augments TRAIL Apo2L-induced apoptosis. *J.Biol.Chem* 2002;277:28504–28511. [PubMed: 12029096]
53. Essers MA, Offner S, Blanco-Bose WE, et al. IFN α activates dormant haematopoietic stem cells in vivo. *Nature* 2009;458:904–908. [PubMed: 19212321]
54. Jorgensen HG, Copland M, Allan EK, et al. Intermittent exposure of primitive quiescent chronic myeloid leukemia cells to granulocyte-colony stimulating factor in vitro promotes their elimination by imatinib mesylate. *Clin.Cancer Res* 2006;12:626–633. [PubMed: 16428509]
55. Smith BD, Kasamon YL, Kowalski J, et al. K562/GM-CSF immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate. *Clin.Cancer Res* 2010;16:338–347. [PubMed: 20048335]
56. Krause DS, Lazarides K, von Andrian UH, Van Etten RA. Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. *Nat.Med* 2006;12:1175–1180. [PubMed: 16998483]
57. Jin L, Tabe Y, Konoplev S, et al. CXCR4 up-regulation by imatinib induces CML cell migration to bone marrow stroma and promotes survival of quiescent CML cells. *Mol Cancer Ther* 2008;7:48–58. [PubMed: 18202009]
58. Godar S, Ince TA, Bell GW, et al. Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 2008;134:62–73. [PubMed: 18614011]

Figure 1.

Stemness of CML stem cells. Like the normal hematopoiesis, the CP CML stem cell is capable of self-renewal and producing all other lineages of hematopoietic cells but the size of the myeloid compartment grows due to Bcr-Abl tyrosine kinase promoted proliferation in the HSC. Additional genetic and epigenetic alterations result in transition to BC. Progression to BC also results in the acquisition of self-renewal potential of a GMP population via the activation of β -catenin. Dotted circles, BCR-ABL-positive cells; CMP, common myeloid progenitors.

Figure 2. Death receptor mediated extrinsic and mitochondrial mediated intrinsic apoptotic pathways. ABT-737 promotes apoptosis by antagonizing Bcl-2 and Bcl-xL and triptolide induces apoptosis in part by decreasing XIAP and Mcl-1.

Figure 3.

Combination of ABT-737 and imatinib significantly enhances cell death not only in proliferating CD34⁺ CML cells but also in quiescent CD34⁺ CML progenitor cells. The sample was obtained from a BC CML patient who had T315I mutation and failed imatinib and dasatinib (58% blasts). ABT, ABT-737; IM, imatinib. CI, combination index at 24 hours.