Pharmacokinetic and Pharmacological Profiles of Free and Liposomal Recombinant Human Erythropoietin After Intravenous and Subcutaneous Administrations in Rats

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Purpose. Recombinant human erythropoietin (Epo) is used frequently through intravenous (i.v.) and subcutaneous (s.c.) administration for the clinical treatment of the last stage of renal anemia. We encapsulated Epo in liposomes to develop an alternative administration route. The purpose of our study was to evaluate the pharmacokinetics and the pharmacological effects of liposomal Epo in comparison with the Epo after i.v. and s.c. administration to rats.

Methods. Epo was encapsulated in liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and soybean-derived sterol mixture (SS) prepared by the reversed-phase evaporation vesicle method. After filtration through a 0.1 μm polycarbonate membrane, liposomes were gel filtered (Epo/liposomes).

Results. Epo/liposomes showed higher pharmacological activity than Epo/liposomes before gel filtration after i.v. administration to rats. Non-encapsulated Epo lost its activity, whereas encapsulated Epo in liposomes retained it. The pharmacological effects of Epo/liposomes were greater than those of Epo after i.v. administration. Epo/liposomes afforded 3–9 times higher AUC, lower clearance and lower steady-state volume of distribution than Epo after both i.v. and s.c. administrations. Epo/liposomes had an improved pharmacokinetic profile compared with Epo. S.c. administration of Epo/liposomes at 7 h may penetrate primarily (40% of dose) through the blood as a liposome and partly (7% of dose) in lymph.

Conclusions. Epo/liposomes may reduce the frequency of injections required for a certain reticulocyte effect in comparison to Epo. The lower clearance of Epo/liposomes may increase the plasma concentrations of Epo, which increases the efficacy.

KEY WORDS: recombinant human erythropoietin; liposome; intravenous administration; subcutaneous administration; pharmacokinetics; pharmacological effect.

INTRODUCTION

Recombinant human erythropoietin (Epo) is a glycoprotein produced primarily in the kidneys and to a lesser extent in the liver. Epo is a single-chain polypeptide with a molecular weight of about 30000, about 40% of which is ascribed to sugar moiety (1). The physiological function of Epo is to regulate the proliferation and differentiation of erythroid precursor cells to red blood cells (RBC). Epo is produced on a large scale by recombinant DNA technology and has been proven to effectively treat renal anemia (2). Epo, which is a peptide medicinal drug, is currently limited to intravenous (i.v.) and subcutaneous (s.c.) administrations at 2–3 times a week.

The use of liposomes as carriers of cytokines, interleukin-2 (3), interleukin-7 (4), interferon (5) and insulin (6) has been reported. The liposomes with a prolonged circulation time in the blood have been developed by coating them with materials, such as polyethyleneglycol (PEG) (7,8) or ganglioside G_{MI} (9), having sialic acid that inhibits uptake by the galactosyl receptor in the liver. The liposomal Epo (Epo/liposomes) may evade the reticuloendothelial system (RES) without PEG or G_{MI} if the terminal sialic acid residues from the sugar moiety of Epo project out from the liposomes. Therefore, pharmacokinetic behavior of Epo/liposomes and their use as delivery systems could extend the therapeutic possibilities of Epo, for example, for parenteral and non-parenteral administration.

Recently, we reported that the small (≤0.2 μm) dipalmitoylphosphatidylcholine (DPPC) liposomes with soybean-derived sterol mixture (SS) were useful Epo carriers and these liposomes prolonged circulation time due to the rigidity of the liposomal membrane (10–13). The purpose of this study was to evaluate the pharmacokinetics and pharmacological effects of Epo/liposomes compared with those of Epo after i.v. and s.c. administrations in rats (14). Pharmacological effects of Epo were evaluated by sysmex and smear methods.

MATERIALS AND METHODS

Materials

DPPC was purchased from NOF Corporation (Tokyo, Japan). SS was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) and was kindly provided by Ryukakusan Co., Ltd. (Tokyo, Japan). Epo was a gift from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals used were of reagent grade. Male Wistar rats were purchased from Saitama Experimental Animal Supply (Saitama, Japan) (11).

Preparation of Epo/liposomes

Liposomes were prepared according to the reversed-phase evaporation vesicle method (13). DPPC (70 μmol) and SS (20 μmol) in chloroform were deposited in a flask, and the organic solvent was removed. Epo (180000 IU/ml) preparation was serially diluted with 1/10 phosphate-buffered saline solution (1/10 PBS, pH 7.4) to make 10800, 32400 and 54000 IU/ml solutions. The lipid film was redissolved in chloroform and isopropyl ether. To the resulting organic phase, the aqueous phase including 3 ml of each Epo was added. The mixture was sonicated to become a homogeneous w/o emulsion, and then the organic solvent was removed. The preparation was extruded successively through polycarbonate membranes with pore sizes of 0.2 and 0.1 μm at about 50°C by Extruder(Lipex Biomembrane Inc., Canada). After extrusion, 0.5 ml of the preparation was passed through a Sephadex G-25 column (1.8 × 35 cm, Pharmacia, Sweden) with 1/10 PBS to remove non-encapsulated

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Epo. The dilution factor of liposome suspension after gel filtration was 9. Epo encapsulated in liposomes after gel filtration is referred to as Epo/liposomes.

Determination of Epo Concentration by HPLC

The Epo concentration of Epo/liposomes was determined by HPLC (13). A liposome suspension (0.3 ml) was shaken with 0.09 ml of chloroform to disrupt the liposomes. After centrifugation at 3000 rpm for 10 min, 0.2 ml of the aqueous phase containing Epo or standard solution of Epo was injected into HPLC. Retention of Epo in Epo/liposomes was calculated from the Epo concentration in Epo/liposomes after gel filtration corrected by the lipid recovery is determined using a Wako phospholipid B test (Wako Pure Chemical Ind., Ltd., Osaka, Japan).

Animal Experiments

Nine-week-old male Wistar rats (about 300 g) were used in all experiments. A dose of each Epo/liposomes (corresponding to 179, 538 or 896 IU/kg, for example, 538 IU/kg = 32400 (IU/ml) × 0.9 (ml/kg) × 0.166 × 1/9) was i.v. administered through the cervical vein and s.c. through the dorsal neck, and animals were returned to cages for further blood collections. Blood (10 or 20 μl) was collected from the dorsal metatarsal vein before and on 2, 4 and 7 d after administration of Epo/liposomes to evaluate the pharmacological effect. Blood (0.3 ml) was collected from the cervical vein before and on 0.5, 1, 2, 4, 7 and 24 h after administration of Epo/liposomes to evaluate the pharmacokinetics effect (14,15). In addition, lymph was collected from ductus thoracicus lymph nodes before and on 2, 4, 7, 9 and 12 h after s.c. administration of 1/10 PBS (untreated), Epo and Epo/liposomes to evaluate the pharmacokinetics effect.

Epo concentration in serum and lymph was measured by radioimmunoassay (RIA, Erythropoietin RIA CHUGAI.). Collected blood was centrifuged immediately to harvest serum (0.1 ml) (14).

Analysis of Pharmacokinetic Data

In the case of i.v. administration, the serum concentration data of Epo were fitted to the biexponential equation by a nonlinear regression program using MULTI (16). Then, the steady-state volume of distribution (Vd), half-lives ($t_{1/2}$, α and β phases) and total body clearance (Cl_{tot}) were calculated from hybrid parameters.

In the same way, the serum concentration profile of Epo following its s.c. administration was fitted simultaneously to the mean serum profile following its i.v. administration in the two-compartment model with the first-order input and output, by use of the MULTI program (16). Then, Ka was determined at low and middle doses. The area under the blood concentration curve (AUC) following i.v. and s.c. administrations was calculated by numerical integration using a linear trapezoidal formula. The Epo concentrations minus inherent serum Epo level were used.

Measurement of Percentage of Circulating Reticulocytes of RBC (Smear Method)

Collected blood (10 μl) was immediately put into a microplate well, mixed gently and allowed to stand for staining with new methylene blue. The smears were further treated with Giemsa's solution (E. Merck A. G., Germany). Numbers of reticulocytes and RBC were counted on the stained blood smear microscopically using a Miller ocular disc. Percentage of circulating reticulocytes of RBC was calculated as follows: 100 × (reticulocyte count in large squares)/(RBC count in small squares × 9) (14,15,17).

Measurement of Residual Circulating Reticulocyte Counts (Sysmex Method)

The stromatolized blood cells were counted using an automatic microcell counter (Sysmex F-500) and a cell monitor (Sysmex CM-5) after Quicklizer treatment. The difference in numbers counted at discriminator levels 1 and 5 was calculated as the residual circulating reticulocyte counts (14,15,17). mAUC and sAUC are the area under the pharmacological time-effect curve of rats measured by the smear and sysmex method, respectively calculating according to the trapezoidal rule.

RESULTS

Epo Activity and Concentration in Epo/liposomes

Liposomes passed through a polycarbonate membrane with a pore size of 0.1 μm showed 155.4 (low dose), 156.7 (middle dose) and 150.7 nm (high dose) as the mean diameter. Encapsulation efficiency was defined as the fraction of the aqueous compartment sequestered by bilayers (13). The ratio of Epo concentration after gel filtration to that before gel filtration was corrected by lipid recovery. Thus, % retention of Epo in Epo/liposomes was 11.5 (low dose), 16.6 (middle dose) and 13.1% (high dose) (data not shown). Low, middle and high doses of Epo/liposomes corresponded to 179, 538 and 896 IU/kg, respectively were administered.

Fig. 1 shows that effects of a single i.v. administration of Epo/liposomes before gel filtration and Epo/liposomes at low and middle doses on the circulating reticulocyte evaluated in rats by the sysmex method. Epo/liposomes before gel filtration contained free and liposomal Epo, but Epo/liposomes contained

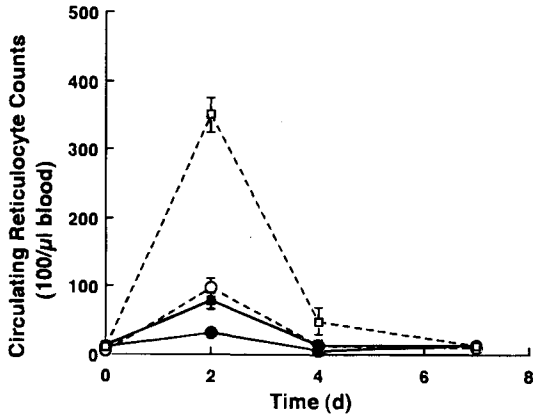

Fig. 1. Effects of a single i.v. administration of Epo/liposomes before gel filtration and Epo/liposomes at low and middle doses on the circulating reticulocyte evaluated in rats by the sysmex method. Data are expressed as means ± S.E. (n = 4–7). Epo/liposomes before gel filtration: (—●—) 179 IU/kg; (—■—) 538 IU/kg. Epo/liposomes: (- -○- -) 179 IU/kg; (- -□- -) 538 IU/kg.

only liposomal Epo. Dose of 538 IU/kg of Epo/liposomes before gel filtration and 538 IU/kg of Epo/liposomes were administered. Two doses of Epo/liposomes showed higher pharmacological activity than Epo/liposomes before gel filtration.

Serum Epo Level

Mean whole serum concentration profiles of Epo (100, 300 and 1000 IU/kg) or Epo/liposomes (low, middle and high doses) after i.v. (a) and s.c. (b) administrations are shown in Fig. 2. The whole serum concentration profiles after i.v. administration were best fitted by biexponential equations for both Epo and Epo/liposomes. Epo/liposomes resulted in higher serum levels of Epo than Epo after i.v. and s.c. administrations.

Lymph Epo Level

Mean whole serum concentration profiles of Epo (300, 1000 IU/kg), lymph concentration of 1/10 PBS (untreated) and Epo (538 IU/kg), and serum and lymph concentration of Epo/liposomes (middle dose) after s.c. administration are shown in Fig. 3. The lymph level of Epo did not change in untreated rats, whereas it increased after s.c. administration of Epo and Epo/liposomes. The lymph level of Epo after s.c. administration of Epo/liposomes (538 IU/kg) was higher than that after s.c. administration of Epo (538 IU/kg). In Epo/liposomes, the serum level of Epo was almost three times higher than the lymph level after s.c. administration.

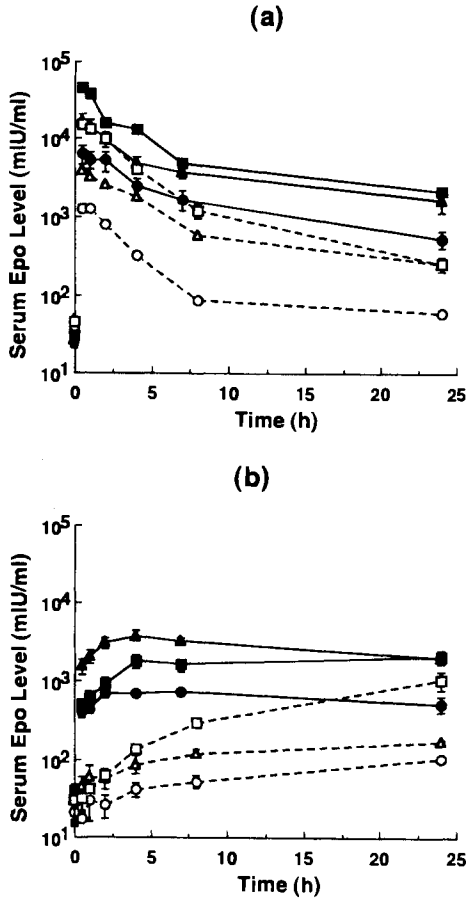

Fig. 2. Time course of serum Epo level after a single i.v. (a) or s.c. (b) administrations of Epo and Epo/liposomes at doses of 100–1000 IU/kg in rats by RIA. Data are expressed as means ± S.E. (n = 2–6). Epo: (- -○- -) 100 IU/kg; (- -△- -) 300 IU/kg; (- -□- -) 1000 IU/kg. Epo/liposomes: (—●—) 179 IU/kg; (—▲—) 538 IU/kg; (—■—) 896 IU/kg.

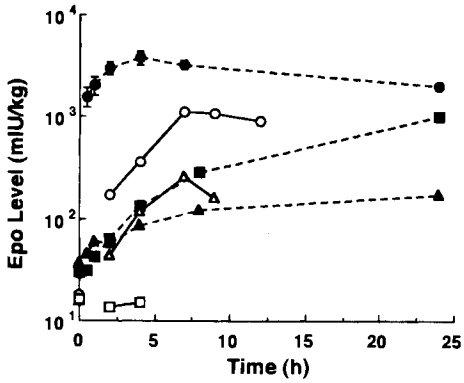

Fig. 3. Time course of serum and lymph Epo level after a single s.c. administration of Epo and Epo/liposomes at middle dose in rats by RIA. Data are expressed as means ± S.E. (n = 2–6). Serum Epo level: (- -●- -) 538 IU/kg of Epo/liposomes; (- -▲- -) 300 IU/kg of Epo; (- -■- -) 1000 IU/kg of Epo. Lymph Epo level: (—○—) 538 IU/kg of Epo/liposomes; (—△—) 538 IU/kg of Epo; (—□—) untreated.

Epo Activity

Fig. 4 shows that the change in residual circulating reticulocyte counts and percentage of circulating reticulocytes of RBC as evaluated by the sysmex and smear methods after a single i.v. (a) and s.c. (b) administrations of Epo/liposomes and Epo. Residual circulating reticulocyte counts (sysmex) rose significantly and dose-dependently on day 2 but returned to the pre-administration level on days 4 and 7 after i.v. and s.c. administrations of Epo in rats.

Percentage of circulating reticulocytes (smear) in rats which received Epo intravenously and subcutaneously on day 0 rose significantly on the day 2, showed a peak on day 4, and returned to the pre-administration level on day 7. Those that received Epo/liposomes showed similar patterns but higher residual and percentage of circulating reticulocyte counts than those that received Epo. Epo activity after i.v. administration of Epo/liposomes was almost comparable to those after s.c. administration.

DISCUSSION

Epo Activity and Concentration in Epo/liposomes

The peak height of circulating reticulocyte counts on day 2 was an indicator of Epo activity since it rose dose-dependently on day 2. Assuming that the liposomal Epo keeps 100% of its Epo activity, we estimated the retention of Epo activity in Epo/liposomes before gel filtration by comparing peak heights of circulating reticulocyte counts on day 2 in Fig. 1. Epo activity in Epo/liposomes was estimated about 17.2% for low dose and 27.7% for middle dose, and was almost comparable to the encapsulation efficacy of Epo/liposomes as measured by protein quantity in liposomes (11.5 for low dose and 16.6% for middle dose) (18). The encapsulation efficiency of calcein, a water-soluble maker, is 15.3%. These values correspond well to each

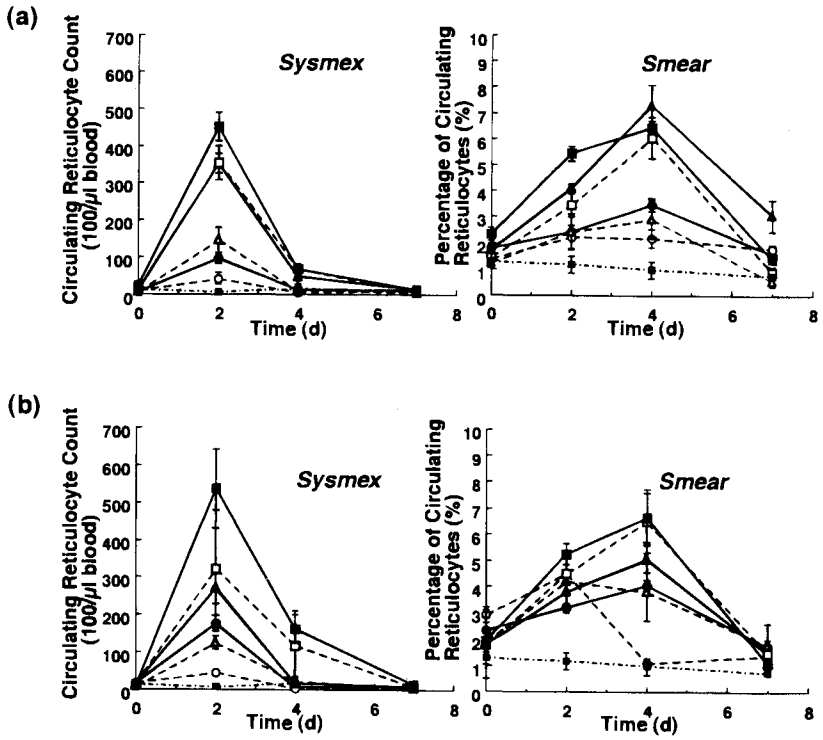

Fig. 4. Effects of a single i.v. (a) or s.c. (b) administrations of Epo and Epo/liposomes at doses of 100–1000 IU/kg on the circulating reticulocyte evaluated in rats by the sysmex and smear methods. Data are expressed as means ± S.E. (n = 4–8). Epo: (- -○- -) 100 IU/kg; (- -△- -) 300 IU/kg; (- -□- -) 1000 IU/kg. Epo/liposomes: (—●—) 179 IU/kg; (—▲—) 538 IU/kg; (—■—) 896 IU/kg; (-·-·-■-·-·-) control.

other. The retention of Epo concentration in Epo/liposomes (16.6%) was used for three doses of Epo/liposomes.

These results are supported by the finding that 1/9 (a reciprocal of dilution factor after gel filtration) of Epo/liposomes before gel filtration showed the same Epo activity as Epo/liposomes following s.c. administration (13). The Epo activity is retained in liposomes since Epo activity of Epo/liposomes was consistent with the encapsulated amount of protein in liposomes. Therefore, the middle dose of Epo/liposomes before gel filtration consists of about 83.4% of free inactive Epo and about 16.6% of encapsulated active Epo in the preparation. Epo activity may have been lost by sonication and the organic solvent in the course of preparation of Epo/liposomes, whereas Epo activity in Epo/liposomes was protected by the bilayers.

Dose vs AUC

Fig. 5 shows the dose-AUC relationship after i.v. (a) and s.c. (b) administrations of Epo and Epo/liposomes by RIA, sysmex and smear method. AUC increased dose-dependently after i.v. administration; the mean AUC/dose of Epo/liposomes (0.20 ± 0.015 kg·h/ml) was about 3 times greater than those of Epo (0.06 ± 0.009 kg·h/ml) (Table 1). sAUC and mAUC appear sigmoidal vs dose after i.v. administration, but they seem linear after s.c. administration. Epo concentration may be too high to induce corresponding pharmacological effects and seems to be saturated after i.v. administration of liposomal Epo.

The mean AUC/dose of Epo/liposomes (0.082 ± 0.0327 kg·h/ml) was about 9 times higher than that of Epo (0.009 ± 0.0017 kg·h/ml) (Table 2) after s.c. administration. The slope of AUC vs doses of Epo following i.v. administration was higher than that following s.c., but the sAUC and mAUC were almost the same value. AUC after s.c. injection of Epo was lower than AUC after i.v. injection but sAUC and mAUC were almost the same. Low Epo concentration after s.c. may be suitable to induce corresponding pharmacological effects.

I.V. Administration of Epo and Epo/liposomes

Table 1 lists the pharmacokinetic parameters based on fitting the whole serum concentrations to biexponential equations and pharmacological effects after i.v. administration for both Epo and Epo/liposomes. Epo/liposomes showed almost a similar distribution of half-lives (α and β) to Epo, except half-lives (β) of Epo at 100 IU/kg. These were because Cl_{tot} (4.0–5.9 ml/h/kg) decreased in comparison with Epo (12.2–16.7 ml/h/kg), and the Vd of Epo/liposomes was about 1/2–1/4 times lower than those of Epo. The first phase might represent the Epo associated with the liposome membrane. The second phase may be characterized by a slower rate constant and may represent a second pool of the small fraction of Epo entrapped in the aqueous phase of liposomes.

While pharmacokinetic studies in human following i.v. administration of Epo (50 IU/kg) indicated that the plasma clearance was monoexponential with a half-life of 5.4 h, an apparent volume of distribution of 69.8 ml/kg and a clearance of 10.09 ml/h/kg (19). Kinoshita *et al.* (20,21) reported that the pharmacokinetic parameters of Epo in rats were $t_{1/2}(\beta)$ of 2.2–2.3 h, Vd of 74.3 ml/kg, AUC of 8.3–9.4 IU·h/ml and Cl_{tot} of 19.2–21.9 ml/h/kg after i.v. administration of Epo (180 IU/

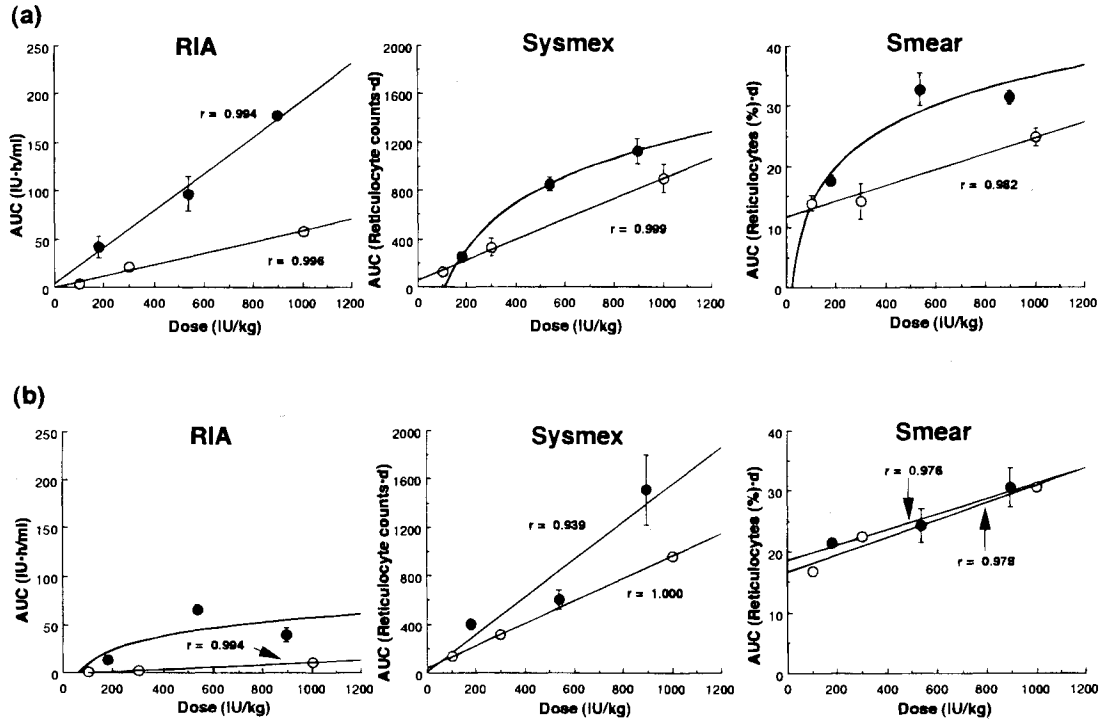

Fig. 5. Correlation between AUC and doses after a single i.v. (a) or s.c. (b) administrations of Epo (- -○- -) and Epo/liposomes (- -●- -) in rats by RIA, sysmex and smear methods. Data are expressed as means ± S.E. (n = 2–8).

kg). In contrast, we showed that Epo in Epo/liposomes provides a sustained blood Epo level and extends the Epo residence time in rats (Fig. 2, Table 1).

The mechanism for decreases in clearance is unclear. Epo/liposomes may be due to high stability in serum, minimizing nonspecific tissue and protein binding, which occur with Epo as reported for liposomal daunorubicin (22).

S.c. Administration of Epo and Epo/liposomes

The pharmacokinetic parameters and the pharmacological effects for s.c. administration of Epo/liposomes and Epo are listed in Table 2. As well as i.v. administration, low and middle doses of Epo/liposomes were compared with 300–1000 IU/kg of Epo, respectively, after s.c. administration.

The absorption rate constant (K_a) of Epo/liposomes was significantly higher than that of Epo. The AUC following s.c. administration of Epo/liposomes increased since the Cl_{tot} decreased. sAUC and mAUC also increased with dose in the case of Epo/liposomes. Epo activity in smear method after s.c. administration of Epo/liposomes showed large deviations. Each deviation may be when Epo/liposomes are transferred from skin to blood and Epo is released from the liposomes. Liposomes in the high dose might be unstable through the s.c. tissues.

I.v. and S.c.

Epo/liposomes following s.c. administration showed a lower concentration than i.v. administration. S.c. administration results in a mean bioavailability of 15.0 ± 2.8% for Epo and 41.0 ± 1.6% for Epo/liposomes calculating (AUC/Dose) after s.c. administration (Table 2) divided by (AUC/Dose) after i.v. administration of Epo and Epo/liposomes (Table 1), respectively. It was reported that the mean bioavailability was 43.7% in human following s.c. administration of Epo (50 IU/kg) (19). Despite its 41.0% bioavailability, Epo/liposomes following s.c. administration showed almost the same Epo activity as Epo/liposomes did following i.v. administration (Figs. 4 and 5).

The pharmacokinetics of Epo/liposomes were compared with those of Epo after s.c. administration. Epo/liposomes following s.c. administration showed a higher Epo concentration compared with Epo and similar to that following i.v. administration of Epo (Fig. 5). Epo after s.c. administration was reported to be degraded by peptidases in the skin, and the size of the Epo molecule may impede absorption (23). It is likely that subcutaneously administered Epo, localized primarily at the injection site, results in the retention of Epo with a sustained release from the tissue (24). Our findings suggested that Epo/liposomes protected degradation of Epo by peptidases and promoted permeation intactly through the s.c. injection site by hydrophobic liposome-encapsulated Epo after s.c. administration. This hypothesis is supported by the finding that low and middle doses of Epo/liposomes showed higher Ka values than those of Epo after s.c. administration, and Cl_{tot} values of Epo/liposomes were almost the same as those after i.v. administration of Epo/liposomes (Table 1, 2).

A sustained serum Epo level was detected following s.c. administration of Epo/liposomes compared with that after i.v. of Epo (Fig. 2). Liposomes have been utilized as carriers for the delivery of therapeutic and diagnostic agents to the lymphatic system. Therefore, we examined whether or not a sustained Epo level of Epo/liposomes results from liposomes passing through the lymphatic system and reaches the general circulation after i.v. route.

About 40% of the dose was released from liposomes in the serum and about 7% of the dose was in lymph 7 h after s.c. administration of the middle dose of Epo/liposomes. The

Table 1. Pharmacokinetic and Pharmacodynamic Parameters of Epo and Epo/liposomes Following i.v. Administration to Rats

	Pharmacokinetics									Pharmacodynamics		
	Dose (IU/kg)	n	$t_{1/2}(\alpha)$ (h)	$t_{1/2}(\beta)$ (h)	AUC[a] (IU·h/ml)	AUC/Dose (kg·h/ml)	Vd (ml/kg)	CL_{tot} (ml/h/kg)	K_{el} (h^{-1})	n	sAUC[b] (Sysmex)	mAUC[c] (Smear)
Epo	100	4	1.3 ± 0.1	26.2 ± 2.5	4.1 ± 0.1	0.04 ± 0.001	304.2 ± 33.2	14.3 ± 0.5	0.27 ± 0.03	3	129.9 ± 32.6	13.9 ± 1.3
	300	2	1.8	12.5	20.6	0.07	152.5	12.2	0.17	3	329.3 ± 75.5	14.2 ± 3.0
	1000	3	1.2 ± 0.1	8.3 ± 2.7	58.7 ± 2.4	0.06 ± 0.002	100.2 ± 13.5	16.7 ± 0.5	0.35 ± 0.02	3	901.1 ± 117.1	24.9 ± 1.5
						(Av. 0.06 ± 0.009)						
Epo/liposomes	179	5	1.8 ± 0.4	10.9 ± 1.2	41.7 ± 10.9	0.23 ± 0.061	73.4 ± 37.9	5.7 ± 1.9	0.21 ± 0.03	7	254.4 ± 44.2	17.7 ± 0.9
	538	6	1.1 ± 0.1	13.7 ± 2.6	97.0 ± 17.6	0.18 ± 0.033	99.2 ± 38.4	5.9 ± 1.9	0.19 ± 0.03	4	852.4 ± 58.2	32.7 ± 2.7
	896	2	0.6	9.1	177.6	0.20	49.5	4.0	0.28	6	1125.5 ± 100.9	31.4 ± 1.1
						(Av. 0.20 ± 0.015)						

Note: Each value represents mean ± S.E.
[a] Measured by RIA method (0–24 h).
[b] Circulating reticulocyte counts · day (0–7 d) measured by the sysmex method.
[c] Circulating reticulocytes (%) · day (0–7 d) measured by the smear method.

Table 2. Pharmacokinetic and Pharmacodynamic Parameters of Epo and Epo/liposomes Following s.c. Administration in Rats

	Pharmacokinetics							Pharmacodynamics		
	Dose (IU/kg)	n	AUC[a] (IU·h/ml)	AUC/Dose (kg·h/ml)	CL_{tot} (ml/h/kg)	K_{el} (h^{-1})	K_a ($\times 10^2\ h^{-1}$)	n	sAUC[b] (Sysmex)	mAUC[c] (Smear)
Epo	100	3	1.0 ± 0.09	0.010 ± 0.0009	35.8 ± 0.3	0.6 ± 0.005	0.9 ± 0.03	2	135.0	16.8
	300	3	2.1 ± 0.08	0.007 ± 0.0003	66.4 ± 51.0	0.2 ± 0.01	0.4 ± 0.3	2	315.3	22.5
	1000	2	10.9	0.011	18.6	0.3	—	2	965.3	29.3
				(Av. 0.009 ± 0.0017)						
Epo/liposomes	179	5	14.2 ± 0.6	0.079 ± 0.0046	5.9 ± 0.1	0.2 ± 0.005	9.6 ± 4.2	4	401.1 ± 45.2	21.5 ± 0.3
	538	4	66.5 ± 3.9	0.124 ± 0.0072	6.1 ± 1.3	0.2 ± 0.05	12.8 ± 6.4	8	603.3 ± 76.4	24.3 ± 2.8
	896	4	39.7 ± 7.5	0.044 ± 0.0083	3.9 ± 0.1	0.3 ± 0.01	0.8 ± 0.1	5	1510.8 ± 294.7	30.6 ± 3.2
				(Av. 0.082 ± 0.0327)						

Note: Each value represents mean ± S.E.
[a] Measured by RIA method (0–24 h).
[b] Circulating reticulocyte counts · day (0–7 d) measured by the sysmex method.
[c] Circulating reticulocytes (%) · day (0–7 d) measured by the smear method.

Epo level in lymph after s.c. administration of the middle dose of Epo was very low compared with that of Epo/liposomes (Fig. 3). Furthermore, Kato *et al.* (24) reported that Epo level is high in blood and very low in lymph after s.c. administration of Epo at 180 IU/kg. Liposomal Epo may penetrate easily into the lymph as suggested by the Epo/liposomes being partly accumulated in the local draining lymph nodes (Fig. 3), which would also provide a similar sustained rate of release into the systemic circulation.

When Epo/liposomes were injected intravenously, Epo/liposomes may have sustained gradual release of Epo from liposomes in blood in comparison with Epo. Therefore, Epo/liposomes resulted in higher serum levels of Epo than Epo. Epo/liposomes has a higher AUC, lower clearance and lower steady-state volume of distribution compared with Epo.

CONCLUSIONS

We found that the Epo activity was retained in liposomes since the pharmacological activity of Epo/liposomes was consistent with the encapsulated amount of protein in liposomes. In addition, AUC of Epo/liposomes was about 3 (i.v.)–9 (s.c.) times greater than those of Epo after i.v. and s.c. administrations, and s.c. administration of Epo/liposomes at 7 h may penetrate primarily (40% of dose) through the blood as liposomes and partly (7% of dose) in lymph. Furthermore, the pharmacological effect of Epo/liposomes was greater than those of Epo after i.v. administration. The lower clearance of Epo/liposomes may increase the plasma concentrations of Epo, which increases the efficacy.

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