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Pharmacokinetics and Pharmacological Effect of Recombinant Human Granulocyte Colony-stimulating Factor Conjugated to Poly(styrene-co-maleic acid) in Rats

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Abstract

A new derivative of recombinant human granulocyte colony-stimulating factor (rhG-CSF) has been synthesized by conjugating rhG-CSF to poly(styrene-co-maleic acid) (poly(styrene-co-maleic acid)-rhG-CSF) to try to avoid glomerular filtration and thus potentiate the neutrophil-proliferating activity of rhG-CSF.

Poly(styrene-co-maleic acid)-rhG-CSF was highly bound to bovine serum albumin (BSA) and the molecular weight of the poly(styrene-co-maleic acid)-rhG-CSF–BSA complex was estimated to be about 90 000 by gel filtration.

Intravenous administration of poly(styrene-co-maleic acid)-rhG-CSF to normal rats resulted in a dose-dependent increase in neutrophil count. The neutrophil-proliferating activity of poly(styrene-co-maleic acid)-rhG-CSF was about 10 times greater than that of rhG-CSF. After intravenous injection at a dose of 5 μg protein kg^{-1}, the total clearance of rhG-CSF fell from 71·0 to 32·1 mL h^{-1} kg^{-1} following poly(styrene-co-maleic acid) modification. An isolated perfusion study in rat kidney showed that the filtered fraction of rhG-CSF was reduced by conjugation with poly(styrene-co-maleic acid).

These results suggest that poly(styrene-co-maleic acid)-conjugation can potentiate the neutrophil-proliferating activity of rhG-CSF by reducing, at least in part, its renal clearance.

Recombinant human granulocyte colony-stimulating factor (rhG-CSF), derived from Chinese hamster ovary cells, is a glycoprotein with a molecular weight of about 19 000; it consists of 174 amino acids and an O-glycoside carbohydrate chain (Oheda et al 1988). This peptide is used clinically to treat various types of neutropenia and is especially useful in bone marrow transplantation and cancer chemotherapy (Lazarus & Rowe 1994; Kornek et al 1998). However, clinical use of rhG-CSF is limited because of its rapid renal clearance and elimination from the circulation (Tanaka et al 1989).

To avoid glomerular filtration, a new derivative of rhG-CSF (poly(styrene-co-maleic acid)-rhG-CSF) was synthesized by conjugating rhG-CSF to poly(styrene-co-maleic acid). Maeda et al (1979) and Inoue et al (1989) reported that poly(styrene-co-maleic acid) bound to superoxide dismutase or neocarzinostatin delayed elimination from the body, enhancing the pharmacological effect.

In this study, we have evaluated the neutrophil-proliferating effect and pharmacokinetics of poly(styrene-co-maleic acid)-rhG-CSF in rats. In addition, the renal handling of poly(styrene-co-maleic acid)-rhG-CSF was evaluated in isolated perfused rat kidney.

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Materials and Methods

Materials

rhG-CSF was obtained from Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). rhG-CSF was labelled with Na-[^{125}I] by the lactoperoxidase method (Hanazono et al 1990). The radiochemical purity of [^{125}I]rhG-CSF was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and found to be more than 96·8% pure. Poly(styrene-

co-maleic acid)-[^{125}I]rhG-CSF was synthesized from [^{125}I]rhG-CSF. [^{3}H]Inulin was purchased from Amersham International (Buckinghamshire, UK) and poly(styrene-co-maleic acid) anhydride from Polyscience Incorporation (Warrington, PA). Resins for chromatography were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and other reagents from Nacalai Tesque (Kyoto, Japan).

Animals

Male Wistar albino rats (200–250 g) were used. Animals were maintained in metabolic cages before the experiments with free access to food and water.

Preparation of poly(styrene-co-maleic acid)-rhG-CSF and poly(styrene-co-maleic acid)-[^{125}I]rhG-CSF

Poly(styrene-co-maleic acid) anhydride (19 μg) and 2,2′-dithio bis(5-nitropyridine) (DTNP; 2 μg) were dissolved in 10 μL dimethylsulphoxide and incubated at room temperature for 24 h. After this time 1 mL 10 μM rhG-CSF solution containing 0·1% Tween 20 was added, followed by 3 h incubation at room temperature. The reaction mixture was subjected to Sephadex G-25 gel chromatography, using 0·1 M phosphate-buffered saline (PBS, pH 7·4) containing 0·1% Tween 20 as an eluent for desalting.

Unmodified rhG-CSF was removed by DEAE (diethylaminoethyl) Sepharose CL-6B gel chromatography using an increasing salt gradient (NaCl: 0–0·3 M).

The protein concentration of poly(styrene-co-maleic acid)-rhG-CSF was determined by the method of Bradford (1976).

Gel filtration of poly(styrene-co-maleic acid)-rhG-CSF–bovine serum albumin complex

Bovine serum albumin (BSA, 0·1%) and poly-(styrene-co-maleic acid)-[^{125}I]rhG-CSF (0·01%) were incubated in PBS with 0·1% Tween 20 at room temperature for 1 h. The reaction mixture was subjected to Sephadex G-50 gel chromatography using PBS with 0·1% Tween 20 as eluent.

Radioactivity in each fraction was measured using an Autowell Gamma System, ARC-300 (Aloka Corp., Mitaka, Japan).

In-vivo haematopoietic activity of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF

rhG-CSF or poly(styrene-co-maleic acid)-rhG-CSF was injected into the tail vein of rats (6 animals per group) at doses of 0·5, 5 or 50 μg protein kg^{-1} in a volume of 100 μL. Blood (50 μL) was collected via the tail vein using a micropipette before and 6, 12, 24 and 48 h after administration. Blood was also collected after 3, 5 and 7 days for the 50 μg protein kg^{-1} dose. Each blood sample was diluted with 1 mL lysing reagent (150 mM NH_4Cl, 10 mM $KHCO_3$ and 100 mM EDTA-4Na solution). After 10 min, white blood cells and platelets were counted using a microcell counter (CC-180A; Toa Medical Electronics, Kobe, Japan). In the same samples, neutrophils were counted using a flow cytometer (Cytron Absolute; Ortho Diagnostic Systems Corp. Tokyo, Japan).

Pharmacokinetics of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF

[^{125}I]rhG-CSF or poly(styrene-co-maleic acid)-[^{125}I]rhG-CSF was mixed with cold rhG-CSF or cold poly(styrene-co-maleic acid)-rhG-CSF at a 19-fold protein concentration, respectively, and 5 μg protein kg^{-1} rhG-CSF was injected into the tail vein of rats (6 animals per group). Blood (100 μL) was collected into microtubes via a catheter placed in the rat carotid artery before and 5, 10 and 30 min and 1, 2 and 4 h after administration. This was centrifuged at 18 000 *g* for 5 min, and 50 μL plasma was used for further investigation. The kidney, liver and spleen were removed 4 h after drug administration, washed with saline and weighed. These organs were mixed with 4 vols 0·1 M PBS containing 0·1% Tween 20, homogenized on ice, and then 1 mL homogenate was used subsequently. Each sample was mixed with the same volume 10% trichloroacetic acid solution and centrifuged. The radioactivity in the sediment was determined. To obtain the pharmacokinetic parameters of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF, the plasma concentrations were analysed using the non-linear least squares program (Yamaoka et al 1981).

Distribution of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF in isolated perfused rat kidney

Isolated perfused rat kidney was prepared as previously reported (Hori et al 1988). [^{125}I]rhG-CSF solution (100 ng in 100 μL) was rapidly administered via the left renal artery to rats (4 animals per group), and the kidney was perfused for 15 min using perfusate containing 5% BSA. After termination of the perfusion, the kidney was removed and weighed. The kidney was then mixed with 4 vols 0·1 M PBS containing 0·1% Tween 20 and

homogenized. A sample of the homogenate was obtained and ^{3}H radioactivity was measured using a liquid scintillation spectrophotometer (Tri-Carb-3385; Packard Instrument, Chicago, IL). The remaining homogenate was mixed with the same volume of 10% trichloroacetic acid, centrifuged, and the ^{125}I radioactivity in the sediment was measured.

Results

Preparation of poly(styrene-co-maleic acid)-rhG-CSF

The yield of poly(styrene-co-maleic acid)-rhG-CSF synthesized was over 90%. The mixture of poly-(styrene-co-maleic acid)-[^{125}I]rhG-CSF and BSA solution was incubated for 1 h and subjected to gel filtration. As shown in Figure 1, Sephadex G-50 chromatography revealed peaks with estimated molecular weights of about 20 000 and 90 000. The former peak was considered to represent poly-(styrene-co-maleic acid)-[^{125}I]rhG-CSF, and the second peak poly(styrene-co-maleic acid)-[^{125}I]rhG-CSF–BSA complex. The area ratio of these peaks was 1 : 15. The latter peak was considered to represent free ^{125}I ion. The authentic binding ratio of rhG-CSF to BSA was less than 5% in a preliminary experiment.

In-vivo haematopoietic activity of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF

The effects of intravenous injection of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF at doses of 0·5, 5 and 50 μg protein kg^{-1} were evaluated in rats. The neutrophil count did not increase after administration of rhG-CSF at 0·5 μg protein kg^{-1} but markedly increased after poly(styrene-co-maleic acid)-rhG-CSF administration at the same dose, reaching a peak after 6 h (Figure 2). The neutrophil count slightly increased after rhG-CSF administration at 5 μg protein kg^{-1}, but markedly increased after poly(styrene-co-maleic acid)-rhG-

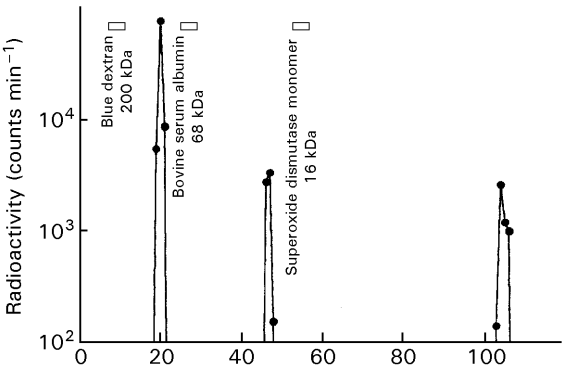

Figure 1. Gel filtration chromatography of the poly(styrene-co-maleic acid)-rhG-CSF preparation. Radioactive poly(styrene-co-maleic acid)-rhG-CSF (100 000 counts min^{-1}) with bovine serum albumin was subjected to gel filtration on a Sephadex G-50 column (1·5 × 50 cm). Chromatography was carried out with 0·1 M phosphate-buffered saline (pH 7·4) at room temperature. One-millilitre fractions were collected and radioactivity was determined. Open symbols are eluted points of molecular markers.

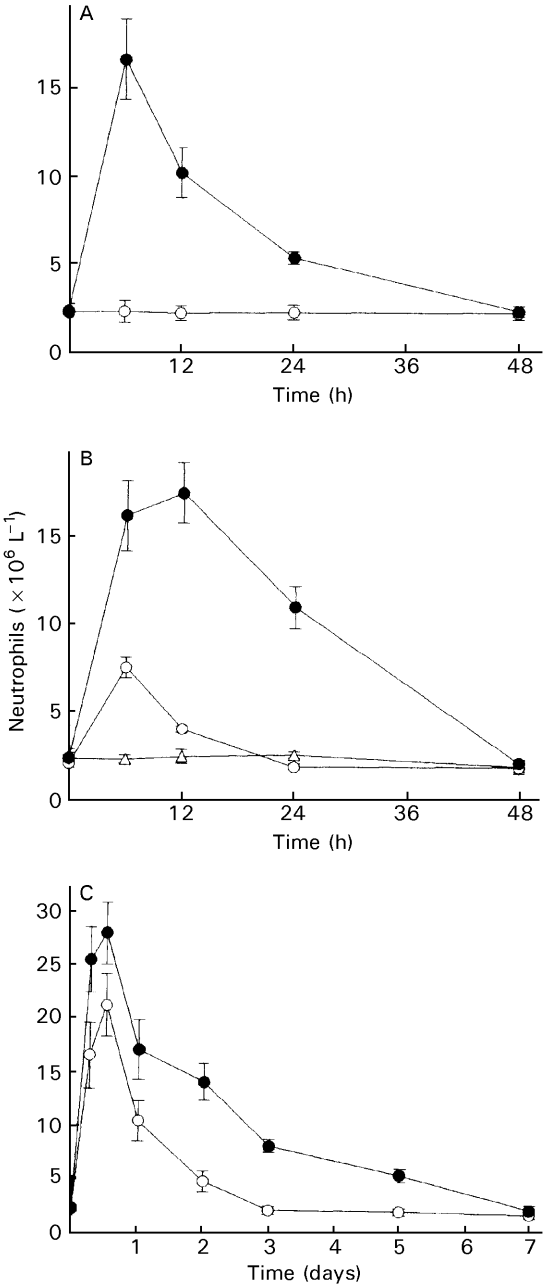

Figure 2. Time-course of neutrophil counts in rats given injections of rhG-CSF (○) or poly(styrene-co-maleic acid)-rhG-CSF (●) or vehicle (△). Doses were 0·5 (A), 5 (B), or 50 (C) μg protein kg^{-1}, intravenously. Each value represents the mean ± s.e.m. of six rats.

CSF administration at the same dose, reaching a peak after 12 h.

At a dose of 50 μg protein kg^{-1}, the neutrophil count markedly increased both after rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF administration, reaching a peak after 12 h but falling to pre-administration levels at 3 and 7 days, respectively. The area under the neutrophils–time curve after administration of rhG-CSF for 48 h at doses of 0·5, 5 and 50 μg protein kg^{-1} was 1·1, 1·5 and $5{\cdot}5 \times 10^{11}$ h L^{-1}, respectively. That of poly(styrene-co-maleic acid)-rhG-CSF at doses of 0·5, 5 and 50 μg protein kg^{-1} was 3·2, 4·9 and $8{\cdot}9 \times 10^{11}$ h L^{-1}, respectively.

No changes were observed in the platelet count after rhG-CSF or poly(styrene-co-maleic acid)-rhG-CSF administration at a dose of 50 μg protein kg^{-1} (Figure 3). Similar results were observed in the platelet count at doses of 0·5 and 5 μg protein kg^{-1} (data not shown).

Pharmacokinetics of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF

The plasma rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF concentrations after intravenous administration at a dose of 5 μg protein kg^{-1} were measured in rats (Figure 4). Since the changes in the plasma concentrations showed a clear delay in the disappearance of poly(styrene-co-maleic acid)-rhG-CSF from the circulating blood compared with rhG-CSF, the pharmacokinetic parameters of both drugs were calculated (Table 1). The total clearance of poly(styrene-co-maleic acid)-rhG-CSF was reduced to 45·1% that of rhG-CSF. The rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF concentrations in the kidney, liver and spleen 4 h after administration were determined and compared with the plasma concentrations (Figure 4). The rhG-CSF concentration was slightly lower in the kidney than in the plasma; the poly(styrene-co-maleic acid)-rhG-CSF concentration was considerably lower in the kidney and other organs than in the plasma.

Distribution of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF in isolated perfused rat kidney

Differences in renal handling between rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF were evaluated in isolated perfused rat kidney. Inulin is a marker of glomerular filtration and is distributed only in the extracellular fluid. In renal tissue, the distribution ratio of the [^{125}I]rhG-CSF was 2·0 ± 1·1% of dose, but the distribution ratio of the poly(styrene-co-maleic acid)-[^{125}I]rhG-CSF was 0·15 ± 0·04% of dose and the distribution ratio of [^{3}H]inulin was 0·14 ± 0·05% of dose.

Discussion

rhG-CSF is one of the most frequently used cytokines in clinical practice and has various pharmacological

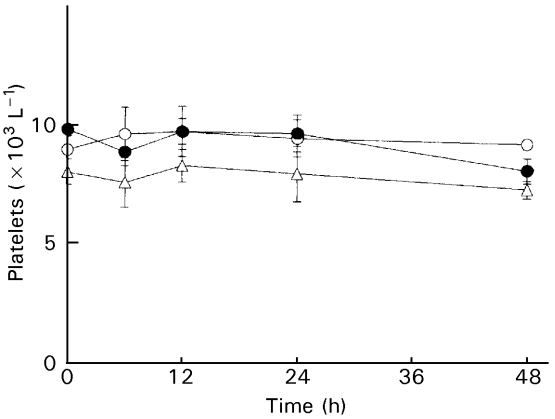

Figure 3. Time-course of platelet counts in rats given injections (50 μg protein kg^{-1}, i.v.) of rhG-CSF (○) or poly(styrene-co-maleic acid)-rhG-CSF (●) or vehicle (△). Each value represents the mean ± s.e.m. of six rats.

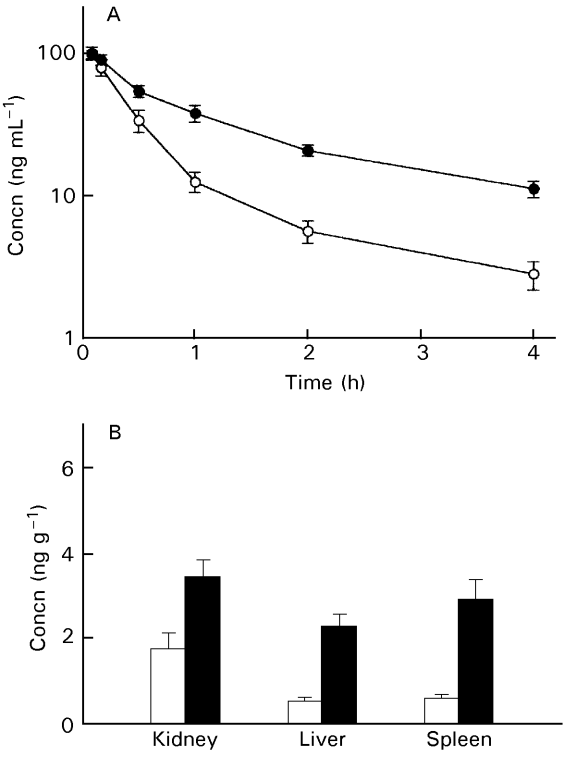

Figure 4. A. Serum rhG-CSF (○) and poly(styrene-co-maleic acid)-rhG-CSF (●) concentrations 4 h after 5 μg protein kg^{-1} intravenous administration to rats. B. Tissue rhG-CSF (open column) and poly(styrene-co-maleic acid)-rhG-CSF (closed columns) concentrations 4 h after 5 μg protein kg^{-1} intravenous administration to rats. Each value represents the mean ± s.e.m. of six rats.

Table 1. Pharmacokinetic parameters of rhG-CSF and poly-(styrene-co-maleic acid)-rhG-CSF in rats after 5 μg protein kg^{-1} intravenous administration.

Parameters	rhG-CSF	Poly(styrene-co-maleic acid)-rhG-CSF
Elimination half-life (h)	2·27 ± 0·34	3·13 ± 0·31
Area under the plasma concentration–time curve (ng h mL^{-1})	70·4 ± 9·2	155·9 ± 24·2
Mean residence time (h)	0·84 ± 0·14	1·58 ± 0·20
Total clearance (mL h^{-1} kg^{-1})	71·0 ± 9·9	32·1 ± 4·4

Each value represents the mean ± s.e.m. of six rats.

actions. There are three major pharmacological effects, the first being the differentiation and proliferation of immature neutrophils in the bone marrow into mature neutrophils (Lord et al 1989). The second is mobilization of neutrophils in the bone marrow to peripheral blood (Lieschke et al 1994). The third is enhancement of neutrophil function in peripheral blood (Hoglund et al 1997). When the systemic neutrophil count reaches a nadir after high doses of anti-cancer drugs or radiotherapy, rhG-CSF is administered to return the neutrophil level to normal (Kennedy et al 1993). However, neutrophils once recruited to peripheral blood have a short life and disappear after several hours (Begley et al 1986). Therefore, neutrophil production in the bone marrow should be continuously promoted. For neutropenia, rhG-CSF is usually administered once daily, and subcutaneous administration is more effective than intravenous administration (Tanaka et al 1989). This suggests that rhG-CSF retention at blood concentrations above a certain level is necessary. Tanaka et al (1991) synthesized rhG-CSF conjugated to polyethylene glycol (PEG-rhG-CSF) and reported that it was retained in rat blood, and that its effects were maintained. Polyethylene glycol was conjugated to rhG-CSF at the amino groups of the four lysine residues and NH_2-terminal methionine residue. Some of the lysine residues may play an important role in biological activities. The dose of PEG-rhG-CSF in-vivo was 100 μg protein kg^{-1}, therefore the effect of PEG-rhG-CSF on neutrophil count at clinical doses (2–5 μg protein kg^{-1}) was unknown.

We selected poly(styrene-co-maleic acid) to increase the molecular weight of rhG-CSF while maintaining its pharmacological activity even at low dose. In poly(styrene-co-maleic acid), the styrene group reversibly binds to the warfarin site of albumin (Kragh-Hansen 1985). Maeda et al (1979) and Inoue et al (1989) conjugated neocarzinostatin and superoxide dismutase, respectively, to poly(styrene-co-maleic acid) and observed enhancement and maintenance of drug efficacy. Therefore, we bound DTNP, which selectively binds to the thiol group, to the maleic acid of poly(styrene-co-maleic acid) by acid-amide binding and incubation with rhG-CSF. Since the free thiol group of rhG-CSF is present only on the cysteine at position 17 (Demetri & Griffin 1991), 1 mol rhG-CSF is expected to bind 1 mol poly-(styrene-co-maleic acid) using DTNP as a spacer. Since drug effects have been reported not to decrease after substitution of the cysteine at position 17 of rhG-CSF with serine (Kuga et al 1989), this cysteine is unrelated to the activity of rhG-CSF.

In this study, the pharmacokinetics and pharmacological effect of poly(styrene-co-maleic acid)-rhG-CSF were evaluated in-vivo and renal handling of poly(styrene-co-maleic acid)-rhG-CSF was studied in-situ. The effect of poly(styrene-co-maleic acid)-rhG-CSF was clearly different from that of rhG-CSF (Figure 2). The changes in the neutrophil count after intravenous administration of poly-(styrene-co-maleic acid)-rhG-CSF at a dose of 0·5 μg protein kg^{-1} were very similar to that of rhG-CSF at a dose of 5 μg protein kg^{-1}. Likewise, the change in the neutrophil count after intravenous administration of poly(styrene-co-maleic acid)-rhG-CSF at a dose of 5 μg protein kg^{-1} was similar to that of rhG-CSF at a dose of 50 μg protein kg^{-1}. This suggested that the effect of poly(styrene-co-maleic acid)-rhG-CSF was approximately 10 times greater than that of rhG-CSF. In addition, comparison between changes in the white blood cell count and neutrophil count at each dose of rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF showed negligible changes in white blood cells other than neutrophils (data not shown). Therefore, rhG-CSF and poly(styrene-co-maleic acid)-rhG-CSF only selectively increased neutrophils.

The plasma concentrations after administration differed significantly between rhG-CSF and poly-(styrene-co-maleic acid)-rhG-CSF (Figure 4). Injection of poly(styrene-co-maleic acid)-rhG-CSF resulted in a prolonged presence in plasma. The slower disappearance of poly(styrene-co-maleic acid)-rhG-CSF resulted in greater values for the area under the plasma concentration–time curve (Table 1). Therefore, when used clinically, poly-(styrene-co-maleic acid)-rhG-CSF could be used at a reduced dose and the administration interval could be prolonged compared with rhG-CSF.

To clarify the mechanism of the advantage offered by poly(styrene-co-maleic acid)-rhG-CSF, the renal handling of poly(styrene-co-maleic acid)-rhG-CSF was evaluated in isolated perfused rat kidney. Among cytokines, rhG-CSF has a relatively low molecular weight and is eliminated mainly by the kidneys (Kuwabara et al 1995). rhG-CSF

accumulated in renal tissue while the accumulation of poly(styrene-co-maleic acid)-rhG-CSF was negligible. The estimated molecular weight of poly(styrene-co-maleic acid)-rhG-CSF is approximately 21 000 kDa. Its styrene group may bind to albumin in-vivo, forming a complex with a molecular weight of about 90 000 kDa. Figure 1 shows the high in-vitro affinity of poly(styrene-co-maleic acid)-rhG-CSF for albumin. Due to this high molecular weight, the glomerular filtration rate of poly(styrene-co-maleic acid)-rhG-CSF and distribution in renal tissue may be reduced. Though distribution of poly(styrene-co-maleic acid)-rhG-CSF in isolated perfused rat kidney showed low affinity for renal tissue, concentration of poly-(styrene-co-maleic acid)-rhG-CSF in the kidney and other organs 4 h after administration in-vivo was higher than that of rhG-CSF (Figure 4). It seemed that most of the rhG-CSF could not be distributed to each organ due to its rapid metabolism and elimination from the circulation. Relationships between plasma concentration and tissue distribution, and duration of drug effects are very important for designing suitable drug delivery systems and should be further evaluated.

In conclusion, in-vivo poly(styrene-co-maleic acid)-rhG-CSF was effective as a drug delivery system for rhG-CSF. Poly(styrene-co-maleic acid)-rhG-CSF may markedly improve the treatment of neutropenia.

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