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IN VITRO SENSITIVITY OF HUMAN ERYTHROID PROGENITORS TO HEMOPOIETIC GROWTH FACTORS: STUDIES ON PRIMARY AND SECONDARY POLYCYTHEMIA

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ABSTRACT

Background. **Primary proliferative polycythemia is a clonal disease characterized by excessive hemopoiesis and associated with a lower than normal erytropoietin plasma level; *in vitro* colony studies may reveal increased sensitivity of the abnormal clone to hemopoietic growth factors.**

Materials and Methods. **We studied the *in vitro* formation of erythroid colonies (BFU-E derived clone) in cultures set up with a serum-free medium and containing Epo, interleukin 3 (IL–3) and stem cell factor (SCF), in various combinations. The clonogenic test was performed by plating non adherent mononuclear cells from the peripheral blood of normal subjects and from patients with PPP and secondary polycythemia (SP).**

Results. **SCF is a major amplifier of erythroid colony growth, in the presence of Epo; in cultures from PPP patients, however, the presence of SCF, in addition to Epo, enhances colony formation at about the same rate as in cultures from normal subjects. When SCF is omitted, the presence of even modest amounts of Epo and IL-3 is sufficient to obtain a statistically significant difference between colony formation from PPP patients on the one side, and SP patients and normal subjects on the other.**

Conclusions. **Our results show that in vitro culture studies may contribute an additional diagnostic criterion for distinguishing between PPP and SP in uncertain cases. It is also possible that hypersensitivity to erythropoietic factors may play a role in the pathogenetic mechanism of primary proliferative polycythemia.**

Key words: primary proliferative polycythemia, stem cell factor, BFU-E derived clone, erythropoietin, interleukin-3

The diagnostic criteria for primary proliferative polycythemia (PPP), or polycythemia vera, have remained for a long time those established by the *ad hoc* Study group,[1] namely a true increase of the red cell mass (RCM), with normal oxygen saturation, plus splenomegaly and/or a combination of two among parameters such as leukocytosis, raised platelet count, raised leukocyte alkaline phosphatase (LAP) and raised serum vitamin B12 or B12-binding capacity. However, in recent years additional criteria have been suggested, like a low erythropoietin (Epo) level and the formation of unstimulated erythroid colonies *in vitro*.[2,3] Concerning the latter point, it is assumed that the finding of so-called *endogenous erythroid colonies* (EEC) is a reliable indication of a pathological clone in PPP, although in other conditions there might be a partial expression of such a phenomenon.[4]

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The value of the EEC finding is enhanced if serum is not used in the culture medium, since the presence of unknown amounts of Epo may undermine the results; it is therefore necessary to design media with a chemically defined composition, the so-called *serum-free media*.[5,6] In this situation it is possible to test the response of hemopoietic stem cells to Epo as well as to other growth factors, and we and other groups found that in PPP there might be a peculiar sensitivity of such progenitors to cytokines like interleukin 3 (IL-3) and interleukin 4 (IL-4).[7-9]

In view of the importance of such findings, not only for diagnostic purposes but also as a contribution to the elucidation of pathogenetic mechanisms, we extended the investigation, looking for combinations of growth factors, which may selectively stimulate colony formation in PPP.

Materials and Methods

Blood samples were obtained from normal adult donors, who served as control group, and from patients with PPP and secondary polycythemia (SP) as listed in Table 1. PPP patients were identified on the basis of the criteria outlined recently by one of us[3] and described in the introduction, with the obvious exception of the *in vitro* culture test. Patients with PPP had been treated mainly with venesection; three of them previously received α-interferon treatment, which did not modify packed cell volume (PCV) values. Patients with SP had severe chronic obstructive disease; 200 mL of peripheral blood (an excess amount, necessary for other investigations) were obtained from both normal subjects and PPP or SP patients, and collected in CDP-adenine. For *in vitro* studies preparations of mononuclear, non-adherent cells were obtained as follows: mononuclear cells were separated by Lymphoprep (d=1.077) gradient centrifugation at 800 g for 20 minutes; the fraction thus obtained was incubated overnight in plastic dishes at 37°C and the adherent cells discarded. This procedure has been shown to result in a post-incubation monocyte count of 1-2%.[6] Further purification was not performed in this investigation, since the contribution of accessory cells like T-lymphocytes was considered important for the formation of colonies in serum-free medium; however, selection of CD34-positive cells by immuno-magnetic methods was carried out for a series of experiments now in progress.

Cell culture

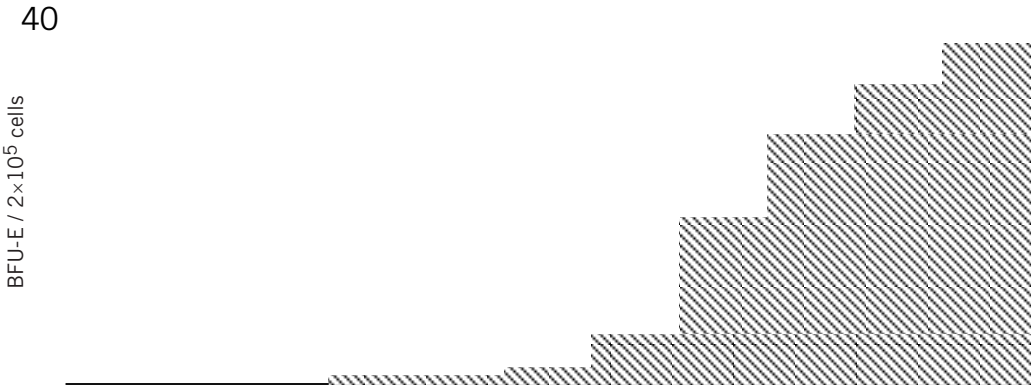
Serum-free cultures were prepared by suspending the non-aherent mononuclear cell fraction in Iscove's modified Dulbecco medium (IMDM); the basic culture medium contained previously deionized 1% bovine serum albumin (Sigma), 8 mcg/mL of L α-dipalmitoil phosphatidylcholine (Sigma), 7.8 mcg/mL cholesterol (Sigma), 270 mcg/mL of purified, fully iron-saturated human apo-transferrin (Sigma), 1.7×10^{-4} M insulin (Sigma), 10 mg/mL sodium piruvate, 2×10^{-4} β-mercaptoethanol (Sigma) and antibiotics (penicillin, streptomycin, gentamicin). Additional components were also added as a coadjuvant mixture, including 5.6 mcg/mL oleic acid, 3×10^{-8} mcg/mL retinyl acetate, and 7×10^{-7} M d-α-tocopherol acetate (all from Sigma). Non adherent cells were plated onto 1 mL Petri dishes at 2×10^{5} cells/dish, growth factors added as indicated in the Figures, and the cultures were incubated at 37°C in 5% CO_2 and air, in a fully humidified incubator for 14 days. Growth factors included: recombinant human stem cell factor (r-hu SCF Genzyme), recombinant human interleukin 3 (r-hu-IL-3, Genzyme) and recombinant erythropoietin (r-hu-Epo, Cilag) at concentrations shown in the Figures. BFU-E's (a term now commonly accepted to indicate BFU-E derived clones) were defined as 2 or more aggregates, each consisting of at least 50 hemoglobinized cells, with a distinctive orange-red coloration. Other aggregates like granulocyte-macrophage colonies were easily recognized and not counted. Colony counts were always performed by the same investigator.

Results

The early part of this investigation, aimed at establishing an optimal synthetic medium for erythroid colony growth, showed a poor colony

Table 1. Summary of clinical data on PPP patients at the time blood was taken for progenitor cell culturing.

Age/sex	Year of diagnosis	Spleen	Hb g/dL	RBC $\times 10^{12}$/L	Hct %	Total Blood volume mL/kg	Arterial O_2 saturation %	WBC $\times 10^9$/L	Platelets $\times 10^9$/L	LAP	MCV fl	Epo mU/mL	Therapy
71/M	1987	normal	16	7	50.1	99	91-96	11.7	130	214	71	<10	phlebotomy
69/M	1992	normal	21.5	7.7	66.6	101	88-96.6	7.6	489	138	86	<10	phlebotomy
47/M	1988	enlarged	15	13.3	44	74	90-97	5.9	867	307	74	10	phlebotomy
43/M	1981	normal	18.3	6.3	53.1	88	97-107	11.4	254	218	83	<10	phlebotomy
79/M	1988	enlarged	20.6	7.1	62.4	86	83-96.1	9.3	283	209	86.8	10.4	phlebotomy
63/F	1981	normal	20.7	8	61.3	70	91.4-96	12.9	428	205	77.6	<10	phlebotomy
61/M	1986	enlarged	22.6	3.1	68.1	85	94-97.3	6.9	140	193	108	<10	phlebotomy
44/M	1987	enlarged	20	6.9	50.3	91	97.7-99	6	220	185	84.5	<10	phlebotomy
80/M	1992	normal	19.1	6.2	59.2	72	94-97.2	8.7	260	180	94.3	10.4	phlebotomy
52/M	1993	normal	19	6.7	57	100	83-96	9.5	187	23	87	<10	phlebotomy
Normal values			M 14-18 F 12-16	M 4.7-6.1 F 4.2-5.4	M42-52 F37-47	60-80	94.9-97.4	4.8-10.8	130-400	60-80	M80-94 F81-99	15-19	



Basic medium	+	+	+	+	+	+	+	+	+	+	+
Coadjuv. mixture	0	0	0	0	+	+	+	+	+	+	+
Lecithin 8 ug/mL	0	0	+	+	+	+	+	+	+	+	+
IL3 ng/mL	0	10 +	0	10 +	10 +	5.5 +	10 +	0	5.5 +	10 +	10 +
SCF ng/mL	0	0	0	0	0	0	0	10 +	25 +	10 +	25 +

Figure 1. Erythroid colony growth from normal subjects in serum-free conditions. Mean values obtained from 5 subjects. Basic medium includes IMDM, insulin (10^{-4}M), transferrin (270 mg/mL), cholesterol (7.8 mg/mL), erythropoietin (1 U/mL). Coadjuvants include oleic acid (5.6 mg/mL), α-tocopherol acetate (7×10^{-7} M) and retinyl acetate (3×10^{-8} M).

yield from normal peripheral blood stem cells when only the basic medium was used (including a relatively low Epo concentration), or even when a coadjuvant mixture, including oleic acid, α-tocopherol and retinol, was added (Figure 1). The addition of IL-3 induced only a modest increase in BFU-E derived clones, while the addition of stem cell factor caused considerable stimulation of colony growth; maximal values were obtained through a combination of IL-3 and SCF at concentrations, respectively, of 10 and 25 ng/mL (and Epo at unchanged dosage). Serum-containing cultures from normal progenitors had a colony yield similar to serum-free cultures containing SCF and IL-3 (data not shown). It should be mentioned that colonies from serum-containing media presented a brighter red-orange color, evidence of more pronounced hemoglobinization. Using serum-free cultures it was not possible to obtain endogenous erythroid colonies from cultures set up with preparations from PPP patients, neither in experiments lacking all growth factors, nor in cultures with growth factors other than Epo added. However, using serum-free media, sensitivity to Epo was tested for normal progenitors and for stem cells from PPP patients (Figure 2). In the presence of stem

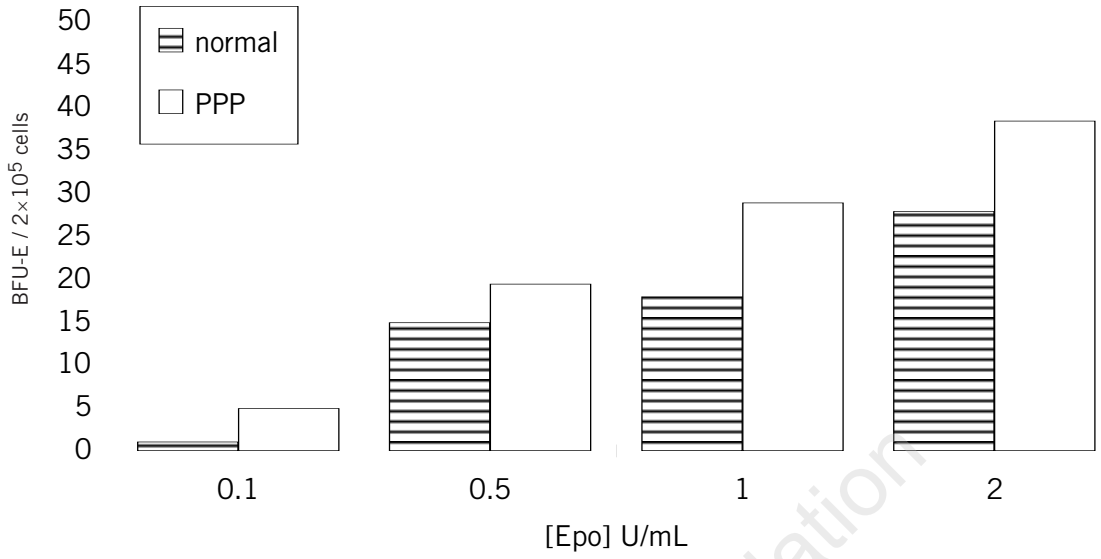


Figure 2. Erythroid colony growth from 3 normal subjects (striped column) and 5 PPP patients (white column). Epo dose-response relationship with constant SCF dosage (10 ng/mL) in serum-free medium. The difference between normal and PPP subjects is not significant at any Epo concentration.

cell factor, PPP peripheral blood stem cells produced a better response than those from normal progenitors: the difference was not significant at any Epo concentration.

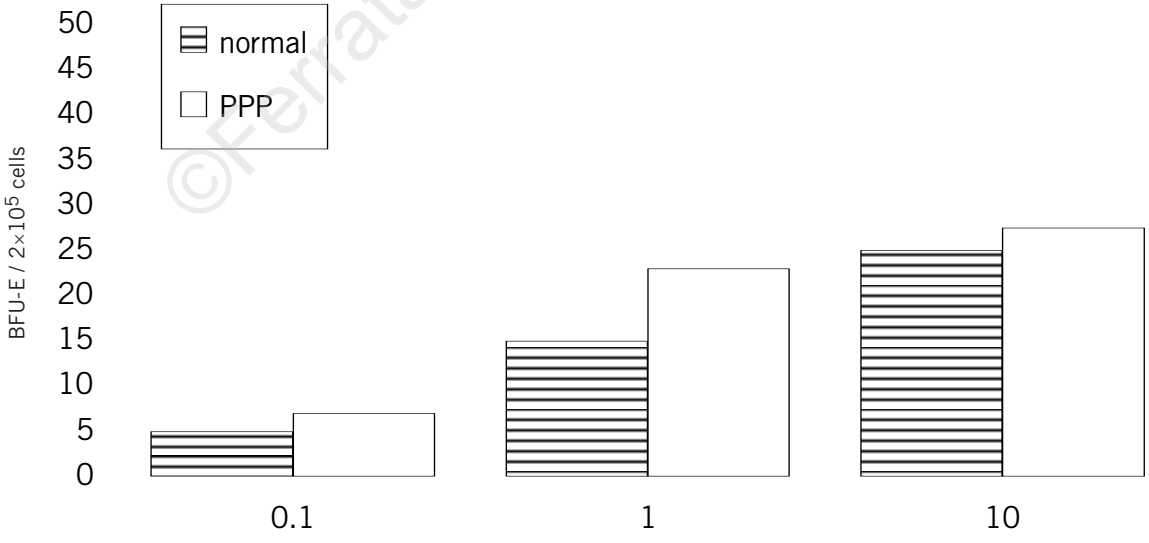


Figure 3. Erythroid colony growth from 3 normal subjects (striped column) and 5 PPP patients (white column). IL-3 dose relationship with constant SCF dosage (10 ng/mL) and Epo 1 U/mL in serum-free medium. The difference between normal and PPP subjects is not significant at any IL-3 concentration.

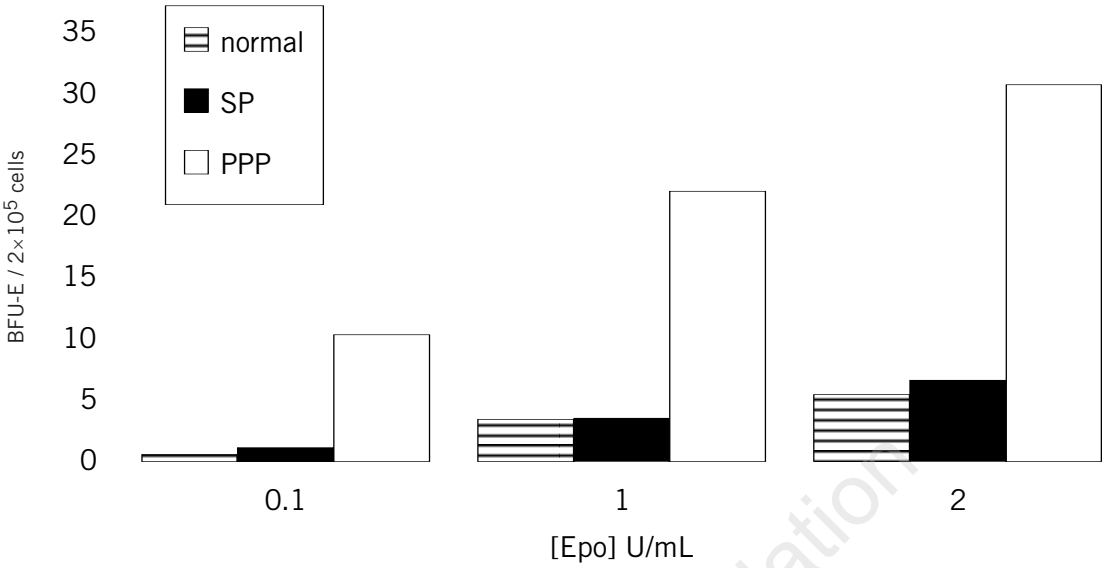


Figure 4. Erythroid colony growth from 3 normal subjects (striped column), 10 SP patients (dark column) and 7 PPP patients (white column). Cultures included IL-3 10 ng/mL and Epo at concentrations shown. Statistical evaluation was carried out using two-way Anova: general linear model with Student-Newman-Keuls test. A significant difference was found between normal and PPP patients, as well as between PPP and SP patients. No difference was found between normal and SP patients. A significant difference was also found in all groups between Epo 0.1 U/mL and 1 U/mL, as well as between 1 U/mL and 2 U/mL ($p<0.05$).

The next step of the investigation was a comparative study of the sensitivity to IL-3 of normal and PPP stem cells in the presence of constant concentrations of both Epo and SCF; it appeared that increasing doses of IL-3 induced a higher yield of BFU-E derived clones, but the difference between normal and PPP samples was not significant (Figure 3). It was therefore decided to exclude from the synthetic medium a component like SCF, which is such a powerful enhancer of stem cell activation that it does not allow clear discrimination, in the presence of Epo, among different experimental conditions. It was thus apparent, after this exclusion and with a constant IL-3 concentration, that a remarkable and statistically significant difference emerged between cultures from normal subjects and PPP patients (Figure 4). It is of particular interest that at a very low Epo concentration (0.1 U/mL) practically no erythroid colonies were obtained from normal subjects, while a fair number of bursts were seen in PPP cultures even at this Epo dosage. Cultures from patients with secondary polycythemia showed smaller colony formation than those from PPP; in the presence of SCF results were almost identical with those from normal subjects (data not shown), while in the absence of SCF but with Epo and IL-3, SP colonies showed intermediate values between normal and PPP cultures (Figure 4). The difference between normal and SP colony formation was not significant, while a statistically significant difference was found between SP and PPP samples, an additional criterion for discrimination.

Discussion

A prerequisite for any investigation on colony formation by hemopoietic stem cells is the establishment of optimal conditions for colony growth, possibly in a serum-free environment. The present research was mainly aimed at studying the effect of growth factors like stem cell factor and interleukin 3, which, along with Epo, have been shown to enhance erythropoietic colony growth. A culture medium was therefore designed which in basic conditions would

not produce a major amount of growth, thus allowing us to visualize the effects of the above mentioned factors. In cultures from normal human subjects SCF in combination with Epo shows remarkable stimulating activity, thus confirming early reports that suggested a complementarity between SCF, which causes an expansion of the progenitor cell pool, and Epo, the effector of red cell differentiation.[10] IL-3 is also a powerful stimulant of erythroid colony formation and it appears that the combined effect of Epo, SCF and IL-3 can produce an optimal amount of erythroid differentiation in culture. It should be pointed out, however, that other factors not tested in the present investigation, like insulin-like growth Factor 1, may induce an enhancement of *in vitro* erythropoiesis.[11]

A comparison between the colony forming activity of normal stem cells and those from primary proliferative polycythemia patients shows a significant increase in PPP cultures with serum-containing media, but not with serum-free media, including SCF. This finding seemed to suggest that SCF may act as a general stimulant on hemopoietic stem cells with little discrimination between normal and pathological clones, although this is still a controversial issue. While some investigators have obtained similar results on Epo-dependent and Epo-independent clones in PPP,[12] another group has very recently reported a significantly better response to SCF by PPP hemopoietic progenitors than by stem cells from normal subjects.[13]

Since in our experimental setting no real distinction could be seen between normal and PPP clones with SCF present in the medium, as shown by results from the early part of this investigation, SCF was excluded from the culture medium: in this situation the addition of modest amounts of both Epo and IL-3 was sufficient to show significant enhancement of PPP erythropoietic colonies with respect to those from normal subjects. It is of interest that in cultures obtained from patients with secondary polycythemia the colony yield was significantly lower than that from PPP patients, thus confirming the peculiar response found in PPP patients. This may become a useful laboratory test for the differential diagnosis between the two conditions.

It is thus established that stem cells from PPP are endowed with an exquisite sensitivity to Epo and IL-3, despite the fact that in cell lines derived from a variety of myeloproliferative disorders Epo did not affect, even at high concentrations, either clonogenic growth or DNA synthesis.[14] The present finding throws more light on the so-called Epo-independent clones of PPP, which are probably as Epo-dependent as the normal ones, but which require much less hormone concentration to activate the differentiative process.[6, 15] Sensitivity to IL-3, confirming previous data,[7,8] also gives an interesting clue to the peculiar biochemical requirement of the pathological clone; in the same context the recent finding of an increased sensitivity of PPP progenitors to other growth factors, like insulin-like growth Factor 1 (IGF-1), independently of Epo, is of interest.[16] Such pathogenetic considerations may have an obvious clinical relevance: it was for instance observed some time ago that endogenous (*Epo-independent*) or Epo-hypersensitive colonies could be found in cultures from patients with pure erythrocytosis, as well as in cases of autosomal dominant polycythemia[17-19] in association with low plasma Epo levels. More recently, a large family with familial erythrocytosis was described with low or low-normal Epo levels and increased *in vitro* sensitivity of hemopoietic progenitors to Epo.[20] It was later found that in this family, which includes an Olympic gold medal winner, there is a definite mutation of the Epo receptor, a change apparently associated with the excessive red cell proliferation.[21]

It appears that more attention is now being given to a constellation of growth factors (as well as their receptors) which seem to play an important role in the pathogenetic process of polycythemia. In particular, it is indeed possible, as suggested by a good portion of recent results, that hyperproliferation of the erythropoietic compartment in PPP is associated with a peculiar sensitivity of the pathological clone to a variety of growth factors, including Epo, SCF and several interleukins. It is also worthwhile to consider the role of receptors and their activity in signal transduction; an important

part of the Epo mechanism of action, namely the prevention of apoptosis, is actually mediated through its receptor.[22]

It is thus apparent that present efforts in the areas of both molecular biology and in vitro stem cell culture will help to elucidate the etiopathogenetic mechanisms of polycythemia, with special emphasis on the role played by growth factors and their receptors.

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