Erythroid colony growth from peripheral blood and bone marrow in polycythaemia

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Abstract

Erythroid colony growth in the presence and absence of erythropoietin was compared in 23 patients with primary proliferative polycythaemia (PPP), nine with idiopathic erythrocytosis, 10 with secondary polycythaemia, 15 with pseudopolycythaemia and in 76 normal subjects. Erythroid colonies growing without erythropoietin stimulation (endogenous erythroid colonies) from peripheral blood (BFU-E) were found in 20 of 22 patients with PPP and in two of seven with idiopathic erythrocytosis. None was found in secondary polycythaemia, pseudopolycythaemia, or in normal subjects. Small numbers of endogenous colony forming units-erythroid (CFU-E) (though not BFU-E) were cultured from the bone marrow of three of 24 normal subjects, suggesting that peripheral blood cultures provide a more specific indicator of clonal erythropoiesis.

Peripheral blood endogenous erythroid colony growth is an effective and convenient means of distinguishing patients with clonal erythrocytosis and may be of particular value when iron deficiency obscures the diagnosis of PPP on conventional criteria.

True erythrocytosis may be the result of increased concentrations of erythropoietin, caused—for example, by hypoxia or a renal carcinoma (secondary polycythaemia)—or it may be due to the clonal expansion of an abnormal haemopoietic stem cell which can also result in an increase in the numbers of leucocytes and platelets (primary proliferative polycythaemia) (PPP). Patients who do not meet the standard criteria for PPP¹ and who do not have a demonstrable cause for their erythrocytosis are grouped together as having

idiopathic erythrocytosis. This is a heterogeneous group, some of whom may be in the early stages of PPP, and who go on to develop all the features of this disease. In 1974 Prchal *et al* reported that cultured marrow erythroid progenitors from patients with PPP formed colonies in vitro without the addition of erythropoietin—endogenous erythroid colonies (EEC).² EEC were shown to be produced by the abnormal clone³ and have been found in other myeloproliferative disorders.^{4,5} As well as being used to distinguish PPP from secondary polycythaemia, particularly in cases where the standard criteria are not satisfied,⁶ EEC have recently been used to identify a subgroup of patients with idiopathic erythrocytosis who have clonal disease and who may therefore be at greater risk of developing PPP.^{7,8}

We compared peripheral blood with bone marrow cultures to detect EEC growth, because peripheral blood is more easily obtained. These studies were carried out in patients with true erythrocytosis (PPP, idiopathic erythrocytosis, secondary polycythaemia), those whose increased haematocrit was due to reduced plasma volume (pseudopolycythaemia),⁹ and in normal subjects to identify the different patterns of growth in clonal and non-clonal erythropoiesis. We also report the finding of EEC in four patients with iron deficiency who did not meet the criteria for PPP.

Methods

The clinical details of the patients and normal subjects included in this study are summarised in table 1. Most patients were studied at presentation and none had received any treatment other than venesection before testing.

PRIMARY PROLIFERATIVE POLYCYTHAEMIA

The criteria for this diagnosis were based on those suggested by the Polycythaemia Study

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Table 1 Summary of clinical details of patients and normal controls

	Number of subjects	Median (range)					
		Age (years)	Haemoglobin (g/dl)	% RCV	Platelets	White cells ($\times 10^9/l$)	% PV ($\times 10^9/l$)
Primary proliferative polycythaemia	23	66.5 (29-87)	17.0 (13.8-23.2)	159.5 (129.4-198)*	90.7 (79-111)	12.3 (8.2-29.8)	638 (185-1024)
Idiopathic erythrocytosis	9	66.0 (32-75)	18.3 (16.1-20.6)	127.0 (125.0-161)	97.0 (69-123)	8.9 (6.5-11.7)	233 (182-294)
Secondary polycythaemia	10	58.0 (21-74)	17.9 (14.1-20.9)	143.0 (127.0-190)	100.1 (74-120)	7.1 (5.3-9.8)	264 (175-333)
Pseudo-polycythaemia	15	69.0 (34-81)	17.1 (15.7-18.4)	94.5 (76.5-119)	79.0 (66-87)	8.1 (4.0-11.3)	251 (104-560)
Normal controls	76	70.0 (14-91)	13.7 (11.9-16.3)	ND	ND	6.8 (3.8-11.8)	264 (150-494)

*Iron deficient patients excluded.
RCV, red cell volume; PV, plasma volume; ND, not done.

Table 2 Clinical details and peripheral blood endogenous erythroid colony growth from four patients with iron deficient PPP

Case No	Haemoglobin (g/dl)	Haematocrit	Red blood cells ($\times 10^9/l$)	MCV (fl)	White cells ($\times 10^9/l$)	Platelets ($\times 10^9/l$)	RCV (%)	PBEEC (BFU-E/ml)
1	15.1	0.469	7.04	67	16.2	1024	103.0	242
2	16.8	0.485	6.21	78	8.2	811	113.0	45
3	14.4	0.457	6.69	68	10.4	595	91.5	11
4	13.8	0.471	6.83	69	11.7	494	89.7	76

MCV, Mean corpuscular volume; PBEEC, Peripheral blood endogenous erythroid colonies; BFU-E, Burst-forming units-erythroid; RCV, Red cell volume.

Group¹ and the Royal College of Physicians Research Unit⁹: a red cell volume of more than 125% of the predicted normal value for the patient's height and weight (more than 130% for women),¹⁰ with splenomegaly, or leucocytosis of more than $12 \times 10^9/l$ and thrombocytosis of more than $400 \times 10^9/l$. Nineteen patients met these criteria. Four patients with a red cell volume less than 125% associated with iron deficiency, but who otherwise met the criteria for PPP cited above, were also investigated (table 2). Bone marrow aspirates were available in 13 of 23 cases.

IDIOPATHIC ERYTHROCYTOSIS

Diagnosis of idiopathic erythrocytosis required a red cell volume of more than 125% (more than 130% for women), without splenomegaly, leucocytosis, or thrombocytosis, or a recognised cause of secondary polycythaemia. Nine patients met these criteria: bone marrow aspirates were available in five cases and peripheral blood samples in seven.

SECONDARY POLYCYTHAEMIA

This was diagnosed on the basis of a red cell volume of more than 125% (more than 130% for women) where there was a recognised cause, such as cardiac disease (one patient), chronic respiratory disease (six patients), associated with reduced arterial oxygen concentration, or renal disease (two patients), or a non-renal tumour (one patient), associated with increased erythropoietin production. Peripheral blood samples only were obtained from these 10 patients.

PSEUDOPOLYCYTHAEMIA

The criteria for the diagnosis of pseudopolycythaemia, based on the recommendations of the Royal College of Physicians Research Unit,⁹ were a reduced plasma volume of less than 87.5% of the predicted value for the patient's height and weight¹⁰ with a normal red cell volume and no other haematological abnormality. Peripheral blood samples were obtained from 15 patients.

NORMAL SUBJECTS

Peripheral blood samples were obtained from 57 haematologically normal volunteers who were either patients attending an eye clinic or donors for allogeneic bone marrow transplants. Twenty bone marrow aspirates were obtained from the bone marrow transplant donors and four bone marrow samples were from patients undergoing hip replacement

surgery who were haematologically normal.

All samples were obtained in accordance with the guidelines of the South Glamorgan Health Authority Joint Ethics Committee.

Bone marrow samples were collected into Eagle's Minimal Essential Medium (EMEM, Wellcome Laboratories) containing 25 U/ml preservative free heparin, and peripheral blood into preservative free heparin alone.

Erythroid colony-forming cells were assayed using a modification of the technique of Iscove *et al.*¹¹ Mononuclear cells were prepared from bone marrow or peripheral blood by centrifugation over Ficoll Hypaque (density 1.077 g/cm^{-3} ; Pharmacia). Mononuclear cells were washed in EMEM and cultured at $2.0 \times 10^5/\text{ml}$ (bone marrow) or $5.0 \times 10^5/\text{ml}$ (peripheral blood) in Alpha medium with nucleosides (Gibco) containing 1.0% methylcellulose (A4 premium; Dow), 2.1 g/l sodium bicarbonate, 10^{-4} M β -mercaptoethanol, 10^{-7} M sodium selenite, 10 mg/ml vitamin E (Sigma), 10 μM saturated human transferrin (Sigma), antibiotic-antimycotic solution ($\times 100$; Gibco) and 33% pooled heat inactivated human AB serum. Optimal concentrations of 5637 human bladder carcinoma conditioned medium were added to provide burst promoting activity and human urinary erythropoietin (Terry Fox Laboratories, Vancouver) in addition, where indicated. Triplicate cultures were incubated at 37°C in 5% carbon dioxide in air in a fully humidified incubator.

Colony forming units-erythroid (CFU-E), consisting of more than eight haemoglobinised cells, were counted at seven days and burst forming units-erythroid (BFU-E) consisting of more than 50 haemoglobinised cells, or three or more subunits, were counted at 14 days using an inverted microscope. Peripheral blood erythroid colony results were expressed per millilitre of blood as the original volume of each sample was known. These values are independent of the blood count and reflect total circulating progenitors. It was not possible to calculate absolute values for bone marrow samples, which are therefore expressed per 10^5 mononuclear cells.

The χ^2 test was used to compare the numbers of patients and normal subjects who grew EEC. The 95% confidence intervals for CFU-E and BFU-E from normal subjects at 1.0 U/ml erythropoietin were calculated on logarithmically transformed data. Erythroid colony growth at 1.0 U/ml erythropoietin from patients and normal subjects was compared using the non-parametric Mann-Whitney U test.

Table 3 The frequency of EEC growth in patients with polycythaemia and normal subjects

	Number with EEC (%)		
	Peripheral blood		Bone marrow
	BFU-E	CFU-E	BFU-E
PPP	20/22 (91)***	13/13 (100)***	8/13 (61.5)***
Idiopathic erythrocytosis	2/7 (29)	4/5 (80)**	0/5 (0)
Secondary polycythaemia	0/10 (0)	ND	ND
Pseudo-polycythaemia	0/15 (0)	ND	ND
Normal controls	0/57 (0)	3/24 (12.5)	0/24 (0)

Patients and normal controls compared by χ^2 .
p < 0.01, *p < 0.001, ND not done.

Figure 1 Peripheral blood BFU-E with and without 1.0 U/ml added erythropoietin (EPO). The boxed areas indicate BFU-E numbers from 57 normal subjects with no added EPO and at 1.0 U/ml (95% confidence limits). The dotted line indicates the median value for normal subjects at 1.0 U/ml EPO. Arrows indicate median values for each patient group.

Figure 2 Bone marrow erythroid colonies with and without 1.0 U/ml added erythropoietin: (A) CFU-E; (B) BFU-E. The boxed area indicates the 95% confidence limits for normal subjects at 1.0 U/ml erythropoietin. Arrows indicate median values.

Results
ENDOGENOUS ERYTHROID COLONIES IN PERIPHERAL BLOOD
The numbers of patients and normal subjects who grew EEC from bone marrow or peripheral blood are shown in table 3. Endogenous BFU-E were observed in peripheral blood cultures from 20 of 22 patients with PPP, including the four with a red cell volume of less than 125% due to iron deficiency whose results are shown in table 2. Only two of seven patients with idiopathic erythrocytosis had endogenous peripheral blood BFU-E, a significant difference compared with the PPP group ($p < 0.001$). No endogenous peripheral blood BFU-E were found in patients with secondary polycythaemia, pseudopolycythaemia, or in 57 haematologically normal controls ($p < 0.0005$ compared with PPP).

ENDOGENOUS ERYTHROID COLONIES IN BONE MARROW
A small number of normal subjects (three of 24) grew low numbers of CFU-E, but not BFU-E, from their bone marrow without added erythropoietin. Endogenous CFU-E were observed from all 13 patients with PPP where bone marrow was available, including the two patients who did not grow EEC from their peripheral blood, and this is significantly different from normal ($p < 0.001$). Growth of endogenous BFU-E from bone marrow was less common in PPP (eight of 13 patients) than either endogenous CFU-E or peripheral blood BFU-E, but still significantly different from normal ($p < 0.001$).
A significantly greater proportion of patients with idiopathic erythrocytosis (four of five) grew endogenous CFU-E than normal subjects

($p < 0.01$), but no endogenous bone marrow BFU-E were seen in this group.

ERYTHROID COLONY GROWTH AT 1.0 U/ML ERYTHROPOIETIN FROM PERIPHERAL BLOOD

Peripheral blood erythroid colony numbers in the presence and absence of added erythropoietin are shown in fig 1. In PPP, the addition of 1.0 U/ml erythropoietin to peripheral blood cultures resulted in increased numbers of BFU-E in all except one case, where the colonies were already too numerous for accurate counting (BFU-E without added erythropoietin: median 36, range 0–>10 000/ml; BFU-E with 1.0 U/ml erythropoietin: median 385, range 91–>10 000/ml). This was significantly higher than the numbers of erythroid colonies produced by normal subjects at 1.0 U/ml erythropoietin (111, 21–436 BFU-E/ml), $p < 0.0001$.

Peripheral blood BFU-E at 1.0 U/ml erythropoietin from patients with idiopathic erythrocytosis (median 241, range 146–499 BFU-E/ml) fell within the normal range as did those from patients with secondary polycythaemia (114, 24–365 BFU-E/ml) and pseudopolycythaemia (146, 35–437 BFU-E/ml).

ERYTHROID COLONY GROWTH AT 1.0 U/ML ERYTHROPOIETIN FROM BONE MARROW

The number of CFU-E at 1.0 U/ml erythropoietin was significantly higher in patients with PPP than in normal subjects ($p = 0.023$) (fig 2A), although the range of results was similar (PPP, median 424, range 97–562 CFU-E/ 10^5 mononuclear cells; normal controls, median 163, range 84–557 CFU-E/ 10^5 mononuclear cells). The growth of CFU-E at 1.0 U/ml erythropoietin from patients with idiopathic erythrocytosis was not significantly different from that of normal controls.

In contrast to the peripheral blood results, bone marrow BFU-E at 1.0 U/ml erythropoietin from patients with PPP were not significantly increased compared with those of normal controls (fig 2B), and those from patients with idiopathic erythrocytosis were also within the normal range.

Discussion

Patients with PPP are more likely to bleed or have thrombosis than patients with other forms of erythrocytosis,¹² and it is therefore helpful to be able to identify those at risk at an early stage. Prchal *et al* showed that the marrow EEC found in PPP are products of the abnormal haemopoietic clone,³ and EEC have recently been increasingly used as a means of identifying true myeloproliferative disease in patients with erythrocytosis.^{6–8} We investigated the feasibility of using EEC growth from peripheral blood, as this is more readily available than bone marrow samples, to distinguish clonal erythrocytosis from non-clonal disease on an individual basis in patients with erythrocytosis.

EEC have been reported in some normal peripheral blood samples¹³ using serum free conditions and adherence-depleted cells, and

as we were concerned with the specificity of this test we studied a large number of normal controls covering the same age range as our patients, but using culture conditions likely to reflect those pertaining *in vivo*. We found no evidence of EEC in normal peripheral blood, but a small proportion of normal subjects grew low numbers of endogenous CFU-E from bone marrow. In contrast, all the bone marrow samples from patients with PPP gave rise to endogenous CFU-E. A higher proportion of patients with idiopathic erythrocytosis (four of five) gave rise to endogenous CFU-E than normal, but the numbers were within the normal range and therefore could not be used to identify clonal disease with certainty in these patients. CFU-E are relatively mature erythroid progenitors and known to be the most sensitive to erythropoietin.¹⁴ Therefore, the low numbers of endogenous CFU-E we observed in normal subjects probably either matured in response to the small amounts of erythropoietin present in the culture or had already been stimulated *in vivo*.

Endogenous bone marrow BFU-E were observed in only eight of 13 (61.5%) patients with PPP and no patients with idiopathic erythrocytosis or normal subjects. In contrast, endogenous peripheral blood BFU-E were cultured from 20 of 22 (91%) of patients with PPP and two of seven (29%) of patients with idiopathic erythrocytosis, and as we detected no EEC in blood from patients with secondary polycythaemia or pseudopolycythaemia or from normal subjects, circulating BFU-E seem more useful for detecting myeloproliferative disease in patients than bone marrow EEC under these culture conditions. This may be of particular relevance when the strict criteria for PPP are not met,¹⁵ as for example where this is complicated by iron deficiency and the red cell volume may not exceed 125% of normal, or in idiopathic erythrocytosis, a heterogeneous condition, where the presence of endogenous peripheral blood BFU-E may help identify the subgroup at greatest risk of developing PPP.¹⁶

Why a higher proportion of patients with PPP should grow endogenous BFU-E from peripheral blood is not clear. These results may reflect a different balance of stimulatory and inhibitory accessory cells in the two sources of mononuclear cells,^{17,18} some of which may themselves be products of the abnormal clone. De Wolf *et al* examined the effects of recombinant interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on peripheral blood EEC from patients with PPP and found that whereas IL-3 enhanced EEC growth from mononuclear cells and adherence-depleted mononuclear cells, GM-CSF only had an enhancing effect when the adherent cells were removed.¹⁹ Experiments are under way to explore the role of accessory cells in erythropoietin sensitivity in myeloproliferative disease.

Recombinant DNA techniques are now available to test for clonality in haematological disease,²⁰ but at present they are only applicable to about 40% of female patients. Six patients from this study, four with PPP who all had

endogenous peripheral blood BFU-E, and two with idiopathic erythrocytosis who did not have EEC, were eligible for analysis with X-chromosome DNA probes.²¹ There was agreement between the two techniques in five out of six cases, and failure to find clonality in the DNA of one patient with PPP may have been due to dilution with normal cells.

Until more generally applicable molecular methods of detecting clonality become available the detection of endogenous blood BFU-E seems to be the most effective way of diagnosing clonal erythropoiesis.

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