Characterization of Erythropoietin Isolated from Rat Serum: Biochemical Comparison of Rat and Human Erythropoietins

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We isolated erythropoietin (Epo) from anemic-rat serum with 1.3 x 10⁶-fold purification and 38% recovery using immunoaffinity chromatography. The isolated Epo migrated in SDS polyacrylamide gel with a molecular size of 37 kDa. Biological properties of rat Epo were compared with those of human Epo using target cells of primate and murine origins. When murine cells were used as target cells for assaying Epo, rat Epo stimulated proliferation of the cells with a 50% lower potency than did human Epo. The activity of rat Epo on human cells was only 25% of that of human Epo. Studies of Epo binding to the receptor indicated that rat and human Epo were not distinguishable in binding to murine cells; however, rat Epo bound to the receptor on human cells with an affinity much lower than that of human Epo. Rat Epo was digested with N-glycanase. Complete removal of N-linked sugars converted the native Epo to the deglycosylated form with 18 kDa. The in vitro activity of deglycosylated Epo was 2.5-fold higher than that of the native Epo.

Erythropoietin (Epo) is a major physiological stimulator of erythropoiesis. Human Epo has been isolated from urine of anemic patients and its properties including the primary structure have been well characterized. Cloning of the cDNA and the gene of human Epo has made it possible to produce a large amount of recombinant Epo, which has greatly contributed to further characterization of human Epo. The Epo cDNA of monkey has also been cloned.

The primary structures of rodent Epos have been deduced from the nucleotide sequence of the cloned mouse gene or that of rat cDNA. The amino acid sequence is highly conserved between primate and rodent Epos but some differences have been confirmed. When we compare the primary structures of rodent and human Epos, replacement of 29 and 35 amino acid residues of the mature Epo consisting of a total of 166 residues is found in rat and mouse Epo, respectively. Although mouse Epo has been purified extensively from the serum of anemic animals or isolated from the culture medium of erythroleukemia cells, only limited information on its biological properties is available. Human and mouse Epos act on target cells from both human and mouse sources but their biological properties including binding to the receptor have never been compared quantitatively. Thus it is not known whether these structural differences between primate and rodent Epos influence their biological properties.

Most of our knowledge of Epo has come from human urinary Epo and recombinant human Epo. Little is known of the serum Epo that functions in erythropoietic tissues in vivo. Tam et al. have shown, using isoelectric focusing, that there are differences between serum and urinary Epos in human, rat, and mouse. Thus the isolation and characterization of serum Epo are important for better understanding the biological properties of Epo. We, therefore, decided to isolate Epo from rat serum, because rats are of far greater use than mice in providing a large volume of serum as a source for Epo purification. The finding that the monoclonal antibodies against human Epo bind to mouse and rat Epos with high affinities has made it possible to isolate serum Epo from these animals with a high yield using immunoaffinity chromatography.

In this paper, we describe the isolation of the Epo protein from sera of anemic rats and its properties. These properties were compared with those of human Epo using Epo-responsive cells from mouse and human sources.

Materials and Methods

Recombinant human Epo, antibodies, and Epo protein assay by ELISA. Production of recombinant human Epo, isolation, and radioiodination were as previously described. Monoclonal antibodies (R2 and R6) against recombinant human Epo were prepared as previously described. R2 was used for the preparation of an immunosorbent column, and both antibodies were used for ELISA. Rabbit anti-human Epo antiserum was obtained by administration of the isolated recombinant human Epo as an antigen. Epo protein was measured by a sandwich-type ELISA as previously described using recombinant human Epo as the standard, which had been standardized with the Second International Standard Reference Preparation for human Epo.

Purification of rat Epo and amino acid acidized Epo. Wistar rats aged three weeks were fed an Fe-deficient diet (Oriental Yeast, Japan) and deionized water for three weeks; their hematocrit values were 20-25% and serum Epo concentrations were 5-10 U/ml. Animals were anesthetized with ether.

Abbreviations: Epo, erythropoietin; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetate; PBS, 10 mM NaPi, pH 7.2, containing 0.15 M NaCl; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; GM-CSF, granulocyte/macrophage colony-stimulating factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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and blood was withdrawn from an abdominal vein. After incubation of blood for 1 h at 37°C and for 12 h at 4°C, the serum was obtained by centrifugation. To 600 ml of anemic rat serum, 60 ml of a mixture containing 0.5% Tween 20, 0.1 M EDTA, and 0.2% NaN₃ was added. The serum was mixed with the antibody (R2)-fixed Affi-Gel 10 in a volume ratio of 40 to 1. Tubes, each containing 40 ml of serum, were rotated slowly for 6 h at 4°C, allowing Epo to bind with the antibody. The gel was washed with 5 volumes of PBS, 0.15 M NaCl, and 0.5 M NaCl in this order. All washings contained 0.05% Tween 20 and 0.02% NaN₃. Epo bound to the gel was eluted with 15 ml of 0.2 M acetate, pH 2.5, containing 0.15 M NaCl and 0.05% Tween 20. The eluate was immediately neutralized with 3 M Tris. The neutralized solution was mixed with 15 ml of PBS containing 0.05% Tween 20 and then the mixture was mixed again with 0.4 ml of the antibody-fixed gel. The eluate containing Epo was obtained by repeating the above procedures. The neutralized eluate (2.5 ml) was put on a phenyl-silica reverse-phase HPLC column (4.6 × 250 mm, Cosmosil 5 Ph column from Nacalai Tesque, Japan) that had been equilibrated with water containing 0.1% (v/v) trifluoroacetic acid and 0.05% (v/v) triethylamine (equilibration buffer). The column was developed by increasing the concentration of acetonitrile from 0 to 60% in the equilibration buffer at a flow rate of 1 ml/min at a gradient rate of 1%/min. The fractions containing Epo protein were collected and concentrated with a Centri-Concentrator (TOMY, Japan). The concentrated solution was lyophilized twice with an addition of 50% acetonitrile. The lyophilized material was dissolved in PBS containing 0.05% Tween 20. For assay of the biological activity, the Epo solution was diluted by PBS containing 0.1% bovine serum albumin. For analysis of the amino acid sequence, the lyophilized material was dissolved in 50% acetonitrile. The amino acids in the NH₂-terminal region of rat Epo were sequenced with an ABI 477A automatic sequencer (Applied Biosystems, U.S.A.), using about 10 μg of rat Epo.

Electrophoresis and Western blotting. To examine the purity of the Epo preparations, proteins were separated by electrophoresis in SDS-polyacrylamide gel with a 10–20% acrylamide gradient and stained with 2D-Silver Stain II (Dai-ichi Chemicals, Japan). Native and N-glycanase-digested proteins on the SDS–polyacrylamide gel were identified by Western blotting. Samples (each about 8 ng of protein) were fractionated by electrophoresis, and the fractionated proteins were electrically transferred to nitrocellulose paper with a semi-dry blotting cell (Trans-Blot SD from Bio-Rad, U.S.A.) containing a buffer that consisted of 48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol. The blot was allowed to react first with rabbit anti-recombinant human Epo antiserum that had been diluted 1000-fold, and then with sheep anti-rabbit IgG antiserum (Vector Lab., U.S.A.). The Epo protein was detected with a ECL detecting kit according to the manufacturer's directions (Amersham, Japan).

N-Glycosidase digestion of rat Epo. N-Linked sugars of rat Epo were removed by incubating Epo (20 ng) with 100 mU N-glycanase (Genzyme, U.S.A.) for 36 h at 37°C in 10 μl of 100 mM NaPi, pH 8.0, containing 20 mM EDTA and 0.1% Tween 20. Completion of the digestion was confirmed by altered mobility during SDS–polyacrylamide gel electrophoresis; Epo was detected by Western blotting after electrophoresis.

In vitro biological activity of Epo. The in vitro biological activity of Epo was tested with both murine and human cell lines. A murine cell line, EP-FDC-P2, the growth of which was dependent on Epo or murine IL-3, was maintained in RPMI 1640 medium (GIBCO, U.S.A.) containing 10% fetal calf serum (Whittaker M. A. Bioproducts, U.S.A.) and 20 ng/ml recombinant human Epo. A human cell line, TF-1, the growth of which was dependent on Epo or GM-CSF, was maintained in RPMI 1640 medium containing 10% fetal calf serum and 5 ng/ml recombinant human GM-CSF (Genzyme, U.S.A.). The cells maintained in the growth medium were washed four times with RPMI 1640 medium containing 10% fetal calf serum to remove the growth factor and were suspended in the same medium. The cells were cultured in microtiter wells (5 × 10⁴ cells/well for 18 h of EP-FDC-P2 and for 36 h of TF-1) containing either rat Epo or human Epo at various concentrations (1–100 pg/ml). Cell growth and survival upon Epo addition were assayed colorimetrically using the cleavage of MTT (Sigma, U.S.A.) by living cells as previously described. Three wells per assay were cultured, and the averaged absorbance values at 600 nm were plotted against the logarithmic concentrations of Epo. The activity was estimated from a parallel line assay.

Results and Discussion

Isolation of rat Epo

Rat Epo was purified from 600 ml of serum of anemic animals with the immunosorbent gel on which the recombinant human Epo-directed monoclonal antibody was fixed. It has been confirmed that this antibody binds with rodent Epo as well as human Epo. After two rounds of purification with the immunosorbent gel, the Epo preparation was chromatographed with a phenyl-silica reverse-phase HPLC column. Figure 1 shows the elution profiles of Epo and protein, which were detected by ELISA and by measurement of absorbance at 280 nm, respectively. Epo was eluted as a single but somewhat broad peak, probably due to a microheterogeneity of the attached sugar chains. The ratio of Epo to protein in each fraction was nearly constant, indicating that the sample was homogeneous. Homogeneity of the sample was confirmed by SDS–polyacrylamide gel electrophoresis (lanes 3 and 4). Rat Epo migrated with a size of 37 kDa, a similar size to that

Fig. 1. HPLC of Rat Epo.

The Epo preparation, purified after two cycles of the immunosorbent column chromatography, was put on an HPLC column and Epo was eluted by increasing acetonitrile concentrations. Solid bar indicates Epo protein measured with ELISA. (---) absorbance at 280 nm, and (---) acetonitrile concentration.

Fig. 2. SDS–Polyacrylamide Gel Electrophoresis of the Purified Rat Epo.

Lane 1, rat serum (1.4 μg protein); lane 2, an eluate (3 μg protein) from the first immunosorbent gel in two rounds of purification using the monoclonal antibody–fixed immunosorbent gel; lane 3, the final Epo preparation (50 ng Epo); lane 4, the final Epo preparation (500 ng Epo). The gel was silver-stained.
of human Epo. The Epo protein appears to be somewhat insensitive to staining with silver; we had to apply 500 ng of the protein to find a distinct band (compare lanes 3 and 4). Purification with an immunosorbent gel was very effective; an Epo band could be clearly seen in the eluate from the first immunosorbent gel (lane 2). The recovery of Epo through all purification procedures was 38%, based on an assay by ELISA, with 1.3 x 10^6-fold purification. Approximately 12 μg of the pure Epo was obtained.

Amino acid sequences of rat Epo

Fifty amino acid residues in the NH_2-terminal region of rat Epo were NH_2-APPRILXDSRVLEYILEKEAEEXVTMGXAEGRPLSEIXTVPDKTYNFYA, where X could not be identified. This sequence was identical to that deduced from the nucleotide sequence of the cloned cDNA, which validated the cloned cDNA. The unidentified X residues were deduced from the nucleotide sequence; positions 7 and 29 were cysteine, and positions 24 and 38 were Asn. These two Asn residues and Asn at position 83 are N-glycosylation sites.

Enzymatic removal of N-linked sugars from rat Epo

Human Epo has three N-glycosylation sites at positions 24, 38, and 83, and these sites for N-glycosylation are conserved in mouse and rat Epos. Human Epo has one O-glycosylation site at position 125, but this site is missing in rodent Epos. Ser-126 has been replaced with Pro in mouse Epo and Glu in rat Epo. To find whether rat Epo was indeed N-glycosylated, rat Epo was digested by N-glycanase. Native and digested Epos were detected by Western blotting after SDS-polyacrylamide gel electrophoresis (Fig. 3). After digestion, the native Epo of 37 kDa completely disappeared, indicating that the deglycosylation reaction was completed. One new band of 18 kDa in rat Epo appeared. This size was nearly equal to that calculated from the amino acid sequence based on cDNA.

The in vitro biological activity of rat Epo before and after N-glycanase digestion, was compared using the MTT assay of target cells. When mouse cells were used for the assay, removal of N-linked sugars from rat Epo yielded a product with 2.5-fold higher activity than the undigested Epo. Deglycosylation of recombinant human Epo causes an increase in affinity to the receptor. We have not examined the binding of deglycosylated rat Epo to the receptor but it is likely that the deglycosylation-induced increase of rat Epo in the activity is due to an increase in affinity for the receptor.

It has been established that N-linked sugars of Epo are essential for expression of the in vivo activity but it has been a debatable subject whether these sugars are required for the in vitro activity. The results presented here with rat Epo also are consistent with our view that N-linked sugars in Epo affect binding affinity of the ligand to the receptor but do not play a key role in expression of the in vitro activity. Recent papers support our conclusion.

In vitro biological activity of recombinant human Epo and rat Epo

Human Epo is biologically active in mice and rats and recombinant mouse Epo produced by COS-1 cell acts on human erythroid precursor cells. The cross-reactivity between primate and rodent Epos, however, has not been measured. Isolation of rat Epo allowed us to address this subject using erythroleukemia cells, the growth of which was dependent on Epo. We here used an Epo-dependent mouse cell line (EP-FDC-P2), because of the lack of a rat cell line. The amino acid sequence of rat Epo receptor is highly homologous to mouse receptor (94% homology). The in vitro activity of rat Epo on mouse cells was approximately 50% of that of human Epo. When human cells (TF-1) were used as target cells, rat Epo was even less active.

![Fig. 4. Binding of Rat and Human Epo to Murine and Primate Target Cells.](image)

Binding of 125I-recombinant human Epo to the receptor was assayed with murine (mouse TSAB) and primate (human TF-1) erythroid cell lines. Inhibition of the binding by the unlabeled forms of pure rat Epo and human Epo was examined to estimate their affinity. The ordinate indicates the binding of 125I-human Epo in the presence of unlabeled Epo at concentrations shown in the abscissa. (□) TSAB and rat Epo, (□) TSAB and human Epo, (●) TF-1 and Epo, and (●) TF-1 and human Epo. Total bindings of 125I-human Epo measured in the absence of the unlabeled human Epo were 9913 cpm of TSAB and 8977 cpm of TF-1. The nonspecific bindings measured in the presence of 200-fold unlabeled human Epo were 1132 cpm of TSAB and 503 cpm of TF-1; the specific bindings were 8781 cpm of TSAB and 8472 cpm of TF-1, which were defined as 100% of specific binding. The concentration of 125I-Epo was 1.2 nM. Concentrations of the unlabeled Epo that gave 50% inhibition of the specific binding were estimated from curves and are shown in the inset. The IEP value (aeratiek in the inset) of rat Epo on human cells was calculated from the extrapolated curve.
potent, only 24% of the human Epo activity. To identify one of the possible causes for these differences in biological activity, the binding of Epo to its receptor on the cells was examined. As shown in Fig. 4, apparent affinities of Epos to target cells were estimated from the inhibition curves of binding of 125I-recombinant human Epo to the receptor by unlabeled Epos. In binding with mouse cells, rat Epo had an affinity similar to that of human Epo; the difference in the biological activity between rat Epo and human Epo on mouse cells was not due to the difference in their affinity to the receptor. In contrast, rat Epo bound to human cells with a much lower affinity than did human Epo; the affinity of rat Epo to human receptor was about one-fourth that of human Epo. This lower affinity of rat Epo to human receptor could be at least partly responsible for the low biological activity of rat Epo on human cells compared with human Epo. Human Epo bound to mouse and human cells with a similar affinity. These differences between human and rat Epos may reflect the hierarchy of species in the ligand-receptor relationship. Further investigation is needed to assign the specific structural differences to the different biological properties between murine and primate Epos.

There are differences in electric charges between serum and urinary Epos in human, rat, and mouse, suggesting that the urinary Epo may be a modified form of the serum Epo. Thus the isolation procedures of serum Epo with a high efficiency will be beneficial to future comparison with urinary Epo.

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References