Myeloproliferative Diseases

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A Unique Activating Mutation in JAK2 (V617F) Is at the Origin of Polycythemia Vera and Allows a New Classification of Myeloproliferative Diseases

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Myeloproliferative disorders (MPDs) are heterogeneous diseases that occur at the level of a multipotent hematopoietic stem cell. They are characterized by increased blood cell production related to cytokine hypersensitivity and virtually normal cell maturation. The molecular pathogenesis of the MPDs has been poorly understood, except for chronic myeloid leukemia (CML), where the Bcr-Abl fusion protein exhibits constitutive kinase activity. Since some rare MPDs are also related to a dysregulated kinase activity, a similar mechanism was thought to be likely responsible for the more frequent MPDs. We investigated the mechanisms of endogenous erythroid colony formation (EEC) by polycythemia vera (PV) erythroid progenitor cells and found that EEC formation was abolished by a pharmacological inhibitor of JAK2 as well as an siRNA against JAK2. JAK2 sequencing revealed a unique mutation in the JH2 domain leading to a V617F substitution in more than 80% of the PV samples. This mutation in the pseudokinase autoinhibitory domain results in constitutive kinase activity and induces cytokine hypersensitivity or independence of factor-dependent cell lines. Retroviral transduction of the mutant JAK2 into murine HSC leads to the development of an MPD with polycythemia. The same mutation was found in about 50% of patients with idiopathic myelofibrosis (IMF) and 30% of patients with essential thrombocythemia (ET). Using different approaches, four other teams have obtained similar results. The identification of the JAK2 mutation represents a major advance in our understanding of the molecular pathogenesis of MPDs that will likely permit a new classification of these diseases and the development of novel therapeutic approaches.

Polycythemia Vera and the Other MPDs

The myeloproliferative disorders (MPDs) were initially thought to include four different diseases: chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (IMF). Subsequently, hypereosinophilic syndrome (HES) and chronic neutrophilic leukemia (CNL) were included in the classification of MPDs. These disorders are hematological malignancies that arise from the transformation of a multipotent hematopoietic stem cell. Clonal hematopoiesis is characteristic of CML, PV, IMF and, at least, some cases of ET. Except for IMF, all MPDs are characterized by increased levels of blood cell production with predominance of one myeloid cell lineage and no marked alterations in cellular maturation. Each of the MPDs has a propensity to progress towards acute leukemia, yet they differ with respect to the rate of transformation to blast crisis. Many complications of the MPDs, such as thrombosis in ET and PV, are related to the overproduction of mature blood cells.

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Pathogenesis of MPDs

The initial classification of the MPDs was based on clinical, morphological and biological criteria. After the discovery of the Philadelphia chromosome (translocation t:9;22) and the identification of the Bcr-Abl fusion protein, a new classification based on the Bcr-Abl molecular marker was developed. Several lines of evidence established that the constitutive Bcr-Abl kinase activity was at the origin of CML, as demonstrated by the successful cytogenetic response obtained in patients treated with imatinib mesylate (Gleevec), an inhibitor of Kit, PDGFR and Abl tyrosine kinase activities. Subsequently, a constitutive tyrosine kinase activity was also implicated in other MPDs or related disorders: activating mutations in *c-kit* in systemic mastocytosis (SM),[1] chimeric *FGFR1* transcripts in some atypical form of CML[2] and the FIP1L1-PDGFRA fusion protein in a subpopulation of patients with HES.[3]

In most instances, the molecular pathogenesis of the MPDs was identified by the examination of recurrent chromosomal translocations (i.e., CML or atypical CML)[4] or as the consequence of a clinical response to tyrosine kinase inhibitors.[3] By contrast, recurrent chromosomal translocations were not found in patients with PV, ET and IMF. Nevertheless, a clinical response to imatinib was described in some PV patients, but it is not known whether this response is due to an inhibition of Kit signaling.[5]

Hypersensitivity to Cytokines in PV and Other MPDs

A constant hallmark of MPDs is an abnormal response to cytokines. As early as 1974, it was demonstrated that a subpopulation of late erythroid progenitors in PV could proliferate in vitro in the absence of erythropoietin (Epo).[6] This assay was subsequently used to distinguish primary polycythemia from secondary forms of erythrocytosis. Nevertheless, EEC formation is not specific for PV since it is also seen in some ET, IMF and CML patients or in patients suffering from thrombotic diseases, such as the Budd Chiari syndrome but no evidence of an MPD. A small fraction of erythroid progenitors in PV are truly Epo-independent, but the major abnormality is hypersensitivity to numerous growth factors including Epo, GM-CSF, IL-3, SCF and IGF1.[7,8] Similarly, megakaryocyte (MK) progenitors are hypersensitive to thrombopoietin (Tpo) in ET and IMF.[9] Numerous studies have ruled out autocrine stimulation or a cytokine receptor defects as causes of the abnormal cytokine response: EEC formation in PV was not blocked by neutralizing anti-Epo antibodies; no significant Tpo synthesis by MK was documented in ET and IMF; no quantitative EpoR defect was found in PV; no genomic mutation in *EpoR* or *c-mpl* (TpoR) was seen in PV, ET and IMF. Together, these results suggested that the cytokine hypersensitivity was likely due to a downstream signaling defect and that the *Bcr-Abl* negative MPDs (PV, ET and IMF) were accompanied by a constitutive kinase activity. Alternatively, a mutation (activating or loss of function) in a phosphatase gene, as illustrated by the juvenile myelomonocytic leukemia model,[10] could also result in a similar phenotype.

Among the three main subtypes of *Bcr-Abl* negative MPDs, PV is certainly the most straightforward model to utilize in order to define the underlying molecular pathogenesis since cytokine-independent terminal erythroid differentiation only depends on Epo signaling. In contrast, the main abnormality in ET and IMF involves MK proliferation and differentiation which are dependent upon not only Tpo, but also other cytokine signaling events.

Epo/EpoR Signaling

Epo and the EpoR are crucial for mature red blood cells formation. In the absence of Epo or EpoR, committed erythroid progenitors undergo apoptosis at the CFU-E and proerythroblast level.[11] Equally important is the Janus kinase JAK2 bound to EpoR and activated upon Epo binding to the receptor.[12] The knock out of JAK2 in mouse embryos is lethal at embryonic day 12.5 due to the absence of mature red cell formation in the fetal liver. Thus, Epo, EpoR and JAK2 are absolutely required for red blood cell formation. The number of EpoRs on the cell surface of erythroid precursor cells is low (typically 500-2000 receptors/cell) and the EpoR has apparently evolved to be poorly expressed at the cell surface. Thus, the EpoR must be efficiently activated by Epo in conditions where receptor numbers at the cell surface are low. One strategy by which this may be achieved is the ligand-independent dimerization of the EpoR on the cell surface.[13] Upon Epo binding, the dimer conformation changes in such a way that a rotation in the transmembrane and cytosolic juxtamembrane α-helix is required to activate JAK2[14] (**Figure 1**; see Color Figures, page 545). JAK2 is bound to the EpoR cytoplasmic domain in the absence of ligand activation. In fact, binding of the N-terminus FERM domain of JAK2 to sequences encompassing Box1 and Box2 of the EpoR cytoplasmic domain is required for trafficking the EpoR to the cell surface.[15] The presence of JAK2 promotes receptor trafficking to the Golgi and acquisition of a mature glycosylation pattern.

Signaling by EpoR starts with JAK2 activation. Three EpoR cytoplasmic hydrophobic residues located before Box1 seem to be crucial for activating JAK2.[14] These residues however are not required for the chaperone effect of JAK2 on EpoR trafficking. Upon receptor activation, JAK2 becomes phosphorylated at the activation loop Y1007, Y1008 and at many other Y residues, such as at Y570 (with inhibitory effect) and at Y221 and Y813 (with positive effects on signaling).[16,17] JAK2 also phosphorylates the EpoR cytoplasmic Y residues. All these p-Y residues in EpoR and JAK2 become recruitment sites for STAT5, STAT3, STAT1, SH-PTP1, CIS and shc/grb2. As a consequence, EpoR signaling activates STAT, MAP-kinase, PI-3-kinase and AKT. The biological consequences of this cascade of events are survival, proliferation and differentiation of erythroid progenitors.

JAK2 as a Candidate Gene Involved in PV Pathogenesis

Several lines of evidence suggested that JAK2 was the most likely candidate gene involved in the pathogenesis of PV. First, JAK2 is directly involved in the intracellular signaling following the exposure to cytokines to which PV progenitors display hypersensitivity (Epo, GM-CSF, IL-3,TPO and more or less SCF and IGF-1). Second, spontaneous activation of STAT3 was found in the granulocytes of 30% of PV patients[18] and an increased AKT phosphorylation was observed in erythroid progenitors after cytokine stimulation[19] (**Figure 1**; see Color Figures, page 545). In erythroblasts derived from EEC, the anti-apoptotic mitochondrial protein Bcl-xL regulated by Epo signaling through STAT5 activation was shown to be increased.[20] Moreover, different kinase inhibitors, including a JAK2 inhibitor are known to block EEC formation.[21] Similarly in IMF, spontaneous MK growth was blocked by a STAT5 dominant negative.[22] Third, JAK2 is involved in EpoR[15] and Mpl[23] trafficking from the endoplasmic reticulum to the cell membrane, and the processes involving Mpl trafficking/maturation are altered in PV and IMF.[24] Fourth, although not recurrent, a common cytogenetic abnormality in PV is a gain in 9p where the JAK2 gene is localized. In addition, loss of heterozygosity (LOH) of chromosome 9p was found in 30% of PV patients identifying a large genomic region of DNA as a target for the search for a potential candidate disease associated genetic defect.[25]

A Unique Acquired Clonal JAK2 Mutation Is Found in PV, ET and IMF

A recurrent unique acquired clonal mutation in JAK2 was recently found by five different research teams. This mutation was observed in a majority of PV patients and a significant proportion of patients with other MPDs, including ET, IMF and some rare MPDs. Our group worked on the mechanisms leading to EEC formation and identified JAK2 as a potential candidate gene because JAK2 kinase inhibitors as well as a *JAK2* siRNA inhibited EEC.[26] Three other teams sequenced *JAK2* as a consequence of kinase gene sequencing in MPDs.[27-29] Finally, one team mapped the minimal region on chromosome 9p to a 6.2 Mbp interval that contains several genes including *JAK2*.[30]

A unique valine to phenylalanine substitution at position 617 (V617F) in the pseudokinase JAK2 domain has been identified. The incidence of this mutation in PV patients is very high (78% of 506 patients), ranging from 65% to 97% depending on the study. These variations in percentage of patients involved is likely due to the clinical criteria used for diagnosis, either the Polycythemia Study Group (PSVG) or World Health Organization (WHO) classifications, and also the sensitivity of the assay used to detect JAK2 V617F. The *JAK2 V617F* mutation was, however, not found in some patients who were shown to have both monoclonal hematopoiesis and to form EEC, demonstrating that some cases of true PV are related to other molecular defects (our own unpublished results). Sequencing of genomic DNA from granulocytes was used in most studies to detect the mutation.[26-30] The sensitivity of this technique is difficult to assess precisely, but it is likely that a threshold of 5% to 15% cells harboring the mutation is required for such an analysis to be positive. In one study, the mutation was detected in 97% of the PV samples (71/73) with a sensitive PCR assay.[27] The variable prevalence estimates of the JAK2 V617F in MPD patients therefore may be related to the fact that in some patients with JAK2 V617F, less than 10% of granulocyte JAK2 alleles are JAK2 V617F alleles, suggesting that MPD patients could retain normal stem cell clones. These data are surprising since PV is considered to be a clonal disease with the majority of myeloid cells, including granulocytes, belonging to the clone. Further studies are, therefore, required to confirm these data.

The *JAK2* mutation is somatic and occurs at the level of a hematopoietic stem cell. In all studies, the mutated *JAK2* was found in myeloid cells, i.e., bone marrow cells, granulocytes, platelets and erythroblasts derived from $CD34^+$ cells, but not in T cells. In addition, mutant *JAK2* was found in hematopoietic colonies derived from hematopoietic progenitor cells.[27] In contrast, the mutation was not present in non-hematopoietic cells in most patients.[28,30] Since familial MPDs have been reported, it was important to know if this mutation can be germ-line. Linkage analysis did not show co-segregation with the 9p locus suggesting that the V617F substitution occurred as a somatic mutation even in these families. The similar frequency of the V617F mutation in familial and sporadic PV is in favor of this assumption[31] (Najman, personal communication). In one study, however, the mutation was detected in buccal swabs of 4% of the patients.[28] This finding may be due to contamination by granulocytes because in another study two patients positive for the mutation after analysis of buccal swabs were negative in cells within hair follicles.[30]

The *JAK2 V617F* mutation has been found in other *Bcr-Abl* negative MPDs with a frequency of about 50% in IMF and more variably in ET (from 25% to 57%).[26-28,30] In many cases, the mutation was detected only at the threshold of the sequencing technique in ET. This may signify that hematopoiesis is not entirely clonal in many ET patients and that the mutation must be searched by more sensitive techniques or directly in platelets. The *JAK2* mutation was recently found in other MPDs with a frequency of about 20% in atypical CML and unclassified MDS and at a lower frequency (2%-3%) in HES, CMML or SM.[32] By contrast, the mutation was not detected in AML, except for AML secondary to an MPD or megakaryocytic leukemia.[32,33] Thus, the presence of *JAK2 V617F* mutation provides a means of developing a new classification of MPDs that includes the majority of PV cases, a large fraction of cases of IMF and some cases of ET (the precise frequency remains to be determined) and some atypical MPDs. This mutation opens new avenues both for the diagnosis and the classification of MPDs.

Direct causative relationship between this mutation and PV

JAK kinases are composed of four conserved domains which include a N-terminal FERM domain required for interaction with cytokine receptors, an SH2 domain followed by a pseudokinase (JH2, from JAK-homology 2) and a C-terminal kinase (JH1, from JAK homology 1) domain. The V617F mutation is located in the JH2 pseudokinase domain of JAK2, which is involved in inhibition of the kinase activity.[34,35] Several point mutations in the JH2 domain, like Y570F and E665K lead to constitutive JAK2 kinase activity.[16,17,36] A model structure of JAK2 attributes an important function to amino acid residues around position 618 in the interaction between JH1 and JH2 and thus in the auto-inhibitory function of JH2.[37] It is likely that the V617F substitution leads to a gain of function as confirmed by two observations: when transfected into factor-dependent cell lines, the mutant JAK2 is auto-phosphorylated and STAT5, ERK/MAP kinase and PI3K/AKT are activated in the absence of any cytokines.[26] As a result, factor-dependent cells become hypersensitive to cytokine, with only a minority of cells acquiring cytokine independence.[26,28,30] The effects of V617F JAK2 are even more striking in cell lines expressing the EpoR suggesting that this mutation has a more dramatic effect on the Epo/EpoR signaling.[26]

A direct link between the *JAK2 V617F* mutation and PV is suggested by at least three lines of evidence: i) the mutation is found in all spontaneous erythroid colonies cloned from the marrow or peripheral blood of PV patients; ii) the mutation induces IGF-1 hypersensitivity in cell lines and a defect in Mpl trafficking (unpublished observation) and iii) introduction of a *JAK2 V617F* DNA into murine HSC followed transplantation into recipient mice and hematopoietic reconstitution leads to erythrocytosis with splenomegaly. In these mice, significant increases in granulocyte or platelet counts were not initially observed.[26]

How does the JAK2 V617F mutant induce EPO hypersensitivity?

At present, it is unknown whether the mutant JAK2 requires a cytokine receptor to scaffold and signal or whether it signals independently of receptor association. In JAK2-deficient cells, the mutant JAK2 could initiate a signal alone but it also transduces a signal from the EpoR upon Epo addition,[26] demonstrating that the mutant JAK2 binds to the EpoR cytoplasmic domain and transduces a ligand-dependent signal. Since both the mutant JAK2 and EpoR are expressed normally at low levels, it is likely that the mutant JAK2 uses and amplifies the signal normally triggered by EpoR. In the same cells or in retrovirally transduced Ba/F3 cells, the mutant JAK2 was able to promote trafficking of the EpoR to the cell-surface, albeit at a lower efficiency than a wild-type JAK2. Further experiments are still required to establish whether the mutant JAK2 also promotes down-regulation of activated EpoRs and whether the lower enhancement of trafficking is not due to this effect.

Our model would predict that the mutant JAK2 binds to EpoR while the receptor is in the endoplasmic compartment, the Golgi apparatus and the cell-surface. This binding promotes EpoR maturation but may also lead to a novel mechanism of cytokine receptor activation from the cytoplasmic domain (inside out signaling), which could occur before the EpoR reaches the surface. Bound JAK2 V617F would thus phosphorylate discrete tyrosine residues and would be autophosphorylated. These events would attract SH2-containing proteins to the EpoR-JAK2 complex and continuously activate STAT5, MAP-kinase, PI-3-kinase and AKT pathways **(Figure 1**; see Color Figures, page 545**)**. The dimeric conformation of the receptor might be switched on by this intracellular-to-extracellular activation. Additional experiments are needed to explore the ligand-independent EpoR tyrosine phosphorylation in cells that express the mutant JAK2. It remains possible that receptor signaling and phosphorylation might not require the "switch" residues of the cytoplasmic juxtamembrane domain, which are required for ligand-dependent activation of JAK2.[14]

The present evidence suggests that the JAK2 V617F binds to the cytoplasmic domain of the intracellular and cell-surface EpoR and that this event promotes ligand-independent signaling. Since constitutively active EpoR mutants have been shown to produce in vivo not only erythrocytosis but also thrombocytosis,[38] it is tempting to speculate that the interaction between EpoR and the mutant JAK2 may contribute not only to the pathobiology of PV but also to ET.

How does a single JAK2 mutation give rise to different phenotypes and diseases?

These data raise the intriguing question of how a single mutation might give rise to at least three different diseases (PV, ET and IMF) and also to some other atypical MPDs. Evidence from clinical, biological and pathology data indicates that ET, PV and IMF are related disorders. The evolution of ET to PV has been well documented, although some authors have stated these ET patients have been falsely diagnosed and represent in reality a forme fruste of PV. About 15% of ET patients progress to myelofibrosis and approximately 20% of PV progress to IMF (spent phase).[39] The boundaries between IMF and ET are not well defined during the so-called pre-fibrotic form of IMF.[40] In addition, biological features of PV, i.e., EEC, high PRV1 mRNA in granulocytes are also found in ET and IMF,[41] while diminished membrane expression of Mpl (TpoR) by platelets has also been described in ET and IMF.[24] Recent results suggest that these criteria except Mpl expression correlate with the *JAK2 V617F* mutation.

Three different events may explain how a single mutation might give rise to different phenotypes: transformation of different hematopoietic stem cells/progenitor cells, different genetic backgrounds and additional somatic mutations. It is unlikely that PV, ET and IMF reflect the trans-

formation of hematopoietic progenitors at different stages of differentiation. Indeed, there are many similarities between the biological properties induced by Bcr-Abl and V617F JAK2 abnormalities, both of which mimic cytokine signaling. There is evidence that Bcr-Abl exclusively transforms HSC and not later progenitor cells, because, in contrast to other oncogenes, it does not confer self-renewal properties to hematopoietic progenitors.[42] It is expected that the situation will be identical for the mutant JAK2, although it has been demonstrated that a constitutively active JAK2 can cooperate with Kit signaling to enhance self-renewal of multipotent progenitors.[43] The absence of the *JAK2* mutation in T cells from PV does not rule out that the transformation event can occur in an HSC. Indeed, this observation is reminiscent of the situation in CML, where Bcr-Abl is hardly detected in T cells. The possibility that a spontaneously active JAK2 is deleterious to mature T cells cannot, however, be excluded. Further studies are required to directly demonstrate that the *JAK2* mutation occurs at the level of the HSC.

It seems likely that some as yet unknown factors might modulate the activity of the mutant JAK2. The V617F substitution is a subtle mutation that only changes the basal JAK2 activation, otherwise all the biological properties of JAK2 are maintained including its binding to cytokine receptors. However, it is not known if the mutant JAK2 requires receptor binding or spontaneously oligomerizes on the membrane or in the cytosol to induce signaling. The highly transforming TEL-JAK2 protein that has lost all the regulatory and cytokine receptor elements of JAK2 induces direct signaling by oligomerization. Thus, it seems likely that slight changes in the V617F JAK2 kinase activity might profoundly change the phenotype of the disease. In favor of this hypothesis, the V617F mutation was found to be homozygous in about 30% of the PV, which correlates with the 9p LOH, which is due to a mitotic recombination and leads to a duplication of the mutated allele with a deletion of the normal allele.[28,30] This implies that the cells homozygous for the mutation have a clonal advantage over the heterozygous and non-mutated cells.[30] Usually, LOH is a mechanism of oncogenesis by which tumor suppressor genes are inactivated. To our knowledge, LOH has never been associated with gain of function mutations. Thus, gene dosage and the presence of a normal JAK2 may influence the activity of the mutant. Indeed, normal JAK2 is capable of inhibiting STAT5 activation induced by the mutant. Furthermore, expression of normal JAK2 in a cell line made factor-independent by the mutant restores cytokine dependency.[26] The competition between the two JAK2s, wild type and mutated, is not directly due to inhibition of the mutant since wild type JAK2 does not inhibit auto-phosphorylation of the mutant[28] but may occur on their substrate(s) by competing for cytokine receptor binding. One might hypothesize that the level of kinase activity modulates the phenotype of the disease. The level of kinase activity could be modified by gene dosage, by polymorphisms of JAK2 or by cooperating genetic events, such as changes in a phosphatase or a SOCS gene, which encode for proteins that normally attenuate JAK2 signaling from cytokine receptors. We hypothesize that a low kinase activity would lead to ET, an intermediate level to PV and a very high level to IMF. Alternatively, the phenotype of the disease may be dependent on genetic events not related to the V617F JAK2 kinase activity. These different hypotheses can now be tested both in patients and in animal models of MPD.

Conclusion

The discovery of the *JAK2 V617F* mutation is a major advance in enhancing our understanding of both the molecular pathogenesis and the clinical aspects of PV and several other *Bcr-Abl* negative MPDs. This observation opens new avenues for fundamental and clinical researches and has direct implications for the diagnosis and classification of MPDs. Research can now focus on MPDs that are negative for the V617F mutation, which might constitute a distinctive entity. Further work is also needed to determine how a single mutation in JAK2 gives rise to three different diseases. Moreover, the *JAK2 V617F* mutation offers a molecular target for new drug discovery.

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