Separation of an Inhibitor of Erythropoiesis in "Middle Molecules" from Hemodialysate from Patients with Chronic Renal Failure

Akira Saito,¹ Iyoko Suzuki,¹ Tai Gi Chung,¹ Takeshi Okamoto,² and Tomomitsu Hotta³

A "middle molecule" inhibitor of erythropoiesis in patients with chronic renal failure was separated from a large amount of hemodialysate. Hemodialysate was passed through Amberlite XAD-4 resin, Sephadex G-50 gel, and DEAE-Sephadex A-25, followed by reversed-phase liquid chromatography. The erythroid colony assay (CFU-E) was used to detect the inhibitory effect of the sample solution. Certain fractions from the DEAE-Sephadex A-25 column showed a dose-related inhibition of CFU-E formation as great as that of standard spermine. The inhibitory effect of these fractions decreased to the control value after proteolytic digestion. The inhibitor was eluted from the liquid-chromatographic column by a solvent gradient containing 390 to 425 mL of methanol per liter. These results suggest there is an inhibitor of CFU-E with a relative molecular mass of 1000 to 10 000 and an active site composed of peptide. This technique may prove useful for separation of the inhibitor of erythropoiesis in uremic body fluid.

Patients with chronic renal failure usually show severe anemia, but the mechanisms responsible for it are incompletely understood. Although the cause of anemia in chronic renal failure is multifactorial, a clinically important factor may be erythropoietic suppression. Previous reports confirm that the serum of such patients contains substances that inhibit erythropoiesis in vitro (1–3). Various investigators have reported the inhibitor(s) to be a lipid material (4), parathyrin (5), sormine (6), a low-molecular-mass peptide (7), certain protein fractions separated by Sephacryl S-200 column chromatography (8), and a purified ribonuclease (9).

Clinical therapy resulting in greater clearance of large molecules, such as continuous ambulatory peritoneal dialysis (10) and partly protein-permeating hemodiafiltration (11), has been effective in alleviating the anemia. These observations led us to investigate in patients an inhibitor of erythropoiesis with a molecular mass >1000 Da.

We describe an efficient method for treating a large volume of body fluids from patients with chronic renal failure and for separating an inhibitor of erythroid colony formation (CFU-E) in the middle range of molecular mass.

Materials and Methods

Reagents

All reagents were of analytical grade and were obtained from commercial sources. Human urinary erythropoietin (specific activity 72 kilo-immunochemical units/g, dry weight) was obtained from Toyo-bo Co., Ltd., Osaka, Japan. "RPMI 1640" solution and fetal calf serum for use in cell culture were obtained from Gibco Laboratories Life Technologies Inc., Chagrin Falls, OH 44022. "α-Medium" was purchased from Flow Laboratories Inc., Irvine KA12 8NB, Scotland. Spermine as its hydrochloride was from Sigma Chemical Co., St. Louis, MO 63178. Pronase (EC 3.4.21.4, 3.4.24.4), specific activity of $1 \times 10^8$ tyrosine units per gram dry weight, was supplied as "Actinase E" from Kaken Chemical Co., Ltd., Tokyo, Japan. A purified preparation of standard β₂-microglobulin was isolated as previously described (12).

Samples

From Shinseikai Daiichi Hospital we obtained 10 L of ultrafiltrate from patients with chronic renal failure who were undergoing therapeutic hemodiagnosis by the extracorporeal ultrafiltration method with use of a partly protein-permeating artificial kidney (11). Hemodialysate samples collected from the overflow outlet of the dialyzer throughout dialysis treatment were also used.

Procedures

Fractionation of hemodialysate. Amberlite XAD-4 was purchased from Rohm & Haas Ltd., Philadelphia, PA, as 20–60 mesh beads. The resin beads were successively washed with methylene chloride, acetone, methanol, and water, then packed in a 8 × 40 cm glass column. We passed 10 L of hemodialysate through this column. After washing the column with 10 L of water, 2-propanol/water (30:70, by vol) was introduced, and about 10 L of effluent, all with an absorbance <0.1 at 280 nm, was collected. This solution was concentrated 20-fold at 40 °C under reduced pressure with an evaporator. Finally, the residual solution was lyophilized, yielding 1.2 g of powder. This was redissolved in water, dialyzed against water, and tested by CFU-E.

¹ The Bio-Dynamics Research Institute, 1-3-2, Tamamizu-cho, Mizuho-ku, Nagoya 467, Japan.
² Terumo Corp., 2-44-1, Hatagaya, Shibuya-ku, Tokyo 150, Japan.
³ First Department of Internal Medicine, Nagoya University School of Medicine, 65, Tsuruma-cho, Showa-ku, Nagoya 466, Japan.

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Sephadex G-50 (superfine) and DEAE-Sephadex A-25 were from Pharmacia, Uppsala, Sweden. Sephadex G-50 resin was packed in a 3.5 × 40 cm glass column and equilibrated with potassium phosphate buffer (10 mmol/L, pH 7.0) containing 1 mol of NaCl per liter. The above powder was dissolved in buffer and eluted with it at a flow-rate of 12.6 mL/h. DEAE-Sephadex A-25 resin was packed in a 2 × 35 cm glass column and equilibrated with potassium phosphate buffer (10 mmol/L, pH 7.0). Fractions separated by Sephadex G-50 (see below) were equilibrated in this buffer and eluted with it from the A-25 column at a flow-rate of 13 mL/h. Elution with a linear NaCl gradient (0 to 0.5 mol/L) was begun, and the 10-mL fractions collected were monitored by absorbance at 280 nm.

Liquid chromatography. Fraction C separated by DEAE-Sephadex A-25 was applied to a reversed-phase chromatography column. The system used in this study consisted of a liquid chromatograph LC-4A, spectrophotometric ultraviolet detector SRD-2AS, and Chromatopac C-R2AX recorder, all from Shimadzu Co., Ltd., Kyoto, Japan. For reversed-phase chromatography we used a 0.6 × 15 cm Shimadzu Shimpack C18 column (particle size 5 μm), at a flow-rate of 1 mL/min. Solvent A was a 1 g/L aqueous trifluoroacetic acid solution; solvent B was a 1 g/L solution of trifluoroacetic acid in methanol. The gradient-elution program consisted of two linear steps: 0–25 min at 0–12.5% solvent B, 25–35 min at 12.5%, 35–95 min at 12.5–42.5%, and 95–130 min at 42.5%. Absorbance of the effluent was monitored at 210 nm.

Proteolytic digestion. Each fraction obtained from the DEAE-Sephadex A-25 column chromatography was lyophilized and dissolved in 1 mL of borate buffer (0.1 mol/L, pH 8.0). To this solution we added 4 mL of pronase solution (10 mg/L in the borate buffer plus 10 mmol of calcium acetate per liter) and left the mixture at 37°C, with gentle shaking, for 15 h. This digest was then dialyzed against water, with use of a 1000-Da exclusion-limit membrane ("Spectrapor 6" cellulose dialysis tubing; Spectrum Medical Industries Inc., Los Angeles, CA 90054); the dialysand was lyophilized, redissolved in 2 mL of water, and assayed for CFU-E.

Assay for erythroid colony-forming cells (CFU-E). Eight-to 12-week-old C57BL/6 female mice were killed by cervical dislocation and bone-marrow cells from the femurs were flushed by use of 26-gauge needles into cold RPMI 1640 solution containing 20 mL of fetal calf serum per liter. Cell suspensions were prepared by repeated pipetting. The bone-marrow cells were washed once with the RPMI 1640 calf serum mixture.

The in vitro growth of erythroid colonies was assayed by use of a semisolid culture (13). We suspended 10^6 cells in 0.5 mL of α-Medium containing, per liter, 3 g of agar, 300 mL of fetal calf serum, 0.5 U of erythropoietin, and 0.1 mmol of mercaptoethanol. The mixtures were gently placed on the center of the bottom of polystyrene dishes and allowed to gel at room temperature. Then we poured 0.5 mL of α-Medium supplemented with fetal calf serum (100 mL/L) around each agar gel and incubated the dishes for two days at 37°C in air containing CO2, 50 mL/L. After incubation, the discs of agar were transferred onto a glass slide and covered with filter paper to remove excess water. The dried preparations were fixed with 50 mL/L aqueous glutaraldehyde solution and stained with 3,3'-diaminobenzidine. Aggregates consisting of eight or more 3,3'-diaminobenzidine-positive cells were counted as CFU-E colonies in three replicate culture plates. As a control assay we added sterilized water to the culture dishes instead of sample solution.

Results

Figure 1 shows the results obtained during the different steps of separation. We assayed for CFU-E the solution passing through the Amberlite XAD-4 column, the aqueous effluent, and the 2-propanol solution. No inhibitory effect was observed in sample solution passed through the column. Colony formation from the water effluent and the 2-propanol solution were, respectively, 80% and 53% of that of the control. To resolve the material obtained from the Amberlite XAD-4 column chromatography, we subjected it to gel filtration. Figure 1B shows the pattern of elution from the Sephadex G-50 column. Samples of each fraction (diluted to 1 × 10^-3 of their original absorbance at 280 nm) were tested in the culture marrow system. Colony formation was specifically inhibited by fractions no. 40, 47, 52, and 61; in all cases, the values were the average of triplicate determinations.

Inhibitory activity in the low-molecular-mass fractions was insiginificant in this study. Fractions 40–61 (Fraction A) were collected, desalted by dialysis, and lyophilized. The residue was dissolved in a small amount of potassium phosphate buffer, and applied to the DEAE-Sephadex A-25 column. Figure 1C shows resulting elution pattern. Seven fractions were assayed for CFU-E formation as described for Sephadex G-50 column chromatography. Fractions 40–55 (Fraction B) and 66–88 (Fraction C), in which the inhibitory effect was 20% that of the control, were pooled. The inhibitory effects on the CFU-E formation were also compared before and after proteolytic digestion (Figure 2). After digestion, any inhibitory effects of Fractions B and C were no longer detectable.
To demonstrate directly the inhibitory effects of Fractions B and C on CFU-E formation, we added each to the culture dishes at different concentrations. As shown in Figure 3, there was a dose-related inhibition of CFU-E formation by Fraction C, but not by Fraction B. To prove the specificity of this Fraction C-induced inhibition, we studied the effects of Fraction C and of standard spermine on erythropoietin dose-response. CFU-E colony formation in Fraction C was increased with increasing erythropoietin, but the number of these colonies was always less than in the control (Figure 4). This action of Fraction C in the erythropoietin dose-response was similar to the inhibitory effect of spermine.

Liquid chromatography further resolved Fraction C into mainly the four fractions C-1, -2, -3, and -4 (Figure 5). Testing these fractions (adjusted to $5 \times 10^{-2}$ of the original absorbance at 280 nm) and spermine (3.5 nmol/mL) in the cell culture system showed that the inhibitory effects of Fractions C-1, -2, and -4 were approximately the same as with the control. Fraction C-3 and spermine, however, respectively supported only 41% and 28% of the colony formation seen with the control.

**Discussion**

In earlier reports, inhibition of erythropoiesis was detected with sera from uremic patients in several different tissue-culture systems involving the use of both human and animal target cells: by polychromatophic mouse assay (14), $^{59}$Fe incorporation into heme (3), $^{3}H$thymidine incorporation into marrow cells (7), and erythroid colony formation from CFU-E and BFU-E (erythroid burst-forming units) (6, 15, 16).

Here we describe the in vitro effect of uremic sera on the CFU-E and granulocyte-macrophage progenitor cells, indicating that the CFU-E inhibiting activity appeared in two different effluent fractions of Sephadex G-15 gel column chromatography: one being the void-volume fraction, with a molecular mass >1500 Da, and the other a low-molecular-mass fraction. Among the low-molecular-mass uremic toxins so far reported, polyamine spermine has been examined as an in vitro inhibitor of erythropoiesis in concentrates of uremic sera (6). On the other hand, clinically, regular or continuous ambulatory hemodialysis reportedly leads to recovery from the anemia associated with end-stage renal disease (10, 17).

However, a large proportion of patients regularly receiving hemodialysis remain anemic. Our group has previously demonstrated that a protein-permeable hemofilter was effective in decreasing the concentration of low-molecular-mass proteins and in alleviating symptoms of uremia such as bone pain and anemia (11). Both this treatment and ambulatory dialysis are associated with much greater clearance of substances of high molecular mass. Because the low-molecular-mass inhibitors such as unbound spermine could be dialyzed away in regular dialysis treatment, we focused here on an inhibitor in the "middle molecules" fraction, $M_t$ from 1000 to 10 000.

In the present study, we attempted to separate the inhibitor of erythropoiesis from the body fluids of patients.
The solvent-extraction methods reported so far have some disadvantages, because extraction of a small amount of bioactive substances from a large amount of sample solution is very labor-intensive. A technique involving liquid-exchange chromatography has been reported, but the loading capacity is small, and the presence of a large amount of salt disturbs the chromatographic system. In contrast, Amberlite XAD-4 resin has good loading capacity, is stable, and adsorbs hydrophobic compounds efficiently (18). This method allowed us to handle a large quantity of hemodialysate more readily, and most of the inhibitory activity was adsorbed by this resin. Because standard spermine was completely removed with 1000-Da exclusion limit membrane (data not shown), we used this membrane dialysis procedure throughout the process of separation to eliminate low-molecular-weight compounds.

Proteolytic digestion eliminated the inhibitory effect of the DEAE-Sephadex A-25 column Fractions B and C, suggesting that the active site of the inhibitor is a peptide. Fraction C, which showed dose-response inhibition, did not lose its inhibitory effect by increasing the erythropoietin concentration, and the effect was similar to the action of spermine. We conclude that Fraction C contained a peptide inhibitor, and that its function was not associated with the action of erythropoietin in erythropoiesis directly. Liquid-chromatographic analysis has consequently shown that the inhibitor is eluted with the 390 to 425 mL/L methanol concentration. Under this condition, spermine is eluted within 5 min. We consider it certain that the liquid-chromatographic eluent contains a (the) middle-molecule peptide inhibitor of erythropoiesis (1000–10 000 Da) but no low-mass compound such as spermine.

The proposed system should be useful for separation of inhibitor of erythropoiesis in the hemodialysate of patients with chronic renal failure, and further investigations are now in progress in our laboratory.

References