Erythropoietin receptors in polycythemia vera.

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Research Article

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Erythropoietin Receptors in Polycythemia Vera

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Abstract

The role of erythropoietin (EP) in polycythemia vera (PV) is controversial, with some experiments suggesting that erythroid progenitors in PV are exquisitely sensitive to EP and EP dependent, and others suggesting that PV progenitors are EP independent. We have examined the characteristics of the EP receptor (EP-R) on erythroid colony-forming cells (ECFC) from patients with PV. In contrast to normal ECFC, which have two classes of EP-R, with 20% showing high affinity (K_d = 0.13 nM; range, 0.04–0.20 nM) and the remainder lower affinity (K_d = 0.37 nM; range, 0.28–0.57 nM), PV ECFC show a single class of 851 low affinity EP-R with K_d = 0.72 nM (range, 0.36–0.85 nM). ECFC from patients with secondary (EP driven) polycythemia or anemia show two classes of EP-R (K_d = 0.18 and 1.10 nM, respectively). Attempts to remove tightly bound EP from putative high affinity EP-R in PV did not reveal any higher affinity receptors. Determination of molecular size by crosslinking showed two proteins of 90 and 100 kD similar to those seen with normal EP-R.

These studies indicate the PV ECFC have EP-R that are structurally similar to normal EP-R but lack the higher binding affinity for EP.

Introduction

Polycythemia vera (PV)[1] is a clonal myeloproliferative disorder characterized by trilineage hyperplasia, but a principal clinical manifestation of erythrocytosis (1). In contrast to the erythrocytosis that is a response to tissue hypoxia and consequent secretion of erythropoietin (EP), this erythrocytosis occurs in the presence of EP levels that are either lower than normal (2) or normal (3).

Erythroid progenitors in PV, in contrast to those from normal persons, grow in vitro in the absence of exogenous EP (4, 5). However, these studies used serum, which contains a small amount of EP. Zanjani et al. (6) and Golde et al. (7), using different serum-containing systems, showed that anti-EP antibodies markedly reduced the endogenous growth of erythroid progenitors, which was then restored by the addition of very small amounts of EP. Casadevall and colleagues (8) reported that erythroid progenitors from PV patients would not grow in a serum-free system without exogenous EP, and that the EP dose-response curve showed a 10-fold increase in sensitivity to EP relative to normal progenitors. These studies indicated that erythropoiesis in PV is EP dependent and exquisitely sensitive to EP. On the other hand, Eridani and coworkers (9), also using a serum-free system, reported growth of erythroid progenitors in the absence of exogenous EP.

One method of evaluating erythropoiesis in PV is by studying the EP receptor (EP-R). A method has been developed in our laboratory for obtaining highly purified erythroid colony-forming cells (ECFC) from peripheral blood in sufficient numbers for quantitative studies of EP binding (10). Using this technique ECFC from seven normal individuals were studied and two classes of EP-R were found in each case. Normal ECFC possess a total of 1,021 binding sites per cell (range, 566–1,254), 20% of which show a high affinity for EP, with a K_d of 0.13 nM (range, 0.04–0.20 nM) and the rest of which have a K_d of 0.37 nM (range, 0.28–0.57 nM) (11).

We have applied this technique to ECFC obtained from patients with PV and have found that PV EP-R, unlike normal cells, do not express the higher affinity class of EP-R, but show a single class of EP-R with a K_d of 0.72 nM (range, 0.37–0.85 nM).

Methods

Study of the blood obtained by therapeutic phlebotomy from patients meeting established criteria for PV (9) and secondary polycythemia was approved by the Vanderbilt and Nashville Veterans Administration (V.A.) Committees for the Protection of Human Subjects. Blood studied from patients with anemia due to hemorrhage or hemolysis was obtained as part of an ongoing approved study of mechanisms of anemia.

Clinical features of PV patients. Five PV patients were studied. No patient had received any treatment other than phlebotomy and none showed evidence of transformation to myelofibrosis or leukemia. Duration of disease at time of study was 4 and 7 yr for one patient each, and less than 1 yr for the remaining three patients.

Preparation of ECFC. Burst-forming units-erythroid (BFU-E) were purified from peripheral blood by modification of the method of Sawada et al. (10). Heparinized peripheral blood was centrifuged at 400 *g*, 24°C, for 15 min. The buffy coat cells were then suspended in alpha

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1. *Abbreviations used in this paper:* ECFC, erythroid colony-forming cell; EP, erythropoietin; EP-R, EP receptors; FH, Ficoll-Hypaque; IMDM, Iscove's modified Dulbecco's medium; MC, methylcellulose; PV, polycythemia vera; rEP, recombinant human EP.

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MEM (Sigma Chemical Co., St. Louis, MO) and light density cells were separated over Ficoll-Hypaque (FH; 1.077 g/cm^3; Pharmacia Fine Chemicals, Piscataway, NJ; Winthrop-Breon Laboratories, New York, NY) at 400 *g*, 24°C, for 25 min. These cells were then collected in MEM containing 2% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), washed in MEM, and then washed twice in citrate-buffered saline. The cells were then layered over 10% BSA (Armour Pharmaceutical Co., Tarrytown, NY) in citrated saline, centrifuged at 400 *g*, 25°C, for 10 min, and then washed and resuspended in MEM. The cells were then depleted of T lymphocytes by sheep erythrocyte rosetting (10), and were suspended in Iscove's modified Dulbecco's medium (IMDM; Sigma Chemical Co.) for incubation in the presence of 20% FCS and 10% giant cell tumor conditioned medium (Gibco Laboratories, Grand Island, NY) overnight at 37°C in polystyrene flasks (Becton Dickinson, Oxnard, CA) to remove adherent cells.

On the following day the nonadherent cells were collected by gentle washing with 37°C MEM. If the red cell/nucleated cell ratio exceeded 5:1, cells from blood group A or B donors in 10 ml of MEM were incubated for 10 min with 2 ml of the appropriate standard diagnostic blood typing serum (Ortho Diagnostic Systems, Raritan, NJ) which had been previously dialyzed at 3°C in PBS and filter sterilized. The suspension was then centrifuged at 400 *g* for 5 min. The pellet was suspended in MEM and the red cells were separated over FH. The interface was then collected, washed in MEM, and resuspended in IMDM. Red cells from group 0 donors were lysed in 5 ml sterile ice-cold water for 30 s, the cells were separated from debris by centrifugation through 10% BSA at 400 *g*, 3°C, for 5 min, and washed with MEM. The day 1 cells were then suspended at a concentration of 70 $\times$ 10^6/ml with 25 μl CD11b/OKM*1 (20 μg/ml), 25 μl CD2/OKT*11 (10 μg/ml; Ortho Diagnostic Systems), 50 μl CD45R/My11, and 50 μl CD16/My23 at 3°C for 60 min as previously described (10). The cells were then washed three times and incubated in 100-mm plastic tissue culture dishes that had previously been coated with affinity-purified goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 90 min at 3°C to remove granulocytes, monocytes, granulocyte-macrophage progenitors, T and B lymphocytes, and natural killer cells (10). The day 1 cells were then collected in IMDM.

Methylcellulose (MC) culture of BFU-E. Day 1 cells were cultured at 3 $\times$ 10^5 cells/ml in 0.9% MC (Fisher Scientific Co., Pittsburgh, PA) in IMDM with 30% FCS, 1% human serum albumin (American Red Cross Blood Services, Washington, DC), 10^{-4} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY), 500 U/ml penicillin, 40 μg/ml streptomycin, recombinant human IL-3 (Genetics Institute, Boston, MA) 10^7 U/mg protein at a concentration of 50 U/ml, 10 U/ml recombinant human insulin (Eli Lilly Co., Indianapolis, IN), and 10,000 U/mg recombinant human EP (rEP) (Amgen Biologicals, Thousand Oaks, CA) protein at 2 U/ml. Cells were incubated in flat-bottomed, 12-well tissue culture plates (Linbro, Flow Laboratories, Hamden, CT) at 37°C in a high humidity 5% CO_2/95% air incubator.

Collection of ECFC. On day 8 the cells were collected in MEM and separated over 10% BSA as described above. Adherent cells were removed by incubation for 1 h in plastic flasks at 37°C in IMDM containing 10% FCS. The cells were gently washed with 37°C MEM and resuspended in IMDM containing 0.1% BSA to be incubated 1 h at 37°C to remove any remaining surface-bound EP (11). After serum-free incubation the cell suspension was layered onto 2 ml FH and centrifuged at 600 *g*, 4°C, for 5 min. The interface cells were washed and resuspended in IMDM with 0.1% BSA. This technique reproducibly yielded cells with 94% viability (10).

Plasma clot assay of ECFC. The percentage of post-MC, post-adherent day 8 cells that were ECFC was quantitated by plasma clot assay. Cells were cultured at 10^3/ml in a mixture containing 15% FCS, 15% heat-inactivated pooled AB serum, 1% human serum albumin, penicillin and streptomycin, 2 mg/ml fibrinogen (Fibrinogen Kabi, Grade L; Kabi Diagnostics, Stockholm, Sweden), 0.2 U/ml bovine thrombin (Parke-Davis, Morris Plains, NJ), with or without 1 U/ml rEP. The cells were incubated in 24-well, flat-bottomed tissue culture plates for 7 d at 37°C as described for MC culture above. On the seventh day (day 15 after the blood was collected) they were fixed and stained (12). ECFC were defined as cells giving rise to colonies of 2–49 nucleated hemoglobinized cells (10, 11), and CFU-E as cells giving rise to colonies of 8–49 nucleated hemoglobinized cells (12). CFU-E comprised 55.6±2.4% of ECFC in these studies.

Studies of ^{125}I-rEP binding. ^{125}I-rEP with full biologic activity and sp act of 325–722 Ci/mmol was purchased from Amersham Corp. (Arlington Heights, IL). Purified day 8 cells were suspended in IMDM at 3°C with 0.1% BSA at 0.8–1.4 $\times$ 10^7/ml in the presence of ^{125}I-rEP in concentrations from 0.025 to 1.0 nM. The cells and ^{125}I-rEP were incubated in 50-μl aliquots of 3°C for 48 h in precooled microwell cluster plates (Costar, Cambridge, MA). After 48 h the cells were suspended in 0.25 ml ice-cold PBS. The viability of these cells was 92.8±1.0%. The suspension was layered over 0.9 ml 10% BSA in PBS in a microcentrifuge tube and centrifuged at 15,000 *g* for 4 min at 3°C. The microcentrifuge tubes were then rapidly frozen in dry ice and ethanol, and the tips were counted in a gamma counter (Nuclear Chicago, Chicago, IL) (11). Nonspecific binding was measured in the presence of a 100-fold excess of rEP. Results were corrected for the number of ECFC present. All studies of ^{125}I-rEP binding were performed in duplicate or triplicate.

^{125}I-rEP crosslinking studies. The molecular size of the EP-R was determined by crosslinking as described by Sawyer et al. (13). Intact day 8 cells were incubated with ^{125}I-rEP at 37°C for 2 h and then transferred to an ice bath where disuccinimidyl suberate was added to a final concentration of 0.5 mM. After 15 min incubation at 0°C the crosslinking reaction was quenched by addition of 150 mM Tris HCl (pH 8.0) and unbound ^{125}I-rEP removed by washing. The cells were extracted for 1 min at 0°C with a solution containing 1.0% Triton X-100, 20 mM Hepes, pH 7.4, and a mixture of 11 proteinases (13). Nuclei and cell debris were removed by centrifugation at 1,000 *g* for 1 min, and the supernatant and pellet analyzed by SDS-PAGE and autoradiography. Experiments using normal and PV cells were performed simultaneously.

Acid wash of ECFC. Day 8 cells were washed for 20 s in 50 mM glycine buffer, pH 3.5, (Sigma Chemical Co.) at 3°C to remove any hormone tightly bound to the surface (11, 14). The cell suspension was then layered over 5 ml 10% BSA and centrifuged at 600 *g*, 3°C, for 5 min to separate the cells from the acidic buffer. The cells were collected, washed, and suspended in IMDM with 0.1% BSA for binding studies.

Measurement of surface-bound and internalized rEP. The time course of internalization and release of rEP and ECFC was determined as previously described for normal ECFC (11). Day 8 cells were incubated in 50-μl aliquots at 3°C for 48 h as described above in the presence of 1.0 nM ^{125}I-rEP to saturate all binding sites. The cells were then collected in 2 ml cold MEM, layered over 4 ml 10% BSA in PBS, and centrifuged at 3°C at 600 *g* for 5 min to separate cell-bound radioactivity from that present in the medium. The cells were washed once, resuspended in cold IMDM with 0.1% BSA, and incubated in 50-μl aliquots in prewarmed microwell cluster plates for varying periods of time at 37°C in a high humidity 95% air/5% CO_2 incubator. After incubation the cells were collected in 2 ml cold IMDM with 0.1% BSA and centrifuged at 600 *g* for 5 min at 3°C. The supernatant was collected and saved, and the pellet incubated in 0.5 ml 50 mM glycine buffer/0.1 M HBSS (Sigma Chemical Co.), pH 2.5, for 5 min in ice to remove surface-bound hormone. The cell pellet was centrifuged through BSA in a microcentrifuge tube as described above. The radioactivity associated with the cell pellet represented internalized ^{125}I-rEP, the supernatant collected after the glycine incubation represented surface-bound ^{125}I-rEP, and the initial supernatant represented ^{125}I-rEP released from the cell surface or interior during incubation at 37°C. Experiments were carried out in parallel with a 50-fold excess of unlabeled rEP to measure nonspecific binding.

Results

Growth of ECFC without exogenous EP. The growth of ECFC in plasma clots without exogenous EP relative to plasma clots

containing EP was determined for normal ECFC, for ECFC from patients with PV, and for ECFC from patients with elevated EP levels in vivo (two with secondary polycythemia, one with chronic hemolysis due to a hemoglobinopathy, and one with chronic anemia due to intestinal telangiectasias). In PV patients ECFC growth in the absence of exogenous EP was 10.24±2.27% of that found with exogenous EP versus 0.18±0.32% for normal ECFC. Growth of ECFC from patients with secondary polycythemia or anemia without exogenous EP was 3.55±2.8% of that found with EP.

Specific binding of ^{125}I-rEP. Characteristics of EP binding were studied six times in four PV patients. The mean percentage of ECFC present was 41.2±31.9%. Binding was saturable at 1.0 nM as in normal progenitors (11). Only one class of 851 EP-R (range, 320–1,530) with K_d = 0.72 nM (range, 0.37–0.85 nM) was detected. The mean correlation coefficient of the regression line for Scatchard analysis was −0.82±0.16. An example is shown in Fig. 1. Attempts to unmask a high affinity receptor class through removal of putative surface bound EP by acid wash did not alter the binding characteristics (Fig. 2). Three acid wash experiments showed 603 EP-R (range, 261–801) with a K_d of 0.97 nM (range, 0.53–1.4 nM) and a mean ECFC percentage of 49.2±13.2% (mean correlation coefficient was −0.92±0.03).

ECFC from the four patients with secondary polycythemia and anemia showed two classes of EP-R similar to normal ECFC, with K_d of 0.18 nM (range, 0.04–0.24 nM) and 1.10 nM (range, 0.33–1.7 nM), respectively. The total number of EP-R (727; range, 288–1,285), however, was decreased, primarily due to a reduction in the number of high affinity receptors (109; range, 71–194). The number of low affinity receptors (618; range, 217–1,081) is similar to that seen in normals (11; Fig. 3).

Time course of ^{125}I-rEP internalization. An increased sensitivity to EP in PV, even in the presence of a smaller number of receptors of lower affinity, might be explained if EP was internalized more rapidly or released more slowly. The time course of internalization of ^{125}I-rEP (Fig. 4) in PV, however, is identical to that reported in normals (11). Initially, virtually all ^{125}I-rEP present is on the surface. After 30 min incubation 41% is present inside the cells and 29% in the medium. At 120 min 45% is present in the medium and 34% within cells. Thus, PV ECFC internalize and release rEP in the same manner as normal cells. This also indicates that the 1-h serum-free incubation used in the day 8 purification process is adequate for removal of surface-bound EP.

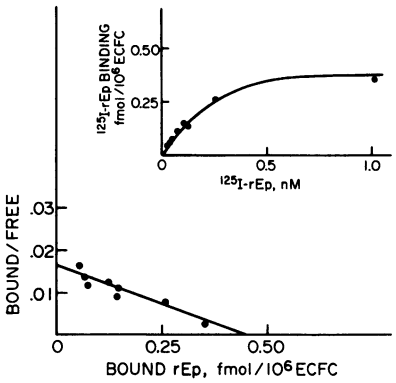

Figure 2. Effect of acidic buffer on ^{125}I-rEP binding to PV cells. After 20-s exposure to glycine buffer, pH 3.5, day 8 cells were incubated with increasing concentrations of ^{125}I-rEP for 48 h. Scatchard analysis showed a single class of 261 EP-R with a K_d of 0.527 nM (correlation = −0.925). Plasma clot assay revealed 54.4±7.6% ECFC.

^{125}I-rEP crosslinking studies. Fig. 5 shows that ^{125}I-rEP was bound to two proteins migrating at 130 and 140 kD in both normal and PV cells. When the molecular size of ^{125}I-rEP on SDS-PAGE is subtracted, molecular masses of 90 and 100 kD are obtained. This is identical to the results previously reported for normal human ECFC (13).

Discussion

Unlike ECFC obtained from normal individuals, patients with polycythemia secondary to increased EP secretion, or patients with anemia due to red cell loss, ECFC from patients with PV lack high affinity EP binding capacity and express only a single class of low affinity EP-R. The receptor is structurally similar to the EP-R on normal ECFC. A similar observation has been reported for ECFC from mice infected with the Friend polycythemia virus, which produces erythrocytosis without elevated EP levels, analogous to PV (13). It is unlikely that these results are a consequence of in vitro downregulation of EP-R in the face of exposure to EP, since high affinity receptors are detected on normal ECFC exposed to the same EP concentrations in vitro. High affinity EP-R are also detected in control

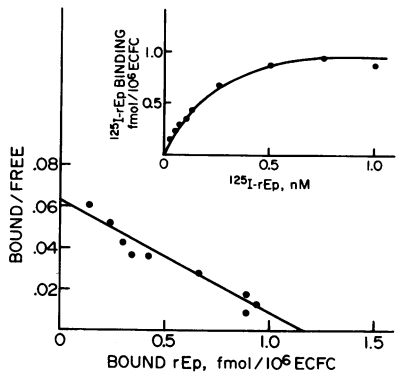

Figure 1. Effect of ^{125}I-rEP concentration on binding to ECFC from a PV patient. Day 8 cells were incubated for 48 h at 3°C with increasing concentrations of ^{125}I-rEP and specific binding was measured. Scatchard analysis showed a single class of 691 EP-R with a K_d of 0.37 nM (correlation = −0.964). Plasma clot assay revealed 85.1±12.7% ECFC. Separate analysis of the four points measured at the lowest rEP concentrations confirmed the absence of a high affinity receptor class.

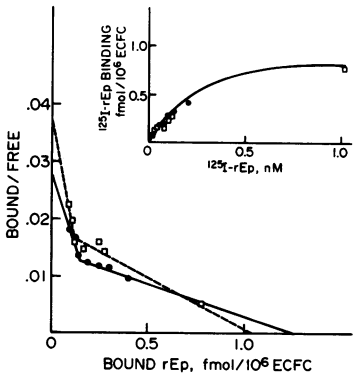

Figure 3. Effect of ^{125}I-rEP concentration on binding to ECFC from a patient with polycythemia secondary to chronic lung disease (*open squares*) and a patient with anemia due to chronic blood loss (*solid circles*). Scatchard analysis of ECFC from the patient with polycythemia showed two classes of 71 and 543 EP-R with K_d of 0.23 (correlation = −0.93) and 1.07 nM (correlation = 0.0724), respectively. Plasma clot assay revealed 58.5±5.2% ECFC. Scatchard analysis of ECFC from the anemic patient showed two classes of 87 and 747 EP-R with K_d 0.24 (correlation = −0.86) and 1.7 nM (correlation = −0.88), respectively. Plasma clot assay revealed 48.4±10.6% ECFC.

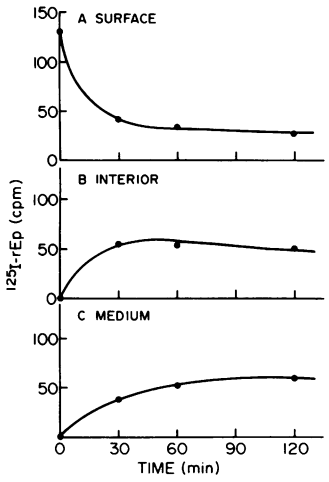

Figure 4. Time course of ^{125}I-rEP internalization in PV. Day 8 cells at a concentration of 1.4×10^7/ml were incubated with 1.0 nM ^{125}I-rEP at 3°C for 48 h and then centrifuged through 10% BSA to remove free ^{125}I-rEP. The cells were incubated to 37°C for 0–120 min and radioactivity that had been internalized, surface-bound, or dissociated was separated by acid wash and measured. The figure shows specific binding of ^{125}I-rEP. (*A*, surface-bound; *B*, internalized; *C*, dissociated radioactivity, respectively.) Plasma clot assay revealed 41.2±8.0% ECFC.

patients with diseases associated with increased EP levels in vivo, although there appears to be a moderate decrease in the number of high affinity EP-R.

It was necessary to add EP to MC cultures to obtain sufficient ECFC for our studies. One could contend that this would favor the growth of normal ECFC over the ECFC belonging to the PV clone. However, Prchal and colleagues, in studies of glucose-6-phosphate dehydrogenase heterozygotes with PV, showed that 71–92% of the colonies grown in vitro in the presence of added EP are progeny of the same clone that grew without exogenous EP (15). Further, if the PV ECFC were contaminated to any considerable extent by normal ECFC, one would have expected to detect high affinity EP-R.

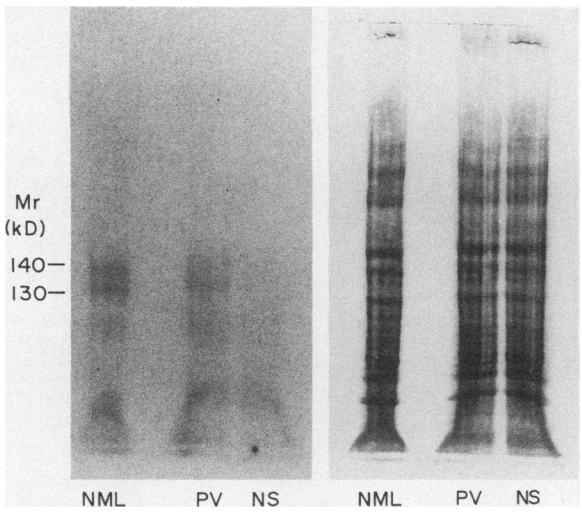

Figure 5. Determination of EP-R molecular size. ^{125}I-rEP was cross-linked to ECFC from a normal individual (*NML*) and a PV patient (*PV*). Nonspecific binding is shown in the third column (*NS*). Two bands at 130 and 140 kD were detected by autoradiography. When the molecular size of ^{125}I-rEP on SDS-PAGE is subtracted, these indicate proteins of 90 and 100 kD. Coomassie blue stains of the gels are shown to indicate that all the bands on the PV gel are shifted slightly due to a difference in protein load, which accounts for the small difference between the NML and PV bands.

Increased numbers of mature BFU-E and CFU-E have been reported in the peripheral blood of PV patients (16), and this might appear to be a potential problem with our culture system. However, such mature progenitors would have become late erythroblasts by day 8 of MC culture, and would have been removed by FH separation of post-MC cells. Assays for such cells on days 1 and 8 did not detect significant numbers, and the distribution of colony size from PV patients and controls was similar (data not shown), suggesting that the maturity of ECFC was similar. Finally, the possibility that high affinity EP-R were masked by tightly bound EP was evaluated by washing the ECFC with acidic buffer to remove surface-bound hormone (11, 14), and no such receptors were detected.

The absence of high affinity EP-R in PV, which appears to be a marker for PV ECFC, and the demonstration that the internalization and dissociation of bound EP is identical to that seen in normals, does not support the concept that the enhanced EP sensitivity reported by some investigators in PV[6–8] is mediated by changes in EP binding. One could postulate a number of potential mechanisms for the growth of PV progenitors in vitro without exogenous EP, such as autocrine production of EP or other growth factors by the cells in PV, or constitutitive production of a putative EP second messenger. However, since the intracellular events that follow EP binding and internalization are unknown, an accurate hypothesis must await an improved understanding of the normal cellular physiology of erythropoiesis.

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