Effects of Erythropoietin Injection on the Anemic Rats with Different Serum Erythropoietin Titer

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ABSTRACT. The effects of administration of purified erythropoietin (EPO) were investigated in four types of anemic model rats made by the injection of phenylhydrazine, iron deficiency, X-ray irradiation and partial nephrectomy. Serum EPO titers of those anemic rats were determined by radioimmunoassay. Partially nephrectomized rats showed chronically uremic anemia and their serum EPO titer remained decreased. These rats were recovered from anemia by injecting EPO. On the other hand, serum EPO titer of other types of anemic rats increased with the progress of anemia and the serum EPO titer showed reverse correlation with hemoglobin concentration. In these rats, curative effect of injections of EPO on anemia was not observed. All those results show that injection of EPO has therapeutic effect on anemia in the animals with renal failure and low serum EPO titer. However, injections of EPO did not show any effect on anemia in the animal with regular renal functions because of their own homeostatic EPO productions under anemic condition.—**KEY WORDS:** anemia, erythropoietin.

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Erythropoietin (EPO) has been known to be a humoral sialoglycoprotein which plays an important role in regulating erythropoiesis. As the purification of EPO demands the complicated processes and the final yield is very low [8], it is very difficult to obtain EPO as a pure form to use it for the improvement of anemia. However, recent usage of specific monoclonal antibody for human EPO on its purification procedures solved the problem [10]. Moreover, cloned EPO gene [5] will realize the mass production of recombinant human EPO in the near future and EPO may be used as the drug for anemia. Previously, we have indicated that purified EPO from human urine worked effectively on the stimulation of erythropoiesis *in vivo* and might be used for the improvement of anemia due to renal failure [7].

In this report, we examine the therapeutic effects of the purified EPO injection in the various types of model anemic rats and

investigate the possibility of using EPO for the improvement of anemia.

MATERIALS AND METHODS

EPO: Human EPO was isolated from urine of anemic patients using an immunoadsorbent column of monoclonal antibody and Sephadex G-100 column [10]. Specific activity of purified EPO was 82,000 units/mg measured by the *in vivo* ^{59}Fe incorporation assay method using starved rat [4] and 88,000 units/mg measured by the *in vitro* ^3H -thymidine incorporation assay method, using fetal mouse liver cells as the target [2].

Animals: Throughout these studies, female Wistar rats, purchased from Charles River Japan Inc., were used and maintained in a constant temperature on a standard diet and water *ad libitum* except maintenance of iron deficiency rats. Rats were weighed and blood for hematological and biological ex-

aminations was collected from the retro-orbital sinus every week.

Normal rats and four kinds of anemic rats were used in this examination. Partially nephrectomized rats were prepared according to the two stage surgical procedure described by Anagnostou *et al.* [1]. Rats of 7 weeks old were anesthetized with ether and the upper and lower poles of the left kidney were removed through a dorso-ventral incision, leaving the pelvis and the hilus intact. Five days later, the right kidney was removed leaving one-sixth of the original renal mass intact. At the end of each operation, the rats were injected intraperitoneally with 1,000 units of penicillin G.

Hemolytic anemia was induced by the injection of phenylhydrazine. Rats received 20 mg phenylhydrazine per kg body weight through the intra-peritoneal injections every day for a week.

Anemic rats with X-ray irradiation were prepared as follows. Seven-week-rats were irradiated with 750R or 1,000R (30R/min) of 150 KV X-rays under 25 cm of source-skin distance. X-ray was generated by the X-ray irradiator (Hitachi X-ray Irradiator Model MBR-1505R) with filters of 0.1 mm Cu and 0.5 mm Al.

For the iron deficiency, weanling rats were maintained for 7 weeks with nutritionally adequate basal ration but iron source and de-ionized water.

Hematological and biological examinations: Hematocrits were determined using microcapillary method. Hemoglobin concentrations were estimated by sodium-lauryl-sulfate hemoglobin method using Hemoglobin B-Test Wako (Wako Pure Chemical Industries Ltd. Japan). Reticulocyte counts were determined by Brecher method [3]. Red blood cell (RBC), white blood cell (WBC) and platelet were measured using microcell counter (Sysmex microcell counter Model CC-120) or platelet counter (Sysmex platelet counter Model

PL-100). Serum was stocked in freezer until biological examinations. Serum iron, creatinine, blood urea nitrogen (BUN) and bilirubin were measured by Ferrozine method, Jaffé method, urease-indophenol method and Michaelson method, respectively, using automatic analyzer (Hitachi autoanalyzer Model 705). Serum EPO titer was determined by the radioimmunoassay developed using highly purified iodinated human EPO as tracer and antisera from rabbits immunized with highly purified human EPO [9].

Administration schedule of erythropoietin: The purified EPO was diluted with phosphate-buffered-saline (PBS) containing 0.1% rat serum albumin for stabilization. Four units of EPO for each 100 g of body weight were injected subcutaneously on each administration. Control group received the same solution used for the dilution of EPO.

Normal rats received EPO every day for a week after 2 times of blood sampling.

Partially nephrectomized rats were injected EPO every day for a week after a blood sampling at 3 weeks after operations.

The rats of hemolytic anemia were given EPO every day for a week simultaneously with intraperitoneal injection of phenylhydrazine after a blood sampling.

The rats with X-ray irradiation of 1,000R received EPO every day for a week at a week after irradiation. One group of 750R X-ray irradiation rats (750R(A)), received EPO every day for a week at 2 weeks after irradiation. Another group of 750R X-ray irradiation rats (750R(B)), was given EPO every day for 2 weeks at a week after irradiation.

Iron deficient rats were given EPO simultaneously with intraperitoneal injection of gluferricon (Nihonzoki Co., Japan) every day for 3 weeks after 4 weeks iron deficient feeding. Sixty-six mg gluffericon for each 100 g body weight were injected every day for 2 weeks and 200 mg for the next one

week. The other group of iron deficient rats was given PBS containing 0.1% rat serum albumin (the solution for diluting EPO) every day for 3 weeks in order to confirm the progress of anemia.

RESULTS

Conditions of anemic animals: Partially nephrectomized rats showed immediate decrease of hematocrit. Three weeks after operations, their hematocrit fell down from 43% to 32%. BUN and creatinine in serum increased and serious uremic anemia was observed (Fig. 1 and Table 1).

The rats injected phenylhydrazine also showed acute hemolytic anemia. Spleen weight and serum bilirubin concentration increased and their hematocrit decreased to 35% after the daily intraperitoneal injection for a week (Fig. 1 and Table 1).

By the X-ray irradiation, gradual decrease of RBC and acute decrease of WBC and platelet in peripheral blood were observed. X-ray irradiation of 750R provided rats a certain damage to their hemopoietic stem cell. Two weeks after irradiation, hematocrit fell down to about 30% and recovered gradually. On the other hand, 1,000R irradiation gave rats serious damage to their hemopoietic stem cell. Apparent decrease of spleen weight was observed and their hematocrits fell down to 11%, 2 weeks later (Fig. 1 and Table 1). Some rats died at 3 weeks after irradiation.

In iron deficient anemic rats, the hematocrits fell down to 14% at 4 weeks after iron deficient feeding. Hematological and biological remarks of those model rats were summarized in Fig. 1 and Table 1.

Effects of EPO injection on improvement of anemia: Table 1 shows the change of hematological parameters when EPO was injected. Hematocrits (42.8%), hemoglobin (14.2 g/dl), RBC ($750 \times 10^4/\text{mm}^3$) and reticulocyte counts (2.3%) of normal rats in-

Fig. 1. Conditions of anemic model rats. Conditions of each model rat were as follows: A. normal rat, 11 weeks old (n=6); B. partially nephrectomized rat, 3 weeks after operation (n=6); C. 750 R of X-ray irradiated rat, 2 weeks after irradiation (n=6); D. 1000 R of X-ray irradiated rat, 2 weeks after irradiation (n=5); E. phenylhydrazine injected rat, after the dairy injection for a week (n=8); F. iron deficient rat, 3 weeks after iron deficient feeding from weanling (n=8).

creased to 49.6%, 16.2 g/dl, $843 \times 10^4/\text{mm}^3$ and 6.0%, respectively, by the injections of EPO, while these values in the control group were not changed.

The erythropoietic effects of EPO were clearly observed on partially nephrectomized rats. Hematocrits (32.8%), hemoglobin (10.4 g/dl), RBC ($621 \times 10^4/\text{mm}^3$) and reticulocyte counts (1.4%) increased to 42.8%, 14.5 g/dl, $762 \times 10^4/\text{mm}^3$ and 8.5%, respectively, while these values in the control group were not changed.

In 750R (A) rats, hematological parameters of EPO injected group significantly increased at 3 weeks after irradiation. In 750R (B) rats, however, the mean values of hematological parameters of EPO injected

Table 1. Effects of EPO injections on the improvement of anemia

Subject	Time ^{a)} (week)	EPO injection schedule (week)	Hematocrit (%)		Hemoglobin (g/dl)		Red blood cell ($\times 10^4/\text{mm}^3$)		Reticulocyte count (%)	
			control	EPO	control	EPO	control	EPO	control	EPO
Normal n=6	0		42.5 $\pm$ 1.5 ^{b)}	41.4 $\pm$ 1.8	14.7 $\pm$ 0.3	14.2 $\pm$ 0.5	748 $\pm$ 39	723 $\pm$ 50	1.9 $\pm$ 0.3	1.9 $\pm$ 0.3
	1 ^{d)}		42.9 $\pm$ 0.8	42.8 $\pm$ 1.7	14.5 $\pm$ 0.1	14.2 $\pm$ 0.6	761 $\pm$ 29	750 $\pm$ 34	2.3 $\pm$ 0.4	2.3 $\pm$ 0.4
	2	1	43.4 $\pm$ 1.0	49.6 $\pm$ 1.9 ^{**c)}	14.7 $\pm$ 0.2	16.2 $\pm$ 0.6 ^{**}	797 $\pm$ 54	843 $\pm$ 61	2.1 $\pm$ 0.5	6.0 $\pm$ 1.2 ^{**}
Partially nephrectomized n=6	2		37.0 $\pm$ 1.4	36.9 $\pm$ 3.1	12.4 $\pm$ 0.6	12.0 $\pm$ 1.2	696 $\pm$ 33	679 $\pm$ 81	0.8 $\pm$ 0.4	1.1 $\pm$ 0.9
	3	1	32.8 $\pm$ 1.0	32.8 $\pm$ 1.6	10.7 $\pm$ 0.5	10.4 $\pm$ 0.3	614 $\pm$ 29	621 $\pm$ 42	1.6 $\pm$ 0.7	1.4 $\pm$ 0.4
	4		32.9 $\pm$ 1.2	42.8 $\pm$ 2.5 ^{**}	11.1 $\pm$ 0.6	14.5 $\pm$ 0.7 ^{**}	620 $\pm$ 39	762 $\pm$ 51 ^{**}	3.0 $\pm$ 1.0	8.5 $\pm$ 1.8 ^{**}
Phenylhydrazine treated n=8	0		41.4 $\pm$ 1.3	41.3 $\pm$ 1.3	14.0 $\pm$ 0.5	13.9 $\pm$ 0.5	710 $\pm$ 28	721 $\pm$ 35	3.0 $\pm$ 0.8	2.9 $\pm$ 0.5
	1	1	35.0 $\pm$ 2.1	35.4 $\pm$ 1.5	7.8 $\pm$ 0.7	8.2 $\pm$ 0.6	330 $\pm$ 34	355 $\pm$ 22	58.2 $\pm$ 4.4	57.0 $\pm$ 4.4
750 R n=12	1		36.4 $\pm$ 1.4	36.5 $\pm$ 2.1	12.5 $\pm$ 0.5	12.2 $\pm$ 0.5	645 $\pm$ 33	638 $\pm$ 41	0.6 $\pm$ 0.6	0.3 $\pm$ 0.3
	2	1	29.7 $\pm$ 2.1	29.6 $\pm$ 2.8	9.9 $\pm$ 1.0	9.8 $\pm$ 1.0	506 $\pm$ 45	487 $\pm$ 42	3.2 $\pm$ 0.7	3.9 $\pm$ 1.3
	3		37.0 $\pm$ 2.2	40.4 $\pm$ 2.1 ^{**}	11.6 $\pm$ 0.7	13.0 $\pm$ 0.6 ^{**}	560 $\pm$ 37	590 $\pm$ 34	9.3 $\pm$ 3.2	10.6 $\pm$ 1.3 ^{**}
750 R n=6	1		37.1 $\pm$ 1.4	36.5 $\pm$ 1.1	12.5 $\pm$ 0.5	12.2 $\pm$ 0.5	634 $\pm$ 28	631 $\pm$ 41	0.5 $\pm$ 0.3	0.7 $\pm$ 0.3
	2	1	27.9 $\pm$ 3.4	27.6 $\pm$ 5.4	9.0 $\pm$ 1.0	8.8 $\pm$ 1.8	475 $\pm$ 44	463 $\pm$ 85	2.0 $\pm$ 1.2	2.3 $\pm$ 1.0
	3		34.0 $\pm$ 6.2	36.9 $\pm$ 6.3	10.6 $\pm$ 2.0	11.2 $\pm$ 2.2	502 $\pm$ 87	535 $\pm$ 103	11.0 $\pm$ 3.0	13.4 $\pm$ 3.0
1000 R n=5	0		40.0 $\pm$ 2.0	40.2 $\pm$ 0.9	13.2 $\pm$ 0.4	13.3 $\pm$ 0.3	655 $\pm$ 29	656 $\pm$ 17	5.3 $\pm$ 0.3	4.4 $\pm$ 0.7
	1	1	37.8 $\pm$ 1.5	37.8 $\pm$ 0.5	12.9 $\pm$ 0.6	12.8 $\pm$ 0.2	641 $\pm$ 48	641 $\pm$ 10	0 $\pm$ 0	0.09 $\pm$ 0.14
	2		11.8 $\pm$ 2.3	10.6 $\pm$ 4.6	4.4 $\pm$ 0.9	3.8 $\pm$ 1.3	203 $\pm$ 58	182 $\pm$ 87	0.4 $\pm$ 0.2	0.8 $\pm$ 0.7
With Gluferricon(Fe) n=8	3		17.6 $\pm$ 0.6	17.5 $\pm$ 1.7	5.0 $\pm$ 0.4	4.8 $\pm$ 0.5	293 $\pm$ 24	286 $\pm$ 33	13.8 $\pm$ 3.6	15.3 $\pm$ 2.3
	4		14.9 $\pm$ 0.3	14.6 $\pm$ 1.6	3.5 $\pm$ 0.4	3.4 $\pm$ 0.4	246 $\pm$ 22	244 $\pm$ 25	18.0 $\pm$ 2.6	18.1 $\pm$ 2.4
	5	3	16.4 $\pm$ 1.3	16.0 $\pm$ 1.5	3.8 $\pm$ 0.2	3.7 $\pm$ 0.4	266 $\pm$ 31	268 $\pm$ 27	26.3 $\pm$ 1.6	26.5 $\pm$ 2.5
	6		17.3 $\pm$ 1.2	16.4 $\pm$ 1.8	3.9 $\pm$ 0.4	3.7 $\pm$ 0.5	278 $\pm$ 38	267 $\pm$ 32	25.3 $\pm$ 4.1	25.0 $\pm$ 3.8
Iron deficiency Without Gluferricon(Fe) n=8	7		24.5 $\pm$ 3.1	22.1 $\pm$ 2.6	5.3 $\pm$ 0.7	4.8 $\pm$ 0.6	412 $\pm$ 58	363 $\pm$ 43	27.2 $\pm$ 2.4	25.3 $\pm$ 3.7
	3		17.9 $\pm$ 0.7	—	4.7 $\pm$ 0.4	—	287 $\pm$ 8	—	13.9 $\pm$ 2.3	—
	4		15.2 $\pm$ 0.9	—	3.5 $\pm$ 0.4	—	245 $\pm$ 13	—	17.5 $\pm$ 1.7	—
	5	3	13.9 $\pm$ 1.1	—	3.3 $\pm$ 0.4	—	218 $\pm$ 11	—	19.2 $\pm$ 2.1	—
Without Gluferricon(Fe) n=8	6		12.6 $\pm$ 1.2	—	3.0 $\pm$ 0.3	—	200 $\pm$ 13	—	21.1 $\pm$ 2.2	—
	7		12.1 $\pm$ 1.8	—	2.4 $\pm$ 0.3	—	198 $\pm$ 20	—	23.9 $\pm$ 2.2	—

a) Time shows weeks after treatment to make anemia.

b) mean $\pm$ SD.c) ** Significant to the each control. ($p < 0.01$)

d) EPO injections were started after the blood sampling at the week.

group were slightly but not significantly higher than those of the control group at 3 weeks after irradiation.

Improvement of anemia by the injections of EPO was not observed in both 1,000R irradiation and phenylhydrazine groups.

The hematological parameters of iron

deficient rats increased by the injections of gluferricon, and simultaneous injections of EPO with gluferricon did not show any additional effect.

Serum EPO titers of anemic rats: Serum EPO titer in those model animals was measured. As shown in Fig. 2, reverse

Fig. 2. Serum EPO titers of anemic model rats. Logarithm of serum EPO titers was plotted against hemoglobin concentrations. Δ normal rat; $\circ$ partially nephrectomized rat; $\blacktriangle$ X-ray irradiated rat (750 R and 1000 R); $\blacksquare$ phenylhydrazine injected rat; $\bullet$ iron deficient rat.

correlation was obtained between serum EPO titer and hemoglobin concentration in the anemic model rats made by the injections of phenylhydrazine, iron deficiency and X-ray irradiation of 750R and 1,000R ($r = -0.807$). On the other hand, serum EPO titer of partially nephrectomized rats did not increase in spite of the decrease of serum hemoglobin concentration.

DISCUSSION

EPO has been known to stimulate erythropoiesis and its clinical use for anemic patients has been long awaited. A very simple isolation procedure with a high yield of EPO using a monoclonal antibody column provides EPO in pure form [10].

In this report, we examined the possibility of use of EPO for the improvement of anemia. Four kinds of model anemic rats were prepared and the therapeutic effects of the injections of purified EPO were examined.

Partially nephrectomized rats showed uremic anemia (Fig. 1 and Table 1) and their serum EPO titers were reduced. In spite of the progress of anemia, serum EPO titer did not increase (Fig. 2) and injections of EPO produced the very effective improvement of anemia (Table 1).

Irradiation of 1,000R X-ray damaged hemopoietic stem cell and made serious anemia (Fig. 1 and Table 1). Injections of EPO did not provide any apparent improvement of anemia (Table 1). In spite of the injection of EPO, anemia of the group irradiated with 750R of X-ray was also aggravated until 2 weeks after irradiation. The degree of irradiation damage was slightly different between 750R (A) and 750R (B), that is, anemic condition of 750R (A) was rather weaker than that of 750R (B). At 3 weeks after irradiation, anemic condition of EPO injected group was slightly but significantly recovered in 750R (A). Although no significant recovery was observed in 750R (B), the characteristic hemopoiesis by EPO injection was present to some degree in both 750R (A) and 750R (B). Therefore, the different effects of EPO injection between 750R (A) and 750R (B) may be due to the degree of irradiation damage to the hemopoietic stem cell [6].

Phenylhydrazine-injected rats showed acute hemolytic anemia and injection of EPO did not show any improvement of anemia in these rats (Table 1).

Iron deficient anemic rats showed low hematocrit value (14%). This anemic condition was recovered only by the injection of glufericon and the injection of EPO did not show any additional effects (Table 1).

In anemic rats made by X-ray irradiation,

phenylhydrazine injection and iron deficiency, serum EPO titer increased following the progress of anemia and reverse correlation was observed between serum EPO titer and hemoglobin concentration as shown in Fig. 2.

These results indicate that injection of EPO is apparently effective on the anemic rats that have renal disease and those serum EPO titers are reduced. In the anemic rats that have regular renal functions, however, injection of EPO does not provide any effective improvement of anemia, possibly because they have the normal homeostatic production of EPO in kidney providing the elevation of serum EPO titer in response to the reduced hemoglobin concentration.

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要 約

貧血モデルラットに対する精製エリトロポエチン投与の効果と貧血ラットの血清エリトロポエチン濃度の関係：升永博明・村上晶彦・後藤雅昭・上田正次（雪印乳業(株)生物科学研究所）——ラットで腎性貧血，鉄欠乏性貧血，X線照射性貧血，フェニルヒドラジン性貧血の病態モデルを作製し，これら貧血ラットの血清エリトロポエチン濃度をラジオイムノアッセイにより測定した。一方，貧血モデルラットに対して尿から精製純化したエリトロポエチンを体重100 g 当り4単位を毎日，皮下に注射し貧血改善効果を観察し，つぎの成績を得た。1) 腎性貧血を除く3種類の病態モデルにおいて，ヘモグロビン濃度の低下にともなう血清エリトロポエチンの著明な上昇が確認され有意な負の相関を示した ($r=-0.807$)。2) 無処理ラット及び腎性貧血ラットではエリトロポエチン投与により著しい増血を示した。鉄欠乏性貧血ラットは鉄剤投与に依存した増血を示し，エリトロポエチンの併用効果は認められなかった。X線照射 (750R) による骨髓抑制性貧血に対して，照射1週から2週にかけての貧血進行期では効果を示さず，2週以降3週にかけては貧血回復がわずかに対照群を上廻った。溶血性貧血ラットではエリトロポエチンの投与効果は認められなかった。