The JAK2 617V>F mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera

Sabrina Dupont,1 Aline Massé,1 Chloé James,1,2 Irène Teyssandier,3 Yann Lécluse,4 Frédéric Larbret,1 Valérie Ugo,5 Patrick Saulnier,6 Serge Koscielny,7 Jean Pierre Le Couédic,1 Nicole Casadevall,1,3,8 William Vainchenker,1 and François Delhommeau1,3,8

Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 790, Université Paris Sud, Institut Gustave Roussy, Villejuif;2INSERM, E217, Université Victor Ségalen Bordeaux 2, Bordeaux;3Assistance Publique–Hôpitaux de Paris, Laboratoire d’Hématologie, Hôpital Hôtel-Dieu et Hôpital Saint-Antoine, Paris;4Institut Gustave Roussy, Service Commun de Cytométrie, Villejuif;5Centre Hospitalier Universitaire de Brest, Laboratoire d’Hématologie, Brest;6Institut Gustave Roussy, Laboratoire de Recherche Translationnelle, Villejuif;7Institut Gustave Roussy, Department of Biostatistics and Epidemiology, Villejuif; and 8Université Pierre et Marie Curie, Paris, France

The JAK2 617V>F mutation is frequent in polycythemia vera (PV) and essential thrombocytopenia (ET) in patients with chronic idiopathic myelofibrosis (IMF). This activating mutation is believed to be a major event contributing to the pathogenesis of these myeloproliferative disorders (MPDs). However, the presence of the mutation in 3 different MPDs raises the question of whether other molecular events or genetic modifiers influence the phenotype. Accumulating evidence suggests that the clonal genotypic changes resulting from mitotic recombination are sufficient to promote erythrocytosis. However, the 2 main biologic characteristics of PV, erythropoietin (Epo) hypersensitivity of erythroid progenitors, and increased amplification of the erythroid lineage, should be studied in detail to evaluate clearly the consequences of clonal genotypic profiles of PV and ET progenitors.

In this study, we investigated the correlation of the levels of hemoglobin, white blood cells, platelets, and endogenous erythroid colonies to the levels of JAK2 617V>F to delineate the biologic features of ET and PV. We systematically genotyped individual erythroid, granulocyte, lymphomyeloid, and long-term culture-initiated colonies derived from bone marrow CD34+ cells isolated from JAK2 617V>F-positive ET and PV patients to study the short arm of chromosome 9 has been observed in approximately one third of PV patients. In this way, clones with the homozygous mutation of JAK2 are generated. Thus, it was hypothesized that patients with low JAK2 617V>F levels (mostly ET patients) would preferentially harbor heterozygous clones and that PV patients would have JAK2 617V>F homozygous clones. It has been demonstrated recently that most PV patients whose granulocytes have up to 50% of their alleles mutated harbor some JAK2 617V>F homozygous clones, whereas ET patients have only heterozygous and wild-type clones. These data suggest that the clonal genotypic changes resulting from mitotic recombination are sufficient to promote erythrocytosis. The 2 main biologic characteristics of PV, erythropoietin (Epo) hypersensitivity of erythroid progenitors, and increased amplification of the erythroid lineage, should be studied in detail to evaluate clearly the consequences of clonal genotypic profiles of PV and ET progenitors.

Introduction

The JAK2 1849G>T (617V>F) mutation has been detected in at least 90% of patients with polycythemia vera (PV), up to 70% of patients with essential thrombocytopenia (ET), and up to 50% of patients with chronic idiopathic myelofibrosis (IMF). This activating mutation is believed to be a major event contributing to the pathogenesis of these myeloproliferative disorders (MPDs). However, the presence of the mutation in 3 different MPDs raises the question of whether other molecular events or genetic modifiers influence the phenotype. Accumulating evidence suggests that the JAK2 mutation directly causes erythropoiesis in patients with PV. However, the JAK2 mutation has not been clearly implicated in ET and IMF pathogenesis. First, mouse models that retrovirally promote erythrocytosis. However, the 2 main biologic characteristics of PV, erythropoietin (Epo) hypersensitivity of erythroid progenitors, and increased amplification of the erythroid lineage, should be studied in detail to evaluate clearly the consequences of clonal genotypic profiles of PV and ET progenitors.

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influence of the clonal genotypic profile on Epo sensitivity and myeloid cell amplification. This work confirms that JAK2 617V→F–positive ET patients harbor mostly heterozygous clones and that PV patients frequently have some homozygous clones. However, some PV patients have a purely heterozygous profile, with no homozygous clones, which is characterized by a prominent heterozygous clone among the committed erythroid and granulocytic progenitors. These results suggest that erythroyctosis in PV not only arises from the emergence of a homozygous clone triggering Epo hypersensitivity or independence and late erythroid amplification but also from the amplification of a heterozygous clone upstream from the committed progenitor cell stage.

Patients, materials, and methods

Patients and samples

We enrolled 541 patients from January to December 2005 based on the suspicion that they had an MPD. The modified Polycythemia Vera Study Group (PVSG)14 and/or World Health Organization (WHO) criteria15 were used to diagnose PV and ET. Two hundred seventeen patients had a suspicion of PV, and 281 had a suspicion of ET. Of the 241 suspected PV patients, 179 (82.5%) harbored the JAK2 617V→F mutation in granulocytes or bone marrow mononuclear cells. Of the 179 suspected ET patients, 157 (55.9%) harbored the mutation. We obtained bone marrow samples taken from healthy subjects during hip surgery. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki, and the study was approved by the Ethics Committee of the Hôtel-Dieu Hospital (Paris, France).

Cell purification

Granulocytes and bone marrow mononuclear cells were isolated as described previously.1 Bone marrow mononuclear cells from 6 ET patients and 20 PV patients were stored in liquid nitrogen in 10% dimethylsulfoxide (Sigma, Lyon, France) in 90% fetal calf serum (FCS) (Stem Cell Technologies, Vancouver, Canada). After rapid thawing, we used a FACSDiva cell sorter (Becton Dickinson, Le Pont de Claix, France) to isolate CD34−, CD38−, and GPA− antibodies (Immunotech, Marseille, France). Colonies grown from burst-forming units–erythroid (BFU-E) and colony-forming units–granulocyte (CFU-G) were counted and picked on day 14. Liquid cultures supporting erythroid differentiation were performed by seeding 4 × 10^5 to 10^6 CD34+CD38− cells in serum-free medium containing 20 ng/mL SCF, 100 IU/mL IL-3, 10^−6 M dexamethasone (Sigma), and 1 IU/mL Epo. Nonadherent cells were collected, counted, and genotyped on day 7. We assessed erythroid differentiation by flow cytometry after labeling with anti-CD36-FITC and anti-GPA-PE antibodies (Becton Dickinson), and by May–Grunwald Giemsa (MGG) staining after cytocentrifugation.

Assessment of simultaneous B, NK, and granulomonoctytic differentiation

Bone marrow CD34+CD38− cells were incubated at one cell per well on a confluent layer of MS-5 cells in 96-well plates in RPMI medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% human serum, 5% FCS, 10 ng/mL IL-3, 50 ng/mL SCF, 50 ng/mL fms-like tyrosine kinase 3 ligand (FLT3-L) (Immunex), 10 ng/mL thrombopoietin (Tpo) (Kyrim laboratories, Tokyo, Japan), 20 ng/mL IL-7, 10 ng/mL IL-15 (Peprotech, London, United Kingdom), and 5 ng/mL IL-2 (Chiron Laboratories, Suresnes, France). The media was changed weekly and cells from wells with significant cell proliferation were collected after 4 to 6 weeks as described previously.16 We labeled part of the cell population with anti-CD15-FITC, anti-CD19-PE (Becton Dickinson), and anti-CD56-APC (Beckman Coulter, Villepinte, France) antibodies and used flow cytometry to determine cell phenotype. The remaining cells were pelletted for genotyping analysis.

Assessment of long-term culture-initiating cells

Long-term cultures were initiated by plating 1, 10, 25, 75, and 200 bone marrow CD34+CD38− cells per well onto a confluent layer of MS-5 cells in 200 µL of alpha MEM (Invitrogen) supplemented with 1% penicillin/streptomycin, 1% l-glutamine, 12% horse serum (Stem Cell Technologies), 12% FCS (Hyclone, Logan, UT), 10^−8 M β-mercaptoethanol. The 96-well plates were maintained at 33°C with 5% CO₂ and one half of the medium was replaced weekly. Five weeks later, we used a standard methylcellulose assay with 20 ng/mL SCF, 100 IU/mL IL-3, 1 IU/mL Epo, and 10 ng/mL G-CSF to evaluate clonogenic progenitors from each well. The proportion of positive wells was recorded and the long-term culture-initiating cell (LTC-IC) frequency was determined as described previously in this paragraph.16 We picked individual colonies from wells containing 1, 10, or 25 cells, based on the LTC-IC frequency, to genotype clonal LTC-ICs.

Nucleic acid extraction

We used the Qiagen DNA extraction kit (Qiagen, Hilden, Germany) to extract DNA from granulocytes, bone marrow mononuclear cells, CD34+ and CD34− cells. DNA from methylcellulose grown colonies and B/NK/myeloid clones was prepared using a 10 mg/mL proteinase K (Invitrogen) buffer containing 0.2% Tween 20 (Sigma).

Genotyping and JAK21849G>T (617V>F) quantification

We did direct sequencing as described previously.1,20 We also analyzed mutational status by using fluorescent competitive probes for quantitative real-time polymerase chain reaction (PCR) on an ABI 7500 (Applied Biosystems, Foster City, CA) as reported previously.20,21 We determined the JAK2 617V→F total JAK2 ratio by the ΔCt procedure as described.21 Standard curves were obtained by diluting mutant DNA from biallelic, 100% homozygous DNA in wild-type diploid DNA. Genotyping of clones grown from single-cell culture of CD34+ cells from patients revealed that JAK2 617V→F total JAK2 values ranged from less than 0.0001 to 0.0101 in 117 wild-type clones (mean ± standard deviation [SD]: 0.0006 ± 0.0015), 0.3636 to 0.5962 in 177 heterozygous clones (0.5023 ± 0.0404), and 0.9817 to 0.9998 in 15 homozygous 617V>F clones (0.9964 ± 0.0046). Seven of the 177 purely heterozygous clone samples (3.95%) exhibited values ranging from 0.5750 to 0.5962. Therefore, we arbitrary set the threshold at 0.60 for the accurate definition of a nonclonal cell population that certainly harbors homozygous cells.

Statistical analysis

Our results are presented as mean plus or minus one SD or the standard error of the mean (SEM). We used paired and unpaired Student t tests to
analyze the data. The correlations between biologic parameters and JAK2 617V>F levels were tested by using nonparametric Spearman rank order correlation tests. Values of *P* less than .05 were considered significant.

### Results

**Quantification of JAK2 617V>F in granulocytes and bone marrow mononuclear cells delineates distinct subgroups of patients**

Of the JAK2 617V>F–positive samples, the JAK2 617V>F/total JAK2 ratio was determined by real-time quantitative PCR for 90 bone marrow and 93 granulocyte samples from 159 PV patients, and 78 bone marrow and 77 granulocyte samples from 149 ET patients (Table 1; Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The levels of mutated JAK2 in bone marrow mononuclear cells and granulocytes were lower in ET samples (0.29 ± 0.193 and 0.270 ± 0.198, respectively) than in PV samples (0.598 ± 0.208 and 0.572 ± 0.243, respectively; *P* < .001) (Table 1). Based on the 0.60 threshold defined in “Patients, materials, and methods” section, 36% of PV samples were found to have cells with duplication of the mutated allele. This was the case for only 4% of ET patients. Ratios below 0.40 were frequent for ET samples (63%). JAK2 617V>F levels ranged from 0.40 to 0.60 for 50% of the PV samples and 33% of the ET samples.

**JAK2 617V>F levels correlate with granulocyte counts, hemoglobin levels, and EEC formation**

We compared the biologic features of PV and ET patients against JAK2 617V>F burden in bone marrow mononuclear cells or granulocytes at the time of diagnosis (Table 2; Figure S1). Age and gender of PV and ET patients did not correlate with JAK2 617V>F levels. PV patients had significantly lower platelet counts and higher hemoglobin, hematocrit, leukocyte, and granulocyte values than ET patients (Table 2). In PV patients, high levels of JAK2 617V>F correlated with high granulocyte counts (Spearman correlation test, *P* = .013) and high hemoglobin values (*P* = .004). The highest platelet counts in PV patients were associated with low JAK2 617V>F levels (*P* = .0029). In ET patients, high JAK2 617V>F levels correlated with high platelet and high granulocyte counts. Notably, all 6 ET patients with a ratio more than 0.60 had more granulocytes (9.9 to 24.5 × 10⁹/L) than other ET patients (Student *t* test, *P* < .001).

Standard plasma clot assays revealed that PV patients had more bone marrow erythroid colonies than did ET patients in the presence of Epo (525 ± 434 versus 346 ± 315 colonies per 2.5 × 10⁵ input cells; *P* = .028). EEC positivity was detected in 100% (84 of 84) of JAK2 617V>F–positive PV patients and 78.0% (46 of 59) of JAK2 617V>F–positive ET patients. The EEC frequency in ET patients was lower than in PV patients (*P* < .001). In PV and ET, the numbers and percentages of EECs were correlated to JAK2 617V>F levels (Table 2).

**Clonal genotypic pattern identifies 2 subsets of PV patients**

We grew bone marrow CD34+CD38+ cells from PV and ET patients in methylcellulose in the presence or absence of Epo or G-CSF. On day 14, we picked a total of 1870 individual erythroid and granulocyte colonies and genotyped them using real-time PCR (Figure 1A). We defined mixed patterns of homozygous, heterozygous, and normal colonies as homozygous profiles, and the mixture of colonies revealing both homozygous and heterozygous profiles as dual hybrid profiles. There were 77 homozygous, 107 heterozygous, and 71 dual hybrid colonies in PV samples, whereas there were 63 homozygous, 59 heterozygous, and 21 dual hybrid colonies in ET samples. The proportions of homozygous colonies were 90% (84 of 93) in PV patients and 46% (59 of 127) in ET patients (*P* = .001). The proportions of heterozygous colonies were 78% (92 of 117) in PV patients and 25% (32 of 127) in ET patients (*P* < .001). The proportion of dual hybrid colonies were 18% (21 of 117) in PV patients and 39% (46 of 117) in ET patients (*P* < .001).

Table 1. JAK2 617V>F quantification in bone marrow and granulocyte DNA from ET and PV patients

<table>
<thead>
<tr>
<th>JAK2 617V&gt;F/total</th>
<th>Bone marrow, n = 90</th>
<th>Granulocytes, n = 93</th>
<th>Bone marrow, n = 78</th>
<th>Granulocytes, n = 77</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (SD)</strong></td>
<td>0.598 (0.208)</td>
<td>0.572 (0.243)</td>
<td>0.298 (0.193)</td>
<td>0.270 (0.198)</td>
</tr>
<tr>
<td><strong>Median (range)</strong></td>
<td>0.50 (0.03–1)</td>
<td>0.50 (0.1–1)</td>
<td>0.30 (0.02–0.75)</td>
<td>0.30 (0.02–0.85)</td>
</tr>
<tr>
<td><strong>Less than 0.20, no. (%)</strong></td>
<td>1 (1.1)</td>
<td>4 (4.3)</td>
<td>19 (24.3)</td>
<td>25 (32.5)</td>
</tr>
<tr>
<td><strong>0.20 to 0.40, no. (%)</strong></td>
<td>9 (10.0)</td>
<td>12 (12.9)</td>
<td>27 (34.6)</td>
<td>27 (35.1)</td>
</tr>
<tr>
<td><strong>0.40 to 0.60, no. (%)</strong></td>
<td>47 (52.2)</td>
<td>44 (47.3)</td>
<td>29 (37.2)</td>
<td>22 (26.6)</td>
</tr>
<tr>
<td><strong>More than 0.60, no. (%)</strong></td>
<td>33 (36.7)</td>
<td>33 (35.5)</td>
<td>3 (3.8)</td>
<td>3 (3.9)</td>
</tr>
</tbody>
</table>

The numbers (percentages) of patients with various JAK2 617V>F total JAK2 ratios are shown.

Table 2. Correlation of biological features of PV and ET patients with respect to the JAK2 617V>F/total JAK2 ratio at the time of diagnosis

<table>
<thead>
<tr>
<th>JAK2 617V&gt;F/total</th>
<th>PV, n = 100</th>
<th>ET, n = 95</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Correlation value</td>
</tr>
<tr>
<td>JAK2 617V&gt;F/total JAK2</td>
<td>0.613</td>
<td>0.213</td>
</tr>
<tr>
<td>Age, y</td>
<td>65</td>
<td>14</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>189</td>
<td>17</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>58.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Platelet count, × 10⁹/L</td>
<td>475</td>
<td>192</td>
</tr>
<tr>
<td>Leukocytes, × 10⁹/L</td>
<td>12.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Granulocytes, × 10⁹/L</td>
<td>9.4</td>
<td>4.9</td>
</tr>
<tr>
<td>Number of colonies (+ Epo)* per 2.5 × 10⁵ input cells</td>
<td>525</td>
<td>434</td>
</tr>
<tr>
<td>Number of EEC (− Epo)* per 2.5 × 10⁵ input cells</td>
<td>173</td>
<td>218</td>
</tr>
<tr>
<td>Proportion of EEC*</td>
<td>0.306</td>
<td>0.181</td>
</tr>
</tbody>
</table>

Spearman correlation test values are indicated. All parameters shown were significantly different when comparing overall ET and PV values by the Student *t* test (*P* < .01).

*A total of 84 of 100 PV patients and 66 of 95 ET patients had cultures at the time of diagnosis.

†Significant *P* values (<.05) for Spearman correlation test values.
of heterozygous and normal colonies as heterozygous profiles. A preliminary study of 14 PV patients showed that 11 had a homozygous profile and 3 a heterozygous profile. Thus, we analyzed the clonal genotype of 6 additional patients whose granulocytic colonies exhibited a JAK2 617V>F total JAK2 ratio around 0.50. Among them, 3 had a heterozygous profile. Results were confirmed by sequencing for 2 PV patients with a homozygous profile and 2 with a heterozygous profile (data not shown). The clonal genotypic pattern of the 6 ET patients was a mix of heterozygous and wild-type colonies. Homozygous colonies were detected in only 2 ET patients at a very low frequency (1 of 44 and 1 of 47 total erythroid colonies). In the presence of Epo, the percentage of mutated erythroid colonies from PV patients with a heterozygous profile (88.5% ± 17.5%; n = 6) was greater than that of other PV patients (48.7% ± 21.4%; n = 14; P = .001), and ET patients (44.4% ± 35.1%; n = 6; P = .027) (Figure 1B). Similarly, the proportion of mutated CFU-G was slightly greater in PV patients with a heterozygous profile than in other PV or ET patients (Figure 1B). In the absence of Epo, the percentages of mutated colonies reached 95.1% ± 6.3%, 96.9% ± 5.1%, and 77.3% ± 24.5%, respectively, with a greater proportion of homozygous colonies (74.5% ± 16.0% versus 23.1% ± 14.4% in the presence of Epo) in PV patients with a homozygous profile. Nine of 251 colonies from PV patients and 7 of 46 colonies from ET patients were wild type (Figure 1B). The erythroid lineage of these colonies could not be ascertained due to the small numbers of cells. Thus, we cannot rule out that these colonies may not be true EECs.

These findings show that ET patients have a heterozygous profile and delineate 2 subsets of PV patients: (1) heterozygous PV patients with a high proportion of heterozygous committed progenitors and no detectable homozygous colonies, and (2) homozygous PV patients with homozygous subclones that are predominant in the absence of Epo.

JAK2 617V>F homozygosity triggers hypersensitivity and independence to Epo

We investigated the Epo sensitivity of homozygous, heterozygous, and wild-type erythroid progenitors to elucidate the relationship between phenotype and the genotypic profiles. We did this by growing bone marrow CD34+ CD38− progenitors from normal subjects (n = 3), and from JAK2 617V>F-positive ET (n = 5) and PV (homozygous, n = 4; heterozygous, n = 1) patients in methylcellulose containing SCF, IL-3, and various concentrations of Epo (0 to 3 IU/mL). A preliminary analysis with 5 samples showed that the number of BFU-E on day 14 reached a plateau with Epo concentrations from 0.1 to 0.5 IU/mL (data not shown). Thus, for further experiments, we used Epo at serial concentrations between 0 and 0.5 IU/mL. With the highest Epo concentration (0.5 IU/mL), the total numbers of erythroid colonies were similar whether cultures originated from PV, ET, or normal bone marrow (81.9 ± 22.1, 67.3 ± 10.5, and 72.3 ± 16.5 colonies per 1500 plated cells, respectively; P > .05) (Figure 2A). However, we found that PV and ET erythroid progenitors were hypersensitive to low concentrations of Epo. There were 34.2 (± 8.2) erythroid colonies in cultures from PV patients and 30.1 (± 5.8) erythroid colonies in cultures from ET patients stimulated with 0.01 IU/mL Epo, significantly more than the 11.2 (± 0.6) colonies in cultures from normal subjects (P < .05). This difference was even more accentuated in the presence of 0.001 IU/mL Epo: there were 16.7 (± 4.4) colonies in cultures from PV patients, 11.2 (± 3.2) colonies in cultures from ET patients, and 1.33 (± 0.3) colonies in cultures from normal donors (P < .02). Although virtually no spontaneous colonies developed from normal samples (0.33 ± 0.13), Epo-independent erythroid colonies were found in PV and ET samples. The percentage of erythroid-independent colonies in PV usually exceeded that seen in ET samples (17.4 ± 5.9 and 8.7 ± 4.4, respectively; P = .046) (Figure 2A).

Then we investigated the involvement of the JAK2 mutation in the Epo hypersensitivity of erythroid progenitor cells. We did this by genotyping individual erythroid colonies that were grown with various concentrations of Epo (709 colonies grown with 0.5 IU/mL Epo, 289 colonies grown with 0.05 IU/mL Epo, 217 grown with 0.01 IU/mL Epo, 143 grown with 0.001 IU/mL Epo, and 157 grown with 0 IU/mL Epo) (Figures 2 and S2). There were fewer normal and heterozygous colonies from PV and ET samples with lower concentrations of Epo. In contrast, the numbers of homozygous colonies from PV samples were only moderately affected by lowering Epo concentrations (Figure 2A). Homozygous colonies were detected in samples from 3 of 5 ET patients. However, they were infrequent (1 of 83, 1 of 98, and 2 of 131 total erythroid colonies) whatever the Epo concentration. We then analyzed the relative frequency of erythroid colonies from each genotype for

Figure 1. Clonal genotypic patterns of erythroid and granulocytic progenitors from ET and PV patients. Bone marrow CD34+ cells from PV and ET patients were seeded in methylcellulose in the presence or absence of Epo or G-CSF. Erythroid and granulocytic colonies were picked on day 14 and genotyped by real-time PCR. Mixed colonies from JAK2 617V>F homozygous, heterozygous, and normal colonies were called homozygous profiles. Patterns with heterozygous and normal colonies and without any homozygous colonies were called heterozygous profiles. (A) Detection of the JAK2 1849G>T (617V>F) mutation by real-time PCR. The results of 3 representative erythroid colonies from one PV patient with a homozygous JAK2 617V>F profile (homozygous PV), one PV patient with a heterozygous profile (heterozygous PV), and one ET patient (ET) are shown. The plots represent the fluorescence curves resulting from the amplification of the JAK2 mutated (T) and JAK2 wild-type (G) alleles. The genotype of each colony is indicated to the left of the amplification plots: G/G, wild-type colony; G/T, colony with heterozygous mutation; T/T, colony with homozygous mutation. (B) Histograms showing the mean percentages of JAK2 617V>F homozygous (), heterozygous (), and JAK2 wild-type () colonies from 14 PV patients with a homozygous profile (homozygous PV), 6 PV patients with a heterozygous profile (heterozygous PV), and 6 ET patients. Numbers above the bars indicate the total number of colonies that were genotyped. Bars indicate SEM. Note the high percentage of mutated erythroid and granulocytic colonies in heterozygous PV samples.
each Epo concentration used (Figure 2B). The Epo dose-response curves for wild-type erythroid progenitors from PV and ET patients were similar to those for normal subjects. In contrast, relative to the number of PV and ET wild-type colonies that grew in the presence of the plateau concentration of Epo, only 26.0% (± 2.7%) grew in the presence of 0.01 U/mL Epo, whereas 73.8% (± 3.5%) of the homozygous mutant colonies and 53.1% (± 4.3%) of the heterozygous mutant colonies grew under these conditions (Figure 2B). In addition, 22.5% (± 3.0%) of heterozygous progenitors and 69.5% (± 3.3%) of homozygous progenitors grew in the absence of Epo. These data indicate that the JAK2 617V→F mutation triggers Epo hypersensitivity and that homozygosity increases Epo independence.

**Increased in vitro erythroid expansion of bone marrow CD34+/CD38− derived cells from ET and PV patients**

We studied the consequences of the JAK2 617V→F mutation on progenitor cell amplification with a plateau dose of Epo (1 IU/mL). Methylcellulose assays revealed that erythroid colonies from patient samples tended to be larger than those from normal bone marrow on day 14 (data not shown). We seeded 4 × 10⁴ to 10⁵ CD34+/CD38− cells from normal, ET, and PV bone marrow for liquid culture assays in serum-free medium containing SCF, IL-3, dexamethasone, and 1 IU/mL Epo. On day 7, cells were enumerated and phenotyped by MGG and CD38/GPA staining, which demonstrated that more than 90% of the cells belonged to the erythroid lineage (data not shown). Overall, total cell expansion in patient cultures was significantly higher than that in healthy donors (57.0 ± 15.3- and 21.7 ± 7.7-fold expansion, respectively; P = .001) (Figure 3A). Next, we compared the JAK2 617V→F/total JAK2 ratio in initial BFU-E, as determined by clonal genotyping, to that of their progeny after 7 days in liquid culture (Figure 3B). In 2 of 2 ET and 3 of 5 PV patients, we observed that the JAK2 617V→F/total JAK2 ratio was higher in the progeny than in the input cell suspension. The 2 remaining PV patients had heterozygous profiles with 90% and 97% of heterozygously mutated BFU-E, respectively. Nearly all of the erythroid progenitors in the input cell suspension were mutated. Thus, we could not observe a further enrichment of mutated alleles. These data show that mutated erythroid progenitors have an in vitro selective advantage over wild-type cells and suggest that the JAK2 617V→F mutation provides a proliferative advantage to erythroid cells in the presence of an optimal concentration of Epo.
Low frequency of JAK2 617V>F mutation in immature lymphomyeloid progenitors and LTC-ICs from ET and PV bone marrow

We examined the frequency of the JAK2 617V>F mutation during the early stages of hematopoiesis. Bone marrow CD34+CD38−cells from 7 PV patients (1 heterozygous and 6 homozygous), 6 ET patients, and 3 healthy subjects were cultured at one cell per well in a medium supporting B/NK/myeloid differentiation. All clones were phenotyped and genotyped after 4 to 6 weeks in culture. There were no overall differences between the variety and differentiation potentials for clones from PV patients, ET patients, and healthy subjects (data not shown). We genotyped lymphomyeloid clones (ie, clones with B/myeloid, NK/myeloid, or B/NK/myeloid potentials). Similar percentages of lymphomyeloid clones from PV and ET patients were mutated (47 of 339, 13.9%, and 35 of 220, 15.9%, respectively) (Figure 4A). The biallelic mutation was detected in 6 NK/myeloid clones and 1 B/NK/myeloid clone from 4 of 6 homozygous PV patients, but never in lymphomyeloid clones derived from the heterozygous PV patient (73 clones) and ET patients (220 clones). We obtained similar results in our analysis of LTC-ICs from 6 PV patients, 1 PV patient with a heterozygous profile, and 6 PV patients with a homozygous profile (ET, PV (heterozygous)) (Figure 4B). We found no significant difference between patient and control LTC-IC frequencies (0.211 ± 0.104 and 0.20 ± 0.149, respectively; P = .431, data not shown). We analyzed 767 LTC-IC-derived individual colonies from 6 PV and 4 ET patients. Thus, we were able to genotype 59 to 144 clonal LTC-ICs per patient. The percentage of mutated LTC-ICs ranged from 0% (0 of 59) to 45% (65 of 144). Homozygous LTC-ICs were detected in 3 of 6 homozygous PV patients, but not in heterozygous PV or ET patients (Figure 4B).

These findings indicate that only a minority of PV and ET immature progenitor cells harbor the mutation and suggest that mitotic recombination occurs in a multipotent hematopoietic stem cell in PV patients.

JAK2 617V>F confers a selective advantage during terminal erythroid and granulocytic differentiations

An increase in genomic JAK2 617V>F/total JAK2 ratio reflects an enrichment in mutated cells during an amplification process and can thus be used to evaluate a selective advantage. Therefore, we measured the JAK2 617V>F/total JAK2 ratio by real-time PCR in immature and mature hematopoietic cells from 5 ET, 4 heterozygous, and 9 homozygous JAK2 617V>F PV patients. Bone marrow CD34+CD38−, CD34+CD38+, GPA+ cells and circulating granulocytes were genotyped (Figures 5 and S3). Despite heterogeneity among homozygous PV patients, the JAK2 617V>F/total JAK2 ratio was substantially higher in mature erythroblasts (0.82 ± 0.10) and granulocytes (0.64 ± 0.17) than in CD34+CD38+ cells (0.32 ± 0.19) and in CD34+CD38− (0.25 ± 0.23) cells (P <.05). Similarly, there was more of the mutated allele in GPA+ cells from ET patients (0.48 ± 0.12) than in the CD34+CD38+ cells (0.26 ± 0.13) and in the CD34+CD38− (0.22 ± 0.18) populations (P <.05). In contrast, the amount of the mutated allele was not different between granulocytes (0.28 ± 0.18) and CD34+CD38− cells (P = .325). The mean JAK2 617V>F levels from heterozygous PV patients were significantly lower in CD34+CD38− cells (0.15 ± 0.08) than in CD34+CD38− cells (0.42 ± 0.12; P = .013), GPA+ cells (0.44 ± 0.04; P = .001; granulocytes (0.41 ± 0.09; P = .018), suggesting a selective growth advantage for cells with the mutation occurring upstream from the CD34+CD38− cell stage.

Discussion

The JAK2 617V>F mutation is an essential molecular event in MPDs and has been implicated in the pathophysiology of PV in animal models.1,6,7 High JAK2 617V>F levels and the presence of homozygous progenitors have been associated with PV, whereas low levels of JAK2 617V>F and the absence of homozygous
progenitor cells have been associated with ET.5,9 Thus, the disease phenotype might be linked to the clonal genotypic pattern.

By systematically genotyping granulocytes and bone marrow mononuclear cells, we confirmed that there are higher levels of JAK2 617V/F in PV samples than in ET samples. Only a minority of ET patients with high granulocyte counts had amounts of JAK2 617V/F more than 0.60. It is unknown whether the disease in these patients is likely to become polycythemic or myelofibrotic. High JAK2 617V/F levels were observed much more frequently in PV samples and were associated with large numbers of white blood cells and EECs and high hemoglobin values. We investigated the frequency of JAK2 617V/F–positive cells at various stages of hematopoiesis, the sensitivity of erythroid progenitors to Epo, and the potential for the erythroid progenitors to proliferate to understand these differences between PV and ET.

Genotyping erythroid and granulocytic colonies from PV and ET bone marrow CD34+CD38− cells revealed various genotypic profiles. We defined homozygous profiles as the mixture of normal, heterozygous, and homozygous colonies, and heterozygous profiles as the combination of normal and heterozygous colonies in the absence of homozygous clones. We observed homozygous and heterozygous profiles in both ET and PV samples. However, homozygous colonies were extremely rare (< 2%) in ET samples and there were no homozygous immature LTC-ICs and lymphomyeloid progenitors. These results are in accordance with a previous study.5,9 They suggest that either mitotic recombination had occurred in a progenitor cell that was already committed in these ET patients or that its frequency in these primitive cells was too low to be detected by our methods. The presence of homozygous clones in these ET patients might mean that they could develop PV. Clinical follow-up of these patients should facilitate the determination of the influence of JAK2 617V/F gene dosage on the pathogenesis of MPDs.

The most striking result in our study was the observation of both heterozygous and homozygous profiles in PV. Consistent with a previous study,9 most PV patients had a mixed pattern of normal, heterozygous, and homozygous committed or immature progenitor cells. However, our data reveal the existence of PV patients with a normal karyotype (ie, without trisomy 9; data not shown) who do not have any homozygous progenitor cells and in which most erythroid progenitor cells carry the mutation. These findings suggest that the homozygous PV clone was selectively amplified upstream from the committed erythroid progenitor cell (BFU-E) stage. This indicates that PV may require at least 2 genetic events, the first event being the heterozygous JAK2 617V/F mutation and the second one being mitotic recombination for the majority of cases. In other cases, the second event would modify the regulation of early stages of hematopoiesis upstream from the BFU-E stage. However, this does not rule out that either this event or a third molecular defect may also modify Epo sensitivity.

We investigated the Epo sensitivity of JAK2 617V/F homozygous, heterozygous, and nonmutated erythroid progenitors. We demonstrate that JAK2 617V/F mutated progenitor cells are more sensitive than normal cells to Epo. Moreover, we show that cells with the homozygous mutation are much more sensitive than heterozygous cells to Epo. We found that 69.5% of JAK2 617V/F homozygous BFU-E and 22.5% of heterozygous BFU-E were Epo independent. Epo dose-response curves for nonmutated colonies from MPD patients and from healthy bone marrows were similar. These results show that the JAK2 617V/F mutation is a key molecular event leading to Epo hypersensitivity or independence, and that the biallelic mutation increases independence of erythroid progenitors. However, the fact that not all mutated cells were Epo independent suggests that other associated molecular defects may contribute to Epo hypersensitivity or independence. In addition, the low percentage of Epo-independent heterozygous clones may account for the EEC negativity in 20% of ET patients. Indeed, the plasma clot assay may not be sufficiently sensitive to detect EEC from ET patients with low proportions of heterozygous clones. Under low serum Epo conditions, mutated erythroid cells should have a selective advantage over normal erythroid cells. Furthermore, bone marrow CD34+CD38− cells from JAK2 617V/F–positive ET and PV samples proliferated at a high rate if cultured with optimal concentrations of Epo (1 IU/mL). This suggests that JAK2 617V/F not only promotes G1/S phase transition,22,23 but also increases the number of mitoses or impairs apoptosis even in the presence of Epo as suggested previously.13,17

In addition, we observed an in vivo selective advantage for mutated cells during granulopoiesis in PV samples, which is similar to what happens during terminal erythroid differentiation. Therefore, we hypothesize that selective proliferative advantage for the mutated cells may lead to the elevated granulocyte counts we observed, which correlate strongly to the JAK2 617V/F level. Our findings differ from those reported in a recent study suggesting that wild-type cells have a growth advantage over mutated cells during vitro amplification.24 However, these experiments were done with circulating CD34+ cells and high Epo concentrations (4 IU/mL) and may not accurately represent the amplification process that takes place in the bone marrow of PV patients. Thus, it would be useful to compare the amplification potentials of blood and bone marrow CD34+ cells from the same patient. Another work essentially performed on circulating CD34+ cells demonstrated that the differentiation potential of PV was already skewed toward the erythroid lineage at the hematopoietic stem cell level.25 Although our results suggest that the main effect of the JAK2 617V/F mutation targets terminal erythroid differentiation, they do not exclude the hypothesis of a cooperative early predisposition of PV hematopoietic stem cells toward erythroid differentiation.

Accumulating evidence suggests that other genetic events occur in some PV or ET patients and that these events precede the JAK2 617V/F mutation.7,26 PV patients with a heterozygous JAK2 617V/F profile have a predominant heterozygous clone at the BFU-E and CFU-G stages of hematopoiesis and a low number of mutated lymphomyeloid progenitors. These observations suggest that there is an additional molecular event that initiates the onset of clonality earlier than the JAK2 617V/F mutation. Recent quantitative and clonality studies demonstrated that the clone harboring the JAK2 mutation represents a small number of cells in some ET patients and in fewer PV patients.21,26,27 The malignant clone in these particular patients is clearly larger than the JAK2 617V/F clone. The malignant clone is more likely than the JAK2 mutant, suggesting that, at least in some patients, other events must precede clonality. It is possible that the same event occurs before the JAK2 mutation in clonal patients with low levels of JAK2 617V/F or occurs after the mutation in heterozygous PV patients (Figure S4). Thus, genetic comparison of PV patients with homozygous and heterozygous profiles may be a powerful approach to characterize this event.

In conclusion, our data suggest a 2-step model of amplification for the erythroid lineage in JAK2 617V/F–positive ET and PV, with putative additional molecular defects that may affect the early stages of hematopoiesis or the terminal erythroid differentiation (Figures 6 and S4). First, an unknown molecular defect could result in a dramatic clonal amplification upstream from the committed progenitor stage. In heterozygous JAK2 617V/F PV, this could
terminal erythroid amplification of the predominant mutated clone results in severe PV patients with a heterozygous profile (hatched diagram) are mutated. Thus, gous subclones is observed in homozygous PV (black diagram). Most BFU-E from committed progenitor cells. In comparison with normal erythropoiesis (white diagram), a moderate late amplification driven by a low percentage of heterozygous committed progenitor cells. In comparison with normal erythropoiesis (white diagram), a moderate late amplification driven by a low percentage of heterozygous subclones is observed in homzygous PV (black diagram). Most BFU-E from PV patients with a heterozygous profile (hatched diagram) are mutated. Thus, selective amplification may occur upstream from this stage of differentiation. Further terminal erythroid amplification of the predominant mutated clone results in severe erythrocytosis. The right diagram shows the selective pressure due to the decrease in the level of serum Epo caused by erythrocytosis.

result in a prominent JAK2 617V>F heterozygous clone, the terminal amplification of which would lead to erythrocytosis. Second, in the absence of such an event, a minority of JAK2 617V>F heterozygous cells could acquire increased Epo hypersensitivity from JAK2 617V>F homozygosity or an alternative unknown molecular defect. Then, a huge terminal amplification of the erythroid lineage, irrespective of Epo depletion, could take place leading to major erythrocytosis, as in homzygous PV patients. In contrast, the mild amplification of the erythroid lineage observed in ET may be due to the absence of these additional hits that favor the erythroid lineage.

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Authorship

Contribution: S.D. and A.M. performed cellular and genotyping experiments, analyzed the data, and contributed to the writing of the paper; C.J. performed cellular experiments and contributed to the writing of the manuscript; I.T. contributed to diagnostic assays and collection of the samples; Y.L. designed and performed cell sorting experiments; F.L. contributed to cell sorting experiments; V.U. contributed to cellular experiments; P.S. and J.P.L.C. performed sequencing analyses; S.K performed statistical analysis; N.C. contributed to the design of the study and the recruitment of the patients; W.V. supervised the study and contributed to the recruitment of patients and the writing of the paper; and F.D. designed the study, conducted cellular and molecular experiments, analyzed the data, and wrote the paper.

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Correspondence: François Delhommeau, Institut National de la Santé et de la Recherche Médicale, Unité 790, PR1, Institut Gustave Roussy, 39 rue Camille Desmoulin, 94805 Villejuif, France; e-mail: fdelhommeau@igr.fr.

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The *JAK2 617V>F* mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera

Sabrina Dupont, Aline Massé, Chloé James, Irène Teyssandier, Yann Lécluse, Frédéric Larbret, Valérie Ugo, Patrick Saulnier, Serge Koscielny, Jean Pierre Le Couédic, Nicole Casadevall, William Vainchenker and François Delhommeau

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