Differential Mechanisms in the Regulation of Endogenous Levels of Thrombopoietin and Interleukin-11 During Thrombocytopenia: Insight into the Regulation of Platelet Production

By Mei Chang, Yu Suen, Gloria Meng, Jeffrey S. Buzby, Jim Bussel, Violet Shen, Carmella van de Ven, and Mitchell S. Cairo

The regulation of megakaryocytopoiesis and thrombopoiesis appears to be under the control of an array of hematopoietic growth factors. To determine the relationship of endogenous thrombopoietic cytokine levels and circulating platelet (PLT) counts, we measured the levels of thrombopoietin (TPO), interleukin-11 (IL-11), and interleukin-6 (IL-6) in patients with significant thrombocytopenia secondary to both marrow hypoplasia and increased PLT destruction. Increased endogenous levels of TPO and IL-11, but not IL-6, were detected in bone marrow transplant patients with thrombocytopenia following myeloblastic therapy (BMT/MAT) (TPO: 1,485.5 ± 87.3 pg/mL, PLT 39,600 ± 7,800/μL, P < .001, n = 12; IL-11: 227.9 ± 35 pg/mL, PLT 32,900 ± 5,700/μL, P < .05, n = 19; IL-6: 25.8 ± 8.4 pg/mL, PLT 32,800 ± 5,057/μL, P > .05, n = 4) vs normal donors (TPO <150 pg/mL, n = 8; IL-11 <50 pg/mL, n = 12; IL-6 < 10 pg/ml, n = 5). TPO levels were significantly increased (328.0 ± 92.8 pg/mL, PLT 20,900 ± 3,000/μL, P < .05, n = 25). However, endogenous TPO levels remained undetectable (<150 pg/mL, PLT 30,500 ± 5,500/μL, n = 15). These results suggest that there may be differential mechanisms regulating endogenous TPO, IL-11, and IL-6 levels during acute thrombocytopenia and suggest that the absolute number of circulating PLTs may not always be the sole regulator of endogenous TPO levels. Other mpl-expressing cells of the megakaryocyte lineage may contribute to regulation of circulating TPO levels as well. Our results also suggest IL-11 levels may in part, be regulated by a negative feedback loop based on circulating PLT counts, but also may, in part, be regulated by a variety of inflammatory agonists. Both TPO and IL-11, therefore, appear to be active thrombopoietic cytokines regulating, in part, megakaryocytopoiesis during states of acute thrombocytopenia.

T HE LIGAND FOR THE c-Mpl proto-oncogene, which is predominantly produced in the liver and kidney, has recently been purified, cloned from several species, and expressed in mammalian cells. Recombinant Mpl ligand (thrombopoietin) (TPO) has been shown to both enhance megakaryocyte development and to increase the size, number, and ploidy of developing megakaryocytes. Intra- or intraperitoneal injection of mice or neonatal rats with purified ligand results in a 400% increase in the circulating platelet (PLT) count. TPO, like many other hematopoietic growth factors, exerts its biologic effects through binding of a specific receptor, the product of the c-Mpl proto-oncogene.

Expression of c-Mpl in mice and humans appears to be restricted to PLTs megakaryocytes, and late progenitors of megakaryocytic lineage, and TPO binding induces tyrosine phosphorylation of a number of substrates, such as Jak2, Shc, and the Mpl receptor itself in human platelet and megakaryocytic cell lines. In addition to its importance in signal transduction, both in vivo and in vitro data suggests that Mpl receptor may be involved in the uptake and metabolism of TPO by PLTs.

A family of pleiotropic hematopoietic growth factors, with a common signal transduction pathway including interleukin-11 (IL-11), IL-6, and leukemia inhibitory factor, has been shown to have various stimulatory effects on megakaryocytopoiesis and platelet production. Within this family, IL-11 represents a unique polypeptide, initially cloned from a primate marrow stromal cell line (PU-34). Preclinical studies in both rodents and nonhuman primates have demonstrated that IL-11 induces significant enhancement of megakaryocytopoiesis. Administration of IL-11 also results in a significant elevation in the PLT count of neonatal rats, and splenectomized mice. Recently, a randomized placebo-controlled trial of recombinant human IL-11 (50 μg/kg) in adult cancer patients with a history of PLT transfusions during severe thrombocytopenia secondary to chemotherapy demonstrated a significant reduction in patients requiring PLT transfusions during subsequent cycles of chemotherapy.

Several lineage-dominant humoral factors that regulate the homeostasis of specific peripheral blood cells have been shown to have an inverse correlation between their circulating level or activity and the respective/responsive blood cell mass. We have previously demonstrated a significant inverse correlation of circulating granulocyte colony-stimu-
TPO AND IL-11 LEVELS DURING THROMBOCYTOPENIA

Table 1. Demographics of BMT Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Diagnosis/ Disease Status</th>
<th>Conditioning Regimen*</th>
<th>BMT</th>
<th>Cytokine Administration†</th>
<th>GVHD Prophylaxis†</th>
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Abbreviations: AA, aplastic anemia; ALL, acute lymphoblastic leukemia; ALLO/MDR, allogeneic BMT/matched related donor; ALLO/MUD, allogeneic BMT/matched unrelated donor; ANLL, acute nonlymphoblastic leukemia; ARA-C, cytosine arabinoside; AS, anaplastic astrocytoma; AUTO, autologous BMT; AUTO/PSCT, autologous BMT/peripheral stem cell transplant; BSG, brain stem glioma; BU, busulfan; CPL, carboplatin; CR, complete remission; CSF, cyclic syphillis; CY, cyclophosphamide; Ewing’s, Ewing’s sarcoma; FBSC, fibrosarcoma; NB, neuroblastoma; NHL, non-Hodgkin’s lymphoma; G, granulocyte; GEM, glioblastoma; GM, granulocyte-macrophage; Mel, melphan; MTX, methotrexate; PNET, primitive neuroectodermal tumor; R/D., residual disease; T-cell Depl, T-cell depletion; TBI, total body irradiation; TT, thiotaepa; VP, etoposide; VPL, verapamil; NA, not applicable.

* Conditioning Regimens: TBI 1,200 cGy, BID × 3d; VP 1,800 mg/m² d; VPL 0.05 mg/kg/min; CY 60 mg/kg/d × 2d. TBI 1,200 cGy, BID × 3d; VP 1,800 mg/m² d; CY 60 mg/kg/d × 2d. TBI 1,200 cGy, BID × 3d; VP 750 mg/m² d × 2d; ARA-C 3 g/m² BID × 3d; CY 45 mg/kg/d × 2d. TBI 1,200 cGy, BID × 3d; VP 1,800 mg/m² d; MEL 50 mg/m² d × 4d; CY 250 mg/m² d × 4d; CPL 300 mg/m² d × 4d; VP 2,000 mg/m² d CI × 4d. CPL 500 mg/m² d × 3d; TT 10 mg/kg/d × 3d; VP 8.3 mg/kg/d × 3; CY 750 mg/m² d × 2d; ARA-C 3 g/m² BID × 3d; CY 1,350 mg/m² d × 2d; TT 250 mg/m² d × 3d. TBI 1,200 cGy, BID × 3d; BUICY 4 mg/kg/d × 3d; ARA-C 3 g/m² BID × 3d; BCNU 100 mg/m² d; TT 300 mg/m² d × 3d; VP 250 mg/m² d × 3d; CY 60 mg/kg/d × 3d; TT 250 mg/m² d × 3d; BUICY 4 mg/kg/d × 3d; CY 80 mg/kg/d × 2d; VP 250 mg/m² d × 3d; BCNU 100 mg/m² d; TT 300 mg/m² d × 3d; VP 250 mg/m² d × 3d; CY 60 mg/kg/d × 3d; TT 300 mg/m² d × 3d.

† Cytokines: G-CSF 10 µg/kg/d; GM-CSF 250 µg/m² d/d followed by G-CSF 10 µg/kg/d (WBC >300 x 10⁹/L); GM-CSF 250 µg/m² d/d.

‡ GVHD Prophylaxis: MTX 10 mg/m² d, days 1, 3, 6, 11. CSP 3 mg/kg/d; MTX 10 mg/m² d, days 1, 3, 6, 11. T-cell depletion: E-Rosette + soybean agglutination.

The data presented in Table 1 indicates the demographics of BMT patients, including their age, sex, diagnosis, disease status, conditioning regimen, BMT type, cytokine administration, and GVHD prophylaxis. The table summarizes the clinical and laboratory data of 24 patients who received bone marrow transplant (BMT) after myeloablative therapy (MAT). The table highlights the characteristics of their disease status and treatment, which are listed in Table 1. The table further specifies the circulating levels of TPO, IL-11, and IL-6 in patients with significant thrombocytopenia secondary to marrow hypoplasia and increased PLT destruction.

Materials and Methods

MAT/BMT patient plasma samples. Between January 1992 and May 1995, a total of 481 plasma samples from 24 patients, all of whom received MAT and BMT as part of the treatment for their malignancies, were collected for subsequent analysis. The characteristics of their disease status and treatment are listed in Table 1. Plasma samples from MAT/BMT patients were obtained from each patient before administration of myeloablative therapy; on the day of BMT; every other day thereafter until absolute neutrophil count was ≥500/µL; every week thereafter until PLT count was ≥50,000/µL; and every month thereafter until 12 months post-BMT.

Submyeloablative therapy. Between April 1994 and July 1994, plasma samples from seven pediatric cancer patients who received submyeloablative therapy (SMAT) were collected for subsequent analysis. The patients, four women and three men, ranged from 2 to 27 years of age (median age, 8 years). Three patients had acute lymphoblastic leukemia (ALL) and the other four patients had either acute nonlymphoblastic leukemia (ANLL), astrocytoma, Wilms’s tumor, or rhabdomyosarcoma. Plasma samples from SMAT patients were obtained during acute thrombocytopenia episodes (PLT < 50,000/µL).

Immune thrombocytopenia purpura patient plasma samples. Between May 1991 and November 1994, a total of 55 plasma samples from 29 patients with immune thrombocytopenia purpura (ITP) were collected for subsequent analysis. The clinical and laboratory data

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for those patients are listed in Table 2. Plasma samples from newly-diagnosed ITP patients were obtained at diagnosis, 3 to 21 days after diagnosis while PLT counts recovered (>100,000/µL), and 6 months after diagnosis. Plasma samples from recurrent ITP patients were obtained during thrombocytopenia episodes, as well as after PLT recovery. Clinical data, and plasma sample collection. Eight ITP patients were treated at New York Hospital, Cornell Medical Center, New York, NY, between May 1991 and September 1994. All other patients were treated at Children’s Hospital of Orange County, Orange, CA. Informed consents for blood samples were obtained from the patients or parents/guardians as approved by the Institutional Review Board of both hospitals. Clinical and laboratory data of the patients were obtained from the patient’s medical records. Plasma was separated, aliquoted, and stored frozen at ~80°C immediately after blood samples were collected, until assay.

Enzyme-linked immunosorbent assay (ELISA) for TPO. The TPO levels in plasma were measured by a TPO receptor-antibody mediated sandwich ELISA. A full description of the assay will be published elsewhere. Briefly, microtiter plates were coated at 4°C overnight with 100 µL of rabbit Fab’ anti-human Fc (2 mg/mL, Jackson ImmunoResearch, West Grove, PA) and then incubated for 2 hours at room temperature with 100 µL of a chimeric molecule consisting of human TPO receptor fused to the Fc portion of human Ig G (100 ng/mL, Genentech, S. San Francisco, CA). Twofold serial dilution (initial dilution 1:5) of plasma samples and standards were added to the wells and incubated for 1 hour. Bound TPO was quantitated colorimetrically by incubation with 100 µL of biotinylated affinity-purified polyclonal rabbit anti-human TPO (Genentech) followed by streptavidin-conjugated peroxidase (Vector, Burlingame, CA) and substrate. Full-length, glycosylated, recombinant human TPO produced by mammalian cells was used to generate standard curves, which were subjected to four parameter nonlinear regression curve fitting. The sensitivity range of the assay was 150 to 1,000 pg/mL of TPO. The assay does not cross react with human IL-6 and IL-11. The performance characteristics of the ELISA were similar in plasma and serum and independent of the type of anticoagulant used. The ELISA preferentially detects full-length TPO. TPO levels measured by ELISA were further validated by comparable results using the megakaryoblastic HU-3 cell proliferation assay. There is a significant positive correlation (P < .001) between the stimulatory activity of thrombocytopenic sera determined by HU-3 bioassay and TPO levels determined by the ELISA.

Mixing rhTPO at a concentration of 1,000 pg/mL with representative plasma samples and measuring the recovery of rhTPO did not show any interfering substances in the plasma.

ELISA for detecting human IL-11. Levels of IL-11 were measured by a sandwich ELISA. Briefly, microtiter plates were coated with monoclonal anti-rhIL-11 antibody (Genetics Institute, Cambridge, MA). RhIL-11 standards, blanks, and the test samples were added and incubated at room temperature. Biotinylated mouse anti-rhIL-11 monoclonal antibody (Genetics Institute) was added and incubated at room temperature. Plates were washed and avidin-conjugated horseradish peroxidase (Vector, Burlingame, CA) was added and incubated for 1 hour at room temperature. Plates were washed and o-phenylenediamine (Sigma, St Louis, MO) was added as the substrate. The reaction was stopped after 5 minutes by addition of 2.25 mol/L sulfuric acid. Optical density of the samples was measured at 490 nm with a Bio Rad (Richmond, CA) EIA reader. Sensitivity of the assay ranged from 40 to 1,000 pg/mL of IL-11. Various concentrations of rhIL-11 (0 to 5,000 pg/mL) were used for the standard curve. All samples were run in duplicate. IL-11 levels by ELISA were previously validated by correlating the standard curve with a B 9/11 proliferation assay (performed by Frann Bennett, Genetics Institute).

ELISA for detecting human IL-6. Levels of IL-6 were measured by a sandwich ELISA (Biosource International, Camarillo, CA) according to manufacturer’s instructions. Sensitivity of the assay ranged from 2 pg/mL to 500 pg/mL. All samples were run in duplicate.

Statistical analysis. Results are expressed as mean values ± standard error of the mean (SEM) of three or more samples. Where appropriate, the probability of significant differences between two groups was determined using the unpaired Student’s t-test. The relationship between platelet and cytokine levels was analyzed by linear correlation (Spearman Correlation Test) and regression analysis. Statistical analyses were performed using the InStat statistical program (Graph Pad, San Diego, CA) for the Macintosh computer. P values < .05 were considered significant.

RESULTS

TPO levels in the plasma of MAT/BMT patients. To determine if plasma levels of TPO protein were altered during thrombocytopenia, 215 plasma samples from 12 patients undergoing MAT/BMT were assayed for TPO. The mean concentrations of plasma TPO from the 12 patients and the corresponding mean number of PLTs as a function of time following MAT are shown in Fig 1A. Before MAT (day -7) with normal PLT counts (211,500 ± 26,100/µL), plasma TPO levels were undetectable (<150 pg/mL). TPO levels
increased promptly during MAT (day -7 to 0) while PLT counts started to decrease (day 0: TPO: 707.89 ± 188.2 pg/mL; PLT: 86,700 ± 13,200/μL, P < .01). TPO levels increased further during the early post-BMT period and peaked at day +10 (1259.4 ± 138.77 pg/mL; PLT: 53,500 ± 14,400/μL, P < .001) coinciding with severe thrombocytopenia and a hypoplastic bone marrow. TPO levels remained elevated and by day +40, as the mean PLT counts rose to 80,600/μL, average TPO levels had decreased to 468.7 ± 80 pg/mL. Three months post-BMT, TPO levels (376 ± 73.9 pg/mL; PLT: 131 ± 68.4 k/μL) were still above the limit of detection, but most returned to baseline levels at 6 months post-BMT, and all patients were below detectability after 9 months (data not shown). As shown in Fig 1B, a significant inverse correlation (r = -0.57, P < .0001, n = 188) could be demonstrated between TPO plasma levels and PLT counts in MAT/BMT patients. There were no differences in TPO levels and PLT counts between autologous and allogeneic BMT at various days post-MAT/BMT (data not shown). The median peak value of TPO was 1451 pg/mL (range, 989 to 1839 pg/mL). Individual peak values were found at a median of 11.5 days post-BMT (range, day +2 to day +21).

**IL-11 levels in the plasma of MAT/BMT patients.** Plasma levels of IL-11 were determined in 266 samples from 19 pediatric cancer patients undergoing MAT/BMT. The mean concentrations of plasma IL-11 from those patients and the corresponding mean number of PLTs as a function of time following
eventually dropped to their nadir (IL-11 post-BMT period, while PLT counts started to decrease and circulating IL-11 levels were below detectability. BLT. There was no significant difference in endogenous IL-11 levels with PLT counts in MATBMT patients was also demonstrated plasma levels of IL-6 and the corresponding mean number of PLTs as a function of time following MAT are shown in Fig 3A. Before MAT (day -7) with normal PLT counts, circulating IL-11 levels increased further after this point and peaked at day 33 post-BMT (467.4 pg/mL; PLT: 32,900 ± 5,700/µL, P < .05) at day 5. In contrast to TPO, IL-11 levels increased further after this point and peaked at day 33 post-BMT (467.4 ± 54.9 pg/mL) despite the progress of PLT recovery (PLT: 81,750 ± 9,700/µL, P < .001, n = 12). The circulating IL-11 levels gradually decreased in 3 months and returned to near baseline levels by 9 months post-BMT. There was no significant difference in endogenous IL-11 levels between autologous and allogeneic BMT at various days post-MAT/BMT (data not shown). A significant correlation of circulating IL-11 levels with PLT counts in MAT/BMT patients was also demonstrated (r = -0.329, P < .001, n = 249) (Fig 2B).

IL-6 levels in the plasma of MAT/BMT patients. Plasma levels of IL-6 were determined in 70 samples from four pediatric cancer patients undergoing MAT/BMT. The mean plasma levels of IL-6 and the corresponding mean number of PLTs as a function of time following MAT are shown in Fig 3A. Before MAT (day -7) with normal PLT counts, circulating IL-6 levels were less than 5 pg/mL. Circulating IL-6 levels did not significantly change during the course of thrombocytopenia and platelet recovery (Fig 3A). There was no correlation between the endogenous IL-6 levels and circulating PLT counts (r = -0.1147, P > .371, NS) (Fig 3B).

TPO levels in the plasma of SMAT. A total of nine plasma samples collected from seven patients with thrombocytopenia (median PLT counts 62,000/µL; range, 36,000/µL to 118,000/µL) secondary to SMAT were assayed for TPO. Significantly elevated TPO levels were observed in all but one sample (median TPO level, 689 pg/mL; range, 361 to 1,227 pg/mL; P < .01).

IL-11 levels in ITP patients. The plasma levels of IL-11 from children with acute ITP at diagnosis were significantly increased when compared to age-matched controls (IL-11: 328.0 ± 92.6 pg/mL; PLT: 19,000 ± 2,700/µL, n = 25, P < .05) (Fig 4). Fourteen days after diagnosis, PLT counts recovered following treatment. The endogenous IL-11 levels, however, remained elevated (IL-11: 550.4 ± 159.7 pg/mL; PLT: 241,000 ± 38,200/µL, n = 13, P < .05), similar to the BMT patients. Six months after diagnosis, the circulating IL-11 levels had returned to normal levels (66.4 ± 20.3 pg/mL; PLT: 282,800 ± 46,200/µL, n = 7).

TPO levels in the plasma of ITP patients. A total of 15 plasma samples collected during thrombocytopenia (median PLT counts 30,500/µL; range, 6,000/µL to 52,000/µL) from 15 ITP patients were also assayed for TPO. In contrast to the results with IL-11, all of the ITP patients at diagnosis had undetectable circulating TPO levels (<150 pg/mL) (Fig 4). Eleven plasma samples were also obtained from six ITP patients, either after PLT recovery for the newly-diagnosed ITP patients or during clinical remission stage for the recurrent ITP patients (median PLT counts, 154,000/µL; range, 98,000/µL to 355,000/µL) (Fig 4). None of these samples had detectable TPO levels either. To rule out the possibility that the plasma of ITP patients contained an inhibitor(s) interfering with the TPO ELISA, plasma samples of ITP patients (Table 2, patient nos. 11 through 15) were mixed with rhTPO at a concentration of 1,000 pg/mL. The amount of rhTPO measured by ELISA in these samples ranged from 915 pg/mL to 1,173 pg/mL (median, 947 pg/mL), which was not significantly different from that of controls without patient plasma, ruling out the presence of an interfering inhibitor(s).

DISCUSSION

Circulating levels of TPO in patients with thrombocytopenia secondary to various pathological conditions were as-
Fig 3. (A) Time course of IL-6 levels and PLT counts in four pediatric cancer patients undergoing BMT after MAT. Pre-MAT day corresponds to day -7. MAT days correspond to day -7 to -1. BMT day corresponds to day 0, and the posttransplantation period corresponds to day +1 to +120. Mean ± SEM of the values are presented. (B) Correlation between plasma IL-6 levels and PLT counts in pediatric cancer patients undergoing BMT after MAT. The results of 62 samples from four patients are plotted. No correlation between IL-6 levels and PLT count was found \( r = -0.1147, P > 0.05 \) (NS) using Spearman correlation analysis.

assessed to evaluate the relationship of endogenous TPO levels to the circulating platelet count. Our patient population could be divided into two major groups: MAT/BMT and SMAT patients with thrombocytopenia, decreased PLT production, and bone marrow hypoplasia; and ITP patients with thrombocytopenia, decreased PLT survival, and increased PLT production based on increased bone marrow megakaryocytes. Elevated TPO levels from patients with severe thrombocytopenia following myeloablative therapy indicated an inverse relationship between circulating TPO levels and the circulating PLT count. In a recent study, TPO bioactivity in serum from patients undergoing MAT and autologous peripheral blood progenitor cell transplantation was measured by a semiquantitative 32D/huMpl+ bioassay system. Although the patients in that study had a shorter duration of severe thrombocytopenia (about 5 days compared with about 30 days in our patients), an inverse correlation between TPO levels and PLT count, over time, was demonstrated and is consistent with the results presented here.

Conversely, the TPO levels observed in ITP patients with severe thrombocytopenia remained undetectable (Fig 4). A mechanism was recently proposed suggesting that in patients with normal liver function, TPO production is constitutive and circulating TPO levels are controlled by the circulating...
been observed, as our assay did not detect TPO levels below We and others have also reported that IL-11 production can
pressed on megakaryocytic and bone marrow stromal cells.4x
nally, it is possible that small differences in the TPO levels
it is possible that circulating TPO levels had briefly surged
sessed after diagnosis, this surge may have been missed. Fi-
Consistent with our hypothesis is the recent observation that
levels may be regulated by the total cell mass of the megakar-
cept, enabling the continuous production of new PLTs,
levels may be regulated by the total number of Mpl receptors, and consequently the TPO
contrast, despite similar degrees of acute thrombocytopenia, the total number of Mpl receptors, and consequently the TPO
catabolism capacity in ITP patients, may be higher due to
levels may be regulated by both circulating PLT count and possibly other in-
cytopenic patients with bone marrow hypoplasia due to low
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