

# Identification of Masked Polycythemia Vera From Patients With Idiopathic Marked Thrombocytosis by Endogenous Erythroid Colony Assay

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We used the methylcellulose-culture technique to determine the utility of the erythroid progenitor growth in vitro from nonadherent T-depleted bone marrow and peripheral blood cells in distinguishing polycythemia vera (PV) from essential thrombocythemia. Thirty patients with PV (group A) and 30 patients who presented with idiopathic marked thrombocytosis with platelet count greater than  $1,000 \times 10^9/L$  and a normal or reduced hemoglobin (Hb) level (group B) were studied at initial presentation. Endogenous (erythropoietin-independent) erythroid colonies (EEC) were found in all patients in group A and 13 in group B. The numbers of EEC were comparable between patients in group A and the 13 patients with EEC in group B, 11 of whom with initial Hb levels ranging between 6.4 g/dL and 12.6 g/dL were found to have PV 2 to 45 months after initial evalua-

tion. The number of EEC did not correlate with the time to the progression of polycythemia, whereas myelosuppression delayed the subsequent development of PV. Of the two patients with EEC in group B who did not develop PV, both received chemotherapy soon after presentation, which might preclude the evidence of polycythemia evolution. None of the other patients in group B who did not form EEC developed PV with a median follow-up of 24 months. This study indicates that the assessment of EEC in bone marrow or blood is helpful in early identification of PV or prediction of polycythemia evolution in patients with marked thrombocytosis in whom polycythemia has been initially masked or anemia is present.

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**I**N EACH SUBTYPE of the myeloproliferative disorders, a different lineage of hematopoietic cells appears to be preferentially involved. Using the diagnostic criteria of the Polycythemia Vera Study Group (PVSG), the distinction between polycythemia vera (PV) and essential thrombocythemia (ET) is usually readily apparent in most patients.<sup>1,2</sup> However, in some cases, overlap conditions may be encountered and a clear categorization would be difficult. The PVSG found that apart from the measurement of red blood cell (RBC) mass, it is difficult to identify any single clinical, hematologic, or biochemical variable that reliably differentiates these two disorders.<sup>2,3</sup> Masked polycythemia caused by iron deficiency or bleeding may lead to normal or even reduced RBC masses and poses a diagnostic challenge. In addition, antiplatelet therapies with aspirin/dipyridamole do not prevent thrombotic complication in patients with PV and are associated with an increased incidence of serious hemorrhage;<sup>4</sup> whereas patients with ET who had digital and cerebrovascular occlusive syndromes are likely to benefit from aspirin in conjunction with myelosuppressive agent.<sup>5,6</sup> It would be helpful if the distinction between PV and ET could be made at the time of initial presentation.

In vitro erythroid progenitor growth from the bone mar-

row (BM) or peripheral blood (PB) has been extensively studied in patients with PV; and the formation of endogenous erythroid colonies (EEC) without the addition of erythropoietin (EPO) has been a consistent feature in PV.<sup>7-15</sup> Compared with PV, relatively small number of patients with ET had been studied and the results were conflicting.<sup>15-22</sup> Moreover, T cells were not removed from the target cells in virtually all of the previous studies, the majority of which were performed without depleting the adherent cells.<sup>16,17,19,20,22</sup> Thus, the possible generation of burst-promoting activity by the presence of these accessory cells needed to be taken into consideration regarding the assessment of EEC growth.<sup>23-26</sup>

In this large study, in vitro erythroid progenitor growth, after depleting both the adherent cells and T-cells from the target cells, was compared between PV and ET. Particular attention was paid to the utility of EEC assay in distinguishing between these two disorders. Our study indicated that the assessment of EEC in BM and/or PB could help to make an early diagnosis of PV and predict the subsequent development of PV from patients with idiopathic marked thrombocytosis in whom polycythemia has been masked or anemia is present at their first presentation.

## MATERIALS AND METHODS

**Patient population.** All patients were newly diagnosed and previously untreated, including 30 patients with PV (group A) and 30 patients with idiopathic marked thrombocytosis (group B). All patients in group A fulfilled the diagnostic criteria adopted by the PVSG at initial presentation.<sup>1</sup> All patients in group B had a sustained platelet count of greater than  $1,000 \times 10^9/L$  with no known underlying cause of reactive thrombocytosis, no significant fibrosis or leukoerythroblastic reaction, no Philadelphia chromosome, a normal or reduced hemoglobin (Hb) levels, and normal to elevated leukocyte alkaline phosphatase scores at the time of initial evaluation. Thirty normal BM samples and 20 normal PB from healthy volunteers served as normal controls. In addition, 50 patients with anemia of various causes were also studied for comparison, including 12 patients with iron deficiency anemia, 3 with thalassemia, 4 with autoimmune hemolytic anemia, 5 with paroxysmal nocturnal hemoglobinuria, 6 with anemia and thrombocytosis of chronic inflammation, and 20 with malignancies. Informed consents were ob-

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**Table 1. Results of BFU-E Growth With or Without EPO in Patients With Polycythemia Vera (Group A) and Idiopathic Thrombocytosis (Group B)**

Group	Sample	No.	No. EEC(+)	No. of BFU-E* Median (range)		% of EEC to EPO-Stimulated BFU-E Colonies Range (median)
				EPO(-)	EPO(+)	
Group A	BM	30	30	21 (2-235)	465 (79-1,669)	0.3-43.2 (4.7)
	PB	30	30	16 (2-147)	189 (62-1,017)	0.9-44.6 (8.2)
Group B with EEC	BM	13	13	25 (2-180)	423 (21-1,100)	0.5-62.1 (9.8)
	PB	13	13	21 (2-110)	177 (24-741)	2.2-41.7 (14.8)
Group B without EEC	BM	17	0	0	280 (38-1,500)	—
	PB	17	0	0	97 (5-570)	—
Controls	BM	30	0	0	176 (28-705)	—
	PB	20	0	0	70 (20-227)	—

\* Per  $5 \times 10^4$  nonadherent T-depleted cells.

tained from each normal subject and patient; investigations were approved by the Human Research Committee at Chang Gung Memorial Hospital.

**Erythroid progenitor cell assays.** Mononuclear cell fractions of heparinized BM or PB were isolated by Ficoll-Paque density gradient centrifugation (Pharmacia, Piscataway, NJ) and the nonadherent (NA) fraction was separated by adherence technique.<sup>27</sup> The NA cells were depleted of E-rosette-forming cells by second Ficoll-Paque centrifugation of a mixture of NA cells and sheep RBCs. T-depleted cells were obtained at the interface and cultured in dishes using Iscove's technique with modification.<sup>28,29</sup> Briefly,  $5 \times 10^4$  NA-T-depleted (NANT) cells were plated in 0.9% methylcellulose, supplemented with Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY),  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol, 1% bovine serum albumin, and 30% fetal calf serum, with or without addition of 1 U EPO (Connaught Laboratory, Canada). Dishes were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Erythroid colonies were scored by their red color on the inverted microscope on day 14 for burst forming unit-erythroid (BFU-E). Well-hemoglobinized colony, visible at 14 days of incubation in the absence of added EPO, was classified as EEC.

**Determination of serum EPO concentration.** Serum EPO levels were measured by radioimmunoassay using EPO-Trac <sup>125</sup>I RIA Kit (Incstar Corp, Stillwater, MN). Duplicate tests were performed for each serum sample.

**Statistical analysis.** The statistical differences between groups were determined by the Mann-Whitney U test.

**RESULTS**

Thirty normal BM samples gave rise to a median of 176 BFU-E colonies per  $5 \times 10^4$  NANT cells (range, 28 to 705). The normal PB cells grew BFU-E colonies ranging from 20 to 227 per  $5 \times 10^4$  NANT cells (median 70) in the presence of EPO. The results of BFU-E growth with or without EPO in patients of both group A and group B are shown in Table 1. There was a considerable variation in the number of EPO-stimulated BFU-E colonies in both group A and group B as well as in normal controls. No endogenous BFU-E colonies were found in any of BM and/or PB cultures of the normal subjects and patients with anemia of various causes. EEC were found in all patients in group A, and 13 in group B. BM and PB cultures were performed simultaneously in all patients, and the results of the EEC growth were parallel in both cultures. The number of BFU-E was increased in the

presence of EPO in cultures of all patients who had EEC. Of the cases that grew EEC, the number of EEC and the ratio of EEC to EPO-stimulated BFU-E in each group also varied greatly. The number of EPO-stimulated BFU-E colonies for group A was significantly higher than those of normal controls ( $P = .0001$  for BM, and  $P = .0001$  for PB). There were no statistical differences in the number of EPO-stimulated BFU-E between group A and patients who formed EEC in group B ( $P = .45$  for BM, and  $P = .43$  for PB). No significant differences were seen in the numbers of BFU-E between group B without EEC and normal subjects ( $P = .25$  for BM, and  $P = .38$  for PB). Of the 13 patients in group B who had EEC, the numbers of EEC in BM and PB were comparable with those for group A ( $P = .98$  for BM, and  $P = .65$  for PB).

The serum EPO levels of the 30 patients in group B ranged from 0.66 to 39.7 mU/mL, with a mean of  $3.59 \pm 4.7$  mU/mL (range, 0.66 to 17.8 mU/mL) for patients with EEC and a mean of  $8.84 \pm 9.16$  mU/mL (range, 1.02 to 39.7 mU/mL) for patients without EEC (normal range, 4.17 to 19.0 mU/mL; mean  $\pm$  SD,  $11.6 \pm 3.83$  mU/mL). The hematologic values at the time of initial investigation in the 13 patients with positive EEC in group B are shown in Table 2; polycythemia was not apparent in all. In 11 of them, PV developed in their subsequent courses as confirmed by RBC mass measurement 2 to 45 months after the initial assessment. The initial Hb levels of these 11 patients ranged from 6.4 g/dL to 12.6 g/dL; 4 patients (cases 1, 2, 3, 11) had acute gastrointestinal bleeding at presentation and another 5 (cases 4, 6, 8, 9, 10) had iron deficiency without active bleeding. Of the 11 patients, 7 received myelosuppressive therapy (hydroxyurea and/or busulfan) before PV evolution; the duration of time from presentation to the diagnosis of PV ranged from 7 to 45 months. In the remaining four patients who did not receive myelosuppressive therapy, PV became evident within 6 months after iron supplementation. As of this writing, cases 12 and 13 had not developed PV 6 months and 8 months, respectively, after the initial evaluation. Both patients required myelosuppressive therapy soon after their presentation because of the presence of serious thrombotic and hemorrhagic complications related to thrombocytosis. Case 13 also had prominent splenomegaly and a slight increase in reticulín content, but without collagen fibrosis in

**Table 2. Hematologic Values at Initial Study and Time to Polycythemia Evolution in Idiopathic Marked Thrombocytotic Patients With Positive EEC**

Patient	Age/Sex	Hb/Ht (g/dL/%)	WBCs ( $\times 10^9/L$ )	Platelet ( $\times 10^9/L$ )	Splénomegaly	Polycythemia Evolution (mos)	Therapy Before Evolution	
							Myelosuppression	Iron
1	75/F	9.0/28.4	45.7	2,320	—	+ (2.0)	—	—
2	67/M	9.4/35.9	64.4	1,870	5 cm*	+ (2.0)	—	+
3	69/M	6.4/20.2	38.6	1,162	—	+ (2.5)	—	+
4	71/M	9.6/29.4	62.7	1,350	—	+ (6.0)	—	+
5	56/F	11.7/38.8	7.3	1,120	Splenectomized	+ (7.0)	+	—
6	69/F	11.5/34.8	28.1	1,008	7 cm	+ (14.0)	+	+
7	62/M	12.6/37.9	17.8	1,402	—	+ (25.0)	+	—
8	46/F	10.5/30.7	16.2	1,088	3.5 cm	+ (29.0)	+	+
9	61/F	9.6/31.9	38.8	1,940	3.5 cm	+ (30.0)	+	+
10	56/M	10.6/30.5	24.4	1,460	3.5 cm	+ (45.0)	+	—
11	68/F	7.3/23.1	23.6	1,842	3.5 cm	+ (9.0)	+	+
12	68/F	10.4/32.8	13.5	1,173	—	—	+	+
13	55/F	9.3/29.2	13.1	1,391	7 cm	—	+	+

Abbreviation: WBCs, white blood cells.

\* Below left costal margin.

her BM biopsy section. On the contrary, none of the patients who did not form EEC in group B, developed PV with a median follow-up of 24 months.

The time to the progression of polycythemia did not correlate with the number of EEC observed on initial examination. Patients receiving chemotherapy required a longer evolution time as compared with those without myelosuppressive therapy. Six patients had EEC assay on PB again after PV evolution (Table 3). The number of EEC after clinical diagnosis of PV was decreased in all five patients who had received chemotherapy before evolution.

#### DISCUSSION

EEC is a biologic marker for PV;<sup>7-15</sup> because EEC may also be present in diseases other than PV such as familial and congenital polycythemia,<sup>30</sup> this biologic marker is being substituted for a clonal marker. However, EEC assay is still a useful tool for differentiating PV from secondary erythrocytosis.<sup>9-11,15</sup> Our results confirmed the earlier findings that EEC were inevitably present in either BM or PB from patients with PV. We also found that the in vitro growth of erythroid progenitors from PB, a readily available source, can be as valuable as from BM. This finding was in agreement

with those of other investigators,<sup>12,14</sup> but was contradictory to the observation of Weinberg et al,<sup>13</sup> who found that the endogenous BFU-E colonies were not uniformly present in PB cultures of patients with PV.

Our culture results showed two distinct BFU-E growth patterns for the patients with idiopathic marked thrombocytosis but without polycythemia at diagnosis; one was similar to the pattern of PV and the other was comparable with that of normal subjects. Thirteen patients in group B had positive EEC, and their serum EPO levels were normal or low; therefore, endogenous stimulation of erythroid progenitors by high EPO levels in these patients was unlikely. Of these 13 patients, 11 developed polycythemia later. In contrast, none of the patients without EEC had a polycythemia evolution. Most of the 13 patients had anemia caused by iron deficiency or acute bleeding at presentation. Eleven of 13 patients had bleeding and/or thrombotic complications and required long-term chemotherapy in their subsequent courses compared with 6 of 17 patients without EEC requiring chemotherapy. The disease course of the 13 patients was very similar to that of patients in group A.

If we excluded the 11 patients in group B who actually had masked PV, then only 2 of 30 patients with idiopathic thrombocytosis did produce EEC. Of the two cases with EEC but without the development of polycythemia, one (case 12) had gangrenous change of right big toe and active bleeding from a big gastric ulcer at her initial presentation recently, which necessitated immediate myelosuppression. The other (case 13) had prominent splénomegaly with possible hypersplenism, which in conjunction with the myelosuppressive therapy, might contribute to the failure of clinical evidence of excessive erythroid proliferation.

The present study showed that ET differed from PV in the response of erythroid progenitors to EPO. EEC were consistently observed in patients with PV, whereas they were not present in ET, with the exception of rare cases in whom

**Table 3. Number of EEC at Initial Evaluation and After Polycythemia Evolution**

Patient	No. of EEC in PB ( $5 \times 10^4$ NANT cells)		Chemotherapy Before Evolution
	At Presentation	After PV Evolution	
3	2	3	—
4	40	28	+
8	8	3	+
9	98	6	+
10	10	5	+
11	48	10	+

masked PV could not be completely excluded. In most prior studies that showed EEC in patients with ET, the diagnosis of ET could not be confirmed because of the inadequate exclusion of PV. Partanen et al<sup>17</sup> found that EEC were present in BM and/or PB in all 4 ET patients studied; notably, 3 of them clearly had had a history of bleeding before their presentation. In addition, almost all patients with EEC in the earlier studies had been treated with chemotherapy<sup>17-20,22</sup>; after the initiation of myelosuppressive therapy, a clear distinction between PV and ET would not be made. Thus, the diagnosis of PV in these patients could not be adequately excluded. Moreover, Shabbad et al<sup>26</sup> observed that adherent cells might induce EEC growth even in normal controls; the failure to deplete the adherent cells and/or T-lymphocytes from the target cells in the previous studies could contribute, at least in part, to the presence of EEC in ET. There were only two series that showed the absence of EEC in ET.<sup>16,21</sup> Mazur et al<sup>21</sup> found no EEC in PB of four untreated patients and one treated patient with ET. Croizat et al,<sup>16</sup> using a plasma clot method, failed to detect circulating EEC in six ET patients who had been treated with busulfan for 1 year or more; it was noteworthy that two of their patients did not even have EPO-stimulated colonies after chemotherapy.

The EEC number at initial evaluation did not correlate with the time to progression to PV. Iron therapy alone was associated with a short time to evolution, whereas initiation of myelosuppressive therapy for thrombocytosis delayed or even precluded later progression to PV. Of the patients examined after PV evolution, the EEC number in PB was lower than that of initial assessment in all patients after myelosuppression.

In conclusion, the results presented here show that the presence of EEC is a constant marker for patients with PV; and EEC are only observed in rare ET patients, in whom, however, PV could not be adequately excluded because of the presence of mild myelofibrosis with possible hypersplenism and the myelosuppressive effect of chemotherapy. This study indicates that in the absence of all standard criteria of PV or even in the presence of anemia, the assessment of EEC may serve as a useful tool to discriminate masked PV from ET as well as for predicting subsequent course.

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