

IMPAIRED EXPRESSION OF THE THROMBOPOIETIN RECEPTOR BY PLATELETS FROM PATIENTS WITH POLYCYTHEMIA VERA

ALISON R. MOLITERNO, M.D., W. DAVID HANKINS, PH.D., AND JERRY L. SPIVAK, M.D.

ABSTRACT

Background The cause of polycythemia vera, which originates from a multipotent hematopoietic progenitor cell, is unknown. Thrombopoietin is a hematopoietic growth factor that regulates the production of multipotent hematopoietic progenitor cells and platelets. To evaluate the possibility that an abnormality in thrombopoietin-mediated signal transduction might be involved in the pathogenesis of polycythemia vera, we examined thrombopoietin-induced tyrosine phosphorylation of proteins and the expression of the thrombopoietin receptor in platelets from patients with the disease.

Methods Platelets were isolated from the blood of patients with polycythemia vera or other chronic myeloproliferative disorders and control subjects. The platelets were exposed to either thrombopoietin or thrombin and then lysed for analysis of tyrosine phosphorylation of platelet proteins and the expression of the proteins by means of immunoblotting. Expression of the thrombopoietin receptor (Mpl) by platelets and megakaryocytes was also assessed.

Results Thrombopoietin-mediated tyrosine phosphorylation of proteins was impaired in platelets from 20 patients with polycythemia vera and 3 with idiopathic myelofibrosis, but not in 4 patients with essential thrombocytosis, 3 with chronic myelogenous leukemia, 6 with secondary erythrocytosis, 2 with iron-deficiency anemia, 4 with hemochromatosis, or 5 normal subjects. Thrombin-mediated tyrosine phosphorylation of proteins was intact in platelets from patients with polycythemia vera, and the tyrosine kinases and substrates involved in the process were present in normal amounts. However, expression of the platelet thrombopoietin receptor Mpl was markedly reduced or absent in 34 of 34 patients with polycythemia vera and in 13 of 14 patients with idiopathic myelofibrosis. Impaired thrombopoietin-induced tyrosine phosphorylation of proteins in patients with these two diseases was uniformly associated with markedly reduced expression of Mpl or the lack of its expression. In patients with polycythemia vera, reduced expression of Mpl by platelets was associated with reduced expression of Mpl by megakaryocytes.

Conclusions Reduced expression of the thrombopoietin receptor Mpl is characteristic of polycythemia vera and idiopathic myelofibrosis. The abnormality appears to distinguish polycythemia vera from other forms of erythrocytosis. (N Engl J Med 1998; 338:572-80.)

©1998, Massachusetts Medical Society.

POLYCYTHEMIA VERA, idiopathic myelofibrosis, chronic myelogenous leukemia, and essential thrombocythemia are classified as chronic myeloproliferative disorders because they arise through the clonal expansion of a multipotent hematopoietic progenitor cell, with subsequent overproduction of one or more of the formed elements of the blood.¹⁻⁴ With the exception of chronic myelogenous leukemia, these disorders lack a clonal marker, their pathogenesis is unknown, and the diagnosis depends on clinical criteria.

Polycythemia vera is characterized by increased numbers of erythrocytes, granulocytes, and platelets. Since erythrocytosis is the most prominent manifestation and the defining clinical criterion of polycythemia vera, most studies of the disease have focused on erythropoiesis. The erythroid progenitor cells in patients with polycythemia vera proliferate and differentiate in vitro in the absence of added erythropoietin,^{5,6} and they are hypersensitive to other growth factors under certain conditions.⁷⁻¹⁰ However, although erythropoietin-independent formation of erythroid colonies in vitro is characteristic of polycythemia vera, it is not specific for the disease^{7,11} and does not define the limits of the affected clone, since not all erythroid progenitors in polycythemia vera are independent of erythropoietin.⁶

Given these attributes of erythroid progenitor cells in polycythemia vera, there has been substantial interest in the function of growth factor receptors in this disease. However, the expression of the erythropoietin receptor and its ability to bind to erythropoietin are normal in polycythemia vera,¹² and abnormalities of the erythropoietin receptor gene have not been identified.^{11,13,14} Mutations of this gene have been found in families with inherited erythrocytosis,^{15,16} but affected family members do not have disorders of thrombopoiesis and granulopoiesis.

In contrast to these studies of the erythropoietin receptor, recent studies suggest that an alteration of thrombopoietin-mediated signal transduction might be implicated in polycythemia vera. The best-characterized effect of thrombopoietin appears to be on the proliferation and differentiation of megakaryocyte precursors,¹⁷ but thrombopoietin also acts directly on primitive hematopoietic stem cells in vitro,¹⁸ and mice with a deficiency of thrombopoietin or its receptor (designated Mpl) have reduced numbers of both mul-

From the Hematology Division, Department of Medicine, Johns Hopkins University School of Medicine, Traylor 924, 720 Rutland Ave., Baltimore, MD 21205, where reprint requests should be addressed to Dr. Spivak.

TABLE 1. CLINICAL AND LABORATORY CHARACTERISTICS OF THE STUDY SUBJECTS.*

DISEASE	MEAN AGE (RANGE)	FEMALE SEX	MEAN HEMOGLOBIN (RANGE)	MEAN MCV (RANGE)	MEAN WHITE-CELL COUNT (RANGE)	MEAN PLATELET COUNT (RANGE)	CURRENT THERAPY
	yr	%	g/dl	fl	cells/mm ³	×10 ⁻³ /mm ³	
Polycythemia vera (n = 34)	64 (33–86)	56	13.8 (8.2–16.9)	78.6 (59.8–106.8)	28,234 (6600–145,437)	717 (108–2082)	Phlebotomy in 34; hydroxyurea in 4
Idiopathic myelofibrosis (n = 14)	64 (47–83)	29	9.9 (7.2–12.7)	90.3 (77.8–115.0)	19,562 (2800–96,600)	633 (106–2005)	None in 9; hydroxyurea in 2; erythropoietin in 1; prednisone in 1; anagrelide in 1
Chronic myelogenous leukemia (n = 7)	59 (36–84)	86	13.0 (11.3–14.6)	93.3 (86.9–101.9)	19,200 (6000–54,900)	785 (303–1551)	None in 4; interferon alfa in 2; hydroxyurea in 1
Essential thrombocytosis (n = 9)	50 (33–72)	56	13.5 (11.2–16.1)	95.7 (77.4–118.0)	7,200 (2400–9100)	927 (592–1739)	None in 5; hydroxyurea in 4
Erythrocytosis (n = 14)	55 (25–81)	21	16.3 (13.0–20.0)	80.8 (76.0–95.7)	7,000 (3300–10,800)	193 (61–335)	Phlebotomy in 9; none in 5
Hemochromatosis (n = 8)	53 (26–70)	13	14.2 (13.0–15.8)	94.6 (84.5–100.9)	7,100 (4500–9700)	234 (132–338)	Phlebotomy in 8
Iron deficiency (n = 2)	36 (31–41)	100	8.4 (6.8–10.0)	67.4 (57–77.8)	8,400 (5600–11,200)	420 (278–562)	None

*Complete blood counts were unavailable for one patient with erythrocytosis and one with hemochromatosis. MCV denotes mean corpuscular volume.

lineage and committed hematopoietic progenitor cells.¹⁹ Overexpression of thrombopoietin in mice leads to extramedullary hematopoiesis and myelofibrosis,²⁰ whereas ectopic expression of *Mpl* produces a fatal erythroblastosis.²¹ Moreover, the retrovirus MPLV, which carries a truncated *Mpl* gene, induces erythrocytosis, thrombocytosis, granulocytosis, and splenomegaly in mice,²² and hematopoietic progenitor cells infected with MPLV in vitro are not dependent on growth factors.²³

For these reasons, we hypothesized that an abnormality of the thrombopoietin–*Mpl* signal-transduction pathway might be present in patients with polycythemia vera. To evaluate this possibility, we investigated thrombopoietin-induced signal transduction and the thrombopoietin receptor in patients with polycythemia vera. Since platelets express *Mpl*²⁴ and because the blood cells in polycythemia vera are derived from an abnormal clone,¹ we studied thrombopoietin-induced signal transduction in platelets. We observed that thrombopoietin-induced tyrosine phosphorylation of proteins in vitro was impaired in platelets from patients with polycythemia vera, as compared with normal platelets, and that this signaling defect was associated with diminished expression of the thrombopoietin receptor in platelets and megakaryocytes. In contrast to previously described abnormalities of platelets in patients with polycythemia vera, which can vary from patient to patient,^{25–27} *Mpl* deficiency was observed in the platelets from all 34 patients with polycythemia vera whom we studied. Furthermore, this abnormality distinguished platelets from patients with polycy-

themia vera from those from patients with benign (secondary) erythrocytosis.

METHODS

Subjects

We studied 64 patients with chronic myeloproliferative disorders: 34 with polycythemia vera, 14 with idiopathic myelofibrosis, 9 with essential thrombocytosis, and 7 with chronic myelogenous leukemia. The study protocol was approved by our joint committee on clinical investigation, and informed consent was obtained from each patient. The diagnosis of polycythemia vera was based on the criteria of the Polycythemia Vera Study Group²⁸ and included an elevated red-cell mass. The diagnosis of the other chronic myeloproliferative disorders was based on standard clinical criteria.²⁹ Control subjects included 14 patients with erythrocytosis (due in 2 patients to renal transplantation, to congenital heart disease in 1 patient, to a hemoglobin variant in 1 patient, and to unknown causes in 10 patients), 8 patients with hemochromatosis who were undergoing periodic therapeutic phlebotomy, 2 patients with uncomplicated iron deficiency, and 10 normal subjects (Table 1).

Tyrosine Phosphorylation of Platelet Proteins

Venous blood collected in 3.8 percent sodium citrate was centrifuged at 160×*g* for 10 minutes to obtain platelet-rich plasma. The platelet-rich plasma was spun at 750×*g* for 12 minutes, and the platelet pellet was washed three times with phosphate-buffered saline containing 0.5 percent bovine serum albumin and 0.6 percent sodium citrate (wash buffer). The platelets were resuspended in the wash buffer and enumerated in an electronic particle counter, and the volume of buffer was adjusted to yield a platelet concentration of 10⁹ cells per milliliter. Platelets were exposed to thrombin or thrombopoietin at selected concentrations for the indicated times at room temperature and then lysed in 20 mM TRIS buffer (pH 7.5) containing 1 percent Nonidet P 40, 137 mM sodium chloride, 10 percent glycerol, 1 mM EDTA, 50 mM sodium fluoride, 5 mM magnesium chloride, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per milliliter, 2 μg of leupeptin per milliliter, and 1 μg of pepstatin per milliliter (lysis buffer). The protein

concentration of the platelet lysates was quantitated by the bicinchoninic acid technique.³⁰

Immunoblotting and Immunoprecipitation of Platelet Lysates

Equal aliquots of platelet lysate protein were subjected to sodium dodecylsulfate–7.5 percent polyacrylamide-gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane at 20 V for 16 hours. The membrane was blocked with a solution of 10 mmol of TRIS per liter, 150 mmol of sodium chloride per liter (pH 7.6), and 1 percent Tween 20 (TBST) and 5 percent bovine serum albumin at room temperature for one hour, washed three times with TBST, and then incubated in TBST with 1 μ g of primary antibody per milliliter for one hour. The membrane was washed three times, incubated for one hour with the appropriate horseradish peroxidase-linked secondary antibody diluted 1:10,000 in TBST, and then washed three times. Detection by enhanced chemiluminescence was performed according to the manufacturer's specifications (Amersham, Arlington Heights, Ill.). Membranes were reprobbed with different antibodies after stripping in 62.5 mmol of TRIS per liter (pH 6.7) with 2 percent SDS and 100 mmol of mercaptoethanol per liter at 70°C for 30 minutes. For immunoprecipitation, antibody was added to the platelet lysate (200 μ g of lysate diluted to 1 μ g per microliter in lysis buffer) at a concentration of 1 μ g per 0.1 ml and incubated at 4°C for two hours. Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden), 0.1 ml of a 50 percent slurry in lysis buffer, was added to the lysate and incubated for an additional hour at 4°C with rocking. The sample was then washed four times with 10 mM TRIS buffer (pH 7.4) containing 1 percent Triton X-100, 150 mM sodium chloride, 1 mM ethyleneglycol-bis-(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 2 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride; resuspended in SDS-PAGE sample buffer; boiled for five minutes; subjected to SDS-PAGE; and transferred to a nitrocellulose membrane for immunoblotting as described above.

Densitometric Analysis

Autoradiographs of immunoblots were scanned with a Hewlett-Packard ScanJet IIc (Hewlett-Packard, Palo Alto, Calif.) and densitometry was performed with NIH Image software (version 2.3). Absorbance of the 85-kd band (Mpl) was quantified as the area under the peak for the band and expressed in arbitrary absorbance units. Pilot experiments identified the amount of platelet lysate protein (35 μ g) that produced a linear response. Protein loading was controlled by reprobbed the membrane with glycoprotein IIIa antiserum to confirm that similar amounts of platelet lysate were present in each sample. The expression of Mpl was determined by dividing the values for the patients by the average values for the control subjects on the same immunoblot. The variation in responses among the control subjects on a given immunoblot never exceeded 15 percent.

Immunohistochemical Analysis of Bone Marrow Biopsies

Sequential bone marrow-biopsy sections from patients with polycythemia vera or control subjects were either exposed to the affinity-purified rabbit Mpl antiserum at a dilution of 1:300 or incubated first with protease XXVII (Sigma) for 20 minutes and then with goat antihuman von Willebrand antiserum (Dako, Carpinteria, Calif.) at a dilution of 1:100, followed by exposure to the appropriate peroxidase-labeled secondary antibody and substrate.

Plasma Thrombopoietin Assay

Thrombopoietin was measured in EDTA-treated plasma samples from patients and controls with an enzyme-linked immunosorbent assay (Amgen, Thousand Oaks, Calif.).³¹ The mean value in normal subjects was 133 pg per milliliter (range, 55 to 377). The interassay variation was 15 percent, and the intraassay variation was 10 percent.

Platelet Flow Cytometry

Platelet-rich plasma prepared as described above was incubated with nonspecific rabbit IgG or Mpl antiserum for 30 minutes at 4°C, washed once, exposed to a goat antirabbit fluorescein conjugate F(ab')₂ fragment (Boehringer Mannheim, Indianapolis) for 30 minutes at 4°C, and washed again with wash buffer. Then 10,000 labeled platelets were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) according to the manufacturer's specifications.

Reagents and Antibodies

The antiphosphotyrosine antibody PY20 and STAT5 antiserum were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The JAK2 antiserum was purchased from Upstate Biotechnology (Lake Placid, N.Y.). Affinity-purified, polyclonal rabbit IgG antibodies to the soluble extracellular domain of human Mpl was a gift from Kirin Brewery (Maebashi, Gunma, Japan). Platelet glycoprotein IIIa antiserum was kindly supplied by Dr. Paul Bray (Hematology Division, Johns Hopkins University School of Medicine, Baltimore). Full-length thrombopoietin was purchased from R & D Systems (Minneapolis). Bovine thrombin was purchased from Armour Pharmaceutical Company (Collegeville, Pa.).

Statistical Analysis

Plasma thrombopoietin levels are not normally distributed. Therefore, the significance of the differences between group means of the thrombopoietin assay was assessed by the Kruskal-Wallis analysis of variance in ranks and Dunn's method for pairwise multiple comparisons.

RESULTS

Tyrosine Phosphorylation of Platelet Proteins

Incubation of normal platelets with thrombopoietin or thrombin induced tyrosine phosphorylation of numerous proteins in a dose-dependent and time-dependent manner. After the exposure of platelets from five normal subjects to a saturating concentration of thrombopoietin (100 ng per milliliter), proteins with apparent molecular masses of 125, 95, and 85 kd underwent tyrosine phosphorylation (Fig. 1). By contrast, incubation of platelets from patients with polycythemia vera in a similar concentration of thrombopoietin failed to induce significant tyrosine phosphorylation of these proteins. This phenomenon, which was found in 20 of 20 patients with polycythemia vera, and in 3 of 3 patients with idiopathic myelofibrosis, was not caused by a global impairment of tyrosine phosphorylation, since thrombin induced similar patterns of tyrosine phosphorylation in platelets from both normal subjects and patients with polycythemia vera. Neither increasing the concentration of thrombopoietin (up to 1000 ng per milliliter) nor increasing the duration of exposure to thrombopoietin (up to 30 minutes) induced substantial tyrosine phosphorylation in platelets from patients with polycythemia vera (data not shown).

The thrombopoietin receptor Mpl lacks a kinase domain.³² Instead, thrombopoietin-induced signal transduction by Mpl involves activation of JAK2 and TYK2, which are members of the Janus family of tyrosine kinases.³³⁻³⁷ We examined tyrosine phospho-

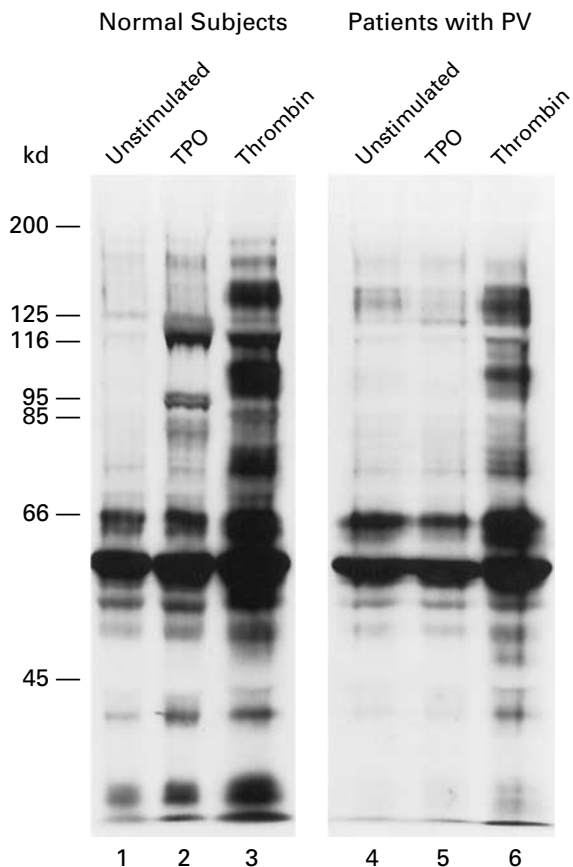


Figure 1. Tyrosine Phosphorylation of Proteins in Platelet Lysates from Normal Subjects and Patients with Polycythemia Vera (PV).

Platelets were either unstimulated or stimulated with 100 ng of thrombopoietin (TPO) per milliliter for 10 minutes at room temperature or 2 U of thrombin per milliliter for 2 minutes at room temperature.

rylation of JAK2 after exposure of platelets from normal subjects and patients with polycythemia vera to thrombopoietin. As shown in Figure 2A, thrombopoietin-induced tyrosine phosphorylation of JAK2 was impaired in platelets from patients with polycythemia vera, even though immunoblotting showed that the amount of JAK2 protein present in these platelets was similar to that in the normal platelets. Tyrosine phosphorylation of TYK2 was also impaired in platelets from patients with polycythemia vera after exposure to thrombopoietin despite the presence of equivalent quantities of TYK2 protein in the two types of platelets (data not shown).

To determine whether impaired tyrosine phosphorylation of JAK2 also impaired phosphorylation of its substrate proteins,³⁸ we examined the electrophoretic mobility of one of these substrates, STAT5,

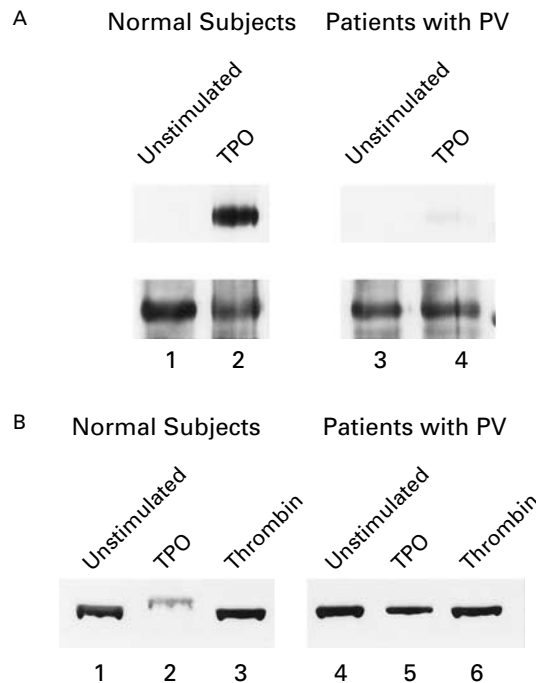


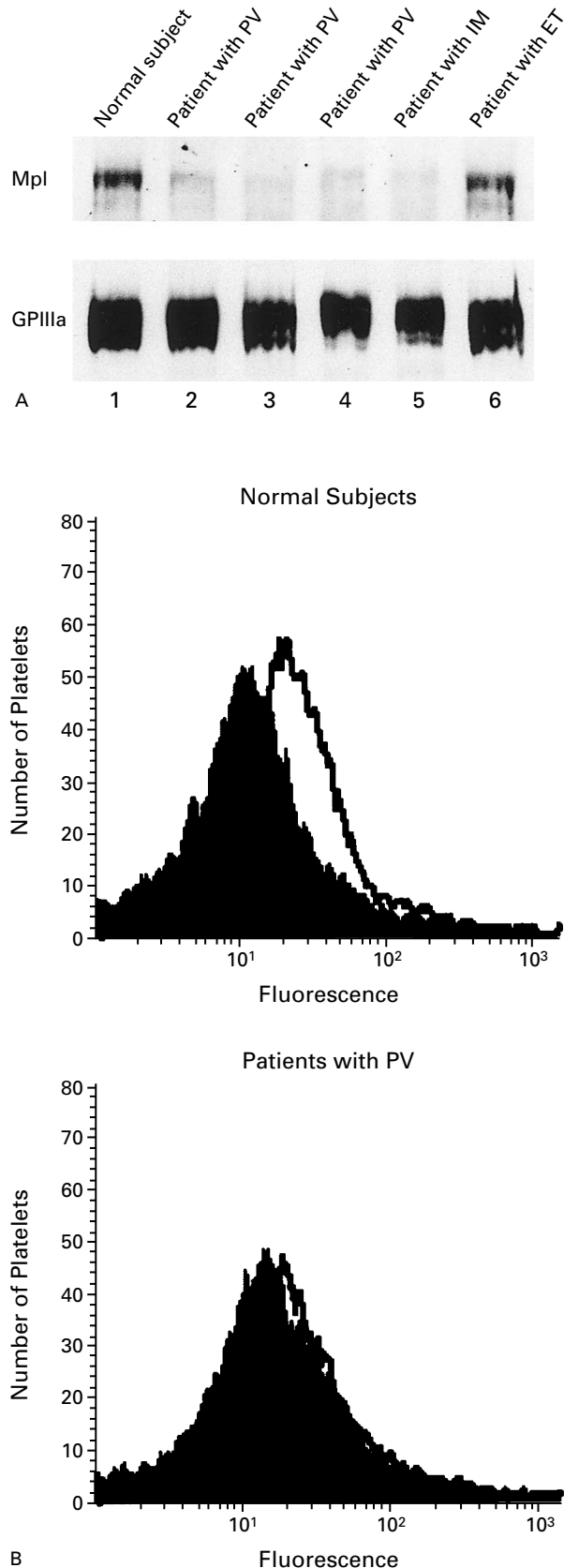
Figure 2. Tyrosine Phosphorylation of JAK2 (Panel A) and Its Substrate STAT5 (Panel B) in Platelet Lysates from Normal Subjects and Patients with Polycythemia Vera (PV).

In Panel A, platelets were either unstimulated or stimulated with thrombopoietin (TPO) (upper blot). The membrane was then stripped and reprobed with JAK2 antiserum to show that protein loading was similar in the samples (lower blot). Panel B shows anti-STAT5 immunoblotting of lysates of platelets that were either unstimulated or stimulated with thrombopoietin or thrombin.

in platelets from normal subjects and patients with polycythemia vera after exposure to thrombopoietin. As shown in Figure 2B, the addition of thrombopoietin to normal platelets significantly reduced the electrophoretic mobility of STAT5, an effect that correlated with an increase in its tyrosine phosphorylation (data not shown). In platelets from patients with polycythemia vera, however, the mobility of STAT5 was unchanged after incubation with thrombopoietin, and it did not undergo tyrosine phosphorylation. The absence of a mobility shift is further evidence of impaired thrombopoietin-mediated signal transduction in patients with polycythemia vera. These data indicate that the diminished tyrosine phosphorylation of STAT5 was due to an upstream defect in JAK2, Mpl, or other components of the thrombopoietin signaling pathway.

Expression of Mpl by Platelets

The abnormalities that we found in tyrosine phosphorylation of platelet proteins in patients with polycythemia vera could reflect an abnormality in



the Janus kinases or Mpl. Since the amount and electrophoretic mobility of JAK2 (and TYK2) appeared to be normal in these platelets, we examined the expression of platelet Mpl using immunoblotting. As shown in Figure 3A and Table 2, for equal quantities of platelet lysate protein, expression of the thrombopoietin receptor was markedly reduced in platelets from patients with polycythemia vera as compared with platelets from normal subjects. Flow-cytometric analysis of fresh, unfixed platelets yielded a concordant finding, indicating that the result with immunoblotting was not due to manipulation of the platelets (Fig. 3B). To ensure that the impaired expression of Mpl by platelets from patients with polycythemia vera was not a consequence of proteolysis during the isolation of platelets or of down-regulation by thrombopoietin or by as yet undefined agents in the plasma of the patients, we examined the expression of Mpl in lysates prepared from a mixture of equal quantities of platelets from normal subjects and patients with polycythemia vera, after incubation of normal platelets in plasma from patients with polycythemia vera, or after prolonged exposure of normal platelets to a saturating concentration of thrombopoietin. In no instance was the expression of Mpl impaired in the normal platelets (data not shown).

Reduced Expression of Platelet Mpl as a Marker for Polycythemia Vera and Idiopathic Myelofibrosis

To assess the specificity of these observations, we examined the expression of Mpl by platelets from 34 patients with polycythemia vera, 14 patients with idiopathic myelofibrosis, 9 patients with essential thrombocytosis, 7 patients with chronic myelogenous leukemia, 14 patients with secondary or undefined erythrocytosis, 8 patients with hemochromatosis who were undergoing periodic therapeutic phlebotomy, 2 patients with uncomplicated iron deficiency, and 10 normal subjects. The expression of Mpl was decreased in platelets from all 34 patients with polycythemia vera, whereas the expression of

Figure 3. Loss of the Thrombopoietin Receptor in Platelets from Patients with Polycythemia Vera (PV).

Panel A shows immunoblotting of lysates of platelets from normal subjects and patients with polycythemia vera, idiopathic myelofibrosis (IM), and essential thrombocytosis (ET) with an antiserum against Mpl (upper blot). The membrane was stripped and reprobbed with antiserum against glycoprotein IIIa (GPIIIa) to show that protein loading was similar in the samples (lower blot). Panel B shows the results of flow-cytometric analysis of the expression of Mpl by platelets from normal subjects and patients with polycythemia vera. The solid curve represents fluorescence due to binding of a nonspecific rabbit IgG; the open curve represents fluorescence due to the binding of the Mpl antiserum. Values are log-transformed.

TABLE 2. DENSITOMETRIC ANALYSIS OF THE EXPRESSION OF Mpl BY PLATELETS FROM 10 PATIENTS WITH POLYCYTHEMIA VERA AND 2 PATIENTS WITH IDIOPATHIC MYELOFIBROSIS.

PATIENT No.	DIAGNOSIS	EXPRESSION OF Mpl (% OF CONTROL VALUE)
1	Polycythemia vera	0
2	Polycythemia vera	0
3	Polycythemia vera	23
4	Polycythemia vera	9
5	Polycythemia vera	14
6	Polycythemia vera	9
7	Polycythemia vera	16
8	Polycythemia vera	4
9	Polycythemia vera	19
10	Polycythemia vera	24
11	Idiopathic myelofibrosis	19
12	Idiopathic myelofibrosis	9

Mpl was not diminished in platelets from the patients with essential thrombocytosis, chronic myelogenous leukemia, erythrocytosis, hemochromatosis, or iron deficiency or in the normal subjects. There was no correlation between decreased expression of Mpl in the patients with polycythemia vera and the platelet count, hemoglobin level, leukocyte count, duration of disease, presence or size of the spleen, the presence of iron deficiency, aspirin use, or concurrent use of hydroxyurea or interferon alfa therapy (data not shown). Interestingly, platelet expression of Mpl was also decreased in 13 of the 14 patients with idiopathic myelofibrosis. The concordance for decreased thrombopoietin-induced tyrosine phosphorylation of proteins and decreased expression of Mpl was 100 percent in the 18 patients (16 with polycythemia vera and 2 with idiopathic myelofibrosis) with sufficient platelet samples for both assays.

Expression of Mpl by Megakaryocytes

To determine whether expression of the thrombopoietin receptor was reduced in megakaryocytes from patients with polycythemia vera, bone marrow-biopsy specimens were examined by immunohistochemical staining with affinity-purified rabbit antibodies against Mpl. As shown in Figure 4, as compared with results in a control patient with essential thrombocytosis, in a patient with polycythemia vera the expression of Mpl was markedly reduced in 13 of the 14 megakaryocytes identified in a section stained for von Willebrand factor. Similar results were obtained in megakaryocytes from nine other pa-

tients with polycythemia vera and five patients with idiopathic myelofibrosis.

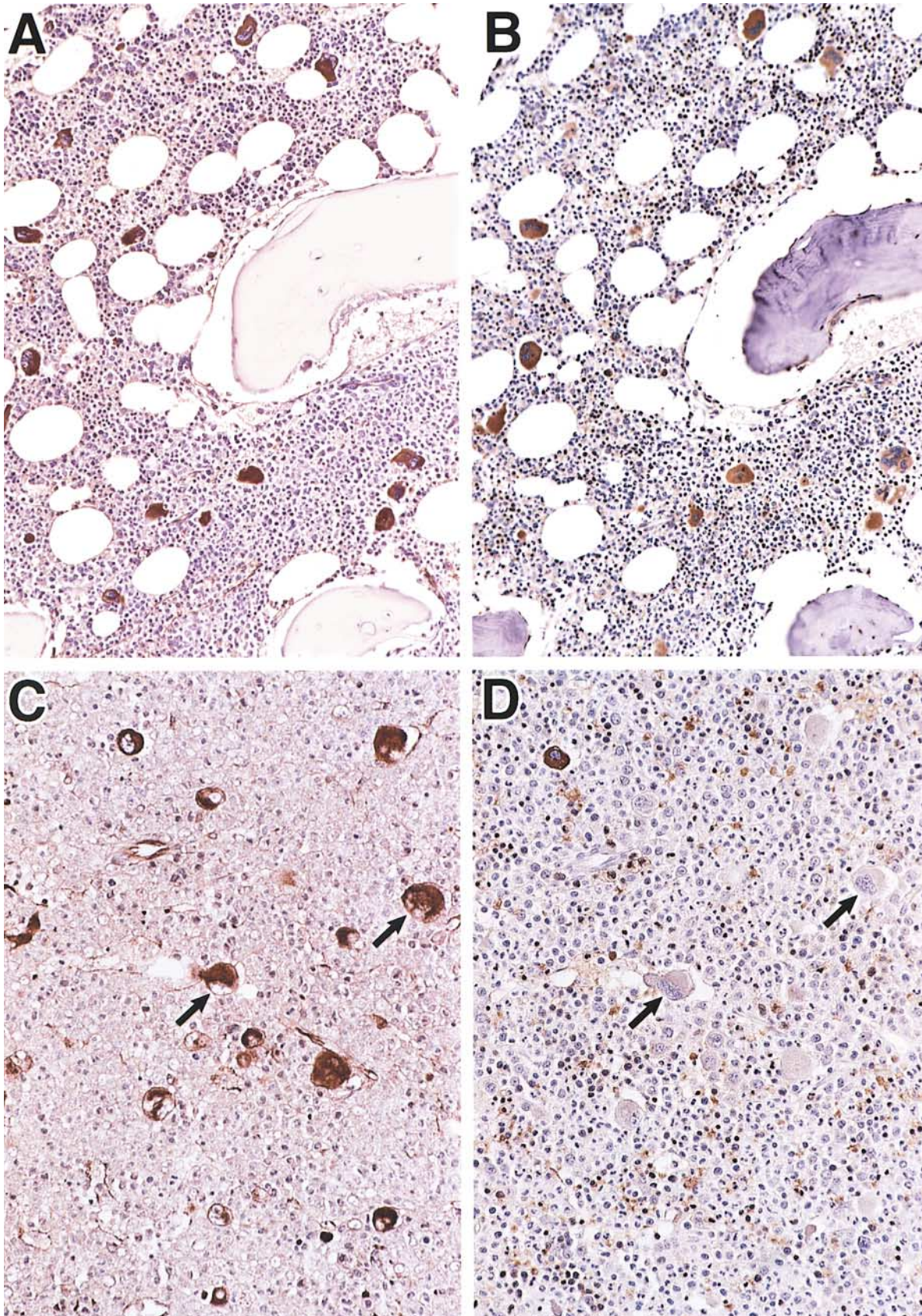
Plasma Thrombopoietin Levels in Patients with Polycythemia Vera

Previous studies have established that there is an inverse correlation between megakaryocyte and platelet mass and plasma thrombopoietin levels, which presumably results from the down-regulation of thrombopoietin due to receptor-ligand interactions.^{39,40} We therefore measured plasma thrombopoietin levels in patients with polycythemia vera and idiopathic myelofibrosis and normal subjects. We found that the mean plasma thrombopoietin concentration was significantly higher ($P < 0.05$) in patients with polycythemia vera (407 pg per milliliter) and patients with idiopathic myelofibrosis (292 pg per milliliter) than in normal subjects (133 pg per milliliter). There was no correlation, however, between the platelet count and the plasma thrombopoietin level in the patients with polycythemia vera.

DISCUSSION

The chronic myeloproliferative disorders polycythemia vera, idiopathic myelofibrosis, and essential thrombocytosis originate in a multipotent hematopoietic progenitor cell, and all are characterized by an increase in one or more types of blood cells. However, clonal markers have not been identified for these disorders, and their pathogenesis is not understood. Furthermore, polycythemia vera may present with an isolated leukocytosis, thrombocytosis, or even myelofibrosis and myeloid metaplasia and may eventually evolve into idiopathic myelofibrosis, making it difficult to distinguish among the three disorders. This problem is not trivial, since the natural history, treatment, and prognosis of polycythemia vera, idiopathic myelofibrosis, and essential thrombocytosis differ.²⁹

Recent experimental data suggest that an abnormality of the thrombopoietin-mediated signal-transduction pathway might be involved in polycythemia vera.¹⁷⁻²¹ Assessment of the expression of Mpl, the thrombopoietin receptor, by platelets provided a way to test this hypothesis. Binding of thrombopoietin to Mpl causes oligomerization of Mpl and activation of members of the Janus family of tyrosine kinases, in particular JAK2 and TYK2.³⁴⁻³⁷ Activation of these kinases is associated with tyrosine phosphorylation of a number of proteins, including members of the STAT family (in platelets, STAT3 and STAT5 specifically) and Shc.³⁵⁻³⁸ We found that exposure of platelets from patients with polycythemia vera to thrombopoietin failed to activate JAK2 or TYK2, and as a consequence STAT5 did not undergo tyrosine phosphorylation, even though the two kinases and the STAT5 substrate were expressed normally and thrombin-induced tyrosine phosphorylation of plate-



let proteins was intact. In contrast to findings in patients with chronic myelogenous leukemia, in whom growth factor-independent tyrosine phosphorylation of platelet proteins occurs as a consequence of the constitutively active kinase Bcr-Abl,⁴¹ we found no constitutive tyrosine phosphorylation of platelet proteins in patients with polycythemia vera.

The basis of the abnormality in thrombopoietin-mediated signal transduction in polycythemia vera appears to be diminished expression of the receptor for thrombopoietin in platelets. The findings of diminished expression of platelet prostaglandin D₂ receptors in some patients with chronic myeloproliferative disorders⁴² and α -adrenergic receptors in the platelets of some patients with essential thrombocytosis⁴³ parallel our observations, but the remarkable consistency of the reduction in the expression of Mpl in platelets and megakaryocytes from patients with polycythemia vera is novel. It is of interest that platelets from 13 of 14 patients with idiopathic myelofibrosis expressed a similar defect in the expression of Mpl. This finding suggests a close biologic relation between polycythemia vera and idiopathic myelofibrosis, which are known to be related clinically. The reduced expression of Mpl was specific for polycythemia vera (as defined by the currently accepted clinical criteria)²⁸ and was not a consequence of iron deficiency, splenomegaly, prior therapy (including periodic phlebotomy), duration of disease, or a hyperactive bone marrow. Nor was it found among patients with erythrocytosis unrelated to polycythemia vera.

The diminished expression of Mpl by platelets and megakaryocytes from patients with polycythemia vera was unexpected in a disease characterized by an excess of hematopoietic cells and hypersensitivity to growth factors. We found no evidence that the lack of Mpl was due to accelerated degradation of the receptor or its down-regulation by thrombopoietin. Overexpression of either thrombopoietin or Mpl in murine models produces many of the clinical features of polycythemia vera and idiopathic myelofibrosis,^{20,21} whereas mice with disabled *Mpl* genes have thrombocytopenia and reduced numbers of

hematopoietic progenitor cells.¹⁹ However, it may be misleading to draw parallels between polycythemia vera and genetically manipulated animal models. First, since polycythemia vera is an acquired disorder and presumably heterozygous, it may not be appropriate to compare it with a homozygous knockout model in mice. Second, loss of expression of a growth factor receptor may reflect the independence of the clonal progenitor cell in polycythemia vera from growth factors that are normally essential. Third, reduced expression or function of these receptors may produce unanticipated effects in cells that express a variety of other growth factor receptors. For example, inherited or experimentally induced mutations of the erythropoietin-receptor gene that cause carboxy-terminal truncations are associated with erythrocytosis due to hypersensitivity to insulin-like growth factor I but not to erythropoietin.⁴⁴ There is, moreover, the intriguing *in vitro* observation that erythroid progenitor cells in polycythemia vera have heightened sensitivity to a variety of hematopoietic growth factors but principally to insulin-like growth factor I.⁷

We cannot state that the loss of the thrombopoietin receptor is the cause of polycythemia vera, but the uniformity of this abnormality in platelets and megakaryocytes from affected patients suggests that it is intimately connected with the disease process. Whether reduced expression of Mpl represents a late consequence of the abnormality causing polycythemia vera or an obligatory early event, our finding provides a new avenue for investigation of the pathogenesis of this disease.

Supported by a grant from the National Institutes of Health (R01-H158589, to Dr. Spivak) and a Stetler Research Fellowship (to Dr. Moliterno).

We are indebted to Mrs. Evelyn Connor, Mrs. Mary Ann Isaacs, Ms. Karen Siebel, and Mr. Albert Sun for expert technical assistance.

REFERENCES

1. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L. Polycythemia vera: stem-cell and probable clonal origin of the disease. *N Engl J Med* 1976;295:913-6.
2. Jacobson RJ, Salo A, Fialkow PJ. Agnogenic myeloid metaplasia: a clonal proliferation of hematopoietic stem cells with secondary myelofibrosis. *Blood* 1978;51:189-94.
3. Fialkow PJ, Jacobson RJ, Singer JW, Sacher RA, McGuffin RW, Neefe JR. Philadelphia chromosome (Ph1)-negative chronic myelogenous leukemia (CML): a clonal disease with origin in a multipotent stem cell. *Blood* 1980;56:70-3.
4. Fialkow PJ, Faguet GB, Jacobson RJ, Vaidya K, Murphy S. Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. *Blood* 1981;58:916-9.
5. Prchal JF, Axelrad AA. Bone-marrow responses in polycythemia vera. *N Engl J Med* 1974;290:1382.
6. Cashman J, Henkelman D, Humphries K, Eaves C, Eaves A. Individual BFU-E in polycythemia vera produce both erythropoietin dependent and independent progeny. *Blood* 1983;61:876-84.
7. Correa PN, Eskinazi D, Axelrad AA. Circulating erythroid progenitors in polycythemia vera are hypersensitive to insulin-like growth factor-1 *in vitro*: studies in an improved serum-free medium. *Blood* 1994;83:99-112.
8. de Wolf JT, Beentjes JA, Esselink MT, et al. In polycythemia vera human interleukin 3 and granulocyte-macrophage colony-stimulating factor en-

Figure 4. Immunohistochemical Staining of Sequential Bone Marrow-Biopsy Sections with von Willebrand Factor Antiserum (Panels A and C) or Mpl Antiserum (Panels B and D) in a Patient with Essential Thrombocytosis (Upper Panels, $\times 64$) and a Patient with Polycythemia Vera (Lower Panels, $\times 100$).

In the patient with essential thrombocytosis, the megakaryocytes stained with the von Willebrand factor antiserum (Panel A) are also stained with the Mpl antiserum (Panel B). The arrows point to megakaryocytes stained with the von Willebrand factor antiserum (Panel C) but not the Mpl antiserum (Panel D) in the patient with polycythemia vera.

- hance erythroid colony growth in the absence of erythropoietin. *Exp Hematol* 1989;17:981-3.
9. Dai CH, Krantz SB, Dessypris EN, Means RT Jr, Horn ST, Gilbert HS. Polycythemia vera. II. Hypersensitivity of bone marrow erythroid, granulocyte-macrophage, and megakaryocyte progenitor cells to interleukin-3 and granulocyte-macrophage colony-stimulating factor. *Blood* 1992;80:891-9.
 10. Dai CH, Krantz SB, Green WF, Gilbert HS. Polycythemia vera. III. Burst-forming units-erythroid (BFU-E) response to stem cell factor and c-kit receptor expression. *Br J Haematol* 1994;86:12-21.
 11. Emanuel PD, Eaves CJ, Broudy VC, et al. Familial and congenital polycythemia in three unrelated families. *Blood* 1992;79:3019-30.
 12. Means RT Jr, Krantz SB, Sawyer ST, Gilbert HS. Erythropoietin receptors in polycythemia vera. *J Clin Invest* 1989;84:1340-4.
 13. Le Couedic JP, Mitjavila MT, Villeval JL, et al. Missense mutation of the erythropoietin receptor is a rare event in human erythroid malignancies. *Blood* 1996;87:1502-11.
 14. Hess G, Rose P, Gamm H, Papadileris S, Huber C, Seliger B. Molecular analysis of the erythropoietin receptor system in patients with polycythemia vera. *Br J Haematol* 1994;88:794-802.
 15. de la Chapelle A, Traskelin AL, Juvonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci U S A* 1993;90:4495-9.
 16. Sokol L, Luhovy M, Guan Y, Prchal JF, Semenza GL, Prchal JT. Primary familial polycythemia: a frameshift mutation in the erythropoietin receptor gene and increased sensitivity of erythroid progenitors to erythropoietin. *Blood* 1995;86:15-22.
 17. Kaushansky K. Thrombopoietin: the primary regulator of platelet production. *Blood* 1995;86:419-31.
 18. Ku H, Yonemura Y, Kaushansky K, Ogawa M. Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood* 1996;87:4544-51.
 19. Carver-Moore K, Broxmeyer HE, Luoh SM, et al. Low levels of erythroid and myeloid progenitors in thrombopoietin- and c-mpl-deficient mice. *Blood* 1996;88:803-8.
 20. Yan XQ, Lacey D, Fletcher F, et al. Chronic exposure to retroviral vector encoded MGDF (mpl-ligand) induces lineage-specific growth and differentiation of megakaryocytes in mice. *Blood* 1995;86:4025-33.
 21. Cocault L, Bouscary D, Le Bousse Kerdiles C, et al. Ectopic expression of murine TPO receptor (c-mpl) in mice is pathogenic and induces erythroid proliferation. *Blood* 1996;88:1656-65.
 22. Wendling F, Varlet P, Charon M, Tambourin P. MPLV: a retrovirus complex inducing an acute myeloproliferative leukemic disorder in adult mice. *Virology* 1986;149:242-6.
 23. Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F. A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell* 1990;63:1137-47.
 24. Debili N, Wendling F, Cosman D, et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood* 1995;85:391-401.
 25. Berger S, Aledort LM, Gilbert HS, Hanson JP, Wasserman LR. Abnormalities of platelet function in patients with polycythemia vera. *Cancer Res* 1973;33:2683-7.
 26. Raman BK, Van Slyck EJ, Riddle J, Sawdyk MA, Abraham JP, Saeed SM. Platelet function and structure in myeloproliferative disease, myelodysplastic syndrome, and secondary thrombocytosis. *Am J Clin Pathol* 1989;91:647-55.
 27. Schafer AI. Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. *N Engl J Med* 1982;306:381-6.
 28. Berk PD, Goldberg JD, Donovan PB, Fruchtman SM, Berlin NI, Wasserman LR. Therapeutic recommendations in polycythemia vera based on Polycythemia Vera Study Group protocols. *Semin Hematol* 1986;23:132-43.
 29. Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, eds. *Hematology: basic principles and practice*. 2nd ed. New York: Churchill Livingstone, 1995.
 30. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76-85.
 31. Marsh JCW, Gibson FM, Prue RL, et al. Serum thrombopoietin levels in patients with aplastic anaemia. *Br J Haematol* 1996;95:605-10.
 32. Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* 1995;268:251-5.
 33. Vignon I, Mornon JP, Cocault L, et al. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc Natl Acad Sci U S A* 1992;89:5640-4.
 34. Tortolani PJ, Johnston JA, Bacon CM, et al. Thrombopoietin induces tyrosine phosphorylation and activation of the Janus kinase, JAK2. *Blood* 1995;85:3444-51.
 35. Drachman JG, Griffin JD, Kaushansky K. The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation of Jak2, Shc, and c-Mpl. *J Biol Chem* 1995;270:4979-82.
 36. Ezumi Y, Takayama H, Okuma M. Thrombopoietin, c-Mpl ligand, induces tyrosine phosphorylation of Tyk2, JAK2, and STAT3, and enhances agonists-induced aggregation in platelets in vitro. *FEBS Lett* 1995;374:48-52.
 37. Miyakawa Y, Oda A, Druker BJ, et al. Recombinant thrombopoietin induces rapid protein tyrosine phosphorylation of Janus kinase 2 and Shc in human blood platelets. *Blood* 1995;86:23-7.
 38. Miyakawa Y, Oda A, Druker BJ, et al. Thrombopoietin induces tyrosine phosphorylation of Stat3 and Stat5 in human blood platelets. *Blood* 1996;87:439-46.
 39. Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. *Blood* 1995;85:2720-30.
 40. Emmons RVB, Reid DM, Cohen RL, et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. *Blood* 1996;87:4068-71.
 41. Oda A, Miyakawa Y, Druker BJ, et al. Crkl is constitutively tyrosine phosphorylated in platelets from chronic myelogenous leukemia patients and inducibly phosphorylated in normal platelets stimulated by thrombopoietin. *Blood* 1996;88:4304-13.
 42. Cooper B, Ahern D. Characterization of the platelet prostaglandin D2 receptor: loss of prostaglandin D2 receptors in platelets of patients with myeloproliferative disorders. *J Clin Invest* 1979;64:586-90.
 43. Kaywin P, McDonough M, Insel PA, Shattil SJ. Platelet function in essential thrombocythemia: decreased epinephrine responsiveness associated with a deficiency of platelet α -adrenergic receptors. *N Engl J Med* 1978;299:505-9.
 44. Damen JE, Krystal G. Early events in erythropoietin-induced signaling. *Exp Hematol* 1996;24:1455-9.