High-Level Expression and Purification of a Recombinant Human Erythropoietin Produced Using a Baculovirus Vector

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Conditions presently have been established for the high-level expression and simplified purification of recombinant human erythropoietin produced in Spodoptera frugiperda cells. Expression, as mediated by infection with a recombinant baculovirus, was accomplished in suspension culture using reduced levels of serum and media supplements experimentally determined to provide optimum levels of factor production (500,000 U/L). Purification of this recombinant human erythropoietin to virtual homogeneity (≥99%) was accomplished via a three-step procedure involving isocratic elution from DEAE-Sepacel, reverse-phase high performance liquid chromatography (HPLC) on a C4 medium, and the single-step elution of purified hormone from concanavalin A agarose. Overall, an 890-fold purification was accomplished with a recovery of 80% as assayed in vitro. Biologically, this purified erythropoietin is highly active, possessing a specific activity in vitro of 200,000 U/mg protein. Chemically, this erythropoietin (molecular weight [mol wt] 26,200) appears exceptionally uniform in its oligosaccharide constitution (30%) as contrasted with heterogeneously glycosylated erythropoietins derived from mammalian cells (mol wt 30,000 to 38,000; 40% to 50% complex-type oligosaccharide). Thus, human erythropoietin as presently produced in an insect cell line comprises not only an abundant source of highly active, readily purified hormone for studies of its mechanism of action and cell surface receptor, but also represents a uniquely homogeneous form that should prove advantageous for direct structural analyses.

MATERIALS AND METHODS

Cell culture and expression of recombinant erythropoietin. Spodoptera frugiperda cells (ATCC) were maintained at 28°C in TMN-FH medium. 16% fetal calf serum (FCS). For the expression of recombinant erythropoietin, cells were grown in spinner flasks to a density of 10^6 cells/mL, collected by centrifugation, and incubated at 10°C cells/mL for one hour with clonal recombinant baculovirus.19 Following subsequent culture for three days in Grace's medium, 4% FCS, 20% TMN-FH, spent medium was collected, concentrated 15-fold (tangential flow system, 10,000 mol wt PLGC filter) and dialyzed against 1 mmol/L Na2EDTA, 10 mmol/L PIPES, pH 6.8.

Purification of erythropoietin. Concentrated media initially was passed over a column of DEAE-Sepacel (Pharmacia, Piscataway, NJ) (1 mL gel/mL dialyzed media) under isocratic conditions in 0.02% octyl-beta-D-glucopyranoside, 10 mmol/L K2HPO4, pH 6.8. Column eluate and two column volumes of buffer wash were collected, pooled, and concentrated 40-fold. Concentrate was subjected directly to reverse-phase high performance liquid chromatography (HPLC) on a C4 medium (Supelcoil LC-304, 25 cm x 1 cm column). Samples were applied in 0.06% trifluoroacetic acid as an initial mobile phase, and were chromatographed using a linear gradient of acetonitrile (30% to 50%) in 0.06% trifluoroacetic acid. Eluted fractions were collected into vials containing 5 μL of 1% octyl-beta-D-glucopyranoside, and were dried under vacuum. HPLC fractions that were shown by bioassay and by Western blot analyses to contain erythropoietin were resolubilized in 1 mol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L MnCl2, 1 mmol/L CaCl2, 0.02% octyl-beta-D-glucopyranoside, 10 mmol/L PIPES pH 6.8, and were applied to a column of concanavalin A agarose (Sigma Chemical Co, St Louis) (approximately 1 mL gel/mg protein). The column was washed with 100 mL of the above buffer, and erythropoietin was eluted in buffer containing 250 mmol/L alpha-methyl-D-mannoside. Subsequently, this eluate was dialyzed at 4°C against 0.01% octyl-beta-D-glucopyranoside.

Biologic assay of erythropoietin. The biologic activity of various preparations was determined by quantifying the erythropoietin-dependent incorporation of [H]thymidine into splenocytes derived from the Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA.

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from mice treated with phenylhydrazine. Assays were standardized against the World Health Organization Second International Reference Preparation, calibrated recombinant human erythropoietin standards, and purified recombinant human erythropoietin produced in CHO cells (160,000 U/A260 Amgen, Thousand Oaks, CA). The activity of purified hormone also was assayed in vitro using a CFU-e colony forming assay and in vivo in ex-hypoxic, polycythemic mice.

Electrophoresis and protein assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli in 12% gels. In specified instances, laser densitometry of silver-stained gels was used to verify purification factors and recoveries. Western blot analyses were performed essentially as described by Towbin using antibodies prepared against recombinant human erythropoietin produced in CHO cells. Protein assays were performed using BCA reagent (Pierce Chemical, Rockford, IL) and chromatographically purified BSA (Cappel, Malvern, PA) as a standard.

RESULTS

Expression of recombinant erythropoietin by S frugiperda cells. In experiments designed to maximize the expression of recombinant erythropoietin in media with reduced protein content, the effects of culturing infected S frugiperda cells in limiting amounts of FCS and media supplements were assessed. Expression in media containing FCS at concentrations of <4% was diminished substantially. Also, erythropoietin produced under these conditions was composed of a mol wt 26,000 form, plus two alternate forms of lower molecular weight (mol wt 24,000 and 22,000). As shown below, this was established in part by Western blot analyses using antibodies prepared against purified human erythropoietin. In contrast, a singular form of recombinant erythropoietin (mol wt 26,000) was expressed by S frugiperda cells cultured in media containing >4% FCS. Also, a significant increase in levels of secreted erythropoietin (ie, 500,000 U/L at 72 hours postinfection; Table 1) was achieved by supplementing media with yeast and lactalbumin hydrolysates at 20% of the nominal levels used in routine cell culture. Evidence that secretion proceeded efficiently under these conditions was given by the lack of detectable amounts of immunoreactive erythropoietin in cell lysates.

Purification of recombinant human erythropoietin. The purification of recombinant human erythropoietin to virtual homogeneity was accomplished via three chromatographic steps. First, erythropoietin-containing media (500,000 U/L, Table 1) was concentrated, dialyzed, and passed over a column of DEAE-Sephalcel under isocratic conditions (0.02% octyl-beta-D-glucopyranoside, 10 mmol/L K2PIPES, pH 6.8). Erythropoietin eluted directly with high recovery (>95% of initial activity), while approximately 98% of the total protein contained in dialyzed media was retained. Thus, this simple procedure provides an initial 45-fold purification.

Material recovered following chromatography on DEAE-Sephalcel was concentrated, and was chromatographed directly on a reverse-phase C4 medium. As established by bioassay, erythropoietin eluted in 44% acetonitrile, with an estimated recovery of >95% of active hormone (Fig 1). No biologically or immunologically active hormone was detected in any other portion of the elution profile. Routinely, the injection of 60,000 U to 100,000 U per run resulted in a similar elution pattern and recovery of hormone (Table 1). Analysis of this material by SDS-PAGE showed that several contaminating proteins co-eluted from C4 medium with erythropoietin, which was estimated by densitometry of silver-stained gels to represent 15% of total protein. By comparison, erythropoietin represented 5% of total protein before C4 HPLC. Thus, this step provided a threefold purification. A sixfold further purification of erythropoietin was accomplished simply by reconstituting this material in buffer containing NaCl at a high molarity (ie, >1 mol/L). Under these conditions the majority of contaminating proteins were insoluble (approximately 85% of total protein) and were removed readily by brief microcentrifugation. In contrast, erythropoietin was fully soluble at concentrations as high as 200,000 U/mL as was established by its reconstitution at increasing concentrations, and subsequent biologi-
Gel Slice Number

B

Fig 2. Analysis of purified erythropoietin by SDS-PAGE. The purity of erythropoietin as isolated by chromatography on DEAE-Sephacel, C4 medium, and concanavalin A agarose was assessed by SDS-PAGE (12% gel, reducing conditions) and silver-staining. Scanning laser densitometry (right panel) was used to confirm purity (>99%). Molecular weight standards (mol wt x 10^-6) are indexed in the left margin.

cal assay. Thus, the purity of this reconstituted preparation typically was >90%, with a recovery of >90% (Table 1).

Final purification of this recombinant erythropoietin to virtual homogeneity was accomplished using a single-step lectin affinity procedure. Reconstituted material derived from C4 HPLC was adsorbed to concanavalin A agarose, and purified erythropoietin was eluted in 250 mmol/L alpha-methyl-D-mannoside. The purity of this material was >99% (200,000 U/mg protein), with a recovery of >95% (Table 1, Fig 2). The activity of this product was determined by in vitro proliferation assays and a CFU-e colony-forming assay. However, preliminary assays using ex-hypoxic, polycythemic mice indicate that this erythropoietin possesses little, if any, activity in vivo.

Characterization of minor forms of erythropoietin produced under alternate conditions of culture. As mentioned above, under optimized culture conditions (ie, 4% serum, 20% TMN-FH), a single form of recombinant human erythropoietin (mol wt 26,000) was produced by S frugiperda cells. However, the nature of the two lower molecular weight forms produced under alternate conditions of culture (see above) was of some interest. First, the biological activity of the mol wt 24,000 form was shown to approximate that of the mol wt 26,000 erythropoietin. This was shown by separating the two forms by SDS-PAGE, eluting each from gel slices, and subjecting gel slice eluates to biological assay and Western blot analysis (Fig 3). Biological assay indicated that approximately equivalent activities were associated with the mol wt 26,000 and 24,000 erythropoietins (Fig 3B). Western blotting confirmed the efficient separation of these forms, plus their approximately equivalent abundance and recovery from gel slices (Fig 3A). Evidence that the difference between these two higher mol wt forms is attributable primarily to differential glycosylation was given by experiments in which these purified erythropoietins were treated with N-glycanase. Treatment with N-glycanase reduced the molecular weight of each form to the approximate molecular

Fig 3. Isolation of mol wt 26,000 and 24,000 erythropoietins and comparison of specific bioactivities. Erythropoietin expressed under low serum conditions (1% FCS) and purified via reverse-phase HPLC (approximately 90% purity) was subjected to SDS-PAGE in the absence of 2-mercaptoethanol. Alternate forms were visualized by Western blotting (A, lane A). A parallel gel lane was sectioned into 1 mm slices, and erythropoietins then were eluted into 0.5 mL of phosphate-buffered saline containing 0.02% Tween 20 and 0.1 mg/mL BSA. Eluates then were subjected to biological assay (B), and to Western blot analysis (A) in order to confirm recoveries and separation.
RECOMBINANT HUMAN ERYTHROPOIETIN

Fig 4. Analysis of N-glycosylation of mol wt 26,000 and 24,000 erythropoietins using N-glycanase. Purified erythropoietins (350 U) expressed under low serum conditions (1% FCS) were incubated with N-glycanase (3 U) in 0.25% SDS, 0.25 mol/L phosphate pH 8.6, at 37°C for 18 hours. Products (+) were analyzed by SDS-PAGE in the absence of 2-mercaptoethanol, and were visualized by silver-staining. A control sample (-) was processed in the same manner in the absence of N-glycanase.

weight of aglycosyl apo-erythropoietin (mol wt 18,400) as determined by SDS-PAGE (Fig 4), thus indicating that each form is N-glycosylated, and is not processed proteolytically beyond its secreted form. However, a minor residual difference in the electrophoretic mobilities of these endoglycolytic products (mol wt 18,900 ν 18,400) was detectable. The basis for this difference presently is unresolved. The third form of erythropoietin (mol wt 22,000, Fig 4, lane A) was found to bind poorly, if at all, to concanavalin A agarose. Due to its low abundance, the specific biological activity of this form has not been estimated directly. However, based on the above observations, this erythropoietin is believed to possess a third distinct oligosaccharide constitution.

In order to provide independent estimates of molecular weight and relative saccharide content, the Stoke’s radius (2.56 nm) and sedimentation coefficient (2.48 x 10⁻¹³ seconds) of the purified mol wt 26,000 form were determined (Fig 5, Table 2). A molecular weight of 26,200 is predicted from these values. Thus, the estimate of relative saccharide content (30%) is in approximate accordance with estimates based on SDS-PAGE.

Fig 5. Determination of Stoke’s radius and sedimentation coefficient of human erythropoietin produced in S. frugiperda cells. The Stoke’s radius (A) of insect erythropoietin was evaluated by HPLC gel filtration chromatography (G2000 SWXL 30 cm x 0.78 cm column, Supelco). Column calibration was performed using DNA (Sigma type III), bovine serum albumin, ovalbumin, and cytochrome C standards (O—O). The elution position of erythropoietin (O) was confirmed by Western blot analysis. Evaluation of the sedimentation coefficient (2.48 x 10⁻¹³ sec; B) was estimated by rate sedimentation in a 5% to 20% sucrose density gradient. Purified erythropoietin (O) was sedimented with standards (O—O; bovine serum albumin and cytochrome C) 18 hours at 40,000 rpm in a SW50.1 rotor. Fractions (0.16 mL) were collected and analyzed by SDS-PAGE.

DISCUSSION

Systems previously developed for the production of recombinant human erythropoietin have depended on the use of mammalian expression systems and a variety of cell lines including COS cells, baby hamster kidney cells, Chinese hamster ovary cells, and psi-2 cells. As is the case with urinary erythropoietin, recombinant hormone produced in mammalian cells is heterogeneously glycosylated, with complex N- and O-linked oligosaccharides comprising as much as 50% of total molecular mass. In contrast, human erythropoietin as presently prepared in S. frugiperda cells is exceptional in both its comparably simple saccharide constitution, and its associated relative homogeneity. In general, the limited capacity of insect cells to process N-linked oligosaccharides to complex types results in a predominance of neutral oligosaccharides of the high mannose type. The prospect that at least certain oligosaccharides of the human erythropoietin presently prepared are of this type is sup-
This conclusion is based first on the observation that treat-ment with 26,000 erythropoietin (Fig 2) is evidenced further by the apparent high uniformity among molecules of this mol-ecule. 

The lack of in vivo activity is a likely explanation for the absence of sialic acid in insect-derived saccharide structures. The absence of sialic acid in insect-deriv- ed saccharide structures is a likely explanation for the lack of in vivo activity. Previously reported procedures for the purification of human recombinant erythropoietins generally have been based on methods developed by Miyake et al for the isolation of human urinary erythropoietin. Commonly, these procedures involve the use of five or more separate conventional chromatographic steps. However, this approach requires the use of a proprietary monoclonal antibody, the denaturation of erythropoietin with SDS for efficient epitope recognition, and at least two additional conventional chromatographic steps. In contrast, the procedure described here achieves >99% purity via three simple chromatographic steps (Table 1). An 890-fold purification is accomplished with ≥80% recovery of bioactive erythropoietin. While this manuscript was in preparation, a procedure for the purification of recombinant human erythropoietin expressed in BHK cells was reported, which involves three distinct chromatographic procedures, i.e., sequential chromatography on CM Affi-Gel blue, Mono Q (FPLC), and a C1/C8 reverse-phase medium. This procedure resulted in a 160-fold purification yielding a product with a specific activity of 84,000 U/mg. In contrast to the present procedure, gradient elution of products was required at each step. In addition, the overall reported recovery of this product was substantially lower (ie, 35%), and its final purity was not fully assessed.

The baculovirus-mediated expression system presently developed for the production of recombinant human erythropoietin provides a convenient source of milligram amounts of highly active growth factor. Whereas the simple glycochemical properties of this erythropoietin have facilitated its purification to virtual homogeneity, it is anticipated that this unique human erythropoietin also should facilitate direct structural analyses aimed at identifying physicochemical and structural features essential to its bioactivity.

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