Bone Marrow Stromal Cells Produce Thrombopoietin and Stimulate Megakaryocyte Growth and Maturation But Suppress Proplatelet Formation

By Hiroshi Nagahisa, Yuka Nagata, Toshio Ohnuki, Mizuhito Osada, Toshiro Nagasawa, Tsukasa Abe, and Kazuo Todokoro

Production of blood cells is regulated by the interplay of various cytokines and bone marrow stromal cells. Recently, a ligand for the orphan receptor Mpl was identified as thrombopoietin (TPO), which specifically regulates megakaryocyte differentiation, and it was reported to be expressed mainly in liver and kidney. As it was found that thrombopoietin is also produced in bone marrow stromal cells, we studied further the roles of bone marrow stromal cells on megakaryocytopoiesis and platelet formation. The stromal cells stimulated growth and maturation of bone marrow-derived megakaryocytes in the presence of thrombopoietin, and also supported growth of BaF3 cells expressing exogenous Mpl without thrombopoietin. Thrombopoietin induces drastic morphological change of megakaryocytes in bone marrow cells in vitro, ie, the formation of lengthy beaded cytoplasmic processes (proplatelet formation). However, when the purified megakaryocytes were cocultured with the stromal cells with or without thrombopoietin, most of the megakaryocytes adhered to the stromal cells and remained unchanged, while free megakaryocytes induced proplatelet formation. These observations indicated that the stromal cells in a hematopoietic microenvironment in bone marrow secrete thrombopoietin and stimulate proliferation and maturation of megakaryocytes, but the interaction of megakaryocytes with the stromal cells may suppress proplatelet formation.

MATERIALS AND METHODS

Expression of Mpl. Human and mouse full-length c-mpl cDNAs were prepared by reverse transcriptase-polymerase chain reaction (RT-PCR). For human c-mpl, 5′ primers (AGAA ATT CAT GTG CTAC CTAGCTGC) and P type-specific 3′ primer (CTCTAGATCAAGGCTGCTGCACAATA) were used. For mouse c-mpl, 5′ primer (GGA ATTCGAGAAAGTGCCCTCTTG) and 3′ primer (TTC TAGACTCCCTCAGGCTG) were used. The obtained cDNAs were sequenced by the M13 dideoxy method and were confirmed to be identical with those already reported. The c-mpl cDNAs (human c-mpl P type, mouse c-mpl and chimera M3 receptor) were inserted downstream of the SRa promoter in the expression vector pME18, which contained the neomycin-resistant gene with the simian virus-40 promoter. The plasmids were transfected into interleukin (IL)-3–dependent mouse proB cells BaF3 by electroporation. Stable transfecants resistant to antibiotic G418 (1 mg/mL) were cloned by limiting dilution, and four of each of the transfecnt clones were analyzed.

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Isolation and expression of TPO. The mouse TPO cDNAs were isolated from a mouse preadipocyte stromal cell line MC3T3-G2/Pα6 (PA6)\(^\text{11,18}\) and from primary mouse stromal cells by screening the expression CDNA libraries and by RT-PCR, and the nucleotide sequences of the isolated cDNAs were determined by the M13 di-deoxy sequencing method.\(^\text{19}\) The full-length cDNAs were inserted in pME18 and expressed in African green monkey kidney COS-7 cells. The conditioned media were prepared by culturing the cells for 3 days without fetal calf serum (FCS). A mock-transfected COS-7 supernatant was prepared as well. All subsequent experiments were done with mouse recombinant TPO derived from COS-7 supernatants.

Cell culture. BaF3 cells and their stable transfectants were maintained in RPMI 1640 medium supplemented with 10% FCS and 500 U/mL of mouse recombinant IL-3 (1.0 \(\times\) 10^3 U/mg; Genzyme, Boston, MA). Mouse stromal PA6 cells or freshly prepared primary mouse stromal cells (3 \(\times\) 10^5 cells in 6 cm dish) were cultured in the same medium for 16 hours, and the transfectants expressing c-mpl genes were cocultured on the feeder cells in the absence of IL-3 for over 1 week and photographed.

Quantitative RT-PCR analysis. The quantitative RT-PCR analysis was performed by the methods of Wang et al.\(^\text{20}\) and Murphy et al.\(^\text{21}\) The total RNAs in total bone marrow, kidney, and liver were prepared by acid guanidium thiocyanate-phenol-chloroform extraction.\(^\text{22}\) The total RNA (5 \(\mu\)g) was reverse transcribed in 40 \(\mu\)L of 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl\(_2\), 0.5 mmol/L of dATP, dGTP, dCTP and dTTP, 2.5 \(\mu\)g of oligo(dT)\(_{12,18}\), 10 mmol/L dithiothreitol, and 200 U of reverse transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD). The reaction conditions were 37°C for 1 hour and 42°C for 1 hour. Endogenous mRNA was used for a housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT), which was used to determine relative levels of TPO specific mRNA. The target sequences for TPO and HPRT were various cycles and in various amounts of reverse transcribed cDNAs. The TPO primers used were: 5' primer, CAGCAGTCGACACATGACTGAT; 3' primer, CGCGCTATGTTTCTCTGAGACA. The HPRT primers were: 5' primer, CAGACCGTAGAAGACACTGC; 3' primer, GCTGGTGAAAAGGACCTTC. The reaction mix contains 10 mmol/L Tris-HCl, pH 9.0, 50 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 0.1% Triton X-100, 0.2 mmol/L of dATP, dGTP, dCTP and dTTP, 1 \(\mu\)Ci of \([^{32}\text{P}]\)dCTP, 2.5 U of Taq polymerase, and 25 pmol of each specific primer pair in a total volume of 50 \(\mu\)L. Aliquots of the PCR products (5 \(\mu\)L) were separated on 1.5% agarose gels in Tris-borate buffer, and the radioactivity of the cDNA synthesized was counted. The radioactivity was plotted against the number of PCR cycles or against the amounts of the reverse transcribed cDNAs, and the best condition was determined before the quantitative analysis. The PCR cycle was used was 25, and 0.5 \(\mu\)g of cDNA was used for each quantitative RT-PCR analysis. The amounts of cDNA synthesized were normalized relative to the amount of PCR product for a housekeeping enzyme HPRT that had been amplified in parallel reactions.

Cell growth assay. Cell proliferation was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).\(^\text{23}\) Cells (1 \(\times\) 10^5) in microtiter plates in 100 \(\mu\)L culture medium in the presence or absence of various concentrations of recombinant TPO prepared from COS-7 supernatants were cultured at 37°C for 48 hours. The TPO concentration required for which the BaF3 cells expressing mouse Mpl grew at one half of the maximal growth rate was defined as 1 U/mL.

In vitro liquid culture of bone marrow cells and of purified megakaryocytes. Bone marrow cells were freshly prepared from BDF1 mice (6- to 8-week-old females) by flushing marrow cavities with Iscove's modified Dulbecco's medium (IMDM) through 26-gauge needles. Cells (1 \(\times\) 10^6 cells/mL) were washed and cultured for up to 3 weeks in IMDM containing 10% FCS with or without the recombinant mouse TPO (50 U/mL) and with or without bone marrow stromal cells, and the morphological changes of the megakaryocytes were monitored. Megakaryocytes were purified from bone marrow by a modified two-step separation technique of Levine et al.\(^\text{24}\) Cells were sedimented at unit gravity in 2% to 16% gradient of bovine serum albumin solution, and the isolated megakaryocytes (100 cells in 8-well chamber slide of 1 cm^2) were cultured in IMDM medium containing 5% FCS with or without 50 U/mL of TPO and with or without stromal cells (3 \(\times\) 10^5 cells/chamber).

RESULTS

Bone marrow stromal cells produce TPO. The cDNAs encoding for human c-mpl (P type),\(^\text{2}\) mouse c-mpl\(^\text{9}\) and a chimeric receptor (M3) constituted of the extracellular domains of human Mpl and the cytoplasmic domains of mouse IL-3 receptor \(\beta\) chain (Aic2B), were transfected into mouse IL-3-dependent proB cell line BaF3. The stable transfectants were then cocultured with mouse stromal PA6 cells, which have preadipocyte characteristics or with mouse primary bone marrow stromal cells freshly prepared. We found that all these transfectants adhere to the stromal cells and grow without IL-3, forming a colony-like structure (Fig 1A), although the parental BaF3 cells did not survive more than 2 days. Furthermore, soluble mouse Mpl inhibited cell growth of all these transfectants. These results suggested that bone marrow stromal cells, including the cell line PA6, also secrete the Mpl ligand (TPO), although TPO was reported to be expressed mainly in liver and kidney.\(^\text{11,12}\) Cell proliferation assay confirmed that the conditioned medium prepared from PA6 cells stimulates the growth of BaF3 cells expressing mouse Mpl (Fig 1B), but not of the parental cells, although the level of secreted TPO in the supernatant was too low to support exponential cell growth in suspension.

Quantitative RT-PCR analysis. We next examined the TPO gene expression in total bone marrow, liver, and kidney by the quantitative RT-PCR analysis, and the relative transcriptional levels were compared. The result shows that TPO transcripts were clearly detected, not only in liver and kidney, but also in total bone marrow cells (Fig 2). The TPO transcripts were most abundant in liver, and the level of TPO transcripts in bone marrow was about one fifth that of liver. It was also observed that the TPO gene was constitutively expressed in the primary bone marrow stromal cells, as well as the PA6 cells (not shown). These results indicate that TPO is indeed expressed, not only in liver and kidney, but also in bone marrow microenvironment in steady-state conditions.

Stromal TPO support growth of Mpl expressing cells. We then isolated TPO cDNAs from primary stromal cells and from PA6 cells and confirmed that TPOs isolated here are essentially identical with that isolated from hepatocyte,\(^\text{12}\) except that Sec\(^\text{22}\) is replaced by Pro. The mouse stromal TPO cDNAs were then expressed in COS-7 cells, and the recombinant TPOs prepared from their conditioned media were used for further analyses. Figure 3 shows that all transfectants expressing human Mpl, mouse Mpl, or M3 (but not cells expressing mouse erythropoietin [EPO] receptor) responded and grew with recombinant mouse full-length TPO in a dose-dependent manner, but that none of these transfectants grew with a mock-transfected COS-7 superna-
STROMAL CELLS AND TPO ON PROPLATELET FORMATION

Both TPO and EPO have strict binding specificity to the receptors. The structural analysis has shown that there exists some sequence similarity in TPO and EPO, and in the receptors for TPO and EPO. It has also been described that high concentration of EPO stimulates megakaryopoiesis. We, therefore, examined the possibility that EPO and TPO cross-react with each other’s receptors. 125I-labeled EPO, however, did not bind to either human Mpl or mouse Mpl (not shown). Further, EPO did not support the growth of the BaF3 transfectants expressing any c-mpl genes, even in the presence of a high concentration of EPO (up to 50 U/mL) (not shown). Conversely, mouse TPO did not stimulate the growth of BaF3 cells expressing mouse EPO receptor (Fig 3). Thus, it is concluded that both TPO and EPO have strict binding specificity to the receptor and thus specific biological function.

In vitro liquid culture of bone marrow-derived megakaryocytes in the presence of TPO. Freshly prepared mouse bone marrow cells were cultured with recombinant mouse TPO in liquid culture for up to 21 days, and their morphological changes were monitored. In 4 days with TPO, a significant number of large megakaryocytes were produced in the liquid culture, although few megakaryocytes were generated without TPO. Acetylcholinesterase staining confirmed that all these large cells are megakaryocytes, and the Romanosky-stained megakaryocytes in this stage clearly showed...
Fig 4. Morphological changes of bone marrow megakaryocytes and purified megakaryocytes in the presence or absence of TPO. (A) Megakaryocyte maturation in liquid culture with recombinant mouse TPO. Total mouse bone marrow cells were cultured with mouse recombinant TPO (50 U/mL) in liquid culture for over 1 week and replated without stromal cells in new dish. The photograph shows several megakaryocytes having lengthy beaded cytoplasmic processes, which are fragmented to yield cytoplasmic pieces the same size as individual platelets. Original magnification × 200. (B) An early-stage of morphological change of purified megakaryocytes in liquid culture with TPO. Mouse megakaryocytes purified from bone marrow cells were cultured with recombinant mouse TPO for 1 to 3 days. Four cytoplasmic processes are growing from a megakaryocyte. Original magnification × 600. (C) A megakaryocyte containing relatively long and partially beaded cytoplasmic processes under the same conditions as (B). Original magnification × 600.
Table 1. Effects of Stromal Cells and TPO on Proplatelet Formation

<table>
<thead>
<tr>
<th>Percentage of Free Megakaryocytes</th>
<th>Percentage of Megakaryocytes Adhered to PA6 Cells</th>
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<tbody>
<tr>
<td>59.5</td>
<td>40.5</td>
</tr>
<tr>
<td>+TPO</td>
<td>-TPO</td>
</tr>
<tr>
<td>5.7 ± 0.9</td>
<td>3.9 ± 0.9</td>
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<td>0 ± 0</td>
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Table 2. Effects of Stromal Cells and TPO on Proplatelet Formation

<table>
<thead>
<tr>
<th>Percentage of Free Megakaryocytes</th>
<th>Percentage of Megakaryocytes Adhered to PA6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.0</td>
<td>78.0</td>
</tr>
<tr>
<td>+TPO</td>
<td>-TPO</td>
</tr>
<tr>
<td>8.9 ± 1.1</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>2.6 ± 0.8</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>-PA6 cells</td>
<td>15.8 ± 3.0</td>
</tr>
<tr>
<td>12.7 ± 1.8</td>
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Proplatelet formation with or without stromal cells and/or TPO after 3 days culture. Number indicates the percentage in the total megakaryocytes. Purified megakaryocytes were cocultured with or without PA6 cells and with or without TPO for 3 days. The percentage of proplatelet formation with or without TPO for 3 days culture. Number indicates the mean values of five independent experiments and the standard deviations.
Fig 5. Morphological changes of purified megakaryocytes in the presence of stromal cells. Original magnification × 300 for (A) through (C). (A) Purified megakaryocytes cultured with stromal cells and TPO. Megakaryocytes adhered to the stromal cells remain unchanged in 3 days of culture. (B) A free megakaryocyte in the process of proplatelet formation and two megakaryocytes bound to the stromal cells in a 15-hour culture with TPO and stromal cells. (C) Two free matured megakaryocytes having cytoplasmic processes and a megakaryocyte adhered to the stromal cells in the presence of TPO and stromal cells in 3 days of culture.
the soluble factor secreted from the PA6 cells inhibited the proplatelet formation was excluded, as the supernatants of PA6 cells did not inhibit proplatelet formation. Thus, it is likely that the free megakaryocytes in the presence of PA6 cells might occasionally interact with the stromal cells during 3 days culture, and the interaction of megakaryocytes and the stromal cells inhibited the proplatelet formation. Taken together, these results suggested that TPO stimulates megakaryocyte growth and maturation in vitro liquid culture, and that the interaction of the stromal cells with the megakaryocytes may suppress the formation of cytoplasmic processes from which the platelets are released.

**DISCUSSION**

Hematopoiesis takes place principally in the bone marrow in adult mammals. The steady-state production of blood cells depends on the interaction between hematopoietic stem cells (and progenitor cells of various lineages) and the various components of the microenvironment present in the medullary cavity. The hematopoietic microenvironment is composed of stromal cells such as fibroblasts, macrophages, endothelial cells and adipocytes, and accessory cells such as T lymphocytes and monocytes, which are surrounded by various extracellular matrixes and cytokines. The regulatory mechanism of megakaryocyte and platelet production has not been identified, but it is likely that the stromal cells in the bone marrow microenvironment of the medullary cavity stimulate megakaryocytopoiesis by interacting with hematopoietic stem cells and/or megakaryocyte progenitor cells and by secreting a key regulatory factor, TPO, with the aid of the liver and kidney TPO. On the other hand, the stromal cells might suppress the production of platelets, by interacting with the matured megakaryocytes in bone marrow and by inhibiting the formation of cytoplasmic processes, from which a number of platelets are released. Therefore, in vivo platelet formation may be triggered by the release of the matured megakaryocytes from the stromal cells in the bone marrow microenvironment. It should be clarified how and why the interaction of the stromal cells with the megakaryocytes blocks the proplatelet formation. The fact that the bone marrow stromal cells function positively on megakaryocytopoiesis and negatively on proplatelet formation is consistent with all the previously known concepts, but further detailed analyses are required to clarify the molecular and cellular regulatory mechanism of megakaryocytopoiesis and platelet formation by bone marrow stromal cells.

Very recently, Choi et al demonstrated that human CD34 progenitor cells obtained from peripheral blood differentiated into platelets in vitro, and their results are consistent with our data obtained with mouse bone marrow and with purified mouse megakaryocytes. The development of platelets from the hematopoietic stem cells is a sequential process of proliferation of the committed megakaryocyte progenitors, nuclear polyploidization and cytoplasmic maturation of the megakaryocytes, and, finally, proplatelet formation followed by platelet release. It is possible to observe the dramatic morphological change of megakaryocytes in liquid culture in vitro in the presence or absence of TPO and/or stromal cells. This in vitro system was found to be a powerful means for the study of the molecular and cellular mechanism of megakaryocyte maturation and platelet release.

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**REFERENCES**


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