RAPID COMMUNICATION

A Model of Myelofibrosis and Osteosclerosis in Mice Induced by Overexpressing Thrombopoietin (mpl Ligand): Reversal of Disease by Bone Marrow Transplantation

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Two types of myelofibrosis have been described in humans. Idiopathic myelofibrosis, also known as agnogenic myeloid metaplasia or primary myelofibrosis, is characterized by bone marrow (BM) fibrosis, splenomegaly, extramedullary hematopoiesis mainly in spleen and liver, and elevated numbers of peripheral blood hematopoietic precursors. Secondary myelofibrosis is usually associated with other identifiable myeloproliferative disorders, cancer (eg, leukemia, Hodgkin's disease, and breast cancer), or viral infections. In both instances, excessive deposition of extracellular matrix proteins that include fibronectin, vitronectin, laminin, and type I, III, and IV collagen is present in the BM. In some patients, myelofibrosis is also associated with the disease osteosclerosis. Myelofibrosis has been hypothesized to be a reactive phenomenon secondary to myeloid disorders. It has been postulated that defective or abnormal megakaryocytes and the release of growth factors such as platelet-derived growth factor (PDGF), and transforming growth factors (TGF-β) are responsible for the development of myelofibrosis. PDGF is a major mitogen for connective tissue cells such as fibroblasts and smooth muscle cells. TGF-β consists of a family of proteins and has broad effects on many types of cells, including fibroblasts and osteoblasts. Although megakaryocytes and platelets are considered to be the major cellular sources of PDGF-BB, PDGF-AB, and TGF-β1 (the most abundant isoform of TGF-β), and expanded populations of megakaryocytes in BM have been described in patients with myelofibrosis, the role of megakaryocytes in myelofibrosis remains to be elucidated due to the lack of correlation between the elevated number of megakaryocytes and BM fibrosis. Recently, monocytes/macrophages in patients with marrow fibrosis have also been shown to produce a wide range of fibrogenic cytokines, such as TGF-β and extracellular matrix proteins, suggesting that monocytes/macrophages are required in the process of BM fibrosis.

We have described a mouse model to study the biological role of thrombopoietin (TPO), a c-mpl ligand, in vivo by retroviral-mediated gene transfer. TPO overexpressing mice were generated by transplanting BM cells infected with TPO retrovirus into lethally irradiated recipient mice. The results of TPO overexpression in the reconstituted animals were reported recently. As expected, TPO stimulated the growth of megakaryocytes and increased platelet levels. Surprisingly, all the TPO overexpressing mice developed syndrome, similar to idiopathic myelofibrosis described in humans. This condition was characterized by marrow myelofibrosis (marked increase in reticulin), osteosclerosis (neotrabecular bone growth), extramedullary hematopoiesis mainly in the spleen and liver, and elevated numbers of hematopoietic precursors in circulation. Important questions raised by our initial study were whether these changes, which were presumably induced by TPO overexpression, were (1) permanent or reversible and (2) associated with the production of growth factors that could stimulate both the fibrotic and osteoclastic responses. To address these issues, we further documented the persistent thrombocytosis, myelofibrosis, and osteosclerosis in TPO overexpressing mice for more than 15 months. In addition, we determined the levels of TGF-β1 and PDGF in the transplanted mice, indicating the possible roles of these cytokines released from megakaryocytes in the pathogenesis of myelofibrosis and osteosclerosis. Finally, we explored the possibility of reversing the syndrome developed in the TPO overexpressing mice by administering to these animals lethal irradiation followed by transplantation of normal BM cells.
MATERIALS AND METHODS

Mice. Eight- to 12-week-old male mice (C57BL/6J × DBA/2J) F1 (BDF1) were used as donors for viral infection. Female BDF1 mice were γ-irradiated (9.5 Gy, Cs137) and used as recipients. All mice were purchased from Charles River Laboratories (Wilmington, MA) and housed at the Amgen vivarium (Thousand Oaks, CA) under pathogen-free conditions.

TPO overexpressing mice. Detailed methods, including construction of murine stem cell virus (MSCV) and TPO-MSCV retroviral vectors, BM infection, and BM transplantation, have been described previously.16 Briefly, the control vector MSCV contains a neomycin phosphotransferase (neo) gene driven by an internal mouse phosphoglycerate kinase (PGK) promoter. The TPO-MSCV vector carried a full-length murine TPO cDNA (1.5 kb) under the transcriptional control of a viral LTR promoter.18 Retroviruses were obtained by transfecting retroviral vectors into a GP + E86 packaging cell line.18 Clones with the highest titers were selected. Both MSCV and TPO-MSCV retroviral-producing cells had titers of more than $1 \times 10^9$ G418 CFU/mL. BM cells were harvested from mice 4 days after 5-fluorouracil (5-FU) treatment (150 mg/mL) and infected with viral supernatant in the presence of growth factors including 100 ng/mL recombinant rat stem cell factor (rrSCF), 100 ng/mL recombinant human interleukin-11 (rhIL-11), 100 ng/mL rhIL-6, and 2.5 ng/mL recombinant murine IL-3 (rmIL-3; all factors from Amgen Inc, Thousand Oaks, CA) and 6 μg/mL polybrene. Culture media were replaced every 24 hours with fresh viral supernatant and growth factors. After a 3- to 4-day infection period, cells were harvested and transplanted into γ-irradiated (9.5 Gy, Cs137) mice. Each recipient received cells harvested from virus-infected cultures originally initiated with donor BM from 1 to 2 femurs. Blood samples were obtained by retro-orbital bleeding and diluted (1:2 or 1:3) with 1% bovine serum albumin (BSA) saline and analyzed on a Technicon H1E blood analyzer (Miles Inc, Tarrytown, NY). At 9 months after transplantation, some of the TPO overexpressing mice were γ-irradiated (10.0 Gy, Cs137, at $2 \times 5.0$ Gy split dose) and transplanted with $3 \times 10^6$ normal BM cells from BDF1 mice.

Colony assay. BM cells (2.0 to $5.0 \times 10^5$/mL), spleen cells (1.0 to $3.0 \times 10^7$/mL), and low-density peripheral blood cells (1.0 to $5.0 \times 10^8$/mL) were plated in 1.0 mL Iscove’s modified Dulbecco’s medium (IMDM) plus methylcellulose containing 10% fetal bovine serum (FBS), 1% BSA, lipids, transferrin, insulin,20 2.5 ng/mL rhIL-3, 2.5 ng/mL rhIL-1β, 100 ng/mL rrSCF, 100 ng/mL rhIL-6, 100 ng/mL rhIL-11, and 2 µg/mL recombinant human erythropoietin (rhEpo). Colonies were scored at day 9.

Analysis of the provirus. Genomic DNA was prepared from BM and spleens of reconstituted animals by digestion of samples with proteinase K (50 µg/mL), followed by phenol/chloroform extraction. DNA samples were then restriction digested with Asp718 to generate viral LTR ends. Ten-microgram DNA digests were electrohoresed in agarose gel and blotted onto Hybound-N+ (Amersham, Arlington Heights, IL) membrane and hybridized to 32P-labeled neo probe by conventional methods.21

Assays for TGF-β1 and PDGF. Platelet-poor plasma (PPP) was prepared by centrifugation and filtration. Whole blood was obtained from transplanted mice in EDTA-containing tubes and centrifuged at 3,000 g for 10 minutes at room temperature. Plasma was then carefully removed and centrifuged through 0.45-μm acetate cellulose filter (Spin-X; Costar, Cambridge, MA) in an Eppendorf centrifuge at 10,000 rpm for 1 minute. PPP was collected and stored at −20°C. TGF-β1 levels in PPP were measured by immunoassay using a human TGF-β1 immunoassay kit (R&D Systems Inc, Minneapolis, MN) as recommended by the supplier. PDGF was analyzed for its
Fig 3. Elevated TGF-β1 and PDGF in TPO overexpressing mice. TGF-β1 was measured by ELISA (A). ■ and □ represent control and TPO overexpressing mice (n = 5 to 10), respectively. PDGF activity was measured in a serum-free culture of NIH3T3 cells by [3H]-thymidine incorporation (detailed in the Materials and Methods) (B). □ and ○ represent cultures without or with anti-PDGF antibodies (AB) at 3 μg/mL, respectively. The number of mice is indicated by n. Ten nanograms per milliliter of rhPDGF-BB was added as the positive control for this assay.

Fig 4. Effects of transplantation of normal BM cells into TPO overexpressing mice on circulating platelet (A), RBC (B), and WBC (C) levels after transplantation (n = 6 to 10).
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Table 1. Retransplantation of TPO Overexpressing Mice With Normal BM Cells

<table>
<thead>
<tr>
<th>Items</th>
<th>Before Transplantation</th>
<th>After Transplantation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>PB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet (×10^6/μL)</td>
<td>3.31 ± 0.286</td>
<td>1.258 ± 0.94</td>
</tr>
<tr>
<td>RBC (×10^6/μL)</td>
<td>8.95 ± 0.13</td>
<td>9.45 ± 0.27</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>10.47 ± 0.20</td>
<td>13.85 ± 0.26</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>31.29 ± 0.89</td>
<td>44.92 ± 1.81</td>
</tr>
<tr>
<td>WBC (×10^3/μL)</td>
<td>16.39 ± 1.53</td>
<td>14.03 ± 2.33</td>
</tr>
<tr>
<td>CFC/mL blood</td>
<td>5.43 ± 2.370</td>
<td>107 ± 35*</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>8.22 ± 0.04</td>
<td>0.072 ± 0.03</td>
</tr>
<tr>
<td>Cells/spleen (×10^11)</td>
<td>8.46 ± 1.61</td>
<td>8.47 ± 0.94</td>
</tr>
<tr>
<td>CFC/spleen</td>
<td>87,820 ± 60,902</td>
<td>5,932 ± 1,977</td>
</tr>
<tr>
<td>BM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells/femur (×10^6)</td>
<td>ND</td>
<td>12.5 ± 0.08</td>
</tr>
<tr>
<td>CFC/femur</td>
<td>ND</td>
<td>71,998 ± 8,006</td>
</tr>
</tbody>
</table>

Results in the before transplantation group were obtained from TPO overexpressing mice before the retransplantation. Results in the after transplantation group were obtained from retransplanted TPO overexpressing mice with normal BM cells at 13 and 15 weeks after retransplantation. Results were mean ± 1 SEM.

Abbreviations: ND, not determined due to the difficulty in BM harvesting; HGB, hemoglobin; HCT, hematocrit.

*n = 3.

formalin fixation. The tissues were then processed using standard histologic methods to yield 3-mm paraffin sections that were subsequently stained with hematoxylin and eosin. The bone sections were also analyzed histochemically using Masson’s trichrome and Gomori’s reticulum stains. To enhance the identification of megakaryocytes and platelets, immunohistochemistry for factor VII and von Willebrand’s factor (Dako, Carpinteria, CA) was also performed using an automated immunostainer (BioTek, Santa Barbara, CA).

RESULTS

Long-term TPO overexpressing mice. Long-term follow-up of transplanted mice resulted in a persistent threefold to fivefold elevation of platelet levels in all animals from 8 independent experiments (data not shown). Figure 1 shows a representative experiment in which 32 TPO overexpressing mice and 30 control mice were reconstituted. Peripheral blood (PB) was analyzed continuously for more than 65 weeks. Platelet levels in TPO overexpressing mice had rapid recovery after irradiation, continued to increase, and reached a peak of approximately 4,500 × 10^9/mL at 6 to 7 weeks. Platelet counts then declined slowly and maintained a steady level at about 3,000 × 10^9/mL from 20 weeks to 73 weeks (Fig 1).

The decline of platelet levels at 6 to 7 weeks after transplantation was possibly due to diminished expression of TPO. If so, by transplantation of vector-transduced cells from these animals into secondary lethally irradiated recipient mice, a pattern of continuous decline of platelets would be expected. Therefore, a group of 5 animals was killed at 8 months after transplantation. Spleen cells (1.5 × 10^6) were pooled and transplanted into 10 lethally irradiated BDF, re-
mice were anemic, with 20% less red blood cells (RBC); were thrombocythemic, with threefold to fourfold elevated platelets; and had dense bone radiographs compared with control mice. After transplantation, platelet numbers in these mice decreased sharply to a normal level of about 1,000 \( \times 10^9/\)mL at 2 weeks after transplantation (Fig 4A). RBC increased to control levels at 5 weeks after transplantation (Fig 4B). White blood cells (WBC) returned to preirradiation levels at 4 weeks after transplantation and did not change significantly (Fig 4C). These data show that the thrombocytosis and anemia in TPO overexpressing mice are reversed by transplantation of normal BM cells.

The retransplanted mice were killed and analyzed at 12 and 15 weeks after transplantation. WBC, platelet, RBC, hemoglobin, and hematocrit levels in the retransplanted animals were normal. When BM cells were harvested, marrow plugs could be easily flushed out with a 23-gauge needle. In contrast, a 27-gauge needle could not be introduced into the bone cavity of TPO overexpressing mice before retransplantation due to severe osteosclerosis. BM plugs were red, indicative of normal erythropoiesis, and had normal cellularity (Table 1). Spleen size, weight, and cellularity of the retransplanted mice returned to the normal range. Hematopoietic precursors in the spleen and peripheral blood, measured by colony assay, were decreased by 15- and 88-fold, respectively, resulting in a similar colony-forming frequency as in the MSCV control mice (data not shown). The number of colony-forming cells (CFC) per femur in retransplanted mice were comparable to that of control mice (data not shown).

Histological analysis of TPO overexpressing animals at 40 to 42 weeks after primary transplantation showed lesions similar to what we had observed in the first series of experiments.\(^{16}\) Radiographically, the animals had abnormal bones (Fig 5B and F) that exhibited marrow fibrosis (Fig 6H), osteosclerosis (Fig 6E), increased marrow megakaryocytes,
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Fig 6. The splenic, bone, and BM changes induced by overexpression of TPO are reversed after retransplantation with normal marrow. Irradiated BDF1 mice were transplanted with marrow infected with a control retrovirus (A, D, and G) or a TPO retrovirus (B, C, E, F, H, and I) and aged. At 9 months, the TPO overexpressing animals were irradiated and retransplanted with normal marrow. The tissues shown above were obtained just before (A, B, D, E, G, and H) and at 13 (C, F, and G) weeks after BM transplantation. Sections of spleen stained immunohistochemically for factor VIII/von Willebrand’s factor from TPO overexpressing animals (B) show a marked increase in megakaryopoiesis and sinusoidal platelets compared with MSCV-expressing animals (A). After transplantation with normal marrow, the splenic changes reverted to normal (C). Sections of femurs from TPO overexpressing animals show a marked increase in bone (E, Masson’s trichrome stain) and reticulin (H, Gomori’s reticulin stain) in the medullary space compared with control animals (D and G, respectively). After transplantation, both the amount of bone and the level of reticulin deposition normalized (E and F, respectively). Photomicrographs were taken using 20× (A through C), 10× (D through F), and 40× (G through I) objectives. Calibration bars represent 100, 200, and 50 μm, respectively.

and platelets and/or megakaryocyte fragments present in the marrow interstitium. Factor VIII-positive material could be seen in the bone matrix in the medullary space. The spleens in the TPO overexpressing animals were expanded by increased megakaryocytes and other hematopoietic lineages (Fig 6B). The sinuses contained numerous platelets. Interestingly, the spleens had increased reticulin deposition similar to the BM. All 10 TPO overexpressing animals had osteosclerosis before their retransplantation with normal marrow. After retransplantation, the skeletal lesions cleared based on radiographic assessments (Figure 5C, D, G, and H). Histologically, the bony abnormalities had normalized significantly using the TPO overexpressing animals as positive controls. There were residual trabeculae in the diaphysis, but the excess bone had been largely removed. The marrow fibrosis had essentially disappeared, except for focal changes in one animal. The spleens had largely returned to normal size. However, there was mildly increased connective tissue in the red pulp areas and septae detected in some animals. Hematopoietic activity had reverted to normal levels.

DISCUSSION

In the current studies, we have shown overexpressing TPO in mice for more than 17 months. Continued elevated platelet levels have been maintained at about threefold in these mice compared with control animals. Such long-term over-expression of TPO extended our original description of marked myelofibrosis, osteosclerosis, extramedullary hematopoiesis in the spleen and liver, and elevated circulating hematopoietic precursors. These effects were seen in all of the TPO overexpressing animals that have been analyzed. Despite the prolonged thrombocytosis, severe marrow fibrosis, osteosclerosis, and extramedullary hematopoiesis, TPO overexpressing mice had the same (80%) survival as control mice at 60 weeks after transplantation.

The syndrome that developed in the TPO overexpressing mice is similar to idiopathic myelofibrosis described in humans, which is occasionally associated with osteosclerosis. The pathogenesis of idiopathic myelofibrosis in humans is unknown. Therapies for the disease involved cytotoxic drugs, radiotherapy, cytokines such as erythropoietin, or splenectomy to relieve symptoms. Recently, there have been a few case reports showing the reversal of myelofibrosis by allogeneic BM transplantation. We hypothesized that, if the experimental myelofibrosis and osteosclerosis were reversible, TPO overexpressing mice may serve as a useful model to explore therapeutic treatment for these diseases. We initially thought to block cytokine production in these
mice by the administration of monoclonal antibodies to TGF-β1 and PDGF-B. The major obstacle for this approach was to maintain high levels of antibodies in vivo to neutralize the continuous production of PDGF and TGF-β1 for a period of time. Therefore, we asked whether the myelofibrosis and osteosclerosis in TPO overexpressing mice could be reversed by blocking overproduction of TPO and normalizing megakaryocyte and platelet levels. A simple BM transplantation approach was taken. At 9 months after the initial transplantation with TPO viral-transduced cells, TPO overexpressing mice were lethally irradiated and transplanted with BM cells harvested from normal mice. As expected, platelet and megakaryocyte levels were normalized after transplantation. Interestingly, all the abnormalities that developed in TPO overexpressing mice, including BM fibrosis, osteosclerosis, anemia, splenomegaly, extramedullary hematopoiesis, and high levels of circulating hematopoietic precursors, were corrected. These results strongly suggest that BM transplantation would be an efficient therapy for patients with myelofibrosis and osteosclerosis.

We initially suggested that the pathogenesis of myelofibrosis and osteosclerosis in the TPO overexpressing mice was not a primary effect of TPO and that elevated cytokines such as PDGF and TGF-β released from megakaryocytes and platelets in TPO overexpressing mice could directly stimulate the fibrogenic and osteogenic response. In the current study, we showed high levels of PDGF and TGF-β1 in the PPP of TPO overexpressing mice. The high levels of TGF-β and PDGF in these mice suggested that both substances were released from megakaryocytes and/or platelets. Our observations support an earlier hypothesis that the release of cytokines from megakaryocytes and platelets played a critical role in the development of myelofibrosis and osteosclerosis. Although monocytes/macrophages, which are capable of producing PDGF and TGF-β, may play a role in the development of myelofibrosis, it is unlikely that monocytes/macrophages contribute significantly to the production of high levels of TGF-β1 and PDGF in the TPO overexpressing mice, because there is no significant increase in these cells in TPO overexpressing mice (data not shown).

Although both TGF-β and PDGF are able to stimulate fibrogenic and osteogenic responses in vivo, the definitive roles of TGF-β and PDGF in the pathogenesis of myelofibrosis and osteosclerosis remain to be further elucidated, eg, by using mice lacking the TGF-β1 gene. In addition, whether TPO also acts directly on BM stromal cells and is involved in bone remodeling in vivo remains to be evaluated. It is also possible that other cytokines that are induced by TPO, PDGF, TGF-β, or their combinations may contribute to the development of myelofibrosis and osteosclerosis. These issues are currently being addressed further in our laboratory.

REFERENCES


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A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation

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