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Quantitative analysis of thrombopoietin receptors on human megakaryocytes

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Abstract Thrombopoietin (TPO), or c-MPL ligand, is the primary regulator of megakaryocyte and platelet production. TPO receptors expressed on human megakaryocytes derived from peripheral blood (PB) and cord blood (CB) progenitors cultured in the presence of TPO have now been analyzed quantitatively. Like those on human PB platelets, TPO receptors on the cultured megakaryocytes exhibited a molecular mass of approximately 80 kDa. Various characteristics of PB- and CB-derived megakaryocytes indicated that the former were more mature than the latter. Both PB- and CB-derived megakaryocytes expressed a single class of high-affinity TPO receptors, with 1933 ± 772 ($n = 3$) and 184 ± 48 ($n = 4$) sites per cell, respectively. These data indicate that the number of TPO receptors on human megakaryocytes increases with cell maturation.

Key words: Thrombopoietin; c-Mpl; Megakaryocyte; Scatchard analysis; Cross-linking

1. Introduction

Thrombopoietin (TPO), or c-MPL ligand, is the recently isolated hematopoietic factor that primarily regulates megakaryopoiesis and platelet production [1–5]. In vitro, recombinant TPO (rTPO) stimulates the proliferation and maturation of megakaryocyte progenitor cells [6–10]. In addition to acting on megakaryopoiesis, rTPO enhances the growth of committed erythroid progenitors [11] and primitive hematopoietic progenitors [12]. rTPO also acts on platelets to activate several signal transduction pathways [13,14] and to enhance their function [15]. Furthermore, the administration of rTPO to mice or non-human primates markedly increases both platelet production and the numbers of megakaryocytes and their progenitors [3,16] as well as those of other hematopoietic progenitors in the bone marrow [16,17].

These effects of TPO are mediated through interaction with its specific cell surface receptor, c-MPL [18], originally identified by its homology to the product of the v-*mpl* oncogene of myeloproliferative leukemia virus. The human c-MPL protein comprises 635 amino acids, and does not contain consensus sequences for kinase-related or nucleotide-binding domains in its cytoplasmic region [18]. Transcripts encoding c-MPL have been identified in normal CD34$^+$ cells [19], in megakaryocytic cell lines [19,20], and in blast cells from individuals with acute myelogenous leukemia [21,22].

Recent studies have shown that platelets express a small number of high-affinity TPO receptors [23–25] with a molecular size of 97 kDa, as assessed by cross-linking analysis [24]. Furthermore, it has been suggested that the plasma concentration of TPO is regulated through the binding of TPO to receptors on circulating platelets [23,26]. However, little is known about TPO receptors on megakaryocytes because of the difficulty in obtaining a sufficient number of purified cells for detailed studies. We have now performed a quantitative analysis of TPO receptors on human megakaryocytes produced by culturing megakaryocyte progenitors obtained from two different sources in the presence of recombinant human TPO (rhTPO).

2. Materials and methods

2.1. Cytokines

Full-length rhTPO and recombinant mouse interleukin-3 (rmIL-3) were expressed in Chinese hamster ovary cells and in *Escherichia coli*, respectively, and were purified to homogeneity by the Production Technology Group at Kirin. Protein concentrations were determined by analysis of amino acid composition.

2.2. Platelet preparation

Blood was collected by venipuncture from healthy volunteers into a syringe containing 10% acid-citrate-dextrose anticoagulant and was gently mixed. Within 1 h after blood collection, platelet-rich plasma (PRP) was obtained by centrifuging the whole blood at 200×*g* for 20 min at room temperature. For preparation of washed platelets, platelets in the PRP were washed twice with binding buffer (see Section 2.4). For preparation of gel-filtered platelets, PRP was fractionated on a Sepharose CL-2B (Pharmacia, Uppsala, Sweden) column that had been equilibrated with binding buffer, as previously described [27].

2.3. Cultured megakaryocytes and megakaryocytic cell line

Low-density mononuclear cells from human umbilical cord blood (CB) and CD34$^+$ cells from peripheral blood (PB) were prepared as described [28], and cultured for 12–14 days in the presence of rhTPO (10 ng/ml) in serum-depleted medium [Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY) supplemented with 1% bovine serum albumin (BSA), 1.7 μM recombinant human insulin (Sigma, St. Louis, MO), human transferrin (300 μg/ml) (Boehringer Mannheim, Mannheim, Germany), and 50 μM 2-mercaptoethanol (Merck, Darmstadt, Germany)]. The resultant cells were highly enriched for megakaryocytes, as assessed by flow cytometry for the expression of CD41 [28]. Megakaryocyte ploidy was analyzed by flow cytometry as described [28]. To yield sufficient numbers of CB-derived megakaryocytes for quantitative analysis of TPO receptors, low-density mononuclear cells were used as the starting cell population in place of CD34$^+$ cells. Megakaryocytes grown from the two

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Abbreviations: TPO, thrombopoietin; rhTPO, recombinant human TPO; rmIL-3, recombinant murine interleukin-3; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; PRP, platelet-rich plasma; CB, cord blood; PBS, phosphate-buffered saline; PB, peripheral blood; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; EPO, erythropoietin

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different cell fractions showed similar ploidy distribution (Table 2 and [28]), indicating a similar stage of cell differentiation.

Dami cells, a human megakaryoblastic cell line [29], were obtained from American Type Culture Collection (Rockville, MD) and maintained in IMDM supplemented with 10% heat-inactivated horse serum. For differentiation of Dami cells, they were incubated for 20 days with 5 nM phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA).

2.4. Equilibrium binding analysis

rhTPO (3 μg) was labeled with ^{125}I by incubation in a final volume of 28 μl with 18.5 MBq of Na^{125}I (Amersham, Braunschweig, Germany), lactoperoxidase (0.45 U/ml) (Calbiochem, La Jolla, CA), glucose oxidase (0.05 U/ml) (Wako, Tokyo, Japan), 9.2 μM NaI, and 0.24% β-D-(+)-glucose (Sigma). Free iodide was removed by passage through a PD-10 gel-filtration column (Pharmacia). The specific activity of ^{125}I-rhTPO was determined by self-displacement analysis with FDCP-hMpl5 cells which are mIL-3-dependent mouse myeloid leukemia FDCP-2 cells genetically engineered to express human c-MPL constitutively [30].

Before binding experiments with CB- and PB-derived megakaryocytes, the cells were washed twice with IMDM containing 10% fetal bovine serum (FBS) and resuspended in the same medium. They were further incubated for 6 h at 37°C without the addition of rhTPO to remove rhTPO that bound to c-MPL on the cell surface during the primary culture, as reported for quantitative analysis of the erythropoietin (EPO) receptor on EPO-responsive murine cell lines [31]. The cultured megakaryocytes were then passed through a 10% glucose solution by centrifugation at 250×*g*. To quantify the binding affinity and number of TPO receptors, we incubated platelets or megakaryocytes for 1 h at 37°C with ^{125}I-rhTPO, in the absence or presence of a 100-fold or more excess of unlabeled rhTPO, in binding buffer [Hanks' balanced salt solution without phenol red, $CaCl_2$, $MgCl_2$, and $MgSO_4$ (modified HBSS) (Gibco), supplemented with 0.35% BSA, 20 mM HEPES, 5 mM EDTA, and 0.02% NaN_3]. Bound radioactivity was separated from unbound ^{125}I-rhTPO by centrifugation of each sample through bovine serum. Non-specific binding was determined by incubating the cells with ^{125}I-rhTPO in the presence of unlabeled ligand. The binding data were subjected to Scatchard analysis.

2.5. Affinity cross-linking

Washed platelets and PB-derived megakaryocytes were incubated in binding buffer for 1 h at 37°C with ^{125}I-rhTPO in the absence or presence of a 200-fold excess of unlabeled rhTPO. After washing twice with ice-cold PBS, the cells were incubated for 30 min at 4°C with 4 μM BS^3 (Pierce, Rockford, IL) in modified HBSS. The cross-linking reaction was quenched by the addition of 0.2 M glycine solution. After two washes with ice-cold PBS, the cells were solubilized at 4°C for 30 min in lysis buffer [modified HBSS containing 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.3 TIU/ml), 2 mM EDTA, and 2 mM EGTA]. The lysates were centrifuged at 15 000×*g* for 20 min to remove nuclei and other debris, and the resulting supernatants were subjected to SDS-polyacrylamide gel electrophoresis on a 6% gel under reducing conditions. Affinity-labeled protein was detected by autoradiography of the dried gels.

3. Results

3.1. Molecular size of TPO receptors on platelets and megakaryocytes

The molecular sizes of TPO receptors on washed platelets and PB-derived megakaryocytes were compared by cross-linking ^{125}I-rhTPO to the receptors with the use of the homobifunctional cross-linker BS^3. A labeled complex of ~170 kDa was apparent when washed platelets or PB-derived megakaryocytes were incubated with ^{125}I-rhTPO in the absence of unlabeled rhTPO, but not in its presence (Fig. 1). Given that the molecular mass of rhTPO is 80–100 kDa, the TPO receptors on both cell types showed a molecular size of approximately 80 kDa.

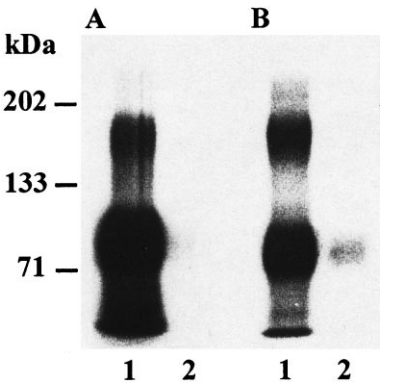

Fig. 1. Cross-linking of ^{125}I-rhTPO to human PB-derived megakaryocytes (A) and platelets (B). PB-derived megakaryocytes and washed platelets were exposed to the cross-linker BS^3 after incubation with ^{125}I-rhTPO in the absence (lane 1) or presence (lane 2) of a 200-fold excess of unlabeled rhTPO. Cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The upper band represents the cross-linked complex and the lower band is free ^{125}I-rhTPO. The positions of molecular size markers are indicated in kilodaltons.

3.2. Quantitative analysis of TPO receptors on human platelets and megakaryocytes

The specific TPO receptors on platelets obtained from normal human PB were characterized by Scatchard analysis of equilibrium binding of ^{125}I-rhTPO. Normal human platelets expressed a single class of high-affinity TPO receptors as previously described [21,22]. Washed human platelets exhibited 22 ± 7 receptors per cell, with a K_d of 28 ± 8 pM (n = 10) (Table 1). Similar results were obtained with gel-filtered platelets [23 ± 12 receptors per cell, with a K_d of 50 ± 39 pM (n = 5)].

Culture of CB mononuclear cells or PB CD34$^+$ cells in the presence of rhTPO for 12–14 days resulted in selective expansion of megakaryocytes (87.1–97.3% and 92.1–95.8% CD41$^+$ cells, respectively) (Table 2). Cell size, ploidy, and expression of CD42 antigen were greater for PB-derived than for CB-derived megakaryocytes (Table 2). Scatchard analysis revealed that CB-derived megakaryocytes expressed 184 ± 48 TPO receptors per cell, with a K_d of 125 ± 12 pM (n = 4) (Table 3), whereas the corresponding values for PB-derived megakaryocytes were 1933 ± 772 receptors per cell and 90 ± 14 pM (n = 3), respectively (Fig. 2, Table 3).

Table 1
Analysis of TPO receptors on normal human washed and gel-filtered platelets

Platelet preparation	Receptors per cell		K_d (pM)		n
	Mean ± S.E.	Range	Mean ± S.E.	Range	
Washed	22 ± 7	15–35	28 ± 8	20–41	10
Gel-filtered	23 ± 12	15–43	50 ± 39	26–112	5

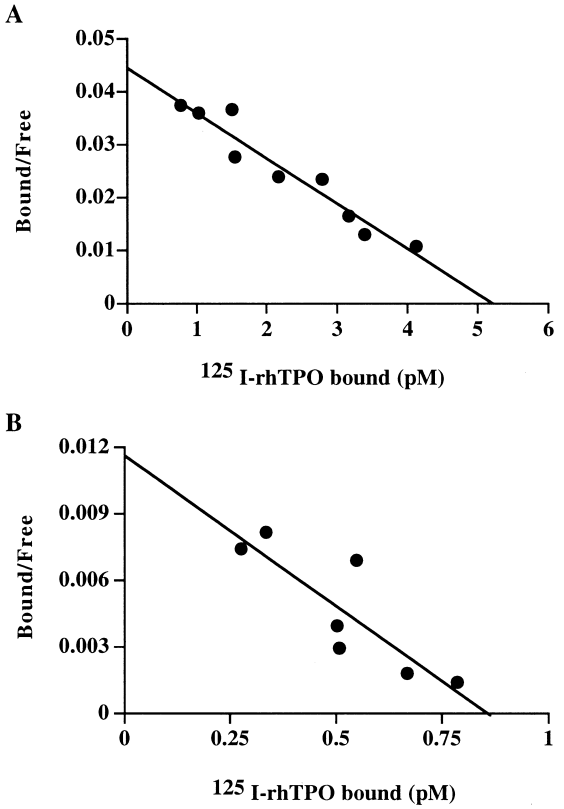

Fig. 2. Scatchard analysis of ^{125}I-rhTPO binding to CB-derived (A) and PB-derived (B) megakaryocytes. Cells were incubated with various concentrations of ^{125}I-rhTPO in the absence or presence of a 100-fold or more excess of unlabeled rhTPO for 1 h at 37°C. Data represent Scatchard analysis of representative experiments.

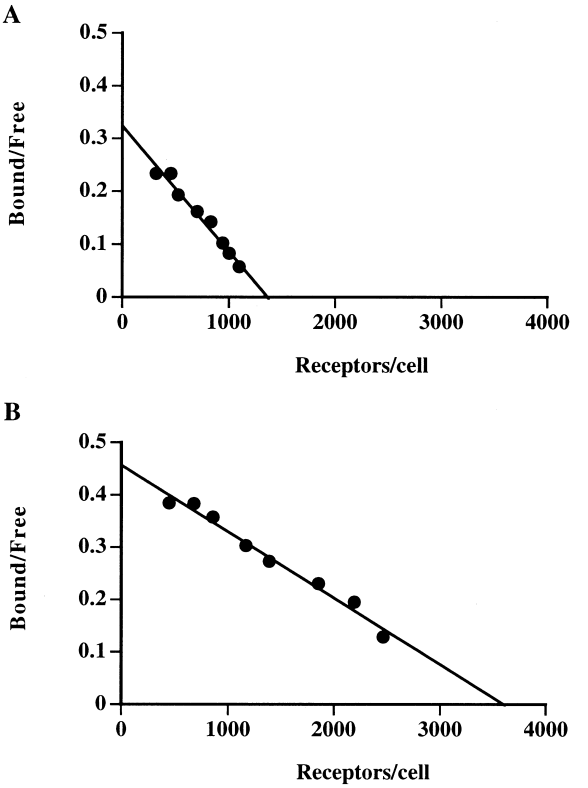

Fig. 3. Scatchard analysis of ^{125}I-rhTPO binding to Dami cells cultured in the absence (A) or presence (B) of TPA. Cells were cultured for 20 days in the absence or presence of 5 nM TPA, after which the binding of ^{125}I-rhTPO was assessed by Scatchard analysis. Data are from a representative experiment and revealed 1351 (A) and 3508 (B) receptors per cell.

3.3. Effect of differentiation on the number of TPO receptors on Dami cells

Finally, we investigated the effect of culture of Dami cells for 20 days with TPA on the expression of TPO receptors. TPA induced megakaryocytic differentiation of Dami cells as characterized by an increase in both size and ploidy (data not shown). Scatchard analysis of equilibrium binding of ^{125}I-rhTPO to TPA-treated and untreated Dami cells revealed the presence of 3508 and 1351 receptors per cell, respectively (Fig. 3). Thus, megakaryocytic differentiation of Dami cells was associated with an increase in the number of TPO receptors.

4. Discussion

Analysis of the molecular events that control the development of hematopoietic cells depends on the availability of sufficient numbers of purified cells. In this study, we have examined the expression of TPO receptors on both human PB platelets and cultured megakaryocytes. Selective enrichment of megakaryocytes was achieved by culture of CB- and PB-derived hematopoietic cells in the presence of TPO as the sole hematopoietic factor. These highly enriched preparations allowed us to conduct quantitative analysis of TPO receptors on human megakaryocytes. Equilibrium binding experiments showed that megakaryocytes as well as platelets express a single class of high-affinity TPO receptors and that

Table 2
Analysis of purity and maturation of CB- and PB-derived megakaryocytes (four and two preparations, respectively)

Source of megakaryocytes	Perimeter (μm)	CD41$^+$ (%)	CD42b$^+$ (%)	Ploidy (%)			
				2N	4N	8N	>8N
Cord blood	ND	87.1	46.8	70.1	10.3	3.5	1.4
	105	97.3	74.8	75.4	11.4		
	ND	92.7	66.8	89.3	7.8		
	ND	88.9	70.3	90	9.9		
Peripheral blood	169	95.8	85.8	32.2	23.2	28.9	12.4
	125	92.1	75.3	56.4	21.9	14.5	5

ND, not determined.

Table 3
Scatchard analysis of TPO receptors on CB- and PB-derived megakaryocytes

Source of megakaryocytes	Preparation	Receptors per cell	K_d (pM)
Cord blood	1	178	113
	2	170	131
	3	137	116
	4	251	139
Peripheral blood	1	2622	97
	2	2078	74
	3	1098	98

the number of TPO receptors on megakaryocytes increases as the cells mature. Furthermore, cross-linking analysis indicated that the molecular mass of TPO receptors on human megakaryocytes is similar to that of those on platelets.

Whereas Fielder et al. [25] showed that ^{125}I-rhTPO was cleaved and degraded to multiple polypeptide fragments on incubation with human platelets, under our experimental conditions human platelets that had been incubated with ^{125}I-rhTPO displayed only the full-length radioligand (data not shown). Therefore, our affinity analysis directly reflects the interaction of c-MPL with full-length TPO, and not that with truncated molecules. In addition, the truncation of rhTPO by thrombin-mediated cleavage [32] should not occur in our system because platelets and megakaryocytes were incubated with ^{125}I-rhTPO in the absence of free Ca ions, which blocks thrombin-catalyzed cleavage. Equilibrium binding experiments with washed platelets revealed a single class of high-affinity TPO receptors with $\sim$20 receptors per cell, similar to results obtained with gel-filtered platelets, which are treated more gently during preparation. Thus, expression of TPO receptors by the washed platelets did not appear to be affected by treatment during their preparation. Previous studies with washed human platelets also detected a single class of high-affinity TPO receptors with $\sim$30 receptors per cell [24,25]. However, murine platelets have been shown to display 220 TPO receptors per cell [23]. The difference in species or in experimental techniques likely contributes to these different results.

Scatchard analysis of our binding data showed that PB-derived megakaryocytes express a greater number of TPO receptors than do CB-derived megakaryocytes. In addition, TPA-induced differentiation of Dami cells was accompanied by an increase in the number of TPO receptors on these cells. These observations indicate that TPO receptor expression increases with terminal maturation of megakaryocytes. Expression of the EPO receptor has been shown to change with differentiation and maturation of cells committed to the erythroid lineage. The EPO receptor has been detected on burst-forming unit-erythroids, which are early, EPO-responsive progenitors. The number of EPO receptors increases with cell maturation, reaching a peak at the colony-forming unit-erythroid and proerythroblast stages, and thereafter decreases with further maturation [31,33].

TPO stimulates not only proliferation of megakaryocyte progenitor cells but also generation of polyploid mature megakaryocytes which result in formation of extended cytoplasmic processes termed proplatelets. The physiological significance of the maturation-associated increase in the number of TPO receptors on megakaryocytes is unclear. However, since the formation of proplatelets from mature megakaryocytes has been shown to be inhibited by the addition of TPO [10], TPO receptors on mature megakaryocytes may participate in the regulation of platelet production. In addition, the plasma concentration of TPO is thought to be regulated, to some extent, by TPO receptors on megakaryocytes [34]. Our data indicate that such regulation of the plasma TPO concentration might be possible given the expression of high-affinity TPO receptors by these cells.

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