Thrombopoietin (TPO) is implicated as a primary regulator of megakaryopoiesis and thrombopoiesis. However, the biologic effects of TPO on human acute myeloblastic leukemia (AML) cells are largely unknown. To determine if recombinant human (rh) TPO has proliferation-supporting and differentiation-inducing activities in AML cells, 15 cases of AML cells that were exclusively composed of undifferentiated leukemia cells and showed growth response to rhTPO in a short-term culture (72 hours) were subjected to long-term suspension culture with or without rhTPO. Of 15 cases, rhTPO supported proliferation of AML cells for 2 to 4 weeks in 4 cases whose French-American-British subtypes were M0, M2, M4, and M7, respectively. In addition to the proliferation-supporting activity, rhTPO was found to induce AML cells to progress to some degree of megakaryocytic differentiation at both morphologic and surface-phenotypic levels in 2 AML cases with M0 and M7 subtypes. The treatment of AML cells with rhTPO resulted in rapid tyrosine phosphorylation of the TPO-receptor, c-mpl, and STAT3 in all of cases tested. By contrast, the expression of erythroid/megakaryocyte-specific transcription factors (GATA-1, GATA-2, and NF-E2) was markedly induced or enhanced in only 2 AML cases that showed megakaryocytic differentiation in response to rhTPO. These results suggested that, at least in a fraction of AML cases, TPO could not only support the proliferation of AML cells irrespective of AML subtypes, but could also induce megakaryocytic differentiation, possibly through activation of GATA-1, GATA-2, and NF-E2.

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In normal hematopoietic tissues, the expression of c-mpl is detected primarily in megakaryocytes, their precursors, and their progeny (ie, megakaryocytes, CD34+ cells, and platelets). In the case of hematologic disorders, the c-mpl expression is observed in half of samples from patients with acute myeloblastic leukemia (AML) and in one-third of those from patients with myelodysplasia, including those with refractory anemia with excess of blast (RAEB), RAEB in transformation, and chronic myelomonocytic leukemia. We have recently shown that the short-term (72 hours) culture with TPO led to proliferation of AML cells in approximately 70% of c-mpl-positive cases and not in any of the c-mpl-negative cases examined. Furthermore, it was found that c-mpl expression and proliferative response to TPO were observed in all subtypes of AML and did not correlate with French-American-British (FAB) classification. These observations suggested that c-mpl may be functional in terms of supporting proliferation of AML cells and that the functional expression of c-mpl may not be restricted to AML cells that display characteristics of megakaryocytes. AML cells appear to be progenitors frozen at early stages of differentiation and are known to express a variety of hematopoietic growth factor receptors. Like normal hematopoietic stem/progenitor cells, proliferation of AML cells can be stimulated in most cases by one or more hematopoietic growth factors, such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), IL-6, and stem cell factor (SCF). However, each of these growth factors usually appears to be incapable of inducing differentiation of AML cells, with the exception of only rare instances in which IL-3, GM-CSF, G-CSF, or M-CSF induce morphologic changes that are indicative of monocytic or granulocytic, but not of megakaryocytic maturation. Because TPO is a novel hematopoietic growth factor that has a potent activity in inducing both proliferation and megakaryocytic differentiation of normal hematopoietic progenitor cells, it is important to clari-
ify the effects of TPO on proliferative potential and phenotype of AML cells for a better understanding of mechanisms underlying the excessive proliferation and aberrant differentiation of AML cells. Furthermore, because severe thrombocytopenia is a common feature of AML, clear information concerning the long-term effects of TPO on AML cells is necessary to construct therapeutic procedures of TPO in patients with AML. In this study, we have therefore examined the effects of recombinant human (rh) TPO on the proliferation and differentiation of AML cells in a long-term suspension culture. Furthermore, we have investigated the effects of rhTPO on tyrosine phosphorylation of c-mpl and STAT3 and also on the expression of erythroid/megakaryocyte-specific transcriptional factors (GATA-1, GATA-2, and NF-E2) in AML cells.

MATERIALS AND METHODS

Reagents and antibodies. Highly purified rhTPO and rhIL-3 were provided by Kirin Brewery Co Ltd (Tokyo, Japan). Rabbit anti-c-mpl IgG was purified by using a protein A Sepharose (Pharmacia Biotech, Upssala, Sweden) from antisera against extracellular domain of c-mpl. Rabbit anti-STAT3 antisera raised against C-terminal portion of STAT3 was generously provided by Dr K. Nakajima (Osaka University, Osaka, Japan). Antiphosphotyrosine, a murine monoclonal antibody (MoAb) generated against phosphotyrosine, was supplied by Dr B. Drucker (Oregon Health Science University, Portland, OR). A murine MoAb YB5.88, which reacts with the extracellular domain of c-kit protein, was a kind gift from Dr L.K. Ashman (University of Adelaide, Adelaide, South Australia). Anti-CD34 MoAb (HPCA-1) was purchased from Becton Dickinson (San Jose, CA); My-anti-CD14, My-anti-CD13, and My9/anti-CD33 from Coulter Immunology (Hialeah, FL); and OKIal/anti-HLA-DR from Ortho-mune (Raritan, NJ).

Patients and leukemic cells. Peripheral blood (PB) or bone marrow (BM) samples were obtained from 59 patients with AML. All samples were obtained after informed consent was given. The diagnosis of leukemia was made by morphologic, cytochemical, and immunologic analyses, and each case was classified according to FAB criteria. Mononuclear cells were isolated with Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and cryopreserved in 10% dimethylsulfoxide in the vapor phase of liquid nitrogen until use. Samples used in this study contained greater than 95% leukemia cells and had viability greater than 90% even after thawing.

Cell proliferation assay and long-term cultures. The frozen cells were thawed, washed, and suspended in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Flow, North Ryde, Australia). To quantitate the proliferation of AML cells in a short-term culture (72 hours), MTT [3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St Louis, MO) rapid colorimetric assay was used as previously reported. This assay was found to give equivalent results obtained by H-thymidine incorporation or cell enumeration, as described previously. In long-term (>1 week) cultures, AML cells (2 x 10^5/mL) were suspended in RPMI 1640 medium supplemented with 10% FCS or in serum-free Cosmedium 001 (Cosmobio, Tokyo, Japan) in the presence or absence of rhTPO (20 ng/mL) or rhIL-3 (10 ng/mL). The concentrations of rhTPO and rhIL-3 induced almost maximal proliferation of human leukemia cells.

RESULTS

Morphologic and phenotypic characteristics of AML cells. In a total of 59 AML cases tested, c-mpl mRNA transcripts were detected in 31 cases by Northern blot analysis, and rhTPO induced proliferation of AML cells in 22 of 31 cases in a short-term (72 hours) culture. Of 22 cases showing both
cells are summarized in Table 1. Three cases were classified
The FAB classification, intensity of c-mpl expression, prolif-  
ner cases were exclusively composed of undifferentiated AML  
cases because of the following reasons. First, cytokine-  
with

Table 1. FAB Classification and Phenotypes of AML Cells Subjected to Long-Term Cultures

<table>
<thead>
<tr>
<th>Case No.</th>
<th>FAB*</th>
<th>Source</th>
<th>c-mpl Expression</th>
<th>Proliferative Response to rhTPO</th>
<th>CD34</th>
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<th>HLA-DR</th>
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* Types of leukemia were determined by clinical, morphologic, and cytochemical criteria according to FAB classification.
† Expression of c-mpl was analyzed by Northern blot analysis: 1+, faint expression; 2+, intensive expression.
‡ Proliferative responses to rhTPO in short-term cultures were classified into three groups according to the stimulation index (SI); SI = (OD540 with TPO)/(OD540 without TPO): 1+, SI < 2; 2+, SI 2 to 5; 3+, SI > 5.
§ Surface phenotype was analyzed by flow cytometry, and the levels of expression were classified into four groups according to the proportion of positive cells: −, <10%; +, 10% to 30%; 2+, 30% to 60%; 3+, >60%.

Even 4 weeks after the initiation of culture, proliferation or survival of AML cells was sustained by rhTPO in 3 cases (cases no. 1, 9, and 14) and by rhIL-3 in 6 cases (cases no. 1, 3, 5, 6, 9, and 14; Fig 1). In those cases showing proliferative response to each factor, the number of viable cells reached a maximum level at 1 to 3 weeks after the initiation of cultures and decreased thereafter.

Effect of rhTPO on megakaryocytic differentiation of AML cells. We have next examined the changes in morphology of AML cells before and after treatment with rhTPO or rhIL-3. In the absence of growth factors, AML cells died without differentiation in all of AML cases tested. The treatment of AML cells with rhIL-3 resulted in expansion of undifferentiated blast cells in more than half of AML cases, but failed to induce morphologic differentiation of the cells in any cases (data not shown). By contrast, in the vitro culture of AML cells with rhTPO for 1 to 4 weeks led to morphologic alterations indicative of megakaryocytic differentiation in 2 AML cases (case no. 1, M0 type; case no. 14, M7 type; Fig 2). At day 0, freshly prepared AML cells of cases no. 1 and 14 were composed of small undifferentiated blast cells (data not shown). However, at days 7 to 10, a substantial proportion of AML cells (case no. 1, 10% to 20%; case no. 14, 40% to 50%) became large in size and had two to seven small nuclei or one large nucleus in the culture with rhTPO, whereas morphologic alteration was not observed in the absence of growth factors (Fig 2).

To determine further the phenotypic changes of AML cells, flow cytometric analysis was performed 7 days after the initiation of cultures with rhTPO, rhIL-3, or medium alone (Fig 3). In case no. 1, rhTPO induced expression of GPIbb/IIa as well as GPIb, and the expression levels of CD34 and c-kit were decreased by the rhTPO treatment. In case no. 14, the treatment with rhTPO led to a slight increase.
Fig 1. Changes of number of total viable cells during long-term culture with rhTPO (●), rhIL-3 (■), or medium alone (○). The number of total viable cells was counted by trypan blue dye exclusion every week after the initiation of culture. The results are shown as the mean values of triplicate cultures, and the standard deviation in this experiment was less than 10% of the mean values.

Fig 2. Light micrograph of AML cells obtained after culture with rhTPO or medium alone for 7 days. Cytocentrifugation preparations of each sample were stained with May-Grünwald-Giemsa (original magnification × 400). The in vitro culture of AML cells (cases no. 1 and 14) with rhTPO for 7 days led to morphologic alterations indicative of megakaryocytic differentiation, whereas morphologic alteration was not observed in the absence of growth factors.
in GPIb expression and to a decrease in CD34 and c-kit expression, although the expression level of GPIlb/IIla was not augmented by rhTPO. In contrast to rhTPO, the culture with rhIL-3 resulted in little or no alteration of the expression of CD34, c-kit, GPIb, and GPIlb/IIla antigens (Fig 3). These findings were basically comparable with the data on morphologic analyses. In addition, the similar morphologic and phenotypic changes of AML cells in cases no. 1 and 14 were observed in serum-free cultures with rhTPO (data not shown).

The expression level of transcription factor GATA-1, GATA-2, and NF-E2 genes in AML cells and the tyrosine phosphorylation of c-mpl and STAT3 before and after treatment with rhTPO. In an effort to characterize the mechanism of TPO-induced proliferation and megakaryocytic differentiation of AML cells, we have investigated the effects of rhTPO on the expression of transcription factors GATA-1, GATA-2, and p45 NF-E2, all of which are known to play a role in megakaryopoiesis and/or thrombopoiesis as well as erythropoiesis. Before and after incubation of AML cells in the presence or absence of rhTPO or rhIL-3 for 24 hours, the expression of GATA-1, GATA-2, and NF-E2 mRNA was examined by means of Northern blot analysis. Before incubation, the expression of GATA-1 and NF-E2 mRNA was preferentially detected in AML cases of M6 and M7 types (cases no. 13, 14, and 15) and was absent or minimal in other types of AML cases (cases no. 1 through 12), whereas that of GATA-2 mRNA was observed in all of the AML cases at a similarly low level (data not shown). The expression levels of GATA-1, GATA-2, and NF-E2 mRNA were not affected by incubation of AML cells with either rhIL-3 or medium alone (Fig 4). By contrast, the treatment of AML cells with rhTPO induced or augmented the expression of GATA-1, GATA-2, and NF-E2 mRNA in cases no. 1 and 14, both of which showed megakaryocytic differentiation in response to rhTPO (Fig 4). However, the induction or augmentation of GATA-1, GATA-2, and NF-E2 by rhTPO was not observed in AML cases that did not exhibit rhTPO-induced differentiation, as shown by 2 representative cases (cases no. 13 and 15) in Fig 4.

We have also examined the effects of rhTPO on tyrosine phosphorylation of c-mpl and STAT3 in AML cases. As shown by 2 representative cases of M7 type (cases no. 14 and 15) in Fig 5, rhTPO was found to induce tyrosine phosphorylation of c-mpl and STAT3 proteins in AML cells that showed proliferative response to rhTPO in the short-term and/or long-term cultures. Furthermore, it was noted that there was no significant difference in the levels of tyrosine phosphorylation of c-mpl and STAT3 proteins between 2 M7 cases: one (case no. 14) showed TPO-induced megakaryocytic differentiation and another (case no. 15) did not.

The tyrosine phosphorylation of c-mpl or STAT3 or the induction of GATA-1, GATA-2, or NF-E2 was not observed in c-mpl-negative AML cases (data not shown).

DISCUSSION

By using different strategies, several groups of investigators have purified and cloned the c-mpl ligand, TPO, that has NH2-terminal domain homologous to erythropoietin and COOH-terminal domain unrelated to any known protein.5,11 A number of recent studies have clearly shown that TPO plays a fundamental role in both megakaryopoiesis and thrombopoiesis.12,15 By contrast, the precise role of TPO in abnormal growth of hematopoietic cells remains to be determined. In this study, we have investigated the effect of rhTPO on proliferation of AML cells by direct measurement of viable cells and have found that rhTPO is capable of supporting proliferation of AML cells more than 2 weeks in 4 of 15 c-mpl-positive cases, including M0, M2, M4, and M7 cases. This finding is largely consistent with our previous results obtained from an MTT rapid calorimetric assay19 and suggests that TPO could support self-renewal capacity of AML cells in those cases. Furthermore, these results support the idea that TPO has a proliferation-inducing activity in a substantial fraction of AML cases, and this activity does not correlate with subtypes of AML.

TPO has recently reported to be able to initiate, but not to complete, the process of megakaryocytic differentiation
EFFECT OF THROMBOPOIETIN ON HUMAN LEUKEMIA CELLS

Fig 4. Northern blot analysis of GATA-1, GATA-2, and NF-E2 mRNA isolated from AML cells after 24 hours of culture with rhTPO, rhlL-3, or medium alone. Fifteen micrograms of total cellular RNA was electrophoresed in formaldehyde agarose gels. Each filter was hybridized with $^32$P-labeled probe, as indicated. The filters were rehybridized with β-actin probe to confirm that equal amounts of RNA were loaded in each lane.

In a human megakaryocytic cell line CMK and a murine IL-3-dependent cell line FDC-P2, TPO was shown to induce the expression of megakaryocytic surface antigens, although morphologic maturation of these cell lines was absent or only minimal. In this study, we have shown that, albeit in only 2 AML cases, rhTPO can induce AML cells to progress to some degree of megakaryocytic differentiation, converting into morphologically identifiable megakaryocytes. In 2 cases of AML cells with phenotypic features of M0 and M7 types, the treatment with rhTPO shown that, albeit in only 2 AML cases, rhTPO can induce AML cells to progress to some degree of megakaryocytic differentiation, converting into morphologically identifiable megakaryocytes. In 2 cases of AML cells with phenotypic features of M0 and M7 types, the treatment with rhTPO

Fig 5. TPO-induced tyrosine phosphorylation of c-mpl (A) and STAT3 (B) in AML cases. AML cells were either unstimulated or stimulated by rhTPO. Cell lysates were immunoprecipitated with anti-c-mpl or anti-STAT3 antisera. The immunoprecipitated proteins were analyzed by Western blotting. The blots were probed with antiphosphotyrosine MoAb. The filters were then stripped and reprobed with anti-c-mpl or anti-STAT3 antisera.
for 7 to 10 days resulted in the development of polyploid megakaryocytes with one to several nuclei, whereas the morphologic alterations indicative of megakaryocytic differentiation were not induced by medium alone or rhIL-3. In addition to the morphologic changes, the rhTPO treatment of the AML cells led to decreased expression of stem cell antigens CD34 and c-kit and/or the increased expression of megakaryocytic antigens GPIb/IIIa and GPIb in these cases. Although it was not clearly shown that the differentiated megakaryocytes formed in response to rhTPO were of clonal origin, rhTPO was unlikely to induce residual normal rather than leukemia precursor differentiation into megakaryocytes, because the number of normal hematopoietic stem/progenitor cells in the PB of AML patients appeared to be very low. Furthermore, it was noted that the rhTPO treatment of PB mononuclear cells prepared from normal healthy volunteers (n = 5) or the patients with acute lymphoblastic leukemia (n = 5) for 7 days did not result in development of megakaryocytes from any samples. These results suggested that differentiated megakaryocytes may be of leukemia precursor origin and that TPO may have an activity in inducing megakaryocyctic differentiation of AML cells, albeit in a limited number of AML cases.

Hematopoietic growth factors such as TPO may activate certain transcription factors that are essential for controlling hematopoietic cell proliferation, commitment, and differentiation. Among numerous transcription factors, erythroid/megakaryocytic-specific transcription factors GATA-1, GATA-2, and p45 NF-E2 have been described to be coupled to megakaryocytic differentiation and to transactivate megakaryocytic-specific gene expression. For example, although loss of GATA-1 prevents primitive and definitive erythroid development in vivo, the forced expression of GATA-1 induces megakaryocytic differentiation in a murine myeloid cell line 416B. In addition, the megakaryocytic differentiation of the 416B cells was similarly elicited by expression of GATA-2 transgene. Furthermore, loss of the p45 subunit of NF-E2 affects megakaryocytic maturation, thereby leading to severe thrombocytopenia in vivo. In the case of AML cells, the expression of GATA-1 and NF-E2 was observed primarily in cells of erythroid (M6) and megakaryocytic (M7) types but barely in those of other types; the low, but detectable, level of GATA-2 expression was noted in all of AML cases. The expression levels of GATA-1, GATA-2, and NF-E2 were not influenced by either rhTPO or rhIL-3 in any of AML cases that did not show megakaryocytic differentiation. By contrast, the treatment with rhTPO for 24 hours resulted in a marked induction or enhancement of GATA-1, GATA-2, and NF-E2 expression in 2 AML cases that did exhibit megakaryocyctic differentiation in response to rhTPO. These results suggested that rhTPO may activate GATA-1, GATA-2, and NF-E2 transcription factors in the AML cases, thereby leading to some further differentiation of AML cells toward megakaryocytic lineage.

Although rhTPO has activities in inducing proliferation and megakaryocytic differentiation of AML cells in a fraction of AML cases, it is not known yet as to which signaling molecules are involved in the processes. Recently, it has been reported that TPO induces tyrosine phosphorylation and activation of various signaling molecules, including Janus family of protein tyrosine kinases (JAKs), signal transducers and activators of transcription (STATs), and Shc.

Furthermore, TPO was suggested to induce tyrosine phosphorylation of its receptor c-mpl through activation of JAK2 tyrosine kinase. In all of the c-mpl-positive AML cases tested, we have found that rhTPO was found to induce tyrosine phosphorylation of c-mpl and STAT3 in a ligand-dependent manner. In addition, there was no significant difference in the level of tyrosine phosphorylation of c-mpl and STAT3 between 2 M7 cases; one showed TPO-induced differentiation and the other did not. These results suggest that the signaling cascade from c-mpl to STAT3 may be undamaged in AML cells and raise the possibility that STAT3 might be involved in TPO-induced proliferation of AML cells, but not in differentiation processes such as activation of GATA-1, GATA-2, and NF-E2. A clear and decisive information concerning the precise mechanism underlying the TPO-induced proliferation and megakaryocytic differentiation of AML cells will not only provide important insights into neoplastic transformation of megakaryocyte progenitor cell, but will also provide novel therapeutic strategies for thrombocytopenia in the patients with AML.

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The biologic properties of recombinant human thrombopoietin in the proliferation and megakaryocytic differentiation of acute myeloblastic leukemia cells

I Matsumura, Y Kanakura, T Kato, H Ikeda, Y Horikawa, J Ishikawa, H Kitayama, T Nishiura, Y Tomiyama, H Miyazaki and Y Matsuzawa