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Stem cell biomarkers in chronic myeloid leukemia

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Abstract. Chronic myeloid leukemia (CML) is a clonal multi-step myeloproliferative disease that is initially produced and ultimately sustained by a rare subpopulation of *BCR-ABL*$^+$ cells with multi-lineage stem cell properties. These *BCR-ABL*$^+$ CML stem cells are phenotypically similar to normal hematopoietic stem cells which are also maintained throughout the course of the disease at varying levels in different patients. Defining the unique properties of the leukemic stem cells that produce the chronic phase of CML has therefore had to rely heavily on access to samples from rare patients in which the stem cell compartment is dominated by leukemic elements. Here we review past and ongoing approaches using such samples to identify biologically and clinically relevant biomarkers of *BCR-ABL*$^+$ stem cells that explain their unusual biology and that may help to design, or at least predict, improved treatment responses in CML patients. These studies are of particular interest in light of recent evidence that chronic phase CML stem cells are not only innately resistant to imatinib mesylate and other drugs that target the BCR-ABL oncoprotein, but are also genetically unstable.

Keywords: Hematopoietic stem cells, chronic myeloid leukemia, imatinib, BCR-ABL-targeted therapies, stem cell biomarkers, drug resistance

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that is usually first identified clinically by symptoms that are caused by an inappropriately increased production of granulocytes and monocytes. Biologically, CML is now recognized to represent a multi-step, multi-lineage, clonal hematopoietic disorder that is initiated and propagated by a rare population of CML stem cells that have acquired a *BCR-ABL* fusion gene. During the chronic phase (CP) of the disease, these CML stem cells possess many properties typical of normal hematopoietic stem cells (HSCs), including an ability to differentiate into almost all blood cell types.

In most CML patients, the *BCR-ABL* fusion gene reflects a simple reciprocal translocation between the long arms of chromosomes 9 and 22, resulting in the formation of the hallmark Philadelphia (Ph) chromosome. The *BCR-ABL* fusion gene encodes a chimeric oncoprotein that displays constitutively activated tyrosine kinase activity and is inappropriately localized in the cytoplasm. These features deregulate cellular proliferation and apoptosis control through effects on multiple signaling pathways [1,2]. Direct evidence that the *BCR-ABL* rearrangement is a key transforming event in the generation of CML has been provided by gene transfer experiments [3,4] and the ability of specific inhibitors of the *BCR-ABL* oncoprotein to induce remissions in patients [5–9].

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An understanding of the role of the *BCR-ABL* oncogene in the genesis and maintenance of CML has led to the development of novel "targeted" therapies that selectively inhibit the tyrosine kinase activity of *BCR-ABL*-encoded fusion proteins. The first of these tyrosine kinase inhibitors was imatinib mesylate (IM), also known as Gleevec (Novartis Pharmaceuticals). Clinical trials have shown "complete" cytogenetic response rates of 87% in CP CML patients treated with IM for 60 months with 93% progression-free survival [10]. Nevertheless, despite these remarkable initial responses to IM therapy, molecular remissions are rare and the emergence of IM-resistant subclones continues to pose a significant clinical problem [11,12]. Indeed, approximately 10–15% of patients with early CP CML will either present with or later show the appearance of IM-resistant cells. Some of these patients respond to higher doses of IM [13] or to second generation tyrosine kinase inhibitors like dasatinib (Sprycel, Bristol Myers Squibb) or nilotinib (Tasigna, Novartis). However, a proportion does not and the disease in this latter group might be better managed by treatment with an intensive conditioning regimen and an appropriately matched transplant of normal HSCs.

In most patients, loss of responsiveness to IM appears gradually - as indicated by a slow increase in the proportion of *BCR-ABL*$^+$/Ph$^+$ cells detectable in the marrow (and/or blood) over a period of several months. However, in a small group (1% to 2%) of IM-treated CP patients, blast phase disease appears within 3 months of a previously documented complete cytogenetic remission [14,15] and the salvage rate for this important subgroup, even with myeloablative treatments supported by an allogeneic transplant, is low [16]. Thus, there is an urgent need to develop prognostic tests to identify those patients who will not respond to IM and for whom alternative treatments would be important to initiate before disease progression occurs.

In the pre-IM era, several clinical and laboratory features allowed responses to existing therapies to be predicted. The Sokal score, initially developed to predict response to busulfan [17], still retains some predictive value for IM therapy. Patients with high-risk Sokal scores have a 69% chance of achieving a complete cytogenetic remission within 12 months, compared to 82% and 89% for intermediate and low-risk scores, respectively. However, once a complete cytogenetic remission has been obtained, the Sokal score loses its prognostic importance and the progression-free survival for all 3 groups remains equal. Therefore, even a high-risk Sokal score at diagnosis does not, on its own, justify the adoption of an alternative front-line therapy until after an initial trial of IM.

The appearance of karyotypic abnormalities in addition to the Ph chromosome has also been associated with an adverse prognosis and is usually accompanied by other manifestations of more advanced disease. However, with the higher doses of IM now in use, evidence of clonal evolution alone has not been associated with an inferior outcome [18,19]. Similarly, deletions involving the derivative 9 chromosome that were predictive of a poor outcome to interferon [20] have not been found to have similar significance for patients receiving IM and preliminary reports suggest little or no relationship of this parameter to the achievement or durability of an IM response [21,22].

The failure of these historical clinical and laboratory parameters to identify patients who are likely to be unresponsive to *BCR-ABL*-targeted therapies has focused attention on a need for new biomarkers that can serve this purpose. A logical starting point is the leukemic stem cell compartment of these patients, since it is the number and properties of these cells that ultimately determine the growth characteristics and evolution of the leukemic clone, as well as the size of the residual normal HSC population upon which autologous regeneration of the hematopoietic system depends.

2. Biologic definition of CML stem cells

Much evidence indicates that the leukemic clone in patients with CML originates in a multi-potent HSC whose ability to generate normal blood cell progeny is not detectably perturbed by the acquisition of a *BCR-ABL* fusion gene, although the number of differentiating cells produced from the small pool of *BCR-ABL*$^+$ stem cells is markedly altered. As a result, during the CP of the disease, the same hierarchy of primitive and mature compartments of cells that characterize normal hematopoiesis can be readily discerned within the expanding leukemic clone and these differentiating "leukemic" cells are functionally, morphologically, and phenotypically almost indistinguishable from their normal counterparts [23,24] (see also Fig. 1). Accumulating evidence indicates that this hierarchy is established in normal hematopoietic cells by mechanisms that progressively restrict hematopoietic differentiation and proliferative potential in a highly co-ordinated fashion that spans many cell generations [25–28]. Sequential stages within this hierarchy can thus be distinguished both by the maximum number and types of

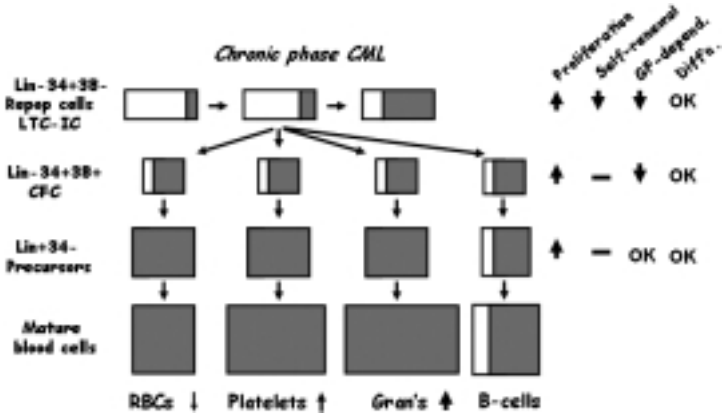

Fig. 1. Schema showing the relative sizes of different subpopulations of normal and leukemic cells present in CP patients. The shared phenotypes and functional endpoints that identify similar types of normal and leukemic elements are listed on the left side. The boxes in the central part of the figure illustrate diagrammatically the relative numbers of each of these that are normal (white) and leukemic (grey). As shown, dominance of the system by CML cells is typically achieved only in the later stages of differentiation. The very slow rate at which the CML stem cells accumulate in spite of their competitive advantage at later stages of differentiation may be explained, at least in part, by the activation in the most primitive CML cells of an autocrine IL-3/G-CSF mechanism that reduces their growth factor dependence [67]. This, in turn, allows the leukemic cells at intermediate stages of differentiation to amplify but, at the stem cell level, this effect may be offset by a decreased self-renewal ability caused by same autocrine growth factors (as summarized on the right hand side of the schema). Diff'n. = differentiation; GF depend. = growth factor dependence.

mature progeny produced when the cells are optimally stimulated either *in vitro* or *in vivo* and by their corresponding phenotypic profiles [23,29].

Normal human HSCs are identified by their ability to repopulate sublethally irradiated immunodeficient (e.g., nonobese diabetic severe combined immunodeficiency, NOD/SCID) mice with both lymphoid and myeloid progeny – indicative of their multipotent status as well as their *in vivo* regenerative activity. Limiting dilution approaches can then be applied to quantify their numbers using endpoints that incorporate all of these features [30,31]. Accordingly, such cells are often described operationally as NOD/SCID-repopulating cells. At least some of these cells can also generate multipotent progeny with similar secondary NOD/SCID mouse repopulating activity – indicative of their possession of some self-renewal potential. Mice with even greater immunodeficiencies (e.g., resulting from the additional inactivation of either the β2-microglobulin gene or the gene for the γ chain of the interleukin-2 [IL-2] receptor, or by injection of anti-asialo GM1 or anti-CD122 antibodies that also the residual target NK cells present in NOD/SCID mice) show selectively enhanced efficiency of engraftment by downstream multipotent human hematopoietic cells with short term repopulating activity [32–34]. However, these latter cells lack sustained repopulating activity and are also unable to generate progeny that can repopulate secondary NOD/SCID recipients. These features suggest that many of the cells able to repopulate more permissive strains of immunodeficient mice for periods of 4–20 weeks are not equivalent to murine cells identified as HSCs on the basis of their sustained self-renewal potential (Fig. 2 – sequential RC's detected in mice) [35].

At the other end of the spectrum are the various lineages of terminally differentiating blood cells. These cells already display some recognizable features of the particular lineage they belong to and typically undergo a small number of amplifying divisions before their maturation is complete. Intermediate between the terminally differentiating cells and the HSC compartment are so-called "progenitor" cells that lack overt morphological features of a particular lineage but are readily detected and quantified by their ability to generate colonies of from 8 to $\sim 10^5$ mature blood cells when plated in a semi-solid medium containing an appropriate cocktail of growth factors. Cells that can generate colony-forming cells (CFCs) in the presence of stromal feeder layers *in vitro* for at least 5 weeks represent a more primitive compartment than most CFCs and, based on their mode of assay, are referred to as longterm culture-initiating cells (LTC-ICs) [23]. Some LTC-ICs are clearly not HSCs, but the extent to which these 2 operationally defined cell types may overlap is not yet clear [29].

In patients with CP CML, *BCR-ABL*$^+$/Ph$^+$ CFCs, LTC-ICs and NOD/SCID repopulating cells are detect-

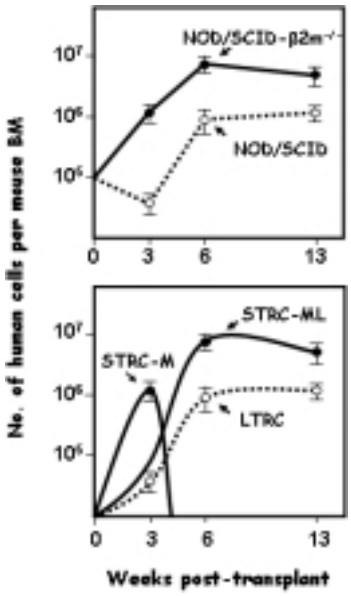

Fig. 2. Different patterns of regenerated human hematopoietic cells in mice with variable immunodeficiencies reflect different engraftment efficiencies of human cells with short and long term repopulating activities. As shown in the upper panel, transplants of bulk $CD34^+$ human cells produce more progeny and faster in NOD/SCID-*β2-microglobulin* null mice (solid line) as compared to standard NOD/SCID hosts (dotted line). Experiments with more purified cell transplants have demonstrated that the increased output of human cells obtained in the more NK-deficient NOD/SCID-*β2-microglobulin* null mice reflects the additional activity of 2 types of short term repopulating cells (STRCs) that do not engraft NOD/SCID mice. One of these STRC subsets is made up of cells that are myeloid-restricted (STRC-Ms). These cells produce a rapid but very short-lived wave of predominantly myeloid cells (up to 4 weeks post-transplant). The second subset of STRCs are cells that have dual lymphoid and myeloid potential (STRC-MLs) but do not self-renew. As a result, their output of mature cells is also transient, albeit for a slightly longer period (up to 13 weeks post-transplant). In contrast, LTRCs repopulate NOD/SCID mice as efficiently as NOD/SCID-*β2-microglobulin* null mice and have extensive self-renewal activity. However, LTRCs are much rarer than STRCs and hence their output of mature progeny in NOD/SCID-*β2-microglobulin* null recipients of unseparated $CD34^+$ cell suspensions is initially obscured by the output of cells from the co-transplanted STRC-Ms and STRC-MLs [32,33]. The lower panel shows a diagrammatic breakdown of the output patterns expected from each of 3 types of repopulating cells expected to be present in the $CD34^+$ cells transplanted in the upper panel. When all 3 subsets engraft (as occurs in the NOD/SCID-*β2-microglobulin* null mice), they produce a combined pattern that is the same as shown by the solid line in the upper panel. In contrast, the dotted line in both panels indicates an exclusive contribution of cells from the LTRCs which is the same in both types of host.

ed using the same procedures and endpoints as have been devised for normal hematopoietic cells. However, whereas the leukemic clone dominates all later myeloid compartments in most patients, including the uncommitted cells that produce colonies of erythroid, granulopoietic and megakaryocytic cells, the most primitive compartments are typically still dominated by normal cells (Fig. 1) [23,36].

3. Phenotypic biomarkers of CML stem cells

Flow cytometry has been used extensively to characterize functionally defined subsets of primitive normal and CML cells. These studies have shown that both share the same low forward and side light scattering characteristics indicative of a small size and agranular cytoplasm and a cell surface phenotype that includes the expression of CD34, Thy1/CD90 and AC133/CD133, and a lack of expression of CD38, CD45RA and CD71 as well as several lineage (lin) markers that characterize different types of mature blood cells. The latter include the T-cell markers: CD3, CD4 and CD8, the NK cell marker: CD56, the B-cell markers: CD19, CD 20 and CD45RA, the monocyte and granulocyte markers: CD13, CD14 and CD66b, the megakaryocyte and platelet marker: CD61, and the erythroid cell markers: CD36 and Glycophorin A [37–43]. Notably, the $lin^-CD34^+CD38^-$ fraction of *BCR-ABL*$^+$/Ph^+ cells in CP CML patients contains the majority of transplantable leukemic stem cells, as defined functionally by their activity *in vitro* as LTC-ICs and by their sustained repopulating cell activity *in vivo* in immunodeficient mice. These findings mirror results accumulated for normal adult human cells with these functionally defined activities. Similarly, the phenotypically more differentiated $CD34^+CD38^+$ CML cells contain the majority of transplantable progenitors detectable *in vivo* as short term repopulating cells (STRCs) and *in vitro* as CFCs [24,34,44].

Other phenotypic markers used to distinguish murine HSCs from closely related cells include an ability to efflux Rhodamine-123 (Rho) and Hoechst 33342 [45, 46]. In primitive hematopoietic cells, these 2 properties appear to be mediated by different members of the ATP-binding cassette (ABC) family of transporters: P-glycoprotein [47] and Abcg2/Bcrp1 [48], respectively. Detection of verapamil-inhibited fluorescence is typically used to relate lack of Rho retention to P-glycoprotein activity in primitive hematopoietic cells. Similarly, Abcg2 activity in these cells has been related to their verapamil-sensitive ability to efflux Hoechst 33342 – often visualized as the generation of a verapamil-sensitive side population (SP) in FACS profiles that depict fluorescence emissions in 2

wave lengths [49]. However, in the mouse, it is now clear that both Rho and Hoechst 33342 efflux activities in HSCs vary with their cycling status [50], a phenomenon that extends to many other surface antigens originally thought to be characteristic of these cells in the normal adult (e.g., CD34 and CD11b/Mac1) (reviewed in [51]). Therefore many markers that are useful indicators of HSCs in normal adult mouse bone marrow are no longer applicable when these cells are perturbed. Normal adult human HSCs also show an ability to efflux Rho [52], consistent with their quiescent status, whereas, many primitive CML cells do not efflux Rho [37]. However, this may simply reflect the fact that a higher proportion of CML LTC-ICs are actively cycling [23].

The situation in the case of the SP phenotype is more complex. In the mouse, HSCs can also be seen to vary in their possession of a SP phenotype according to their cycling status, although the precise relationship between these 2 parameters has not been delineated. Indeed, it is likely that other factors play a role in controlling the possession of an SP phenotype since changes in this property may not precisely mirror changes in cycling activity. Moreover, in humans, all NOD/SCID repopulating cells in the fetal liver have an SP phenotype [53], whereas the only SP cells in adult human tissue that have any repopulating activity appear to be lymphoid restricted. [54]. Interestingly, SP cells have recently been identified in several solid tumors, including samples of neuroblastoma, breast cancer and lung cancer [55,56]. In the case of AML, expression of CD123 (the alpha chain of the IL-3 receptor) has been described as a reliable marker of leukemic stem cells, including $CD34^+CD38^-$ CML cells, but not their normal counterparts [57,58].

4. Deregulated biological properties of CML stem cells

Thus functionally defined CML stem cells appear very similar phenotypically to their normal counterparts with certain exceptions. These exceptions can generally be related to the expression of markers that vary according to the activation or cycling status of the cells and hence may simply reflect differences in the proportion of CML stem cells that are proliferating at any given time. Evidence that primitive CML cells have a higher proportion of their members in cycle was first provided by studies that measured the sensitivity of primitive clonogenic cells to a brief exposure to high specific activity ^{3}H-thymidine. These experiments showed that primitive normal CFCs are predominantly quiescent whereas their CML counterparts are predominantly cycling [59,60]. Later, evidence of an increased turnover of CML LTC-ICs was obtained by examining their distribution in different phases of the cell cycle defined using Hoechst 33342 and Pyronin Y staining [61]. The more discriminating power of these latter experiments also revealed that, at any given moment, a significant fraction of the $CD34^+$ leukemic cells from CP CML patients are quiescent. This quiescent population includes some members of the leukemic $CD34^+CD38^+$ compartment as well as many of the leukemic $CD34^+CD38^-$ cells and many of the leukemic LTC-ICs [62]. Nevertheless, when these quiescent CML cells were stimulated by growth factors in single-cell suspension cultures, they rapidly entered the cell cycle and produced leukemic progeny. Entry of these cells into a proliferative mode was also demonstrated when they were placed in culture with stromal cells under conditions used to detect LTC-ICs, or when they were transplanted into sublethally irradiated NOD/SCID mice. Together these observations clearly demonstrate that the quiescent status of primitive CML cells is transient and reversible. The fact that this reversibility can be demonstrated *in vivo*, points to the likely importance of these cells in sustaining and spreading the disease in patients. Nevertheless, the existence of such primitive, transiently quiescent CML cells may explain the inability of chemotherapy to eradicate CML, as well as the late relapses occasionally seen many years after a transplantation procedure has been performed [63,64]. Indeed, primitive quiescent CML cells have now been found to also be highly resistant to IM and other *BCR-ABL*-targeted therapies [65, 66].

We have also provided evidence that CML stem cells have an intrinsically determined, reduced self-renewal ability [23,67] which is recapitulated in the leukemias that are caused by *BCR-ABL*-transduction of primitive murine HSCs [68]. This reduced self-renewal ability results in an increased rate of differentiation of CML stem cells as compared to their normal counterparts under conditions where their proliferation is stimulated. Thus, under "normal" conditions when the majority of normal HSCs are quiescent, CML stem cells depend on their increased rate of turnover to accumulate. However, when conditions that are potently mitogenic to all HSCs are induced, either *in vitro* or *in vivo*, the net growth of normal HSCs is initially greater than that achieved by CML stem cells [23,69]. In contrast,

at subsequent stages of leukemic progenitor differentiation (primitive CFCs), continuation of a heightened proliferative activity gives the leukemic cells a marked advantage thus offering an explanation for the ability of the *BCR-ABL*$^+$ cells to acquire clonal dominance in spite of a prevalence of normal HSCs.

Multiple mechanisms that contribute to a heightened proliferative activity of primitive CML CFCs involving both positive and negative regulators of their normal counterparts have been described. For example, we have shown that primitive CML cells are unresponsive to certain chemokines (MIP-1α, MCP-1 and SDF-1) that inhibit the cycling of primitive normal cells [70–74]. These findings have been interpreted as indicating a common pathway used by all of these chemokines to suppress proliferation in different types of primitive hematopoietic cells which the *BCR-ABL*-encoded oncoprotein can subvert or override. However, regardless of the ultimate mechanism identified, it is important to note that primitive CML cells are not resistant to all inhibitors of normal progenitor cycling activity. The turnover of both is similarly regulated by TGF-β [75,76], thus providing a possible explanation for the finding that many of the most primitive CML cells are quiescent at any given moment.

5. Molecular biomarkers based on specific deregulated gene expression in CML stem cells

As described above, leukemic stem cells from chronic phase CML patients have been difficult to study owing to their rarity and phenotypic overlap with normal HSCs. Most research on the effects of *BCR-ABL* expression has therefore focused on identifying the various signal transduction pathways that are activated in *BCR-ABL*$^+$ murine and human cell line model systems or in clones generated from primitive murine bone marrow cells that have been transduced with a *BCR-ABL* cDNA and only a few studies have been able to investigate *BCR-ABL*-mediated alterations in gene expression in primary CML stem cells or their closely related derivatives. Nevertheless, understanding the molecular mechanisms of deregulated gene expression in these critical cells may well provide important clues for developing new diagnostic and therapeutic strategies, including strategies for understanding and treating disease progression. For example, a recent unexpected finding is the relative lack of responsiveness of highly enriched populations of primitive CML cells to IM and other *BCR-ABL*-targeted drugs as compared to their more differentiated progeny (Fig. 3) [36,77]. A second finding is the evidence of a heightened genomic instability in primitive CML cells of likely relevance to their accumulation of IM resistant mutations and other changes that cause the disease to progress [78–80]. Both of these findings have intensified interest in more fully delineating the properties (biomarkers) of primitive CML cells that may explain these novel features.

5.1. Deregulated expression of BCR-ABL in CML stem cells

Three different groups have independently reported that *BCR-ABL* expression is highly elevated in the most primitive subset of lin$^-$CD34$^+$CD38$^-$ CML cells and is then rapidly and progressively reduced as these cells differentiate [66,77,81]. Interestingly, the levels of *BCR-ABL* transcripts present in the most primitive CML stem cells are much higher than those present in the G_0 fraction of CML CD34$^+$ cells (by a factor $\sim$ 50) [77]. In addition, the pronounced changes in *BCR-ABL* expression seen in different subsets of chronic phase CML stem cells do not mirror the changes seen in expression of *BCR*. Expression of *BCR*, like *BCR-ABL*, decreases progressively as CML cells differentiate, but with a maximum change overall of only 10 to 15-fold (as compared to $>$ 100-fold for *BCR-ABL*) [77]. This disparity suggests the operation of a novel mechanism that leads to a marked increase in the levels of *BCR-ABL* transcripts in very primitive CML cells. Investigating the basis of this deregulated expression of *BCR-ABL*, the possible role of the primitive cell context in which it occurs, and its possible generality to other fusion oncogenes will be of considerable interest in future studies.

The observed changes in *BCR-ABL* transcript levels also result in changes in p210$^{BCR-ABL}$ oncoprotein expression and activity, although these appear to be less pronounced [77]. Since CRKL is a major target of p210$^{BCR-ABL}$ kinase activity [82–84], it would then be expected that the levels of phosphorylated CRKL in the most primitive CML cells would also be highest, as is, in fact observed [77]. Subsequent studies in *BCR-ABL*$^+$ cell line models have provided definitive evidence that the level of *BCR-ABL* expression alone can be a determinant of IM responsiveness [77,85].

5.2. Activated autocrine mechanism in CML stem cells

One of the earliest findings from studies of *BCR-ABL*-transduced hematopoietic cell lines was the acti-

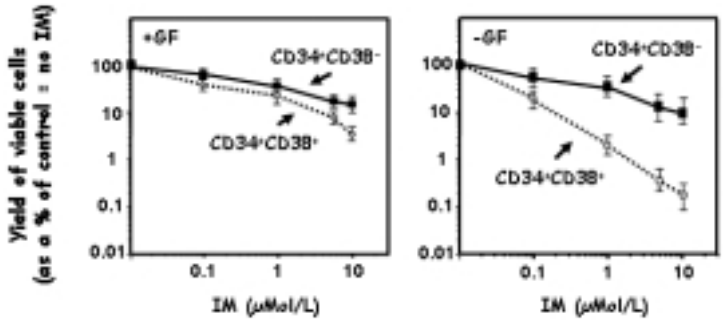

Fig. 3. Intrinsically determined lack of responsiveness of CML-stem cell enriched populations to IM. The graphs show a comparison of the differential recovery of viable cells from suspension cultures initiated with purified $CD34^+CD38^-$ (solid lines) and $CD34^+CD38^+$ (dotted lines) CML cells and then incubated for 3 weeks in the presence of varying concentrations IM, either in the presence (left panel) or absence (right panel) of growth factors. Under either condition, the leukemic $CD38^-$ subset is more resistant than the $CD38^+$ subset to IM (relative reduction in viable cells recovered due to IM is less). Moreover, this differential is enhanced when cell survival and proliferation is forced to depend on the autocrine mechanisms possessed by both sets of primitive leukemic cells used to initiate the cultures. Data is taken from Ref 77. GF = growth factors.

vation of an autocrine mechanism which could confer partial or complete growth factor autonomy in cells that were previously factor-dependent [86,87]. However, for many years, this finding was considered a cell line anomaly because a similar abnormality was not evident in studies of primary CML cells. Then, when it became feasible to look for alterations in gene expression in small numbers of cells, we discovered that most $CD34^+$ CML cells display a constitutively activated production of IL-3 and granulocyte-colony stimulating factor (G-CSF) [67]. This gives these cells the expected factor-independent phenotype and accounts for their increased levels of phosphorylated STAT5. Consistent with these latter observations was the finding that the autocrine production of IL-3 and G-CSF in primitive CML cells is silenced when they become quiescent, and is then reversed when they begin to proliferate [62]. Activation of this autocrine mechanism appears to be an intrinsically determined property of *BCR-ABL*$^+$/Ph^+ cells, since it reappears in the $CD34^+$ progeny of CML cells that are regenerated in transplanted sheep [88] and mice, and is rapidly induced when primitive normal human [89] or murine [90–93] hematopoietic cells are transduced with *BCR-ABL*.

The combination of an increased turnover and decreased self-renewal ability of primitive CML cells has been recognized for many years, as noted above [23]. However, a molecular mechanism that might account for this duality has remained elusive. The simultaneous discovery that very primitive normal hematopoietic cells exposed to excessive concentrations of IL-3 and G-CSF *in vitro* show an intense proliferative response that is coupled to diminished self-renewal activity was therefore of great interest [94]. Accordingly, we speculated that the self-stimulation of primitive CML cells by autocrine IL-3/G-CSF contributes to both of these abnormal features of their biology. Similarly, at later stages of normal $CD34^+$ cell differentiation, the same 2 growth factors would be expected to simply promote cell survival and proliferation. It would thus be anticipated that an autocrine IL-3/G-CSF loop active in $CD34^+CD38^+$ CML cells might, likewise, contribute to their competitive expansion potential.

Quantification of the levels of IL-3 and G-CSF transcripts in different subsets of CML cells has shown that these are both highest in CML stem cells and then progressively decrease together, even before the cells leave the $CD34^+CD38^+$ compartment [77]. Thus during the differentiation of primitive CML cells, the level of expression of *BCR-ABL* correlates with the expression of IL-3 and G-CSF. This observation is of some practical relevance given the reduced IM sensitivity displayed by $lin^-CD34^+CD38^-$ CML cells, as compared with the $lin^-CD34^+CD38^+$ cells *in vitro* in the absence of exogenously provided growth factors. Recently, it was found that *in vitro* pulsing of initially quiescent CML progenitors with G-CSF prior to and following treatment with IM promotes the re-entry of these IM-insensitive cells into the cell cycle and their significantly enhanced elimination as compared to IM treatment alone [95]. Autocrine production of GM-CSF in CML cells has also recently been reported together with evidence suggesting that this mechanism may contribute to IM and nilotinib-resistance in *BCR-ABL*$^+$ progenitors through activation of the JAK/STAT pathway [96]. Taken together, the autocrine production of cytokines in primitive CML cells is likely to be a multifaceted, but important mechanism contributing to

responses to *BCR-ABL*-targeted therapies and itself a potential additional therapeutic target.

5.3. Deregulated expression of ABC transporters in CML stem cells

Many members of the ABC transporter superfamily promote the uptake or efflux of specific drugs and can therefore be clinically important determinants of intracellular drug concentrations achievable *in vivo* or *in vitro* [97]. One particularly intriguing property of stem cells is that they express high levels of 2 particular ABC transporters. One of these is ABCB1 (also called P-glycoprotein) and the other is ABCG2 (also called breast cancer resistance protein, or BCRP) [48,98,99]. IM is a substrate of ABCB1 and overexpression of the *ABCB1* gene has been implicated as a cause of IM resistance in CML [100–105]. However, this finding has not been universally supported [106,107]. In cell lines engineered to overexpress *ABCG2*, IM has been found to interact with the protein product [108–111] by competitive attachment of IM to the ATP-binding site on ABCG2. Thus, IM is not a substrate of ABCG2 and ABCG2 does not efflux IM. Rather, IM is an inhibitor of ABCG2 activity.

In primitive CML cells isolated directly from chronic phase CML patients, *ABCG2*, like *ABCB1*, is overexpressed relative to the same subsets of primitive normal cells (Fig. 4) [77,112]. However, mice deficient in either *Abcb1* or *Abcg2* are viable, fertile and have normal HSC compartments [113,114]. This indicates that none of these genes are individually required for normal HSC growth or maintenance, although mice lacking these genes do show increased sensitivity to the lethal effects of drugs such as vinblastine, ivermectin and mitoxantrone, consistent with a role of these ABC transporters in protecting cells from toxins.

OCT-1, another transporter, appears to be a major regulator of IM uptake [115,116]. Interestingly, lower OCT1 transcript levels have been reported in IM nonresponders by comparison to IM responders in a study cohort of 30 CML patients [117]. In addition, it has been found that primary CML cells with lower than average OCT1 activity display reduced sensitivity to IM *in vitro*. The combination of low expression of OCT1 and elevated expression of *ABCB1* and *ABCG2* might thus be expected to contribute to a poor therapeutic response. We have found that this transporter phenotype is exactly what is seen in the most primitive CP CML cells by comparison both to their normal counterparts and to their more differentiated progeny (Fig. 4) [77]. Conversely, transcript levels for *ABCB1* and *ABCG2* are highest in the normal HSC-enriched subset and lowest in the most mature normal cells and this difference is further exaggerated in the corresponding subsets of CML cells. Taken together, these findings indicate a variety of perturbations that likely contribute to the relative IM resistance of the most primitive CML cells.

5.4. Deregulated expression of AHI-1 in CML stem cells

Another gene that may cooperate with *BCR-ABL* at the stem cell level is a novel oncogene, *AHI-1*, (*Abelson helper integration site-1*) [118]. Ahi-1, the murine gene, was originally discovered by analysis of the site of proviral insertions in various v-abl and myc-induced murine leukemias and lymphomas [119–124]. Subsequent demonstration of a high frequency of Ahi-1 mutations in certain virus-induced murine leukemias provided more direct evidence that *Ahi-1* has leukemogenic activity [118]. This gene encodes a modular protein with a SH3 domain, SH3 binding domains, and multiple WD-repeat domains, suggesting novel signaling activities. Interestingly, the conserved homologous human gene (*AHI-1*) encodes an additional coiled-coil domain in the N-terminal region of the protein which is entirely missing from the mouse Ahi-1 protein.

Ahi-1/AHI-1 is normally expressed in the most primitive murine and human hematopoietic cells and then shows similar specific patterns of downregulation in different lineages [125]. Thus downregulation of *AHI-1* expression appears to be an important conserved step in the normal regulation of early hematopoietic cell differentiation. Interestingly, *AHI-1* transcripts levels are abnormally elevated in K562 cells, a line derived from a CML patient in blast crisis, and also in primary CML cells from patients at all stages of their disease, but not in many types of patients with Ph^- leukemias. Moreover, in CML, the highest levels of *AHI-1* transcripts are found in the leukemic stem cells. Thus, there is a similar pattern of upregulated *BCR-ABL* and *AHI-1* expression in primitive CML cells. This may, in fact, be important to the loss of stem cell self-renewal capacity and accelerated activation of differentiation programs that is characteristic of CP CML cells. Accordingly, mechanisms that impede the normal downregulation of expression of *AHI-1* in primitive hematopoietic cells, or that inappropriately stimulate its expression in immediate cells, might be predicated to have leukemogenic sequelae. Indeed, we have recently found that overexpression of Ahi-1 alone in primitive murine

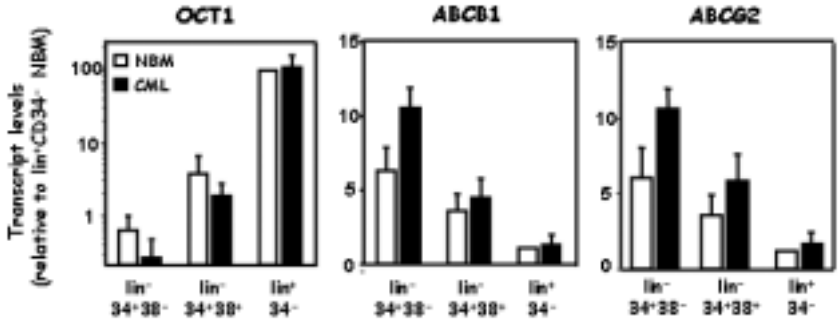

Fig. 4. Altered expression of certain transporter genes in primitive CML cells. The reduced expression of *OCT1* and the increased expression of *ABCB1* and *ABCG2* in primitive normal human bone marrow (NBM) cells is exaggerated in their CML counterparts, as shown by quantitative real-time PCR measurements of the RNAs present in extracts of the purified cell fractions indicated ($\text{lin}^-\text{CD34}^+\text{CD38}^-$ = stem cell enriched, $\text{lin}^-\text{CD34}^+\text{CD38}^+$ = intermediate progenitors, $\text{lin}^+\text{CD34}^-$ = terminally differentiating cells). These findings suggest that the most primitive CML cells express reduced levels of the transporter required to bring IM into the cell, and increased levels of transporters that can export and/or bind IM. Data is taken from Ref 77.

hematopoietic cells confers on them a proliferative advantage *in vitro* and a lethal leukemogenic activity *in vivo*. Moreover, both of these effects are enhanced by co-transduction of the cells with *BCR-ABL* [126]. Taken together, these findings suggest a potential role of *AHI-1* in mediating or enhancing the transforming activity of *BCR-ABL* in CML cells and possibly in contributing to the subsequent progression of the disease to a more acute form.

5.5. *Global gene expression analyses of chronic phase CML stem cells*

Kronenwett R and co-workers reported the first comparison of the total transcriptome of CD34^+ CML cells with their normal counterparts from adult bone marrow and mobilized peripheral blood using both cDNA arrays and oligonucleotide arrays [127,128]. Evidence of activation of the MAPK, PI3K/AKT, JAK/STAT, MYC and RAS pathways in the primitive CML cells were detected as predicted. These studies also demonstrated that the expression of several genes involved in DNA repair, including *RAD23A*, *ERCC1*, *ERCC3* and *ERCC5*, was significantly reduced (2 to 4-fold) in the CML cells supporting the concept that the DNA repair machinery in CML cells is abnormal [129,130]. Abnormal adhesion and homing are additional reported features of primitive CML cells [131–134], and it is therefore interesting that *N-CADHERIN*, *L-SELECTIN*, *CXCR4* and *CCR2* transcripts were also found to be downregulated in CD34^+ CML cells as compared to their normal counterparts. Another gene implicated in CML and that showed altered ($\sim$ 5-fold lower) expression in these studies was *IRF8* (*ICSBP1*). The biological importance of reduced *IRF8* expression in the pathogenesis of CML has been suggested by studies showing that *Irf8* deletion in mice causes the development of a CML-like myeloproliferative syndrome [135] and forced expression of *Irf8* inhibits the growth of *BCR-ABL*-transformed primary murine bone marrow cells [136]. Conversely, transcripts for the leptin receptor (LEPR), SKI (a transcriptional co-repressor) and the adenosine A receptor (a neurobiological receptor gene also expressed in G-CSF-mobilized normal blood progenitors [137]) were increased in CML cells. In this regard, it is interesting that treatment of CML cells *in vitro* with DPCPX, an antagonist of the adenosine A1 receptor, suppressed the formation of erythroid and granulopoietic colonies by CML progenitors more than by normal bone marrow progenitors. Finally, evidence of activation of the proteasome-ubiquitin protein pathway in primitive CML cells was also evident from the same gene expression comparisons also consistent with biological evidence of a selective effect of proteasome inhibitors on CML progenitors [138,139].

Although the *BCR-ABL* gene is believed to be both necessary and sufficient to initiate the development of CP CML, it is not known whether changes in other genes not evident at a cytogenetic level may also commonly contribute to this process. Thus, differentially expressed genes revealed by previous studies might not necessarily be deregulated as a direct result of expression $\text{p210}^{\text{BCR-ABL}}$, but reflect additionally acquired mutations or epigenetic abnormalities. To identify genes whose expression in primitive human hematopoietic cells are directly upregulated by expression of *BCR-ABL*, Verfaillie's group prepared a cDNA library from extracts of *BCR-ABL*-transduced cells from which cDNAs present in control human CD34^+ cord blood cells had been subtracted [140]. About half

of the several dozen clones thus identified were homologous to known genes. One of these was *NUP98*, which has been implicated in many translocations associated with the development of AML [141]. Others appeared to encode novel proteins. Although some of these findings were confirmed by gene expression analysis of primary CML cells, not all of the changes identified were reversed by IM treatment.

Transcriptome profiling has also been used to try to identify biomarkers of IM responsiveness. Such studies have identified genes associated with NFκB activation, cell adhesion [142], DNA damage repair, oxidative stress responses [143] and drug metabolism [144]. One study attempted to generate a risk assessment method based on the application of a subset of genes selected from initial array data to a test group of 12 IM-treated patients (10 responders and 2 nonresponders). The results assigned all of the responders and one of the nonresponders to the low-risk group, with the other nonresponder being assigned to the intermediate-risk group. These data suggested that clinical responses to IM treatment might be predictable by certain gene expression parameters.

5.6. Indicators of disease progression

The onset of disease progression from CP to blast crisis is well known to be highly variable if cytogenetic remissions are not achieved, in spite of the absence of other mutations detectable by standard cytogenetics. The classic view has been that the progression of CML is caused by the acquisition of a rare second event. Such events have been thought to occur stochastically, but at a higher rate within the CP clone than in their normal counterparts due to the greater proliferative activity of the primitive CML cells in concert with their genomic instability [36]. Predicting such changes is clearly of considerable clinical importance in selecting an optimal therapeutic option since the presence of even undetectable levels of blast phase disease is likely to prevent durable remissions from being achieved unless myeloablative chemotherapy and a stem cell transplant is given. An interesting attempt to address this question was recently reported by Yong et al. [145]. They first compared the $CD34^+$ leukemic cells from 3 CP patients who progressed to blast crisis within 3 years of diagnosis and 10 whose blastic transformation took place more than 7 years after diagnosis. This comparison revealed 20 genes whose expression was significantly different between the 2 groups and this difference was confirmed by quantitative reverse transcriptase PCR measurements for 18 of these. Application of a multivariate Cox regression model to a complete cohort of 68 patients then showed an association of 2 of these with more prolonged survival; i.e., a low expression of CD7 in combination with a high expression of either proteinase 3 or elastase.

6. Future directions

Accumulated knowledge of the unique biological properties of CP CML stem cells underscores the need for more comprehensive information about their molecular alterations. This is crucial to allow newer therapies to be devised and deployed with greater predictive power of the risk-benefit for individual patients. We anticipate that much of this anticipated progress will come from large scale comparisons of the genome, transcriptome and proteome of more highly purified populations of normal and CML stem cell populations. However, such studies will also need to be accompanied by technical improvements for performing such studies with adequate depth and fidelity on the very small numbers of cells that can realistically be isolated from an individual sample ($< 10^3$). As a first step towards this goal, we have recently developed a modified Serial Analysis of Gene Expression (SAGE) protocol based on the PCR-LongSAGE method [146]. This innovation allows global transcriptome profiling to be usefully applied to as few as 10^3 primary hematopoietic cells. As a first proof-of-principle experiment, we further showed how this methodology could be used to identify novel transcripts in a 200,000 tag LongSAGE library prepared from extracts of FACS-purified lin^-CD34^+ normal human adult bone marrow cells [146]. More recently, we have constructed and sequenced similar libraries from the $CD38^+$ and $CD38^-$ subsets of the $CD34^+$ CML population from several CP patients and their phenotypic counterparts in normal adult human hematopoietic tissues. We anticipate that the results of comparative analyses of these datasets and additional meta-comparisons with published transcriptome data will provide important new clues about key molecular properties of CML stem cells.

7. Conclusion

The discovery of IM has revolutionized the treatment of CML. While the vast majority of early CP patients continue to do well with an excellent quality of life,

there is a smaller cohort of patients that are not well served by treatment with this agent. It is these patients along with more advanced phase patients that require alternative therapeutic options. To date, the most sensitive method for assessing the effectiveness of IM therapy is to monitor peripheral blood *BCR-ABL* transcript levels using real-time PCR. This strategy can usually forecast the emergent expansion of resistant subclones but is not able to detect the original presence of the mutant or progressed CML stem cells from which such subclones are presumed to arise. Here we describe a number of properties of CP CML stem cells that distinguish them from normal HSCs and from the bulk of the CML clone which is comprised of leukemic cells that have begun to differentiate into various mature blood cell types. Some of these features of CML stem cells can already account for their abnormal growth characteristics, their innate insensitivity to *BCR-ABL*-targeted agents (as well as conventional chemotherapeutic drugs), and their tendency to generate mutant derivatives. New approaches for investigating CML stem cell features and their diversity in individual patients should greatly facilitate the goal of predicting individual patient responses and the development of more effective therapies for those not optimally served by current regimens that rely on single *BCR-ABL*-targeted agents.

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