Studies of the Site and Distribution of CD34+ Cells in Idiopathic Myelofibrosis

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Abstract

The frequency and distribution of CD34+ cells in the bone marrow (BM) of patients with idiopathic myelofibrosis (IM) were determined using an immunohistochemical technique. The percentage and absolute number of circulating CD34+ cells were enumerated. Patients with IM exhibited a continuum of number of BM CD34+ cells ranging from 1 to 85 per 5 mm². The frequency of BM CD34+ cells was inversely related to the number of circulating CD34+ cells. The BM biopsy specimens obtained from 4 patients with IM who underwent allogeneic stem cell transplantation were examined sequentially. Quantitative measurement revealed that the reticulin fiber volume was progressively reduced after allogeneic stem cell transplantation. All 4 patients had normocellular marrow with normal numbers of BM CD34+ cells after transplantation. These findings suggest that the BM fibrosis and abnormal hematopoietic stem cell distribution in patients with IM is a consequence of the progeny of a malignant hematopoietic stem cell clone.

Idiopathic myelofibrosis (IM) is a chronic Philadelphia chromosome (Ph)-negative myeloproliferative disorder (MPD) originating at the level of the hematopoietic stem cell (HSC), characterized by cytopenias, splenomegaly, a leukoerythroblastic blood picture, poikilocytosis with teardrop RBCs, varying degrees of bone marrow (BM) fibrosis, extramedullary hematopoiesis, and an increased number of CD34+ hematopoietic progenitor cells (HPCs) in the peripheral blood (PB). The HSC defect in IM results in profound hyperplasia of abnormal BM megakaryocytes and monocytes, which have been shown to release fibrogenic growth factors locally, resulting in BM fibrosis.2-4

The number of HSCs/HPCs circulating in the PB of patients with IM has been reported to be several fold higher than in healthy control subjects.5 Recently Barosi and coworkers6 further studied the number of HSCs/HPCs in the PB of patients with IM by quantitating the absolute numbers of CD34+ cells in a large, well-defined population. They reported that the median absolute number of PB CD34+ cells in patients with IM was 360 times greater than the median number in healthy control subjects and 18 to 30 times greater than the number in patients with other Ph– MPDs.6 These investigators also provided evidence that the number of CD34+ cells in the PB in excess of $300 \times 10^6$/L was characteristic of a patient with an exceedingly poor prognosis and was associated with an increased risk of transformation to acute leukemia.6

The BM in IM ranges from hypercellular with few fat cells and a focal increase of reticulin fibers to markedly hypocellular with dense reticulin and collagenous fibrosis.7 Significantly different numbers of BM CD34+ cells have been observed in patients with IM with hypercellular BM compared with patients with profoundly fibrotic BM.7,8 Increased numbers of BM CD34+ cells have been reported predominantly in patients with hypercellular, mildly fibrotic BM. A reduced numbers of CD34+
cells has been observed in the BM of patients with greater degrees of BM fibrosis. However, the relationship between the number of circulating CD34+ cells and the number of bone marrow CD34+ cells remains poorly defined.

We studied the distribution and frequency of CD34+ cells in the BM of patients with IM and attempted to determine whether there was a relationship between the number of circulating CD34+ cells and the number of BM CD34+ cells. In addition, we systematically studied the frequency of CD34+ cells and extent of reticulin fibrosis before and after allogeneic stem cell transplantation.

Materials and Methods

Patients

We studied 31 patients with IM with a median age of 57 years (range, 35-78 years). Four patients underwent allogeneic stem cell transplantation. The diagnosis of IM was established according to the Italian Diagnostic Criteria, which include 2 required and 6 optional criteria. The required criteria are as follows: (1) diffuse BM fibrosis and (2) absence of Ph chromosome or a bcr-abl rearrangement in PB cells. The optional criteria include the following: (1) splenomegaly of any degree, (2) anisopoikilocytosis with teardrop RBCs, (3) presence of circulating immature myeloid cells, (4) presence of circulating erythroblasts, (5) presence of clusters of megakaryoblasts and anomalous megakaryocytes in BM sections, and (6) myeloid metaplasia. The diagnosis of myelofibrosis is established when there is BM fibrosis and lack of evidence of the bcr-abl rearrangement in conjunction with any 2 of the 6 optional criteria if patients have splenomegaly. If the patients do not have splenomegaly, in addition to the required criteria, 4 of the optional criteria are needed to make the diagnosis of IM.

BM biopsy specimens were obtained from the pathology archives of the University of Illinois College of Medicine, Chicago, and Policlinico San Matteo, Pavia, Italy, under a protocol approval by the institutional review board of each of these institutions. Twenty control BM specimens were obtained from people with solid tumors who had undergone staging BM biopsies. The median age of the control subjects was 54 years (range, 31-76 years). The control subjects had normal hematologic parameters and normal BM morphologic features without evidence of tumor involvement.

Immunohistochemical Staining for CD34+ BM Cells

Formalin-fixed, paraffin-embedded sections of BM from patients with IM were analyzed for the presence of CD34+ cells. The tissue sections were deparaffinized in xylene and hydrated in graded alcohols. The sections were stained with a monoclonal mouse antihuman CD34 antibody (CD34 QBend 10, DAKO, Carpinteria, CA) followed by incubation with a peroxidase-labeled polymer conjugated to a goat antimouse immunoglobulin. Staining was performed with a histostainer (DAKO) using a peroxidase detection kit with the chromogen 3,3’-diaminobenzidine tetrahydrochloride. All staining procedures used an isotypic antibody as a negative control.

Quantification of CD34+ HSCs/HPCs

Following immunostaining, the total number of CD34+ cells was enumerated in each BM biopsy section using a light microscope at ×400 magnification. The marrow area was measured using an ocular micrometer. The frequency of CD34+ HSCs/HPCs was calculated and expressed as the number of CD34+ cells per 5 mm² marrow areas.

Quantification of Reticulin Fibers

A Gomori silver stain was performed on the BM biopsy specimens. The semiquantitative grading of reticulin fibrosis was performed as previously described. The volume of reticulin fiber was measured using a multipurpose morphometric analysis test system proposed by Weibel. Two photographs were taken from the central portion of each BM section. Each photograph was coded and shuffled and then randomly examined, with the observer performing the analysis unaware of the source of the specimen. Random sampling was achieved using a multipurpose test system that was superimposed on each photomicrograph. This system consists of 84 lines/168 points on a field of 145.5 cm². The presence of reticulin fibers at each sample point was recorded. The reticulin fiber volume was estimated by counting the points lying over reticulin fibers, and the final result was expressed as a volume percentage of reticulin fiber present in the BM using the following formula:

\[ V = \frac{P_a}{P_c} \times 100\% \]

where \( P_a \) represents point number on reticulin fibers; \( P_c \), test point number; and \( V \), volume of the reticulin fibers.

Enumeration of Circulating CD34+ Cell Numbers

In 13 patients from Pavia, the number of CD34+ cells circulating in the PB was enumerated at the same time the BM biopsy was obtained. Enumeration of numbers of circulating CD34+ cells was carried out as previously described. Briefly, PB samples were collected into an EDTA-anticoagulated tube. Cells were incubated for 15 minutes at room temperature with fluorescein isothiocyanate (FITC)-conjugated CD45 monoclonal antibody (Becton Dickinson, San Jose, CA) and phycoerythrin (PE)-conjugated CD34 monoclonal antibody. Laser dye styryl (LDS751) for nuclear identification and ammonium chloride for lysing RBCs were added to the tubes.

Analyses were performed according to the cell-gating guidelines recommended by the International Society of Hematotherapy and Graft Engineering. The cells first were gated into region 1 so that the CD45+ cells would include all nucleated WBCs and exclude RBCs, platelets, and cellular debris. CD45+ cellular events in region 1 then were analyzed for
expression of CD34 antigen, and positive events were gated into region 2. The number of CD34+ cells was derived from the number of events representing specific staining, as determined by CD45-FITC/CD34-PE/LDS751 staining, minus the number of events representing nonspecific staining, as determined by the CD45-FITC/isotype-PE control. The absolute number of CD34+ cells was calculated from the corrected number of CD34+ cells multiplied by the absolute WBC count. A minimum number of 100 CD34+ cellular events and 100,000 CD45+ cellular events were collected for the CD34+ cell quantification.

Statistical Analysis

The frequency of BM CD34+ cells was correlated with the number of circulating CD34+ cells. Correlations were determined by linear regression analysis, and the strength of the correlation was evaluated by the linear regression coefficient, r. P values were determined based on the value of the linear correlation coefficient and the number of data points used in the linear regression. A probability of less than 0.05 was considered statistically significant.

Results

Quantification of CD34+ Cells in BM Biopsy Specimens

The BM CD34+ HSCs/HPCs could be distinguished from CD34+ endothelial cells based on morphologic features. CD34+ cells were distributed in a random manner located...
in the paratrabecular (endosteal) and nonparatrabecular regions within the BM in control subjects and patients with IM. The mean ± SD number of BM CD34+ cells in control subjects was 15.18 ± 6.95 per 5 mm². The number of CD34+ cells in patients with IM ranged from 1 to 128 per 5 mm². There was a continuum of the numbers of CD34+ cells within the BM of patients with IM; some patients had greater numbers of CD34+ cells than those observed in control subjects, others had numbers similar to those of control subjects, and the remainder had reduced numbers compared with control subjects (Figure 1).

Distribution of CD34+ Cells in BM and PB

In 13 patients with IM, the absolute number of circulating CD34+ cells and the percentage of circulating CD34+ cells were enumerated at the time of the BM biopsy. The distribution of numbers of CD34+ cells in the BM and PB is summarized in Table 1. The frequency of BM CD34+ cells was inversely related to the number of circulating CD34+ cells ($r = -0.56591; P = .043$). One group of patients with IM had higher numbers of BM CD34+ cells (mean ± SD, 45.71 ± 23.10 per 5 mm²; cases 7-13), cellular marrow, and modestly increased numbers of circulating CD34+ cells. A second group of patients with IM was characterized by decreased numbers of BM CD34+ cells (mean ± SD, 4.83 ± 4.21 per 5 mm²; cases 1-6), decreased marrow cellularity with extensive BM fibrosis, and markedly increased numbers of circulating CD34+ cells (Table 1 and Figure 1).

Quantification of Reticulin Fibrosis and CD34+ Cell Numbers in BM of Patients With IM After Allogeneic Stem Cell Transplantation

Four patients with IM underwent allogeneic stem cell transplantation with reduced intensity of the conditioning regimen that has been previously described. Quantitative analysis revealed that the mean ± SD volume of reticulin fiber gradually decreased from 51.3% ± 19.8% before transplantation to 6.0% ± 1.4% 12 months after transplantation. Regardless of the numbers of BM CD34+ cells before transplantation, all 4 patients had a normocellular BM and normal numbers of BM CD34+ cells (mean ± SD, 13.8 ± 2.3 per 5 mm²) 12 months after transplantation. After transplantation, all 4 patients were observed to have near normal numbers of PB CD34+ cells (1.8, 0.74, 0.3, and 2.1/µL, respectively).

Discussion

IM is a neoplastic stem cell disorder in which a multipotent HSC acquires a clonal proliferative advantage, and its progeny inappropriately releases fibrogenic and angiogenic factors into the BM microenvironment. We found a continuum of the numbers of CD34+ cells within the BM of patients with IM. Some patients had greater numbers of marrow CD34+ cells than the number observed in control subjects; others had normal numbers of BM CD34+ cells, and the remainder had decreased numbers of marrow CD34+ cells compared with control subjects. The increased number of CD34+ cells within the BM was associated with increased BM cellularity, whereas the reduced number of CD34+ cells in the BM was associated with dense fibrosis.

A controversy regarding the progression of BM fibrosis in patients with IM remains. Wolf and Neiman undertook a study of 35 cases of IM. Sequential BM biopsies were done in 21 cases at intervals ranging from 2 to 10 years. They were unable to demonstrate a correlation between the extent of BM fibrosis and duration of disease. On the other hand, Thiele and coworkers studied a group of patients with the prefibrotic form of IM. Sequential BM biopsy specimens in 22 of 80 patients revealed progressive BM fibrosis that was associated with changes in hematologic parameters toward
Table 1
Distribution of CD34+ Cells in the Bone Marrow and Peripheral Blood of Patients With Idiopathic Myelofibrosis

<table>
<thead>
<tr>
<th>Case No.</th>
<th>No. of Bone Marrow CD34+ Cells (/5 mm²)</th>
<th>No. of Circulating CD34+ Cells (/µL)</th>
<th>CD34+ Cells in the Peripheral Blood Mononuclear Cell Fraction (%)</th>
<th>Reticulin Fibrosis</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>307</td>
<td>3.76</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>528</td>
<td>2.75</td>
<td>++++</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>23</td>
<td>0.80</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>18</td>
<td>0.78</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>240</td>
<td>7.49</td>
<td>++++</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>345</td>
<td>2.27</td>
<td>++++</td>
</tr>
<tr>
<td>7</td>
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<td>0.24</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>4</td>
<td>0.10</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>1</td>
<td>0.11</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>1</td>
<td>0.01</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>6</td>
<td>0.09</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>61</td>
<td>5</td>
<td>0.05</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>85</td>
<td>1</td>
<td>0.02</td>
<td>++</td>
</tr>
</tbody>
</table>

+, occasional fine individual fibers; ++, fine fiber network throughout most of the section, no coarse fibers; ++++, diffuse fine fiber network with scattered thick, coarse fibers; ++++, diffuse fine and coarse fiber network with areas of collagen fiber deposition.

Image 2
The degree of reticulin fibrosis in a patient with idiopathic myelofibrosis before and after allogeneic stem cell transplantation. A, A bone marrow biopsy specimen before transplantation (Gomori silver stain, ×200). B, A bone marrow biopsy specimen 12 months after transplantation (Gomori silver stain, ×200).

Table 2
Volume Density of Reticulin Fibers in Bone Marrow Biopsy Specimens From Patients With Idiopathic Myelofibrosis Following Allogeneic Stem Cell Transplantation

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pretransplantation</th>
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<th>3</th>
<th>6</th>
<th>12</th>
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<tbody>
<tr>
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<td>44</td>
<td>25</td>
<td>20</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>21</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>69</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>51.3 ± 19.8</td>
<td>31.2 ± 26</td>
<td>11.5 ± 6</td>
<td>8 ± 5.4</td>
<td>6 ± 1.4</td>
</tr>
</tbody>
</table>

* Data are given as percentages.
Table 3

Frequency of CD34+ Cells in the Bone Marrow of Patients With Idiopathic Myelofibrosis Following Allogeneic Stem Cell Transplantation*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pretransplantation</th>
<th>Posttransplantation (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>9.6</td>
</tr>
<tr>
<td>2</td>
<td>11.5</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>16.3</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td>10.2</td>
<td>21.6</td>
</tr>
</tbody>
</table>

* Data are given per 5 mm² of bone marrow area.

the typical clinical features of classic IM. Recently, Buhr et al studied sequential BM biopsy specimens from patients with IM and found a stepwise evolution of the morphologic features of classic IM in 13 of 41 patients and minimal reticulin fibrosis in 16 of 41 patients with no fibrosis at initial diagnosis. A large prospective histologic study of BM is required to further document the stepwise evolution in IM.

IM is characterized by an increased number of circulating CD34+ HSCs/HPCs. Barosi et al reported that the median absolute number of circulating CD34+ cells in healthy control subjects was 0.25 × 10⁶/L. The median absolute number of circulating CD34+ cells in the overall population of patients with IM was 360 times greater than the median number in healthy subjects. In addition, the numbers of circulating CD34+ cells were higher in patients with IM than in other subtypes of Ph− MPD. In the present study, we demonstrated that the increased number of circulating CD34+ cells was inversely correlated with the number of BM CD34+ cells. A decreased number of BM CD34+ cells was associated with a greater degree of reticulin fibrosis, whereas a greater number of BM CD34+ cells was associated with cellular BM. These results indicate that the absolute number of circulating CD34+ cells likely can serve as an informative biomarker to assess progression of disease within the BM.

It has been hypothesized that the increased number of circulating CD34+ cells in the PB is caused by the activation of quiescent stem cells that have been dormant in the organs outside the BM compartment, such as the spleen and liver. However, additional data also have suggested that a rise in CD34+ cells in the PB might result from an efflux of CD34+ cells from the fibrotic BM into the circulation. Our results support the latter hypothesis and indicate that the increase in number of circulating CD34+ cells is associated with a relative reduction in the number of BM CD34+ cells.

The mechanisms by which CD34+ cells egress from the BM to the circulation remains unknown. The alteration of the BM architecture, including dilated sinusoidal vessels and intraluminal hematopoiesis, might facilitate easy access of HSCs/HPCs into the circulation. However, the observation that patients with IM have more circulating CD34+ cells than do those with secondary forms of myelofibrosis suggests that the physical disruption of the normal BM microenvironment due to fibrosis is not sufficient to explain this phenomenon. Studies have suggested that the migration of CD34+ cells from the BM into the circulation might be due to an intrinsic defect of the neoplastic HSCs/HPCs that significantly impairs their capability to interact with the BM microenvironment.

Clinical studies have shown that allogeneic stem cell transplantation can serve as a potential curative treatment for patients with IM. We systematically studied the frequency of CD34+ cells and the extent of reticulin fibrosis before and after allogeneic stem cell transplantation. Our data suggest that allogeneic stem cell transplantation normalizes the number of CD34+ cells in the PB and results in the gradual resolution of BM fibrosis. These findings further support the hypothesis that BM fibrosis and abnormal HSC/HPC distribution in patients with IM is a consequence of the progeny of a malignant hematopoietic stem cell clone that leads to IM.

References


