Chronic myeloproliferative disease with and without the Ph chromosome – some unresolved issues

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Introduction

Progress in understanding the biology of the chronic myeloproliferative disorders (MPD) continues apace. Whereas there was a tendency in recent years to regard Ph-positive CML as an entity quite distinct from the Ph-negative myeloproliferative disorders, increasingly the categorisation proposed originally by Dameshek in 1951, whereby all the co-called myeloproliferative diseases form part of a spectrum with perhaps more similarities than differences, proves to be 'correct'. In this paper we have considered very recent developments in five closely related areas, namely the molecular mechanisms that might underlie progression of CML from chronic phase to blastic transformation, some of the approaches that might be useful to target residual CML stem cells in treated patients on the assumption that they may have the capacity to regenerate the full clinical picture of CML, the issue of what sort of molecular or epigenetic lesions might define the clinical phenotype in $JAK2^{V617F}$-positive disorders, and finally newer approaches to targeting JAK2 for in clinical practice. In each case there seem to be lessons that could apply equally to the Ph-positive and Ph-negative diseases.

What causes CML to progress?

Treatment of early chronic phase CML with imatinib significantly reduces the rate of progression (1) but transformation is still a major therapeutic challenge as in the majority of those patients who do progress to blast crisis the response to tyrosine kinase inhibitor (TKI) therapy is not durable (2,3). This is often attributed to the heterogeneous nature of the advanced phase of CML (4) in which a variety chromosomal and molecular abnormalities are usually present, some of which may play a role in maintaining the transformed state, such as by inactivation of tumor suppressor genes (5,6). In the TKI era the molecular changes

cause disease progression. The precise molecular events predisposing to blastic transformation are also unknown although activation of Wnt and Hedgehog signalling and inhibition of the protein phosphatase 2A (PP2A) may all make major contributions (9-16).

The molecular changes mentioned above could be secondary since evidence is now accumulating to support the concept that the greatly increased BCR-ABL1 kinase activity in $CD34^+$ granulocyte-macrophage progenitors (GMP) could be the primary determinant of disease progression (4-6), as a series of epigenetic changes which determine the phenotype of blast crisis $CD34^+/CD38^+/CD45RA^+$ GMP seems to depend on increased BCR-ABL1 activity (4,17). Differentiation arrest of GMPs depends on the ability of high BCR-ABL1 activity to activate MAPK(ERK1/2) that, in turn, enhances the translation inhibitory effect of the RNA binding protein hnRNP E2 on C/EBPα, the major regulator of myeloid maturation (17,18). Likewise, the self renewal ability of GMP results from BCR-ABL1-independent (19) and –dependent (9, 20) signals leading to inhibition of glycogen synthase kinase 3β (GSK3β) and consequently to activation of β-catenin, and mutations that generate a mis-spliced and thereby inactive GSK3β mRNA have been detected in ~50% of blast crisis patients (11). Furthermore, BCR-ABL1 dramatically perturbs the CML transcriptome resulting in altered expression of genes, of which some (e.g. PRAME, MZF1, EVI-1, WT1 and JUN-B) might also play a role in disease progression (5, 10, 21, 22).

The post-transcriptional, translational and post-translational effects of high levels of BCR-ABL1 may also be important. They may result in activation of factors with reported mitogenic and anti-apoptotic activity (e.g. MYC, JAK2, LYN, STAT5, BMI-1, PI-3K/AKT and BCL-2-related proteins) and as well as inhibition of major key regulators of cellular processes such as p53 and PP2A (4-6). Indeed suppression of PP2A is essential for induction and maintenance of BCR-ABL1-generated oncogenic signals and also allows BCR-ABL1 to be expressed at high levels (23). These postulated BCR-ABL1 dose-dependent mechanisms of altered gene regulation, clearly not limited to those cited above, may explain blastic transformation in CML patients who do not appear to have any chromosomal or molecular abnormalities, and may also explain the relative sensitivity at least initially of blast crisis cells to TKIs. Interestingly, the BCR-ABL1 oncoprotein increases the incidence of point mutations and chromosomal aberrations by simultaneously inducing pathways that lead to accumulation of free radicals

the SRC family member LYN kinase has also been reported (27), TKI-resistance might also be a factors that facilitates disease progression.

Furthermore, it is quite possible that genetic instability of CML blast crisis depends both on an increased propensity of CML chronic phase progenitors to undergo genetic changes and on the probability that one of the mutations induced by BCR-ABL1 or by some other mechanism functions as an "amplifier" of a genetically unstable phenotype. Indeed, there is also a cohort of CML patients (~15%) presenting with deletions of the derivative chromosome 9, whose leukemia may be more prone to genomic instability *ab initio* (28); such patients used to progressed to blast crisis much more rapidly than CML patients lacking the der9 deletion, though this may not be so in the 'imatinib era'. The same considerations may apply to the recently described GATA-2 L359V mutation and the loss of chromosome Y observed in CML patients undergoing blastic transformation (29, 30).

To date, strong evidence supports the idea that dosage of BCR-ABL1 kinase activity plays a pivotal role in many CML patients undergoing progression and that in some cases secondary genetic or chromosomal abnormalities can facilitate transformation, while in other cases they just influence the aggressiveness of the already transformed CML blast crisis progenitor cell clone. Indeed, ~30% (pre-imatinib era) (31,32) and ~50% (post-imatinib era) (33) of blast crisis patients do not exhibit chromosomal abnormalities and presumably only a fraction of these patients have molecular inactivation of the p53 gene, yet their overall survival is only marginally better than that of patients with chromosomal abnormalities. Thus, the crucial answered question is "What controls BCR-ABL1 expression and activity during progression?" A possible scenario might include a BCR-ABL1 autoregulatory loop that amplifies signals which positively influence BCR-ABL1 gene transcription and enhances its protein stability by preventing its proteasome-dependent degradation. Conversely, we cannot exclude the possibility that there could be a single genetic lesion, still unidentified, that occurs in chronic phase CML with high frequency and predisposes to blastic transformation.

Targeting leukemia stem cells

Various lines of evidence favor the conclusion that leukemia stem cells (LSC) are rarely if ever eradicated by treatment with TKIs. Although TKIs induce rapid hematologic and cytogenetic responses in the majority of chronic phase (CP) CML patients (1), relapse of the disease is generally observed when TKI treatment is interrupted. A recent study conducted on 14 CML patients treated with imatinib (IM) for a minimum of 4 years showed that BCR-ABL1 levels were maintained in the primitive HSC (CD34+, CD38- fraction) despite continued IM treatment (34). Mathematical models have been used to analyse the *in vivo* kinetics of disease response

which promotes enhanced proliferation and malignant transformation. A FTI made by Bristol-Myers Squibb, BMS-214662, significantly reduces quiescent CML stem cell numbers through induction of apoptosis (42). It synergises with TKIs to overcome the anti-proliferative effect exerted by these agents on CML stem and progenitor cells (42). Although BMS-214662 exerts a strong FTI activity *in vitro*, its overall mechanism of action in killing the LSC fraction is still unclear.

Another drug with strong potential for targeting quiescent CML stem cells is the sphingosine

When the mutated JAK2^{V617F} is expressed in bone marrow cells by retroviral transduction (45-49) or in transgenic mice (50-54) MPD phenotypes ranging from thrombocytosis to polycythemia and in some cases myelofibrosis can be observed. These results have been interpreted as evidence that MPD is initiated by JAK2^{V617F} as a single step process. However, several observations suggest that in patients with MPD the situation is more complex and that

including regulation of genes at the epigenetic level or mediated by microRNAs, and post-translational protein modification.

Epigenetic abnormalities in cancer cells point to cell-heritable defects that affect gene expression and occur as a result of two main mechanisms: DNA methylation and modifications (acetylation or methylation) of histones (71). A large body of information exists concerning both hypermethylation at specific gene loci, particularly of tumor suppressor genes, and global DNA hypomethylation in cancer cells from solid tumors or hematologic neoplasia. However, studies on epigenetics in MPDs are still scanty, but there is increasing evidence indicating that some genes, which are supposedly involved in MPD pathogenesis, can be abnormally regulated at the epigenetic level. For example, reduced levels of SOCS3, a member of the family of suppressors of cytokine signaling which function as negative regulators of the JAK2 signaling pathway, have been reported in cells from MPD patients, particularly those with primary myelofibrosis (PMF), and have been ascribed to promoter gene hypermethylation (72,73). Another example is SDF1-receptor CXCR4, that is abnormally down-regulated in the $CD34^{+}$ hematopoietic progenitor cells that constitutively circulate in PMF patients. Reduced transcriptional activity of CXCR4 is caused by hypermethylation at specific CpG islands of the promoter, that reverted to normal after short-term incubation with the demethylating agent 5-aza-deoxycitidine; furthermore, a significant reduction in the proportion of *in-vitro* generated $JAK2^{V617F}$ mutated cells was observed after long-term incubation of $CD34^{+}$ cells with a combination of 5-azacitidine and an HDAC inhibitor (74). These treatments also resulted in correction of the abnormal *in vitro* migratory characteristics of $CD34^{+}$ cells (75) and in their

JAK2^{V617F} mutated cells, including EEC formation, through post-transcriptional down-regulation of JAK2 (82).

Orchestrating gene expression in normal adult cells and during development is one role of microRNAs (miRNAs), a large family of small non-coding RNAs. However, miRNAs can also function either as oncogenes or oncosuppressors (83) in human cancer, including acute or chronic leukemias. There are data indicating that miRNAs may be abnormally regulated in MPD cells, usually by a general down-regulation as reported in other cancer cells. Conversely, some miRNAs were found over-expressed and this apparently correlated with JAK2^{V617F} mutation (84,85); in particular, there are preliminary data suggesting that miRNA-16 can be involved in the abnormal expansion of erythroid compartment in PV (86). However, we need more information about miRNA gene targets and the mechanisms underlying their differential expression in MPDs.

Additional complexity can originate from abnormal post-translational protein modification, as recently discovered by the group of Green (87) who studied deamidation of the anti-apoptotic protein Bcl-x_L. Bcl-x_L is known to be upregulated in both CML and PV, and recent data indicate that a BH3 mimetic peptide induces apoptosis in JAK2^{V617F} high-allele burden PV erythroblasts, preventing their proliferation and inhibiting the generation of EECs (88). In normal cells, Bcl-x_L deamidation in response to etoposide or radiation-induced DNA damage is a mechanism for deleting mutated cells through a DNA-damage induced apoptotic pathway. It was found that induced Bcl-x_L deamidation is prevented in JAK2^{V617F} or BCR-ABL mutated cells; the fact that incubation of PV or CML myeloid cells with JAK2 inhibitors or imatinib, respectively, restored the Bcl-x_L deamination pathway, would support a causal link between the defective deamination response and the aberrant tyrosine kinase activity. It is tempting to speculate that accumulation of clonal cells harboring damaged DNA due to inadequacy of Bcl-x_L deamination might facilitate stepwise progression of PV or CML to acute leukemia. Additional support for the role of defective modifications of key proteins in MPD cells comes from the observation that SOCS3 is unable to exert its negative regulation of JAK/STAT signaling because the turnover of the protein is reduced, unlike SOCS1; in fact, exogenous SOCS3 was actually found to promote, rather than to reduce, the proliferation of murine cell lines expressing JAK2^{V617F}, possibly aas f5a0.0488 TD MTmb4 re pos

the other hand there is rapid evolution of targeted therapies designed to block these mechanisms. Until now the treatment of the Ph- MPDs has been by far most effective in the earliest phases of disease. Specifically, for PV and ET agents such as anagrelide and hydroxyurea have clearly reduced the risk of both thrombotic and hemorrhagic events. Such drugs have not however been very valuable in the later phases of disease, especially for those patients with late phase primary myelofibrosis or myelofibrosis arising after earlier4 ET or PV (post ET/PV/MF) (90). Indeed, current therapies have been unable to prevent progression to either these phases or to acute leukemia (90). Allogeneic stem cell transplant can cure patients with advanced myeloproliferative disorders but is still associated with an appreciable risk of short and long-term morbidity and mortality; moreover the increasing average age of patients with MPDs means that only a minority are really good candidates for transplant procedures.

The discovery of several key MPD associated mutations has broadened the therapeutic horizons for these disorders significantly (91). Starting with the discovery of the $JAK2^{V617F}$ mutation in exon 14 of JAK2, there are now 10 mutations described in JAK2 exon 12. Additionally five mutations thus far have identified in the thrombopoietin receptor MPL. All of these mutations seem to feed into a final common pathway of cellular activation through the PI3 kinase pathway, the STAT pathway, and the MAP kinase pathway (91).

Various therapeutic strategies are being developed to try to block the proliferative stimulus associated with these MPD associated mutations. Current testing of therapeutic inhibition of these inhibitors can be divided up into three groups, specifically pre-clinical (based on in vitro activity against $JAK2^{V617F}$ containing cells), those with ongoing testing in murine models, and those undergoing testing in clinical trials. Although there is a pipeline of between 10 to 20 agents with reported in vitro or murine model activity, we will focus on those agents in which clinical activity has already been reported in the public forum. JAK2 inhibition clinical results can be divided into 3 categories of agents. The first comprises novel small molecules designed and tested for specificity and selectivity against JAK2 (INCB 018424, XL019, TG101348). The second comprises agents which inhibit a variety of kinases including JAK2 (ITF2357, CEP-701). The third comprises agents which have previously demonstrated clinical activity in Ph-MPDs in which their impact on JAK2 (and $JAK2^{V617F}$) allele burden is being measured, such as pegylated interferon alpha-2

JAK2 Inhibitors for myelofibrosis. The most mature clinical experience for a JAK2 inhibitor is for INCB018424 (Incyte Co, Wilmington, DE) (selective against JAK1 and JAK2) with the largest PMF trial in history (>120 patients). This agent leads to significant reduction in

Table 1: Currently reported efficacy for JAK2 inhibitors from clinical trials in patients with myelofibrosis

	Anemia	Splenomegaly	Constitutional symptoms	Pruritus	⇓JAK2 burden	Myelo-proliferation	Reference
INCB018424	<10%	X	X	X	10%	X	(92)

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