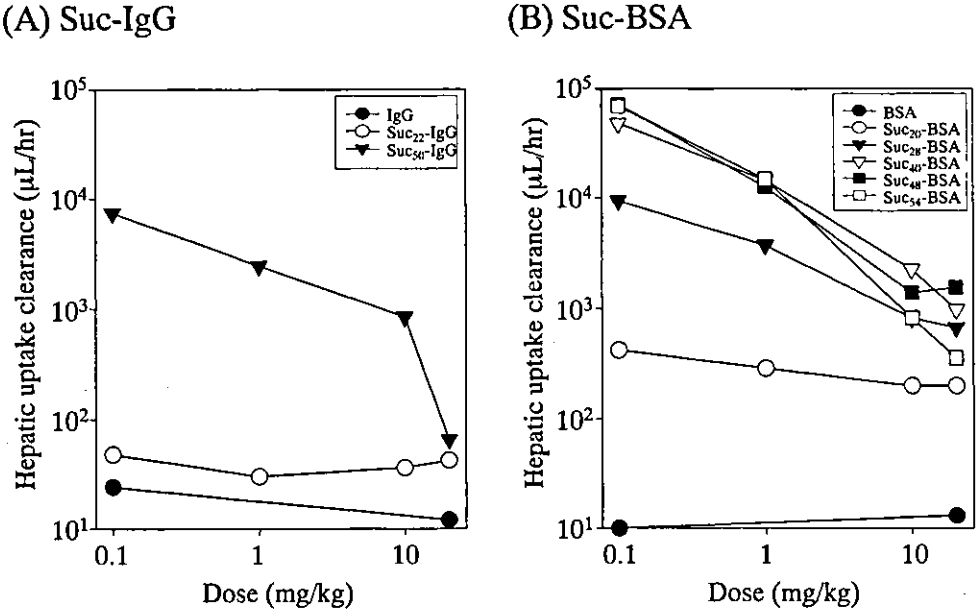

Fig. 7. Hepatic and renal uptake clearances of ^{111}In-labeled succinylated proteins after intravenous injection in mice at the doses of 0.1, 1, 10, and 20 mg/kg. A, IgG (●), Suc_{22}-IgG (○), and Suc_{50}-IgG (▼); B, BSA (●), Suc_{20}-BSA (○), Suc_{28}-BSA (▼), Suc_{40}-BSA (▽), Suc_{46}-BSA (■), and Suc_{54}-BSA (□)

imum degree of modification should be selected. Retrospectively, these considerations are supported by our previous successful approach using Suc-catalase (Fig. 5A) (molecular mass, 250 kDa; 1.5 × 10^3 succinylated molecules/Å^2), with a relatively high remaining enzymatic activity. This compound has been shown to be effectively targeted to liver nonparenchymal cells and has important therapeutic potential in the treatment of hepatic injuries induced by ischemia/reperfusion (Yabe et al., 1999).

Previous studies have demonstrated that a charged collagen-like domain containing a lysine cluster of the SRA forms a positively charged groove that specifically interacts with negatively charged ligands (Doi et al., 1993). It has also been suggested that the spatial distribution of the negatively charged residues or the negative charge density of ligands plays an important role in electrostatic interactions (Pearson et al., 1993). Recently, Suzuki et al. (1999) proposed a hypothesis that ligand binding to SRs, sufficient to allow cellular uptake, requires not only a high density of negative charges but also an increase in the apparent affinity by numerous interactions between one ligand and multiple SR molecules. It is likely that larger succinylated proteins offer a better chance of multiple binding compared with smaller ones with a higher curvature. The low affinity of Suc-SODs for SRs might be supported by this hypothesis, that both a negative charge density and multiple binding would be a prerequisite for efficient recognition in vivo, assuming that SRA or other receptors with similar characteristics play a major role. The detailed molecular mechanisms await further investigation.

Suc-SOD significantly accumulated in the kidney. This, however, should be primarily ascribed to tubular reabsorption after efficient glomerular filtration of this small protein. Interestingly, the degree of succinylation significantly affected the renal handling of SOD. Efficient and saturable renal uptake was observed for Suc_9-SOD, whereas the renal accumulation of Suc_{22}-SOD was significantly lower. This finding, suggesting the importance of the free amino groups on protein derivatives in the uptake by renal tubular epithelial cells (Sumpio and Maack, 1982; Christensen et al., 1983), is in good agreement with our previous observations involving various chemically modified small proteins (Mihara et al., 1994).

Unexpectedly, we found marked renal accumulation of highly succinylated BSA, which is not susceptible to glomerular filtration due to their size under a normal physiological condition (Fig. 2), and our confocal microscopic studies revealed that Suc_{54}-BSA localized predominantly in the luminal side of the proximal renal tubule in the kidney (Fig. 3). This phenomenon was clearly observed only at higher doses in which the uptake via hepatic SRs was saturated and plasma concentrations were maintained for long periods.

TABLE 3

Pharmacokinetic parameters of ^{111}In-labeled succinylated proteins in mice after intravenous injection

Each parameter was calculated based on the model shown in Fig. 1 and was obtained by fitting the differential equations 1 to 3 to the experimental data of the plasma concentration and liver accumulation time-courses of each ^{111}In-labeled succinylated protein at four doses. $K_{m,l}$ and $K_{m,p}$ are the Michaelis constants for liver and plasma, respectively. $V_{max,l}$ and $V_{max,p}$ are the maximum rate of uptake for liver and plasma, respectively. Parameter values are expressed as the mean ± computer-calculated S.D.

Compound	$K_{m,l}$	$V_{max,l}$	$K_{m,p}$	$V_{max,p}$
	nM	*nmol/hr*	*nM*	*nmol/hr*
Suc_{20}-BSA	2501 ± 1736	0.98 ± 0.56	23552 ± 89932	18.92 ± 68.30
Suc_{28}-BSA	52.16 ± 20.24	0.78 ± 1.65	917.32 ± 516.69	4.75 ± 1.69
Suc_{40}-BSA	9.00 ± 0.05	2.99 ± 0.32	675.76 ± 31.48	3.42 ± 0.61
Suc_{46}-BSA	12.33 ± 0.05	1.99 ± 0.20	136.89 ± 39.75	5.46 ± 0.58
Suc_{54}-BSA	5.29 ± 0.08	1.31 ± 0.16	229.13 ± 66.49	10.63 ± 1.65
Suc_{50}-IgG	83.97 ± 26.37	0.84 ± 0.16	278.19 ± 104.26	2.60 ± 0.62

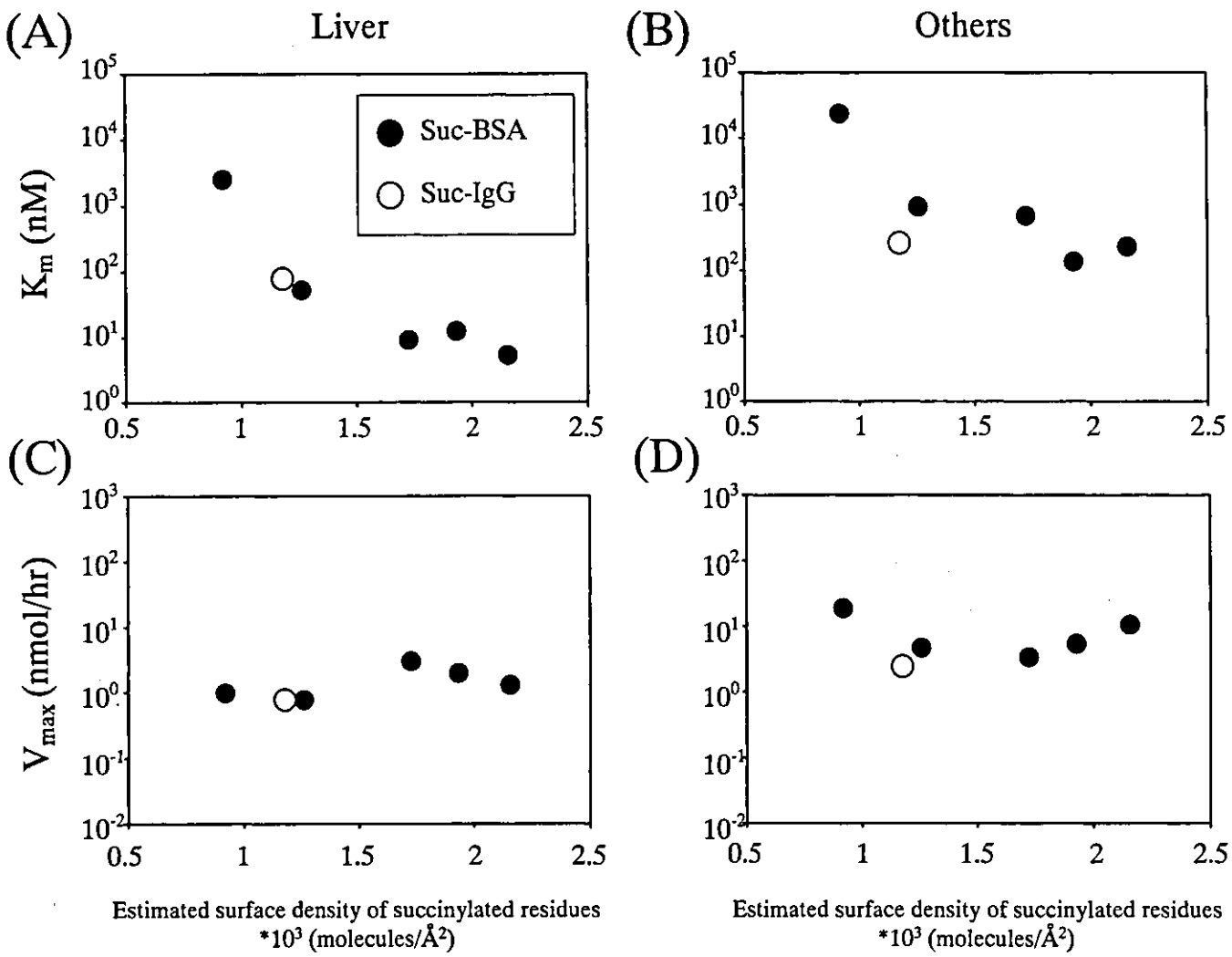

Fig. 8. Relationship between $K_{m,l}$ (A), $K_{m,p}$ (B), $V_{max,l}$ (C), and $V_{max,p}$ (D) of ^{111}In-labeled succinylated proteins and the degree of succinylation. Parameters of ^{111}In-labeled Suc-BSA (●) and ^{111}In-labeled Suc-IgG (○) were plotted against the estimated surface density of the succinylated amino groups.

These results suggested that, following intravenous injection, the BSA derivative underwent glomerular filtration to a significant extent regardless of its large size, and subsequent uptake by the renal tubular epithelial cells (i.e., reabsorption) occurred in a similar manner to smaller proteins like SOD. It is reasonable that neither poly I nor poly C showed obvious inhibitory effects on renal accumulation of ^{111}In-Suc_{54}-BSA in the competition experiments (Fig. 4), assuming that the accumulation was ascribed mainly to protein reabsorption. Increased renal uptake of ^{111}In-Suc_{54}-BSA after an excess dosing of cold Suc-BSA in the same experiment also can be explained.

Although Kuipers et al. (1997) also reported that a certain amount of radioactivity was located in the kidney, the mechanism is unknown. It is postulated that glomerular permeability might be enhanced through an unknown action of highly succinylated protein in the kidney. Although the mechanism for this phenomenon is not clear, we speculate that mesangial cells might play an important role. It has been reported that negatively charged BSA enhances production of nitric oxide (NO) from macrophages (Alford et al., 1998), and following that, glomerular mesangial cell relaxation is enhanced by NO (Stockand and Sansom, 1997). Although further studies are needed, NO may be involved in the phenomenon that causes Suc-BSA to be passed through glomeruli and accumulated in the proximal renal tubule.

In conclusion, the present study has demonstrated that the hepatic uptake of succinylated proteins is determined by the affinity for SRs expressed on the liver nonparenchymal cells, and the affinity depends on the molecular size of the protein and the surface density of the succinylated amino groups of the protein. Furthermore, we have shown that highly succinylated proteins are also accumulated in the kidney probably, due to altered glomerular permeability. Thus, the present study has provided useful basic information for therapeutic strategies and the molecular design of succinylated proteins for use as drug carriers and therapeutic agents for SR-mediated targeting in vivo. Based on the finding, we are currently developing the targeted delivery system of antigen proteins through the SR-mediated endocytosis. To control antigen-specific immune responses by effective delivery of antigen, direct succinylation of the antigen and conjugation of epitope peptide derived from the antigen to Suc-BSA have been used.

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Journal of Controlled Release 80 (2002) 283–294

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GENE DELIVERY

Inhibition of liver metastasis by targeting of immunomodulators using mannosylated liposome carriers

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Received 26 September 2001; accepted 14 December 2001

Abstract

Mannosylated liposomes were prepared by incorporating cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosylethyl)amino)butyl)formamide (Man-C4-Chol) into small unilamellar liposomes consisting of cholesterol and distearoyl phosphatidylcholine (DSPC). The biodistribution of liposomes labeled with [^{3}H]cholesteryl hexadecyl ether was examined in mice. The rate and extent of the hepatic uptake of those [^{3}H]liposomes increased proportionally on increasing the mixing ratio of Man-C4-Chol. Their hepatic uptake was reduced by increasing the administered dose due to the limited number of mannose receptors. The liver uptake of [^{3}H]Man-liposomes was preferentially mediated by liver non-parenchymal cells (NPC) and significantly inhibited by co-injection with an excess of Man-BSA, indicating the involvement of a mannose receptor-mediated mechanism in the hepatic uptake of Man-liposomes. Muramyl dipeptide (MDP), an immunomodulator, was also incorporated into the liposomes and its inhibitory effect in an experimental liver metastasis model was examined. In contrast to free MDP treatment, which showed little effect on the inhibition of metastasis, liposomal MDP significantly reduced the number of metastatic colonies in the liver. Active targeting of MDP to liver NPC by Man-liposomes resulted in more effective inhibition than delivery of MDP by liposomes without mannose. Treatment with MDP/Man-liposomes further increased the survival of the tumor-bearing mice. These results suggest that Man-liposomes are effective carriers for targeted delivery of bioactive compounds to liver NPC.

Keywords: Mannosylated liposome; Mannose receptor; Muramyl dipeptide; Tumor metastasis; Kupffer cells

1. Introduction

The liver is a major target organ of tumor metastasis, and almost any cancer can spread to the liver [1]. Liver metastases are frequently associated with a fatal outcome since there is as yet no effective treatment. Surgical hepatic resection, one of the most effective treatments for hepatic tumors, can be applied to only 10–15% of patients with hepatic metastases [2]. In recent years, numerous studies have demonstrated that stimulation of anti-tumor defense systems in the body may be of particular importance in patients undergoing resection of primary and/or metastatic tumors [3].

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0168-3659/02/$ – see front matter
PII: S0168-3659(02)00006-8

Kupffer cells (KC), tissue-fixed macrophages localizing in the hepatic sinusoids, play key roles in arresting circulating tumor cells although the number of tumor cells arrested is limited [4]. They are also involved in clearing tumor cells from liver tissue, suppressing tumor growth in the very early stages of metastasis, and modulating the host immune response to tumor cells. To effectively kill tumor cells, thus reducing the tumor burden, KC should be activated to a tumoricidal state by stimulation with biological response modifiers. Activated KC have been found in liver sinusoids adjacent to tumor cells and in tumors forming foci within the hepatic parenchyma [5]. Previous studies have shown that KC can be activated to a tumoricidal state by the administration of immunomodulators, such as interferon-γ or muramyl dipeptide (MDP) [6].

MDP is an immunomodulatory component of bacterial cell walls and has been shown to stimulate macrophages both in vitro and in vivo. Although soluble, free MDP has been shown to influence many functions of murine macrophages, such as the production of prostaglandins and collagenase, superoxide anion-generating ability, proliferation in response to lymphokines, and cytolytic activity against tumor cells [7]. However, MDP administered parenterally in the free form is rapidly cleared from the body within 60 min and excreted in urine [8]. Several approaches have been examined to increase its adjuvant activity against tumor metastases. These include encapsulation into multilamellar liposomes [9,10], conjugation to neoglycoproteins [11,12], gelatin [13], derivatization to form a lipophilic analogue [14] or MDP-lysine conjugate [15]. Recently, Srividya et al. [16] reported that scavenger receptor-mediated delivery of MDP is able to activate the antitumor efficacy of macrophages by using maleylated bovine serum albumin or polyguanylic acid. Efficient delivery of MDP to KC is required to obtain its optimal therapeutic efficacy, and the use of liposomes possessing ligands for receptors on the cells is a promising approach. Of the various receptors on the surface of KC, the macrophage mannose receptor is an encouraging candidate for liposomal targeting of drugs to the cells. We and others have reported targeted delivery of drugs, genes, antigens and immunomodulators to macrophages by using mannose-bearing liposomes [17–19]. The mannose receptor is a 175-kDa type I transmembrane protein and belongs to the C-type lectin family containing multiple carbohydrate recognition domains. It is expressed on KC, alveolar, peritoneal and splenic macrophages, monocyte-derived dendritic cells, and subsets of vascular and lymphatic endothelial cells [20].

In a previous study, we synthesized a novel mannosylated cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosylethyl)-amino)alkyl)formamide (Man-C4-Chol). A liposomal formulation consisting of Man-C4-Chol, distearoyl phosphatidylcholine (DSPC), and cholesterol is rapidly delivered to liver non-parenchymal cells (NPC) after intravenous injection into mice due to the mannose receptor-mediated uptake [21]. A cationic liposomal formulation containing Man-C4-Chol was successfully used for in vivo gene transfer in liver NPC by intravenous injection after complex formation with plasmid DNA [19]. As shown in these studies, Man-C4-Chol-containing liposomes can be widely used for targeted delivery of drugs and genes to liver NPC such as KC. In the present study, we have examined the targeted delivery of MDP to KC using Man-C4-Chol/DSPC/Chol liposomes (Man-liposomes) to inhibit experimental liver metastasis of colon carcinoma cells. Firstly, the tissue distribution of Man-liposomes with varying ratios of Man-C4-Chol was examined in mice. Then, MDP was incorporated into liposomes and the ability of MDP formulations to inhibit liver metastases was examined in mice with experimentally induced liver metastases of colon carcinoma cells, by measuring the number of metastatic nodules in the liver as well as the tissue weight of the liver. Finally, the survival of the mice injected with tumor cells was monitored. We found that the targeted delivery of MDP by the Man-liposomes is an effective approach to increasing the antitumor activity of MDP against liver metastases in mice.

2. Materials and methods

2.1. Materials

DSPC, cholesteryl chloroformate and MDP were purchased from Sigma (St. Louis, USA). Cholesterol

was obtained from Nacalai Tesque (Kyoto, Japan). [^{3}H]Cholesteryl hexadecyl ether (CHE) was purchased from NEN Life Science Products (Boston, USA). Mannosylated bovine serum albumin (Man-BSA) with 46 mannose/BSA on average was synthesized as reported previously [22]. All other chemicals were reagent-grade products obtained commercially.

2.2. Animals

Male ddY and CDF1 mice (25–28 g) were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All procedures were examined by the Ethics Committee on Animal Experiment at Kyoto University and animal care was in accordance with the National Institutes of Health Guidelines for Animal Experiment and Japanese law.

2.3. Tumor cells

A murine colon carcinoma cell line CT-26 obtained from the Cancer Chemotherapeutic Center of the Cancer Institute (Tokyo, Japan), was cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin G/streptomycin at 37 °C in a 5% CO_2 incubator. The cells were harvested from 2-day-old subconfluent cultures by trypsin and washed with phosphate buffered-saline (PBS, pH 7.4). The concentration of the cells was adjusted to 10^6 cells/ml of Hank's balanced salt solution (HBSS). The tumor cells were injected into mice within 1 h of their preparation.

2.4. Preparation of liposome formulations

Man-C4-Chol was synthesized and DSPC/Chol (Bare-liposomes, 60:40 in molar ratio) and DSPC/Chol/Man-C4-Chol (Man-liposomes, 60:40-X:X with X=1, 2.5, 5 for the molar ratio) were prepared as reported previously [21]. These formulations were radiolabeled with a trace amount of [^{3}H]CHE for tissue distribution experiments. For encapsulation of MDP into liposomes, lipid mixtures were hydrated with a solution containing MDP at a ratio of 20 μg MDP/mg lipid. The suspension was sonicated and then extruded using polycarbonate membranes (100-nm pore size) at 65 °C. The cholesterol concentration was measured by a cholesterol E-test (Wako Pure Chemical, Osaka, Japan) and the lipid concentration was adjusted to 2.5 mg/ml. The mean particle diameter was measured by using a laser light scattering particle size analyzer (LS-900, Otsuka Electronics, Osaka, Japan).

2.5. In vivo distribution experiment

Each [^{3}H]liposome formulation was injected intravenously into male ddY mice (25–28 g) via a lateral tail vein. At predetermined time points after injection, blood was collected from the vena cava under ether anesthesia. Then the mice were sacrificed and the liver, spleen, kidney, lung and heart were excised, rinsed with saline, and weighed. Tissue samples were digested with 0.7 ml Soluene-350 (Packard, Meriden, CT, USA) by incubation overnight at 45 °C. To the digested samples were added 0.2 ml isopropanol, 30% hydrogen peroxide, 0.1 ml 5 M HCl, and, finally, 5 ml Clear-Sol I (Nacalai Tesque, Kyoto, Japan). The radioactivity of samples was assayed by a Beckman Model LS5000TA liquid scintillation counter (Beckman, Tokyo, Japan).

2.6. Calculation of pharmacokinetic parameters

The distribution data of [^{3}H]liposome formulations were analyzed in terms of the organ uptake clearance (CL_{org}) [22]. After injection of [^{3}H]liposomes, the change in the amount of radioactivity in a tissue with time can be described as

$$\frac{dX_i}{dt} = CL_{org}C_p - K_{efflux,i}X_i \quad (1)$$

where X_i (normalized to the % of the dose) represents the amount of radioactivity in tissue i after the administration of the [^{3}H]liposomes; C_p (% of the dose/ml) is the concentration of radioactivity in the plasma; CL_{org} (ml/h) represents the apparent tissue uptake clearance from the plasma to tissue i; and $K_{efflux,i}$ (h^{-1}) represents the efflux rate constant from tissue i. Since little efflux of [^{3}H]radioactivity from tissues was observed throughout the entire experimental period, the efflux process can be ignored and Eq. (1) is simplified to

$$\frac{dX_i}{dt} = CL_{org}C_p. \quad (2)$$

When Eq. (2) is integrated from time 0 to t, CL_{org} can be expressed as

$$CL_{org} = \frac{X_{i,t}}{\int_0^t C_p dt} = \frac{X_{i,t}}{AUC_t} \quad (3)$$

where AUC_t (% of dose.h/ml) represents the area under the plasma concentration–time curve from time 0 to t, calculated by fitting a mono-exponential equation to the plasma concentration–time data of the [^{3}H]radioactivity–time profile using the non-linear least-squares program MULTI. Therefore, CL_{org} can be easily calculated from Eq. (3) at several time points after administration. The total body clearance (CL_{total}) can be calculated by dividing the administered dose by the AUC up to infinity.

2.7. Cellular distribution of [^{3}H]liposomes in the liver after intravenous injection

At 30 min after injection of [^{3}H]liposomes (25 mg/kg), the liver of each mouse was perfused with a collagenase buffer [23] and the liver cells were separated into parenchymal cells (PC) and non-parenchymal cells (NPC) by differential centrifugation. Radioactivity of the cell suspensions was assayed as above.

2.8. Co-administration of Man-BSA or Man-liposomes

[^{3}H]Liposomes were injected at a dose of 25 mg/kg into mice with or without an excess amount of ligands for the mannose receptor, i.e. Man-BSA (20 mg/kg) or Man-liposomes (250 mg/kg). At 5 min after injection, blood and liver were sampled and their [^{3}H]radioactivity measured.

2.9. Effect of liposomal MDP against liver metastases

Experimental liver metastases were induced by injecting 1×10^5 CT-26 tumor cells in 0.1 ml HBSS into the portal vein of CDF1 mice. On days −3, 0, 3, 7, 10 of tumor cell injection, free MDP (100 μg), empty liposomes (25 mg/kg) with 10 μg free MDP, or liposomes (25 mg/kg) containing 10 μg MDP were intravenously injected into the mice (six to eight mice per group). On day 14, mice were sacrificed and the number of tumor nodules on the liver surface and the liver weight were measured. In a different set of experiments, survival of mice was recorded up to 30 days after tumor cell inoculation.

2.10. Statistical analysis

Differences were statistically evaluated by one-way ANOVA followed by the Tukey-H.S.D. test. Differences in the survival of mice were evaluated by using the log-rank test.

3. Results

3.1. Physicochemical properties of liposomes

The mean particle sizes of the prepared DSPC/Chol liposomes (Bare-liposomes) and DSPC/Chol/Man-C4-Chol liposomes were almost identical for all formulations. The size of each liposome preparation was ~95 nm in diameter and remained stable for at least 1 month.

3.2. Tissue distribution of [^{3}H]liposomes after intravenous injection

Fig. 1 shows the blood concentration- and liver accumulation-time courses of [^{3}H]liposomes with varying amount of Man-C4-Chol. [^{3}H]Bare-liposomes (DSPC/Chol/Man-C4-Chol = 60:40:0) showed a prolonged circulation in plasma and an increasing amount of Man-C4-Chol augmented the uptake of [^{3}H]liposomes by the liver. The liposomes containing 5 mol% of Man-C4-Chol showed the highest hepatic uptake among the formulations examined and this uptake reached ~75% of the injected dose at 10 min after injection. In the following experiments, therefore, this liposome formulation with 5 mol% of Man-C4-Chol was used as Man-liposomes.

Fig. 2 shows the blood concentration- and liver accumulation-time courses of [^{3}H]Bare-liposomes

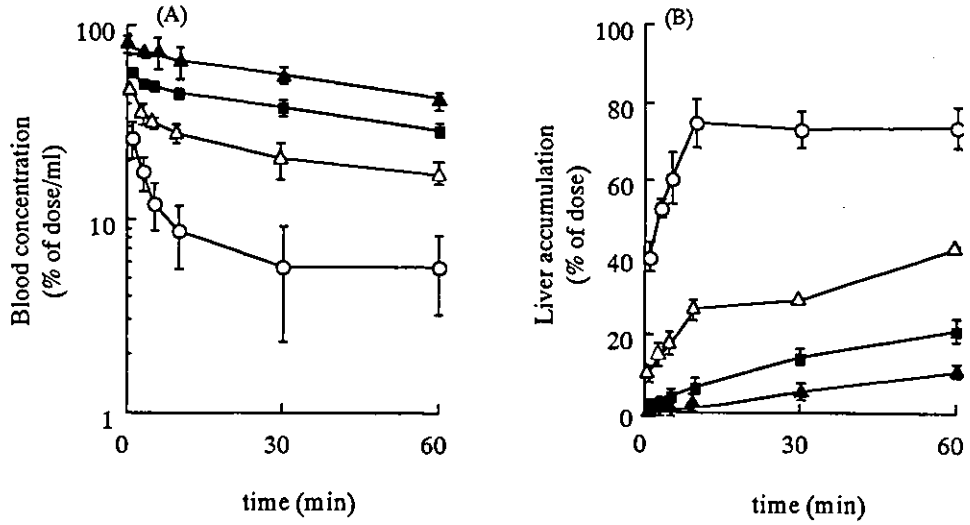

Fig. 1. Blood concentration (A) and liver accumulation (B) of [^{3}H]liposomes after intravenous injection into mice at a dose of 25 mg/kg. Mixing ratios are: DSPC/Chol/Man-C4-Chol, 60:40:0 (▲, Bare-liposomes), 60:39:1 (■), 60:37.5:2.5 (△), and 60:35:5 (○). Results are expressed as the mean±S.D. of three mice.

and [^{3}H]Man-liposomes following intravenous injection in mice at various doses. When [^{3}H]Man-liposomes were injected at low doses of 2.5 and 25 mg/kg, more than 75% of the radioactivity was recovered in the liver. However, increasing the dose reduced the amount and rate of liver accumulation of [^{3}H]Man-liposomes due to saturation of the mannose receptor-mediated hepatic uptake. The elimination from the blood circulation was approximately inversely correlated with their hepatic uptake. The incorporation of MDP into [^{3}H]Man-liposomes hardly altered the tissue disposition of the liposomes (data not shown). On the other hand, the tissue distribution of [^{3}H]Bare-liposomes was only slightly affected by the administered dose. Except for the liver, none of the other tissues exhibited a significant uptake for either [^{3}H]Bare-liposomes or [^{3}H]Man-liposomes (data not shown).

Table 1 summarizes the area under plasma concentration–time curve (AUC), total clearance (CL_{total}), hepatic uptake clearance (CL_{liver}), and tissue uptake rate index (tissue uptake clearance per unit tissue weight) for representative tissues of [^{3}H]liposomes after intravenous injection. At any dose, the CL_{liver} of [^{3}H]Man-liposomes was close to the corresponding CL_{total}, indicating that the liver is the main organ determining the biodistribution of [^{3}H]Man-liposomes.

3.3. Inhibition of hepatic uptake of liposomes

Neither Man-liposomes nor Man-BSA inhibited the hepatic uptake of [^{3}H]Bare-liposomes simultaneously injected (Fig. 3). On the other hand, when [^{3}H]Man-liposomes were co-administered with Man-BSA (20 mg/kg), a well-known macromolecular ligand for the mannose receptors [24], the amount of radioactivity in the liver was slightly but significantly reduced. These results indicate that recognition by the mannose receptor is involved in the hepatic uptake of Man-liposomes.

3.4. Hepatic cellular localization of liposomes

Fig. 4 shows the intrahepatic distribution of [^{3}H]liposomes at 30 min after intravenous injection into mice at a dose of 25 mg/kg. [^{3}H]Bare-liposomes were recovered in both parenchymal cells (PC) and non-parenchymal cells (NPC) with a PC/NPC ratio of 1.1. On the other hand, [^{3}H]Man-

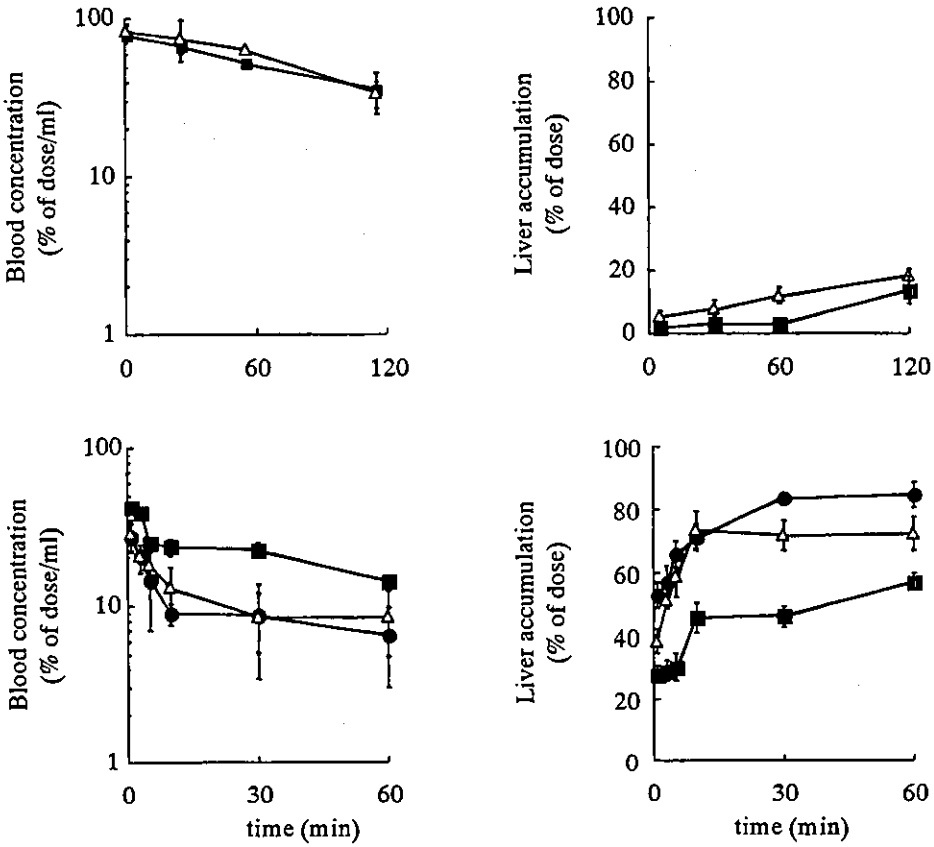

Fig. 2. Blood concentration (left) and liver accumulation (right) of [^{3}H]Bare-liposomes (top, DSPC/Chol, 60:40) and [^{3}H]Man-liposomes (bottom, DSPC/Chol/Man-C4-Chol, 60:35:5) after intravenous injection into mice at various doses: 2.5 mg/kg (●), 25 mg/kg (△), and 250 mg/kg (■). Results are expressed as the mean±S.D. of three mice.

liposomes were preferentially accumulated in NPC with a PC/NPC ratio of 0.39, clearly indicating that the incorporation of Man-C4-Chol into DSPC containing liposomes enhanced their uptake by liver NPC. Although Fig. 4 showed that [^{3}H]Man-liposomes had greater recoveries in both PC and NPC

Table 1
AUC, clearances, and tissue uptake rate index of liposomes after intravenous injection into mice

Compound	Dose (mg/kg)	*AUC* (% of dose h/ml)	Clearances (ml/h)		Tissue uptake rate index (ml/h per g)			
			CL_{total}	CL_{liver}	Liver	Kidney	Spleen	Lung
DSPC/Chol	25	70.85	0.52	0.19	0.12	0.01	0.65	0.01
(Bare-liposome)	250	70.96	0.80	0.13	0.12	0.01	0.60	0.01
DSPC/Chol/Man-C4-Chol	2.5	10.37	11.82	10.88	8.9	0.40	8.41	0.01
(Man-liposome)	25	10.16	10.24	9.85	8.26	0.27	8.38	0.03
	250	37.08	2.69	2.32	1.94	0.08	3.66	0.01

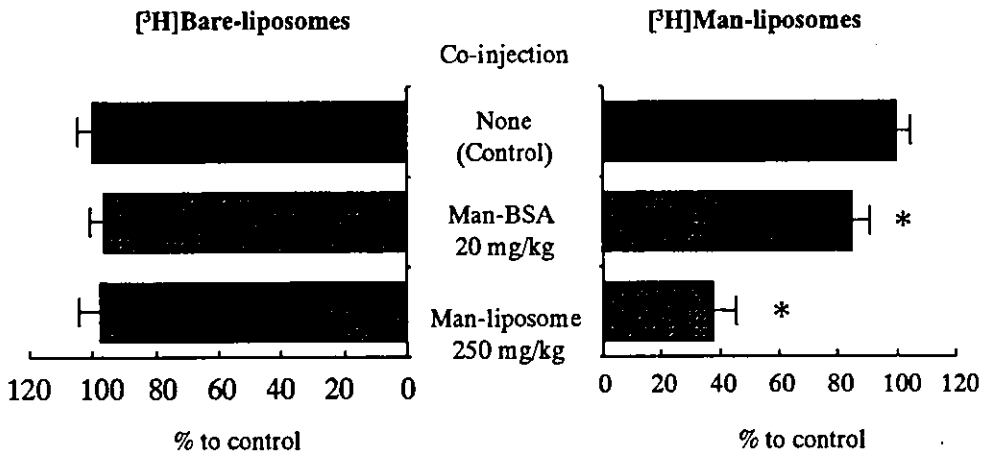

Fig. 3. Liver accumulation of [³H]Bare-liposomes (left, DSPC/Chol, 60:40) and [³H]Man-liposomes (right, DSPC/Chol/Man-C4-Chol, 60:35:5) 5 min after intravenous injection into mice at a dose of 25 mg/kg. [³H]Liposomes were co-injected with none (control), 20 mg/kg Man-BSA, or 250 mg/kg Man-liposomes. Results are expressed as the mean±S.D. of three mice. *Statistically significant from the control group ($P<0.01$).

than did [³H]Bare-liposome, only the amounts of Man-liposome in NPC were statistically significant ($P<0.05$).

3.5. Effect of liposomal MDP against liver metastases

On day 14, the number of metastatic colonies greater than 50 on the liver surface were counted in the control mice injected with saline. Mice injected with liposomal MDP had significantly fewer tumor colonies than mice treated with saline or free MDP (Fig. 5A). MDP encapsulated in Man-liposomes showed the most prominent effect as far as inhibition of liver metastases was concerned. MDP plus empty Bare- or Man-liposomes had no effect on reducing the number of colonies (data not shown). The weight of the liver correlated with the number of the metastatic colonies in the liver (Fig. 5B).

When tumor cells were intravenously injected, all mice treated with saline died by 23 days after tumor injection. The treatments with free MDP or MDP/Bare-liposomes marginally increased the survival time of mice. On the other hand, the survival time of mice treated with MDP/Man-liposomes was significantly different from those of all other groups ($P<0.05$).

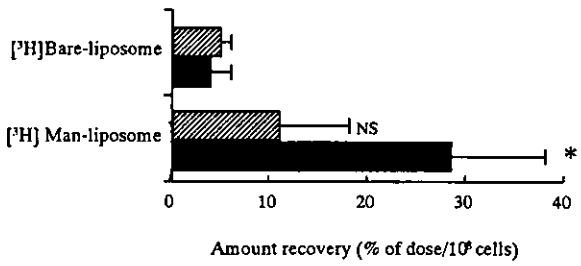

Fig. 4. Hepatic cellular localization of [³H]liposomes 30 min after intravenous injection into mice at a dose of 25 mg/kg; (square with diagonal line) PC and (filled square) NPC. Results are means±S.D. of five mice. *Statistically significant from [³H]Bare-liposome ($P<0.05$); NS, not significant.

4. Discussion

Targeted drug delivery to macrophages is an important strategy for achieving diverse aims such as the treatment of lysosomal storage diseases [25], the activation of macrophages with immunomodulators [26], cell or cell product depletion [27], and the blockade of macrophage functions [28]. To date, various liposomal formulations have been widely used for targeted drug delivery to macrophages [29].

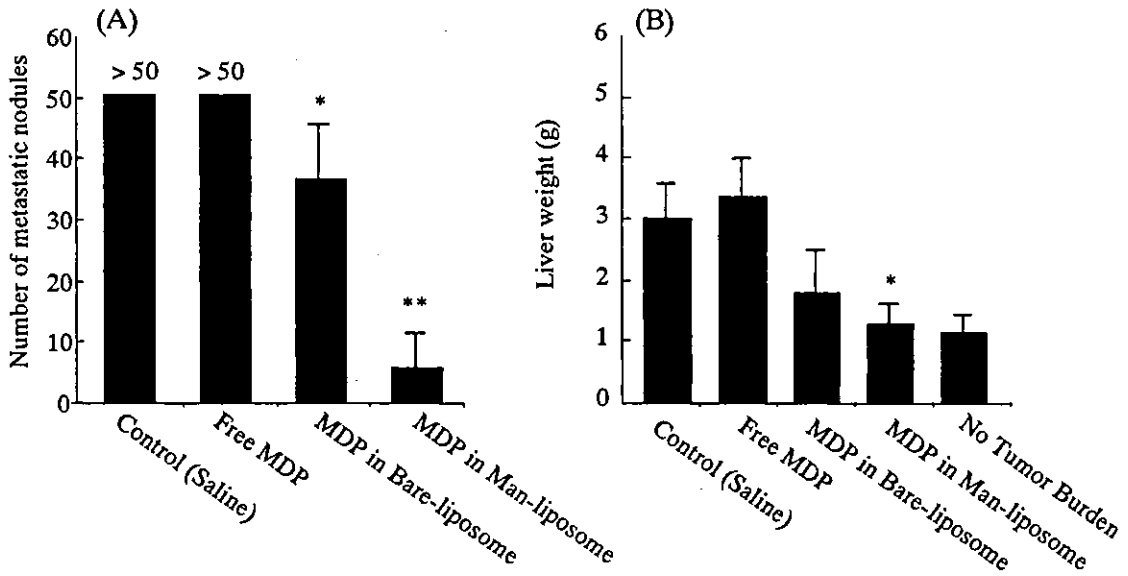

Fig. 5. (A) Number of metastatic nodules of CT-26 tumor cells on the liver surface and (B) liver weight of mice intraportally injected with the cells. Mice were sacrificed at 14 days after tumor injection and the number of nodules counted. Free MDP (100 μg), empty liposomes plus 10 μg MDP, or liposomes containing 10 μg MDP were injected into a tail vein of tumor-inoculated mice on days −3, 0, 3, 7, and 10. Results are expressed as the mean±S.D. of at least seven mice. $*P<0.01$, $**P<0.005$, statistically significant from the control group.

Although liposomes tend to be captured by macrophages following in vivo administration and are used as carriers to the mononuclear phagocyte system (MPS), active targeting of liposomes to the cells can improve the rate and extent of their uptake by the target cell population. Cell-specific targeting using carbohydrate recognition mechanisms of the body itself has attracted great interest as far as drug and gene delivery are concerned, and we have reported the development of various polymeric or particulate carriers in this context [19,30].

In general, small liposomes composed of DSPC and cholesterol with diameters of 100 nm or less are known to have long half-lives in the blood circulation. This is because such liposomes avoid trapping by MPS, do not significantly activate the complement system, and are not destroyed to any extent by serum components [31]. These characteristics are suitable for targeted drug delivery since this can be achieved by the addition of a ligand specifically recognized by target cells. In the present study, therefore, we selected small DSPC/Chol liposomes and modified them with Man-C4-Chol to obtain mannosylated targeting liposomes for MDP delivery to KC. We have previously reported the in vitro binding and uptake properties of these Man-liposomes via the macrophage mannose receptor using cultured mouse peritoneal macrophages [32]. Man-liposomes were recognized by macrophages via the mannose receptor. In addition, they were bound to serum mannan binding protein (MBP), a C-type soluble multimeric serum lectin specific for D-mannose, L-fucose, and *N*-acetylglucosamine, and this binding facilitated their uptake by macrophages. Therefore, Man-liposomes are good candidates for targeted delivery of drugs to macrophages.

The binding affinity of ligands with the mannose receptor has been reported to depend on the clustering and geometric organization of the mannose residue on a branched sugar chain [33]. The mannose receptor is a type I transmembrane protein with eight different C-type carbohydrate-recognition domains (CRD) in a single polypeptide. Of the eight CRDs, CRDs 4–8 are required for binding and endocytosis. The requirement of multiple CRDs for high affinity ligand binding and endocytosis probably reflects the requirement for multivalent binding [20]. Therefore, it is well known that the affinity of synthetic

mannosylated ligands, such as neoglycoproteins and mannosylated liposomes, for the receptor depends on the density of mannose residues [24,34,35]. In the present study, similar effects of the density of mannose on in vivo targeting to the liver were observed. Increasing the molar ratio of Man-C4-Chol (5 mol% in maximum) greatly increased the hepatic uptake of the liposomes. Since the amount delivered to the liver exceeded 75% of the injected dose, no further increase in the ratio of Man-C4-Chol was expected.

The in vivo biodistribution profiles clearly indicated that the Man-liposomes are mainly taken up by liver NPC. Because of the limited number of mannose receptors, increasing the dose of Man-liposomes saturates the receptors. The saturation would be followed by a decrease in their hepatic uptake when normalized with respect to the administered dose (Figs. 2 and 3) even though the recycling rate of the mannose receptors is reported to be as short as 15 min [36], as observed with glycosylated polymeric macromolecules [22]. The CL_{liver} values in Table 1 more clearly indicate the saturation of the hepatic uptake of [^{3}H]Man-liposomes at the highest dose than do the accumulation amounts in the liver (Figs. 2 and 3), based on the relation of these parameters shown in Eq. (3). In contrast, Bare-liposomes appeared to undergo uptake irrespective in the increasing doses, probably in a non-specific manner [37]. Man-liposomes were significantly inhibited by pre-administration of an excess amount of Man-BSA or Man-liposomes (Fig. 3) and a rather low PC/NPC ratio (0.39) compared with Bare-liposomes (1.1). As observed in our previous study [32], MBP-mediated uptake of Man-liposomes by KC is also involved in the total in vivo uptake by NPC.

Intraportal injection of CT-26 colon carcinoma cells resulted in the formation of a number of metastatic nodules on the surface of the liver, indicating that some of the tumor cells injected survive, adhere to the endothelial cells, extravasate and proliferate in the liver. To form colonies in the liver, the tumor cells intraportally injected need to be arrested in the liver sinusoid, adhere to endothelial cells, extravasate, invade liver tissue, and then proliferate [38]. In a previous study of our group, when [^{111}In]tumor cells were injected into the portal vein, ~50–60% of the radioactivity was recovered in the liver with much less activity in the kidney, lung, spleen and heart (Tamada, submitted for publication). Although the precise mechanism of such tumor arrest in the liver sinusoid remains to be elucidated, physical trapping of the cells within the microvasculature is involved. In addition, hepatic sinusoidal cells play important roles in the early phase of liver metastasis. Depletion of KC functions prior to tumor cell challenge increases metastatic growth in the liver [39], indicating that these cells form a defense system against liver metastases. However, during the early period (1 h) following tumor cell infusion into a mesenteric vein, Bayon et al. [4] showed that the number of tumor cells entrapped in the liver was greater in KC-free rats than in control animals. These results suggest that KC can arrest tumor cells circulating within blood vessels and then kill them directly or by recruiting inflammatory cells. Therefore, activation of KC represents a method of inhibiting tumor metastasis to the liver, although the capacity of KC killing tumor cells is somewhat restricted to a small number of tumor cells [6]. Recently, a role for natural killer cells similar to that of KC in preventing liver metastases has been reported [3]. The tumoricidal activities of KC are mediated by several factors, such as cytokines (IFN-γ, TNF-α, IL-6), reactive intermediates of oxygen or nitrogen, enzymes metabolizing essential amino acids (e.g. arginase), prostanoid metabolites (e.g. PGE2) and nucleotides (e.g. thymidine) [40].

Various immunomodulatory compounds, such as interferons, interleukins, colony stimulating factors, and various bacterial products, can augment selective activation of macrophages to tumoricidal states. MDP, a synthetic peptidoglycan derived from mycobacterial cell walls, possesses various immunopotentiating activities by stimulating fixed macrophages of the mononuclear phagocyte system (MPS). The phagocytic activity of KC has been shown to be particularly increased after MDP treatment [41]. However, because of its rapid clearance from the body, the activation of antitumor properties of macrophages by free MDP was poor. Attempts to augment the antitumor activity of MDP have been made using liposomes, lipoproteins, neoglycoproteins, and synthetic polymers but with limited success [12,16]. Therefore, it is of interest to examine the possibility of modulating the antitumor activity

of macrophages by specific delivery of MDP through the mannose receptor-mediated endocytosis.

In the present study, the tissue disposition of Man-liposomes was investigated in mice following a single injection. Abra et al. reported that when the two doses of a liposome formulation were separated by 24 h, the first dose had a minimal effect on the disposition of the second dose [42]. To improve the dosing schedule of MDP/Man-liposomes, however, possible effects of multiple dosing on the tissue disposition need to be further investigated. When formulated in Man-liposomes, MDP exhibited excellent activity in preventing liver metastases and a significant increase in survival time (Figs. 5 and 6). Yamashita et al. [43] also reported that cetylmannoside-modified multilamellar liposomes (Man-MLV) are far more effective than unmodified liposomes as a carrier to deliver biological response modifiers to human blood monocytes. Therefore, it appears that Man-liposomes are valuable carriers for specific therapeutic agents, superior to conventional multilamellar liposomes, removed by phagocytes [29]. The results of the present study have shown that delivery of MDP using Man-liposomes via the mannose receptor-mediated endocytosis pathway increases the antitumor efficacy of the KC.

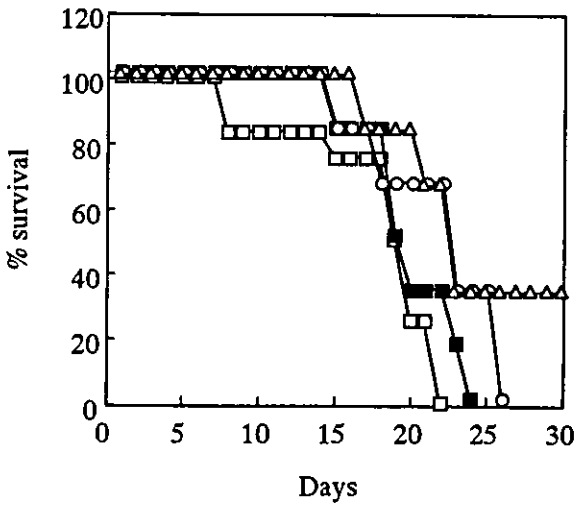

Fig. 6. Survival time of mice injected with CT-26 tumor cells followed by intravenous treatment with saline (control; □), 100 μg free MDP (■), MDP (10 μg)/Bare-liposomes (○) or MDP (10 μg)/Man-liposomes (△). Survival of mice was observed daily for 30 days.

5. Conclusion

In conclusion, MDP can be selectively targeted to liver NPC, including KC, using Man-liposomes. Mannose receptor-mediated endocytosis is involved in the uptake of the liposomes by liver NPC. MDP encapsulated in Man-liposomes is a useful modification for administering this drug in order to inhibit tumor metastasis to the liver by augmenting the tumoricidal activity of Kupffer cells.

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Serum mannan binding protein inhibits mannosylated liposome-mediated transfection to macrophages

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Received 9 October 2001; received in revised form 25 January 2002; accepted 4 February 2002

Abstract

The effects of serum mannan binding proteins (MBP) in the transfection of plasmid DNA/Man–liposome complex via mannose receptor-mediated endocytosis was studied in vitro using cultured mouse peritoneal macrophages. Plasmid DNA encoding luciferase gene was complexed with cationic mannosylated liposomes (Man–liposomes), composed of cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol) and dioleoyl phosphatidylethanolamine (DOPE). The transfection efficiency, as well as the binding and uptake of the plasmid DNA/Man–liposome complex, was investigated with or without serum MBP. The in vitro transfection efficiency of the complex was significantly reduced on increasing the amount of serum MBP. In addition, the cellular association of the complex was also reduced. These results indicate that serum MBP specifically binds to the mannose moieties on the complex and suppresses its cellular uptake, resulting in inhibition of the gene transfection in macrophages. Such an interaction is an obstacle to mannose receptor-mediated in vivo gene transfer to mannose receptor-positive cells using mannosylated gene carriers. © 2002 Published by Elsevier Science B.V.

Keywords: Cationic liposome; Gene transfer; Mannose receptor; Mannan binding protein; Peritoneal macrophage

1. Introduction

Ineffective and variable in vivo gene transfer by plasmid DNA/cationic nonviral carrier complexes leads to unfavorable biodistribution profiles of the complex following systemic administration and the interaction of the complex with blood components should play some important roles in it. Serum proteins, erythrocytes and other blood cells can bind to the complex leading to an alteration in its physicochemical properties such as particle size and electrical charge. Aggregated complex is entrapped in capillary beds and very little reaches target cells and/or is rapidly cleared by the mononuclear phagocyte system. Actually, nonspecific, charge-mediated interaction of plasmid DNA/cationic liposome complexes with serum proteins has been reported [1,2]. We have also shown that erythrocytes are important for embolization of plasmid DNA/cationic liposome complex in the lung following intravenous injection [3].

In contrast to the nonspecific nature of cationic carrier-mediated gene transfer, receptor-mediated gene delivery systems are a promising tool to achieve a target cell-selective gene transfer. Ligands currently being investigated include asialoglycoproteins [4], lactose [5], transferrin [6], epidermal growth factor [7], insulin [8], antibody [9] and mannose [10]. Recently, we have developed galactosylated and mannosylated nonviral carriers [11–14] to deliver plasmid DNA to hepatocytes and macrophages via asialoglycoprotein receptors and mannose receptors, respectively. In these approaches, the ligands attached are believed to transport genes to cells which have the corresponding receptors.

Liver nonparenchymal cells (NPC), such as sinusoidal endothelial cells and Kupffer cells are involved in various diseases and disorders of the liver: Gaucher disease [15], hepatic ischemia/reperfusion injuries [16], and viral infection [17]. Several strategies have been developed to transfer genes directly into these cells. Since they have mannose receptors on their surface, targeted delivery of drugs, genes and antigens to these cells have been successfully achieved by using mannose-bearing liposomes [14,18]. Transfection efficiency in macrophages, however, is commonly much

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0304-4165/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0304-4165(02)00199-X

lower than other types of cells [19,20], probably because of their high degradation activity.

Besides mannose receptors, the serum-type mannan-binding proteins (MBP), another mannose-specific lectin circulating in mammalian serum, also recognizes D-mannose, *N*-acetyl-D-glucosamine and L-fucose on a variety of pathogens [21] and glycosylated macromolecules and particulates [22,23]. Therefore, such recognition could affect the targeting of plasmid DNA to the cells if mannosylated carriers are used as a targeting vector. We have reported that the uptake of mannosylated liposomes and proteins by mouse peritoneal macrophages was influenced by the presence of MBP in the medium [22]. These results suggest the possibility that the serum MBP might affect the mannose receptor-mediated gene delivery to macrophages too.

In this study, therefore, cationic mannosylated liposomes (Man–liposome), consisting of cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol) and dioleoyl phosphatidylethanolamine (DOPE) were prepared and evaluated as a targetable carrier of plasmid DNA to macrophages. The transfection efficiency, as well as the binding and uptake of plasmid DNA/Man–liposome complex was investigated in mouse peritoneal macrophages, with or without serum MBP.

2. Materials and methods

2.1. Materials

DOPE was purchased from Avanti Polar-lipids (Alabaster, AL, USA). Man-C4-Chol was synthesized as previously reported [14]. [α-^{32}P]dCTP was obtained from Amersham (Tokyo, Japan). Serum MBP was isolated from ICR mouse serum (Japan Bio-supply) using an affinity column of mannosylated bovine serum albumin (BSA)-bound Sepharose as described previously [23]. Opti-MEM I and other culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were reagent grade products obtained commercially.

2.2. Animals

Male ICR mice (25–28 g) were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All animal procedures were examined by the Ethics Committee for Animal Experiments at the Kyoto University.

2.3. Preparation of DNA

Plasmid DNA encoding firefly luciferase cDNA was prepared as previously reported [14]. For the uptake experiment, plasmid DNA was labeled with [α-^{32}P]dCTP using a nick translation kit (Takara, Japan). No fragments were detected in an agarose gel electrophoretic analysis of [^{32}P] plasmid DNA.

2.4. Harvesting and culture of macrophages

Elicited macrophages were harvested from the peritoneal cavity of male ICR mice 4 days after intraperitoneal injection of 1 ml of 2.9% thioglycolate medium (Nissui Pharmaceutical, Tokyo, Japan). Cells were washed, then suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, UK), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The cells were plated on 6- or 12-well culture plates (Falcon®, Becton Dickinson Labware, USA) at a density of 1×10^6 cells/cm^2. After incubation for 24 h at 37 °C in 5% CO_2–95% air, non-adherent macrophages were washed off with culture medium and then cultured under the same conditions for another 48 h.

2.5. Preparation of DNA/liposome complexes

Mannosylated liposomes (Man–liposome) were prepared by mixing Man-C4-Chol and DOPE at a molar ratio of 6:4 in chloroform. The mixture was dried as a thin film in a round-bottomed flask using a rotary evaporator, and the remaining solvent was completely removed in a vacuum desiccator. The film was then hydrated in sterile 20 mM HEPES buffer (pH 7.8), and the dispersion was sonicated for 10 min in a bath sonicator to produce Man–liposomes followed by filtration using a 0.45-μm pore filter. Plasmid DNA/Man–liposome complexes were prepared by mixing plasmid DNA solution (1 μg/ml) and Man–liposomes (1 mg/ml) at various mixing ratios (w/w), then left to stand for 30 min at room temperature before experiments were carried out.

2.6. Particle size and zeta potential measurements

The particle size of the complexes was measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan), and the zeta potential was measured with a laser electrophoresis zeta-potential analyzer (LEZA-500T, Otsuka Electronics).

2.7. Transfection to macrophages

After 2 days in culture, the culture medium of the mouse peritoneal macrophages was replaced with 2 ml Opti-MEM I containing plasmid DNA (1 μg/ml) complexed with varying amounts of Man–liposomes. Six hours later, the medium was changed to plasmid DNA-free RPMI 1640 supplemented with 10% FBS, and incubated for an additional 18 h. Then the cells were scraped off from the plates and subjected to three cycles of freezing (liquid N_2 for 3 min) and thawing (37 °C for 3 min), followed by centrifugation at 14,000 rpm for 3 min at 4 °C. Before centrifuga-

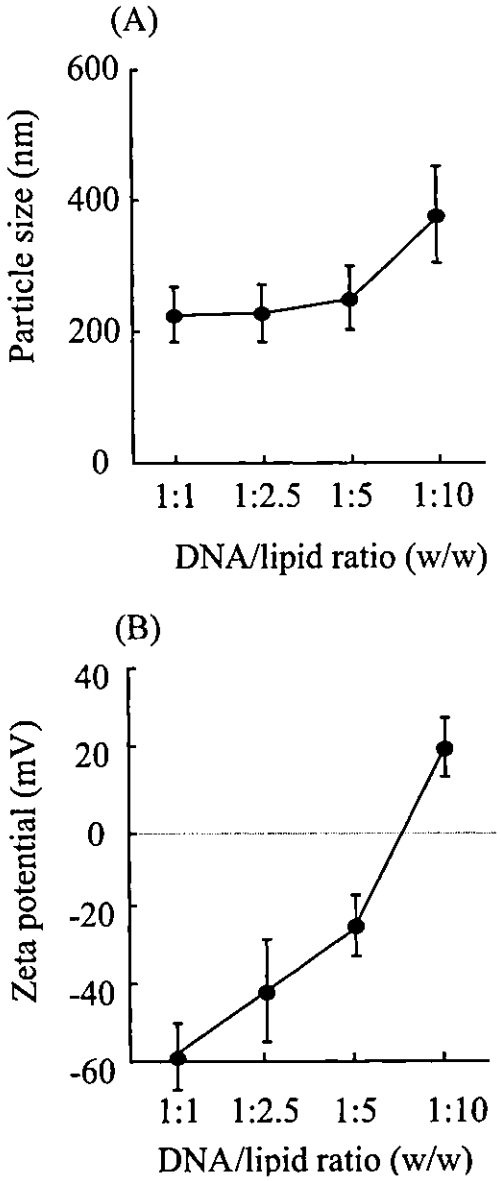

Fig. 1. The particle size (A) and zeta potential (B) of the plasmid DNA/Man–liposome complex at different mixing ratios. For the particle size, plasmid DNA (5 μg/ml) was complexed with Man–liposome at ratios of 1:1, 1:2.5, 1:5 and 1:10 (w/w) in saline. For the zeta potential, plasmid DNA (2 μg/ml) was complexed with Man–liposomes at ratios of 1:1, 1:2.5, 1:5 and 1:10 (w/w) in 5% dextrose solution containing 1 mM NaCl. Each value represents the mean±S.D. of three different measurements.

tion, an aliquot of the suspension was assayed for protein using BSA as a standard [24]. The luciferase activity in the supernatant was assayed by luminometry (Lumat LB 9507, EG&G Berthold, Germany) after mixing with a luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan).

2.8. Uptake by macrophages

The [^{32}P]-labeled DNA (0.5 μg/ml; 1×10^{-13} mol/ml)/Man–liposome complex at a mixing ratio of 1:2.5 was pre-incubated at room temperature for 10 min with various concentrations of purified MBP (0.01–10 μg/ml; 1.6×10^{-9}–1.6×10^{-11} mol/ml) or BSA (10 μg/ml). The amount of MBP was enough for binding to pDNA/Man–liposome complex according to the previous reported the maximum capacity of MBP binding to the mannan was 3.6 pmol of mannan per μg of protein [25]. Then the complex was added to macrophages and incubated at 4 °C or 37 °C. At the end of the incubation period (2 h for binding and 6 h for uptake experiment), the medium was removed and the cells were washed five times with 1 ml PBS. The cells were solubilized with 0.5 ml of NaOH (1 M) overnight and then neutralized with 0.1 ml HCl (5 M). The ^{32}P radioactivity was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan) after addition of 5 ml of Clear-Sol I (Nacalai tesque, Kyoto, Japan). The protein content of the samples was measured by the method described above.

3. Results

3.1. Physicochemical properties of plasmid DNA/liposome complex

A Man-C4-Chol/DOPE liposome formulation was prepared at a ratio of 6:4 (molar ratio) and the particle size and zeta potential were determined to be approximately 200 nm and 20.9 mV, respectively. Cationic mannosylated liposomes were mixed with plasmid DNA to obtain the plasmid DNA/Man–liposome complex. Fig. 1 shows the particle size (A) and zeta potential (B) of the complexes prepared at different mixing ratios. The complexes at ratios of 1:1, 1:2.5 and 1:5 (w/w) were negatively charged, whereas the one at 1:10 was positively charged. The particle size of the complexes at ratios of 1:1, 1:2.5 and 1:5 was approximately 200 nm in all cases while that of the complex at 1:10 was about 400 nm.

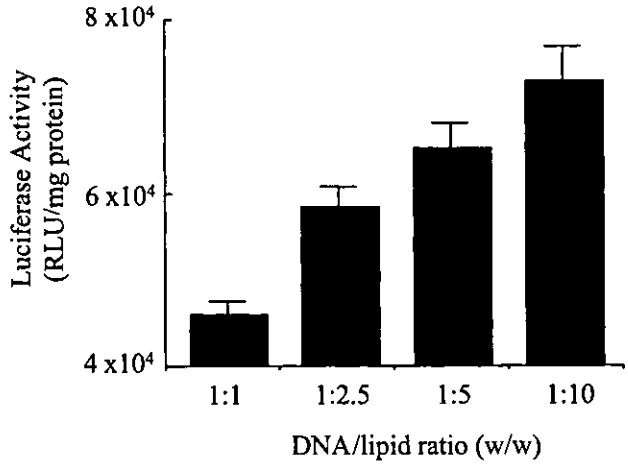

Fig. 2. Luciferase expression in macrophages transfected with plasmid DNA/Man–liposome complex prepared at different mixing ratios. The plasmid DNA concentration was fixed at 1 μg/ml in all experiments. Each value represents the mean±S.D. of three wells.

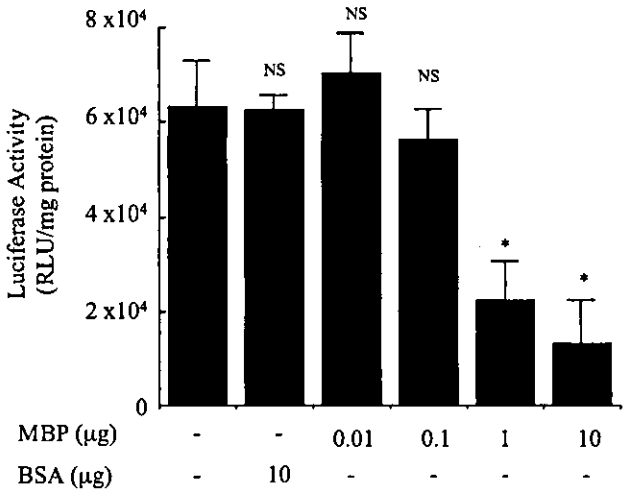

Fig. 3. Effect of co-presence of serum MBP (0.1–10 μg/ml) or BSA (10 μg/ml) on the transfection activity of the plasmid DNA/Man–liposome complex at a ratio of 1:2.5 (w/w) in macrophages. Each value represents the mean±S.D. of three wells. (*) Indicates $p < 0.05$; (NS) not significant.

The effects of adding serum MBP on the properties of these complexes were examined using a complex formulated at 1:2.5. The addition of serum MBP hardly changed the particle size while the zeta potential increased slightly from −42.2 to −26.78 mV.

3.2. Transfection to cultured macrophages

Fig. 2 shows the luciferase expression in macrophages transfected with plasmid DNA/Man–liposome complexes. As the ratio of Man–liposomes in the complex increased, the transfection efficiency also increased. Then the effect of serum MBP on transfection with plasmid DNA/Man–liposome complex (1:2.5) was examined. To this end, plasmid DNA/Man–liposome complex was pre-incubated with serum MBP (0.01–10 μg) or BSA (10 μg). Transfection with the complex was inhibited by the addition of serum MBP in an MBP concentration-dependent manner; the expression decreased to about 10% of the control value at 10 μg MBP (Fig. 3). On the other hand, the addition of 10 μg BSA had no significant effect on transfection.

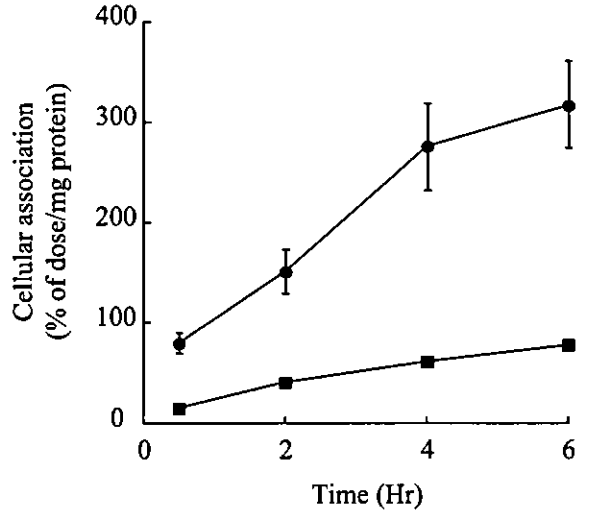

Fig. 4. The cellular association time-courses of [^{32}P] plasmid DNA (0.5 μg/ml)/Man–liposome complex (1:2.5) at 4 °C (■) or 37 °C (●) for 30 min, 2, 4 and 6 h by cultured elicited peritoneal macrophages. Each value represents the mean±S.D. of three wells.

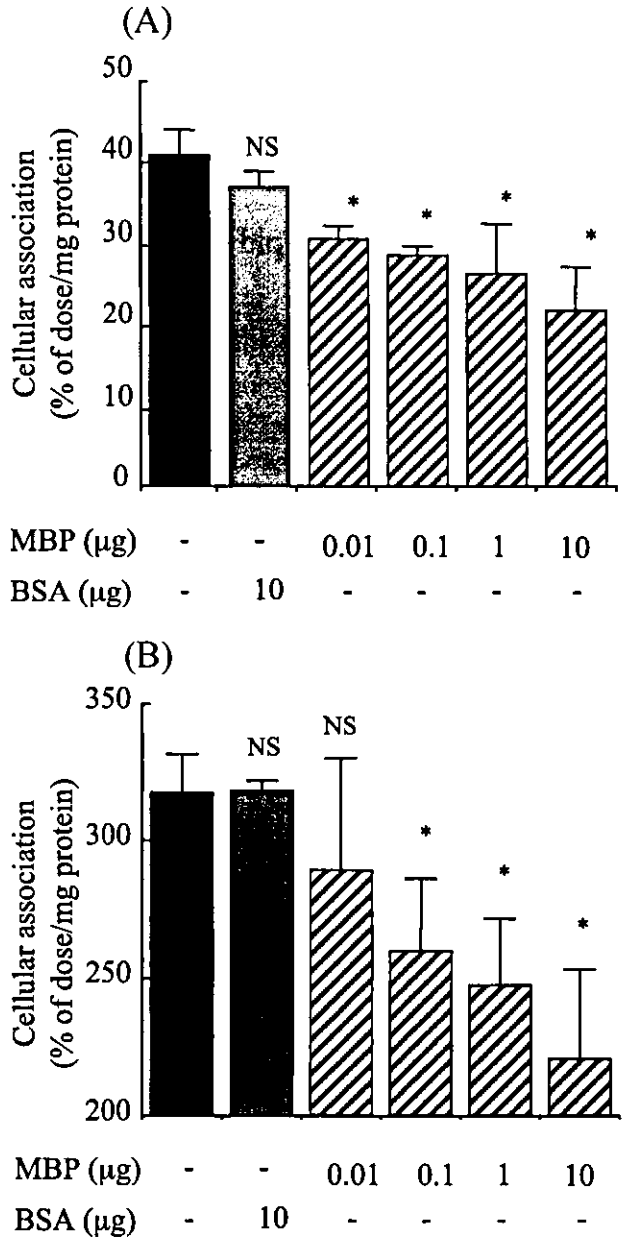

Fig. 5. The effect of serum MBP on the cellular association of [^{32}P] plasmid DNA/Man–liposome complex at 4 °C for 2 h (A) or 37 °C for 6 h (B) by cultured elicited peritoneal macrophages. [^{32}P] plasmid DNA (0.5 μg/ml) was complexed with Man–liposomes at a ratio of 1:2.5 (w/w) and then pre-incubated at room temperature for 10 min before starting the cellular uptake experiment, with and without serum MBP (0.1–10 μg/ml) or BSA (10 μg/ml). Each value represents the mean±S.D. of three wells. (*) Indicates $p < 0.05$; (NS) not significant.

3.3. Uptake by cultured macrophages

A possible inhibitory mechanism of the decreased transfection efficiency by serum MBP is the inhibition of the binding of plasmid DNA/Man–liposome complex to macrophages, through a shielding of mannose on the complex. Then, the cellular association of [^{32}P] plasmid DNA/Man–liposome complex (1:2.5) was examined. Fig. 4 shows the time-course of the amount of [^{32}P] plasmid DNA/Man–liposome complex associated with macrophages. At 4 °C, a significant and time-dependent cellular association of the complex was observed. Increasing the temperature to 37 °C increased the amount of the complex associated, suggesting its uptake by macrophages.

Serum MBP inhibited the association of the [^{32}P] plasmid DNA/Man–liposome complex with macrophages at both 4 °C (Fig. 5A) and 37 °C (Fig. 5B). Increasing the amount of serum MBP significantly decreased the amount of complex associated with macrophages, whereas BSA had no effect. The inhibitory effect of serum MBP was more marked at 37 °C (Fig. 5B), suggesting endocytotic internalization of the plasmid DNA/Man–liposome complex by macrophages at this temperature.

4. Discussion

Delivery of genetic material into cells can be achieved by a variety of methods. Among them, cationic liposomes have been demonstrated as efficient and safe carriers for plasmid DNA in animal experiments as well as in clinical trials [26,27]. Although the positive charge of the plasmid DNA/liposome complex is the driving force of the interaction with and internalization into target cells, the charge also increases the undesirable interaction of the complex with blood components and vascular endothelial cells before reaching the target cells after systemic administration [28]. In addition, a highly cationic complex is more cytotoxic than one that is less cationic [29].

A major drawback of liposome-mediated transfection is its limited transfection efficiency in the presence of serum [30,31]. Serum proteins mask the cationic charge of the plasmid DNA/cationic liposome complex and inhibit its interaction with cell membranes, resulting in a reduced cellular uptake. In addition, Li et al. [32] have demonstrated that serum components can directly inactivate the cationic complex by inducing its aggregation. A prolonged incubation of lipoplex in serum resulted in its disintegration and the release and degradation of incorporated plasmid DNA. Furthermore, we recently reported that the interaction of DOPE-containing lipoplex with erythrocytes induces aggregation and causes embolization in the lung capillaries resulting in the accumulation of lipoplex in the lung [33].

Receptor-mediated gene transfer is a promising approach to target cell-selective transgene expression in vivo. In previous studies, we and others have achieved mannose receptor-mediated in vivo gene transfer by using mannosylated poly(L-lysine) [12,34] or mannosylated cationic liposomes [14]. In such mannose receptor-mediated approaches, mannose moieties need to be presented to the mannose receptors of target cells, such as endothelial cells and macrophages. Although the cationic nature of plasmid DNA/liposome complexes is very important for their interaction with target cells, it could be an obstacle for cell-specific gene transfer based on the specific ligand recognition. Therefore, the charge should be carefully controlled for receptor-mediated gene transfer. In the present study, Man-C4-Chol, a cholesterol derivative with mannose, was used as the targeting molecule for macrophage mannose receptors. This molecule also has a positive charge to give it an affinity for negatively charged plasmid DNA. Therefore, increasing the Man–liposome:plasmid DNA ratio resulted in the formation of positively charged complexes. Although the transfection efficiency of the plasmid DNA/Man–liposome complex increased on increasing the Man–liposome ratio (Fig. 2), such enhancement is due to an increase in the positive charge of complex (Fig. 1), as observed in a previous study [14]. To avoid the effects of charge-mediated gene transfer, the electrical charge of the complex was kept negative (−42.2 mV) at a mixing ratio of 1:2.5.

In addition to the electrical charge, the particle size of DNA complexes is another important factor determining the in vivo fate of DNA. When the target is liver NPC, the DNA complex can directly interact with the cells without passing through the endothelial layer of the liver. However, to be taken up via receptor-mediated endocytotic processes, there should be a restriction on the particle size of the DNA complexes [35]. The results of the present study suggest that the DNA complexes with about 200–300 nm are small enough to access these target cells for in vitro gene transfection.

Serum MBP is a mannose-specific Ca^{2+}-dependent (C-type) lectin of the collectin family [36]. The carbohydrate specificity of MBP is similar to that of the macrophage mannose receptor: D-mannose, *N*-acetylglucosamine, and L-fucose [37]. Therefore, serum MBP might affect the gene transfer of plasmid DNA with mannosylated carriers. In our series of investigations, we examined the effects of MBP on the biodistribution of mannosylated carriers in mice. A pharmacokinetic analysis of the tissue disposition of mannosylated proteins revealed a retardation of their hepatic uptake due to complex formation with serum MBP [23]. Furthermore, the addition of serum MBP resulted in a reduced uptake of mannosylated proteins by peritoneal macrophages (unpublished data).

The in vitro transfection efficiency of the plasmid DNA/Man–liposome complex significantly decreased on increasing the amount of serum MBP added (Fig. 3). Then, the cellular association of the complex was also reduced. These results indicate that, as in the cases of mannosylated proteins, serum MBP interferes with the interaction of the