Purification of Murine Erythropoietin Produced in Serum-Free Cultures of Erythroleukemia Cells

By R.-L. Qian, K. Chin, J.-K. Kim, H.-M. Chin, J. Cone, and W.D. Hankins

We previously documented that several erythroleukemia cell lines released factors that stimulated erythropoiesis in vivo and in vitro. A simple five-step scheme has been devised that allows purification of this erythropoietic activity to apparent homogeneity. The methods employed included lectin affinity chromatography (wheat germ agglutinin), gel filtration (ultra gel ACA44), ion exchange, hydroxylapatite, and high performance liquid chromatography. Following polyacrylamide gel electrophoresis, biologic activity was recovered in an area corresponding to a molecular weight of 35,000 daltons. Silver staining of a polyacrylamide gel after electrophoresis of our most purified preparation revealed a single band at 35,000 daltons.

RESULTS

Lectin chromatography. 2,000 mL of conditioned medium was applied directly to wheat germ agglutinin column (2.5 cm x 27 cm; bed volume was about 100 mL) at a flow rate of 24 mL/hr. When loading was completed, the column was washed with 300 mL of PBS, following which the Epo was eluted with PBS containing N-acetyl glucosamine (0.5 mol/L). The activity and optical density (OD) profile is shown in Fig 1. Routinely, greater than 50% of the activity was recovered in a peak that trailed the second optical density peak. Specific activities for the WGA-purified material was around 27.6 U/μL. The peak fractions were pooled, dialyzed against water, lyophilized, and stored at −70°C. This material was referred to as Step I Epo. In other experiments, Epo was eluted with a gradient of N-acetylglucosamine (not shown). Neither the recovery nor the purity of Epo was improved with the gradient elution.

Gel filtration. Lyophilized Step I Epo was dissolved in 5 mL PBS (PH 7.4) and loaded onto an ACA44 gel filtration column with dimensions of 2.5 cm x 84 cm and a void volume of 130 mL. The column had been standardized with albumin (66,000 d), ovalbumin (43,500 d), and ribonuclease A (13,700 d). As shown in Fig 2, Epo activity was recovered between the ovalbumin and chymotrypsin peaks. The average molecular weight of the Epo peak was 35,000 to 36,000. The Epo peak was divided into three fractions referred to as head (tubes 46 and 47), middle (tubes 48–57), and tail (tubes 58–61). By pooling these three fractions, the recovery of Epo from gel filtration was in excess of 90%, a specific activity of 316.52 U/μL. However, subsequent chromatographic procedures and PAGE analysis revealed that by saving only the middle pool, a high molecular protein of 66,000 (probably albumin) was virtually eliminated as a contaminant. The specific activity in this pool was 652.92 U/μL (Table 1) and the recovery was 70.2%. This material was referred to as Step II Epo and was dialyzed against water, lyophilized, and stored at −70°C.

Ion exchange chromatography. 100 to 200 units of Step II material was dissolved in water and loaded onto a mono Q anion column of Pharmacia’s Fast Performance Liquid
Chromatography instrument. Preliminary analytical runs employing a continuous linear gradient of NaCl revealed that Epo was eluted between 0.14 mol/L NaCl to 0.25 mol/L NaCl with a peak at 0.18 mol/L NaCl. Therefore, in this and subsequent runs, the proteins were eluted with a discontinuous gradient of NaCl in a 0.01 mol/L Tris-HCl buffer (PH 7.5), as shown in Fig 3. Epo was eluted in two unequal peaks. Less than 5% of the erythropoietic activity was found in the small peak (0.1 mol/L NaCl). This material was not further analyzed. Greater than 70% of the activity was routinely recovered in 0.3 mol/L NaCl, and had a specific activity of 1,112.50 units/ml. This material was dialyzed against water, lyophilized, and stored at −70 °C as Step III Epo.

Hydroxylapatite chromatography. 300 to 600 units of Step III material was added to a hydroxylapatite column that had been equilibrated in 0.02 mol/L phosphate buffer, PH 6.8. A gradient of increasing phosphate concentration (0.02 to 0.1 mol/L) was employed to elute the proteins. As shown in Fig 4, three OD peaks were observed. The Epo activity profile was a symmetrical peak that overlapped the second major protein peak. The second small peak of Epo activity at fraction 29 was not reproducible and was, therefore, not included in the pooled fractions. Fractions were pooled, dialyzed, lyophilized, and stored at −70 °C as Step IV Epo. The recovery of activity from this procedure ranged between 50% and 70%.

Neuraminidase treatment and electrophoresis. To characterize the Epo purified to this stage, neuraminidase (0.06 units) was added to a solution of 100 units of Step IV Epo and dissolved in 25 μL of 50 mmol/L sodium acetate buffer, PH 5.2. This mixture was incubated at 37 °C for three hours and then boiled to destroy the neuraminidase. This material was combined with electrophoresis sample buffer, boiled for three minutes, and loaded onto a 10% polyacrylamide gel. A control preparation of Step IV Epo was similarly treated with omission of the neuraminidase. The samples were electrophoresed for four hours at a constant current of 15 mA, after which the gels were cut into 50 horizontal slices (2 mm/slice). Each slice was placed in a 12 × 75 mm glass tube to which was added 1.0 mL PBS, PH 7.4. The gel slices were mashed extensively and allowed to stand overnight at 37 °C. The following morning, the gel matrix was removed by
centrifugation and the supernatant assayed for Epo activity. The activity profile is shown in Fig 5A. The fractions containing Epo activity were pooled, dialyzed, and again subjected to polyacrylamide gel electrophoresis (PAGE). The results, shown in Fig 5b, demonstrate that the biologic activity comigrates with a single band of silver stained material in the molecular weight range of 35,000 daltons. Yanagawa et al observed that neuraminidase treatment of human Epo yielded molecules that migrated faster than nontreated Epo. As seen in Fig 5, neuraminidase-treated murine Epo also migrated slightly ahead of nontreated Epo.

High performance liquid chromatography. 200 units of Step IV material was dissolved in 0.1% TFA and applied to a Supelco LC-8-DB HPLC column. Protein was eluted with a gradient of 30% to 60% acetonitrile in 0.1% trifluoroacetic acid. More than 50% of the activity was recovered in fractions 66–68 (Fig 6). When this material was subjected to PAGE and silver stained (Fig 7), a single band of protein was recovered in a region that corresponded to 35,000 daltons. It should be noted that this gel was “overstained” in order to visualize the Epo bands. Even so, no other proteins were detected although some artifactual staining was observed in

Fig 4. Purification of Step II Epo with hydroxyapatite column (1.5 cm x 5 cm). Flow rate: 2 mL/min.

Fig 5. (A) SDS-polyacrylamide gel electrophoresis and erythropoietin step IV before (solid line) and after (dashed line) neuraminidase treatment. The slices containing maximum erythropoietic activity were eluted, pooled, dialyzed, concentrated, and applied to a second SDS-polyacrylamide gel electrophoresis. In addition, Lane 1. Standards: albumin, egg, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100. Lane 2. Approximately 20 units of neuraminidase treated Step IV murine Epo recovered from 6A in the area from 5.0 to 5.4 cm. Lane 3. Approximately 20 units of untreated Step IV Epo recovered from 6A in the area from 5.4 to 5.8 cm. Lane 4. Six units of purified human EPO (Toyoba Inc. Japan). Following electrophoresis the gel was stained with silver.
questions arose concerning the relation of Epo production to observation of Epo production by erythroid cells, conceptual source of murine Epo has long been needed for research purposes. Growth factors provided both an opportunity for erythropoietic control (lane 5), which received only the marker dye. The starting specific activity was 0.21 U/mg. Sequential procedures that would permit isolation of homogeneous erythropoietin structure and function. We therefore undertook to devise a rapid and simple method, using conventional procedures that would permit isolation of homogeneous murine erythropoietin. As starting material, we used supernatants from erythroleukemia cells cultured in the absence of fetal calf serum for 10 to 14 days. Such conditioned media and which produced a single band upon polyacrylamide gel electrophoresis. The apparent molecular weight of the electrophoretic band was 35,000 daltons. The overall recovery of erythropoietic activity through all the procedures was 5% to 15%. A summary of a typical purification is shown in Table 1.

Both human and sheep erythropoietin have been reported to be glycoproteins. Our finding that neuraminidase treatment of hydroxylapatite purified material increased the electrophoretic mobility of the erythropoietic activity and abolished the in vivo activity are consistent with the interpretation that murine erythropoietin is also a glycoprotein.

The data in this report provide further support for our contention that the erythropoietic factors produced by the erythroleukemic lines are indeed erythropoietin. Since human Epo is the only erythroid regulatory factor that has been purified, sequenced, and molecularly cloned, it seems an appropriate molecule for comparison. Our previous results and those of Choppin et al demonstrated many similarities between human Epo and the factors produced by erythroleukemia cells. For example, all the physical and chemical properties assessed for murine and human Epo have been identical. Furthermore, we demonstrated immunologic cross-reactivity between murine Epo and antiserum raised against human Epo. Since the reported molecular weight of human Epo is approximately 35,000, we can now include molecular weight amongst the similarities between murine and human Epo. Nevertheless, for a complete comparative analysis, the amino acid sequence and the nucleic acid sequence of the corresponding genes will be necessary. Experiments to derive this additional information are presently underway.

REFERENCES


5. Krystal G: A simple microassay for erythropoietin based on
3H-thymidine incorporation into spleen cells from phenylhydrazine treated mice. Exp Hematol 11:649, 1983


Purification of murine erythropoietin produced in serum-free cultures of erythroleukemia cells

RL Qian, K Chin, JK Kim, HM Chin, J Cone and WD Hankins