A Translocated Erythropoietin Receptor Gene in a Human Erythroleukemia Cell Line (TF-1) Expresses an Abnormal Transcript and a Truncated Protein

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We previously identified a translocation breakpoint in exon 8 of the erythropoietin receptor (EpoR) gene in TF-1 cells, a cell line derived from a human erythroleukemia. To investigate the potential pathogenetic significance of this abnormality, we more precisely mapped the breakpoint within exon 8 and studied the expression of the translocated gene by S1 nuclease mapping of EpoR transcripts and chemical crosslinking of labeled erythropoietin (Epo) to TF-1 cell surface receptors. Transcripts from the abnormal gene were found to be highly expressed in relation to normal EpoR transcripts in TF-1 cells. The breakpoint predicted by S1 mapping of abnormal EpoR transcripts agreed closely with that determined by Southern analysis. Chemical cross-linking of 125I-Epo to TF-1 cells showed an abnormal, low-molecular-weight cross-linked species directly recognized by anti-Epo antibodies and present in considerable excess over the normal EpoR. Karyotype analysis showed that each of 10 TF-1 cell metaphases had, in addition to multiple other alterations, one chromosome 19 with additional chromosomal material translocated onto the short arm at 19p13.3, the location of the EpoR gene. We conclude that the structurally abnormal EpoR gene in TF-1 cells is highly expressed and produces an abnormal protein. We speculate that the chromosomal material brought into the EpoR locus by translocation is responsible for the high level of expression. We hypothesize that this translocation participated in the evolution of the erythroleukemia from which TF-1 cells were derived.

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MATERIALS AND METHODS

Materials. TF-1 cells were provided to us by Toshio Kitamura (DNAX, Palo Alto, CA). These cells were maintained in culture as previously described. 23,24 Human erythroleukemia (HEL) cells 26 were purchased from the American Type Culture Collection (Rockville, MD). Cell culture media, reagents, and sera were purchased from Gibco-BRL (Gaithersburg, MD). Reagents for polymerase chain reaction (PCR) were from Perkin-Elmer Cetus (Norwalk, CT). PCR primers were synthesized by the Microchemical Facility of the Institute of Human Genetics (University of Minnesota, Minneapolis, MN). Radioactive nucleotides were purchased from Amersham (Arlington Heights, IL). Genomic DNA clones from the human EpoR gene were provided by L. Penny and B.G. Forget (Yale University, New Haven, CT). 27 The probes have been described previously. 22 Total cellular RNA from cultured human erythroid progenitor cells

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(CPU-E) was provided by S.B. Krantz and Amitritha Wickrema (Vanderbilt University, Nashville, TN). The plasmid template used to synthesize the S1 nuclease protection probe was ER-2, a previously described human EpoR cDNA. Monoclonal antiphosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc (Lake Placid, NY). Polyclonal anti-Epo and anti-MaiE antibodies were prepared as previously described. Anti-EpoR antibodies were a pool of sera from two rabbits: one immunized with a recombinant glutathione-S-transferase-EpoR extracellular domain fusion protein and the other immunized with a recombinant MaiE-EpoR intracellular domain fusion protein.

Southern analysis. DNA was extracted from TF-1 and peripheral blood leukocytes using standard techniques. DNA (10-20 μg) was digested with restriction enzymes purchased from Bethesda Research Laboratories (Gaithersburg, MD), fractionated by electrophoresis in agarose gels, transferred to Nytran membranes (Schleicher and Schuell, Keene, NH), and hybridized to 32P-labeled DNA probes as described. Autoradiography was performed using Kodak X-OMAT AR film (Kodak, Rochester, NY).

1. Nuclease analysis. Total cellular RNA of cultured TF-1 and HEL cells was isolated using RNazol (Cinna/Biotex, Friendswood, TX). The S1 nuclease protection assay used in this study is a modification of the method described by Berk and Sharp and Sambrook et al. A uniformly labeled, defined-length, double-stranded DNA probe was synthesized by PCR under the following conditions: 5 μL 10X buffer from the GeneAmp kit (Perkin-Elmer Cetus), 5 ng template plasmid DNA, 1 μL 10 mmol/L deoxyadenosine triphosphate (dATP), 1 μL 10-mmol/L deoxythymidine triphosphate (dTTP), 1 μL 10-mmol/L deoxyguanosine triphosphate (dGTP), 20 μL α-32P-labeled deoxyxycytidine triphosphate (dCTP) (100 Ci/mL, specific activity at 400 Ci/mmol), 0.1 μg of each oligonucleotide primer, 2.5 U Taq polymerase enzyme, and water to 2.5 μL. The following temperature cycles were used for amplification: 94°C for 8 minutes, 15 times (94°C, 1 minute, 60°C for 2 minutes, and 72°C, 3 minutes). Unincorporated deoxynucleotide triphosphates were removed by Sephadex G-50 column chromatography (Pharmacia, Piscataway, NJ). The template was the ER-2 plasmid. The primers were Z114:5'-AGATCGGCTGCGATCCGCGGAGCAGCC-3' (EpoR cDNA sequence) and Z1123 5'-TAATAGACTGACTATAGGGCGGA-3' (the T7 promoter sequence).

RNA samples to be studied were coprecipitated with the double-stranded DNA probe (5 × 106 cpm per hybridization) in 70% ethanol and 0.3 mol/L sodium acetate. After washing in 70% ethanol, the RNA/DNA pellet was resuspended in 30 μL hybridization buffer (40 mmol/L PIPES (pH 6.4), 1 mmol/L EDTA (pH 8.0), 0.4 mol/L NaCl, 80% formamide) and overlaid with mineral oil in a microcentrifuge tube. The hybridization was performed in a PTC-100 programmed thermal controller (MJ Research, Watertown, MA) with the following sequence: 94°C for 20 minutes to denature the probe and RNA secondary structure with cooling to the predetermined hybridization temperature (62°C) for 12 to 16 hours. Probe reannealing was minimal at the optimized hybridization temperature. After hybridization, 300 μL ice-cold S1 nuclease solution [0.28 mol/L NaCl, 0.05 mol/L sodium acetate (pH 4.5), 4.5 mmol/L ZnSO4, 20 μg/mL single-stranded carrier DNA, 150 to 200 U/mL S1 nuclease] was rapidly added. S1 nuclease digestion was performed for 90 minutes at 45°C and stopped by adding 80 μL of the stop mixture [4 mol/L ammonium acetate, 50 mmol/L EDTA (pH 8.0), 50 μg/mL yeast tRNA]. The protected fragments were extracted once with phenol/chloroform (1:1), precipitated in ethanol, and analyzed by electrophoresis in a 4% polyacrylamide/7.6 mol/L urea gel. Autoradiography was used to image the signals.

Chemical cross-linking. [32P]-Epo was cross-linked to TF-1 cells as previously described. UT-7 cells, a human leukemia cell line, were used as a control. UT-7 cells express 7000 EpoR with a k.d. of 0.2 nmol/L, whereas TF-1 cells express 1630 EpoR with a dissociation constant of 0.4 nmol/L. UT-7 cells have an amplified EpoR gene, but EpoR structure and function are apparently normal. For the cross-linking experiments, the cells were grown in a minimum essential medium (aMEM) containing 10% fetal calf serum and 2.5 ng/mL recombinant human GM-CSF. The cells were labeled with [125I]-Epo, cross-linked with 10 mmol/L 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) in the presence of 20 mmol/L sulfo-NHS (Pierce, Rockford, IL). After 20 minutes on ice, the remaining cross-linker was blocked with 0.1 mol/L ethanolamine buffered with acetic acid to pH 8.0. The cells were washed and solubilized using 1% Triton X-100 as previously described. Extracts from 25 × 105 cells were immunoprecipitated using either anti-Epo, anti-MaiE, or antiphosphotyrosine antibodies. After boiling in Laemmli sample buffer, the samples were analyzed by electrophoresis in a 7.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) followed by autoradiography. Alternatively, SDS and β-mercaptoethanol-denatured material was chromatographed through a Sephadex G50 column to remove β-mercaptoethanol and reimmunoprecipitated using either anti-Epo or anti-EpoR antibodies.

Karyotype analysis. For cytogenetic analysis the cell line was subjected to in situ culture and robotic harvesting. Metaphases were stained with quinacrine mustard and with Leishman's stain after trypsinization. In addition, some metaphases were stained by

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Figure 1: Restriction endonuclease map of the EpoR translocation breakpoint in TF-1 cell DNA. The maps of the normal and abnormal EpoR genes in TF-1 cells are summarized schematically. Exons are indicated by solid black rectangles. The maps in the upper part of the figure were derived by Southern analysis of TF-1 cell genomic DNA, using a variety of genomic DNA probes. The enlarged maps in the lower portion of the figure were obtained using a probe from exon 8. The positions of selected restriction endonuclease sites are shown. The region of the abnormal EpoR gene to which the translocation breakpoint has been localized is bordered by arrows.
distamycin A/DAPI banding. The karyotypes were expressed in accordance with the International System for Human Chromosome Nomenclature.18

RESULTS

The restriction endonuclease map of the abnormal EpoR gene in TF-1 cells is shown in Fig 1. These data were obtained by Southern analysis of TF-1 genomic DNA using an exon 8 probe (probe 4 in an earlier report15). The restriction map of the abnormal EpoR gene, 5' to exon 8, is identical to that of the normal gene(s), also present in TF-1. The high resolution map (enlarged, lower portion of Fig 1) localizes the breakpoint to a segment of exon 8 between a normally placed Neo I site and an abnormal Hph I site. The breakpoint is, therefore, 5' to the translation termination codon. If this rearranged gene is expressed as a protein, the structure of the carboxyl terminus would be affected.

To assess whether the abnormal EpoR gene is expressed, we studied EpoR transcripts in TF-1 cells by S1 nuclease mapping. Our strategy is shown in Fig 2A. We chose a hybridization probe derived from the 3' end of EpoR cDNA, including the entire region encoded by exon 8 and part of exon 7. This probe was synthesized by PCR as an internally labeled, defined-length, double-stranded DNA molecule, using a plasmid template containing the ER-2 EpoR cDNA (see Materials and Methods). The probe was denatured and hybridized to TF-1 and control RNA samples under conditions to favor DNA:RNA hybrid formation. After S1 nuclease digestion, the protected fragments were separated using denaturing polyacrylamide gel electrophoresis, and autoradiographs were made. Figure 2B shows the results of this experiment. The DNA probe includes some plasmid sequences so that the probe is larger (959 nucleotides) than the fragment protected by full-length EpoR mRNA (911 nucleotides). Lane P contains the undigested probe. Lane C gives the results of hybridization to RNA from normal human CFU-E. The protected fragment is the size predicted for normal full-length EpoR mRNA. Lane t is a negative control hybridization using yeast tRNA. Lane T shows the results for RNA from TF-1 cells. In addition to a faint full-length protected fragment, there is a very prominent signal at 380 nucleotides. Lane H shows the S1 nuclease analysis of HEL cell RNA. Interestingly, the protected probe fragment in lane H is slightly (approximately 20 nucleotides) shorter than the full-length control. The basis for this subtle abnormality is unknown. The nucleotide sequence of the
EpoR gene coding sequence in HEL cells that corresponds to the S1 nuclease probe is normal (data not shown).

The breakpoint position determined by restriction site mapping (Fig 1) led us to predict that, if the abnormal gene is expressed, there would be an abnormal protected fragment on S1 analysis of 340-480 nucleotides. The observed 380 nucleotide species (Fig 2) is in close agreement with our prediction, leading us to conclude that this protected probe fragment represents highly abundant transcripts of the rearranged gene EpoR. Furthermore, the breakpoint can be precisely mapped by the S1 nuclease protection experiment, given that the 5′ end of the S1 nuclease probe is known. The breakpoint determined by this method is 303 nucleotides 5′ to the translation termination codon. Therefore, approximately 100 amino acids at the carboxyl terminus of the EpoR are lost or replaced in the protein encoded by the translocated EpoR gene.

To assess whether the EpoR present on the TF-1 cell surface is the product of the translocated EpoR gene, chemical cross-linking of 125I-Epo to TF-1 cells was performed using EDAC. Figure 3 shows the results of this experiment. EDAC cross-linked two proteins to Epo on the cell surface of UT-7 cells (lane 1): the larger, at 120 kD, represents the 85-kD protein seen in cross-linking experiments with DSS in UT-7 cells and other hematopoietic cell lines; the other, at 105 kD, corresponds to the cloned EpoR chain that exhibits a molecular mass of 71 to 75 kD in UT-7 cells. The latter is immunoprecipitated with anti-EpoR antibodies, whereas the former is not. The 100-kD protein cross-linked to Epo using DSS (140 kD before correcting for Epo) is not detected in experiments using EDAC with UT-7 cells. Control anti-MaIE antibodies did not precipitate a significant quantity of radioactivity (Fig 3A, lanes 2 and 5), whereas antiphosphotyrosine antibodies precipitated both the 85-kD and 71-kD Epo cross-linked proteins (Fig 3A, lane 3). We have previously shown that the 85-kD protein was not tyrosine phosphorylated, but was coimmunoprecipitated with the tyrosine phosphorylated 71-kD protein.

Interestingly, TF-1 cells exhibit very little cross-linking to the 71-kD EpoR protein compared with UT-7 cells (Fig 3A, lane 4). In contrast, cross-linking to the 85-kD protein, not thought to represent the cloned chain but rather an accessory component of the EpoR, is virtually the same as in UT-7 cells. In addition, TF-1 cells possess an abnormal, low-
molecular-weight, cross-linked protein of 80 kD, or 46 kD after correction for Epo. In contrast to UT-7 cells, most EpoR in TF-1 cells are not tyrosine phosphorylated (Fig 3A, lane 6); counting of immunoprecipitates showed that 26% of receptor-bound radioactivity was precipitated by anti-phosphotyrosine antibodies in UT-7 cells and only 4.8% in TF-1 cells. The 46-kD cross-linked protein in TF-1 cells was not precipitated by antiphosphotyrosine antibodies. In TF-1 cells only a faint band (Fig 3A, arrow), corresponding to p85, was detected in antiphosphotyrosine precipitates. This protein could be tyrosine phosphorylated in TF-1 cells. Alternatively, it could be coimmunoprecipitated with the low amounts of p71 EpoR expressed in these cells or with other tyrosine phosphorylated proteins.

We concluded that the 46-kD Epo cross-linked protein is the product of the highly expressed, abnormal EpoR gene in TF-1 cells. To confirm this hypothesis, we performed immunoprecipitations with anti-EpoR antibodies (Fig 3B). Anti-Epo-precipitated material was denatured by boiling in SDS and β-mercaptoethanol-containing buffer. The β-mercaptoethanol was removed by gel filtration, and Triton X-100-containing buffer was added. The extracts were then reimmunoprecipitated using either anti-EpoR (lane 2) or anti-Epo (lane 3) antibodies. Figure 3B shows that the 46-kD and the 71-kD proteins were directly recognized by anti-EpoR antibodies. Therefore, the 46-kD protein corresponds to a shorter form of the cloned EpoR chain. Most likely it represents the product of the translocated EpoR gene in TF-1 cells. As previously reported, the 85-kD protein was not immunoprecipitated by anti-EpoR antibodies (Fig 3B, lane 2) but was precipitated by anti-Epo antibodies (lane 3).19

To confirm our suspicion that the abnormal EpoR gene in TF-1 cells results from a translocation affecting the EpoR locus of chromosome 19, karyotype analysis was performed using TF-1 cell metaphase chromosomes. The karyotype was 50–70.XY,+Y,add(1)(p32),–2,add(3)(p11),add(3)(q12),add(7)(q32),–8,–10,–11,–12,add(14)(p11.2),–17X2, +add(19)(p13.3),add(19) (q13.1),–21,–22X2,+12–21mar. A representative karyotype is shown in Fig 4. In each of 14 metaphase cells analyzed, there was an abnormal chromosome 19 with additional chromosomal material translocated onto the short arm at p13.3, the approximate location of the EpoR gene. This abnormality probably represents the "19p+" that was noted in a previous report.23 Interestingly, this translocation has been stable in TF-1 cells and was seen in every metaphase studied. There were multiple additional chromosome abnormalities in each metaphase. Based on these data, we cannot exclude the possibility that the abnormal EpoR gene has been relocated to another site and, therefore, no longer resides on chromosome 19.

**DISCUSSION**

In this report, we present evidence that the abnormal EpoR gene in TF-1 cells is highly expressed as a transcript and as a protein. We also show that this abnormality is associated with a consistent alteration of chromosome 19p [+add(19)(p13.3)] in TF-1 cells. We speculate that this abnormality was involved in the evolution of erythroleukemia in the patient from whom the cell line was derived.

Another recent report describes an abnormal EpoR gene in a human leukemia cell line, UT-7.18 In UT-7 DNA, the EpoR gene rearrangement maps outside of the coding region. Evidence indicates that the EpoR gene is amplified in UT-7. The authors propose that abnormalities of the EpoR gene are not rare events in murine and human erythroleukemia.

Although the oncogenic potential of the murine EpoR is well established, little evidence suggests that the EpoR gene is commonly abnormal in human erythroleukemia. Cytogenetic analysis of erythroleukemia in humans does not detect frequent abnormalities of chromosome 19p.30 Therefore, it seems doubtful that translocations of the type affecting the EpoR in TF-1 cells are common. Nevertheless, the coincidence of high-level EpoR expression in two human leukemia cell lines with EpoR gene abnormalities is striking. The possibility that more subtle mutations of the EpoR may occur in erythroleukemia has not been excluded. The slight but reproducible, abnormality of the HEL cell EpoR mRNA by S1 analysis (Fig 2) raises the prospect that this third human cell line might have an abnormal EpoR. More study of de novo human erythroleukemia specimens is warranted, to detect both gross and subtle EpoR gene abnormalities.

What accounts for the high level of EpoR expression in TF-1 cells? The multiple copies of chromosome 19 in many metaphases do not explain the high expression of the abnormal gene that is often present in a single copy per cell. We hypothesize that abnormally high expression results from the translocation event itself. Perhaps genetic material introduced into the EpoR locus activates EpoR gene transcription through an ectopic enhancer. Analysis of the DNA beyond the breakpoint will, hopefully, answer this question.

How might the abnormal EpoR in TF-1 contribute to leukemogenesis? Our data suggest that the translocation leads to a frameshift in the translational reading frame and a truncated EpoR protein. S1 protection mapping showed that the last (carboxyl end) 100 amino acids of the resulting EpoR protein would be lost. The primary Epo cross-linked protein in TF-1 cells, p46, of is of a size consistent with this prediction. Moreover, this region of the EpoR was previously shown to contain most, if not all, of the phosphorylatable tyrosines.41 As shown in Fig 3, p46 is not immunoprecipitated by anti-phosphotyrosine antibodies, in contrast with the full-length EpoR of UT-7 cells.

Because the carboxyl end of the EpoR appears to contain a negative regulatory domain,24 p46 might be hyperfunctional. However, this may not be sufficient to explain its role in leukemogenesis. In a Finnish kindred with familial erythrocytosis, there is a mutation in the EpoR gene, leading to a premature termination codon and (presumably) a truncated protein.4544 There is no evidence of leukemia or other adverse phenotypes reported in affected individuals. We speculate that the translocated EpoR gene in TF-1 cells represents one of several steps in leukemogenesis. Perhaps this rearrangement arose after other molecular events had already destabilized the genome. Functional analysis of the abnormal EpoR in TF-1 cells promises to shed light on these questions.

Finally, we emphasize that TF-1 cells are frequently used as an experimental model for normal EpoR structure, function, and signal transduction. In light of our evidence that
the majority of EpoR in TF-1 cells are the product of the abnormal gene, TF-1 cells are not a valid model for the physiologic EpoR.

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