RAPID COMMUNICATION

Concentrations of Thrombopoietin in Bone Marrow in Normal Subjects and in Patients With Idiopathic Thrombocytopenic Purpura, Aplastic Anemia, and Essential Thrombocythemia Correlate With Its mRNA Expression of Bone Marrow Stromal Cells

By Yasuo Hirayama, Sumio Sakamaki, Takuya Matsunaga, Takashi Kuga, Hiroyuki Kuroda, Toshiro Kusakabe, Katsunori Sasaki, Koshi Fujikawa, J unji Kato, Katsuhisa Kogawa, Ryuzo Koyama, and Yoshiro Niitsu

The function of bone marrow (BM) stromal thrombopoietin (TPO) in megakaryopoiesis remains unknown. In the present study we attempted to clarify the pathophysiological implications of stromal TPO in normal subjects (NS) and in patients with idiopathic thrombocytopenic purpura (ITP), aplastic anemia (AA), and essential thrombocythemia (ET) by measuring TPO concentrations in BM and peripheral blood (PB) and by estimating the levels of stromal TPO mRNA with TaqMan fluorescence-based post-reverse transcription-polymerase chain reaction product detection system. The results showed that TPO concentrations in PB were significantly elevated in patients with ITP (34.9 ± 11.7 pg/mL) and AA (364.1 ± 153.5 pg/mL) but within normal range in patients with ET (each 20.0 and 22.1; NS, 22.1 ± 8.2 pg/mL). In all subjects, the TPO concentrations in BM correlated well with the PB levels, and the former were consistently higher than the latter. The concentrations of TPO in BM also correlated with the levels of TPO mRNA in stromal cells. Furthermore, expression levels of TPO mRNA clearly correlated with megakaryocyte counts in NS and patients with ITP, indicating that stromal TPO actually enhances megakaryopoiesis. Thus, our results in the present study indicate that TPO from BM stromal cells is considered to play an essential role for megakaryopoiesis under various pathophysiological conditions.

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amplification of DNA sequences, whereas the reverse TPO primer (P2) was complimentary to the exon 6. The TPO target probe (Pr A) was designed to be specific for exon 6. The TPO target probe (Pr A) was used for amplification of DNA sequences, whereas the reverse TPO primer (P2) was designed to be specific for another portion of exon 6.

**Table 1. Primers and Probes Used for TaqMan Real-Time Quantitative RT-PCR**

<table>
<thead>
<tr>
<th>TaqMan probe: TPO cDNA-518F</th>
<th>5’-GAGAATGGAAATACCA-GATGGA-3’</th>
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<tbody>
<tr>
<td>GAPDH amolicon</td>
<td>5’-GAAGGTGAAGGTTCGAGT-AGT-3’</td>
</tr>
<tr>
<td>PCR primer: TPO cDNA-310F (forward)</td>
<td>5’-TGAGACCTTCTACACAGCATCA-3’</td>
</tr>
<tr>
<td>TPO cDNA-610R (reverse)</td>
<td>5’-TGAGACCTTCTACACAGCATCA-3’</td>
</tr>
<tr>
<td>GAPDH cDNA (forward)</td>
<td>5’-GAAGGTGAAGGTTCGAGT-AGT-3’</td>
</tr>
<tr>
<td>GAPDH cDNA (reverse)</td>
<td>5’-GAAGGTGAAGGTTCGAGT-AGT-3’</td>
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**RESULTS**

**TPO concentrations in BM and PB.** The mean TPO concentrations in BM and PB of NS and patients with ITP, AA, and ET were measured by ELISA as shown in Table 2. Both BM and PB values in ITP were higher (P < .05) than those in NS and returned to near-normal value after steroid therapy. Patients with AA had extremely elevated levels of TPO, ie, 20-fold above normal values. Two patients with ET had TPO values within the normal range.

**Verification of TaqMan RT-PCR for measurement of TPO mRNA in stromal cells.** Before the application of TaqMan real-time quantitative RT-PCR for assessing the stromal TPO mRNA levels in various hematological diseases, we verified the

**Table 2. TPO Concentrations in PB and BM Plasma**

<table>
<thead>
<tr>
<th>TPO Concentration (pg/mL)</th>
<th>PB</th>
<th>BM</th>
</tr>
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<tbody>
<tr>
<td>NS (n = 6)</td>
<td>22.1 ± 8.2</td>
<td>32.2 ± 6.8</td>
</tr>
<tr>
<td>ITP (n = 5)</td>
<td>34.9 ± 11.7*</td>
<td>61.4 ± 13.9*</td>
</tr>
<tr>
<td>ITP after PSL treatment (n = 6)</td>
<td>25.0 ± 4.3</td>
<td>36.0 ± 12.9</td>
</tr>
<tr>
<td>AA (n = 6)</td>
<td>364.1 ± 153.5*</td>
<td>406.9 ± 142.8*</td>
</tr>
<tr>
<td>ET (n = 2)</td>
<td>20.0, 22.1</td>
<td>31.4, 35.7</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD. *P < .05 v NS.
method by using the sample from a normal subject (Fig 2). Two-fold diluted samples of total cell RNA (50 ng, 258 ng, 800 ng) from normal stromal cells showed each distinct amplification curve in both TPO mRNA (Fig 2A) and GAPDH mRNA (internal control) assays (Fig 2B).

For both mRNA, clear inverse correlations (inserted figures) were observed between CT values (cycles at threshold line) and the amount of applied cellular RNA.

Thus, this assay was confirmed to provide a high sensitivity with a nanogram range of sample RNA and sufficient quantitativeness for measuring TPO mRNA in stromal cells.

Expression levels of TPO mRNA of BM stromal cells in NS and in patients with ITP, AA, and ET. The levels of TPO mRNA and GAPDH mRNA expression in stromal cells from 6 NS, 5 patients with ITP, 6 patients with prednisolone treated ITP, 6 patients with AA, and 2 patients with ET were then examined by the TaqMan RT-PCR assay (Fig 3). Two hundred nanograms of total cellular RNA extracted from stromal cells of each patient and normal subject was subjected to TaqMan RT-PCR method. In each group, amplification curves of GAPDH mRNA from each individual overlapped to form almost a single line, and CT values were also almost the same (Fig 3A). Contrarily, amplification curves of TPO mRNA exhibited discrete patterns with different CT ranges indicated by rectangles for each group of disease and NS (Fig 3B), and discrete lines for each individual (Fig 3C).

Because TPO mRNA for standard was not available to quantitate its absolute amount in stromal cells, the relative ratio of TPO mRNA/GAPDH mRNA was used. In Fig 4, levels of stromal TPO mRNA in these diseases and NS which were expressed as relative ratio of CT values for TPO mRNA and GAPDH mRNA were shown. In the patients with ITP, the expression levels were significantly increased compared to those in NS ($P < .05$) and were lowered by steroid treatment. In patients with AA, the levels were as high as those of patients with ITP. Two patients with ET showed normal expression of TPO mRNA.

Relationship between TPO concentration in BM plasma and the TPO mRNA expression of BM stromal cells in NS and in patients with ITP, AA, and ET. In all subjects there was a clear positive correlation between TPO mRNA levels of stromal cells and TPO concentrations in BM (Fig 5). However, the correla-
tion lineairities found in patients with the three disorders (ITP, AA, and ET) were all deviated from the expected lines based on those of normal subjects (indicated as a dotted line). In nontreated ITP patients, actual BM TPO concentrations were lower than one would predict from the regression line of NS who had the same TPO mRNA expression level as nontreated ITP. Conversely, in patients with AA, TPO concentrations deviated toward far higher levels than those predicted from the regression line of the NS.

**Relationship between megakaryocyte count and BM stromal TPO mRNA.** To investigate that stromal TPO indeed enhances megakaryopoiesis in vivo, we analyzed a relationship between stromal TPO mRNA and megakaryocyte counts in NS and in patients with ITP, AA, and ET. As shown in Fig 6, except for the cases of AA and ET whose megakaryopoiesis are not under the control of TPO because of impaired responsiveness to TPO (AA) or autoproliferative property of megakaryocyte (ET), megakaryocyte counts well correlated with the levels of BM stromal TPO mRNA.

**DISCUSSION**

To explore the source(s) of TPO whose expression varies in response to changes in platelet number, in this study we first measured the TPO concentrations in BM and PB to compare them in NS and patients with ITP, AA, and ET. The TPO concentrations in PB of our subjects agreed with those NS reported previously. In all the subjects examined, the TPO concentration in BM was positively correlated with that in PB and always exceeded that of PB with significant difference. This
difference may be even more substantial in vivo because PB invariably contaminates marrow aspirate, resulting in dilution of TPO in BM. These results indicated that TPO in BM was synthesized by some BM constituent(s).

Because we and others have already disclosed the fact that TPO mRNA is expressed in BM stromal cells, we hypothesized that stromal TPO may be the key regulator of megakaryopoiesis.

To evaluate the contribution of marrow stroma cell TPO to megakaryopoiesis, we obtained RNA from stromal cells grown for 4 weeks in Dexter cultures. Previous studies have suggested that such cells may reflect the physiological state of the marrow stroma from which the cultures were derived. This was true in our experiments, at least for the relative proportions of fibroblasts, endothelial cells, adipocytes, and macrophages.

Because RNAse protection assays for stromal TPO or mRNA were insensitive, we turned to a TaqMan real-time RT-PCR assay to determine stromal TPO mRNA levels. This assay has been recently applied for measurement of trace substances in biological fluid and was proven to be quite reliable in terms of reproducibility and accuracy.

By using this assay it was proven that TPO mRNA expression in stromal cells of patients with ITP was significantly increased and returned to normal with steroid treatment. This phenomenon may be explained by our recent observation that transforming growth factor-β (TGF-β), which is released from destructed platelets or megakaryocytes in this disorder, stimulates stromal TPO mRNA, and the expression of stromal TPO mRNA decreases along with TGF-β with steroid therapy. This notion apparently contradict the results by Nagahisa et al, who found TPO mRNA levels in stromal cell was not affected by acute thrombocytopenia or thrombocytosis in mice. However, they have not examined the stromal TPO levels during drastic change in platelet count and therefore most likely failed to detect altered TPO expression.

Levels of TPO mRNA were correlated with TPO concentrations in BM in ITP, suggesting that at least one of the factors that defines the concentration of TPO in BM, ie, factors defining megakaryopoiesis, is the production of TPO by stromal cells. However, the concentration of TPO in the BM may not be solely determined by stromal TPO production because it was lower than the value predicted from the TPO mRNA level of NS (Fig 1). This unexpectedly low level could be caused by the adsorption of TPO on the surface of megakaryocytes that are increased in the BM of this disease.

In AA, stromal TPO mRNA was as high as that of ITP. Although merely speculative, suppression of stromal TPO production by putative factors from nonstromal hematopoietic cells may be relieved by aplasia of hematopoietic cells because the expression of stromal TPO mRNA was enhanced by abolishing hematopoietic cells with high doses of chemotherapy (S. Sakamaki et al, unpublished data). TPO concentrations in BM and stromal TPO mRNA in AA also showed a clear positive correlation, again indicating that TPO in BM is derived from stromal cells. However, patients with AA had extremely high TPO concentrations in their BM (20-fold the normal value) and deviated significantly from the predicted value of mRNA levels of NS. This may be explained in part by the idea that in AA,
megakaryocytes and platelets, on which TPO can be adsorbed, are drastically suppressed, and also by the notion that increased TPO itself may in turn suppress stromal TPO mRNA. Two patients with ET had normal levels of both TPO mRNA and TPO protein, despite the well-known abnormalities in megakaryopoiesis in this disease. This apparent paradoxical observation may be explained by a previous finding that megakaryocytes and platelets in this disease have fewer TPO receptors than their normal counterparts and by the assumption that, in ET, both megakaryocytes and stromal cells may not be under regulation.

To verify that BM stromal TPO actually enhances megakaryopoiesis, the relationship between the stromal TPO mRNA and megakaryocyte counts was also analyzed. However, it is inappropriate to examine the effect of stromal TPO on megakaryopoiesis in AA or ET whose hematopoietic cells, including megakaryocytes, are not under the control of TPO. For this reason, we analyzed the relationship in NS and in patients with ITP, whose hematopoietic cells are supposed to be normal, and found a clear positive correlation between stromal TPO mRNA and megakaryocyte counts.

The present notion that BM stromal cells is a major source for TPO which locally regulates megakaryopoiesis appears to contradict to the fact that TPO mRNA levels in organs such as liver and kidney are substantially high compared with that in stromal cells. We believe that high levels of TPO mRNA expression in liver or kidney do not necessary mean that those organs are mainly involved in megakaryopoiesis because TPO released from these organs may be diluted in the circulation so that the concentration of TPO locally produced by stromal cells exceed that of PB.

The physiological role of liver and kidney, although merely speculative, may be to provide baseline TPO level in circulation preparing for stromal dysfunction.

In conclusion, the results of the present study strongly suggest that TPO in BM is mainly derived from BM stromal cells and that the concentration of TPO may be determined by its production rate from stromal cells and possibly by its absorption rate on receptors of platelets and megakaryocytes. Thus, in humans, TPO from BM stromal cells is considered to play an essential role for megakaryopoiesis under various pathophysiological conditions.

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