Clinical (III)

Use of large-pore-size membrane (BK-F) in the removal of high molecular weight substances

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Introduction

Anemia is one of the major complications in long-term dialysis patients. It is well known that recent treatments using erythropoietin formulations have produced marked improvements in this condition. While there are many possible causes for anemia, the existence of an erythropoiesis inhibitor is suspected to be one of them. While many studies have been performed on erythropoiesis inhibitors, they have not yet led to clear identification of these substances[1),2)]. We discovered a substance that inhibits CFU-E colony formation in mouse hemodialysate collected using a large-pore-size membrane consisting of the newly developed PMMA. This substance is of larger molecular size than previously suspected substances which were thought to possess molecular

weights of several hundred to several thousand. We studied the composition and properties of this substance, and also observed the clinical effectiveness of removing it through dialysis using a large-pore-size membrane.

Subjects and Method

1. Dialysis membrane

The newly developed large-pore-size membrane, BK-F, uses PMMA as its material, and possesses a molecular weight fractionation curve designed to allow the removal of high molecular weight substances to a level that poses no danger. SCAlb is approximately 0.03. A type of membrane possessing an area of 1.6 m^2 was used for the clinical evaluation.

2. Erythropoiesis inhibitor activity evaluation

1) Sample fractionation

Fractionation for the evaluation of erythropoiesis inhibitor activity was performed as follows. First, a large amount of hemodialysate was collected using BK-F. It was then concentrated 200 to 250 times using an ordinary cellulose membrane. Then, following gel filtration (using Sephacryl S-200, made by Pharmacia), the obtained peaks were sampled, freeze-dried, and their effect on CFU-E colony formation observed.

2) Cell evaluation

Bone marrow cells were extracted from the femoral bone of a mouse (C57 BL/6), and the items listed below were added to it using the ordinary methylcellulose method, to produce an α-MEM based mixture with a total volume of 1 ml:

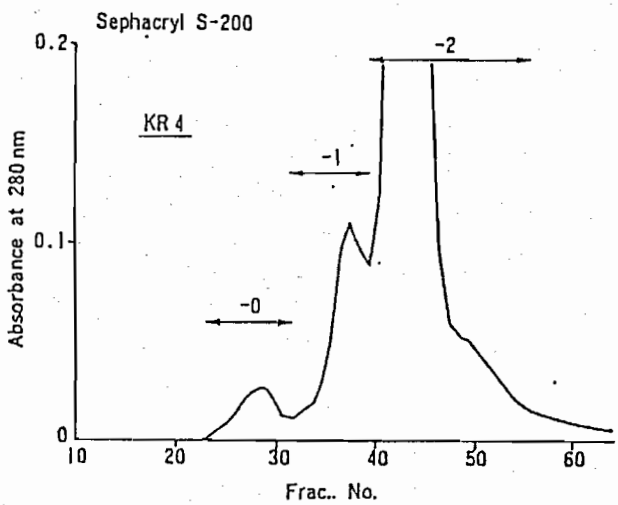
Cells	10^6
Methylcellulose	0.8%
Fetal calf serum	30%
Bovine serum albumin	1%
EPO	0.5 U
Fractionation sample solution	10%

The mixture was cultured in a 35-mm petri dish, at 37°C, under a 5% CO_2 atmosphere, for 48 hours. The number of CFU-E colonies for each 1 cm^2 area was then counted, using an optical microscope. The colony formation rate was calculated by dividing the number of colonies produced when the fractionation sample was added, by the number of cells produced when α-MEM was added in a ratio of 10% to the total volume instead of the fractionation sample.

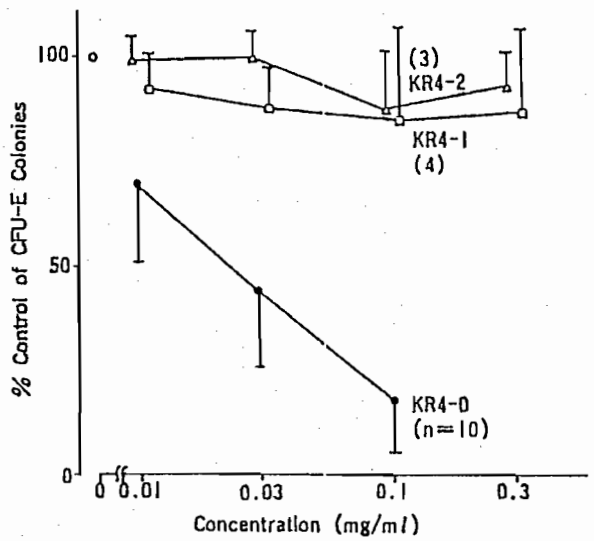
3. Clinical evaluation

Dialysis using the BK-F membrane was performed for 11 months on 8 dialysis patients (5 males, 3 females, average age of 49.5, with an average dialysis history of 10 years). None

of the patients had taken erythropoietin formulations for the year preceding the start of the BK-F membrane dialysis. Changes in hematocrit were observed in these patients. Measurements were also taken on the albumin, immunoglobulin, and total cholesterol levels.



a. Gel filtration fractions



b. Inhibiting effects of various fractions on CFU-E colony formation in mice

Figure 1 Gel filtration of concentrated dialysate obtained using BK-F

Results

1. Fractionation of Erythropoiesis Inhibitior

Figure 1a shows gel filtration fractions of the concentrated dialysate obtained using BK-F. Samples were taken from the three peaks that appeared, which were labeled

KR4-0, KR4-1, and KR4-2, beginning with the one with the shortest elution time. Figure 1b shows the inhibiting effect of each fraction on CFU-E colony formation in mice. Fractions KR4-2, which has the largest peak consisting of mostly albumin, and KR4-1, which consists of mostly IgG, had no inhibiting effect on CFU-E colony formation in mice. In contrast, Fraction KR4-0, which possesses the largest molecular weight, showed a strong inhibiting effect on colony formation. Peaks near fraction numbers 60 to 80, in which substances with molecular weights of 10,000 or less are eluted, also showed weak inhibiting effects on colony formation.

2. Compositions and properties of erythropoiesis-inhibiting fractions

In terms of gel filtration, Fraction KR4-0 possesses an elution time that matches that of IgM, and is presumed to possess a molecular weight of around 1 million. Because its molecular weight is close to that of IgM, attempts to fractionate KR4-0 using gel filtration for high molecular fractionation, ion chromatography, and affinity chromatography were not successful. Thus, a method of separating and purifying KR4-0 has not yet been established. The overall composition of KR4-0 is 40% amino acid and 20% sugar, and the

remaining 40% contains some lipids. When KR4-0 was separated into parts that dissolve in aqueous ammonium acetate buffer solution and in butanol, the parts that dissolved in the former exhibited an erythropoiesis-inhibiting effect. KR4-0 was also separated into parts that dissolve in chloroform and aqueous buffer solution. Again, the part that dissolved in the aqueous buffer solution showed the erythropoiesis-inhibiting effect. Therefore, the erythropoiesis inhibitor was determined to be water-soluble. Figure 2 shows the changes in the erythropoiesis inhibitor activity that are caused by heating. In four repeated trials, the inhibitor still retained its inhibiting effect, even after being heated at 100°C for 10 minutes. However, the inhibiting effect became weaker as the heating time lengthened, completely disappearing after 30 minutes of heating.

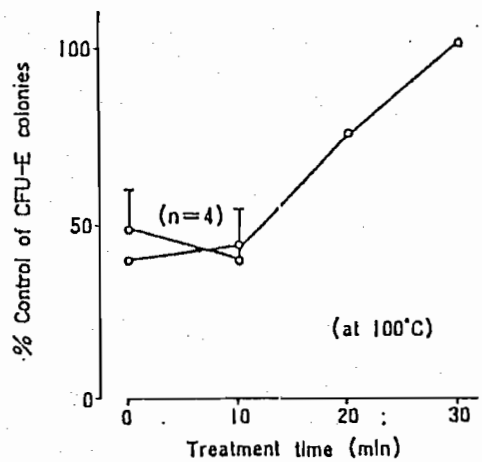


Figure 2 Changes in erythropoiesis-inhibiting activity due to heating

3. Clinical effectiveness

After BK-F membranes were used in dialysis instead of ordinary membranes, four out of six cases showed significant increases in hematocrit level in four months. (See Figure 3.)

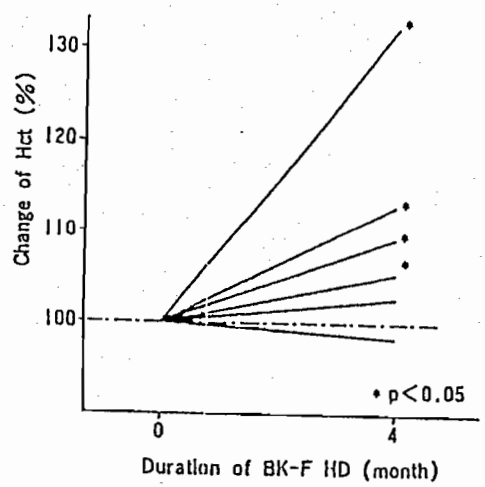


Figure 3 Hematocrit increases when BK-F membranes are used for dialysis

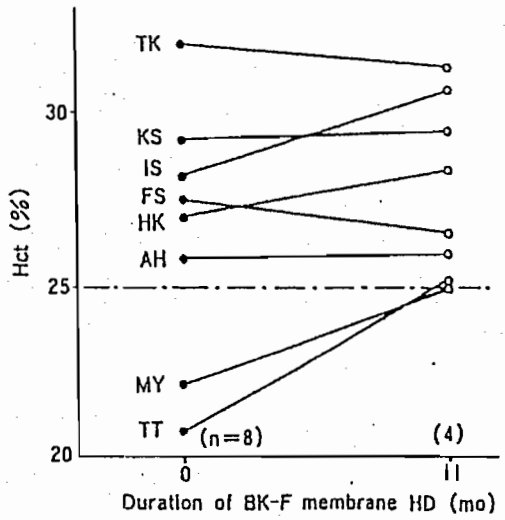


Figure 4 Hematocrit level after 11 months of BK-F membrane usage

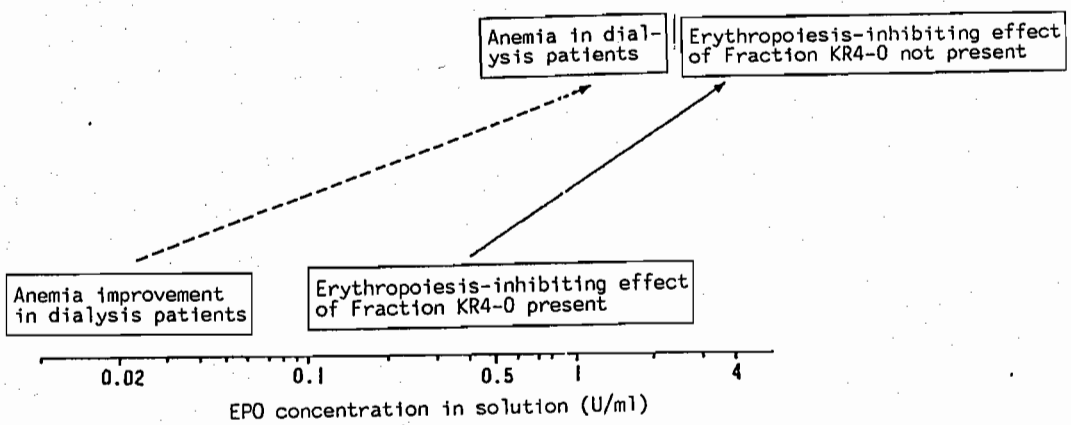


Figure 5 Erythropoiesis-inhibiting effect of Fraction KR4-0 and EPO concentration

Figure 4 shows hematocrit levels after 11 months of BK-F membrane usage in 8 patients (including two who switched from BK-P to BK-F membranes). The group of patients that had shown hematocrit levels of 25% or higher before using BK-F membranes, showed either increases or no change. In contrast, hematocrit levels reached 25% for the two patients who had begun with levels of less than 25%. During this observation period, no changes were detected in the levels of albumin, IgG, IgA, or IgM. Although, overall cholesterol levels did increase significantly between the third and fifth months, the resulting levels were not problematic. Cholesterol levels subsequently declined by the eleventh month, resulting in no significant differences.

Discussion

Various types of erythropoiesis inhibitors have been reported up to this point in time. Some studies have even reported improvements in anemia from dialysis using HPM.[3] However, a method of purifying and identifying the substance has not been established, nor have the effects of HPM usage been fully understood.

The amount of the albumin main fraction and IgG main fraction that permeated the BK-F membrane used for the study, was only twice the amount that permeated the BK-P membrane developed for the purpose of removing β_2-MG. In contrast, the amount of KR4-0, the erythropoiesis-inhibiting fraction, that permeated the membrane, was more than ten times that amount. Therefore, it is safe to conclude that the molecular weight of the substance comprising this fraction is far greater than that of any of the erythropoiesis inhibitors that have been reported so far. Because of its larger molecular size, the purification and identification of this substance have not yet become possible using conventional analysis methods.

In clinical evaluation of the long-term usage of BK-F membranes, the group with hematocrit levels of around 20% showed increases to the 25% level, while the group with the level higher than 25% showed increases or no change. It is

not being suggested that the use of BK-F membranes can increase hematocrit levels indefinitely. Rather, the membrane seems to stabilize hematocrit at levels between 25 and 30%. It is possible that the membrane creates a physiological environment in which the patient's own erythropoiesis ability can function.

In terms of erythropoietin (EPO) studies, it has been reported that anemia in dialysis patients improves when they are given doses of EPO large enough to achieve concentration levels of around 1 U/ml. KR4-0, the fraction possessing erythropoiesis-inhibiting activity that we discovered, exhibited a strong erythropoiesis-inhibiting effect up to the point where EPO concentration levels in the culture solution were 0.5 U/ml. However, it lost this effect at 4 U/ml. (See Figure 5.) This fact substantiates the clinical results in which anemia improved as EPO concentration level increased, even in the presence of erythropoiesis inhibitors such as KR4-0. The above result implies that removing erythropoiesis inhibitors such as KR4-0 may lead to the increased effectiveness of EPO, which in turn could result in the possibility of dosage reduction.

Conclusion

(1) Fraction KR4-0, which possesses powerful inhibiting effects on the formation of CFU-E colonies in mice, was seperated in the dialysate obtained using a large-pore-size membrane (BK-F). This KR4-0 was eluted in gel filtration, in the same location as IgM (1000kD). Furthermore, KR4-0 is water soluble, does not dissolve in chloroform or butanol, and is stable when heated at 100°C for 10 minutes. However, it loses its activity when heated at 100°C for 30 minutes.

(2) After a switch was made from ordinary membranes to BK-F membranes, four out of six patients showed significant increases in hematocrit levels in four months, with no EPO administration.

(3) Even if KR4-0 was present, the administration of large doses of EPO eliminated KR4-0's inhibiting effects on CFU-E colony formation. This result substantiates the clinical results which show improvement in anemia when large doses of EPO were administered, even in the presence of erythropoiesis inhibitors such as KR4-0.