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 dipalmitylophosphatidylcholine (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 reticuloocyte 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 30,000, about 40% of which is ascribed to sugar moiety (1). The physiological function of Epo is to regulate the prolifer-ation 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 polyethylene glycol (PEG) (7,8) or ganglioside GM1 (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 GM1 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) dipalmitylophosphatidylcholine (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 isoprpyl 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 Extrudert/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
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 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 dorsal 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 duct thoracic 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 MULTIT (16). Then, the steady-state volume of distribution (Vd), half-lives (t1/2, α and β phases) and total body clearance (CLtotal) 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 MULT 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 standard 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 Quickizer 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 used.

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