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Differential NOD/SCID mouse engraftment of peripheral blood CD34+ cells and JAK2V617F clones from patients with myeloproliferative neoplasms

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1. Introduction

Chronic myeloproliferative diseases (MPD) are heterogeneous diseases characterized by clonal proliferation at the hematopoietic stem cell (HSC) level [1]. A gain-of-function V617F mutation in the JH2 auto-regulatory domain of the Janus Kinase 2 (JAK2) gene (JAK2V617F) is the commonest molecular abnormality in MPD, occurring in more than 90% patients with polycythemia vera (PV) and 50% patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF); underscoring the pathogenetic role of deregulated Jak2 [2–4]. JAK2V617F transgenic mice [5,6] and mice receiving JAK2V617F-transduced bone marrow (BM) cells [2,7–9] developed PV and other MPD-like diseases. The JAK2V617F mutation has also been found in the primitive hematopoietic cell population [10] as well as the hematolymphoid progenitors from some cases of PV [11], supporting the proposition that JAK2V617F mutation is a stem cell event.

The xenogeneic transplantation of human hematopoietic cells into immunodeficient mice has become a standard model for the study of HSC. Moreover, PV and PMF were different in their lineage differentiation programs and the clone size of the JAK2V617F HSC. Information about JAK2V617F ET is currently lacking.

In this study, we examined HSC activity and JAK2V617F clone in PV by comparing NOD/SCID mouse engraftment by PB CD34+ cells from patients with JAK2V617F-positive PV and secondary polycythemia (SP). The latter was a JAK2V617F-negative non-neoplastic condition in which erythropoiesis is stimulated by increased serum erythropoietin. The change in JAK2V617F clone in PV upon NOD/SCID mouse engraftment was also enumerated. Finally, we also compared the HSC activity between PV, ET and PMF by examining their engraftment and changes in JAK2V617F clone in NOD/SCID mice.

2. Materials and methods

2.1. Patients

CD34+ cells were obtained from PB of patients with JAK2V617F-positive PV and SP. PB mononuclear cells (MNC) were purified by density gradient centrifugation. CD34+ cells were isolated immunomagnetically (Miltenyi, Bergisch, Gladbach, Germany) and were either transplanted directly or stored in liquid nitrogen until use. BM MNCs were also prospectively collected from PV, ET and PMF patients. The investigation was approved by the Institution Review Board in accordance with the Declaration of Helsinki.

2.2. NOD/SCID mouse transplantation and enumeration of engraftment

PB CD34+ cells from PV and SP patients were transplanted into sublethally irradiated (250 cGy) 6–8 weeks old NOD/SCID mice (Jackson Laboratory, Bar Harbor, Maine, USA) by direct intra-femoral injection as described [15]. Engraftment was estimated by flow cytometry analysis.
assessed 6–8 weeks after transplantation. Briefly, BM was flushed out from the injected (right femur) and uninjected (left femur and bilateral tibias) bones and were processed separately. Red cells were lysed (BD Pharm LyseTM Lysing buffer, BD Biosciences, San Jose, CA, USA) and marrow cells were co-stained with human specific fluorescein isothiocyanate (FITC)–conjugated antihuman CD45 antibody (clone 2D1) and mouse specific phycoerythrin (PE)–conjugated antimouse CD45.1 antibody (clone A20) (BD Biosciences, San Jose, CA, USA) for 30 min in ice, followed by flow cytometry analysis. Successful human cell engraftment was defined by the presence of more than 0.1% human CD45+ mouse CD45.1+ cells in the recipient mouse BM. In some experiments, human BM MNC or CD34+ cells from PV, ET and PMF patients were transplanted via an intravenous or intra-femoral route and engraftment was similarly enumerated.

2.3. Real-time quantitative polymerase chain reaction (Q-PCR)

To quantify the JAK2V617F clone in engrafting human cells, genomic DNA was extracted from the mouse BM cells. Q-PCR was set up with the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the genomic DNA (0.5 μL), the primers (200nmol/L) and TaqMan probe (100nmol/L), using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Sequences of primers and probes used in Q-PCR and the PCR conditions have been described previously [16]. Standard curves for the quantification of JAK2 and JAK2V617F clones were constructed by plotting the Ct against the logarithm of the starting amount (0.05–500 fg) of pGEM-T plasmids containing either the JAK2 or JAK2V617F sequence. The quantity of JAK2 and JAK2V617F clones was determined by correlating the Ct values from the standard curves. Percentage of JAK2V617F clone in each sample was calculated as the amount of JAK2V617F/amount of JAK2. The Q-PCR was human specific and non-transplanted mouse marrow invariably gave negative results.

2.4. Amplification refractory mutation system PCR (ARMS-PCR)

In the latter part of the study in which BM MNC and CD34+ cells from PV, ET and PMF patients were transplanted intravenously into NOD/SCID mice, the JAK2V617F clone before and after transplantation was enumerated using ARMS-PCR, performed for 40 cycles as described [17]. Comparison of the JAK2V617F clone relative to total JAK2 was performed semi-quantitatively by comparing the mutant and wild-type PCR bands in the same sample by the ImageJ program (Version 1.38x, National Institutes of Health, USA). The ARMS-PCR was also human specific and non-transplanted mouse marrow invariably gave negative results.

2.5. Correlation between ARMS-PCR and Q-PCR

As the enumeration of JAK2V617F clone by ARMS-PCR was only semi-quantitative, we validated it by concomitant Q-PCR, where DNA was available. In a total of 53 samples, the burden of the JAK2V617F clone was quantified by both methods. There was a significant correlation between ARMS-PCR and Q-PCR ($R = 0.899$; $P < 0.001$) (Fig. 1), validating the use of ARMS-PCR in this study.

2.6. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Comparisons between numerical and categorical data were evaluated by Mann–Whitney’s U-test and $x^2$ test. A P-value of <0.05 was considered statistically significant.

3. Results

3.1. Patient samples

PB samples from 13 PV and 9 SP patients undergoing therapeutic venesection were prospectively collected (Table 1). The two groups of patients were similar in age and hemoglobin concentration. However, patients with SP were predominantly male smokers whereas patients with PV have significantly higher total white cell counts and platelet counts. Serum erythropoietin assay has not been a regular investigation in our institute and the levels were not available. In addition, BM samples from 12 patients with JAK2V617F-positive MPD (W.H.O. classification: PV = 4; ET = 3; PMF = 5) were also prospectively collected (Supplementary Materials: Table 1).

3.2. Engraftment of PV CD34+ cells in NOD/SCID xenogeneic transplantation

To examine if PV CD34+ cells exhibited an engraftment advantage, PB C34+ cells were transplanted in comparable numbers into irradiated NOD/SCID mice from patients with PV (median cell dose = $0.3 \times 10^6$ mouse$^{-1}$, range: $0.075 \times 10^6$ to $1.5 \times 10^6$ mouse$^{-1}$) and SP (median cell dose = $0.3 \times 10^6$ mouse$^{-1}$, range: $0.1 \times 10^6$ to $1.0 \times 10^6$ mouse$^{-1}$) via direct intra-femoral injection. In BM harvested from the mouse femur injected with human cells (injected BM), 8/26 mice transplanted with 5/13 PBCD34+ cells from PV patients engrafted (median: 4.26%, range: 0.3–5.56%), in contrast to 0/14 mice from 9 SP patients ($P = 0.017$) (Fig. 2A). The engrafting PV cells were capable of differentiating into myeloid cells (CD45+CD34+: 31.6 ± 9.0%), B-lymphoid cells (CD3–CD19+: 75.5 ± 11.9%), as well as maintaining the primitive CD34+ phenotype (9.8 ± 1.9%). In BM harvested from the other mouse femur and tibia that had not been injected with human cells (uninjected BM), 4/26 mice transplanted with 3/13 PBCD34+ cells from PV patients engrafted (median: 1.43%, range: 0.11–1.81%), in contrast to 0/14 mice from 9 SP patients ($P = 0.058$) (Fig. 2B, also Table 2).

3.3. Reduction of JAK2V617F clone upon NOD/SCID engraftment

We next investigated if the JAK2V617F mutation conferred engraftment advantage to PV cells in NOD/SCID mice. We compared the JAK2V617F clone size in PV CD34+ cells in the initial graft and 6 weeks after direct intra-femoral transplantation into NOD/SCID mice. When the uninjected BM were analyzed, there was a consistent decrease in the JAK2V617F clone after transplan-

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Table 1

<table>
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<tr>
<th>Clinicopathologic characteristics of patients with polycythemia vera and secondary polycythemia.</th>
<th>Polycythemia vera JAK2V617F in 13/13 cases</th>
<th>Secondary polycythemia JAK2V617F in 0/9 cases</th>
<th>$P$-value</th>
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<tr>
<td>Patient number</td>
<td>13</td>
<td>9</td>
<td>0.045</td>
</tr>
<tr>
<td>Male:female*</td>
<td>6:5</td>
<td>8:0</td>
<td>0.955</td>
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<tr>
<td>Age (range, median) (years)</td>
<td>34–82, 61.5</td>
<td>46–78, 67.0</td>
<td>0.05</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>19.6 ± 0.77</td>
<td>18.8 ± 0.15</td>
<td>0.001</td>
</tr>
<tr>
<td>White cell count (×10$^9$ L$^{-1}$)</td>
<td>17.8 ± 2.29</td>
<td>8.1 ± 1.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet (×10$^9$ L$^{-1}$)</td>
<td>516.1 ± 76.45</td>
<td>288.0 ± 71.43</td>
<td>0.05</td>
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* Identity of two patients with polycythemia vera and one patient with secondary polycythemia was not available.


Fig. 1. Correlation between ARMS-PCR and real-time quantitative PCR. Every point in the graph represents the simultaneous enumeration of JAK2V617F clone by real-time Q-PCR (y-axis) and ARMS-PCR (x-axis) of one sample.
injected BM of NOD/SCID mice. Time Q-PCR in PV samples before and after transplantation in (C) uninjected and (D) injected BM. Human engraftment is higher in PV than SP patients. Mouse BM was harvested from either (A) injected or (B) uninjected BM. Human engraftment is higher in PV than SP patients. The bars represent the mean engraftment. (C and D) Percentages of cells from PV and SP patients. Mouse BM was harvested from either (A) injected or intravenously into NOD/SCID mice (Fig. 3A). When the injected BM was analyzed, the results were highly variable. The median JAK2V617F/(JAK2V617F+JAK2) ratios from 17/26 paired samples were 65.9% and 32.0% in the initial and 6-week samples respectively (P = 0.079) (Fig. 2D).

3.4. Transplantation of BM MNC from PV, ET and PMF patients

To compare the engraftment kinetics of the JAK2V617F clone in PV, ET and PMF HSC, we also transplanted BM samples from these patients into NOD/SCID mice via an intravenous or intra-femoral route. Owing to the small number of cells collected, CD34+ selection was not performed, and only MNC were transplanted in most cases. Successful engraftment was detected in 8/9 recipient mice in all four PV patients (median: 6.50%; range: 1.70–56.0%; also see Supplementary Materials: Table 2). The engrafting PV cells from BM were also capable of differentiating into myeloid cells (CD45+CD33*: 51.5 ± 17.4%), B-lymphoid cells (CD3−CD19*: 23.9 ± 16.4%) as well as maintaining the primitive CD34+ phenotype (11.0 ± 7.63%). Similar to transplantation with PB CD34+ cells, a significant decrease in JAK2V617F clone was also noted when BM MNC or CD34+ cells from 4 PV patients were transplanted intra-femorally or intravenously into NOD/SCID mice (Fig. 3A).

When BM MNC from three ET patients were transplanted, 2/3 cases showed successful engraftment in four mice. Among the four mice engrafting with two patient samples (Supplementary Materials: Table 2), one of them showed multi-lineage engraftment (mouse 6.1: CD33+ cells: 77.7%; CD3−CD19+ cells: 18.5% and CD34+ cells: 13.3%). The other two mice showed only myeloid engraftment (Mouse 5.2: CD33+ cells: 50.0%; Mouse 6.2: CD33+ cells: 69.3%). Lineage analysis was not performed in one mouse. None of the five PMF samples showed successful human engraftment in seven mice. Similar to the PV cases, the frequency of JAK2V617F/(total JAK2) clones in ET markedly diminished in the engrafting cells (Fig. 3B). For PMF, despite unsuccessful engraftment by flow cytometry criteria (≥0.1%), human JAK2V617F could be detectable in the recipient mouse BM and the clone was sustainable in five mice (Fig. 3C).

4. Discussion

In this study, we demonstrated that BM and PB cells from PV patients were able to engraft into NOD/SCID mice via both intravenous and intra-femoral routes and differentiate into multiple hematopoietic lineages. Similar observations have been reported recently, supported the proposition that the JAK2V617F mutation in PV occurred at the HSC level. Both the percentage of PV samples and the absolute level of successful engraftment were remarkably similar to those reported by Ishii et al. [13] but lower than those by James et al. [14], suggesting that either depletion of NK cells or the use of CD34+ cells of BM origin in the latter study might lead to a more robust xenogenic engraftment. This limitation notwithstanding, there are a number of interesting observations which may provide us with further insights into the pathogenesis of JAK2V617F-positive MPD.

First, we demonstrated that PB CD34+ cells from patients with PV but not SP engrafted in NOD/SCID mice. Previous studies have shown that HSC in PV and PMF patients undergo spontaneous mobilization [18], and that PB CD34+ cells could engraft into NOD/SCID mice [13]. On the other hand, although G-CSF mobilized CD34+ cells from health donors consistently engraft in NOD/SCID mice [19], whether unmobilized CD34+ cells from patients with non-neoplastic disease can also engraft is unclear. We addressed this issue by examining venesection samples from SP patients, the closest non-neoplastic control of PV patients. We believed that it presented a valid control to evaluate the impact of JAK2V617F in PV on NOD/SCID mouse engraftment. On the other hand, G-CSF mobilized or bone marrow CD34+ cells are expectedly different from unmobilized CD34+ and may introduce confounding factors in the comparison, irrespective of the presence of JAK2V617F mutation. Surprisingly, none of the 14 mice receiving 0.1–1.0 × 10^6 CD34+ cells from 9 SP patients engrafted, compared with 8/26 mice who...
results suggested that PB CD34+ cells from PV exhibited engraftment superiority when compared with those from SP. Microarray study comparing BM CD34+ cells from PV patients and healthy donors demonstrated differential expression of more than 100 genes [20]. It remains to be defined if the differential gene expression may account for the engraftment capability of PB CD34+ cells from PV patients.

Secondly, we demonstrated that the engraftment capability of PV CD34+ cells was unrelated to changes in JAK2V617F clones. In fact, a diminution of the JAK2V617F clone upon engraftment of PV HSC in NOD/SCID mice has also been reported recently [13,14]. Intriguingly, the reduction in JAK2V617F clones was only consistently seen in un.injected but not the injected BM, which might be explained by defective homing of the JAK2V617F clones to the recipient BM. Overall, the results suggested that the JAK2V617F mutation has not conferred a proliferative advantage to HSC in NOD/SCID mouse model.

Thirdly, in contrast to PV, the JAK2V617F clones in PMF were preserved in the engrafting cells. Similar results have been reported recently, suggesting the proposition that PMF contained more JAK2V617F HSC than PV [14]. In addition, we observed a significant reduction in JAK2V617F clones when BM cells from ET patients engrafted in NOD/SCID. In these experiments, BM MNC from all patients were similarly transplanted in comparable numbers. Therefore, the preservation or loss of JAK2V617F clones in PMF versus PV/ET.

Our findings of an engraftment superiority of PB CD34+ cells in PV and the diminution of JAK2V617F clones upon engraftment suggested that a pre-JAK2V617F mutation event might be of pathogenic significance. They also corroborated with the observations of JAK2V617F-negative clonal hematopoiesis [21–23] and leukemic transformation in JAK2V617F-positive PV [16,17]. Whether the JAK2V617F-negative NOD/SCID repopulating cells were of clonal origin would have to be carefully evaluated. Recent studies have reported that specific TET2 (ten-eleven translocation2) [24] and ASXL1 (additional sex comb-like 1) [25] mutations, karyotypic aberration [26] or specific haplotype [27,28] may also play a pathogenic role in PV. These issues should be vigorously investigated.

Conflict of interest statement

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2010.01.028.
References


