Serum Form of the Erythropoietin Receptor Identified by a Sequence-Specific Peptide Antibody

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The present investigation was undertaken to search for soluble forms of the erythropoietin receptor in human serum using polyclonal antibody against an amino terminal peptide sequence in the extracellular domain. This sequence was located adjacent to the amino terminus at residues 25-38. When this antibody was used for Western blots of solubilized membranes from nucleated bone marrow cells, a protein consistent with native erythropoietin receptor was seen. Purified soluble ectodomain of the erythropoietin receptor displayed appropriate reactivity with this antibody. When sera from normal subjects and patients with a range of hematologic disorders were examined by Western blotting, a protein with a molecular mass of 34 Kd was detected in sera from patients with enhanced erythropoiesis including sickle cell anemia, thalassemia, and megaloblastic anemia. This protein was rarely detected in normal serum but appeared when normal subjects were treated with recombinant erythropoietin and disappeared after full treatment of patients with megaloblastic anemia due to vitamin B12 deficiency. The protein was not detected after myeloablation for bone marrow transplantation but appeared with marrow engraftment. Reactivity of this protein with the peptide antibody was competitively inhibited by the amino terminal peptide sequence. An additional 48 Kd protein was detected that showed minimal variation in intensity with differing degrees of erythropoietic activity. Detection of this protein could not be inhibited by the addition of synthetic peptide. Our findings indicate the presence of a soluble form of the erythropoietin receptor related to the extracellular domain that is highly correlated with enhanced erythropoiesis.

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ERYTHROPOIESIS is dependent on the sequential maturation of stem cells to mature red blood cells. This erythropoietic process includes passage through at least three progenitor stages, the early burst forming unit (BFU-E), a subsequent colony forming unit (CFU-E), and the stage of late progenitors. These maturation stages are subject to regulatory influences of a number of hemopoietic growth factors, including stem cell factor, interleukin-3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF), insulin-like growth factor I, and insulin but one of the most important regulatory factors is erythropoietin (EPO). The effect of EPO is highly dependent on the stage of the erythropoietic development. EPO provides a proliferative signal to BFU-E, a differentiation signal to the CFU-E, and a signal to maintain cellular viability of the late progenitors by limiting apoptosis. These different influences of EPO are mediated by interaction with specific erythropoietin receptors (EPOR) on the erythroblast surface.

Molecular studies have afforded a rapid advance in our knowledge of the structure and function of EPOR. The full length sequence of both murine and human EPOR has been determined by molecular cloning. The full length human erythropoietin receptor (EPOR-α) is a transmembrane protein containing 508 amino acids. The protein is about equally divided by a small 22 amino acid membrane spanning region into an extracellular amino terminal domain containing 236 amino acid residues. The human cDNA encodes a 66 Kd protein. The EPOR shows striking similarities to other receptors belonging to the hemopoietin/growth factor superfamily including those for IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, G-CSF, growth hormone, and prolactin. These similarities pertain in the extracellular domain to four closely spaced cysteine residues and a highly conserved tryptophan-serine-x-tryptophan-serine (WSXWS) motif in the region adjacent to the cell membrane.

Despite the rapid advances in our knowledge of the structure and function of EPOR, clinical applications have not yet been identified. Structural abnormalities in the EPOR have been postulated to occur in polycythemia vera resulting in an exaggerated sensitivity to EPO and there are a number of hematologic disorders with failed or disordered erythropoiesis and elevated serum EPO levels that may be attributable to quantitative or qualitative abnormalities of EPOR. One aspect of EPOR physiology that would be of potential clinical interest is the presence in serum of secreted or soluble forms. For example, measurement of serum transferrin receptor has provided a clinically valuable indirect measure of total erythropoiesis. Several receptors in the hemopoietin superfamily including IL-4, IL-5, IL-6, IL-7, IL-18, G-CSF, GM-CSF, and prolactin have been shown to have naturally occurring alternatively spliced mRNA encoding for potentially secreted soluble forms. A soluble form of the leukemia inhibitory factor (LIF) receptor has been identified in murine serum. In addition, the growth hormone receptor, IL-2 receptor, IL-6 receptor, and the prolactin receptor have all been shown to have naturally occurring soluble forms in serum that provide clinically useful information. For example, the soluble IL-2 receptor has been shown to correlate with tumor mass in lymphoreticular malignancy, the soluble growth hormone receptor has been used to categorize growth hormone deficiency states, and the soluble form of the IL-6 receptor found in the serum of human immunodeficiency virus (HIV)-infected patients has been suggested to have an immune modulatory function. The present study was undertaken to determine whether the presence and nature of a...
human EPOR was kindly provided by Dr Simon Jones of Genetics Institute, Boston.

Preparation of peptide antibody. A peptide sequence was selected from that deduced for the full length EPOR on the basis of regional hydrophilicity, proline richness, and a paucity of intrapeptide lysines. The peptide sequence excluded those in the region of the conserved cysteines, the WSXWS motif, other conserved regions, and the truncation site in the IL-2 receptor amino acid sequence aligned with the murine EPOR sequence. The selected peptide sequence consisted of 14 amino acid residues. The sequence APPPNLPDPKFESK, designated S25, was located at positions 25-38 immediately adjacent to the amino terminus at the cleavage point of the hydrophobic leader sequence. The terminal lysine was chosen to facilitate linkage to KLH by means of glutaraldehyde. The S25 sequence was synthesized, purified, and used to produce polyclonal antisera as previously described.

To characterize the reactivity of the peptide antibody, human bone marrow was obtained from subjects undergoing harvest for allogeneic bone marrow transplantation. Nucleated cells were obtained by density separation on Ficoll-Hypaque. A cell membrane fraction of these cells was obtained by sonication in ice-cold HBSS containing several protease inhibitors including 1 mmol/L PMSF, 50 pmol/L EP475, 50 pmol/L pepstatin A, and 1 mmol/L 1,10 phenanthroline. The cell membranes were pelleted by ultracentrifugation and solubilized in either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer or HBSS containing 0.001 mol/L tris and 1% teric (HBSSTT). A whole cell fraction was also obtained by solubilizing nucleated bone marrow circulating form of EPOR may be helpful in the clinical assessment of erythropoiesis.

MATERIALS AND METHODS

Materials. Hank's buffered salt solution (HBSS), HEPES, phenylmethylsulfonyl fluoride (PMSF), disopropyl fluorophosphat (DIFP), 1,10 phenanthroline, pepstatin A, tris [hydroxymethyl]aminomethane (tris), prestained molecular mass markers, 4-chloro-1-napthol, keyhole limpet hemocyanin (KLH), polyoxyethylene 9 lauryl ether (teric), and Freund's adjuvant were obtained from Sigma (St Louis, MO). EP475 was kindly provided by Dr H. Nagase of the Department of Biochemistry and Molecular Biology at the University of Kansas Medical Center. Additional prestained molecular mass markers were obtained from Bio-Rad Laboratories (Richmond, CA). Peroxidase conjugated goat IgG to rabbit IgG was obtained from Dako (Carpinteria, CA). Polyacrylamide gradient gels and nitrocellulose membranes were obtained from Bio-Rad Laboratories. A purified recombinant soluble ectodomain of the
Fig 3. SDS-PAGE and Western blot of purified recombinant EPOR ectodomain. (A) Electrophoretic mobility of purified EPOR ectodomain (2 µg) under nonreducing (lane 2) and reducing (lane 3) conditions. The gel was stained with Coomassie blue. (B) Western blot with S25 peptide antibody of purified EPOR ectodomain (1 µg) after electrophoresis under nonreducing conditions. Individual lanes had included during the primary antibody incubation incremental amounts of peptide against which the antibodies were produced. The concentrations of peptide (ng/mL) added to respective lanes in parentheses were 0 (2), 100 (3), 250 (4), 500 (5), 1,000 (6), 5,000 (7), 20,000 (8), 50,000 (9), and 100,000 (10). Apparent molecular mass indicators (Kd) are present in lane 1 of both panels.

cells at 4°C in HBSSTT containing the protease inhibitors listed above. Following overnight agitation, the particulate matter was removed by ultracentrifugation. To further characterize the reactivity of the peptide antibody, purified recombinant EPOR ectodomain was used.

Biochemical procedures. Various marrow cell fractions, purified recombinant EPOR ectodomain, and human sera were subjected to SDS-PAGE on 4% to 20% gradient gels as described by Laemmli. The gels were electroblotted as described by Burnette. The nitrocellulose membranes were blocked by incubation with 1% nonfat dry milk in phosphate-buffered saline (PBS) for 1 hour and incubated with peptide antibody (whole antisera diluted 1:200 for all studies except for the recombinant ectodomain evaluation and for the competitive binding studies with the serum) in 0.1% nonfat dry milk in PBS for 2 hours. After washing, the membrane was incubated with goat antirabbit IgG conjugated to horseradish peroxidase (diluted 1:5,000) in 0.1% nonfat dry milk in PBS for 2 hours. After washing the membranes, immunoreactive material was detected following the addition of 4-chloro-1-naphthol as a substrate for the peroxidase. Commercial molecular weight markers were used and apparent molecular weights are indicated on the figures. The staining intensity of one of the proteins detected by Western blotting varied markedly in different human sera. To compare the results with different sera, a scoring system based on staining intensity was used. No visible band was scored 0, a maximum reaction was scored 2, and values of 0.5, 1, and 1.5 were used to represent intermediate intensities.

Specificity of the Western blot findings was established by competitive blots during which incremental amounts of the synthetic peptide were added in the primary antibody incubation step. This approach was used to study both the purified EPOR ectodomain and human serum.

Fig 4. Western blot with S25 amino-terminal peptide antibody of serum from normal subjects before (lanes 2 and 4) and after (lanes 6 and 8) the administration of EPO in therapeutic concentrations. Apparent molecular mass indicators (Kd) are present in lanes 1, 3, 5, and 7. The 34 Kd protein in lanes 6 and 8 were scored 1½.
Fig 5. Western blot with S25 peptide antibody of serum from a patient with sickle cell anemia known to contain the 34 Kd protein. Apparent molecular mass indicators (Kd) are present in lane 1. Individual lanes had included during the primary antibody incubation incremental amounts of peptide against which the antibodies were produced. The concentrations of peptide added (ng/mL) to respective lanes in parentheses were 0 (2), 100 (3), 200 (4), 500 (5), 1,000 (6), 2,000 (7), 5,000 (8), 10,000 (9), 20,000 (10), 50,000 (11), and 100,000 (12).

Amino acid sequence analysis was performed after initial electrophoresis on 7.5% to 20% gradient sequencing grade gels. The samples were then transblotted onto polyvinylidene difluoride membranes by the method of Matsudaira.4 Amino acid sequencing was performed at the Protein Sequencing Facility of the Department of Biochemistry and Molecular Biology at the University of Kansas Medical Center.

Clinical samples. Sera were obtained from normal volunteer subjects and patients with a variety of hematologic disorders. All samples were obtained from participants in investigations that had been approved by the human subjects committee at the University of Kansas Medical Center. Informed consent was provided in accordance with the Declaration of Helsinki. In 21 normal subjects, sera were obtained before and following the administration of 100 U/kg recombinant human EPO subcutaneously on 10 days over a 2-week period. Studies were performed on sera from anemic patients with iron deficiency (17), chronic renal failure on maintenance dialysis (9), chronic inflammatory disease (18), sickle cell anemia (11), β thalassemia major (3), and megaloblastic anemia due to vitamin B12 deficiency (6). In the latter patients, follow-up sera were obtained after correction of the deficiency state. Sera were also obtained from eight patients undergoing bone marrow transplantation. The initial samples were obtained immediately following the myeloablation procedure and subsequent samples following marrow administration. All sera were diluted 1:2 in normal saline before analysis by Western blotting. Two μL of diluted serum was electrophoresed for each sample.

RESULTS

When antisera were reacted with solubilized membranes from nucleated bone marrow cells, a protein with a molecular mass of 65 Kd was detected. An example of the reaction of the S25 antiserum with membrane solubilized in either HBSSTT or sample running buffer is shown in Fig 1.

The supernatant obtained by solubilization of whole nucleated bone marrow cells differed from the results seen with cell membranes. The supernatant contained a protein with a molecular mass of 34 Kd that showed a strong reaction with the S25 antiserum (Fig 2). When this material was subjected to amino acid sequence analysis, its identity with
the EPOR ectodomain was confirmed as indicated below where EPOR residues 25-34 are shown along with the first 10 amino acids of the protein:

Solubilized cells  A X P N L P X P K  
EPOR  A P P N L P D P K  

(X = non-identified residues)

In addition, a small peptide was detected with a sequence identical to that of human EPO at amino acid position 118-121.

Solubilized cells  S P P D  
EPO  A I S P P D A A S A

The purified ectodomain\(^{36}\) on SDS-PAGE migrated as a predominant 30 Kd moiety and a minor 60 Kd protein under nonreducing conditions and as a single 30 Kd protein on reduction (Fig 3A). S25 peptide antibody showed strong reactivity with this purified EPOR ectodomain. This reactivity could be effectively blocked by the addition of synthetic peptide during the primary antibody incubation (Fig 3B). This confirms the specificity of the EPOR ectodomain.

In all human sera examined, a protein with a molecular mass of 48 Kd was readily detected (Fig 4). A second smaller protein band with a molecular mass of 34 Kd was detected in sera from patients with enhanced erythropoietic activity. This band was absent from normal sera but could be readily seen when normal subjects were administered a course of recombinant EPO to enhance erythropoietic activity (Fig 4). When sera containing this protein band were analyzed on SDS-PAGE under reducing conditions, no change in the apparent molecular mass was seen (data not shown). When competitive Western blots were performed, detection of the 48 Kd protein was not inhibited by the addition of synthetic peptide during the primary antibody incubation (Fig 5). Detection of the 34 Kd protein by S25 antiserum was progressively inhibited by the incremental addition of synthetic amino-terminal peptide (Fig 5).

The 34 Kd protein was rarely detected in normal sera and was similarly absent in most patients with iron deficiency anemia (data not shown), the anemia of chronic disease (Fig 6A), and the anemia of chronic renal failure (Fig 6B). When a patient with the anemia of chronic renal failure was treated with recombinant EPO, this protein became markedly apparent (Fig 6B). This 34 Kd protein band was very prominent in patients with enhanced erythropoiesis including those with sickle cell anemia (Fig 6C), thalassemia major (data not shown), and untreated vitamin B\(_12\) deficiency (Fig 7). Interestingly, the band disappeared in the latter when normal erythropoiesis was restored following a course of parenteral vitamin B\(_12\) (Fig 7).

The results of the scoring system to estimate the quantitative reactivity of the 34 Kd protein band in different clinical states is shown in Fig 8. An absent band was scored 0, a maximum reaction was scored 2, and values of 0.5, 1, and 1.5 were used to represent intermediate intensities. Of 29 normal sera, a faint reactivity was observed in only 5. In 18 patients with the anemia of chronic disease, the protein band could be detected in 5 and in 3 of these the band was highly visible. In contrast with these findings in normal subjects and patients with hypoproliferative anemia, an intense reactivity of the 34 Kd peptide was invariably observed in patients with enhanced erythropoiesis.

Serial measurements on sera from patients undergoing a bone marrow transplant permitted studies of the effect of erythropoiesis on anti-EPOR antibody reactive protein within the same patient. The 34 Kd protein was not visible immediately following the marrow ablation procedure, first appearing some weeks following transplantation at the time of engraftment (Fig 9). The patient shown was also evaluated with serum transferrin receptor concentrations. These were measured by our monoclonal enzyme-linked immunosorbent assay (ELISA)\(^{22}\) and are known to accurately reflect the erythroid progenitor mass.\(^{23}\) The appearance of a prominent 34 Kd protein at day 16 post-transplant coincided with a brisk increase in the serum transferrin receptor concentration. Indeed, in the other transplant cases studied, there was a strong correlation between the 34 Kd protein and the serum transferrin receptor concentrations (data not shown).

DISCUSSION

The key observation in the present investigation was the detection of a 34 Kd protein in human serum that is highly correlated with enhanced erythropoiesis. This protein was detected with antibody against a sequence of the amino terminus of the receptor ectodomain in the region most remote from the cell membrane. This antibody was shown in a number of ways to be specific for EPOR, including appro-
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Fig 8. Scatterplot of the visually determined intensity of the 34 Kd protein on Western blot with S25 amino terminal peptide antibody of sera of normal subjects and those with a number of conditions characterized by altered erythropoietic activity.

Recent molecular studies of EPOR in normal bone marrow, small quantities could be detected and this was also true in various hypoproliferative anemias (Fig 8). Very high quantities were observed in hyperproliferative states characterized by either effective erythropoiesis as in patients with sickle cell anemia or in normal subjects or those with the anemia of chronic renal failure following recombinant EPO or by ineffective erythropoiesis as seen in patients with megaloblastic anemia or thalassemia.

Fig 9. Western blot with S25 amino terminal peptide antibody of sera obtained longitudinally from a patient undergoing bone marrow transplantation. With the day of transplant designated day 0, the days studied were -1 (lane 2), 0 (lane 3), 1 (lane 4), 2 (lane 6), 5 (lane 6), 6 (lane 7), 7 (lane 8), 8 (lane 9), 9 (lane 10), 12 to 14 (lanes 12 to 14), 16 (lane 15), 19 (lane 16), 21 (lane 17), 27 (lane 18), 29 (lane 19), and 33 (lane 20). Molecular mass indicators are shown in lanes 1 and 11. From top to bottom, these represent 226, 119, 96, 81, 62, 45, and 38 Kd, respectively. Scores from lanes 15 to 20 of the 34 Kd protein were 2, ½, 1½, 1, ½, 1½.
The body developed to a fusion molecule of the EPOR-ectodomain of EPOR-s mRNA and a proteolytic cleavage fragment. The current data are unable to distinguish between a protein derived from EPOR that provides a quantitative marker of erythropoiesis. Assuming that the late progenitor (CD34+, CD7-), two distinct populations were seen in relation to EPOR expression. Early progenitors (CD34+, CD7+) express predominantly a membrane-bound EPOR with a truncated cytoplasmic domain containing only 56 amino acids (EPOR-t). This form of the receptor apparently conveys a mitogenic signal but not a signal for protection against programmed cell death. This arises as a consequence of a 95 bp α insert corresponding to the intron between exon VII and VIII. Late progenitors (CD34+, CD7+) express predominantly a membrane-bound EPOR with a full-length cytoplasmic domain (EPOR-f) that conveys both a mitogenic signal and a signal protecting against apoptosis. Of relevance to the present study is the finding of a third form of receptor mRNA that encodes a potentially secreted form of the receptor termed EPOR-s. This mRNA is expressed in fixed proportion to the total EPOR mRNA and is produced as a consequence of a 104 bp α insert due to the presence of an imperfect splice acceptor site in the 5′ flanking region of exon V resulting in an additional 46 amino acids after position 195. It would be predicted that the ectodomain of all three forms of EPOR would be detected by the S25 antisera used in the present study because the amino terminus of EPOR-t, EPOR-f, and EPOR-s are all the same. Therefore, the current data are unable to distinguish between a protein product of EPOR-s mRNA and a proteolytic cleavage fragment of either EPOR-f or EPOR-t. This distinction must await isolation of this circulating protein and carboxy terminal amino acid sequence analysis. Likewise, the origins of the 34 Kd moiety detected in solubilized marrow also awaits further biochemical identification.

One limitation of the present study was the reliance on a semiquantitative visual estimate of the amount of the clinically relevant 34 Kd protein. A clearer definition of the role of this protein in assessing erythropoiesis could be obtained by the development of a quantitative ELISA for the serum form of EPOR. The development of such an assay could potentially provide a quantitative measure of different stages of erythropoietic development. The recent introduction of the assay that detects the truncated transferrin receptor in serum has provided a useful index of the size of the late progenitor pool. Assuming that the 34 Kd peptide derived from EPOR provides a quantitative marker of either early progenitor or total progenitor activity, the use in tandem of measurements of the serum transferrin receptor and serum EPOR may be a useful tool to assess the number and stage of maturation of red blood cell precursors. In this regard, a recent preliminary report using a polyclonal antibody developed to a fusion molecule of the EPOR-ectodomain with glutathione-S-transferase in an ELISA system has described the presence of immuno-detectable EPOR in human sera.

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