Missense Mutation of the Erythropoietin Receptor Is a Rare Event in Human Erythroid Malignancies

By Jean-Pierre Le Couedic, Maria-Theresa Mitjavila, Jean-Luc Villeval, Frédéric Feger, Stéphanie Gobert, Patrick Mayeux, Nicole Casadevall, and William Vainchenker

Human erythroid malignancies (polycythemia vera [PV] and erythroleukemia) are associated with erythropoietin (Epo)-independent growth and differentiation. Missense or nonsense mutations in the Epo receptor (Epo-R) have been recently described in experimental erythroleukemia in mice and in cases of erythrocytosis in humans. To search for a similar genetic alteration in erythroleukemia and PV, we entirely sequenced the exons of the Epo-R gene as well as the intron-exon junctions in these disorders using polymerase chain reaction. In 1 of 10 cases of erythrocytosis, a single allele mutation was found in the 8th Epo-R gene exon that changed asparagine 487 into a serine. No Epo-R gene mutation was found in 12 PV cases studied, but the same mutation (N487S) was found in 1 patient with polycythemia that did not fulfill the criteria of PV (polycythemia of unknown origin). We did not detect this mutation after sequencing part of the 8th exon of the Epo-R gene from 21 other patients with polycythemia of unknown origin and 51 normal controls. The Epo-R mutation was also found in Epstein-Barr virus-derived cell lines from both cases, suggesting that it is not related to the malignant clone. Therefore, this mutation does not appear to be somatic, although no familial cases were found. The biologic effect of this mutation was subsequently studied. Erythroid progenitors from the polycythemic patient normally responded to Epo, whereas those from the erythroleukemic patient were Epo-independent due to autocrine stimulation by Epo. The normal and the mutated Epo-R were transfected into the murine Ba/F3 cell line. Both types of cells displayed the same response to Epo for proliferation, differentiation, and inhibition of apoptosis. Although this mutation may destroy a consensus binding site for Grb2, no obvious differences either in the pattern of Epo-induced tyrosine phosphorylated proteins or in the binding of Grb2 to the Epo-R were observed. In conclusion, a somatic Epo-R missense mutation does not appear to be a molecular mechanism involved in the abnormal growth of human erythroleukemia and PV. However, the Epo-R mutation (N487S) that we describe is located in the same tyrosine sequence beginning at AA 485 as the one previously observed (P488S) in a case of polycythemia (Sokol et al. Exp Hematol 22:447, 1994). These results suggest that this phosphopeptide sequence may play an important role in Epo signalling.

© 1996 by The American Society of Hematology.

Erythroid malignancies in animal models are usually associated with factor-independent proliferation or a hypersensitivity to erythropoietin (Epo).1 In humans, the same proliferation abnormalities seem to occur in erythroid hemopathies. Indeed, the occurrence of spontaneous erythroid colony formation in the presence of serum is one of the main biologic hallmarks of polycythemia vera (PV), although it is controversial whether this is due to hypersensitivity to a cytokine present in the culture components (serum or albumin)2 or related to true Epo-independence. This growth abnormality is frequently used for diagnosis.3,4 Early erythroid leukemias are characterized by the presence of erythroid blast cells blocked at a stage of differentiation equivalent to the colony-forming unit-erythroid (CFU-E) and are associated with Epo-independent proliferation.5 Thus, erythroid progenitors from PV and early erythroid leukemia have lost the normal Epo requirement necessary for terminal erythroid differentiation.

In some early erythroid leukemias, it has been shown that autocrine stimulation by Epo is responsible for this impaired regulation of proliferation.6 However, other mechanisms may be involved in this process. Numerous recent experiments have focused on the important role of the Epo receptor (Epo-R) in benign and malignant erythroid pathologies in mice and humans.

In mice, the Epo-R has been implicated in two erythroleukemia models. First, the Epo-R is constitutively activated by the gp55 protein of the Friend spleen focus-forming virus (SFFV).10,11 The interaction between the Epo-R and gp55 mimics the effects of Epo on its receptor12 and this mechanism of growth factor independence has some analogy with autocrine stimulation by Epo.13 Second, a missense mutation at codon 129 resulting in an arginine to cysteine substitution induces Epo-R homodimerization that leads to a constitutive activation of the receptor.14,15 This mutation induces a factor-independent proliferation when expressed in certain permanent cell lines16 and may also be oncogenic in vivo resulting in erythroleukemia.17 However, occurrence of a second genetic event is necessary to induce leukemia.18 Accordingly, it has been suggested that a point mutation in the Epo-R could be involved in the pathogenesis of PV and of human erythroleukemia.19

In humans, four types of Epo-R abnormalities have been described. First, an Epo-R truncated in its cytoplasmic negative regulatory domain is responsible for a benign human erythrocytosis with an autosomal dominant transmission.20 This disease is characterized by a low serum Epo level and Epo-independent erythroid colony formation with Epo hypersensitivity.19 A point mutation in the Epo-R gene gives rise to a premature stop codon that generates an Epo-R pro-
tein lacking the C-terminal 70 amino acids. Recently, another truncated Epo-R lacking the C-terminal 64 amino acids due to an insertional mutation was described in primary familial polycythemia. These situations are reminiscent of murine experimental models in which deletions in the carboxy terminus of the Epo-R induce Epo hypersensitivity. This may be due to the loss of the PTP1C binding domain of the Epo-R. Second, a point mutation in the negative regulatory domain has been found in a case of sporadic erythroleukemia. However, there is presently no direct evidence that this mutation is implicated in the etiology of this disorder. Third, mutations in the coding or noncoding region of the Epo-R have been found in two factor-dependent erythroleukemic cell lines, TF1 and UT7, leading to an upregulation of the Epo-R in UT7 cells and the expression of an abnormal receptor in TF1 cells.

In this study, we have investigated whether a point mutation in the Epo-R gene could be involved in the pathogenesis of PV and erythroleukemia. A point mutation that destroys a potential Grb2 consensus binding site in the negative regulatory cytoplasmic domain was found in a single case of erythroleukemia. However, we could not show that this mutation modifies Epo signalling or Epo-dependent cell growth and differentiation in transfected Ba/F3 cells. The same mutation was also found in one case of polycythemia that did not fulfill the diagnostic criteria for PV.

MATERIALS AND METHODS

Patients. Ten cases of erythroleukemia, 15 cases of PV, 22 cases of polycythemia of unknown origin, and 51 controls were studied. These erythroleukemia are not M6 leukemia but early erythroid leukemia that we have previously described in detail. These leukemias are characterized by blast cells that are blocked at early stages of erythroid differentiation (colony-forming unit-erythroid [CFU-E] or burst-forming unit-erythroid [BFU-E]) and are diagnosed by immunophenotyping and ultrastructural studies.

PV diagnosis was based on the slightly modified criteria defined by the PV study group (PVSG): (1) a true polycythemia (ascertained by the increased red blood cell mass); (2) SaO2 greater than 92%; and (3) either splenomegaly or white blood cells (WBC) greater than 12 × 109/L and platelets greater than 500 × 109/L. We have preferred to use this later value, which is slightly higher than that defined by PVSG (450 × 109/L) because platelet counts of up to 500 × 109/L can be observed in normal subjects. Leukocyte alkaline phosphatase and vitamin B12 levels were not routinely measured. In addition, erythroid cultures and determination of the serum Epo level by an enzyme-linked immunosorbent assay (ELISA; BioMerieux, Lyon, France) were performed in all patients. Some polycythemic patients did not fulfill these criteria and had no other discernible cause for secondary polycythemia (including a normal P50 determination). Consequently, their disease can be classified neither as a myeloproliferative disorder nor as a secondary polycythemia.

DNA sequencing. Genomic DNA was isolated from peripheral blood cells (leukocytes and mononuclear cells) or bone marrow cells using standard methods. Each of the 8 exons of the Epo-R gene was amplified and sequenced using the polymerase chain reaction (PCR) technique with the following oligonucleotide primers: 5'-TCC GGA AGG GCC TGG TCA GC-3' 120/101 exon1 (1); 5'-GTC CAG AAA CAG CAT GGC CC-3' 216/197 exon2 (2); 5'-GCT TGG GAA GAT GGC AGG GC-3' 841/860 exon3 (3); 5'-GCC TAC GAG CTC GAG GGC GC-3' 1180/1161 exon2 (4); 5'-GCC GTG AGC CAT GAT GGC GC-3' 1921/1940 exon3 (5); 5'-GAC CAC TCC TCC ATT CCC AG-3' 2299/2280 exon3 (6); 5'-AGG GTG TCA GTC GTC GCC CA-3' 2307/2326 exon4 (7); 5'-GCC ACC TCC AGA GGC GTG GC-3' 2580/2561 exon4 (8); 5'-TAC GTC CTC CTC GG-3' 2961/2980 exon5 (9); 5'-GCC CTG CCT CTC CCT ACA CC-3' 3182/3163 exon5 (10); 5'-AAT CAC GGG GCA AGC CCA CC-3' 3184/3203 exon6 (11); 5'-TGG AAA GAG GAC GGT TGG CG-3' 3376/3357 exon6 (12); 5'-GCC TCT ATG ACT GGG AGT GC-3' 5336/5355 exon7 (13); 5'-TGG AAG TCC TCT CAG AGC GC-3' 5678/5697 exon7 (14); 5'-GAT ATC CTG GGC ATG CTC AC-3 5737/5767 exon8 (15); 5'-TGC GTC CAT GGA CAC TGT GGC GC-3' 5993/6012 exon8 (16); 5'-GCA CAG TGT CCA TGG AGC CA-3' 6012/5993 exon8 (17); 5'-CCTA TAA TGG TTG GGT CTG CA-3' 6282/6263 exon8 (18).

With the exception of the oligonucleotide primers used to sequence the large exon 8 (from no. 14 to 17), all the other oligonucleotide primers were chosen in the flanking intronic sequences. PCR were performed using 2.5 µg of DNA following standard protocols. The reaction mixture was subjected to 5 minutes of denaturation at 95°C followed by 35 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 60°C, and 1 minute of extension at 72°C, with a final 7 minutes of extension at 72°C in a thermocycler (Perkin-Elmer Co, Roissy, France). Desalting and removal of dNTPs was achieved by centrifugating the sample in a Centricon 100 microconcentrator (Amicon, Danvers, MA). Dideoxy-terminator sequencing reactions were performed with fluorescent dye-labeled terminators according to the manufacturer’s protocols (Applied Biosystems, Roissy, France). Samples were sequenced on a DNA Sequencer (Model 373 A; Applied Biosystems). Sequence analysis was performed using SeqEd software (Applied Biosystems).

Expression vector constructions. After FicolI-metrizoate (Seromed, Berlin, Germany) gradient separation of blood or bone marrow samples, cultures were set up in serum-free medium using a previously described technique. Cultures were performed in triplicate in 1 mL volume petri dishes (Corning Glass Inc, Corning, NY) with cell concentrations ranging from 1 × 104 cells/mL (patient 1) to 5 × 105 cells/mL (patient 2). Cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO2. Colonies were scored at day 7 and 14 under an inverted microscope. Cultures were performed in the absence of any added growth factor or in the presence of Epo alone or different combinations of cytokines, including stem cell factor (SCF), interleukin-3 (IL-3), or granulocyte-macrophage colony-stimulating factor (GM-CSF). Recombinant human Epo was a gift from Cilag (Levallois-Perret, France). SCF, IL-3, and GM-CSF were gifts from Amgen (Thousand Oaks, CA).

In both patients with an Epo-R mutation, cultures were first performed on fresh cells and subsequent studies were repeated on frozen cells.

An Epstein-Barr virus (EBV) cell line was spontaneously obtained from patient 1. In patient 2, an EBV cell line was established after in vitro viral infection (Prof G. Sterkers, Immunology Laboratory, Hôpital Robert Debré, Paris, France).

Expression vector constructions. The human Epo-R cDNA clones, p18R3 and pMT2/Epo-R, were obtained from Drs S.S. Jones and A.D. D'Andrea, respectively. Intron sequences found in the p18R3 plasmid and a 7/1A base change at nucleotide 235 found in the pMT2/Epo-R plasmid were removed by substituting these regions with the corresponding normal ones found in the other plasmid.

To perform the mutagenesis, we first cloned a fragment into the EcoRI fragment of the hEpo-R cDNA from the pMT2/Epo-R plasmid into the Bluescript KS plasmid (Stratagene, La Jolla, CA) to introduce two unique enzyme restriction sites 3' (EcoRI) and 5' (Bal I) of the 4162 A base. Second, PCR amplification was performed using the patient D genomic DNA as a template with the 5'-TGG-
AAGTCCCTCTCAGAGCGC sense oligonucleotide (nucleotides 5678 to 5697 from the Epo gene) and the 3' TGGCGCTCTCATG-AATTCTGGAGAACAGGGA antisense oligonucleotide (nucleotides 6283 to 6290 with two missense modifications: nucleotides 6276 C to A and 6279 A to C, introducing an EcoRI restriction site 3' of the stop codon). Then, the Bal I/EcoRI fragment isolated from the PCR product was ligated in place of the normal Bal I/EcoRI Epo-R cDNA fragment excised from the previous plasmid. Clones were sequenced and one that included the required mutation (nucleotide 6146 A to G of the gene) was selected. Finally, the full-length mutated Epo-R cDNA plasmid was obtained by introducing the missing 5' fragment of the Epo cDNA isolated from the pBRI plasmid by digestion with Xho I.

Both mutated and normal cDNAs were sequenced and Bsr I fragments encompassing the entire coding regions of these cDNAs were isolated. These fragments were substituted to the Bsr I excised neo-mycin resistance gene of the pKJ-l plasmid, such that the expression of these cDNAs were under the control of the phosphoglycerate kinase (PGK-1) gene promoter (Fig 1).

DNA transfection of Ba/F3 cells. Ba/F3 cells were cultured in Iscove's modification of Dulbecco's medium (IMDM; GIBCO/BRL, Grand Island, NY) and 10% fetal calf serum (FCS) in the presence of WEHI-3B conditioned medium (CM). The pKJ mutEpo-R or the pKJ Epo-R cDNA plasmids (50 μg) were cotransfected with the pKJ-1 plasmid (2.5 μg) into 10⁷ Ba/F3 cells by electroporation (Bio-Rad electroporator set at 960 pF and 250 plasmid (2.5 pg) into 10⁷ Ba/F3 cells by electroporation (Bio-Rad pKJ Epo-R plasmids (50 pg) were cotransfected with the PIC-I plasmid by digestion with Bal YEcoRI and the miasonso mutation modifying an Adenine to a Guanine at nucleotide 1462 of the Epo-R cDNA are indicated with arrows.

Binding studies. Epo was iodinated to a specific activity of 20 to 30 mCi/mg using Iodo-gen. Cells (2 to 5 x 10⁶) were cultured in the presence of WEHI-3B CM for at least 3 days, washed, and incubated in duplicate at 37°C in 100 μL IMDM containing 5% FCS, 0.1% sodium azide, 25 mmol/L HEPES, pH 7.4, and various concentrations of 125I Epo. After 2 hours of incubation, cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and cell-bound and free radioactivity was determined. Nonspecific binding was measured by incubating the cells with a 100-fold excess of noniodinated Epo. Binding experiments were performed on 20 clones; for each, 4 x 10⁴ cells were incubated in presence of 2 x 10⁵ cpm of 125I-Epo.

Fig 1. Vectors transfected in Ba/F3 cells to express the normal Epo-R and the N487S mutated Epo-R. PGK-1 pro, promoter of the gene encoding the mouse phosphoglycerate kinase-1. The site of transcriptional initiation at the PGK-1 pro. and the missense mutation modifying an Adenine to a Guanine at nucleotide 1462 of the Epo-R cDNA are indicated with arrows.

Growth characteristics of Ba/F3 clones. Cells were cultured in IMDM and 10% FCS in the presence of Epo or WEHI-3B CM for at least 1 week before the experiment (except when otherwise indicated), washed 3 times, and seeded at 5 x 10⁶ cells/ml in a 24-well plate in the presence of Epo (2 U/mL) or WEHI-3B CM or in the absence of added growth factor, respectively. Cell concentration and cell survival were determined daily using the trypan blue exclusion.

Analysis of Epo responsiveness was determined by cell survival (in the microwell assay) and Epo-dependent proliferation was measured by 3H-thymidine (3Hthy) incorporation. Briefly, transfected Ba/F3 cells were cultured for 12 hours in serum-free medium in the absence of growth factors. Cells were then seeded in 96-well microplates at a concentration of 1.5 x 10⁴ cells/150 μL serum-free medium in the presence of increasing concentrations of Epo. After 48 hours of culture, 0.15 μCi of [3H]thymidine (48 Ci/mmol; Amersham, Les Ulis, France) was added to each well for 6 hours. Cells were harvested onto glass fiber filters and radioactivity was determined by liquid scintillation counting after TCA precipitation.

Immunoprecipitation and Western blotting. Ba/F3 cells transfected with the normal and mutated EpoR were grown in the presence of WEHI-3B CM. The cells were serum-and growth factor-starved for 5 hours before stimulation with Epo. The cells were then lysed as described previously. Extracts were either analyzed directly by Western blot using antiphosphotyrosine antibodies (4G10 monoclonal antibody [MoAb]; Ubi, Lake Placid, NY) or immunoprecipitated using anti-Grb2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) before Western blot analysis.

RESULTS

DNA studies. In the first set of experiments, we investigated whether an Epo-R rearrangement was present in 10 patients with erythroleukemia. Southern blots using BamHI restriction enzyme and a probe encompassing all the cDNA
sequence were performed. No obvious rearrangement compared with the control was observed (data not shown). However, in 1 patient, an EpoR gene amplification was suspected. Previous experiments using the leukemic cells from this patient showed an increase number of Epo-R.

Therefore, we searched for missense mutations in the Epo-R gene. All the 8 Epo-R gene exons including the flanking intronic sequences of the 7 first exons were individually sequenced by PCR. In comparison to the published normal sequence, two mutations were found in two different patients. In one, the mutation was silent (from ACT to ACC) located in the 8th exon, was found in one allele only. This result was confirmed by several independent sequences obtained with different primers. In addition, this mutation generates a new Bpm I restriction site that was confirmed by PCR analysis (Fig 2).

To determine if this mutation was related to the malignant clone, we studied an EBV cell line obtained from the peripheral blood cells of the patient. The same mutation was observed, suggesting that it was inherited. Investigation of relatives was not possible, but there was no history suggestive of polycythemia or erythroleukemia in the family.

Patients with PV were subsequently investigated. In 12 patients, bone marrow or blood cells were obtained and DNA was extracted. The Epo-R gene coding sequence was entirely sequenced. A mutation in the Epo-R could not be shown in any of the patients.

In a second set of experiments, 22 patients with polycythemia of unknown origin were studied by sequencing part of the 8th exon of the Epo-R gene using DNA from peripheral blood cells (mononuclear cells and granulocytes). The N487S mutation was found in 1 patient (patient 2) that was further studied. Sequencing of the other Epo-R exons in this patient did not display any other mutation. As in patient 1, the mutation was also found in marrow cell DNA as well as DNA extracted from an EBV-derived cell line. An examination of a detailed history did not provide any evidence supporting a history of familial polycythemia. Two hematologically normal children were available for investigation, but no mutation in the Epo-R gene could be found in their blood cell DNA.

Finally, 51 normal subjects were used as controls. The mutation in the 8th exon of the Epo-R gene could not be detected by PCR in any of these controls.

In vitro erythroid cultures. Patient 1 with a de novo erythroleukemia had been extensively studied previously\(^1\) (patient D in this study). Briefly, spontaneous leukemic erythroid growth (in the absence of exogenous stimulating factor and in serum-free medium) was present (plating efficiency about 1%). Epo but not GM-CSF significantly increased the number of colonies in comparison to unstimulated cultures. However, this spontaneous erythroid growth appeared to be related to an autocrine stimulation by Epo: (1) Epo-mRNA was present in the erythroid blasts and (2) an Epo antisense oligonucleotide partly blocked this spontaneous growth\(^2\) (patient 4).

In patient 2 with polycythemia of unknown origin and a low serum Epo level (<10 mU/mL), no spontaneous erythroid colonies were detected in blood or marrow cultures in three different experiments performed in two different laboratories. An Epo dose response was determined on blood and marrow erythroid progenitors. The Epo sensitivity of normal CFU-E in the presence of Epo and of blood BFU-E with the combined addition of Steel factor, IL-3, and Epo was normal (Fig 3).

Effects of the mutation in Ba/F3 cells. To analyze the effects of the mutation, we introduced and expressed the normal and mutated Epo-R gene in the murine IL-3–dependent Ba/F3 cell line. Electroporation of the normal and mutated Epo-R expression vector resulted in similar numbers of Epo-dependent clones assayed on methyl cellulose cultures 1 day after transfection. Rare development of growth factor-independent cells could be observed both with the mutated

---

**Fig 2.** Bpm I-digested PCR product amplified from the Epo-R gene of patient 1, patient 2, and normal control blood cells. Size-fractionated digests, by means of electrophoresis in a 2% agarose gel, are stained with ethidium bromide. The nondigested PCR fragment is a 947-bp fragment including the 8th exon of the Epo-R gene and obtained with the primers no. 13 and 18 previously described in the Materials and Methods. Using the control cells, only one Bpm I restriction site is present (position 5758) and leads to the 422-bp and 389-bp digested fragments (control). The mutation introduces a 81-bp (partial digestion) restriction site that was confirmed by DNA analysis (Fig 2).

---

\(^1\) Bpm I-digested PCR product amplified from the Epo-R gene of patient 1, patient 2, and normal control blood cells. Size-fractionated digests, by means of electrophoresis in a 2% agarose gel, are stained with ethidium bromide. The nondigested PCR fragment is a 947-bp fragment including the 8th exon of the Epo-R gene and obtained with the primers no. 13 and 18 previously described in the Materials and Methods. Using the control cells, only one Bpm I restriction site is present (position 5758) and leads to the 422-bp and 389-bp digested fragments (control). The mutation introduces a 81-bp (partial digestion) Bpm I-digested fragments. These new fragments are only detected with the patients.
and the normal Epo-R expression vector. Southern blot analysis of the transfected cells showed the presence of approximately 15 different integration sites (data not shown). Occurrence of the transfected mutated gene in the Ba/F3 cells was checked by PCR analysis, which showed the new Bpm I restriction site created by the mutation.

Cell proliferation and survival were assayed in the presence either of WEHI-3B CM, Epo (2 U/mL), or without the addition of growth factor. No difference was observed between the Ba/F3/Epo-R and Ba/F3/mutEpo-R (data not shown). Furthermore, the delay in proliferation when switching Ba/F3 cells from WEHI-3B CM to Epo was similar in Ba/F3/Epo-R and Ba/F3/mutEpo-R (data not shown). Finally, to investigate whether the mutation modified Epo responsiveness, normal and mutated Epo-R-expressing cells were cultured in the presence of different concentrations of Epo (Fig 4). The data show that the amount of Epo resulting in 50% cell survival after 2 days of incubation did not significantly differ between both types of transfected cells (Fig 4A). These results were confirmed using [3H]ThdR incorporation as a measure of Epo-dependent proliferation (Fig 4B).

Epo-R analysis. Epo-R and mutEpo-R could be detected in the transfected cells using antihuman Epo-R MoAbs by flow cytometry (10% positive cells compared with a control antibody). To determine if the mutation modified Epo-R number and function, we investigated the number of Epo binding sites in the mutated and normal Epo-R Ba/F3 cells. Scatchard analysis (Fig 5) was performed with 3 different mutated or normal Epo-R expressing Ba/F3 cloned or pooled cells. The receptor affinities were not significantly different: Kd = 225, 387, and 222 pmol/L in normal Epo-R expressing cells, and 185, 750, and 192 pmol/L in mutated Epo-R-expressing cells. The number of receptors per cell was 5,200, 1,200, and 300, and 8,000, 5,800, and 600 in the normal and mutated Epo-R-expressing cells, respectively. From these data, it was unclear if the mutation modified the number of receptors. Thus, we analyzed 19 other clones transfected either with the mutated or with the normal Epo-R. No significant difference was observed in total binding and nonspecific binding between the two groups of transfected clones: total binding and nonspecific binding respectively represented 12,540 ± 10,000 cpm and 2,260 ± 160 cpm for the mutated Epo-R-expressing cells (n = 9) and 11,920 ± 7,460 cpm and 2,600 ± 510 cpm for the normal Epo-R-expressing cells, respectively (n = 10).

Epo-induced tyrosine phosphorylation. Resting Ba/F3 cells transfected with the mutEpo-R or with the wild-type human Epo-R were stimulated for 5 minutes with 5 U/mL Epo and tyrosine phosphorylated proteins were analyzed by Western blot. As shown on Fig 6, proteins with molecular masses of 145, 130, 115, 98-90, 60, and 52 kD were tyrosine phosphorylated to a similar extent in response to Epo in both cell types.

Grb2 association with tyrosine-phosphorylated proteins. If tyrosine residue 485 is phosphorylated after Epo stimulation, the sequence Tyr485-Ser-Asn-Pro (YSNP) of the wild-type receptor could be a binding site for the Grb2 SH2 domain, whereas the sequence Tyr485-Ser-Ser-Pro (YSSP) of the mutated receptor should not bind Grb2.49 To test whether Grb2 association with tyrosine phosphorylated proteins was modified in cells transfected with the mutated Epo-R, Grb2 was immunoprecipitated from Epo-stimulated cells or from unstimulated cells and immunoprecipitates were analyzed by Western blot using antiphosphotyrosine antibodies. As shown in Fig 6 (right, Grb2), the same tyrosine-phosphorylated proteins were associated with Grb2 in cells transfected with the normal Epo-R, Grb2 was immunoprecipitated from Epo-stimulated cells or from unstimulated cells and immunoprecipitates were analyzed by Western blot using antiphosphotyrosine antibodies. As shown in Fig 6 (right, Grb2), the same tyrosine-phosphorylated proteins were associated with Grb2 in cells transfected with the normal or the mutated receptor. In both types of cells, Grb2 associated with proteins of 95, 72, and 52 kD. The 72-kD and 52-kD proteins corresponded to the Epo-R and Shc, respectively, whereas the 95-kD protein remains to be identified. Reprobing the blot with the anti-Grb2 antibodies showed that the same quantity of Grb2 was immunoprecipitated in each sample. Thus, mutation of Epo-R Asn487 to Ser did not modify the association of Grb2 with Epo-induced tyrosine phosphorylated proteins. Furthermore, no difference in the phosphorylation pattern induced by Epo in
Fig 4. Epodependent proliferation of Ba/F3 cells expressing the normal Epo-R or the N487S mutated Epo-R. The proliferation of 4 independent cell pools expressing either the normal Epo-R (open symbols) or the mutated Epo-R (bold line, solid symbols) are represented. (A) The proliferation is measured by [3H]thymidine incorporation and results are mean values (±SD) of three determinations. (B) The proliferation is measured by the number of viable cells found after incubating 200 cells during 4 days in the presence of different concentrations of Epo. Results are mean values (±SD) of duplicated cultures.

Ba/F3 cells transfected with the normal or mutated Epo-R could be detected (Fig 6, left, TLC).

DISCUSSION

Because the Epo-R is essential for erythroid differentiation, we investigated whether a missense mutation in the Epo-R could be responsible for the altered growth of PV and erythroleukemic cells. This comprehensive study of the Epo-R was performed on a large number of patients with erythroleukemia, PV, and unclassified polycythemic states.

Previous studies using biochemical techniques have eliminated a gross abnormality in the Epo-R because the number of Epo-R, and its biochemical features (determined by affinity measurements and cross-linking experiments) were identical in PV and normal erythroid cells. In addition, no chromosomal rearrangements or gene amplification were found by Southern blotting. However, these experiments did not exclude the possibility of a point mutation in the Epo-R that might constitutively activate Epo signalling without changing the biochemical characteristics of the receptor. Because such a mutation could involve either the extracellular domain (as in some inherited erythrocytoses in humans), or the cytoplasmic domain (as in some inherited erythrocytoses in humans), we decided to sequence all the Epo-R gene exons as well as the exon/intron junctions.

No mutation in the Epo-R coding sequence was observed in 12 PV patients fulfilling the criteria defined by the PVSG, including a low serum Epo level and spontaneous erythroid colony growth. This negative result is not due to the lack of sensitivity of our PCR technique because in 2 cases with other pathologies, a point mutation in the Epo-R gene was detected. One possible limitation of our study may be the low frequency of cells derived from the malignant clone in the patient marrows. Indeed, PV is not associated with a cytogenetic marker; thus, in contrast to chronic myeloid leukemia, it is difficult to precisely determine the contribution of the malignant clone to the patient’s hematopoiesis. However, previous studies using X-linked markers such as G6PD or methylation sites have shown that the great majority of the marrow cells and mature granulocytes belong to the malignant clone. Thus, if an Epo-R missense mutation was responsible for PV, it would involve one allele but a little less than 50% of Epo-R genes from marrow cells would carry the mutation if the malignant clone largely predominates.
This situation is reminiscent of a genetic monoallelic disease that can be diagnosed by our PCR technique.

Consequently, it appears that a missense mutation in the Epo-R is not usually involved in the pathogenesis of PV and that the molecular mechanism responsible for this disease remains unknown. This result is in agreement with the recent report of Hess et al\(^7\) that did not find any mutation in the Epo-R gene using PCR and single-stranded conformation polymorphism analysis in 24 PV patients in combination with sequencing in 4 of them. It seems more likely that the primary defect in PV may be in a molecule involved in cytokine signalling such as a phosphatase.\(^{23}\) Such a defect would better explain the hypersensitivity of PV cells to several cytokines than would an alteration in a specific cytokine receptor. Interestingly, an abnormality in the Ras pathway is associated with the pathogenesis of other myeloproliferative diseases such as chronic myeloid leukemia (CML)\(^8\) or myelomonocytic leukemia.\(^9\)

We also searched for an Epo-R point mutation in 10 cases of early erythroid leukemia.\(^7\) Despite the study of a fairly large number of patients with a rare disease, with the exception of 1 case we were unable to find either a rearrangement or a mutation in the Epo-R gene. Therefore, this result suggests that an Epo-R molecular defect is rarely involved in true erythroid malignancies.

It is noteworthy that, in 1 case of early erythroid leukemia as well as in 1 case of 22 unclassified polycythemias, the same missense mutation involving 1 allele of the Epo-R gene was found. This mutation consisted of an adenine to guanine substitution in nucleotide 6146 changing an asparagine to serine at position 487. In addition, the mutation creates a new Bpm I restriction site. This N487S mutation is located in the negative regulatory domain of the Epo-R that includes several binding sites for tyrosine kinase or phosphatase\(^{22}\) but does not alter the consensus binding site for PTP1C.\(^{23,24}\) The N487S mutation is adjacent to a recently described mutation in nucleotide 6148 that substitutes a serine for proline at position 488 in the protein.\(^{25}\) This congenital mutation is associated with sporadic primary polycythemia but no biologic relationship between the mutation and the polycythemia could be shown.\(^{25}\)

In the 2 present cases, it remains difficult to determine if the mutation is somatic or genetic. Indeed, in the erythroleukemic patient, no detailed family study could be performed, whereas in the other patient, only his 2 children were available and no mutation in the Epo-R gene was detected. In addition, we were unable to obtain nonhematopoietic tissue from both patients. However, in both cases, the mutation was detected in EBV cell lines derived from their peripheral blood B cells. Because it seems very unlikely that the B cells belonged to the malignant erythroleukemic clone, this result strongly suggests that this mutation is not acquired.
For Western blot using antiphosphotyrosine antibodies before analysis by antiphosphotyrosine probing, the blot was stripped and probed with anti-Grb2 antibodies to ascertain equal immunoprecipitation in each sample (right, bottom).

Therefore, it remained possible that this mutation was a polymorphism. For this reason, we sequenced the Epo-R gene of 51 normal subjects, but could not find a similar mutation. Although this result does not formally exclude a rare polymorphism, it suggests a linkage between the N487S mutation and the erythroid pathology of the patients. Thus, we attempted to determine the biologic relevance of this mutation.

Erythroid growth was totally different in both patients. In the erythroleukemic patients, malignant erythroid colonies were grown without adding any cytokine. In contrast, no difference in the Epo response of erythroid progenitors from the polycythemic patient and normal controls was observed. In fact, we have previously shown that the spontaneous erythroid growth in this erythroleukemic patient was due to autocrine stimulation by Epo. Indeed, Epo transcripts were present in the blasts and the abnormal growth was partly inhibited by Epo antisense oligonucleotides.

Epo-R signalling does not only lead to proliferation but may play an important role in differentiation. To investigate the relevance of the N487S mutation, a biologic model was created by transfecting the wild-type and the mutated Epo-R cDNA into the factor-dependent murine Ba/F3 cell line. However, the mutation did not modify either cell survival and proliferation or the Epo dependency of Epo-R-expressing cells. Using Northern blot analysis, we could show that the β-major globin transcript was also induced by the mutated receptor (data not shown). Further quantitative studies will be required to understand whether the erythroid differentiation signal transmitted by this mutant Epo-R is modified. Recently, several observations have also focused on the possible oncogenic role of Epo-R hyperexpression, and it has been shown that a missense mutation in the Epo-R can induce membrane hyperexpression of the receptor. However, no membrane overexpression of the mutated Epo-R was observed on Ba/F3 or on the blasts of the erythroleukemic patient in our study.

Finally, we investigated whether the N487S mutation changes the signal transduction pathway used by Epo because it may alter a SH2 binding sequence recognized by Grb2. The normal peptidase sequence, YSNP, extending from amino acid 485 to 488, may be sufficient for Grb2 recognition because the SH2 domain of Grb2 binds to proteins on the basis of an asparagine located 2 amino acids carboxy terminal to phosphotyrosine. The Epo-R mutant has a serine in place of an asparagine that abolishes the potential consensus sequence for Grb2 association. In mice, this phosphopeptide (Y485) is not conserved and is not a consensus site for Grb2. The only peptide that may bind Grb2 begins at Y489. In addition, it is not totally clear whether Grb2 directly associates with the tyrosine phosphorylated Epo-R or via a docking protein such as SHC or PTP1D. This type of association may depend on the cellular model because Grb2 binds directly to the Epo-R in the Epo-R+ DA, Epo-R+ Mo-7E, and UT-7 cell lines and also through SHC or PTP1D in Epo-R+ Mo-7E. However, we could not find any difference in the phosphorylation pattern induced by Epo in the Ba/F3 cell line transfected with the normal or mutated Epo-R. Moreover, this Epo-induced phosphorylation pattern was very close to that previously reported for UT-7 cells and for Ba/F3 cells transfected with the murine Epo-R, showing that the human Epo-R was apparently fully functional in murine cells. Despite the possible elimination of a Grb2 binding site in the mutated receptor, Epo-induced Grb2 association with tyrosine phosphorylated proteins was identical in cells transfected with mutated or wild-type receptors. Thus, in Ba/F3 cells transfected with the human Epo-R, the Y485-S-N-P sequence is probably not involved in Grb2 recruitment.
Therefore, we have been unable to show that this N487S mutation has biologic consequences. It is noteworthy that this mutation modifies the same peptide sequence (Y485) as does the mutation described by Sokol et al.25 In that case, it could not be shown that the Epo-R mutation was directly responsible for the polycythemia. However, both reports support the hypothesis that this presumptive phosphopeptide sequence (beginning at tyrosine 485) plays a crucial role in Epo signalling.

Further experiments will be required because we cannot exclude that Ba/F3 cells usually used for hematopoietic receptor biochemical studies is a cellular model that allows the identification of all Epo-R signalling abnormalities.21 For example, Ba/F3 cells do not respond to SCF, which plays a pivotal role in normal erythropoiesis. Possible abnormalities in Epo/SCF synergism could not be investigated in that tissue. Because the Y485 peptide is absent in the murine Epo-R, it cannot be excluded that some signaling pathways are species-specific and require a human cellular environment. Experiments are in progress using retroviral gene transfer to address this question.

In conclusion, this study shows that a mutation in the Epo-R is not directly involved in the pathogenesis of PV and erythroleukaemia. It is noteworthy that a mutation involving the phosphotyrosine sequence beginning at amino acid 485 may be indirectly involved in the pathogenesis of some rare unclassified polycythaemias and may be a predisposing factor for erythroleukaemia.

ACKNOWLEDGMENT

We thank Dr M. Lipinski and C. Scamps for technical expertise concerning the DNA sequencing; Drs P. Hunt and J. Egrie (Amgen, Thousand Oaks, CA) for the generous gifts of recombinant human IL-3, SCF, and GM-CSF; Drs D. Dabour and M. Gilibert (Cilag) for providing us with recombinant human Epo; and Drs A.D. D'Andrea and S.S. Jones (Genetics Institute, Cambridge, MA) for providing the human Epo cDNA. We are indebted to Prof Y. Najean (Hôpital St Louis, Paris, France) and Prof B. Varett (Hôpital Necker, Paris, France) who provided us with the patient's blood samples. We also thank Prof G. Sterkers (Hôpital Robert Debré, Paris, France) for establishing the EBV cell line.

REFERENCES


38. Fraker PJ, Speck JCJ: Protein and cell membrane iodinations with a sparingly soluble chloramide, 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978


51. Liboi E, Carroll M, D’Andrea AD, Mathey-Prevot B: Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. Proc Natl Acad Sci USA 90:11351, 1993


Missense mutation of the erythropoietin receptor is a rare event in human erythroid malignancies

JP Le Couedic, MT Mitjavila, JL Villeval, F Feger, S Gobert, P Mayeux, N Casadevall and W Vainchenker