

Basic Sciences of the Myeloproliferative Diseases: Pathogenic Mechanisms of ET and PV

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Abstract

The molecular pathogenesis of ET and PV is unknown, although the relatively indolent clinical course observed in most patients suggests that the defect may be subtle and difficult to establish. Clonality analysis using X-chromosome inactivation patterns in females on purified CD34+ cells have confirmed that a defect is present in the hematopoietic stem cell. However, at least in ET, a significant proportion of patients have polyclonal hemopoiesis, and this presumably reflects the heterogeneous nature of the disorder(s). Attention has focussed on the potential disruption of the physiological regulators EPO and TPO and their respective receptors. In familial disorders, pathological mutations have been identified in some, but by no means all, cases: EPO receptor mutations in PFCP, TPO mutations in FT and, conversely, TPO receptor (c-mpl) mutations in CAMT. Equivalent ligand or receptor mutations have not been detected in ET or PV patients. However, there is evidence to suggest that c-mpl expression may be dysregulated, with low or absent c-mpl mRNA or protein reported in ET and/or PV patients. At present it is not clear whether this is the cause or consequence of the paradoxically normal/increased TPO levels found with both primary and secondary thrombocytosis. In vitro culture analysis has demonstrated both cytokine independence and hyper-sensitivity as a generalised feature of progenitor cells from many patients, but differences exist depending on the assays used and there is little understanding of the mechanism(s) underlying these responses. Two genes have recently been identified with increased mRNA expression in PV granulocytes: PRV-1, a novel cell surface receptor closely related to the uPAR/Ly6/CD59/snake toxin family of proteins, and NFI-B, a member of the nuclear factor I family which may be associated with TGF-beta resistance. Investigation of their regulation and biological effects may assist in determining the pathobiology of these elusive disorders.

Although the chronic myeloproliferative disorders (MPDs) polycythaemia vera (PV) and essential thrombocythaemia (ET) are characterised by abnormally high levels of cells of a particular haemopoietic cell lineage, respectively erythroid and megakaryocytic cells, there are few clues at present to the underlying mechanisms leading to this increase. The relatively indolent clinical course in most patients suggests that the defect(s) may be subtle and difficult to establish. The two disorders share many common features and attempts to categorise patients are often limited by an inability to group patients together according to defined specific markers. For example, many PV patients have a significant thrombocytosis, conversely a significant proportion of ET patients evolve

into PV, and either may develop myelofibrosis or transform to acute myeloid leukaemia.

It is likely that, at least in part, this cross-over reflects their common origin and developmental programme. For example, in some models of haemopoiesis, an erythroid/megakaryocytic progenitor cell is included in the pathway between the multipotential progenitor cell (CFU-GEMM) and cells committed to a single lineage. Transcription factors involved in directing lineage specificity may be common to both cell types, e.g. GATA-1, originally thought to be the factor responsible for determining expression of erythroid-specific genes, is now known to play an important role in directing expression of megakaryocytic genes. Furthermore, unlike myeloid

cells where there is a certain degree of redundancy in the haemopoietic growth factors responsible for directing their growth and maturation, erythroid and megakaryocytic cells each have a lineage-specific growth factor, erythropoietin (EPO) and thrombopoietin (TPO), which does not appear to cross react with other cells.

Unlike congenital or inherited disorders where pathological situations can often be traced to abnormalities in single genes, PV and ET are almost certainly multifactorial disorders, requiring acquisition of two or more alterations for disease presentation. A number of cellular and molecular differences have now been identified in blood and bone marrow samples from PV and ET patients which may help improve diagnosis and further our understanding of the pathogenesis of these complex disorders.

1. Clonality Studies

Clonal markers have been used to demonstrate the stem cell origin of the MPDs. Cytogenetic abnormalities are relatively uncommon in both PV and ET. Of those that have been detected in more than a single individual, some are not specific to the MPDs, e.g. deletion of chromosome 20q, and/or are present in only a small proportion of patients, e.g. trisomy 8 or 9 [1]. Clonality status in PV and ET patients has therefore predominantly been examined using X-chromosome inactivation patterns (XCIPs) [2]. Although a "broad brushstroke" approach to disease, it has nevertheless yielded useful information. The study is limited to female patients who are informative for an X-linked polymorphism, who do not have a constitutionally skewed pattern nor age-related skewing in their myeloid cells. In practice, this involves comparison of the pattern in the cell of interest (progenitor cells, granulocytes, reticulocytes or platelets) with control tissue of the same origin (T cells), and exclusion of females with a skewed XCIP in their granulocytes but not T cells who are ≥ 65 years (an arbitrary age).

XCIP studies of PV and ET have shown that they are heterogeneous disorders when defined according to clonality status. Although many patients have the expected clonal pattern, some, who fulfil the current PVSG criteria, do not. In PV only a few such patients have been reported [3-5], but the results are particularly striking in ET where 4 different studies have demonstrated a significant proportion of patients with polyclonal myelopoiesis, varying from 35-57% when age and constitutional skewing are taken into account [6-9]. These results raise a number of issues, in particular whether they represent a varying spectrum of the same disease or are different diseases.

Polyclonal myelopoiesis in PV or ET may represent a preliminary disease phase which only progresses to clonal disease with the acquisition of a pathogenic alteration providing the cell with a growth or survival advantage. Certainly in ET, many patients are asymptomatic and are often only picked up by chance through a blood test for unrelated reasons. At present there is

little evidence for progressive disease as prospective analysis of XCIPs in patients from diagnosis with many years of follow-up have not been reported. None of the polyclonal ET patients from our own institution have shown alterations in XCIPs that might reflect a slowly emerging clone (Allen and Gale, unpublished observations). Furthermore, the polyclonal XCIP did not change in the one young patient we studied who evolved from ET to PV. Similarly, no clinical progression to myelofibrosis or acute myeloid leukaemia has been observed in six monoclonal patients over three or four years, nor in nine patients with polyclonal disease. Chuisolo et al ('01) found concordant XCIPs in purified CD34+ progenitor cells, granulocytes and platelets from 18 ET patients, 9 with skewed and 9 with balanced patterns [8], indicating that in most patients clonal selection has already occurred in the earliest identifiable progenitor cell. Although normal polyclonal progenitor cells may remain in patients with clonal disease but be suppressed within the marrow environment, infusion of CD34+ cells from four ET patients into NOD/SCID mice reproduced the same XCIP in the mice as in the patient (Gale, Ings and Harrison, unpublished observations).

For the patient, the most important consequence of clonality status is whether this information can assist in determining the underlying pathology and clinical course of their disease. In ET, for example, although clonal and polyclonal patients do not differ with respect to age, platelet count, splenomegaly and incidence of haemorrhage, studies have shown that patients with polyclonal disease may have a decreased risk of thrombosis [7,8], or that they require less cytoreductive therapy [10]. Clearly, this difference cannot be considered as absolute, as there are many risk factors for thrombosis, but it does provide a base from which to look for more specific markers that might have wider applicability to all patients.

2. Cytokines and Signalling Pathways

Since EPO and TPO are the physiological regulators of erythropoiesis and thrombopoiesis, considerable attention has been focussed on whether abnormalities in these molecules or their signalling pathways are involved in the pathology of PV or ET. Congenital disorders have provided clear evidence that mutations in a single gene can lead to erythrocytosis or thrombocytosis. Approximately 10% of families with primary familial and congenital polycythaemia have EPO receptor (EPOR) mutations which lead to truncation of up to 110 amino acids from the C-terminal tail [11]. This removes a negative regulatory domain that binds the phosphatase HCP-1 and leads to a proliferative hyper-responsiveness of erythroid cells to EPO. In familial thrombocythaemia, 4 different heterozygous mutations have been detected in the upstream regulatory domain of the TPO gene which all lead to increased protein levels [12-15]. The mutations remove the physiological inhibition of TPO translation by competition from non-productive peptides initiated from 1 of the 8 possible start sites and result in

increased translation [16]. Conversely, missense mutations or introduction of premature stop codons in the TPO receptor, c-mpl, can lead to congenital amegakaryocytic thrombocytopenia [17-19]. Total loss of c-mpl expression on platelets has been demonstrated in at least some of these patients, but all were homozygotes or compound heterozygotes, and their heterozygous parents or sibs were asymptomatic.

To date, equivalent mutations have not been identified in patients with PV and ET. In PV and some ET patients, serum EPO concentrations are low. Receptor number, structure and function are normal. Decreased transcript levels of an alternatively spliced EPOR isoform with a truncated intracytoplasmic tail (EPOR-T) and a putative dominant-negative role have been detected by RT-PCR analysis in blood and bone marrow from PV patients [20], transcript levels normally being higher than the full length form in immature haemopoietic cells [21]. In vitro studies have suggested that such truncated receptors may have increased cell surface expression [22]. However, the physiological significance of this finding is unclear.

Normal or increased serum or plasma TPO concentrations have been widely reported in ET, other MPDs and in reactive thrombocytosis (RT), an unexpected finding as TPO synthesis is thought to be constitutive and circulating concentrations regulated by and inversely proportional to the megakaryocyte/platelet mass. No mutations have been detected in the 5' regulatory region of the TPO gene in ET patients which might account for the normal/increased levels [23,24], and current evidence suggests that they are secondary to reduced clearance resulting from decreased c-mpl expression (see below) rather than an intrinsic defect in TPO *per se*. Increased levels of IL-6 may induce the normal/increased TPO levels observed in RT resulting from inflammatory causes [25], but IL-6 levels in ET patients are normal [26,27]. Other factors may, however, influence TPO concentrations, for example, the level of fibroblast growth factor-2 is increased in ET and PV patients [28] and recent studies have suggested that it can induce TPO expression in bone marrow stromal cells [29].

No mutations have been found in the coding sequence of c-mpl from ET or PV patients [30-33], and although transcripts from several alternatively spliced isoforms of c-mpl have been identified, no differences in their relative expression have been detected [30,33]. Increased levels of a c-mpl isoform with a 21bp deletion in the N-terminus of the extracellular domain have, however, been found in 6 of 7 PV patients [31], although the functional consequences have not been reported. Nevertheless, there is widespread evidence to suggest that c-mpl expression may be dysregulated in MPDs. Decreased or absent c-mpl mRNA and protein have been variously reported in platelets and bone marrow megakaryocytes from PV and/or ET patients [34-37]. The pattern and intensity of c-mpl staining of bone marrow megakaryocytes is characteristically heterogeneous in both ET and PV [37,38], and may be useful to discriminate PV from secondary erythrocytosis [39,40] and,

when combined with bone marrow angiogenesis, ET from RT [41]. Studies have shown a four-fold reduction in TPO clearance by ET platelets, which could therefore explain the normal TPO levels [33]. Significantly higher TPO levels than those observed would be required to cause sufficient down-regulation of surface c-mpl, which suggests that there is rather a defect in c-mpl synthesis, either through decreased transcription or mRNA stability, or an abnormal feedback loop [33,35]. In PV, it has been suggested that defective post-translational glycosylation of c-mpl may lead to impaired transport from the Golgi apparatus and reduced surface expression [42]. This defect could be demonstrated in purified CD34+ cells which showed increased viability in the absence of growth factors and were able to proliferate in response to TPO alone [43].

Other abnormalities in signalling pathways have been reported in PV. For example, 4 of 14 PV patients had constitutively activated STAT3 in their granulocytes [44]. This may reflect growth factor independence or hyper-sensitivity (see below). It may also contribute to the increased expression of the anti-apoptotic factor Bcl-X_L observed in PV erythroid precursor cells and its sustained expression in more mature cells [45]. Such results would suggest an increased survival of PV progenitors rather than, or in addition to, increased proliferation.

3. Cytokine Independence, Hyper-sensitivity and Inhibition

A hallmark feature of PV progenitor cells is the ability to form endogenous erythroid colonies (EECs) in the absence of EPO. True EPO independence as opposed to hyper-sensitivity was demonstrated in some, but not all PV BFU-E by the lack of inhibition with neutralizing monoclonal antibodies against EPO or EPOR [46], and by their growth in complete serum-free medium [47]. Instead it probably results from a hyper-sensitivity of PV progenitors to insulin-like growth factor-1 (IGF-1) [47]. Although there are conflicting reports on the basal and IGF-1 induced tyrosine phosphorylation in PV cells, no mutations have been detected in the IGF-1 receptor, and the effect may in fact be mediated through altered levels of IGF binding proteins [48,49]. PV cells demonstrate hyper-sensitivity to other haemopoietic growth factors including IL-3, GM-CSF, SCF and TPO, but although this might indicate a defect in a common signalling molecule, the mechanism is unknown. Often used as a diagnostic marker in PV, EEC formation is also found in a significant proportion of ET patients, although it has been suggested these patients will generally evolve into PV and therefore likely represent masked PV with thrombocytosis rather than true ET [50]. The proportion of patients who are diagnosed with ET and evolve into PV is, however, low. IGF-1 responses and binding profiles have not been reported in ET patients.

In ET, evaluation of cytokine responses still largely depends on the assay and cell source used. Many groups

have reported endogenous megakaryocytic growth, but early studies included plasma and cannot therefore distinguish between true independence and hyper-sensitivity. In serum-free cultures antibodies to IL-3, IL-6 and GM-CSF did not inhibit spontaneous growth, but there are conflicting reports regarding possible autocrine stimulation via TPO and c-mpl [32,51,52]. Several studies have shown that spontaneous CFU-MK growth disappears when purified CD34+ cells are cultured [51,53,54], suggesting that the cells may be hyper-sensitive to TPO. One study has reported that mononuclear cells from peripheral blood of 18 of 20 ET patients had a highly specific hyper-sensitivity to TPO when cultured in a complete serum-free semi-solid medium in the presence of IL-3 and retinoic acid [55], but liquid culture studies in our own laboratory of CD34+ cells purified from bone marrow found no difference between normal controls and 3 ET patients (Walker, Watts and Linch, unpublished observation).

Bone marrow CFU-MK from ET patients have been reported to have decreased sensitivity to the inhibitory effects of transforming growth factor- β (TGF β) (56), although patients have normal circulating TGF β levels of this factor [27]. Since TGF β strongly suppresses TPO- β induced growth of CFU-MK growth in a dose-dependent and differentiation stage-specific manner, possibly through a feedback loop involving induction of TPO by stromal cells and up-regulation of TGF β receptors I and II [57], these results may indicate defective TGF β signalling. Reduced TGF β receptor II mRNA levels have been reported in small numbers of PV and ET patients [58], but no receptor mutations have been detected [59,60]. In our own studies, we observed no difference between normal and ET bone marrow derived CD34+ cells in the degree of TGF β inhibition (Walker, Watts and Linch, unpublished observation). Further studies are therefore required to elucidate the role of aberrant cytokine responses in megakaryocytic proliferation in ET.

4. Recent "Global" Approaches

A number of studies have used a "global" approach to identify changes in PV and ET patients. Comparative genomic hybridization detected new cytogenetic abnormalities in one study, and in another identified gain of chromosomes 8, 9 or 9p but failed to detect 20q deletions [61,62]. A genome wide comparison of microsatellite markers in DNA from granulocytes and T cells or fibroblasts found loss of heterozygosity on chromosome 9p in 6 PV patients due to mitotic recombination [63]. Although no mutations in the coding sequence of 19 candidate genes within this region were detected, elevated expression of the transcription factor NFI-B was detected in granulocytes and CD34+ cells. A member of the nuclear factor 1 transcription factor family whose DNA consensus binding site is widely found in promoter sequences, *in vitro* overexpression of NFI-B caused apparent resistance to TGF β inhibition and it may therefore play a pathological role in PV pro-

genitor cell proliferation.

Overexpression in granulocytes of another gene called polycythemia rubra vera-1 (PRV-1) was obtained by subtractive hybridization of cDNAs from PV patients and normal controls [64]. The gene encodes a novel cell surface receptor which is most closely related to the uPAR/Ly6/CD59 family and its expression appears to be restricted to haemopoietic cells, only being detectable in normal bone marrow and to a lesser extent fetal liver. Of particular interest, though, was its apparent restriction to PV granulocytes. Low or absent expression was found in granulocytes from CML, myelofibrosis and most ET patients. Further studies have indicated that EEC+ but not EEC- ET patients have levels comparable to PV patients, suggesting as above that this may reflect masked PV [65]. The role of PRV-1 in disease pathogenesis is unknown, nor whether it marks a more widespread underlying defect as found in PNH cells; in fact MPD patients, in particular those with ET, have been reported to have slightly increased levels of CD55/CD59 deficient red cells [66]. Nevertheless, it may still provide a suitable diagnostic marker to discriminate PV from other MPDs.

Our current ability to diagnosis and treat PV and ET is severely restricted by the limited understanding of the pathogenesis of these enigmatic disorders. They are clearly complex and heterogeneous, and are likely to require multi-faceted approaches to determine their underlying pathology. This presents a considerable challenge, but provides a rich resource for identifying genes and pathways that are critical in the finely tuned homeostatic balance that determines normal haemopoiesis.

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