ELSEVIER

International Journal of Pharmaceutics 185 (1999) 13–22

international journal of pharmaceutics

www.elsevier.com/locate/promis

Distribution characteristics of entrapped recombinant human erythropoietin in liposomes and its intestinal absorption in rats

Yoshie Maitani [a,*], Hidetaka Moriya [a], Naoto Shimoda [b], Kozo Takayama [a], Tsuneji Nagai [a]

[a] *Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan*
[b] *Development Coordination Department, Chugai Pharmaceutical Co. Ltd., 1-9, Kyobashi: 2-chome, Chuo-ku, Tokyo 104-8301, Japan*

Received 12 January 1999; received in revised form 3 March 1999; accepted 23 March 1999

Abstract

Recombinant human erythropoietin (Epo) is frequently administered by intravenous (i.v.) injection for the clinical treatment of renal anemia. Oral (per os; p.o.) administration is desired as an alternative route to i.v. administration, and liposomes have been chosen as a drug carrier. We found previously that after a p.o. administration to rats of Epo entrapped in liposomes before gel filtration, the Epo was absorbed, but variability in the number of days of appearance and in the levels of pharmacological effects, i.e., the peak of circulating reticulocyte counts (RTC), was observed. The purpose of the present study was to examine the distribution characteristics of Epo in liposomes and intestinal absorption of liposomal Epo in rats by using purified Epo entrapped in liposomes after gel filtration (Epo/liposomes). The distribution characteristics of Epo/liposomes were determined by measuring the Epo in liposomes by a radioimmunoassay, high-performance liquid chromatography and zeta potential measurements. We observed that the protein part of Epo was mostly entrapped in liposomes, and was not adsorbed by the liposomal membrane at middle and high Epo p.o. doses, but the zeta potential of the Epo/liposomes increased negatively with the increase in the Epo p.o. doses. These results suggest that the sialic acid part of Epo entrapped in liposomes may project out from liposomes, depending on the entrapped Epo concentration. Little Epo was adsorbed or penetrated into liposomes when it was added to empty liposomes. After the p. o. administration of Epo/liposomes, the peak of RTC appeared at a 2-day delay on day 6, without variation and without dose dependency in comparison with that after i.v. administration. These results suggest that one of the reasons for the variability may be because the non-entrapped Epo and/or Epo/liposomes itself affected the intestinal absorption of Epo/liposomes. In conclusion, Epo/liposomes without nonentrapped Epo may be clinically useful for the oral administration of Epo.

Keywords: Human erythropoietin; Liposomes; Rats

* Corresponding author. Tel.: + 3-5498-4635; fax: + 3-5498-5782.
E-mail address: yoshie@hoshi.ac.jp (Y. Maitani)

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00143-X

1. Introduction

Human erythropoietin is a glycoprotein produced primarily in the kidneys and to a lesser extent in the liver. Human erythropoietin is a single-chain polypeptide with a molecular weight of about 30 000, about 40% of which is ascribed to its sugar moiety (Imai et al., 1990). The physiological function of Epo is to regulate the proliferation and differentiation of erythroid precursor cells to red blood cells (RBC). Recombinant human erythropoietin (Epo) is now produced on a large scale by using recombinant DNA technology, and has been proven effective for the treatment of renal anemia (Eschbach et al., 1987). Treatment with Epo as a peptide medicinal drug is currently limited to intravenous (i.v.) and subcutaneous (s.c.) administrations; it is clinically administered via an i.v. or s.c. injections two to three times a week. However, these injections are painful and an altemative route of administration is thus desirable. Although nasal route has been examined (Shimoda et al., 1995), the oral (per os; p.o.) route seems a suitable altemative because it offers improved convenience and patient compliance. However, proteins and peptides are usually enzymatically degraded in the gastrointestinal tract after p.o. administration. Liposomes have therefore been chosen as one of drug carriers, for intestinal absorption since liposomes may protect peptide drug from degradation by the acidic pH of the stomach and from metabolism by luminal, brush border and cytosolic peptidases, and may improve poor permeability of drug across the intestinal epithelium.

The use of liposomes as carriers of cytokines, i.e. interleukin (IL)-2 (Kedar et al., 1994), IL-7 (Bui et al., 1994), interferon (IFN) (Killion et al., 1994), insulin (Choudhari et al., 1994) has been reported. Insulin entrapped in liposomes (Muramatsu et al., 1996; Takeuchi et al., 1996) and ovalbumin entrapped in liposomes (Tsume et al., 1996) have been orally administered, but Epo in liposomes has not yet been administered clinically, to our knowledge.

We (Maitani et al., 1996) recently reported that Epo was absorbed after a p.o. administration to rats of Epo entrapped in liposomes before gel filtration (Epo/liposomes-b), i.e. as a mixture of Epo and liposomal Epo, but the variability in the number of days of appearance and in the levels of the pharmacological effects was observed. Such variability was not observed after i.v. and s.c. administrations of Epo/liposomes-b in rats (Moriya et al., 1997). Therefore, the purpose of the present study was to examine the distribution characteristics of Epo in liposomes and intestinal absorption of liposomal Epo in rats by using purified Epo entrapped in liposomes after gel filtration (Epo/liposomes). The degree of adsorption of Epo to the liposomal membrane was quantified and the Epo concentration in serum after a p.o. administration of Epo/liposomes was also measured.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from NOF (Tokyo, Japan). The soybean-derived sterol (SS) used in this study was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%), which were kindly provided by Ryukakusan (Tokyo). Epo (epoetinβ, 180 000 IU/ml, 180 000 mg polypeptide equivalent/ml) was a gift from Chugai Pharmaceutical (Tokyo). All other chemicals used were of reagent grade. Male Wistar rats were purchased from Saitama Experimental Animal Supply (Saitama, Japan).

2.2. Preparation of Epo/liposomes

Liposomes were prepared according to the reverse-phase evaporation vesicle method (Maitani et al., 1996; Moriya et al., 1997). DPPC (70 μmol) and SS (20 μmol) in chloroform were deposited in a flask, and the organic solvent was removed. The Epo preparation was serially diluted with 1/10 dilution of phosphate-buffered saline in distilled water (1/10 PBS, pH 7.4) to make 10 800, 32 400 and 54 000 IU/ml solutions (low, middle, and high doses), respectively. The lipid film was redissolved in chloroform and isopropyl ether. To the result-

ing organic phase, the aqueous phase including 3 ml of each Epo was added. Empty liposomes were prepared using 1/10 PBS without Epo. The mixture was sonicated, and the organic solvent was then removed. The preparation was extruded successively through polycarbonate membranes with pore sizes of 0.2 and 0.1 μm at about 50°C by an Extruder (Lipex Biomembrane, Vancouver, Canada). After extrusion, 0.5 ml of the preparation was passed through a Sephadex G-25 column (1.8 × 35 cm, Pharmacia, Uppsala, Sweden) with 1/10 PBS to remove non-entrapped Epo.

2.3. Adsorption of Epo to empty liposomes

Empty liposomes (30 μmol DPPC/ml and 8.6 μmol SS/ml) were incubated with Epo solution (0, 5400, 10 800, 32 400, 54 000 IU/ml) in 1/10 PBS for 1 h at 25°C. After incubation, the sample was subjected to gel filtration, and liposomes which had adsorbed Epo were obtained. The Epo concentration was determined by a radioimmunoassay (RIA) using Erythropoietin RIA, Chugai, Tokyo as described below. The adsorbed Epo was measured by subjecting the liposomes without further treatment. Total recovered liposomal Epo was measured after the disruption of the liposomes achieved by adding chloroform into aliquots of the fraction.

2.4. Determination of Epo concentration by HPLC and RIA

The Epo concentration of Epo/liposomes was determined by high-performance liquid chromatography (HPLC) (Moriya et al., 1997) and/or RIA 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 the HPLC system.

Collected blood was centrifuged immediately to harvest serum (0.1 ml), and the Epo concentration in the serum was then measured by RIA (Maitani et al., 1996).

2.5. Calculation of Epo molecules in a liposome

The entrapping efficiency of Epo in Epo/liposomes (f) was calculated from the Epo and DPPC concentrations in Epo/liposomes after gel filtration (the ratio of Epo to DPPC, (Epo/DPPC)after) and in Epo/liposomes before gel filtration (the ratio of Epo to DPPC in the preparation, (Epo/DPPC)before) using a phospholipid B test (Wako Pure Chemical, Osaka, Japan):

$$f = (\text{Epo/DPPC})\text{after}/(\text{Epo/DPPC})\text{before} \quad (1)$$

The number of Epo molecules in the Epo/liposome was calculated as:

$$\frac{\text{Epo molecule}}{\text{liposome particle}} = \left(\frac{\text{Epo}}{\text{DPPC}}\right)_{\text{after}} \times \frac{734 \times 269032}{180 \times 18236 \times 10} \quad (2)$$

where Epo is expressed as mg polypeptide equivalent/ml (mg pp. eq./ml, hereinafter) and DPPC as mg/dl, the molecular weights of polypeptide moiety of Epo and DPPC are 18 236 and 734, respectively, the Epo concentration is 180 μg pp. eq./μl, and the number of DPPC molecules (n_{DPPC}) in a liposome particle is 269 032.

The n_{DPPC} based on the liposome diameter (156.7 nm) and on the total surface of the lipid (the outer and inner surface areas per polar head group for DPPC; 0.60 nm^2 (Lis et al., 1982) and 0.49 nm^2 (Huang and Masaon, 1978), respectively), can be calculated assuming that liposomes are unilamellar and form a uniform population of spherical vesicles (Huang and Masaon, 1978). The geometrical capture volume (V) of liposomes per mol of DPPC can be calculated as the internal volume (V_L) from the internal radius of a liposome using the bilayer thickness, 4.4 nm (Huang and Masaon, 1978); $V = V_L N_A / n_{\text{DPPC}} =$ 3.79 l/mol, where N_A represents the Avogadro's number.

2.6. P.o. administration and measurement of pharmacological effects of Epo

Nine-week old male Wistar rats (about 300 g) were fasted for 1 day before the p.o. administra-

tion as described previously (Maitani et al., 1996). Ultrafiltration (Sartorius, Gotingen, Germany) was used to concentrate 9-fold diluted Epo/liposomes by gel filtration, since a maximum of 9–10 ml of liposome suspension per rat can be administered orally. The dose was decided considering the *f* values obtained by HPLC for each dose, and assuming that Epo entrapped in liposomes is active (Qi et al., 1995b). Epo doses corresponding to 5976 IU/kg (low dose), 17 928 IU/kg (middle dose) or 29 880 IU/kg (high dose) were given to rats by the p.o. administration of Epo/liposomes-b and Epo/liposomes, and the animals were then returned to their cages for blood collections. Blood (10 or 20 μl) was collected from the dorsal metatarsal vein before and 2, 3, 4, 5, 6, 7, 8, 9 and 13 days after the p.o. administration to evaluate the pharmacological effect. Blood (0.3 ml) was collected from the cervical vein before and on 18, 24, 30, 42, 48 and 54 h after the p.o. administration to evaluate the Epo concentration.

The pharmacological effects of Epo (Epo activity) were evaluated by counting the percentage of circulating reticulocytes of RBC (reticulocyte counts, RTC) by the smear method, as previously reported (Shimoda et al., 1995).

2.7. Measurement of particle size and zeta potential

The particle size of Epo/liposomes after storage, that of liposomes with adsorbed Epo after the incubation of Epo with empty liposomes, and the zeta potential of Epo/liposomes were determined using an electrophoretic apparatus (dynamic light scattering and electrophoretic light scattering ELS-800, Otsuka Electronics, Osaka, Japan) in 1/10 PBS (pH 7.38) at 25°C. The measurement was in duplicate, and the mean values were used.

3. Results

3.1. Characterzation of Epo/liposomes

The Epo/liposomes that passed through the polycarbonate membrane with a pore size of 0.1 μm had mean diameters of 0.15–0.16 μm, and did not change remarkably after storage for 11 weeks at 4°C at 0.16–0.17 μm for three kinds of doses. The *f* values were 0.12, 0.04 (low dose), 0.17, 0.27 (middle dose) and 0.13, 0.16 (high dose), as determined by measuring the protein amount in the liposomes by HPLC and RIA, respectively. The *f* value shown by HPLC in the high dose of Epo/liposomes was almost the same as that by RIA, whereas that of the middle dose by RIA was higher than that by HPLC.

The data of the distribution of Epo molecules in Epo/liposomes indicated that little Epo was adsorbed, and that protein part of Epo was mostly entrapped in liposomes at the middle and high doses of Epo/liposomes (Table 1). However, the zeta potential of the Epo/liposomes decreased with the number of entrapped Epo molecules in a given Epo/liposome particle (Table 2).

For the examination of the interaction of Epo with liposomes, adsorbed and entrapped Epo molecules in a liposome particle were measured after the incubation of Epo with empty liposomes (Table 3). Little Epo was adsorbed and penetrated into the liposome particles.

Table 1
Adsorbed and entrapped erythropoietin (Epo) molecules in Epo/liposomes measured by radioimmunoassay

Epo/liposomes	Epo (IU/ml)[a]	Epo (mol/particle)[b]	
		Adsorbed[c]	Total[d]
L	10 800	1.4	1.5
M	32 400	1.5	25.5
H	54 000	2.3	28.5

[a] Epo/liposomes were prepared with Epo and 70 μmol DPPC and 20 μmol SS in 3 ml.

[b] Calculated using Eq. (2) and the mean Epo concentration (IU/ml) of duplicate measurements.

$$\left(\frac{\text{Epo}}{\text{DPPC}}\right)_{\text{before}} = \frac{\text{Epo IU/ml}}{1710\ \text{mg/dl}}$$

[c] Fractions containing both adsorbed and entrapped Epo were assayed without any further treatment.

[d] Chloroform was added into aliquots of the fractions. The total recovered liposome Epo (adsorbed and entrapped) was then determined.

Table 2
The zeta-potential of Epo/liposomes and the number of Epo molecules in a particle Epo/liposomes determined by RIA and HPLC, and captured volume of liposomes

Epo/liposomes	Epo (IU/ml) (mg pp.eq./ml)[a]	Zeta-potential of Epo/liposomes (mV)[b]	Epo (mol/liposome particle)		
			HPLC[c]	RIA[d]	Captured volume of liposome[e]
L	10 800	0.23	4.3	1.5	5.0
M	32 400	−5.44	15.8	25.5	14.9
H	54 000	−7.15	23.5	28.5	24.8

[a] Epo/liposomes were prepared with Epo, 70 μmol DPPC and 20 μmol SS in 3 ml.
[b] The mean values measured in 1/10 PBS (pH 7.38) at 25°C.
[c] The mean values calculated using Eq. (2) and $(\text{Epo/DPPC})_{\text{before}} \times f$, f values in text.
[d] From Table 1.
[e] Captured volume using calcein is 5.6 ml/mmol lipid. $\frac{\text{Epo molecule}}{\text{liposome particle}} = \frac{5.6 \times \text{Epo(IU/ml)} \times 269032}{180 \times 3 \times 10^4 \times 10^3}$

Stability of Epo/liposomes cannot be monitored by HPLC and RIA since inactivated Epo and active Epo cannot be discriminated. The percentage of recovered Epo activity of Epo/liposomes during and after an 11-week-storage at 4°C was determined by comparing the RTC counts on day 4 after a single i.v. administration in rats (Fig. 1). In the low-dose liposomes, the percentage of recovered Epo activity of Epo/liposomes was 91.7% at 3 weeks and 43.2% at 11 weeks; in the middle-dose liposomes, it was 112.2% at 3 weeks and 57.4% at 11 weeks and in the high-dose liposomes, it was 103.7 at 3 weeks and 92.5% at 11 weeks though it was almost 100% at 1 week in all-dose liposomes.

3.2. Pharmacological effect and serum level of Epo after p.o. administration of Epo/liposomes-b and Epo/liposomes

Figs. 2 and 3 show the percentage of RTC after a single p.o. administration of Epo/liposomes-b and Epo/liposomes, respectively. Epo/liposomes-b appeared to be absorbed by the p.o. route in none of the three rats at the low dose and in two of the three rats at both the middle and high doses. However, both the number of days of appearance and the percentage of RTC showed variation at the middle and high doses. The doses of Epo/liposomes-b and Epo/liposomes represent the total Epo activity, including entrapped Epo in liposomes. In the case of Epo/liposomes at all doses, the RTC percentage showed an increase on day 6 without dose dependency that remained for 2–3 days and then returned to the pre-administration level on day 13, as shown in Fig. 3.

Table 3
Number of Epo molecules in a liposome after the incubation of Epo and empty liposomes, measured by RIA

Epo (IU/ml) (mg pp. Eq./ml)[a]	Epo (mol/particle)[b]	
	Adsorbed[c]	Total[d]
5400	0.01	0.03
10 800	0.01	0.06
32 400	0.01	0.19
54 000	0.01	0.07

[a] The concentration of Epo incubated with empty liposomes.
[b] The mean values of Epo adsorbed liposomes were obtained after gel filtration. Calculated using Eq. (2).
[c] Assay without further treatment.
[d] Chloroform was added into aliquots of the fractions. The total recovered liposome Epo (adsorbed and entrapped) was then determined.

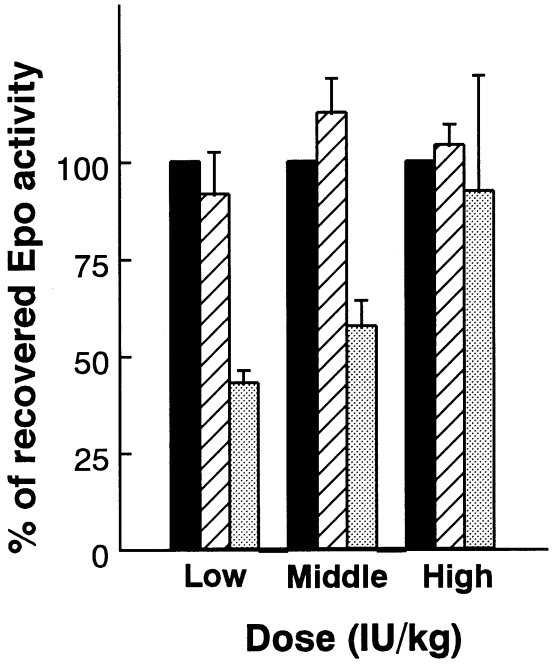

Fig. 1. The percentage of recovered Epo activity of three kinds of Epo/liposomes during and after 11 weeks of storage at 4°C as measured by circulating reticulocyte counts by the smear method on day 4 after a single i.v. administration in rats ($n = 3$–8, mean ± S.E.). Dose; 179 IU/kg for Epo/liposomes (L), 538 IU/kg for Epo/liposomes (M), 896 IU/kg for Epo/liposomes (H). ■, 1 week; ‰, 3 weeks; ▧, 11 weeks.

The time courses of the whole serum concentrations of the controls (no treatment) and the rats given Epo/liposomes (middle dose) by p.o. administration are shown in Fig. 4. The Epo/liposomes resulted in high serum levels of Epo after about 20 and 50 h, but no peaks in three of the five rats.

4. Discussion

We have used Epo/liposomes-b for i.v. and p.o. administrations since non-entrapped Epo is thought to be inactivated in liposome preparations (Qi et al., 1995b; Maitani et al., 1996; Moriya et al., 1997). SS was selected in the component of liposomes since its stabilization effect is stronger than cholesterol (Qi et al., 1995a; Maitani et al., 1996). We suggested that Epo activity was partly lost by sonication and by the effect of organic solvent used in the preparation of Epo/liposomes, whereas Epo activity in Epo/liposomes was protected by the liposome bilayers (Qi et al., 1995b).

A several-day time-lag in the time of the number of days of appearance and individual variation in the pharmacological effect with a lack of dose dependency were observed in rats after the p.o. administration of Epo/liposomes-b (Maitani et al., 1996). The pharmacokinetics and pharmacological effects of Epo/liposomes were evaluated in comparison with those of Epo, after i.v. and s.c. administrations in rats (Moriya et al., 1997). The pharmacological effect of Epo/liposomes was saturated with respect to the Epo concentration, and did not show dose dependency. However, no variation in the time of the number of days of

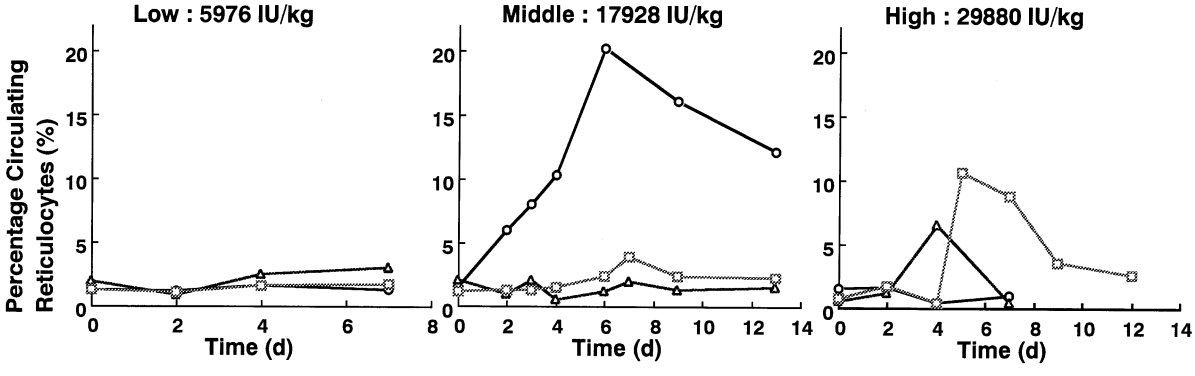

Fig. 2. Effects of a single oral administration of one of three doses of Epo/liposomes-b on the percentage of circulating reticulocytes as evaluated by the smear method in individual rats ($n = 3$).

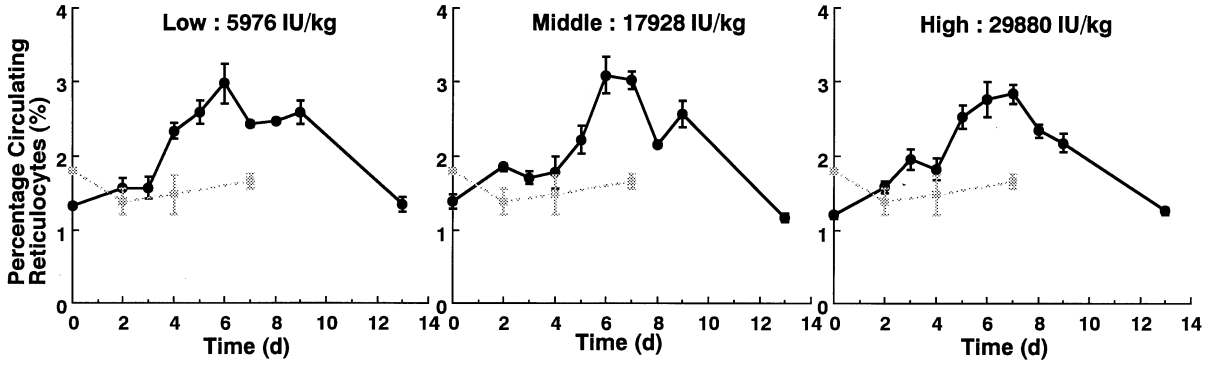

Fig. 3. Effects of a single oral administration of one of the three doses of Epo/liposomes on the percentage of circulating reticulocytes as evaluated by the smear method in rats.—: Each point represents mean ± S.E. ($n = 5$). – – –: Controls p.o.-administered PBS. Mean ± S.E. ($n = 3$).

appearance or in the individual levels of pharmacological effects occurred after i.v. and s.c. administrations of Epo, Epo/liposomes or Epo/liposomes-b (Moriya et al., 1997). The variation after the p.o. administration may be due to Epo/liposomes-b before it penetrates into systemic circulation. We provide the hypothesis that the observed differences between p.o. and i.v./s.c. may be due to contribution of liposomal trapped Epo itself and/or non-entrapped Epo to intestinal absorption of Epo/liposomes.

In the present study, therefore, the distribution characteristics of Epo in Epo/liposomes and the interaction of non-entrapped Epo with liposomes were examined. The f values of Epo/liposomes measured by HPLC as the protein quantity in liposomes were 0.12–0.17 and those by RIA 0.04–0.27. These f values were almost comparable to the retention ratio of Epo activity in Epo/liposomes, about 0.17 for the low dose and 0.28 for the middle dose, measured by the peak heights of RTC on day 4 after administration (Moriya et al., 1997).

The geometrical capture volume of the liposomes was 3.79 l/mol lipid, a value which corresponds well with the 5.6 l/mol lipid obtained experimentally using calcein, as previously reported (Maitani et al., 1996), and the f value of calcein, a water-soluble marker, is 0.15. This finding suggests that the Epo was captured in liposomes passively without specific interaction of Epo with liposomes.

The particle size of Epo/liposomes did not change after storage for 11 weeks at 4°C. The Epo activity of the low and middle doses of Epo/liposomes was retained for 3 weeks, and that of the high dose was retained for 11 weeks (Fig. 1). The high dose of Epo/liposomes was the most stable and showed the most negatively charged zeta potential among the three kinds of Epo/liposomes (Table 2), since electric repulsion of liposomes makes liposome dispersion stable. These results may be due to the protein part of Epo that

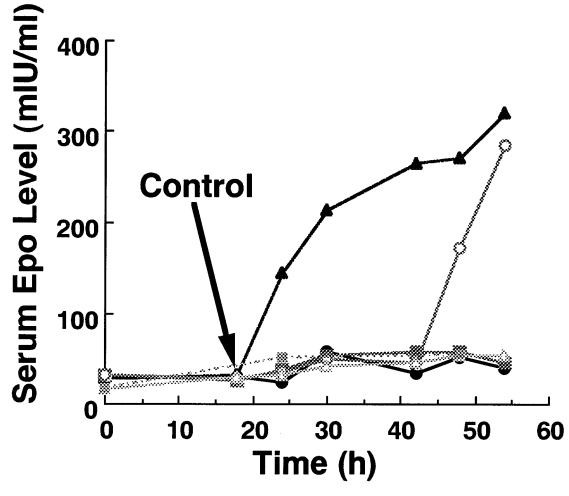

Fig. 4. Time course of serum Epo level after a single oral administration of Epo/liposomes at the middle dose, measured by RIA in individual rats ($n = 5$). – – –: Controls p.o.-administered PBS. Mean ± S.E. ($n = 3$).

concerns its activity, being entrapped mostly in the liposome.

The non-entrapped Epo might interact with the Epo embedded in the hydrophobic regions of the liposome bilayer. The distribution of a peptide drug in liposomes is important to maintain the drug activity in liposomes (Koppenhagen et al., 1998). The distribution of Epo in Epo/liposomes showed that the amount of the Epo adsorbed to liposomes was lower than the Epo entrapped in liposomes at the middle and high doses (Table 1). The molecular number of adsorbed Epo in Epo/liposomes was almost constant as measured by RIA, though the zeta potential of Epo/liposomes became more negative with the increase in Epo concentration (Table 2). These results suggest that the sialic acid part of Epo entrapped in liposomes may project out from liposomes, depending on the entrapped Epo concentration, since hydrophilic part of Epo tends to be outside and inside of liposomes.

To examine the effect of non-entrapped Epo on liposomes, we determined the amount of adsorbed Epo after the incubation of Epo with empty liposomes. Epo was not adsorbed by the liposomal membrane and did not penetrate the liposomes, regardless of the Epo concentration (Table 3). The particle size of liposomes with absorbed Epo was not changed (145.7–153.3 nm) in comparison with the particle sizes of empty liposomes (153.8 nm) before incubation. Epo seems to tend not to penetrate liposomes since Epo is water-soluble.

The peak heights of RTC on day 4 are an indicator of Epo activity, since RTC rose in a dose-dependent manner on day 4 after i.v. and s.c. administrations of Epo, Epo/liposomes-b and Epo/liposomes (Qi et al., 1995b; Maitani et al., 1996; Moriya et al., 1997). In the present study, the peak heights of RTC appeared on day 6 without dose-dependency with a small error of the mean after the p.o. administration of Epo/liposomes (Fig. 3), whereas they showed a large error after the p.o. administration of Epo/liposomes-b (Fig. 2). The control formulation, i.e. 36 000 IU/kg ~ 108 000 IU/kg free Epo solution or the mixture suspension of empty liposomes and free Epo given p.o. did not increase RTC. These findings suggest that one of the reasons for variability may be that Epo/liposomes itself affected the intestinal absorption. Epo leaked from the Epo/liposomes in the GI tract might be responsible for intestinal absorption of Epo/liposomes.

The peak of RTC after the p.o. administration of Epo/liposomes was delayed by 2 days and retained for 2–3 days longer in comparison with that after i.v. administration in the previous study (Moriya et al., 1997). This result suggests that the Epo/liposomes could not penetrate into the blood circulation for 2 days after the p.o. administration, since Epo/liposomes were adsorbed to the intestinal mucosae, and/or Epo/liposomes were transported into the lymph. The sialic acid part of Epo/liposomes projected out from liposomes might interact with intestinal mucus. The intestinal mucosa has a turnover of 3 days and could possibly hold proteins for 3 days before releasing them into the bloodstream. The Epo/liposomes might be released from the intestinal mucosa gradually. Therefore, the pharmacological effect after the p.o. administration continued for a longer time compared to that after i.v. and s.c. administrations.

We measured the Epo concentration in serum after a p.o. administration, but variability of the Epo level was observed; i.e. only two of five rats showed an increase in the Epo level (Fig. 4). The Epo level in serum after the p.o. administration did not reflect the pharmacological effect as it did like after s.c. administration (Moriya et al., 1997). The Epo level in serum might reach the effective one to induce the pharmacological effect 48 h after the p.o. administration. Therefore, Epo/liposomes might tend to be via lymph transport after p.o. administration.

Jenkins et al. (1994) reported that 0.5 μm microparticles were absorbed through Peyer's patches into the mesenteric lymph from the rat intestine. Bargoni et al. (1998) reported the intestinal uptake and transport of 113–143 nm solid lipid nanoparticles in rat lymph, and to a lesser extent in blood. Florence (1997) reported that uptake of micro and nanoparticulate does take place, not only via the M-cells in the Peyer's patches and the isolated follicles of the

gut-associated lymphoid tissue, but also via the normal intestinal enterocytes.

Concerning the lack of dose dependency of Epo/liposomes, the sialic acid group projecting out of the Epo/liposomes might facilitate the interaction of Epo/liposomes with Epo receptors. The amount of Epo adsorbed by liposomes was low, but the zeta potential of Epo/liposomes might have an important role in the interaction of liposomes. The low dose of Epo/liposomes after p.o. administration showed strong and long-acting pharmacological effects compared with that of Epo/liposomes-b. The mechanism of the uptake of liposomes in intestinal mucosa is not clear, and further study is needed.

5. Conclusions

The main findings of the present study were as follows: (a) Epo is not adsorbed by the liposomal membrane in liposome preparations or when added to empty liposomes; (b) Epo/liposomes are stable in terms of Epo activity and liposomal particle size; (c) variability in the number of days of appearance and the levels of pharmacological effects was observed after the oral administration of Epo/liposomes without gel filtration, but not in that of Epo/liposomes after gel filtration; (d) the time of the appearance of pharmacological effects was delayed by 2 days after the oral administration of Epo/liposomes, compared with that after an i.v. administration of either Epo or Epo/liposomes; and (e) Epo activity after the oral administration of Epo/liposomes is not dose-dependent but is retained. In conclusion, Epo/liposomes after gel filtration may be useful for the p.o. administration of Epo in clinical settings.

Acknowledgements

The authors are grateful to Ms Yuko Tominaga and Ms Haruko Miyamoto for their assistance in the experimental work. This work was supported by the Ministry of Education, Science, Sports, and Culture, Japan.

References

Bargoni, A., Cavalli, R., Caputo, O., Fundarò, A., Gasco, M.R., Zara, G.P., 1998. Solid nanoparticles in lymph and plasma after duodenal administration to rats. Pharm. Res. 15, 745–750.

Bui, T., Faltynek, C., Ho, R.J.Y., 1994. Differential disposition of soluble and liposome-formulated human recombinant interleukin-7: effects on blood lymphocyte population in guinea pigs. Pharm. Res. 11, 633–641.

Choudhari, K.B., Labhasetwar, V., Dorle, A.K., 1994. Liposomes as a carrier for oral administration of insulin: effect of formulation factors. J. Microencapsulation 11, 319–325.

Eschbach, J.W., Egrie, J.C., Downing, M.R., Browne, J.K., Adamson, J.W., 1987. Physicochemical and biological comparison of recombinant human erythropoietin with human urinary erythropoletin. New Engl. J. Med. 316, 73–78.

Florence, A.T., 1997. The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. Pharm. Res. 14, 259–266.

Huang, C., Masaon, T.J., 1978. Geometric packing constraints in egg phosphatidylcholine vesicles. Proc. Natl. Acad. Sci. USA 75, 308–310.

Imai, N., Kawamura, A., Higuchi, M., Oh-eda, M., Orita, T., Kawaguchi, T., Ochi, N., 1990. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin; results of a combined phase I and II clinical trial. J. Biochem. 107, 352–359.

Jenkins, P.G., Howard, K.A., Blackhall, N.W., Thomas, N.W., Davis, S.S., O'Hagan, D.T., 1994. Microparticulate absorption from the rat intestine. J. Control. Rel. 29, 339–350.

Kedar, E., Rutkowski, Y., Braun, E., Emanuel, N., Barenholz, Y., 1994. Delivery of cytokines by liposomes. I. Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes. J. Immunother. 16, 47–59.

Killion, J.J., Fishbeck, R., Bar-Eli, M., Chernajovsky, Y., 1994. Delivery of interferon to intracellular pathways by encapsulation of interferon into multilamellar liposomes is independent of the status of interferon receptors. Cytokine 6, 443–449.

Koppenhagen, F.J., Visser, A.J.W., Herron, J.N., Storm, G., Crommelin, D.J.A., 1998. Interaction of recombinant interleukin-2 with liposomal bilayers. J. Pharm. Sci 87, 707–714.

Lis, L., McAlister, M., Fuller, N., Rand, R.P., Parsegian, V.A., 1982. Interactions between neutral phospholipid bilayer membrane. Biophys. J. 37, 657–666.

Maitani, Y., Hazama, M., Tojo, T., Shimoda, N., Nagai, T., 1996. Oral administration of recombinant human erythropoietin in liposomes in rats: influence of lipid composition and size of liposomes in bioavailability. J. Pharm. Sci. 85, 440–445.

Moriya, H., Maitani, Y., Shimoda, N., Takayama, K., Nagai, T., 1997. Pharmacokinetic and pharmacological profiles of free and liposomal recombinant human erythropoietin af-

ter intravenous and subcutaneous administration in rats. Pharm. Res. 14, 1621–1628.

Muramatsu, K., Maitani, Y., Nagai, T., 1996. Dipalmitoylphosphatidylcholine liposomes with soybean-derived sterols and cholesterol as a carrier for the oral administration of insulin in rats. Biol. Pharm. Bull. 19, 1055–1058.

Qi, X.R., Maitani, Y., Nagai, T., 1995a. Effect of soybean-derived sterols on the in vitro stability and the blood circulation of liposomes in mice. Int. J. Pharm. 114, 33–41.

Qi, X.R., Maitani, Y., Shimoda, N., Sakaguchi, K., Nagai, T., 1995b. Evaluation of liposomal erythropoietin prepared with reverse-phase evaporation vesicle method by subcutaneous administration in rats. Chem. Pharm. Bull. 43, 295–299.

Shimoda, N., Maitani, Y., Machida, Y., Nagai, T., 1995. Effect of dose, pH and osmolarity on intranasal administration of recombinant human erythropoietin in rats. Biol. Pharm. Bull. 18, 734–739.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y., 1996. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. 13, 896–901.

Tsume, Y., Take, Y., Sakane, T., Nadai, T., Sezaki, H., Watanabe, K., Kohno, T., Yamashita, S., 1996. Quantitative evaluation of the gastrointestinal absorption of protein into the blood and lymph circulation. Biol. Pharm. Bull. 19, 1332–1337.