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Isolation of erythropoietin by monoclonal antibody*

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Introduction

Two factors, burst promoting activity and erythropoietin (EPO), have been reported which stimulate erythroid cell differentiation. Burst promoting activity acts on early committed erythroid progenitor cells and target of EPO is the more matured yet morphologically unidentified precursor cells. Recently, a factor which is distinct from EPO and acts late in erythroid differentiation, primarily on erythroblasts, has been reported (1). It is believed that EPO plays a predominant role in regulating erythropoiesis because of a good correlation between serum levels of EPO and rates of erythrocyte formation. The problems in studying the physiology and the biochemistry of erythropoiesis have been cumbersome, insensitive, and relatively unspecific assay methods and lack of a simple purification method to provide suitable pure preparation of the hormone. Monoclonal antibodies have been shown to be a powerful tool for purification, characterization, and quantitative analysis of macromolecules. In this paper we report the production of hybridomas that secrete monoclonal antibodies against human urinary EPO and isolation of EPO from an urine concentrate with an immunoadsorbent column.

Materials and Methods

All were described elsewhere (2-4).

Results and Discussion

Purification of human urinary erythropoietin —— Human EPO was purified from the

Table 1 Summary of purification of human urinary erythropoietin

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification
Urine concentrate	30939	1806000	58	1
DEAE-cellulose	16111	1596840	99	2
Phenyl-Sepharose	2323	978750	421	7
Hydroxylapatite	672	836600	1245	21
Sephadex G-100	83	249000	3000	51
Sulfopropyl-Sephadex	16	81600	5100	88
Immunoadsorbent columns against contaminants	3	69000	23000	396
Preparative SDS-polyacrylamide gel electrophoresis	1	38000	38000	655

urine of aplastic anemia patients by column chromatography with DEAE-cellulose, Phenyl-

* A part of this paper has been published (S. Yanagawa et al., Agric. Biol. Chem., 47, 1311-1316 (1983)) and the rest has been submitted for publication.
Abbreviation : EPO, erythropoietin.

Sepharose CL4B, Hydroxylapatite, Sephadex G-100, Sulfopropyl-Sephadex, and antibodies against contaminants and subsequently by preparative SDS-polyacrylamide gel electrophoresis (3). Analysis with SDS-polyacrylamide gel electrophoresis revealed one protein band and the distribution of EPO activity measured after extraction of sliced gels was exactly superimposed to the protein. About 650-fold purification was achieved and one mg of purified EPO was obtained from 600,000 ml urine (Table I). Specific activity of the EPO was determined to be about 38,000 by in vitro ^{59}Fe and ^{3}H-thymidine methods. A similar value was also obtained by in vivo assay method. This value was lower than the value of 70,400 reported by Miyake et al. (5) who isolated human urinary EPO with conventional purification methods. The pure EPO obtained here was prepared by extracting protein from SDS-polyacrylamide gel in the last step of purification and therefore denatured protein with less or no EPO activity was possibly included. It is also very likely that the difference in specific activity is due, at least in part, to different procedures for protein measurement.

Erythropoietin-specific hybridoma —— Fusion of spleen cells from the mice immunized with the pure EPO with NS-1 myeloma cells yielded 258 growing hybridomas of the total seeded 264 wells. Cultures demonstrating cell growth were tested for supernatant antibodies by a solid-phase antibody-binding assay, detecting the presence of an EPO specific antibody as an EPO antibody biotinylated anti-mouse IgG avidin biotinylated peroxidase complex. Seven strongly positive (S1-S7) and 12 weakly positive (W1-W12) cultures were found. It was possible that besides these positive cultures there were hybridomas producing other class of immunoglobulins, because the anti-mouse IgG was used for this screening. Screening of the supernatants by solid-phase antibody-binding assay using the mixture of rabbit anti-mouse IgG, IgM, and IgA excluded this possibility.

All positive cultures were expanded in 24-well tissue culture plates. Rescreening of the supernatants with the binding assay before further enlargement of culture in a T-flask showed that all of S1-S7 and four (W2, W6, W8, and W9) of W1-W9 were still positive. The negative cultures were discarded. Cells from cultures S1-S7, W2, W6, W8, and W9 were injected into mice for production of ascites and each ascitic fluid was examined with respect to immunoglobulin production with SDS-polyacrylamide gel electrophoresis (Fig.1). The strong production was found in cultures S2, S3, S7, and W8.

Small antibody columns were made containing Affi-Gel 10 on which immunoglobulin purified from each ascitic fluid of S2, S3, S7, and W8 was fixed. The purified EPO was

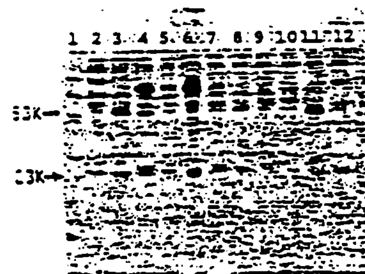

Fig.1. SDS-polyacrylamide gel electrophoresis of ascitic fluids of hybridomas. Hybridoma cells were injected into mouse for production of ascites and each ascitic fluid was subjected to SDS-polyacrylamide gel electrophoresis. Protein bands were revealed with silver staining. Lanes : 1, Mr standards (94,000, phosphorylase B; 67,000, bovine serum albumin; 43,000, ovalbumin; 30,000 carbonic anhydrase; 20,000 soybean trypsin inhibitor; 14,400, α-lactoalbumin); 2, ascitic fluid of hybridoma S1, 3; S2, 4; S3, 5; S4, 6; S5, 7; S6, 8; S7, 9; W2, 10; W6, 11; W8, 12; W9. Arrow heads with 53K and 23K indicate heavy chain and light chain of IgG, respectively.

applied on the columns and then the columns were washed with PBS (phosphate buffered-saline), followed with the pH 2.5 elution buffer (0.2M acetate buffer, pH 2.5/0.15M NaCl). The eluted fractions were neutralized immediately with Tris-base. Erythropoietin activity in the flow-through fractions and in eluted ones was measured. The EPO activity was lowered to 78% of the initial value upon incubation with the elution buffer. When erythropoietin was applied on the control column which contained monoclonal antibody against guinea pig transglutaminase (manuscript in preparation), 80% activity was recovered in the flow-through fractions and no activity was found in the eluted fractions. On the contrary, little EPO activity passed directly through the S2 column but 51% of the applied activity appeared in the eluted fractions, indicating that the antibody produced by hybridoma S2 binds with EPO. Immunoglobulins from hybridomas S3, S7, and W8 does not seem to bind with EPO, because significant activity appeared in the flow-through fractions and no activity was found in the eluted fractions. These observations were validated by analysis of the both fractions on SDS-polyacrylamide gel electrophoresis (figure not shown). When EPO was applied on the columns containing immunoglobulins from transglutaminase-directed hybridoma, S3, S7, and W8, EPO protein was found only in the flow-through fractions but not in the eluted fractions. On the other hand, the EPO band was detected only in the eluted fractions with the column made up from antibody from S2 hybridoma of the mixture of immunoglobulins from all cultures.

Although hybridomas of S1, S4, S5, and S6 poorly produced immunoglobulins when they were injected into mice, immunoadsorbent columns were made with their immunoglobulins and tested for binding of EPO. It was found that EPO activity appeared in the flow-through fractions with S4 and S5 but not with S1 and S6 columns. With S1 and S6 columns the activity was recovered in the eluted fractions. Thus we have obtained three hybridoma cultures producing antibodies capable of binding with EPO.

In parallel with screening and propagation of fused cells, hybridomas (S1-S7) which were strongly positive with the binding assay were cloned by limiting dilution in microtiter plates with mouse thymocyte as feeder layer. Cloning was started at the time of transfer of cells from original 96-well cultures into 24-well cultures. Fifty cells were seeded per 96 wells for cloning. When results were obtained with the immunoadsorbent cloumns, culture of all clones except S1, S2, and S6 was ceased. Hybridoma-growing wells were tested for EPO-antibody production in the culture supernatants with the binding assay. Hybridoma clones from two positive wells of each cell line were propagated in large scale cultures and injected into mouse for production of ascitic fluid. Analysis of ascitic fluids with SDS-polyacrylamide gel electrophoresis revealed that S2 produced in large amount of immunoglobulin, while S1 and S6 were very poor in the production. Recloning did not change the property of each clone with respect to antibody production. One of the S2 clone selected with a criterion of rapid growth has been quite stable in high-antibody production and is used throughout in this laboratory.

Subclass of IgG produced by hybridomas were determined with Ouchterlony double-immunodiffusion method by using rabbit antiserum raised against each subclass of mouse IgGs. It was found that IgG from cloned hybridomas of S1, S2, and S6 were IgG_3, IgG_1, and IgG_1, respectively

Purification of human urinary erythropoietin with immunoadsorbent column

Unless otherwise indicated, all procedures for purification were performed at 0-4°C. urine with high EPO from anemic patients (more than 0.5 unit/ml of urine) was collected in bottles containing sodium azide during cold seasons (September-March). The collected urine was brought into the laboratory every week and frozen at -20°C to store until use. The urine was thawed, filtered under suction, and concentrated by ultra-filtration on a hollow-fiber device (Amicon) with a nominal Mr cutoff of 10,000. Extensive removal of low molecular weight materials in the final stage of concentration was performed by several times repeating addition of cold distilled water and subsequent concentration. About 70 liter of urine was treated at one time and the treatment took about 48 h to complete. The urine concentrate was lyophilized. About 0.5 g of dry materials was obtained from 1 liter of urine.

Table II Summary of erythropoietin purification with immunoadsorbent column
Urine concentrate from ca. 700 liter human urine was used as starting material. Protein was measured with Coomassie Brilliant Blue binding assay (Bio-Rad), using ovalbumin as a standard. EPO activity was measured with in vitro labelled thymidine incorporation using fetal mouse liver as target cells or with in vivo radioactive Fe incorporation method, using starved rats (2). Standard EPO was from Connaught Research Laboratories, Canada (sheep plasma EPO step-III, 4 units/mg protein).

Step	Protein (mg)	Activity in vitro (units) $\times 10^{-3}$	Yield (%)	Specific activity in vitro (units/mg)	Purification	Specific activity in vivo (units/mg)
Urine concentrate	34000	792	100	23	1	14
Immunoadsorbent column	10	594	75	59400	2580	59000
Sephadex G-100	5.5	500	63	88000	3830	81600

Urine concentrate, which came from ca. 700 liters of patients with aplastic anemia, was applied on a column in which EPO-specific monoclonal antibody fixed on Affi-Gel 10 was packed. The column was extensively washed with PBS and then developed with 0.2M acetate buffer, pH 2.5/0.15M NaCl. Fractions eluted with the pH 2.5 buffer were immediately neutralized with 3.4M Tris-base. Purification with the immunoadsorbent column was tremendously effective ; most of protein in the urine concentrate emerged in the flow-through fractions without being adsorbed and EPO was eluted sharply by the pH 2.5 buffer. Twentysix hundred-fold purification was achieved by this single step and 75% of the activity was recovered (Table II).

EPO purified with the immunoadsorbent column was recognized as a main band with Mr 35,000 on SDS-polyacrylamide gel electrophoresis but some other protein bands including the Mr 20,000 protein which was a main contaminant were seen. Furthermore this preparation was tinged with faint brown.

Further purification with a Sephadex G-100 revealed two peaks of protein. It was found by measuring the activity in the pooled preparation of each peak that most of the EPO activity was recovered in the second peak and about 5% of the total recovered activity appeared in void fractions. Faint brown appeared in void fractions and the pooled EPO preparation was quite transparent. Analysis of Sephadex G-100 fractions with SDS-polyacrylamide gel electrophoresis demonstrated that contaminants including

the main contaminant, Mr 20,000 protein, which were found in the EPO preparation purified with the immunoadsorbent column were not detected in any fractions of the second peak. However, they contained faint but detectable protein components in leading side of the EPO main band with Mr 35,000. Activity measurement in the extracts from sliced gels indicated that there is EPO activity in the leading side as well as in the main band, suggesting heterogeneity of EPO protein. As shown in Table II, purification of human urinary EPO with an immunoadsorbent and susequent Sephadex G-100 column provides us pure EPO in a high yield. The specific activity of pure EPO was 88,000 units/mg of protein with in vitro ^{3}H-thymidine incorporation method and 81,600 with in vivo starved rat method (see Table II). These values are higher than 70,400 of the isolated human urinary EPO with conventional purification methods by Miyake et al.(5), although the differences seem unimportant from limited reliability of the EPO assay methods.

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