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Identification of an Erythropoietic Inhibitor from the Dialysate Collected in the Hemodialysis with PMMA Membrane (BK-F) and Its Clinical Effects

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Introduction

Anemia is one of the major complications among hemodialysis patients. Since erythropoietin (EPO) was commercialized, its clinical effect in ameliorating anemia has been widely proven. By the way, it has long been supposed that such renal anemia is multifactorially caused and, as one of those factors, some erythropoietic inhibitor(s) may be accumulated in uremic plasma. Despite some trials [1, 2] to find out such factors, no single substance has been identified yet.

We found that a specific fraction, which shows the inhibiting effect on the colony formation of colony-forming unit-erythroid (CFU-E) from mice was obtained through the gel filtration of the dialysate collected in the hemodialysis with PMMA membrane, BK-F, having a larger pore than that of the other PMMA membrane, BK, developed for the removal of β_2-MG [3]. The nominal molecular weight of this fraction was demonstrated to be bigger than that of the albumin or immunoglobulin (IgG) and the isolated band corresponding to 40,000 daltons and showing the strong inhibiting effect on the CFU-E colony formation was obtained by its sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In parallel with the above analysis on the erythropoietic inhibitor(s), the clinical effect of the long-term use of this membrane, BK-F, was evaluated.

Method

Dialysis Membrane

In this study, PMMA membrane, BK-F, which had a larger pore diameter than that of another PMMA membrane, was used. The sieving coefficient of albumin of BK-F was 0.03 while that of BK was 0.01. In the long-term clinical evaluation, low flux cellulosic membranes were used as a control conventional membrane.

Preparation of Concentrated Dialysate

As the standardized procedure, around 30 liters of dialysate per batch were collected in the hemodialysis with BK-F in 2 or 3 patients and concentrated through the circulative ultrafiltration using low flux cellulosic membrane, AM-SD, to 1/200th to 1/250th of its original volume. Thus the concentrated dialysate that originated from the hemodialysis with BK-F membrane was obtained.

Fractionation of the Concentrated Dialysate

The above-mentioned concentrated dialysate was fractioned through the gel filtration (Sephacryl S-200, Pharmacia Co.). The peaks obtained were individually separated and lyophilized for the evaluation of the inhibiting activity on CFU-E colony formation.

Biological Measurement with Cells

A modification of the methylcellulose technique of Iscove et al. [4] was applied to form CFU-E colonies. Bone marrow cells were harvested from the femur of C57 Black/6 mice and suspended with α-minimum essential medium (α-MEM) at the concentration of 10^7 cells/ml. Then the following substances were put into a 35-mm plastic tissue culture dish followed by mixing. Solutions of methylcellulose, BSA, EPO and the samples were previously prepared with α-MEM:

Cell suspension	0.1 ml
1.8% methylcellulose	0.5 ml
FCS	0.3 ml
10% BSA	0.1 ml
100 units/ml EPO	5 μl
Sample solution (or α-MEM)	0.1 ml

The dish was incubated at 37 °C for 48 h in a CO_2 incubator and then the number of CFU-E colonies was counted using an optical microscope. On the other hand, a blank test was performed by use of α-MEM instead of the sample solution. Dividing the number of colonies with the sample solutions by that obtained in the blank test, the ratio of the CFU-E colony formation was calculated.

Further Trials to Isolate and Purify Erythropoietic Inhibitors

(1) *SDS-PAGE:* The following gel was used for SDS-PAGE: 4–20% T gradient gel, 1 mm thick and 10 wells (TEFCO). Sample fluid was applied to each well and electrophoresed for about 2 h at a current of 15 mA. Proteins isolated were strained with CBB to detect as bands.

(2) *Western blotting:* After electrophoresis, proteins in the gel were blotted on the PVDF membrane (Applied Biosystems). Then, blocking was performed with skim milk and biotin-avidin method using the biotinized antibody was applied for detection.

(3) *Amino acid sequencing:* A band in the SDS-PAGE gel was blotted on the PVDF membrane and then the amino acid sequence from the N-terminal was tried to be analyzed with a sequencer, A 477 (ABI).

(4) *Fractional electrophoresis:* Mini Prep Cell (Bio-Rad), a fractional electrophoresis, was used to purify a few milligrams of the fractionated substance having a molecular weight of about 40,000 from the gel fraction, KR4-0 described below.

Preparation of an Antibody

Antigen peptide and rabbit antiserum were prepared by Sawady Technology Co. using Fmoc MAP resin (Applied Biosystems).

Clinical Evaluation

Study I (the group of patients not treated with EPO): The subjects were 6 patients who had received hemodialysis with conventional cellulosic membrane and had not been administered EPO for 1 year before this study. They were 5 males and 1 female and their age and hemodialysis duration were 55 ± 11 years (range 47–73) and 13 ± 4 years (range 8–20), respectively. Instead of the conventional cellulosic membrane, BK-F membrane (1.6 m^2) was used for 4 months without changing the other hemodialysis operational parameters.

Study II (the group of patients treated with EPO): Six patients who had been dialyzed with conventional cellulosic membrane and administered EPO (6 males, age 62 ± 10 years (range 47–77), dialysis duration 7 ± 5 years (range 2–15)) were the subjects for this study. For 3 months, the conventional cellulosic membrane was replaced with BK-F (1.6 m^2) and the dose of EPO was adjusted to maintain the hematocrit (Hct) level.

Study III (comparison between patients dialyzed with BK-F for 2 years and those with conventional cellulosic membrane for the same period): To match with 17 patients dialyzed with BK-F for 2 years, 17 out of around 200 dialysis patients treated with conventional cellulosic membrane for 2 years in the same hospital (Tohjinkai Hospital) were picked up based upon gender, hemodialysis duration and age as the matching parameters in this priority order:

	BK-F	Conventional cellulosic membrane
Patients, n	17	17
Sex, m/f	6/11	6/11
Age, years	53 ± 11	53 ± 11
Dialysis duration, years	11 ± 7	11 ± 7

Statistical Analysis

Statistical analysis of studies I, II and III were carried out by the Student's t-test, paired t-test and Wilcoxon's rank sum test, respectively.

Results

Isolation of the Fraction, KR4-0

Figure 1 shows the results of the gel filtration of the concentrated dialysate collected in the hemodialysis with BK-F. As can be seen, three peaks were obtained and were fractionated as KR4-0, KR4-1, and KR4-2 according to the elution time. The protein analysis using latex agglutination method revealed that the major components of KR4-1 and KR4-2 are IgG (89%) and albumin (84%), respectively, while IgG and albumin were not detected from KR4-0.

Figure 2 shows the ratio of CFU-E colony formation of each fraction. While KR4-2 and KR4-1 did not show any effect, KR4-0 whose molecular weight was larger than those of the other two peaks in the gel filtration demonstrated a significant erythropoiesis-inhibiting effect dose-dependently. Though not given in the figure, the fraction numbers 60–80 corresponding to molecular weight $< 10{,}000$ revealed a weak erythropoiesis-inhibiting effect.

Composition and Properties of KR4-0

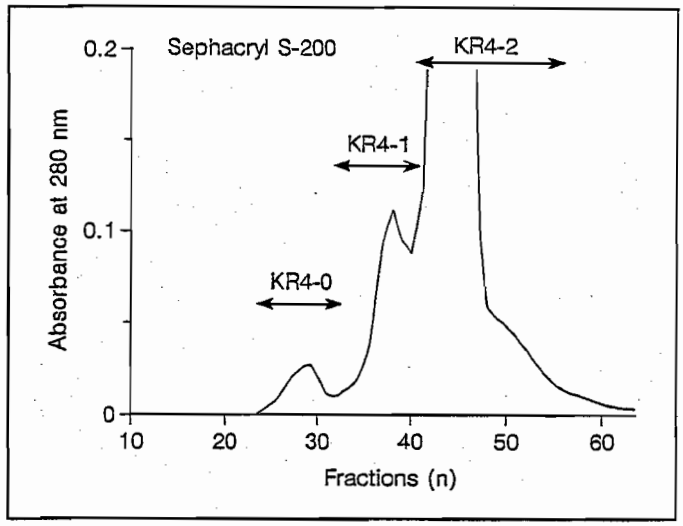
KR4-0 consisted mainly of 40% amino acid and 20% carbohydrate, with lipids partly found in the remaining 40% of components. Extraction test of KR4-0 was performed by use of an ammonium acetate buffer solution and buthanol. The aqueous layer originating from ammonium acetate solution showed an erythropoiesis-inhibiting effect. Furthermore, another extraction test was carried out using chloroform instead of buthanol, and the erythropoiesis-inhibiting effect was again recognized in the aqueous layer.

Figure 3 shows the changes in the ratio of CFU-E colony formation incubated with KR4-0 observed when this fraction was heated at 100 °C. A strong erythroiesis-inhibiting effect was recognized until the heating time did not exceed 10 min. However, longer heating more than 10 min caused a fall in the effect and heating for 30 min thoroughly inactivated the effect.

Isolation and Purification of an Erythropoietic Inhibitor from KR4-0

Under the given condition of the gel filtration, the elution time of KR4-0 coincided with that of IgM. To pinpoint erythropoietic inhibitor(s) from the fraction, KR4-0, the gel filtration of KR4-0 for the isolation of the substances with large molecular weight was attempted. However, no satisfactory fraction was obtained. Ion exchange chromatography and affinity chromatography were also tried but neither succeeded.

Although the nominal molecular weight of the fraction, KR4-0, was equivalent with that of IgM, this fraction was not separated with 2% T PAGE differently from IgM. However, KR4-0 was separated only with SDS-PAGE.



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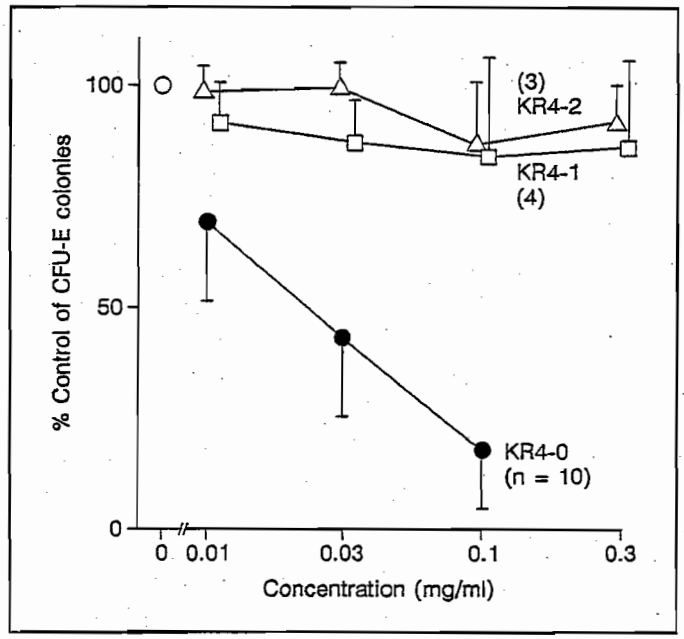

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Fig. 1. Fractionation by gel filtration of concentrated dialysate when BK-F was used clinically.

Fig. 2. Inhibition of CFU-E colony formation by fractions from gel filtration.

Weak linkages of KR4-0 were considered to be destroyed with SDS and bands of molecular weight of 160,000, 70,000, 40,000 and 30,000 were detected.

An appropriate amount of separated gel of each band on SDS-PAGE was cut out and the substance contained was extracted. As SDS in the extract showed cytotoxicity, the substance extracted was purified with a SDS removal column. Figure 4 shows a picture of bands on SDS-PAGE and the results of the

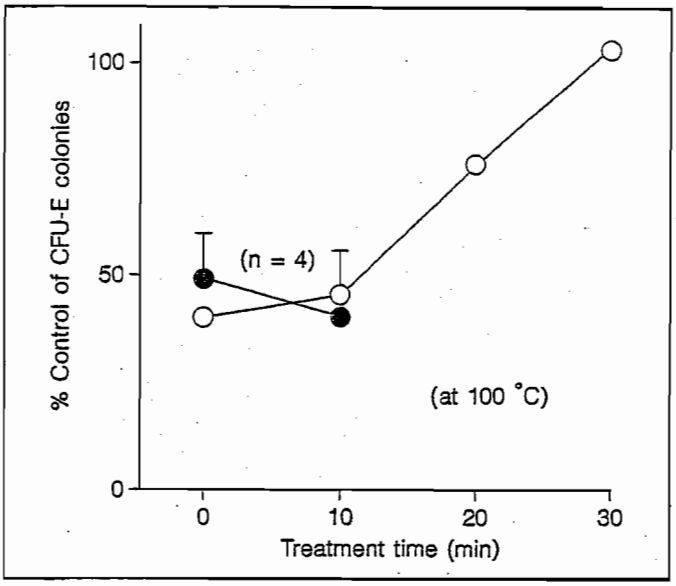

Fig. 3. Changes in CFU-E colony formation rate by heating.

Table 1. The amino acid sequence of YS-1 analyzed from position 1 to 19 from the N-terminal

1					6					11					16			19
Ala-	Val-	Val-	Tyr-	Asp-	Lys-	Asp-	Gly-	Thr-	Ser-	Phe-	Asp-	Ile-	Tyr-	Gly-	Lys-	Val-	Gln-	Ala-

measurement of the inhibiting effect on the colony formation. Erythropoiesis-inhibiting effect was found in the region of molecular weights of 40,000 and 70,000, with the former showing a stronger effect. The main band in the region of molecular weight of 70,000 was blocked in N-terminal, but the amino acid sequence of the main band in the region of 40,000 (tentatively called as YS-1) could be analyzed from position 1 to 19 from the N-terminal. The sequence is shown in table 1. The peptide of this sequence was synthesized and rabbits were immunized with this peptide to obtain antiserum.

Neutralization of Erythropoiesis-Inhibiting Effect of KR4-0 Using the Anti-YS-1 Antibody

IgG was purified from antiserum, which is called an anti-YS-1 antibody. It was observed how colony formation was inhibited due to KR4-0 after the addition of the anti-YS-1 antibody. The results are shown in figure 5, and the change found when IgG was added is also shown as a reference. This antibody was thus demonstrated to neutralize the effect to inhibit colony formation by KR4-0 to some extent.

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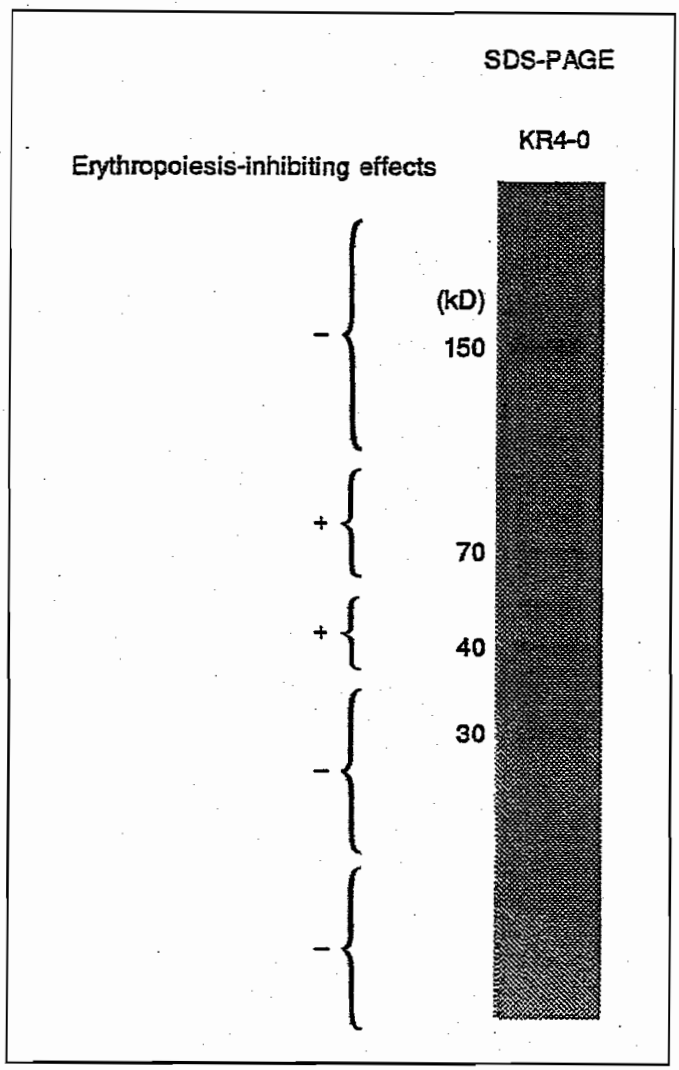
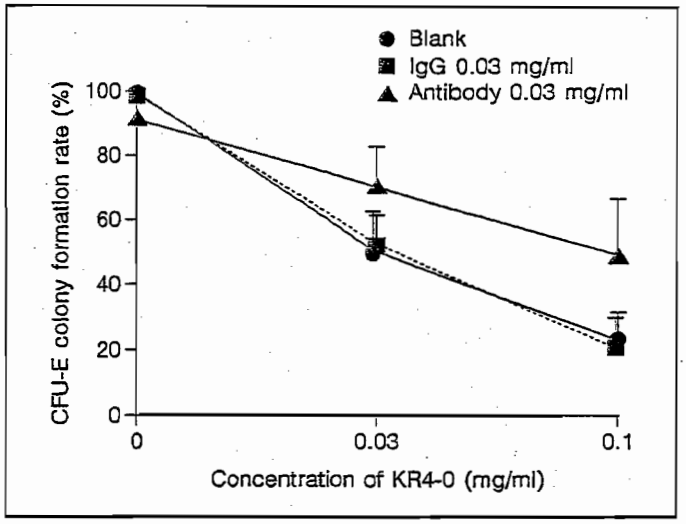


Fig. 4. SDS-PAGE pattern of KR4-0 and evaluation of erythropoiesis-inhibiting effects for extracts from gel slices' erythropoiesis-inhibiting effects (+ or −).

Detection of YS-1 with Anti-YS-1 Antibody

(1) *Detection of YS-1 in KR4-0 from the dialysate individually collected during hemodialysis:* KR4-0 after SDS-PAGE was blotted on a PVDF membrane, and the Western blot technique was applied to obtain YS-1. It was detected as a band at the given position (molecular weight of about 40,000) in 10 out of 10 patients.

(2) *Detection of YS-1 from urine:* Desalted and concentrated urine collected from 2 nonuremic subjects was subjected to gel filtration and the peaks obtained were analyzed. YS-1 was detected from the peak corresponding to



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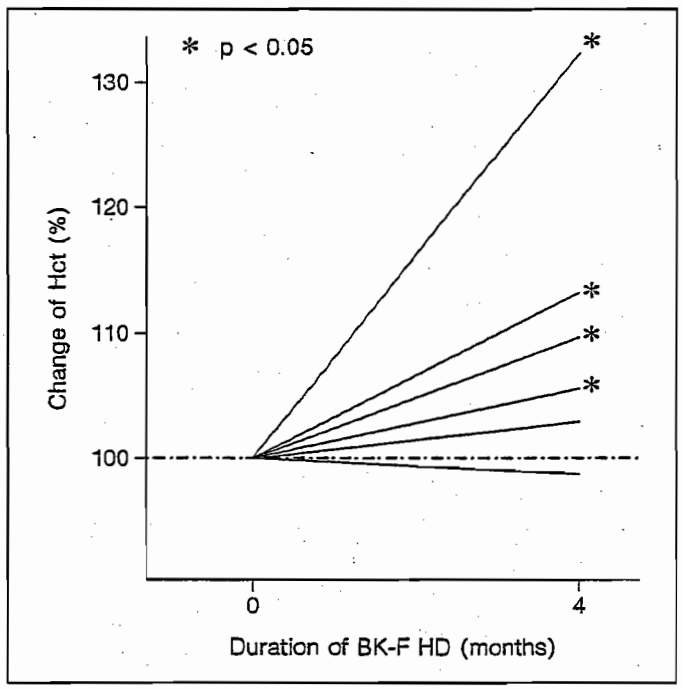

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Fig. 5. Changes in CFU-E colony formation rate of KR4-0 by addition of YS-1 antibody.
Fig. 6. Change of Hct level in 4 months after switching the use of a conventional cellulosic membrane to BK-F.

KR4-0. On the other hand, urine collected from 2 patients who received hemodialysis and maintained the renal function was analyzed in the same way and YS-1 was detected in them.

(3) *Detection of YS-1 from plasma:* Direct detection of YS-1 was not succeeded from plasma. Performing the following pretreatment procedures,

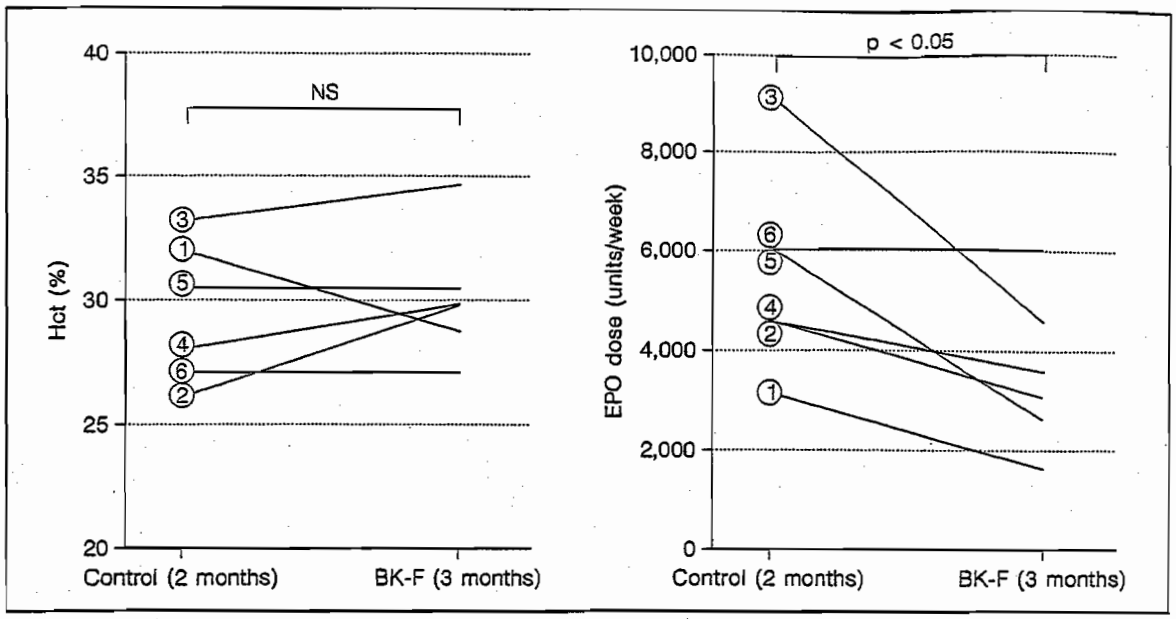

Fig. 7. Changes in Hct level and EPO dose by using BK-F for 3 months (the numbers circled show the patient numbers).

the fraction corresponding to YS-1 was extracted: (a) plasma of the patients was subjected to gel filtration so as to collect KR4-0; (b) the fraction of KR4-0 was electrophoresed to collect the fraction of molecular weight of 40,000, and (c) the fraction of molecular weight of 40,000 was analyzed using the Western blot technique after SDS-PAGE electrophoresis.

Follow-Up on the Clinical Effects

Study I (the group of patients not treated with EPO): Figure 6 shows the change of the Hct level when BK-F was used in 6 patients for 4 months who had used the conventional cellulosic membrane and were not administered EPO since one previous year. Four out of 6 patients showed a significant rise in their Hct level. During this observation period, levels of albumin, IgG, IgA and IgM did not show any significant change at all.

Study II (the group of patients treated with EPO): Figure 7 shows the change in the dose of EPO to maintain the Hct level at around 30% (25–35%) during the use of BK-F for 3 months in 6 patients who had previously been treated with conventional cellulosic membrane. While the Hct was maintained at levels between 29.5 ± 2.8 and $30.0 \pm 2.6\%$, the dose of EPO was significantly reduced by 36% from $5,500 \pm 2,049$ to $3,500 \pm 1,581$ units/week.

Study III (comparison between patients dialyzed with BK-F for 2 years and those with conventional cellulosic membrane for the same period): Figure 8

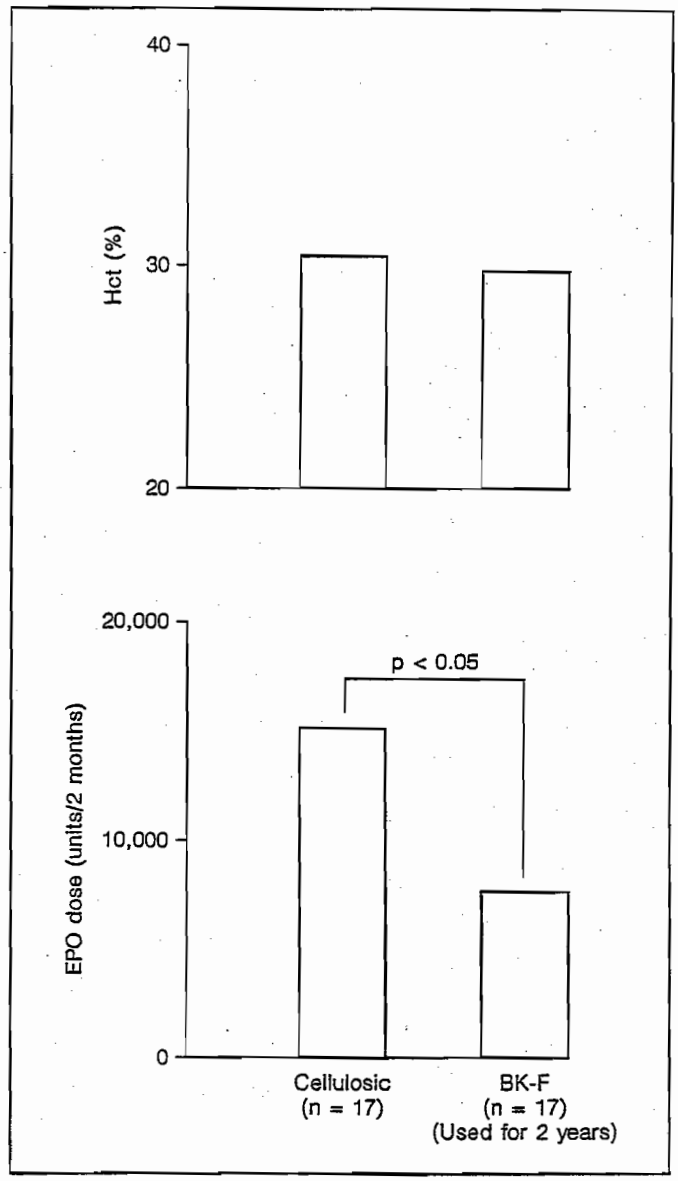

Fig. 8. Comparisons of Hct level and EPO dose between the groups dialyzed with a conventional cellulosic membrane and BK-F.

shows the Hct levels and doses of EPO in 17 patients who were treated with BK-F for 2 years and 17 patients with conventional cellulosic membrane whose age, hemodialysis duration and gender were matched with those of the former. The Hct levels did not differ from each other at the value of 30.2±5.1 and 29.6±4.0% for the groups with conventional cellulosic membrane and BK-F, respectively. On the other hand, a significant difference was observed in the dose of EPO. The dose to the group with BK-F of 7,500 (0–28,500) units/2

months was significantly less than that to the group with the conventional cellulosic membrane of 15,676 units/2 months (0–72,000) by 52%.

Change in plasma albumin level: Figure 9 shows the change in the plasma albumin level during the time BK-F was being used. In comparison to the level between before and after the use of BK-F, it was reduced by 0.1–0.2 g/dl 6 months after the start of the application, but the level rose to the previous level after 2 years. There was no case in which the fall of the plasma albumin level led to a discontinuation of BK-F.

Discussion

Although there is no doubt that EPO, since being commercialized, has played a great role in ameliorating anemia among hemodialysis patients, they still have not thoroughly been relieved from this major uremic complication. Uremic anemia has been reported to be ameliorated in most patients by the administration of EPO whose blood level reaches about 1 unit/ml, as high as 10 times the normal level. Necessity of such a high dose of EPO may be interpreted to be due to the presence of erythropoietic inhibitors accumulated in the uremic plasma. In our study, we found that KR4-0, an erythropoietic inhibitor, showed a strong erythropoiesis-inhibiting effect up to 0.5 units/ml of the EPO level and that it did not show any effect at the 4 units/ml level. This result supports the clinical findings that anemia can be improved at high levels of EPO even if an erythropoietic inhibitor such as KR4-0 exists.

KR4-0, an erythropoietic inhibitor, is eluted in the region where a substance is detected whose molecular weight is larger than albumin or IgG and rather coincides with that of IgM in terms of gel filtration. When the concentrated dialysate collected from the hemodialysis with BK was applied to the same analytical procedure, the peak ratio of KR4-0/KR4-2 was 1/500th to 1/5,000th while that in the case of BK-F was 1/10th to 1/100th. Taking into consideration the supposed molecular weight of KR4-0, its permeation through BK-F seems unlikely to occur. And therefore, it may be interpreted that KR4-0 is detected through the aggregation of fragments permeated through BK-F into the dialysate. However, further isolation of KR4-0 was not achieved until SDS was charged to this fraction. SDS-PAGE of KR4-0 revealed the subfractions 70,000 and 40,000 daltons, both of which showed a strong inhibitory effect on colony formation. In the main band of the region of molecular weight of 70,000, N-terminal was blocked, but amino acid sequence of the main band of molecular weight of 40,000 could be analyzed from position 1 to 19 from N-terminal. This sequence could not be found in a database, which suggests that this might be a novel substance.

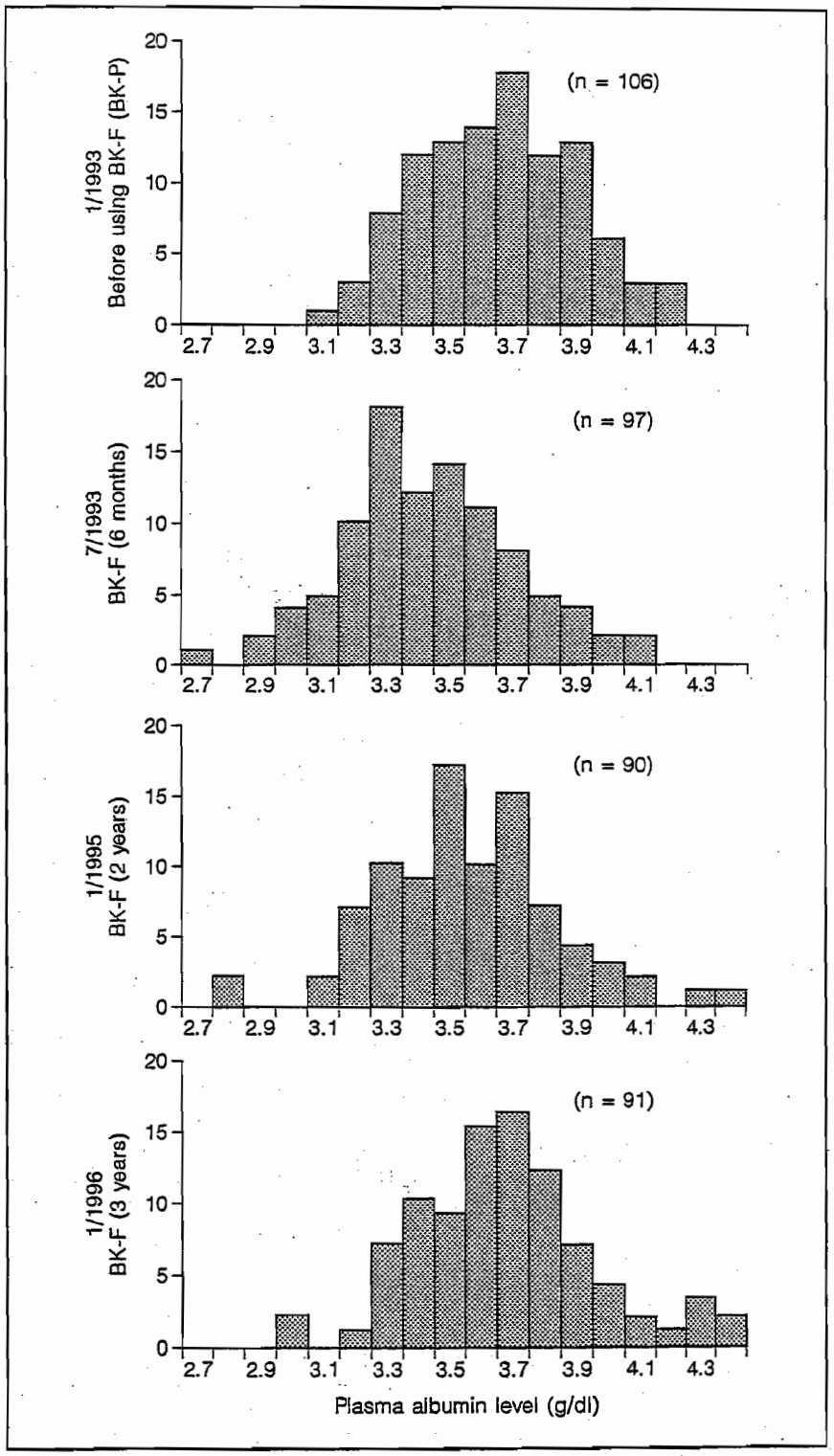

Fig. 9. Changes in plasma albumin level (number of subjects in parentheses).

A peptide having the same amino acid sequence (YS-1) was synthesized and rabbits were immunized to prepare the YS-1 antibody. By the use of anti-YS-1 antibody thus obtained, YS-1 was detected from KR4-0 individually collected in the hemodialysis, and urine and plasma from the patients receiving hemodialysis. These indicated the existence of YS-1, but it was not possible to obtain a sufficient amount of the substance to determine the CFU-E colony formation ratio. Therefore, it could not yet be proven that YS-1 is an erythropoietic inhibitor. Though it is indirect, anti-YS-1 antibody neutralized the erythropoiesis-inhibiting effect of KR4-0, and it can be thought that YS-1 may be an erythropoietic inhibitor.

On the other hand, BK-F was demonstrated to remove KR4-0 and its clinical effect has been proven. We thought the possibility of an overdose of EPO to suppress the effect of the erythropoietic inhibitor and proposed a hypothesis in which the removal of erythropoietic inhibitor such as KR4-0 would reduce the dose of EPO. Clinical evaluation of study II was performed to prove this, and the reduction of the dose of EPO was successfully achieved by keeping the Hct level at a certain level (fig. 7).

BK-F also naturally removed the substances whose molecular weight was less than that of IgG and albumin together with KR4-0. If there might be any erythropoietic inhibitors in this region, they were considered to be also removed. For instance, and erythropoietic inhibitor proposed by Saito et al. [1] is one of them. In addition, it is said that a furancarboxylic acid proposed by Niwa et al. [5] has an erythropoiesis-inhibiting effect. They proved that this carboxylic acid has strong affinity with albumin, but it was actually removed by BK-F [6]. These findings indicate that BK-F can remove KR4-0, a novel erythropoietic inhibitor in addition to erythropoiesis-inhibiting substances previously found. It can be concluded that all these findings might contribute to the clinical effect of BK-F in improving anemia.

Conclusions

An erythropoiesis-inhibiting fraction, KR4-0, and its subfraction, YS-1, with a molecular weight of 40,000, were isolated. They originated from the dialysate collected in the hemodialysis with PMMA membrane, BK-F, having a large pore structure. On the other hand the prolonged use of this membrane suggested its clinical effect in ameliorating renal anemia by replacing the conventional cellulosic membrane.

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