The Constitutive Mobilization of Bone Marrow Repopulating Cells into the Peripheral Blood in Idiopathic Myelofibrosis

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Running title: IM PB contains BM-repopulating cells
Section: Neoplasia

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Abstract

Idiopathic myelofibrosis (IM) is characterized by the constitutive mobilization of CD34+ cells. IM peripheral blood (PB) CD34+ cells had a reduced cloning efficiency and a lower frequency of cobblestone areas as compared to normal G-CSF mobilized PB CD34+ cells. IM CD34+ cells engrafted NOD/SCID mice, demonstrating that they contain bone marrow (BM) repopulating cells. G-CSF mobilized CD34+ cells produced multiple hematopoietic lineages within the NOD/SCID mice with a predominance of CD19+ cells. By contrast, IM CD34+ cells produced predominantly CD33+ cells, increased numbers of CD41+ cells, but fewer CD19+ cells. Transcriptional clonality assays of the engrafted human IM cells demonstrated their clonal origin. CD34+ cells from one patient isolated prior to leukemic transformation were capable of generating acute leukemia in NOD/SCID mice. The engrafted human cells exhibited the same abnormal karyotype as primary cells in a portion of the population. These findings demonstrate that BM repopulating cells and more differentiated progenitor cells are constitutively mobilized into the PB in IM, and that their differentiation program is abnormal. In addition, the NOD/SCID model may be useful in gaining an understanding of the events occurring during the transition of IM to acute leukemia.
Introduction

Chronic idiopathic myelofibrosis (IM) is a hematological malignancy characterized by splenomegaly, a leukoerythroblastic blood picture, teardrop poikilocytosis (dacrocytosis), varying degrees of bone marrow (BM) fibrosis, and extramedullary hematopoiesis. IM is thought to originate at the level of the multipotent hematopoietic stem cell (HSC). The HSC defect results in a profound hyperplasia of morphologically abnormal megakaryocytes and clonal populations of monocytes, which have been shown to locally release fibrogenic growth factors, leading to BM fibrosis.

Rare CD34+ HSC/hematopoietic progenitor cells (HPC) circulate in the peripheral blood (PB) of normal individuals. Increased numbers of CD34+ cells can be mobilized into the PB following the administration of a variety of cytokines and/or chemotherapeutic agents. Barosi et al recently demonstrated that the PB of IM patients contained 360 times greater numbers of CD34+ cells than normal controls and 18 to 30 times greater numbers of CD34+ cells than patients with other Ph negative myeloproliferative disorders (MPDs). In addition, the PB CD34+ cell number was further shown to be related to disease progression and to serve as a biomarker for disease activity. The number of assayable HPC present in the PB of IM patients have also been shown to be increased. Therefore, IM represents a unique situation in which the numbers of CD34+ cells appearing in the PB are frequently markedly increased in the absence of extrinsic stimuli.

Although the CD34 antigen is expressed by both the HSC and HPC, the functional potential of the CD34+ cells in the PB of IM patients has not been well defined. In this report we have further phenotyped the CD34+ cells of IM patients and examined the multilineage differentiation potential of these cells, as well as their ability to repopulate immunodeficient mice. In addition, we have demonstrated the utility of this in vivo model to analyze the cellular and molecular events that occur during the transition of IM to acute leukemia.
Materials and Methods

Patients and Normal Control Subjects

All human tissue samples were obtained after informed consent following the guidelines of the Institutional Review Board of the University of Illinois College of Medicine. PB samples were obtained from (1) normal donors in steady-state hematopoiesis, (2) normal donors mobilized with G-CSF (Amgen, Thousand Oaks, CA at 5 µg/kg/d subcutaneously), (3) patients with IM or polycythemia vera (PV), who met the WHO diagnostic criteria for IM and PV\textsuperscript{1-4,16}, as well as patients with secondary myelofibrosis associated with pulmonary hypertension.\textsuperscript{17} None of the patients were receiving cytotoxic agents at the time of study and none had evidence of transformation to acute leukemia. The specific clinical characteristics of the patients whose CD34\textsuperscript{+} cells were examined for BM-repopulating potential are described in Table 1.

Table 1: Clinical Characteristics of Patients with IM

<table>
<thead>
<tr>
<th>Patient (Sex)</th>
<th>Hgb (g/L)</th>
<th>WBC x10\textsuperscript{9}/L</th>
<th>Platelet count x10\textsuperscript{9}/L</th>
<th>Spleen size, cm below left costal margin</th>
<th>% blasts in PB</th>
<th>RBC transfusion requirement</th>
<th>Severity of BM fibrosis</th>
<th>Transition to acute leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM-1 (F)</td>
<td>106</td>
<td>24.2</td>
<td>275</td>
<td>4</td>
<td>0</td>
<td>Yes</td>
<td>3</td>
<td>No</td>
</tr>
<tr>
<td>IM-2 (F)</td>
<td>86</td>
<td>33.7</td>
<td>95</td>
<td>20</td>
<td>0</td>
<td>Yes</td>
<td>3</td>
<td>No</td>
</tr>
<tr>
<td>IM-3 (F)</td>
<td>75</td>
<td>18.0</td>
<td>26</td>
<td>16</td>
<td>0</td>
<td>Yes</td>
<td>3</td>
<td>No</td>
</tr>
<tr>
<td>IM-4 (F)</td>
<td>140</td>
<td>60.3</td>
<td>356</td>
<td>Splenectomized</td>
<td>0</td>
<td>No</td>
<td>3</td>
<td>Yes (5 Weeks)</td>
</tr>
</tbody>
</table>

Purification of Human PB CD34\textsuperscript{+} Cells

The PB was layered onto Ficoll-Hypaque (1.077 g/mL) (Amersham Biosciences, Piscataway, NJ), and low-density mononuclear cells (MNC) separated after centrifugation. A CD34\textsuperscript{+} cell population was isolated utilizing a magnetic activated cell sorting CD34\textsuperscript{+} Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. The purity of the CD34\textsuperscript{+} cell population was analyzed using
a FACSCaliber Flow Cytometer (Becton Dickinson, Mountain View, CA). Cell fractions showing a CD34+ cell purity of 85% or greater were used for subsequent experiments including HSC phenotyping, in vitro progenitor assays and transplantation into immunodeficient (NOD/SCID) mice.

**Phenotypic Analysis of CD34+ Cell**

Separate aliquots of isolated CD34+ or MNC were double stained with anti-CD34 and a panel of monoclonal Abs against c-kit, CD38, CD90, and CD33 (Becton Dickinson, San Jose, CA). The percentage of CD34+ cells expressing c-kit, CD90, CD33 but not CD38 was then determined by flow cytometric analysis.

Cell cycle analysis of PB CD34+ cells was performed by propidium iodide (PI) (Sigma Chemical) staining as described previously with minor modifications. Briefly, cells were fixed in 70% ethanol; to avoid the formation of aggregates, the cell suspension was added drop wise onto 70% ethanol while vortexing and kept on ice for 20 minutes. After twice washing with PBS, the cells were stained with a staining buffer containing 4mM citrate buffer, 30U of RNAase, 0.1% triton X-100, 5 µg/mL PI and 0.138mM NaCl at 4 °C and the DNA content analyzed with a FACSCalibur. Data files containing forward- and side-scatter peak signals as well as width and areas of the PI signal were collected. Doublet events were excluded by gating on the PI signal-width channel.

**Colony Forming Cell (CFC) and Cobblestone Area Forming Cell (CAFC) Assays**

CD34+ cells were incubated at a concentration of 500 cells per mL of culture mixture. One mL of culture mixture containing 500 cells, 0.9% methylcellulose, 30% FBS, 1% BSA, 0.05 mM 2-ME (Stem Cell Technologies, Vancouver, BC, Canada) and a cytokine cocktail (SCF 100ng/ml, IL-3 100ng/ml, IL-6 100ng/ml, G-CSF 20ng/ml, EPO 4U/ml, and TPO 100ng/ml) (Amgen, Thousand Oaks, CA) was placed in 35 mm non-tissue culture dishes and incubated at 37 °C in 5% CO2. All cultures were performed in triplicate and various colony types enumerated using an inverted microscope at day 12-14 of culture, according to previously described criteria.¹⁹
For the CAFC assays, CD34+ cells were suspended in limiting dilution in IMDM + 10% FBS + cytokine cocktail (SCF 100ng/ml, LIF 100ng/ml, IL-3 100ng/ml, IL-6 100ng/ml, GM-CSF 50ng/ml) and seeded onto pre-established feeder cell layers of an irradiated murine fibroblast cell line M210B4. The cells were then incubated at 37°C and 5% CO2 with half of the incubating volume replaced with fresh media containing the same cytokine cocktail on a weekly basis. After 5 weeks of culture, the number of cobblestone areas was enumerated and the frequencies of CAFC calculated using L-CALC software.

**NOD-SCID Repopulating Assay**

NOD/LtSz-scid/scid (NOD/SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in microisolators under specific pathogen-free conditions at the Biologic Resources Laboratory at the University of Illinois at Chicago. To optimize human cell engraftment, 7- to 8-week-old female NOD/SCID mice recipients received a series of pretreatments consisting of 1) a sublethal dose of whole-body irradiation of 350 cGy with a cobalt radiation source, 2) intraperitoneal injections of 200 µg/mouse TM-β1 (BD Biosciences Pharmingen), a monoclonal antibody (mAb) directed against the murine IL-2Rβ to eliminate remaining NK activity after irradiation at 6-12 hours prior to transplantation, and 3) alternate-day intraperitoneal injections of 10 ng of recombinant human (Rhu) SCF, 6µg of RhuGM-CSF and 6µg of RhuIL-3 per injection, for the first 10 days after transplantation.

Purified CD34+ cells were washed twice in PBS (Cambrex, Walkerville, MD) and injected into the tail veins of NOD/SCID mice at a dose of 0.5 to 3 × 10⁶ cells/mouse in 0.5 mL PBS. In two cases, CD34- cells obtained from the flow through of the CD34 selection columns were collected and injected intravenously at 15-30 × 10⁶ cells/mouse. Nine to 16 weeks after human cell transplantation, the mice were sacrificed and the PB, spleen, femur and tibia harvested for analysis. From these organs, cell suspensions were prepared, the RBCs were treated with a lysing buffer (Sigma), and cell numbers and viability determined.
For analysis of human cells in murine tissues, cell suspensions were preincubated for 30 minutes at 4°C in PBS containing 0.1% BSA, 20% mouse serum, and 20% human serum. Separate cell aliquots were then incubated for 30 minutes at 4°C with a panel of mAbs against the following human markers conjugated to FITC, PE, or APC: CD34, CD45, CD33, CD19, CD41a, IgM, CD38, CD56 and CD3 (Becton Dickinson, San Jose, CA). A separate cell aliquot was stained with isotype-matched mAbs labeled with the same fluorochromes to establish the levels of non-specific immunofluorescence. After washing, 2 μg/ml PI was then added to eliminate dead (PI+) cells from the analysis. The samples were then analyzed flow cytometrically using a FACSVantage. Routinely, 30,000 events were acquired per sample. The lack of cross-reactivity of human-specific antibodies with mouse cells was confirmed in every experiment by staining BM cells from a nontransplanted irradiated control mouse. The percentage of human cells in the recipient was calculated as the number of human CD45+ cells/the number of PI- cells × 100. Engraftment of human cells was defined by the presence of at least 0.1% nucleated cells expressing CD45 over the background fluorescence.

**Immunohistochemical Staining**

Formalin-fixed and paraffin-embedded sections of tibias from the recipient mice were stained with May-Grunwald Giemsa. For immunochemical staining, the tissue sections were deparaffinized in xylene and hydrated in graded alcohol. The sections were then stained with a monoclonal mouse anti-human CD34 antibody (CD34 QBend 10) (DakoCytomation, Carpenteria, CA). Staining was performed on a DakoCytomation Histostainer using streptavidin/horseradish peroxidase detection kit with the chromogen 3,3’-diaminobenzidine tetrahydrochloride (DBA). All staining procedures were performed utilizing an isotypic antibody as a negative control.

**Transcriptional clonality assays**

The genotypes for 5 X-chromosome exonic polymorphisms (MPP1, IDS, G6PD, BTK and FHL1) of the 3 IM females were determined by Real-Time PCR using commercial ABI (Applied Biosystems, Foster City, CA) TaqMan probes. To examine the clonal origin of the human IM hematopoietic cells engrafted in NOD/SCID mice,
RNA was extracted from human CD33+ cells purified from the femurs of these mice utilizing Milteneyi cell selection devices. Transcriptional clonality assays were then performed using SSCP analysis as previously described.22

**Cytogenetic analysis**

Cytogenetic analysis was performed on the BM of patient IM-4 at the time of leukemic transformation (May 2003) and one year prior to that time. The human CD45+ cells isolated from the mice transplanted with PB CD34+ cells obtained from patient IM-4 (5 weeks prior to the leukemia transformation) utilizing Milteneyi cell selection devices were also analyzed. Cells were cultured for approximately 24 h in the absence of mitogens. Chromosome preparation and banding were performed as previously described.23 An attempt was made to analyze at least 20 metaphases. The description of karyotypes follows the recommendations of the International System for Human Cytogenetic Nomenclature.24

**Statistical Analysis**

Data points are expressed as the mean ± standard error of the mean (SEM). Differences between percentages of cells expressing a particular cellular phenotype were calculated using the Wilcoxon test, whereas differences between other variables were compared using either a Student's t-test or analysis of variance (ANOVA).

**Results**

**Phenotypic Analysis of CD34+ Cells Mobilized Into the PB of IM**

Previously a greater number of CD34+ cells have been reported to circulate in the PB of patients with IM as compared to normal controls.12 We compared the percentage of CD34+ cells within the PB MNC of patients with IM, normal donors mobilized with G-CSF, normal individuals and patients with secondary myelofibrosis. As shown in Figure 1, the percentage of CD34+ cells within the PB MNC fraction from both patients with IM and normal donors mobilized with G-CSF were dramatically elevated with a mean±SEM of 8.34±2.47% (p<0.001), and 1.06±0.42% (p<0.01), respectively as
compared to normal volunteers (0.27±0.20%). When three patients with secondary myelofibrosis associated with pulmonary hypertension were similarly studied, the percentage of CD34+ cells within the MNC fraction was not increased. However, a greater percentage of the cells within the MNC fraction in patients with PV were CD34+ than within that fraction in normal controls (p<0.05), albeit not to the same degree as observed in IM. It is important to note the relative degree of CD34+ cell mobilization in IM was dramatically greater as compared to G-CSF mobilized donors (p<0.01) and PV patients. These findings suggest that IM is unique in that the CD34+ cells are preferentially mobilized into the PB.

CD34+ cells in IM PB were further characterized by mAb staining and flow cytometric analysis. Two-color staining and FACS analysis were performed on pre-isolated PB CD34+ cells from normal volunteers or patients with IM. In some cases, IM PB MNCs were used directly for phenotypic analysis. As can be seen in Table 2, the fraction of IM PB CD34+ cells expressing CD117 (c-kit), CD38, CD90, and CD33 are significantly different than that of normal volunteers. A greater percentage of IM PB than normal PB CD34+ cells were c-kit+, CD38+, and CD90+ (Table 2), suggesting that the CD34+ cells in the PB of IM patients might represent a more primitive population of HPC than that which normally circulates in the PB. In addition, the expression of the myeloid lineage marker CD33 was shown to be significantly increased in IM PB CD34+ cells.

Table 2: Phenotype of CD34+ Cells in Normal and IM PB

<table>
<thead>
<tr>
<th>Cell Surface Antigens</th>
<th>Normal PB (% , n=5)</th>
<th>IM PB (% , n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD117 (c-kit)</td>
<td>16.0±7.3</td>
<td>40.8±15.1*</td>
</tr>
<tr>
<td>CD90 (Thy-1)</td>
<td>23.9±4.6</td>
<td>43.5±5.8*</td>
</tr>
<tr>
<td>CD38 (Negative)</td>
<td>0.6±0.4</td>
<td>4.7±3.6*</td>
</tr>
<tr>
<td>CD33</td>
<td>33.0±5.8</td>
<td>57.2±13.5*</td>
</tr>
</tbody>
</table>

* p<0.05

It has been reported that virtually all of the PB CD34+ cells mobilized by G-CSF are non-cycling quiescent cells.22-24 We therefore, performed cell cycle analysis using flow cytometry to determine the percentage of quiescent IM PB CD34+ cells. Similar to
normal PB CD34+ cells and G-CSF mobilized CD34+ cells, greater than 98% of the IM CD34+ cells resided within the G0/G1 phase (data not shown).

**IM PB CD34+ Cells Have Decreased Cloning Efficiency in Vitro**

In order to examine the differentiation potential of IM HSC/HPC, the number and type of HPC assayed from PB CD34+ cells isolated from 6 normal volunteers, 4 normal donors mobilized with G-CSF and 7 patients with IM were enumerated. As shown in Figure 2, about 60% of the colonies formed by normal PB CD34+ cells were derived from BFU-E; while both IM and G-CSF mobilized CD34+ cells generated predominantly CFU-GM-derived colonies (~80%). More importantly, the cloning efficiency of IM CD34+ cells was significantly lower than that of normal PB CD34+ cells (p<0.01). The cloning efficiency of IM CD34+ cells and CD34+ cells isolated from G-CSF mobilized PB were, however, similar (p>0.05), as was the distribution of the colony types formed (Figure 3). Since there are greater numbers of CD34+ cells in the PB of IM patients, the absolute number of assayable HPC are clearly increased in patients with IM as compared to normal individuals or even G-CSF mobilized normal volunteers.

We next assessed the ability of IM PB MNC and CD34+ cells to form CAFC in a stromal cell-based culture system after 5 weeks of incubation. The frequency of CAFC has been previously used as an in vitro surrogate assay for human HSC.19 As can be seen in Table 3, the frequency of CAFC in IM PB MNC was shown to be higher than that observed in G-CSF mobilized normal donors, suggesting that IM is also associated with the mobilization of greater numbers of more primitive HPC (CAFC) than G-CSF mobilization. However, the frequency of CAFC within the CD34+ cells was 3 fold lower in IM PB, as compared to normal PB and G-CSF mobilized PB (Table 4). These findings suggest that the preferential mobilization of HSC/HPC in IM is also accompanied by abnormalities in CD34+ cell function.

**Table 3: Frequency of CAFC in PB MNC from Normal G-CSF Mobilized Donors and Patients with IM.**

<table>
<thead>
<tr>
<th>Source of PB MNC</th>
<th>CAFC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobilized PB (n=2)</td>
<td>1 in 1,386</td>
</tr>
<tr>
<td>IM PB (n=3)</td>
<td>1 in 245</td>
</tr>
</tbody>
</table>
Table 4: Frequency of CAFC in PB CD34+ cells from Normal Volunteers, Normal G-CSF Mobilized donors and patients with IM

<table>
<thead>
<tr>
<th>Source of CD34+ cells</th>
<th>CAFC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PB (n=2)</td>
<td>1 in 206 (157-270)</td>
</tr>
<tr>
<td>Mobilized PB (n=2)</td>
<td>1 in 186 (162-212)</td>
</tr>
<tr>
<td>IM PB (n=3)</td>
<td>1 in 746 (477-955)</td>
</tr>
</tbody>
</table>

IM PB CD34+ Cells Contain NOD/SCID Repopulating Cells

While there is a significant mobilization of CD34+ cells into the PB in IM, one cannot be certain, at present, if these PB CD34+ cells are composed exclusively of more differentiated progenitor cells or also contain HSC. To address this question, the in vivo functional behavior of CD34+ cells isolated from the PB of IM patients and G-CSF mobilized volunteers were studied by transplanting these purified CD34+ cells into NOD/SCID mice. A dose of 0.5-3 × 10⁶ cells/mouse was transplanted. After 9-15 weeks, the mice were sacrificed and the BM analyzed for the degree of human cell engraftment utilizing flow cytometric analysis and monoclonal antibodies against CD45, CD34, CD19, CD33, IgM, CD56, CD41 and CD3.

As shown in Table 5, IM and G-CSF mobilized PB CD34+ cells were capable of engrafting NOD/SCID mice and producing cells belonging to multiple hematopoietic lineages (Figure 3). By contrast, the CD34+ cells from two IM patients did not possess the ability to repopulate the NOD/SCID mice. The differentiation program of IM CD34+ cells was remarkably different from that of G-CSF mobilized normal donors. Although both sources of CD34+ cells were capable of producing hematopoietic cells belonging to multiple hematopoietic lineages, CD19+ B-cells represented the predominant cell population in the BM of mice transplanted with G-CSF mobilized PB CD34+ cells, while PB CD34+ cells from IM patients produced predominantly myeloid cells (CD33+ cells) as well as far greater numbers of CD41+ cells (megakaryocytes) and fewer CD19+ cells (Figure 3 A and 3B). In addition, approximately 30% of the human cells in the BM of the NOD/SCID mice (at weeks 9) after transplantation with IM PB CD34+ cells were
CD34+ in comparison to about 6% observed in the mice transplanted with G-CSF mobilized PB CD34+ cells (Figure 3 A). These data suggest that BM repopulating cells exist within the PB of IM patients, and that they are restricted to the CD34+ cell population. In addition, the differentiation program of the IM CD34+ cells appears to differ from that of normal CD34+ cells mobilized into the PB by G-CSF as demonstrated by greater capacity to generate CD34+, CD41+ and CD33+ cells in vivo.

Table 5: IM CD34+ Cells Contain SCID-Repopulating Cells

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Donor Number</th>
<th>No. of Cells Transplanted</th>
<th>Frequency of Engraftment</th>
<th>Human CD45+ Cells in BM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF Mobilized PB CD34+</td>
<td>N-1</td>
<td>1.9 x 10^6</td>
<td>1/1</td>
<td>12.4%</td>
</tr>
<tr>
<td></td>
<td>N-2</td>
<td>2.5 x 10^6</td>
<td>1/1</td>
<td>21.5%</td>
</tr>
<tr>
<td></td>
<td>N-3</td>
<td>0.5 x 10^6</td>
<td>1/1</td>
<td>0.3%</td>
</tr>
<tr>
<td>IM PB CD34+</td>
<td>IM-1</td>
<td>3.0 x 10^6</td>
<td>6/6</td>
<td>1.9%</td>
</tr>
<tr>
<td></td>
<td>IM-2</td>
<td>1.2 x 10^6</td>
<td>2/2</td>
<td>0.7%</td>
</tr>
<tr>
<td></td>
<td>IM-3</td>
<td>0.5 x 10^6</td>
<td>2/2</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>IM-4</td>
<td>3.0 x 10^6</td>
<td>3/3</td>
<td>83.0%</td>
</tr>
</tbody>
</table>

Engrafted Human Hematopoietic Cells in NOD/SCID Mice Receiving IM CD34+ Cells are Clonal in Origin

Utilizing transcriptional clonality assays,22 we analyzed the clonal origin of those hematopoietic cells which engrafted into the NOD/SCID mice transplanted with CD34+ cells from 3 female IM patients. Patient IM-1 was heterozygous for G6PD; IM-2 was heterozygous for MPP1, IDS and G6PD, and IM-3 was heterozygous for MPP1 and G6PD. We analyzed the exonic allelic transcription of all X-chromosome polymorphic alleles in mice transplanted by CD34+ cells from all three IM patients. CD33+ cells were isolated from the BM cells of each mouse and all 6 individual polymorphic alleles examined; in each case the CD33+ cells expressed only a single allelic transcript of each X-chromosome polymorphism. As shown in the Figure 4 (only the G6PD assay shown), the CD33+ cells from all three mice only expressed either the T or C allele of G6PD, but
not both. This finding demonstrated that the human hematopoiesis present in these mice was derived from the same clone as the human IM cells.

**Development of Leukemia in the Mice Receiving IM PB CD34+ Cells**

Patient IM-4 represented a unique opportunity to observe the evolution of IM to acute leukemia. Although the PB cells from this patient were harvested at a time when the patient’s disease appeared stable, this individual transformed into acute myeloid leukemia 5 weeks following blood collection. The patient developed an acute myeloid leukemia with blast cells that expressed CD34, CD45, CD33, and CD13. As can be seen in Figure 5 A, the mice receiving PB CD34+ cells from this patient achieved an extremely high level of human cell engraftment with ~83% human CD45+ cells in the BM, ~15% in the spleen and ~11% in the PB. Flow cytometric analyses revealed that the CD45+ cells in these hematopoietic organs were predominantly CD34+ and CD56+ (Figure 5 A) and CD33+ (data not shown), a phenotype similar to that observed during the leukemic phase of IM. Furthermore the BM cavities of these NOD/SCID mice were heavily infiltrated with blast-like cells (Figure 4 A) which were shown by immunohistochemical staining to express the human CD34 antigen (Figure 5 B). These data indicate that the leukemic transformation occurred in vivo in the NOD/SCID mice after transplantation, during a time interval virtually identical to that observed in the patient, and that these cells were present in not only the BM but also the spleen and PB of these mice (Figure 5 A).

In order to determine the origin of the human CD45+ leukemic cells isolated from the NOD/SCID mice, their karyotype was compared to that of the primary cells studied one year prior to leukemia transformation and at the time of the leukemic transformation (Table 6). A clone of cells containing the identical karyotypic abnormality (45, XX, ?inv dup(1)(p21p33), der(3)t(3;16)(q11.2;p11.2), del(5)(q15q33), del(7)(q22), -10, -12, der(16)t(16;17)(p11.2;q11.2), -17, +2mar) was detected in each of these cell samples indicating their common origin. Surprisingly, this clone represented the minority of human cells within the NOD/SCID mice. The majority of the donor derived cells, however, possessed a new abnormality (42~44, XX, del(1)(p34.1), -4.add(6)(p2?3), -7, del(8)(q13), -10, der(11)t(?4;11)(q27;q?23), add(12)(q34.3),add(13) (q34), -17, ?dup(17)(p11.2), +mar), which is likely a consequence of genomic instability characteristic of this secondary leukemia.
Table 6: Cytogenetic Abnormalities in Cells from Patient IM-4 Engrafted in NOD/SCID Mice

<table>
<thead>
<tr>
<th>Source Distribution</th>
<th>Karyotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source Distribution</td>
<td>Karyotypes</td>
</tr>
<tr>
<td>IM BM (May 2002)</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>55%</td>
</tr>
<tr>
<td>Leukemic Transformation BM (May 2003)</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>15%</td>
</tr>
<tr>
<td>Engrafted Human Cells In NOD/SCID Mice</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>87%</td>
</tr>
</tbody>
</table>

*Apparent differences between these karyotypes, other than the deletion 7, are the result of differences in interpretation between 2 cytogenetic laboratories. The chromosomes are in fact the same.

Discussion

The CD34 surface antigen has served as a means to identify and separate HSC and HPC from their more differentiated progeny. CD34 is present on ~0.05% of nucleated circulating cells. By quantitating the absolute number of CD34+ cells in the PB of a large well-defined population of patients, Barosi et al have shown that the PB of IM patients contained 360 times greater number of CD34+ cells than normal controls and 18 to 30 times greater numbers of CD34+ cells than patients with other Ph negative MPDs. The CD34+ cell number was further shown to be related to disease progression and to serve as a biomarker for disease activity.

In this study, we characterized the IM PB CD34+ cells by an array of phenotypic and functional assays. We found that IM PB CD34+ cells contained a phenotypically more primitive population of HPC than that of normal PB; with a greater percentage of the IM PB CD34+ cells exhibiting a c-kit+, CD90+ and CD38- phenotype. Most human primitive hematopoietic cells with high potential for self-renewal and multilineage differentiation has been reported to express high levels of c-kit or CD90, and low levels
of CD38. In addition, Barosi et al have previously shown that only 66% (23%-99%) of CD34+ cells expressed CD38. Interestingly, despite containing a phenotypically more primitive population of HPC, IM PB CD34+ cells displayed a significantly lower frequency of CFC and CAFC as compared to normal controls. This reduced cloning efficiency of IM CD34+ cells is either a consequence of the mobilization process or a function of a malignant transformation event that characterizes these cells. A resolution of this question will require additional studies utilizing CD34+ cells isolated from larger numbers of IM patients.

These findings also suggest that the preferential mobilization of CD34+ cells in IM is accompanied by abnormalities in cell function. This discordance between phenotype and function is reminiscent of the behavior of CD34+ cells isolated from patients with myelodysplasia and acute myeloid leukemia, in which increased numbers of PB CD34+ cells have also been associated with reduced CD34+ cell cloning efficiency.

The use of immunodeficient mice as hosts of human HSC transplantations has provided powerful models for both normal and abnormal human hematopoiesis. Subfractionation of the input cells coupled with time course studies of the number and types of the mature progeny produced has revealed the presence of a hierarchy of primitive transplantable progenitors that produce different spectra of progeny for varying, but predictable periods. In this study, we have demonstrated for the first time that NOD/SCID BM repopulating cells exist within the PB CD34+ cells of patients with IM. More importantly, the differentiation program of the IM PB CD34+ cells differ from that of normal CD34+ cells mobilized into the PB with G-CSF. These IM CD34+ cells have the unique ability to generate a greater fraction of CD34+, CD33+ and CD41+ cells. Similarly, Thanopoulou et al have reported that lineage- cells from myelodysplasia patients repopulate NOD/SCID-β2m-/- mice and produce an abnormal differentiation pattern. Thus, our data demonstrate the potential of the NOD/SCID model for future investigations of IM.

IM is believed to originate at the level of the HSC resulting in a profound hyperplasia of morphologically abnormal megakaryocytes and clonal populations of monocytes. It would therefore, be important to know if the engrafted human cells in
the NOD/SCID mice are derived from the malignant clone. Utilizing both transcriptional clonality assays and cytogenetic studies, we have demonstrated that the engrafted human hematopoietic cells in the mice transplanted with PB CD34+ cells from each IM patient were clonal in origin, indicating that they are derived from the abnormal clone of the patient. Although, it has been reported that NOD/SCID mice have been repopulated with both normal and leukemic human cells after transplantation of cells from patients with chronic myeloid leukemia, the vast majority (>90%) of human progenitors present in the BM of the NOD/SCID mice were Ph+ by cytogenetic analysis. Preliminary studies using marker chromosome abnormalities or restriction fragment length polymorphisms have documented that about 80% of circulating CD34+ cells in a patient with IM are clonal.

One of the hallmarks of IM is the inevitable progression of the disease to acute leukemia. In this study, we also observed this leukemic transformation in vivo in NOD/SCID mice transplanted with stable-state IM PB CD34+ cells. The IM patient from whom the sample was derived eventually underwent acute leukemic transformation. This ability to observe the evolution of leukemia from IM in vivo has not to our knowledge been previously reported. Although such events would likely be rare, this model provides an in vivo system in which to analyze the events in individual patients that play a role in the progression of IM to acute leukemia. In addition, IM-engrafted NOD/SCID mice should offer new opportunities for developing and testing novel therapeutic agents for the treatment of IM.

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References


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Figure legends:

Figure 1: The percentage of CD34+ cells in PB MNC fractions of normal subjects (n=10), secondary myelofibrosis (n=3), IM (n=25) and normal donors mobilized with G-CSF (n=14).
Figure 2: Cloning efficiency of normal PB (n=6), G-CSF mobilized PB (n=4) and IM PB (n=7) CD34⁺ cells. The number of CFC is the sum of the number of BFU-E-, CFU-GM- and CFU-GEMM-derived colonies cloned per 2000 CD34⁺ cells plated. Difference in CFC formation between normal PB and IM PB was statistically significant (p<0.005).
Figure 3: (A) Analysis of myelopoiesis, B lymphopoiesis and megakaryocytopoisis in the BM of the NOD/SCID mice transplanted with either normal G-CSF mobilized PB (n=3) or IM PB (n=3) CD34+ cells. Overall distribution of myeloid (CD33+), B-lymphoid (CD19+) lineages, progenitor cells (CD34+) and megakaryocytes (CD41+) among human cells (CD45+). (B) Representative flow cytometric analysis of BM from mice transplanted with PB CD34+ cells from a G-CSF mobilized volunteer or a patient with IM. BM cells were analyzed by flow cytometry at 9-15 weeks after transplantation.
Figure 4: Transcriptional clonality assay of G6PD in human CD33+ cells isolated from mice transplanted with IM PB CD34+ cells. Lane #1 ~ #3: clonal expression of G6PD in human CD33+ cells from three individual mice, each transplanted with CD34+ cells from a different female IM patient: IM-1 (Lane #1), IM-2 (Lane #2) and IM-3 (Lane #3). Lane #4: negative control. Lane #5: positive control for clonal expression of G6PD on T allele. Lane #6: positive control for clonal expression of G6PD on C allele. Lane #7: positive control for polyclonal expression of G6PD on both T and C alleles.
Figure 5: (A) Representative flow cytometric analysis of BM, Spleen and PB from mice transplanted with PB CD34+ cells isolated from Patient IM-4. Cells were analyzed by flow cytometry at 12 weeks after transplantation. (B) May-Grunwald Giemsa staining and anti-CD34 immunochemical staining of a section of tibia from the mouse transplanted with cells isolated from Patient IM-4.

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Figure 5
The constitutive mobilization of bone marrow repopulating cells into the peripheral blood in idiopathic myelofibrosis

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