A novel mutation in the erythropoietin receptor gene is associated with familial erythrocytosis

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Primary familial erythrocytosis (familial polycythemia) is a rare myeloproliferative disorder with an autosomal dominant mode of inheritance. We studied a new kindred with autosomally inherited familial erythrocytosis. The molecular basis for the observed phenotype of isolated erythrocytosis is heterozygosity for a novel nonsense mutation affecting codon 399 in exon 8 of the erythropoietin receptor (EPOR) gene, encoding an EpoR peptide that is truncated by 110 amino acids at its C-terminus. The new EPOR gene mutation 5881G>T was found to segregate with isolated erythrocytosis in the affected family and this mutation represents the most extensive EpoR truncation reported to date, associated with familial erythrocytosis. Erythroid progenitors from an affected individual displayed Epo hypersensitivity in in vitro methylcellulose cultures, as indicated by more numerous erythroid burst-forming unit-derived colonies in low Epo concentrations compared to normal controls. Expression of mutant EpoR in interleukin 3–dependent hematopoietic cells was associated with Epo hyperresponsiveness compared to cells expressing wild-type EpoR. (Blood. 2002;99:3066-3069)

Introduction

Familial erythrocytosis, also known as primary familial and congenital polycythemia (PFPC), is a rare disorder involving isolated proliferation of bone marrow progenitors of the erythroid lineage. This disorder is typically characterized by an autosomal dominant mode of inheritance, and less frequently, by the occurrence of sporadic cases. The clinical features include the presence of isolated erythrocytosis without evolution into leukemia or other myeloproliferative disorders, absence of splenomegaly, normal white blood cell and platelet counts, low plasma erythropoietin (Epo) levels, normal hemoglobin-oxygen dissociation curve indicated by a normal P50, and hypersensitivity of erythroid progenitors to exogenous erythropoietin in vitro. Mutations in the gene encoding the erythropoietin receptor (EPOR) have been described in several families with isolated familial erythrocytosis. We report a new kindred with dominantly inherited familial erythrocytosis associated with heterozygosity for a novel point mutation in the EPOR gene.

Study design

Patients, erythroid colony formation assays, DNA and RNA analyses

Peripheral blood samples from affected and unaffected individuals were obtained under a protocol approved by the Institutional Review Board at Yale University School of Medicine. In vitro erythrocytosis colony formation assays were performed as described. The genomic DNA structure of the EPOR gene and RNA analyses for cloning of mutant EpoR complementary DNA (cDNA) were performed as described. Identity of subcloned polymerase chain reaction (PCR) products containing amplified EPOR genomic DNA and cDNA fragments were confirmed by nucleotide sequencing.

Cloning and expression of mutant EpoR and Epo dose-response assays

Wild-type (WT) and mutant human EPO cDNAs were cloned in pBabe-puro retroviral vector and plasmids were stably transfected into amphotropic producer cell line PA317 using Lipofectamine reagent (Invitrogen, Carlsbad, CA). Puromycin-resistant (3.5 μg/mL) PA317 cells were cocultured with murine interleukin 3 (IL-3)–dependent 32D cells in the presence of 4 μg/mL polymyxin for 10 days and cultured in Epo 2 U/mL (Amgen, Thousand Oaks, CA) instead of IL-3. As negative control, 32D cells transfected with empty pBabe-puro vector were generated. Single-cell clones of 32D cells were isolated by limiting dilution. Expression of EpoR protein in single-cell clones was demonstrated by immunoprecipitation and Western analysis as described and surface expression of EpoR was confirmed by a flow cytometry assay (data not shown) using recombinant human Epo (Amgen) that was biotinylated as described. Negative control 32D cells transfected with vector only do not express EpoR protein, do not proliferate in Epo, and do not demonstrate surface binding of biotin-Epo by flow cytometry assay (data not shown). Epo dose-response assays were performed using MTT reagent (dimethylthiazol-2-yl-2,5-diphenyltetrazolium) as described.

Results and discussion

We studied a 4-generation white family from Maine (Figure 1). The propositus (patient III:24 in Figure 1) was first evaluated at the age of 15 years because of headaches associated with a hemoglobin of 20.7 g/dL and a hematocrit of 62%. The white blood cell and platelet counts and morphology of the red blood cells in peripheral

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blood smear were normal. The plasma Epo level was less than 10 mU/mL and hemoglobin electrophoresis and P50 were normal. The persistent headaches were relieved by phlebotomy. Many affected individuals in this family underwent periodic phlebotomy to control hematocrit levels.

The phenotype of the propositus and other evaluated affected individuals in the pedigree were consistent with clinical features of PFCP. To determine whether erythrocytosis in this family was associated with Epo hypersensitivity of erythroid progenitors, we performed in vitro semisolid medium cultures using peripheral blood mononuclear cells. The results of scoring of day 14 erythroid burst-forming unit (BFU-E)–derived colonies in semisolid methylcellulose medium containing serum and various added Epo concentrations are illustrated in Figure 2A. In the absence of added Epo in the culture medium, 0 to 1 BFU-E–derived colonies were detected in the samples from the affected individual and no erythroid colonies were present in control cultures. At highest added Epo concentration of 1 U/mL, the numbers of BFU-E–derived colonies were similar in cultures of the affected individual and those of the unaffected family member (30 ± 3.7 versus 33 ± 7, respectively). Notably, erythroid progenitors of the affected individual displayed hypersensitivity to Epo when compared to those of an unaffected family member in the presence of relatively low and intermediate range of Epo concentrations (25-100 mU/mL). A significant difference in the number of erythroid colonies was observed in 25 mU/mL, with 1.3 ± 0.5 colonies in the control plates compared to 8 ± 0.5 colonies in the patient cultures (P < .0002 by Student t-test). In 50 mU/mL Epo, 20 ± 5.8 colonies were present in the cultures from the individual with erythrocytosis compared to 11 ± 1.5 in the unaffected family member (not significant). In 100 mU/mL Epo, the difference in colony numbers remained significant with 28 ± 0.5 in the patient versus 17 ± 2.8 in controls (P < .03). The differences in the total numbers of erythroid colonies was accompanied by a visible difference in the size of individual colonies when culture plates from the affected individual and control were examined (data not shown).

Figures in EPO gene mutation in familial erythrocytosis have been found in about 12% of cases in a series of individuals with PFCP and it appears that mutation in a gene(s) other than EPO may account for the polycythemic phenotype in the majority of PFCP cases.4 To determine whether the PFCP phenotype in the new family was associated with a mutation in the EPO gene, we evaluated the structure of the gene with particular focus on the region encoding the C-terminal domain of the receptor that exerts a negative effect on receptor mitogenic function.19–21 Direct nucleotide sequencing as well as sequencing of subcloned genomic PCR amplification products from the propo-
date that is associated with PFCP in heterozygous individuals and confers Epo hypersensitivity in erythroid progenitors and transfected hematopoietic cells. This human EpoR mutant lacks 110 distal amino acid residues, comparable with regard to the extent of the cytoplasmic truncation, to the truncated murine EpoR studied by Zang et al.25 It is possible that specific structural differences between truncated human and murine EpoRs other than the extent of the cytoplasmic truncation play a role in generation of the polycythemic phenotype. For instance, compared to the murine EpoR, a notable difference in the structure of truncated human EpoR mutants is the presence of an additional tyrosine residue in the membrane proximal region of human EpoR that could potentially contribute to the dominant, gain-of-function effect of truncated human receptors resulting in generation of PFCP phenotype in vivo.

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References


Figure 2. Erythroid colony assays, mutation analysis, and Epo-dose response. (A) Erythroid colony formation assays in 2 family members. The vertical axis indicates the numbers of BFU-E-derived colonies per 2.5 x 10^5 peripheral blood mononuclear cells, expressed as the mean ± SD of assays performed in triplicate. The final Epo concentration added to the cultures is indicated on the horizontal axis. (B) DNA sequence of subcloned genomic PCR products from the EpoR gene of the proband. Analysis of individual clones shows the sequences for the normal allele (left) and the mutant allele (right). The bold letter T indicates 5881G>T substitution resulting in introduction of a STOP codon. (C) Upper panel shows diagram of exon 8 of EPOR gene. The coding sequence is shown as solid box and the 3’ untranslated region as an open box. The position and size of genomic PCR amplification products and a Tru9 I(T) restriction map of the 493-base pair (bp) PCR amplification product for the mutant allele are shown. Lower panel shows detection of the mutation by restriction endonuclease digestion of PCR-amplified genomic DNA. Part of the pedigree is shown at the top of the figure. Genomic DNA amplification products were digested with Tru9 I and fractionated by electrophoresis in a 2% agarose gel. The mutation creates a unique Tru9 I site in mutant allele and yields fragments of 369 bp and 124 bp in addition to the 493-bp fragment from the normal allele in infants heterozygous for the new mutation. None of the unaffected individuals (open symbols) have Tru9 I site in their genomic DNA, whereas all affected individuals in the family (filled symbols) are heterozygous for the mutation. (D) Epo dose-response of 32D cells transfected with wild type (WT) or mutant (ME) EpoRs. The cells were cultured in the indicated concentrations of Epo (U/mL) and the results are expressed as a percentage of maximal proliferation in 10 U/mL Epo as determined by MTT assay. Each data point represents the mean of 4 determinations with SE bars shown. Similar results were obtained in several experiments using multiple independent single cell clones of transfected cells.


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