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Efficacy of Recombinant Human Erythropoietin(rhu-EPO)

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Abstract – Efficacy and *in vivo* bioassay of recombinant human erythropoietin (rhu-EPO), was investigated. Efficacy studies were conducted in normal, and cisplatin-induced anemic rats. Normal and anemic animals were treated intravenously with rhu-EPO for 5 days, and the changes in the number of red blood cells (RBC), hemoglobin concentration (Hb), hematocrit value (Hct) and percentage reticulocyte value (Ret, reticulocyte/RBC) were examined. In normal rats, rhu-EPO significantly increased RBC, Hb, Hct and Ret at the doses of 50~1,250 IU/kg/day in a dose-dependent fashion. Cisplatin-induced anemic rats showed significant increase of RBC, Hb, Hct and Ret after administration of rhu-EPO (50-200 IU/kg/day) in a dose-dependent manner. These changes of hematological parameters disappeared gradually after cessation of the treatment. The *in vivo* bioassay results in polycythemic mice showed that rhu-EPO had 90% of bioactivity compared to NIBSC standard rhu-EPO. These results suggest that rhu-EPO might be useful for the therapy of anemia originated from renal failure and chemotherapy-induced anemia.

Keywords □ rhu-EPO, efficacy, bioassay, erythropoiesis, cisplatin, polycythemic

Erythropoietin (EPO) is a glycoprotein hormone produced by kidneys or certain extrarenal tissues and is the primary endogenous regulator of red blood cell formation (Graber and Krantz, 1978). EPO reduction due to the loss of viable renal tissue in cases of renal failure as well as the anaemia from other causes (arthritis, AIDS, cancer) has led to the use of EPO for treating these and other conditions (Abels et al., 1991; Goodnough et. al., 1989; Stone et al., 1988). It is a part of a complex feedback system that ultimately adjusts the size of cell mass to demand of oxygen by the tissues (Bauer and Kurtz, 1989). With the recent production of EPO by recombinant technology, the *in vivo* bioassay of recombinant human erythropoietin (rhu-EPO) has become a necessity for evaluation of potency for production batches (Barbone et al., 1994). Recently, our laboratory has developed the method of producing novel rhu-EPO by mammalian cells transfected with a human erythropoietin gene. Aim of the present study was to investigate the efficacy of rhu-EPO by using normal and cisplatin-induced anaemic rats and to compare the erythropoietic potential of rhu-EPO with that of reference standard using bioassay in polycythemic mice.

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MATERIALS AND METHODS

Test substance

Recombinant human erythropoietin (rhu-EPO; code name DA-3585; Lot No. BTS-003) was purified chromatographically from the conditioned medium of Baby Hamster Kidney (BHK) cells transfected with a vector containing human erythropoietin gene. In this paper, EPO activity is expressed as *in vitro* activity measured by ELISA. rhu-EPO was formulated in 3% mannitol solution adjusted pH 7.0 by sodium hydroxide containing 34.2 mM sodium chloride, 12.5 mM sodium monophosphate (monobasic) and human serum albumin (1.0 mg/ml). For the bioassay test, Standard EPO were obtained from NIBSC (ampoule code 87/684). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Six-week old male SD rats and six-week old female ICR mice obtained from Charles River, Japan were fed on a standard pelleted diet(Cheiljedang Co. Korea) and tap water *ad libitum*. The animal quarters were maintained at 21-25℃ and 45-65% relative humidity. A 12h-light-and-dark cycle was repeated. All the animals were

acclimatized for a week in order to stabilize the hematocrit.

Efficacy test of rhu-EPO in normal rat

rhu-EPO was administered intravenously at the dose of 0, 50, 250 and 1,250 IU/kg once a day for 5 days. Blood samples were obtained before and after 3, 7, 10 and 14 days of first dose of rhu-EPO. On each occasion, blood was withdrawn from the orbital sinus (under light ether anesthesia) and measured for haematological parameters. Number of red blood cells (RBC), hemoglobin concentration (Hb) and hematocrit value (Hct) were determined by cell counter (Minos Vet™, ABX) and percentage reticulocyte value (Ret, reticulocyte/RBC) was counted on smears stained with new methylene blue solution (Brecher, 1949).

Efficacy test of rhu-EPO in cisplatin-induced anemic rat

All rats were received a single intravenous injection of 8 mg/kg cisplatin(cis-diamine-dichloroplatinum, CDDP) and randomized into four groups. Eighteen days later, the animals were administered rhu-EPO intravenously at the dose of 0, 50, 100 and 200 IU/kg for 5 days. Blood samples were obtained before and after 8, 18, 21, 25, 28, 32 and 35 days of cisplatin injection. On each occasion, blood was withdrawn from the orbital sinus and hematological parameters were determined as described above.

Bioassay of rhu-EPO in polycythemic mouse

In vivo activity of newly developed rhu-EPO was measured by the method of Kazal and Erslev (1975). In brief, each mouse was intramuscularly injected with 1mg of iron dextran and placed in low-pressure chamber at 0.4 atmosphere for 16 hrs each day for two weeks. The polycythemic mice were then rested for 5 days prior to the assay. rhu-EPO preparations were injected subcutaneously once a day for 2 days. Then 0.5 Ci of ^{59}Fe was injected intraperitoneally 24 hrs after last dose of rhu-EPO (Table I). Sixty-six hrs after the radioiron injection, the mice were anesthetized with ether and bled from orbital sinus. Data from mice with low hematocrits (less then 55% at autopsy) were discarded. The ^{59}Fe in an aliquot of blood was counted in a well-type gamma counter and the percentage ^{59}Fe utilization was calculated on the basis of blood volume (7% of body weight). This was compared to a value generated with standard rhu-EPO obtained from NIBSC.

Table I. Experimental design of rhu-EPO bioassay in polycythemic micc

Groups	Test substances	Injection dose (IU/head/day)	Total dose (IU/head)	No. of animals
1	vehicle	0	0	8
2	rhu-EPO	0.25	0.5	8
3		0.5	1.0	8
4	standard EPO	0.25	0.5	8
5		0.5	1.0	8

Statistical analysis

The results of efficacy tests were tested by one way analysis of variance (ANOVA). If ANOVA indicated significant difference between treatment and control groups, a Dunnett's test was performed. When the data failed Normality test, non-parametric analysis of variance (Kruskal-Wallis test) was used. Treatment values differing from control at the level of $P<0.05$ are indicated with an astrisk.

Statistical analysis of bioassay in polycythemic mouse followed by 2-dose multiple assay method as described by European Pharmacopeia (1997).

RESULTS

Effects of rhu-EPO in the normal rats

As depicted in Fig. 1, consecutive injection of rhu-EPO to normal rats resulted in dose-dependent increase of RBC, Hb, Hct and Ret.

At the dose of 1,250 IU/kg, rhu-EPO produced a rapid increase of RBC, reaching a maximum on tenth day after first dosing. Hb and Hct were significantly increased between third to tenth day at doses of 1,250 and 250 IU/kg groups compared with control and reached the peak at day 7. On the seventh day, Ret from 1,250 IU/kg group was significantly increased to 8.87% and then decreased on the tenth day.

Effects of rhu-EPO in the cisplatin-induced anemic rats

As shown in Fig. 2, RBC, Hb and Hct were decreased at 18th day in the group of receiving 0 IU/kg of rhu-EPO (control group), and it has returned to the normal value at 5 weeks after the cisplatin injection.

Therapeutic experiment with rhu-EPO was performed on cisplatin-induced anemic rats at doses of 50, 100 and 200 IU/kg. The number of RBC increased after rhu-EPO injection in a dose-dependent manner compared with that of control and above normal range in 200 IU/kg group on 25th and 28th days. The treatment with rhu-EPO caused a significant dose-dependent increase of Hb and Hct

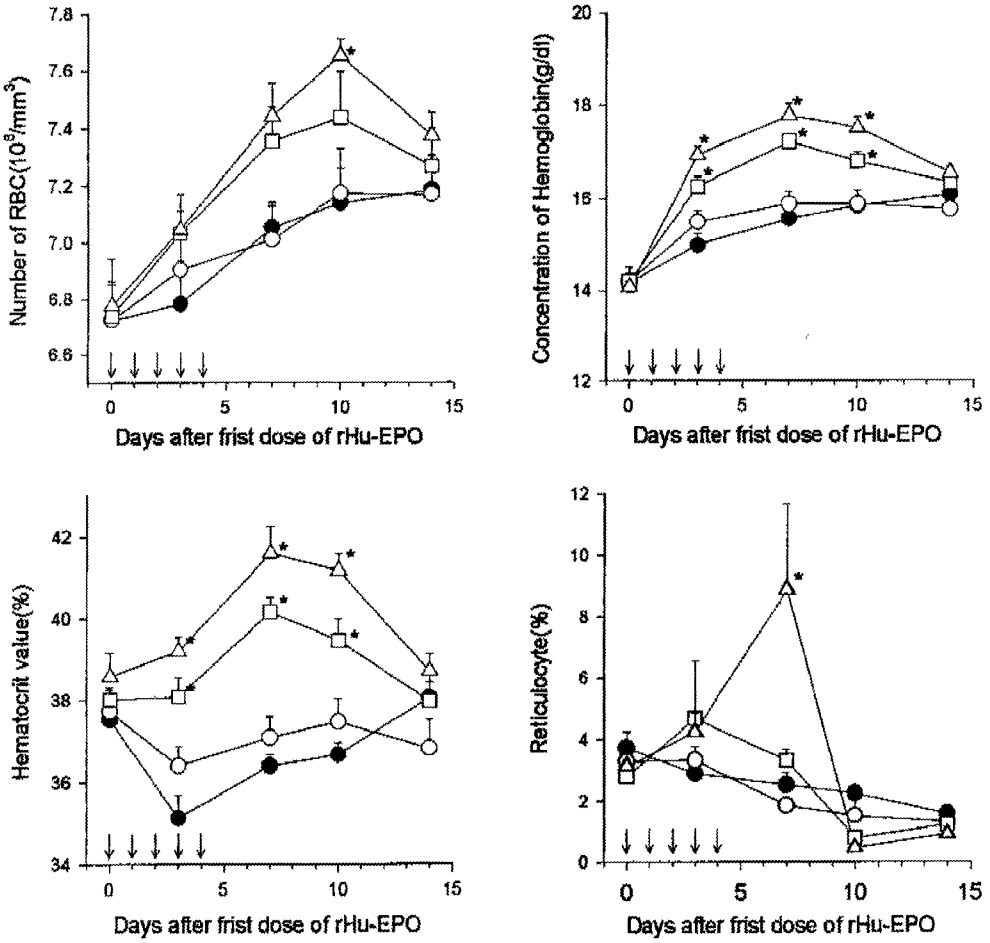

Fig. 1. Effects of rhu-EPO on erythropoiesis in normal rats injected intravenously once a day for 5 days. Each point represents the mean±S.E.M. of 8 rats. The astrisk indicates the value significantly different from control at $P<0.05$. ↓: The date of rHu-EPO administration (0 IU/kg, ●; 50 IU/kg, ○; 250 IU/kg, □; 1,250 IU/kg, △).

resembling RBC change. In 100 and 200 IU/kg dose groups, Ret reached two or three times then that of control at day 7 and rapidly decreased under the control value after tenth day.

Bioassay of rhu-EPO

On the 6th day after the termination of hypoxia, rhu-EPO were injected for 2 days and 24hr later ^{59}Fe was injected for measurement of ^{59}Fe incorporated RBC. New preparation of rhu-EPO (BTS-003) showed 89.3% relative activity compared with NIBSC standard EPO and its fiducial limit was 0.801-0.983.

DISCUSSION

The erythroid system is considered to begin at the lev- of the earliest unipotential erythroid stem cell, which dergoes maturation and proliferation to form the colony forming unit-erythroid (CFU-E), and then the morphologically recognizable precursors (Iscove and Sieber, 1975). This system is influenced by erythropoietin (EPO), a glycoproteic hormone produced by kidneys and other tissues.

In the present study, we examined the effect of newly developed recombinant human erythropoietin (rhu-EPO) on normal and cisplatin-induced anemic rats. In the results, rhu-EPO dose-dependently increased RBC, Hb, Hct and Ret at dose levels of 250 and 1,250 IU/kg in normal rats. The percentage of reticulocyte reached a peak at 7th day and decreased rapidly after 10th day, which indicated the loss of erythropoietic effects of exogenous rhu-EPO. One of the last events in the differentiation and maturation of red blood cells is the production of the reticulocyte. The reticulocyte is the last cell in the generation of erythrocytes to have any nuclear

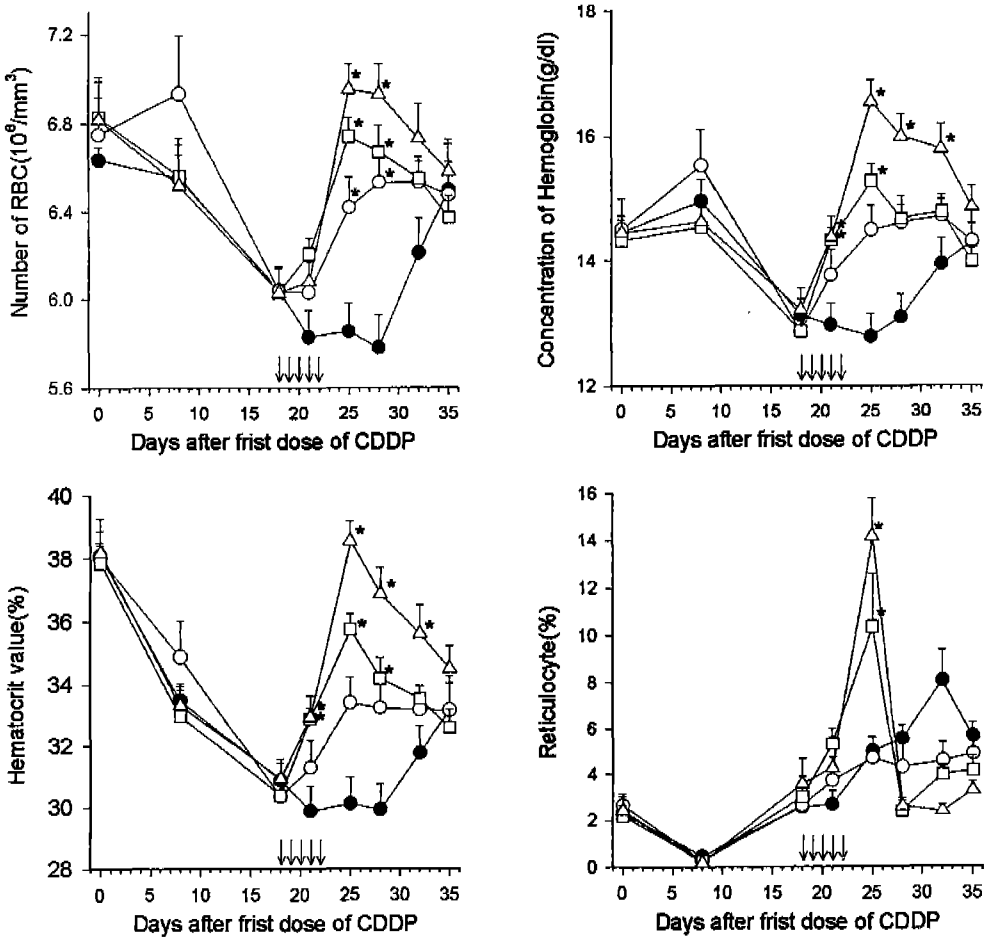

Fig. 2. Effects of rhu-EPO on erythropoiesis in anaemic rats. Animals were administered CDDP (cisplatin 8 mg/kg, i.v.) and injected rhu-EPO intravenously once a day for 5 days. Each point represents the mean±S.E.M. of 8 rats. The astrisk indicates the value significantly different from control at $P<0.05$. ↓ : The date of rHu-EPO administration (0 IU/kg, ●; 50 IU/kg, ○; 100 IU/kg, □; 200 IU/kg, △).

material. EPO is known to stimulate the production of red blood cells and speed up intramarrow transit time. This effect causes more reticulocytes to migrate into the circulation that allows for the determination of erythropoietic activity based on reticulocyte counts in the peripheral blood. RBC reached a peak at tenth day following initiation of EPO treatment. The difference of the peak time of RBC and Ret is thought to be the results of accumulation of matured RBC converted from newborn reticulocytes. It takes 1 or 2 days and RBC lifetime is about 45-70 days in rats. Masunaga et al. (1986) reported that EPO administration to partially nephrectomized rats increased RBC and hemoglobin concentration and the maximal values appeared slightly later than that of reticulocyte count. These reports are consistent with our present result. On the day of 14, the hematological parameters including RBC, Hb and Ret in rhu-EPO-treated rats were lower than those of control. It is thought that intrinsic EPO production was transiently reduced for the maintenance of biological constancy. In addition, this phenomenon was also observed by other investigators (Fuchs and Eder, 1992; Masunaga et al., 1992).

Cisplatin, a potent antitumor drug, is used widespread in cancer treatment protocols. Nausea, vomiting, nephrotoxicity, and progressive normocytic anemia are most commonly known as adverse effects (Kuzur and Greco., 1981; Rossof et al., 1972). Possible mechanisms for the anemia associated with cisplatin therapy include 1) decreased erythropoietin production caused by nephrotoxicity, 2) direct damage of hematopoietic stem cells or their accessory cells, and 3) increased red cell destruction. Of these, the primary defect appears to be decreased stem cell production (Rothmann et al., 1985). In the present study, there was a rapid and marked response to intra-

venous administration of rhu-EPO in cisplatin-induced anemic rats. This response was similar to the results obtained in normal rats. Moreover these results are consistent with the reports of Wood and Hrushesky (1995) and Matsumoto et al (1990). These results clearly demonstrate rhu-EPO is useful for cisplatin-induced anemia. However, it is thought that rhu-EPO has no effect on renal function. There was no difference in serum levels of urea nitrogen and creatinine between rhu-EPO-treated rats and vehicle-treated rats (data not shown).

In bioassay test, newly produced rhu-EPO showed 90% *in vivo* activity of NIBSC standard EPO. This suggests 1 IU *in vitro* potency of the rhu-EPO is equivalent with 0.9 IU *in vivo* activity.

In conclusion, it is suggested that rhu-EPO has hematopoietic effect both in normal and cisplatin-induced anemic rats.

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