VOLUME 26 · NUMBER 17 · JUNE 10 2008

JOURNAL OF CLINICAL ONCOLOGY

REVIEW ARTICLE

Chronic Myeloid Leukemia Stem Cells

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Submitted April 7, 2008; accepted April 9, 2008.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/08/2617-2911/$20.00

DOI: 10.1200/JCO.2008.17.5745

ABSTRACT

Although rare, chronic myeloid leukemia (CML) represents an important paradigm for understanding the molecular events leading to malignant transformation of primitive hematopoietic progenitors. CML was the first cancer to be associated with a defined genetic abnormality, *BCR-ABL*, that is necessary and sufficient for initiating chronic phase disease as well as the first cancer to be treated with molecular targeted therapy. Malignant progenitors or leukemia stem cells (LSCs) evolve as a result of both epigenetic and genetic events that alter hematopoietic progenitor differentiation, proliferation, survival, and self-renewal. LSCs are rare and divide less frequently, and thus, represent a reservoir for relapse and resistance to a molecularly targeted single agent. On subverting developmental processes normally responsible for maintaining robust life-long hematopoiesis, the LSCs are able to evade the majority of current cancer treatments that target rapidly dividing cells. Enthusiasm for the enormous success of tyrosine kinase inhibitors at controlling the chronic phase disease is tempered somewhat by the persistence of the LSC pool in the majority of the patients. Combined therapies targeting aberrant properties of LSC may obviate therapeutic resistance and relapse in advanced phase and therapeutically recalcitrant CML.

J Clin Oncol 26:2911-2915. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Cancer is a leading cause of death for adults in the Western world and its incidence and prevalence are increasing with the rising age of the population.[1] While significant advances have been made in the diagnosis and treatment of cancer, cures have been achieved for only some tumors, mostly when treated in the early stages. The major limitation to developing curative therapy has been an incomplete understanding of the molecular and pathophysiologic mechanisms driving cancer progression, metastasis, and relapse. Chronic myeloid leukemia (CML) is relatively rare, but is one of the best-characterized malignancies. CML research paved the way for increasing understanding of molecular events involved in cancer initiation and progression. CML was the first malignancy to be associated with a characteristic and defining chromosomal translocation called the Philadelphia chromosome (Ph+) that brings together the *c-abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 as t(9;22)(q34;q11). This novel fusion gene gives rise to a constitutively active protein tyrosine kinase product— BCR-ABL.

In CML and other cancers, a growing body of evidence suggests that a primitive population of cancer stem cells (CSCs) has escaped the normal control of self-renewal resulting in cancer propagation. The CSC hypothesis posits that, rather than the heterogeneous group of cells within a cancer each being capable of propagating the cancer, only CSCs have sufficient capacity to make more of themselves in a process termed self-renewal, in addition to surviving and differentiating to recapitulate the tumor. These CSCs have been implicated in the pathogenesis of relapse and therapeutic resistance.[2-5] They have been identified in a number of malignancies, such as acute myelogenous leukemia (AML),[6] breast cancer,[7] brain tumors,[8] colon cancer,[9] as well as CML.[2,10] A number of studies suggest that quiescent CSCs, particularly in CML, may be a reservoir for relapse and as a consequence are recalcitrant to therapies that target rapidly dividing cells.[4,10-12]

The human hematopoietic stem cell (HSC; CD34+CD38−CD90+Lin−)[13] initiates chronic phase CML as a result of acquisition of the Ph+, which can be detected in all the myeloid, erythroid, and lymphoid lineages but rarely in T cells.[14] Although necessary, BCR-ABL is not sufficient for generation of leukemia stem cells (LSCs) involved in blastic transformation. Indeed, the cell type and context-specific events involved in the molecular evolution of CML from the primitive HSC compartment to fully transformed progenitors is important for understanding diagnostic and therapeutic implications of LSC in CML. Activation of BCR-ABL in HSCs leads to preferential expansion of myeloid progenitors and differentiated progeny in the blood and bone marrow of patients in early chronic phase.[3,14] Clinical symptoms are directly related to

the increase in number of mature cells of the granulocytic lineage. Traditionally, reduction of the WBCs with leukopheresis or hydrea was sufficient to palliate the symptoms related to cellular accumulation. Inevitable progression to accelerated phase typically occurred within 3 to 5 years. Within 3 to 7 years of diagnosis, most patients progressed to the development of blast crisis that shares pathologic features with AML in about two thirds of the cases or lymphoblastic leukemia in one third of the cases. This stage is characterized by leukemia resistance to all current cytoreductive multiagent chemotherapeutic agents. As a consequence, most patients succumb to disease.

The only commonly curative strategy is allogeneic HSC transplantation in the chronic phase. Small, but notable numbers of patients have been cured from the disease with administration of interferon alfa,[15] although early transplantation was a preferred long-term treatment strategy. Over the past few years, inhibition of BCR/ABL tyrosine kinase activity with imatinib was found to be sufficient to halt the disease process and reverse the hematologic abnormalities. Close polymerase chain reaction monitoring of the blood of patients treated with imatinib or other BCR-ABL inhibitors demonstrated that a 3- to 4-log reduction in *BCR-ABL* expression correlated strongly with the ability to achieve long-term remissions. However, most of the patients have low-level persistent *BCR-ABL* transcripts and relapse on discontinuation of the drug.

Acquisition of the aberrant BCR-ABL kinase is necessary and may be sufficient to establish chronic phase CML. The fundamental biologic processes that lead to the BCR-ABL chromosomal translocation remain unanswered. This notion is highlighted by the observation of the translocation in the bone marrow of normal subjects who do not develop CML.[16,17] Compelling evidence suggests that it is also not sufficient to promote progression to blast crisis.[3] In seminal work by Radich and colleagues, other genetic and epigenetic changes were shown to play a key role in blastic transformation including genes involved in self-renewal (Cadherin, *MDI1*, Prickle 1, *FZD2*), survival (*GADD45G, BCL2, FOXO3A, MCL1*), cell cycle control (*GASDD45G, FANCG, XRN2, BSZ1A, HIST1H2AE*), differentiation (*CEBA, CEBPE, FOXO3A*) and regulation of innate and adaptive immune responses.[18]

INITIATING ROLE OF BCR-ABL

The oncogenic capacity of *BCR-ABL* was initially documented in vitro through its ability to support factor independent growth of established hematopoietic cell lines and primary bone marrow cells.[19] The first description of an in vivo model of CML came from the transplantation of *BCR-ABL*–transduced murine bone marrow cells into lethally irradiated syngeneic recipients.[20] Retroviral overexpression of BCR-ABL was necessary and sufficient to induce a myeloproliferative syndrome akin to chronic phase CML, as well as acute myeloid and lymphoid leukemias. Refinements of the model led to more efficient generation of the CML-like disease and recapitulation the myeloid skewing of the *BCR-ABL* transformed hematopoietic stem cells.[21] However, progression to blast crisis was observed infrequently.[22] Transgenic models were not particularly successful in recapitulating CML due to either embryonic lethality of BCR-ABL,[23] or predominant acute lymphoblastic leukemia development in the models utilizing inducible BCR-ABL,[24] or embryonic stem cell–derived chimeric mice.[25] A notable insight into the role of transcription factors in CML was gained when a deficiency of a transcription factor, *JunB*, in the myeloid lineage lead to the development of a murine MPD in which serial transplantation potential existed only at the primitive HSC level.[26,27] Moreover, granulocyte-macrophage colony-stimulating factor–mediated survival and proliferation of the *JunB* deficient granulocyte-macrophage progenitors (GMP) was associated with changes in antiapoptotic proteins Bcl2 and Bclx, as well as cell cycle regulators $p16^{ink4a}$ and *c-Jun*.[27]

CML STEM CELLS AND MECHANISMS OF THERAPEUTIC RESISTANCE

The HSC origin of CML was elegantly demonstrated in the late 1970s in studies showing a clonal phenotype of the X-linked G6PD enzyme in the granulocytes, erythrocytes, and platelets of the heterozygous female patients.[14] Further evidence for a stem-cell origin of chronic phase CML was obtained as a result of advances in xenogeneic transplantation models in immunocompromised mice. In fact, serial transplantation in immunocompromised mice has served as a "gold standard" for identification of CSCs in various malignancies including AML,[6] acute lymphoblastic leukemia,[28] brain,[8] colon,[9] breast,[7] head and neck,[29] and melanoma.[30] These models revealed that chronic phase CML patients contained both normal and leukemic stem cells in the CD34+ fraction while blast crisis was predominated by the CD34+ LSC with more vigorous transplantation and blast generating potential recapitulating the aggressiveness of human disease.[2] Human cells with long-term engraftment potential were enriched for CD34+ primitive HCS characterized by CD90 expression.[10] Closer analysis of transplantable and long-term culture initiating cells subpopulations of CD34+ cells demonstrated predominance of quiescent G_0 cells.[11] Phenotypically primitive CD34+CD38− cells from the long-term culture initiating cells of chronic phase patients had a propensity to differentiate along the myeloid lineage on long-term engraftment in immunocompromised mice.[31]

Clinical use of BCR-ABL tyrosine kinase inhibitors (TKI) has revolutionized the treatment of CML. The relatively moderate toxicity of TKI and prolonged responses observed in patients in the chronic phase of the disease has significantly decreased morbidity and mortality of CML.[32] While a hematologic response is observed in more than 95% of patients in chronic phase, the major molecular responses defined by a 3-log decline of the *BCR-ABL* transcript detected by reverse-transcriptase polymerase chain reaction have been achieved in fewer than 5% of patients. Patients in advanced phases of disease often respond to single-agent TKI but inevitably relapse with treatment-refractory disease.

There are three major mechanisms for disease resistance to TKI including *BCR-ABL* amplifications, *BCR-ABL* mutations, and leukemic clonal evolution. Amplification of *BCR-ABL* was first observed in the imatinib-resistant cell lines developed under selective pressure of increasing in vitro drug concentrations.[33] This phenomenon was documented in some patients with acquired resistance.[34] However, the more common causes of TKI resistance have been clonal evolution present in up to a quarter of patients on disease progression[35] and mutations of the *BCR-ABL*.[36] The cells harboring previously mentioned resistance mechanisms are less likely to arise in early chronic phase of the disease and may have preexisted at a more advanced diagnostic stage. More potent TKIs do not change signaling or behavior of CML progenitors[37] Taken together, these observations suggest that new strategies will be needed to eliminate CML.

CML PROGRESSION AND ABERRANT PROGENITOR REPROGRAMMING

Aberrant Self-Renewal

Analysis of CML blast crisis cells showed enhanced canonical Wnt signaling activation through enhanced nuclear localization of the activated b-catenin.[3] Activation of the Wnt signaling in GMP endowed these cells with aberrant self-renewal capacity, normally a property of hematopoietic stem cells, as measured by generation of replatable myeloid colonies. Replating efficiency was reduced with axin— a potent inhibitor of b-catenin. Self-renewal capacity is normally absent in GMP suggesting that they have acquired the function of leukemic stem cell. Work by Radich and colleagues[18] comparing microarray data from CD34+ cells across the disease spectrum identified several critical differentially expressed genes that function in self-renewal, differentiation, survival, and DNA-damage response. The Wnt pathway, critical for HSC self-renewal and interaction with bone marrow niche, was found to be activated during progression to advanced phase. This article linked the abnormality in free b-catenin pool to the decreased JunB expression through the disregulation of MDFI— an inhibitor of myogenic basic helix-loop-helix transcription factors. Disruption of differentiation and enhancement of self-renewal may be a critical component of disease progression.

Aberrant Differentiation

The failure of TKI to eliminate CML in chronic phase is exacerbated by the inevitable progression of CML in advanced stages. In blast crisis and imatinib-resistant CML the GMP pool was significantly expanded.[3] Aberrant activation of HSC self-renewal programs and inhibition of differentiation at an inappropriate developmental stage leads to accumulation and survival of the more mature and typically short-lived CD34+ myeloid progenitors. Emerging evidence suggests that a transcriptional repressor Bmi1 that is normally restricted to the stem-cell compartment is overexpressed in the aggressive forms of CML that progressed to blast crisis within 3 years and during advanced phases of disease.[38] Interestingly, a large family of *Hox* genes with diverse functions in hematopoiesis has been found to be negatively regulated by Bmi1.[39] Some *Hox* family members, such as *HoxA9* and *HoxB4*, as well as the *Hox* gene master regulator MLL are better known for leukemogenic potential through enhanced HSC self-renewal and myelopoiesis skewing on upregulation. Downregulation of *Hox* genes, such as *HoxA5* located at the 3′ end of the gene cluster, has been shown to impair myelopoiesis suggesting differentiation block at HSC. Redundancy and developmental stage–specific functions of *Hox* transcription factors makes it difficult to form concrete conclusions. However, inactivation of the *HoxA4* and *HoxA5* through hypermethylation was found in 59% and 34% of CD 34+ cells from chronic phase CML respectively and in approximately 90% of the CD34+ BC CML patient samples.[40] This study also demonstrated that *HoxA4* and *HoxA5* inactivation was associated with poor prognosis in other myeloid and lymphoid malignancies and may be a marker of more severe disease.

Aberrant Survival

For most cell types the capacity for long-term survival is a luxury that is largely limited to the stem-cell population and diminishes with differentiation. Some cancer cells regain the ability to survive for extended periods of time. These cells constitute the CSC population. Reacquiring genes for survival allows these cells to propagate even as they outgrow blood supplies, detach from the surrounding supportive stroma, and accumulate genetic mutations. Cancer cells regain long-term survival capacity largely by the deregulation of apoptosis, a process that would normally keep them in check. As a means of protection against malignancy, apoptosis is initiated on chromosomal DNA damage, and is disrupted in virtually every cancer. Since cytotoxic chemotherapeutics commonly act by inducing apoptosis in malignant cells, resistance to apoptosis often equals chemotherapeutic resistance. The restoration of long-term survival capacity thus reprograms cancer cells to behave more like stem cells and impacts their ability to proliferate, survive, metastasize, and resist treatment. Therapies aimed at inhibiting these pathways may be able to specifically target and eradicate cancer stem cells.

Cancer cells disrupt the complex and redundant apoptotic pathways in a variety of ways. In CML, resistance to apoptosis begins with BCR-ABL, and in fact the ultimate outcome of BCR-ABL inhibition with imatinib is induction of apoptosis.[41,42] BCR-ABL–mediated apoptosis resistance involves the differential expression of the Bcl-2 family of apoptosis-regulatory proteins including antiapoptotic members such as Bcl-2 and Mcl-1, and pro-apoptotic members such as Bad and Bim. The antiapoptotic members act to inhibit the release of cytochrome c from the mitochondria and subsequent activation of Apaf1. This in turn inhibits caspase 9 activation and the downstream caspase cascade of apoptosis initiation. Pro-apoptotic members have more diverse mechanisms. Some inhibit the activity of the antiapoptotic members by heterodimerization via BH-3 domains (Bad and Noxa) while others act at the outer mitochondrial membrane to form pores (Bax and Bak).[43] Many effects on Bcl-2 family protein expression are directly linked to the activity of BCR-ABL in CML cells. For example, PI3 kinase activation by BCR-ABL leads to activation of Akt and downstream inhibition of Bad.[44] BCR-ABL also activates STAT5 leading to increased expression of Bcl-xl.[45-47] Finally, Bcl-2 expression is increased in cells with BCR-ABL.[48]

Functional resistance to apoptosis in cells harboring the *BCR-ABL* oncogene collaborates the expression data mentioned earlier. First, cell lines expressing BCR-ABL are resistant to stress-induced apoptosis.[49-53] These cells also display delayed apoptosis after the withdrawal of prosurvival cytokines[54-56] and this delay can be attenuated by using antisense oligonucleotide silencing against *BCR-ABL*.[54,56] Furthermore, there is evidence that BCR-ABL contributes to apoptosis-resistance in a dose-dependent manner[57] and BCR-ABL expression increases with disease progression from chronic phase to blast crisis.[58] In a murine model of myeloid leukemia where targeted BCR-ABL expression in myeloid progenitors and myelomonocytic progeny led to a chronic myeloproliferative disorder, coexpression of Bcl-2, but not Ras or Myc, promoted progression to acute myeloid leukemia.[59] We have observed a parallel increase in Bcl-2 expression with disease progression (Jamieson, unpublished data).

Therefore, initiation of CML by BCR-ABL leads to resistance to apoptosis. CML progression from chronic phase to blast crisis through activation of aberrant self-renewal, differentiation, and survival pathways greatly enhances resistance to apoptosis. As discussed earlier, activation of the WNT pathway resulting in nuclear accumulation of b-catenin leads to increased expression of WNT target genes such as *c-Myc*. *C-Myc* protects from apoptosis by upregulation of Bcl-2 family proteins. In addition, GSK3B, a critical negative regulator

of WNT signaling pathway, has been shown to regulate the activity of both pro-apoptotic and antiapoptotic Bcl-2 family proteins. GSK3B can activate Bax, a membrane pore-forming Bcl-2 family member, by phosphorylation at serine 163.[60] GSK3B can also regulate the stability of Mcl-1, one of the antiapoptotic family members, and inhibition of GSK3B via the induction of glucose metabolism leads to stabilized Mcl-1 and resistance to apoptosis.[61]

In addition to WNT-mediated signaling, key apoptosis proteins are highly regulated by the PI3K/Akt pathway. In response to metabolic, survival, and growth signals, PI3K activates Akt which then acts on a number of downstream targets. Akt directly inhibits Bad and Bax[62] and modulates the activity of transcription factors such as those from the nuclear factor κB and FoxO families. Nuclear factor κB protein activation leads to its nuclear localization, where it then activates the transcription of a number of prosurvival molecules including Bcl-2, Bcl-xl, and various caspase inhibitors.[63] The importance of these transcription factors in CML has been highlighted by evidence that NFκB is activated in transgenic models of CML[64] and that BCR-ABL can activate NFκB.[65] In contrast, FoxO transcription factors are targeted for proteolysis via Akt-mediated signaling. These factors normally induce the transcription of prodeath molecules including Bim and FasL. In CML, BCR-ABL causes constituitive repression of *FoxO3a* by continued activation of Akt[66,67] leading to yet another mechanism of BCR-ABL–mediated apoptosis resistance. Finally, Akt inhibits GSK3B activity. Thus the Akt pathway broadly modulates apoptosis and, via regulation of GSK, is perhaps intrinsically linked to the WNT pathway in the dysregulation of apoptosis in CML.

THERAPEUTIC IMPLICATIONS OF CML STEM CELLS

The relative importance of these pathways in CML progression and their impact on clinical outcomes for patients with CML remains an important but unsolved question. The precise sequence of molecular events leading to resistance to BCR-ABL inhibitor therapy, as well as the cellular framework in which they occur, has not been completely elucidated. With our increasing ability to enrich for leukemic stem cells it should be possible to assess the diagnostic, prognostic, and therapeutic relevance of the epigenetic and genetic mechanisms driving leukemic stem-cell self-renewal, survival, and aberrant differentiation prospectively in the context of clinical trials. Research performed to date regarding the cell type and context specific effects of mutations responsible for therapeutic resistance and disease progression suggests that combined therapy targeting the skewed proliferation, survival, differentiation, and self-renewal of CML progenitors will need to be implemented in patients who do not sustain a molecular remission within a year of starting targeted BCR-ABL inhibitor therapy or who are diagnosed with advanced phase disease at presentation. While combination molecularly targeted therapy has been widely and effectively implemented for HIV as highly active antiretroviral therapy, a similar approach has not yet been widely introduced for recalcitrant or advanced phase CML. Clinical trials involving highly active antileukemic stem-cell therapy combining BCR-ABL inhibition and self-renewal pathway inhibitors with or without additional survival pathway antagonists may expunge the LSC population that represents a reservoir for relapse and obviate progression to blast crisis.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Catriona H.M. Jamieson, Wintherix Inc **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** None **Research Funding:** None **Expert Testimony:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

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Final approval of manuscript: Edward Kavalerchik, Daniel Goff, Catriona H.M. Jamieson

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