Serum Thrombopoietin Levels in Patients Receiving High-Dose Chemotherapy with Support of Purified Peripheral Blood CD34+ Cells

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Abstract

In a case control study, serum levels of thrombopoietin (TPO) were determined by a sandwich ELISA in 20 patients (median age, 7 years; range, 2-56 years) with various malignancies who received high-dose chemotherapy and a stem cell rescue operation. The patients received two different transplant modalities: (a) 12 patients received purified autologous peripheral blood CD34+ cells; and (b) 8 patients received cells in the CD34(-) fraction, which still contains many CD34+ cells. No significant differences were observed between the two groups with regard to the duration required to achieve an absolute granulocyte count of >0.5 x 10^9/liter, the duration of dependence on platelet transfusion, or the number of platelet transfusions. In both groups, the serum TPO levels were inversely correlated with the circulating platelet count. Multivariate analysis demonstrated that significant determinants of the serum TPO level included the circulating platelet count (standardized regression coefficient = -0.5179), transplantation with cells in the CD34(-) fraction (0.2414), solid tumor (0.1420), and the age of the patient (-0.1236; r^2 = 0.3021; P < 0.0001). These results suggest that the mode of stem cell support (i.e., the presence of accessory cells in the inoculum), age, or the type of preceding chemotherapy affects serum TPO levels after transplantation.

Introduction

High-dose marrow ablative therapy and PBSCTs have become established options for the treatment of high-risk malignancies. Although the larger number of CD34+ cells in PBSCT grafts compared with a marrow harvest seems to be the primary cause of more rapid hematopoietic recovery in PBSCTs, there is still a possibility that infused accessory cell populations, including monocytes and lymphocytes, enhance engraftment by producing cytokines.

Several reports regarding the endogenous production of TPO in patients receiving intensive chemotherapy have been published (1-4). Moreover, TPO has been shown to lead to clinical amelioration of thrombocytopenia after chemotherapy (5-8). The elegant study of the regulation of TPO by Fielder et al. (9, 10) demonstrated that platelets directly regulate TPO, to which they bind with high affinity, and then internalize and degrade. However, these are not the only determinants of TPO production (11). Whether or not accessory cell populations in the graft, the patient’s age, or the diagnosis affects TPO production after intensive chemotherapy with PBSC rescue is not fully understood.

In this study, we examined the factors that determine serum levels of TPO in a series of patients receiving chemotherapy with PBSC rescue operations.

Materials and Methods

Patients and Therapy. Twenty patients who had been treated at the University Hospital of Tokushima were entered into this study with the written consent of the patients themselves or their parents. This study was approved by the Institutional Review Board. The median age of the patients was 7 years (range, 2-56 years). The diagnosis included ovarian cancer in three patients, neuroblastoma in three patients, yolk sac tumor in three patients, acute lymphoblastic leukemia in two patients, rhabdomyosarcoma in two patients, retinoblastoma in two patients, breast cancer in two patients, and non-Hodgkin’s lymphoma, Wilm’s tumor, and cervical carcinoma of the uterus in one patient each (Table 1).

For the treatment of solid tumors, we performed a pilot feasibility study of consecutive double high-dose therapies, in which each course was followed by transplantation with G-CSF (filgrastim; Kirin Brewery Co., Tokyo, Japan)-mobilized peripheral blood cells that had been separated into CD34+ and CD34- fractions by an Isolx system (Baxter Limited, Dearfield, IL). Positive selection of CD34+ cells has been associated with inevitable cell loss. To overcome this loss, CD34+ cells that had migrated into the negative fraction were saved and used for the first transplant, which was followed by a second transplant after a 3-5-month interval. Some of the clinical data have been published elsewhere (12, 13).

The patients were divided into two different clinical groups as summarized in Table 1. Group A consisted of 12 patients who received high-dose chemotherapy followed by the infusion of purified autologous CD34+ blood cells. The cytoreductive regimen included the MCV regimens (Ref. 14; 450 mg/m^2 MCNU, 16 g/m^2 1-β-o-arabinofuranosycytosine, 1.6 g/m^2 VP-16, and 100 mg/kg cyclophosphamide) in three patients and the combination of 180 mg/m^2 melphalan, 1.6 mg/m^2 VP-16, and 1.6 mg/m^2 carboplatinum in nine patients. Group B consisted of eight patients who received high-dose chemotherapy with 100 mg/kg cyclophosphamide, 1.6 g/m^2 VP-16, and 1.6 g/m^2 carboplatinum followed by the infusion of cells in the CD34(-) fraction. The median duration of chemotherapy was 12 months (range, 10-16 months) in Group A and 10 months (8-10 months) in Group B.

Apheresis. Apheresis were performed in the recovery phase of consolidation chemotherapy that included treatment with 50-200 g/m^2/day of G-CSF. The details of the procedure for collecting blood cells have been described elsewhere (15). A Fenwal CS-3000 Plus cell separator (Baxter Limited) was operated using a small-volume collection chamber and a granulocyte chamber to process 100-350 ml of blood per kilogram of body weight. Cells were recovered in a 50-ml suspension.

Isolation of Blood CD34+ and CD34- Cells. Cells were isolated with the Isolx 50 system (Baxter Limited) according to the manufacturer’s instructions. Briefly, cells were incubated with 0.5% human γ-globulin (Polyglobulin N; Bayer Japan, Tokyo, Japan) containing PBS for 15 min to block intact Fc receptor on the cell surface. After incubation, cells were washed with PBS containing 1% HSA and incubated with anti-CD34 monoclonal antibody (9C5; Baxter Limited) at a cell/antibody ratio of 10^5:0.5 g. After 30 min of incubation at 4°C with gentle rotation (4/min), cells were washed three times with PBS.
Table 1 Characteristics of the 20 patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>5</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>Median</td>
<td>7</td>
</tr>
<tr>
<td>Range</td>
<td>2-56</td>
<td>2-56</td>
</tr>
<tr>
<td>Hematological malignancies</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Solid tumors</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Periods of prior chemotherapy (mo)</td>
<td>Median</td>
<td>12</td>
</tr>
<tr>
<td>Range</td>
<td>10-16</td>
<td>8-10</td>
</tr>
</tbody>
</table>

Table 2 Number of transfused cells and engraftment data

<table>
<thead>
<tr>
<th>Transplant with</th>
<th>CD34+ cells</th>
<th>Cells in CD34(—) fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNCC* (× 10^6/kg)</td>
<td>5.0 ± 4.6</td>
<td>372 ± 228</td>
</tr>
<tr>
<td>CFU-GM (× 10^6/kg)</td>
<td>48 ± 28</td>
<td>40 ± 29</td>
</tr>
<tr>
<td>CD34+ (× 10^6/kg)</td>
<td>3.0 ± 2.0</td>
<td>3.1 ± 3.3</td>
</tr>
<tr>
<td>Posttransplant G-CSF therapy</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Days to ACG* &gt;0.5 × 10^9/liter</td>
<td>12 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Days to PLT* &gt;50 × 10^9/liter</td>
<td>24 ± 6</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>Days with PLT transfusion</td>
<td>12 ± 3</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>No. of PLT transfusions</td>
<td>6 ± 2</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

Data are mean ± SE. *MNC, mononuclear cell. **ACG, absolute granulocyte count. ***PLT, platelet.

and resuspended in Iscove’s modified Dulbecco’s medium with 1% HSA at 1–2 × 10^6/ml. The anti-CD34 antibody-coated cells were incubated with sheep antiserum IgG1-coated microspheres (Dynabeads; Dynal Corp., Oslo, Norway) for 30 min with rotation, and cells that were bound to the beads were then collected with a magnet using an Isolox system (Baxter Limited) or MPR-6 (Dynabead Corp.). Finally, Dynabead-bound cells were incubated with 200 units/ml chymopapain (Chymocell-R; Baxter Limited) for 15 min at 37°C. After incubation, CD34+ cells were isolated from the beads using a magnet. Cells were mixed slowly with an equal volume of a freezing solution containing 5% DMSO and 6% HES. Cells were transferred to 5-ml polypropylene tubes and then placed directly in an electric freezer that maintained a temperature of −135°C (Sanyo Electric Co., Tokyo, Japan). The cells were stored in the same freezer until use.

Transplant Procedure. The detailed procedure has been reported elsewhere (13, 14). Briefly, 36 h after completion of the cytoreductive regimen, the cells were rapidly thawed at 37°C and promptly infused into the patient through a central venous catheter without additional post-thaw washing manipulation. Platelet transfusion was given as indicated to maintain the platelet count >20 × 10^9/liter. No further specific anticancer therapy was given after the transplant.

Measurement of Serum TPO Levels by Sandwich ELISA. Immediately before the cytoreductive regimen, at the completion of therapy (day 0), and on days 1, 3, 5, 7, 14, 21, and 28, blood samples were obtained from the patients. Samples were immediately centrifuged to obtain the serum, which was aliquoted and frozen at −80°C until use. To measure serum levels of TPO, a sensitive sandwich ELISA was applied in triplicate as reported previously (16, 17). The lower sensitivity of the assay is 0.2 μmol/ml. Briefly, each well of flat-bottomed microtiter plates (Maxisorp; Nunc) was coated at 4°C overnight with 100 μl of TN1 at a concentration of 10 μg/ml in 50 mM carbonate buffer (pH 9.4). After preincubation of the wells with 200 μl of a blocking reagent (Super block in TBS; Pierce) for 0.5 h at RT, 100 μl of recombinant human TPO standard, a serum sample, or blank was added to well and allowed to react with the coated TN1 overnight at RT. After washing with 20 mM Tris-Cl containing 0.5 M NaCl, 0.05% Tween 20, and 0.1% NaN3 (pH 7.5; T-TBS), 100 μl of the biotinylated anti-rhTPO F(ab')2 antibody at 500 ng/ml in T-TBS containing 1% BSA and 2% polyethylene glycol 6000 (dilution buffer) were added to each well for 3 h at RT. After washing with T-TBS, 100 μl of streptavidin alkaline phosphate conjugate (1 milliunit/ml in dilution buffer; Boehringer Mannheim) were added for 1 h at RT. The color was developed using an amplification system (Life Technologies, Inc.). After washing with T-TBS, 50 μl of substrate solution were added for 40 min at RT. Subsequently, 50 μl of amplifier solution were added for 30 min at RT. The reaction was stopped by adding 50 μl of 0.3 M H2SO4. The color intensity was measured by a plate reader (Well Reader SK601; Sekiagaku Kogyo Co., Ltd., Tokyo, Japan) with a measuring filter of 492 nm and a reference filter of 630 nm. The absorbance of each sample was reduced by that of the each sample incubated with TN1. The average value of each standard or the high TPO serum sample was reduced by that of the blank for the standard. The sample concentration was calculated by regression analysis for the standard curve.

Statistical Analysis. Fisher’s correlation analysis was used to examine the association between TPO and platelet count. To determine whether or not the circulating platelet count, the patient’s age, the diagnosis, the duration of prior chemotherapy, or transplant cohort affected serum TPO levels, we adopted a multivariate analysis using the stepwise regression method.

Results

Toxicities and Hematopoietic Recovery. Little evidence of therapy-related serious toxicities, whether pulmonary, renal, or hepatic, was found, and none of the patients had significant liver or renal dysfunction at the time of sampling. Seventeen patients developed fever during cytopenia, but all responded promptly to parenteral antibiotic therapy. No evidence of complicated disseminated intravascular coagulation was observed. Seven patients received 50–200 μg/m2/day of G-CSF starting on day +1 from stem cell infusion. The mean numbers of cells infused into the patients and the recovery data...
for hematopoiesis are summarized in Table 2. Because the number of CD34+ cells in the CD34− fraction could not be evaluated accurately due to their scarcity (<0.1%), we analyzed the number of CFU-GM; this value was 48 × 10³/kg in group A and 40 × 10³/kg in group B. No significant differences were observed between the two groups with regard to the duration required to achieve an absolute granulocyte count of >0.5 × 10⁹/liter, the duration of dependence on platelet transfusion, or the number of platelet transfusions.

**Serum Levels of TPO.** The platelet count was inversely associated with serum TPO. In general, there were no meaningful differences in the transition kinetics of serum levels of TPO between the two cohorts that received different modes of stem cell rescue operation (Fig. 1). When both groups were combined, we observed a strong negative correlation between serum TPO levels and the circulating platelet count (r = −0.469148; P < 0.0001; Fig. 2).

Multivariate analysis demonstrated that significant determinants of the serum TPO level included the circulating number of platelets (standardized regression coefficient = −0.5179), transplantation with cells in the CD34(−) fraction (0.2414), solid tumor (0.1420), and age of the patient (−0.1236; r@ = 0.3021; P < 0.0001).

**Discussion**

To clarify the clinical benefit of TPO in stem cell therapy, this study was performed as part of a preclinical study. To examine whether infused accessory cell populations enhance engraftment by producing cytokines, including TPO, we analyzed samples from cohorts of 12 or 8 patients who received intensive chemotherapy with PBSCs, which were separated into CD34+ and CD34(−) cells. The rationale for the use of the CD34(−) fraction is as follows. Positive selection of blood progenitor cells enhances engraftment by producing cytokines, including TPO, which are associated with inevitable cell loss, which prevents effective consecutive transplants from increasing the total intensity of chemotherapy. To overcome this problem and to maximally increase the total intensity of chemotherapy, we developed a new concept of consecutive transplantation strategy (12, 13). Our pilot feasibility study showed that the median number of infused CFU-GM and engraftment data in the two transplants were identical and suggested an improved cost:benefit ratio.

The kinetics of endogenous TPO observed in our patients seem to closely agree with those in patients who received regular chemotherapy with carboplatinum (3) or myeloablative therapy with PBSCTs (1, 2, 4). However, these studies only reported the significant role of endogenous TPO in the regulation of thrombopoiesis after chemotherapy. Several studies have noted that an inverse correlation exists between the plasma TPO level and the platelet count (1, 4, 16–19). In this study, our results suggest that younger patients or those with solid tumors responded better to myelosuppressive stimulation by chemotherapy by producing higher levels of TPO. Thus, age or the type of preceding chemotherapy may affect the endogenous production of TPO.

In addition, we demonstrated the possibility that the mode of stem cell support, i.e., the presence of accessory cells or unknown cytokine(s) produced by them in the inoculum, is the second-most important determinant of serum TPO levels. A similar possibility was reported by Chang et al. (11) with pediatric patients. It is well recognized that in vivo hematopoiesis is regulated by a cytokine network at multiple levels. The various cytokines act individually and in concert at different stages of hematopoiesis. Our observations will be useful for future studies of the regulation of thrombopoiesis and for the development of a therapeutic protocol with TPO for patients receiving high-dose chemotherapy and support with hematopoietic stem cells.

**References**


12. Kajiume, T., Kawano, Y., Hoshi, T., Abe, T., Matsuanga, K., Saito, S., Hiroa, A., Kawano, Y., Kuroda, Y., Ninomiya, T., Koyama, T., Suzue, T., Shimokawa, T., and...
THROMBOPOIETIN DURING TRANSPLANT WITH CD34+ CELLS


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