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

By Xiao-Qiang Yan, Dave Lacey, Dave Hill, Ying Chen, Frederick Fletcher, Robert G. Hawley, and Ian K. McNiece

We have previously shown that mice induced to overexpress thrombopoietin (TPO) by retroviral-mediated gene transfer into bone marrow (BM) cells develop myelofibrosis and osteosclerosis. It was speculated that these effects were secondary to TPO, resulting from high levels of megakaryocytes and platelets. Also, it was proposed that these mice represent a model for myelofibrosis and osteosclerosis. In this report, we show that levels of both transforming growth factor-β1 and platelet-derived growth factor are increased twofold to fivefold in the platelet-poor plasma of TPO overexpressing mice compared with control mice. These data suggest that the increased megakaryocytes produce elevated levels of these cytokines that lead to the pathogenesis of disease. Further, we retransplanted TPO overexpressing mice, at 40 to 42 weeks after primary transplantation, with normal BM cells. After the secondary transplantation, megakaryocytes and platelets returned to normal levels and the myelofibrosis and osteosclerosis were completely corrected. These data extend our initial studies of the effects of overexpression of TPO and show the potential use of this model to explore the underlying cause of myelofibrosis and osteosclerosis and potential treatments for these diseases.

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A MODEL OF MYELOFIBROSIS AND OSTEOSCLEROSIS

Fig 1. Effects of long-term overexpression of TPO on peripheral platelet levels after transplantation. Dotted line (○) and solid line (●) represent MSCV control and TPO overexpressing mice (n = 10 to 15), respectively. Dashed line (■) represents 10 secondary transplanted mice that received spleen cells (1.5 × 10⁷ per mouse) from 5 TPO respectively. Dashed line 1.1 represents 10 secondary transplanted overexpressing mice at 32 weeks after transplantation.

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. 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. Retroviruses were obtained by transfecting retroviral vectors into a GP + E86 packaging cell line. Clones with the highest titers were selected. Both MSCV and TPO-MSCV retroviral-producing cells had titers of more than 1 × 10⁶ G418⁵ CFU/mL. BM cells were harvested from mice 4 days after 5-fluorouracil (5-FU) treatment (150 ng/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 × 5.0 Gy split dose) and transplanted with 3 × 10⁶ normal BM cells from BDF₁ mice.

 Colony assay. BM cells (2.0 to 5.0 × 10⁷/mL), spleen cells (1.0 to 3.0 × 10⁷/mL), and low-density peripheral blood cells (1.0 to 5.0 × 10⁷/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, recombinant human interleukin-1 (rHL-l, 100 ng/mL; Amgen, Thousand Oaks, CA) recombinant rat SCF (rrSCF) 100 ng/mL rhIL-6, 100 ng/mL rhIL-11, and 2 U/mL recombinant human erythropoietin (rEpo). 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 and used for analyzing host-provirus DNA junctions and with Asp718 for excision of provirus at LTR ends. Ten-microgram DNA digests were electrophoresed in agarose gel and blotted onto Hybond-N+ (Amersham, Arlington Heights, IL) membrane and hybridized to 32P-labeled neo probe by conventional methods.

 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).
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 routinely stained with hematoxylin and eosin. The bone sections were fixed in formalin and processed for histologic methods to yield 3-mm paraffin sections that were subsequently stained with hematoxylin and eosin.

**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 × 10^6/mL at 6 to 7 weeks. Platelet counts then declined slowly and maintained a steady level at about 3,000 × 10^6/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 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 lethally irradiated BDF, recipients. Platelet levels in the secondary recipients recovered rapidly and reached a level greater than 4,000 × 10^6/mL (Fig 1, dashed line), whereas the primary TPO overexpressing mice maintained platelets at about 3,000 × 10^6/mL. These results showed that long-term reconstituting stem cells were transduced by TPO retroviral vector and that the LTR promoter was functional for more than 17 months in vivo.

**Analysis of provirus in TPO overexpressing mice.** The sustained high levels of platelets in TPO overexpressing mice (Fig 1) indicated the persistence of functional provirus in the reconstituted animals. In a previous report, we documented that the 65% to 81% of hematopoietic precursors in the reconstituted mice were neo-positive, the presence of the expected levels of TPO RNA transcripts in the BM and spleen, and detectable levels of TPO activities in the circulation. We further analyzed here the clonal make-up in the hematopoietic system of the reconstituted animals. Because of the development of myelofibrosis and osteosclerosis in the BM of TPO overexpressing mice, retroviral-marked clones were only analyzed in the spleens of reconstituted mice. Genomic DNA was isolated from spleens of animals at 10 weeks and at 8 to 9 months after transplantation. DNA was restriction-digested with Bgl II, which has a single cut in the provirus, and hybridized to a 32P-labeled neo probe, generating proviral-host junction fragments. Multiple viral-marked clones were present in the spleens of TPO overexpressing animals and control animals at 10 weeks and at 8 to 9 months after transplantation (Fig 2). These findings reflect an efficient gene transfer into multiple stem cells.

**Elevated TGF-β1 and PDGF levels in TPO overexpressing mice.** PPP was prepared from reconstituted animals. Latent TGF-β1 in PPP was activated to an immunoreactive form by acetic acid/urea treatment and was measured by enzyme-linked immunosorbent assay (ELISA) shown in Fig 3A. After transplantation, TGF-β1 levels in TPO mice increased threefold to fivefold over time. Compared with control animals, the levels remained twofold higher for more than 1 year after transplantation. PDGF activities in PPP were measured in a serum-free assay on NIH3T3 fibroblasts. Under serum-free condition, NIH3T3 cells would not grow unless serum, PPP, or PDGF was added. The growth of NIH3T3 cells was measured by the amount of 3H-thymidine incorporation (Fig 3B). PPP from TPO overexpressing mice had twofold higher mitogenic activities on fibroblasts than PPP from control mice. The mitogenic activities could be neutralized by antihuman PDGF-B monoclonal antibodies. The neutralizing activity of anti-PDGF monoclonal antibody could be overridden by the addition of a higher concentration of PDGF-BB (data not shown).

**Reversal of the fibrosis and osteosclerosis syndrome in TPO overexpressing mice by normal BM transplantation.** Attempts were made to explore possible treatments for the syndrome of myelofibrosis and osteosclerosis in association with extramedullary hematopoiesis that developed in TPO overexpressing mice. Ten TPO overexpressing mice from two experiments at 40 to 42 weeks after transplantation were lethally irradiated again and transplanted with 3 × 10^6 normal BM cells. Before irradiation, all 10 TPO overexpressing

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**Table 1. Retransplantation of TPO Overexpressing Mice With Normal BM Cells**

<table>
<thead>
<tr>
<th>Items</th>
<th>Before Transplantation (n = 5)</th>
<th>After Transplantation (n = 5)</th>
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<tbody>
<tr>
<td>PB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet (x10^6/μL)</td>
<td>3,310 ± 286</td>
<td>1,258 ± 94</td>
</tr>
<tr>
<td>RBC (x10^6/μL)</td>
<td>8.95 ± 0.13</td>
<td>9.46 ± 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 (x10^9/mL)</td>
<td>18.39 ± 1.53</td>
<td>14.03 ± 2.33</td>
</tr>
<tr>
<td>CFC/mL blood</td>
<td>5,463 ± 2,370</td>
<td>107 ± 35*</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.226 ± 0.04</td>
<td>0.072 ± 0.03</td>
</tr>
<tr>
<td>Cells/spleen (x10^9)</td>
<td>4.86 ± 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 (x10^6)</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>
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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.
mice were anemic, with 20% less red blood cells (RBC); were thrombocytemic, 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. Radiographically, the animals had abnormal bones (Fig 5B and F) that exhibited marrow fibrosis (Fig 6H), osteosclerosis (Fig 6E), increased marrow megakaryocytes,
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 20x (A through C), 10x (D through F), and 40x (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.16 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.1,2 Recently, there have been a few case reports showing the reversal of myelofibrosis by allogenic BM transplantation.23,24 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 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 (D. Lacey, unpublished data, August 1994), 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.

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