Thrombopoietin stimulates proliferation and megakaryocytic differentiation of mouse pro-B cell line BF-TE22

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Abstract

We have isolated and characterized a thrombopoietin (TPO)-dependent BF-TE22 cell line endogenously expressing murine Mpl, which is a subclone of murine pro-B Ba/F3 cells. TPO stimulated the proliferation of BF-TE22 cells in a dose-dependent manner, and also induced the expression of megakaryocyte lineage-specific AP-51 and CD61 cell surface antigens. The results indicate that the murine Mpl on BF-TE22 cells can transmit both proliferation and megakaryocyte lineage-specific differentiation signals to cells. Furthermore, it was shown that IL-3 inhibits the TPO-induced differentiation signals of BF-TE22 cells. These results suggest that the signals mediated by IL-3 predominate over those of TPO in BF-TE22 cells. Thus, BF-TE22 cells will be useful for the biological and biochemical studies of the TPO-Mpl signal transduction mechanism.

Introduction

Thrombopoietin (TPO), recently identified by our group and others, is one of the hormones required for the survival, proliferation, and differentiation of megakaryocyte progenitor cells (Kato et al., 1995; De Sauvage et al., 1994; Lok et al., 1994; Bartley et al., 1994). The Mpl receptor belongs to the cytokine receptor superfamily that includes the receptors for other hematopoietic growth factors such as interleukins (ILs), colony-stimulation factors (CSFs) and growth hormone (Skoda et al., 1993; Vigon et al., 1992). The Mpl and other members of the cytokine receptor family do not contain kinase-related or nucleotide-binding consensus sequences in their cytoplasmic domains, and their intracellular signaling pathways have yet not be fully elucidated.

Although the mechanism by which the Mpl transduces the signals has not yet been clarified, recent studies by our group and others demonstrated that the Mpl receptor transduced into IL-3-dependent mouse

leukemia cell lines transmits TPO-triggered mitogenic signals (De Sauvage et al., 1994; Lok et al., 1994; Bartley et al., 1994; Ohashi et al., 1995). Moreover, it has been showed that the chimeric receptor carrying the extracellular domain of the growth hormone receptor (GH-R) linked to the cytoplasmic domain of the human Mpl can also transduce the mitogenic signals in these cells (Gurney et al., 1995). These findings indicate that the Mpl intracellular domain is necessary and sufficient to transmit proliferative signals. Moreover, these also suggest that ligand-induced dimerization of the intracellular domain of the Mpl is a key step for activation of the Mpl as well as other members of cytokine receptor families and receptor tyrosine kinase groups.

Compared with mitogenic signals, little is known regarding differentiation signals in cytokine receptors. It has been reported that erythropoietin (EPO) induced erythroid/megakaryocyte-specific transcriptional factor GATA-1 expression and beta-globin synthesis in Ba/F3 cells transformed with the EPO receptor (Liboi et al., 1993). It was also shown that granulocyte colony stimulating factor (G-CSF) activated the neutrophil-specific genes including myeloperoxidase and leuko-

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cyte esterase in FCD-P1 cells expressing the G-CSF receptor (Fukunaga et al., 1993). In the Mpl receptor system, however, few studies concerning the Mpl-mediated cellular differentiation of hematopoietic cell lines were reported (Drexler et al., 1996; Morita et al., 1996; Nagata et al., 1995; Porteu et al., 1996; Quentmeier et al., 1996).

In this report, we have isolated a TPO-dependent Ba/F3 subclone cell line BF-TE22, which expressed murine Mpl and could grow in response to TPO. We found that murine Mpl on BF-TE22 cells could transduce the megakaryocyte lineage-specific differentiation signals. Thus, TPO not only supported the proliferation of BF-TE22 cells but also induced the expression of megakaryocyte/platelet cell surface antigens.

Materials and Methods

Cell lines and reagents

The TPO-dependent subclone cell line BF-TE22 was cultured in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO/BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS) and 10 ng/ml murine TPO. Mouse IL-3 was prepared by using the *E. coli* expression system in our laboratory. Murine TPO was homogeneously purified from the culture supernatant of Chinese hamster ovary (CHO) cells transfected with an expression plasmid carrying full-length murine TPO cDNA (Bartley et al., 1994). Rabbit anti-murine Mpl polyclonal antibody and anti-phosphotyrosine monoclonal antibody were obtained from Amgen Inc. (Thousand Oaks, CA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. Anti-murine CD61 antibody was purchased from Pharmingen (San Diego, CA). Anti-murine megakaryocyte/platelet AP-51 monoclonal antibody (Morita et al., 1996) which reacts to the unidentified cell surface antigen on mouse megakaryocyte and platelet was kindly provided by Dr. Nagasawa of Tsukuba University. The antibodies for murine platelet surface markers were obtained from Pharmingen. Fluorescence isothiocyanate (FITC)-conjugated second antibodies for rat, hamster or rabbit IgG were from Biosource International Inc. (Camarillo, CA).

Flow cytometric analysis

The cell surface expression of murine Mpl or hematopoietic cell surface antigens (AP-51, CD61,

CD31, CD51, CD62P, and GPV) in IL3- or TPO-stimulated BF-TE22 cells was analyzed by using a flow cytometry method. BF-TE22 cells cultured with IL-3 or TPO (1×10^6 cells) were washed and resuspended in phosphate-buffered saline (PBS) containing 5% FCS and 0.1% NaN₃. They were then incubated for 30 min at 4 °C with first antibody in a final volume of 0.1 ml. Cells were then washed and incubated with FITC- or PE-conjugated second antibody for a further 30 min at 4 °C. All measurements were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell proliferation assay

Cell proliferation was measured by a colorimetric assay using ³H-thymidine or MTS reagent (Buttke et al., 1993) (Promega, Madison, WI). Exponentially growing cells were extensively washed three times with phosphate-buffered saline. After washing, cells (2.5×10^3 cells/well) were plated onto microculture plates in the presence or absence of various concentrations of murine TPO or mouse IL-3, and cultured for 3 days. Cell growth was determined by monitoring the incorporated radioactivity or the optical density at 492 nm.

Immunoprecipitation and immunoblotting

Preparation of cell lysates, immunoprecipitation and immunoblotting were performed as described previously (Ohashi et al., 1994). In brief, cells were solubilized with Noidet P-40 (NP-40) lysis buffer (0.5% NP-40/10 mM Tris-HCl, pH 7.6/150 mM NaCl/5 mM EDTA/2 mM Na₃VO₄/1 mM phenylmethylsulfonyl fluoride/5 mg of aprotinin per ml), and clear lysates obtained by centrifugation were incubated with anti-murine Mpl polyclonal antibody. Immunoprecipitates using Protein G-Sepharose FF (Pharmacia, Uppsala, Sweden) were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Figure 1. Expression analysis of the murine Mpl receptor on BF-TE22 cells. (A) Flow cytometric analysis of the BF-TE22 cells with anti-murine Mpl antibody. Cells were incubated for 30 min at 4 °C with control rabbit IgG (thin line) or rabbit anti-murine Mpl polyclonal antibody (thick line). After washing twice with staining buffer, cells were then stained for 1 h with FITC-conjugated anti-rabbit IgG. Fluorescence intensity was determined with a FACSscan analyzer. (B) Western blotting analysis of BF-TE22 cell lysates not stimulated or stimulated by TPO. Cell lysate and immunoprecipitates of BF-TE22 with unstimulation or TPO-stimulation were prepared as described in Materials and Methods. Western blotting analysis was performed by using anti-phosphotyrosine antibody 4G10 (upper lane) and anti-murine Mpl antibody (lower lane).

Results

Growth character of TPO-dependent BF-TE22 cells

By extensive screening of many hematopoietic cell lines in our laboratory, we found that a subclone of the murine IL-3-dependent murine pro-B Ba/F3 cell line (Palacios et al., 1985), BF-TE22, could proliferate in TPO-containing medium in the absence of IL-3. This cell line was established by transfection of the pEFneo plasmid (Ohashi et al., 1994) and selection in G-418-containing medium. As shown in Fig. 1, we observed by flow cytometric analysis using anti-murine Mpl polyclonal antibody that BF-TE22 cells expressed the Mpl receptor on their cell surfaces. The Mpl receptor on their cell surface was tyrosine-phosphorylated in the response to TPO stimulation. We next examined the mitogenic activity of murine TPO on BF-TE22 cells. Fig. 2 shows the growth profiles of the BF-TE22 cells. Murine TPO stimulated the DNA synthesis and proliferation of the BF-TE22 cells in a dose-dependent manner. TPO dose-response curve on BF-TE22 cells exhibited the typical sigmoid curve and reached a plateau at 1 ng/ml of murine TPO, whereas the original Ba/F3 cells did not respond to murine TPO. Murine TPO also supported the long-term proliferation of BF-TE22 cells (data not shown). These results indicated that Mpl receptor expressing on BF-TE22 cell surface

binds to TPO and mediates the proliferative signals to cells in response to TPO.

TPO not only supported the proliferation of BF-TE22 cells, but also induced megakaryocyte-lineage differentiation signals

To examine whether TPO induces the megakaryocyte lineage-specific differentiation of BF-TE22 cells, we next investigated the induction of several megakaryocyte/platelet cell surface antigens including AP-51, CD61, CD31, CD51, CD62P and GPV on BF-TE22 cells cultured in the presence of IL-3 or TPO. Fig. 3 shows the results from flow cytometric analysis of cell surface antigens in BF-TE22 cells. The results demonstrated that the expression of AP-51 and CD61 antigens significantly increased in BF-TE22 cells stimulated by TPO compared with the cells stimulated by IL-3. Thus, these findings indicated that TPO activates the megakaryocyte lineage-specific differentiation on BF-TE22 cells. However, TPO didn't enhance the expression of platelet-specific cell surface antigens such as CD31, CD51, CD62P and GPV on BF-TE22 cells. Therefore, these results may suggest that TPO-induced the megakaryocyte lineage-specific differentiation signals but not the terminal differentiation signals of BF-TE22 cells.

Figure 2. Growth profiles of BF-TE22 cells. Cells were extensively washed three times with phosphate-buffered saline. The cells were then cultured with various concentrations of TPO for 3 days. DNA synthesis and the viable cell number were determined by ³H-thymidine uptake assay (open circles) and colorimetric MTS assay (closed circles), respectively.

It is known that IL-3 also acts on the megakaryocyte progenitor cells and supports their cell proliferation (Kaushansky et al., 1995). To investigate whether IL-3 can induce the enhanced expression of AP-51 and CD61 antigens in combination with TPO, the BF-TE22 cultured in TPO-containing media were cultured for 10 days in the presence or absence of IL-3. As shown in Fig. 4, the presence of IL-3 in addition to TPO resulted in poor induction of both cell surface antigens. These results indicated that, in response to TPO, the Mpl receptor could transduce a specific signal that caused the active expression of megakaryocyte lineage-specific proteins, whereas the signals mediated by the IL-3 receptor system were inhibitory to the signal evoked by the Mpl. This inhibitory effect of IL-3 on Mpl-mediated expression of both cell surface antigens was dose-dependent (data not shown).

Discussion

The results reported here demonstrate the characterization of BF-TE22 cells, which endogenously expressed murine Mpl and can responded to TPO. TPO stimulated not only the DNA synthesis of BF-TE22 cells but also short- (Fig. 2) and long-term cell proliferation (data not shown). Further, TPO also enhanced the expression of megakaryocyte-lineage specific cell surface antigens including AP-51 and CD61 (Fig. 3). These findings indicated that the Mpl on BF-TE22

cells could mediate both proliferation and differentiation signals to cells in response to TPO. The Mpl expression level in BF-TE22 cells was higher than that of other TPO-dependent subclones derived from the Ba/F3 cell line. These subclones also showed the mitogenic responses by TPO stimulation, but were not as sensitive as BF-TE22 cells in their TPO responsibility. No significant expression of megakaryocyte lineage-specific cell surface antigens was observed in these subclones (data not shown). Moreover, it has been reported that the chimeric receptor composed of the extracellular domain of the IL-4 receptor and the transmembrane and cytoplasmic domain of murine Mpl could transduce the ligand-dependent proliferative signals in Ba/F3 cells, and that the proliferative responsibilities in transformants corresponded to the receptor expression levels on their cell surfaces (Skoda et al., 1992). Thus, the potent transducing activity of Mpl receptor concerning TPO-dependent proliferative and differentiation signals on BF-TE22 cells may be due to the high level of Mpl expression.

It has been shown that the Mpl receptor is expressed in the megakaryocyte lineage from progenitors to platelets (Debili et al., 1995). TPO acts on the megakaryocyte progenitor cells and supports their cell growth and development alone or in combination with IL-3, erythropoietin, stem cell factor, or IL-11 (Kaushansky et al., 1995; Broudy et al., 1995). However, it was recently demonstrated that TPO inhibited the proplatelet formation from developed megakary-

Figure 3. Flow cytometric analysis of megakaryocyte/platelet-specific cell surface antigens in BF-TE22 cells stimulated by IL-3 or TPO. Cells cultured with IL-3 (thin line) or TPO (thick line) were incubated for 30 min at 4 °C with the indicated antibody. After washing twice with staining buffer, cells were then stained for 30 min with FITC- or PE-conjugated secondary antibody. Fluorescence intensity was determined with a FACScan analyzer.

ocytes (Choi et al., 1995; Nagahisa et al., 1996). These results indicate that TPO may stimulate the growth and development of megakaryocyte progenitors, but not induce the terminal differentiation from developed megakaryocytes to platelets. In our present study, TPO stimulated the growth and the expression of megakaryocyte lineage-specific cell surface antigens in BF-TE22 cells, but did not enhance the expression of platelet-specific cell surface antigens, suggesting that TPO could not induce terminal differentiation of BF-TE22 cells (Figs. 3 and 4). Moreover, TPO did not induce the morphologic changes of BF-TE22 cells such as polyploidization and platelet release in the late stage of megakaryocyte development (data not shown).

It is well known that IL-3 supports the growth of megakaryocytes from bone marrow cells in both semisolid and suspension culture systems alone or in combination with TPO, but has little effect on megakaryocyte maturation (Segal et al., 1988; Broudy et al., 1995). This indicates that IL-3 is a potent growth stimulation factor for megakaryocyte progenitors but is not a differentiation factor for megakaryocyte maturation. In the present study, we demonstrated that IL-3 could not induce the expression of megakaryocyte lineage-specific cell surface antigens in combination with TPO and strongly inhibited the TPO-triggered

differentiation signals in BF-TE22 cells. Moreover, inhibitory effect of IL-3 on the megakaryocytic differentiation of BF-TE22 cells was dose-dependent. These results suggested that the megakaryocytic differentiation of BF-TE22 cells evoked by TPO could be reversed by IL-3, and that the signal mediated by IL-3 predominated over that of TPO.

Very recently, it was reported that several human hematopoietic cell lines including UT-7/TPO, HU-3/TPO, M-O7e/TPO, M-MOK/TPO, and TF-1/TPO express the endogenous Mpl protein on their cell surfaces and exhibit the TPO-dependent characteristics (Komatsu et al., 1996; Drexler et al., 1997). TPO supported not only the proliferation of these human hematopoietic cells, but also induced an up-regulation of the megakaryocytic cell surface markers CD41, CD42, and CD61 in some of these cells, suggesting some differentiation along megakaryocytic lineage. As shown in Figs. 3 and 4, the expression of megakaryocytic cell surface markers AP-51 and CD61 on BF-TE22 cells significantly increased when cells were cultured for only 10 days in the continuous presence of TPO. However, TPO appears both a proliferative and a differentiative effect on these human TPO-dependent cell models when cells were exposed to TPO in long-term. This suggests that it takes a lot of time to investigate the differentiative effects of TPO by us-

Figure 4. IL-3 inhibited the expression of AP-51 and CD61 antigens induced by TPO stimulation. Cells were cultured for 10 days with 10 ng/ml TPO in the presence (thin line) or absence (thick line) of 1 ng/ml IL-3, or IL-3 alone (broken line). Cells were then stained with anti-AP-51 or anti CD61-antibody, and then analyzed with a FACScan analyzer.

ing these human models. Therefore BF-TE22 cells reported here have TPO-specific signal transduction pathway to mediate the megakaryocytic differentiation signals and would be useful to study the specific action of TPO.

It is known that several intracellular signaling pathways such as JAK-STAT and MAP kinase pathways are involved in the postreceptor signalling of the cytokine receptor system (Ihle et al., 1995). Komatsu et al. reported that TPO induced the tyrosine-phosphorylation of JAK2 tyrosine kinase, STAT5-related protein, and MAP kinases (Komatsu et al., 1996; Yamada et al., 1996). Moreover, we and other investigators reported that many intracellular signaling proteins including JAK2, Tyk2, STAT1, STAT3 and STAT5, Vav, SHPTP2, Cbl and Shc were tyrosine-phosphorylated after TPO stimulation on platelet and TPO-dependent cell lines such as hematopoietic cell lines which endogenously or exogenously express the Mpl protein (Gurney et al., 1995; Nagata et al., 1995; Miyakawa et al., 1996; Morita et al., 1996; Oda et al., 1996; Porteu et al., 1996). These results indicate that the TPO/Mpl signals as well as other cytokine/cytokine receptor signals were transduced through JAK-STAT and MAP kinase pathways. However, these signaling molecules and signal transduction pathways involved in the TPO/Mpl system were common in part to those in other members of the cytokine receptor superfamily. Therefore, these studies may be not sufficient to explain the difference between the Mpl receptor-specific signaling and other cytokine receptor-specific signaling. Very recently, it was demonstrated that the knock-out of NF-E2 megakaryocyte lineage-specific transcriptional factor gene in mice results in the dramatic inhibition of cellular differentiation from mature megakaryocytes

to platelets (Shivdasani et al., 1995). Cellular differentiation of megakaryocytes is controlled by many transcriptional factors that induce a set of genes that are expressed specifically in differentiated cells. AP-51 and CD61 antigens are expressed in cells restricted to the megakaryocyte-lineage. The significant increase of these antigens in BF-TE22 cells by TPO suggests that the promoter of these genes carries cis elements to which putative transcriptional factors responsible for megakaryocyte differentiation bind. Detailed analysis of the promoters for these genes and their transcriptional factors may reveal the mechanism for megakaryocyte-specific differentiation signal transduction mediated by the Mpl receptor. Finally, the BF-TE22 cells reported here will be useful to study the molecular mechanism of TPO-induced cell proliferation and megakaryocyte-specific differentiation.

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