blood cells such as monocytes, macrophages and dendritic cells (DC) (Lewis and Andre, 1980, 1981; Thiele et al., 2003).

In this study, therefore, we focused on fetuin and tried to evaluate its possible involvement in the SRs-mediated hepatic uptake of NS-50 in rats.

2. Materials and methods

2.1. Materials

Poly inosinic acid (poly I), poly cytidylic acid (poly C) and bovine fetuin were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Iwai Chemical Company (Tokyo, Japan). Goat anti-rat/mouse class A scavenger receptor (SR-A) antibody (A-20), goat anti-rat villin antibody (C-19) and goat anti-rat/mouse fetuin antibody (F-20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals were of the finest reagent grade available.

2.2. Nanospheres

Monodispersed, non-ionized polystyrene nanospheres (NS-50) covalently linked with fluorescein isothiocyanate, 50 nm in diameter, were used as received (Polysciences, Warrington, PA, USA). For pre-coating of NS-50 with fetuin or BSA, the suspension of NS-50 was incubated with an initial concentration of 10 μg/ml fetuin or BSA (NS-50:protein = 5:1, w/w) at 37 °C for 5 min in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4). The amount of coated fetuin (234.8 ± 47.3 μg/mg NS-50) or BSA (198.9 ± 29.1 μg/mg NS-50) was constant for at least 1 h in KRB solution (pH 7.4) at 37 °C, except for the uptake experiments described in Fig. 4 where various amounts of fetuin are present in the medium. Zeta-potential was determined in phosphate buffered saline (PBS, pH 7.4) by an electrophoretic light scattering spectrometer (ELS-6000, Otsuka Electronics, Osaka, Japan) and obtained values were −21.8 ± 2.3, −26.6 ± 1.3 and −7.6 ± 1.6 mV for NS-50, NS-50 pre-coated with fetuin and NS-50 pre-coated with BSA, respectively.

2.3. Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25 °C and 55% of humidity, were allowed to free access to standard laboratory chow (Clea Japan, Tokyo) and water prior to the experiments. Rats weighing 220–240 g were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with "Interdisciplinary Principles and Guidelines of the Use of Animals in Research".

2.4. Western blot analysis

After nanospheres were incubated in KRB buffer containing rat serum (5%, v/v, pH 7.4) for 20 min at 37 °C, nanospheres were separated by ultra centrifugation using a Beckman Optima XL-90 (Beckman Instruments Inc., Palo Alto, CA, USA) at

40,000 × g for 15 min at 4 °C. After solubilizing with 10% SDS solution, the resulting protein solution was subjected to SDS-PAGE using 12.5% polyacrylamide gel (Ready Gel, Bio-Rad, Hercules, CA, USA) and blotted onto cellulose nitrate membranes (Advantec, Tokyo). For the detection of fetuin, goat anti-rat/mouse fetuin antibody (F-20) and the peroxidase-linked anti-goat IgG antibody (Santa Cruz Biotechnology Inc.) were used at 1:1000 or 1:5000 dilution in blocking buffer, respectively. The protein band was visualized with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. In vivo disposition experiments

In vivo disposition experiments were examined according to the method reported previously (Furumoto et al., 2004). In brief, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg). At 5 min after injection of poly I or poly C (10 mg/kg), NS-50-fetuin (12.5 mg/kg) was injected from a femoral vein. Blood samples (0.2 ml) were withdrawn from the jugular vein at fixed time periods. At 1 h after the intravenous injection of NS-50-fetuin, liver, spleen, lung and kidney were excised, rinsed with saline, and weighed. Blood concentrations of NS-50-fetuin (C_b) versus time curves were analyzed by Eq. (1) using the non-linear least-square regression program MULTI (Yamaoka et al., 1981):

$$C_b = Ae^{-\alpha t} + Be^{-\beta t} \tag{1}$$

The area under the blood concentration–time curve from 0 to time t (AUC_0^t) was calculated by Eq. (2):

$$AUC_0^t = \int_0^t C_b \, dt \tag{2}$$

Total body clearance (CL_{total}) and tissue uptake clearance (CL_{tissue}) were calculated by Eqs. (3) and (4), respectively:

$$CL_{total} = \frac{dose}{AUC_0^t} \quad (t = \infty) \tag{3}$$

$$CL_{tissue} = \frac{X_{tissue}^t}{AUC_0^t} \quad (t = 60\,min) \tag{4}$$

where X_{tissue}^t represents the amount of NS-50-fetuin in a tissue at time t.

2.6. Liver perfusion experiments

Liver perfusion was carried out with KRB buffer (pH 7.4) following the recirculating perfusion procedure as reported previously (Furumoto et al., 2002). Nanospheres, an initial concentration of 50 μg/ml, were recirculated in the isolated liver preparation at a flow rate of 13.0 ml/min. In our preliminary experiments, the viability of the liver was checked by the glutamic–oxaloacetic transaminase (GOT) activity in the outflow, confirming that the viability of liver was maintained throughout the perfusion study. The perfusion was performed for 50 min and the perfusate concentration of nanospheres in

the reservoir was fluorometrically determined (excitation maximum 458 nm, emission maximum 540 nm) (RF-540 Fluorescent Spectrometer, Shimadzu, Kyoto, Japan) until 50 min. Hepatic clearance (CL_h) was calculated according to the following equation:

$$CL_h = \frac{X^t_{liver}}{AUC^t_0} \quad (t = 50\,min) \tag{5}$$

where X^t_{liver} and AUC^t_0 mean the amount of nanospheres in the liver at time t and the area under the concentration of nanospheres in the perfusate versus time curve from 0 to time t, respectively. X^t_{liver} was estimated by subtracting the remaining amount of nanospheres in the reservoir from the total amount of dose. AUC^t_0 was calculated according to the trapezoidal rule.

Pre-treatment of the perfused liver with trypsin was performed by following the method reported by Furumoto et al. (2002). The effect of poly I, an inhibitor of SRs (Peiser and Gordon, 2001), or poly C, a negative control (Peiser and Gordon, 2001), was examined according to the method previously reported (Furumoto et al., 2002).

2.7. Cell isolation and culture

Rat hepatocytes and Kupffer cells were isolated after collagenase perfusion following the conventional procedures (Knook and Sleyster, 1976). Then, the freshly isolated hepatocytes were cultured in collagen-coated 24-well plates (ASAHI Techno Glass, Tokyo) in Williams E medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), insulin (10^{-7} M), dexamethazone (10^{-7} M), penicillin G (100 U/ml), streptomycin (100 μg/ml) and gentamycin (50 μg/ml). The isolated hepatocytes were maintained in a humidified 5% CO_2/95% air atmosphere at 37 °C. The medium was refreshed after 6 h and thereafter every 24 h. Experiments with isolated hepatocytes were performed on the third day after isolation of the cells. Kupffer cells were grown in 24-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml). After the incubation for 2 h at 37 °C and 5% CO_2/95% air atmosphere, the medium was refreshed and then cultured at 37 °C and 5% CO_2/95% air atmosphere for 48 h before experiments.

2.8. Uptake experiments

After washing cultured rat hepatocytes and Kupffer cells with FBS-free medium, uptake studies were started by adding nanospheres (100 μg/ml) suspended with phosphate buffered saline (PBS) (pH 7.4) to the cells. In the case of trypsin pre-treatment study, Kupffer cells and hepatocytes were incubated for 30 min at 37 °C with PBS containing 1 and 10 μg/ml trypsin, respectively, before the addition of nanospheres. In the case of poly I- or anti-SR-A antibody-treatment study, poly I (50 μg/ml), anti-SR-A antibody (1 μg/ml) or anti-villin antibody (1 μg/ml) as a negative control was simultaneously added to Kupffer cells together with nanospheres. The incubation with nanospheres for the uptake was performed at 37 °C for 6 or 1 h for hepatocytes

or Kupffer cells, respectively. Then, the cells were washed with PBS.

2.9. Determination of blood, tissue and cell concentrations of particles

Concentrations of NS-50-fetuin were determined by following the method described in our previous report (Furumoto et al., 2002).

In the case of tissue and blood, tissue or blood samples were homogenized with the equivalent volume of distilled water and then frozen, thawed and mechanically shaken. This procedure was repeated three times to completely solubilize cells. In the case of the measuring the amount of NS-50-fetuin in hepatocytes and Kupffer cells, cells washed with PBS were first solubilized with 10% SDS solution for 12 h.

Then, solubilized samples were lyophilized over 36 h, and the resulting dried samples were re-suspended in accurately measured volume of chloroform and mechanically shaken for 18 h. After the resulting suspension was filtered through a 0.22 μm solvent-resistant membrane filter (Millex HV Milopore, Bedford, MA, USA), the fluorescence of the filtrate (excitation wavelength 458 nm, emission wavelength 540 nm) was fluorometrically determined (RF-540 Fluorescent Spectrometer). The extraction of nanospheres into chloroform from the cells was almost complete in the presence of biological substances, but the standard curve was prepared to more precisely estimate their amounts in the biological specimen. The squared correlation coefficient for each standard curve was over 0.995.

2.10. Statistical analysis

Results are expressed as the mean ± S.D. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance was evaluated by using Student's t-test or Dunnett's test for the single or multiple comparisons of experimental groups, respectively.

3. Results

To confirm the adsorption of fetuin on the surface of NS-50 in the blood circulation, Western blot analysis was performed for proteins adsorbed on NS-50 after incubation with rat serum. After incubation with rat serum, a variety of serum proteins adsorbed onto the surface of NS-50 (Fig. 1A), but fetuin was certainly one of the serum proteins associated on the surface of NS-50 (Fig. 1B).

To investigate the role of surface associated fetuin on the hepatic disposition characteristics of NS-50, we prepared NS-50 pre-coated with fetuin (NS-50-fetuin) and evaluated whether scavenger receptor-mediated uptake is involved in the hepatic disposition of NS-50-fetuin in vivo. Fig. 2 shows the effect of pre-injection of poly inosinic acid (poly I), an inhibitor of SRs, or poly cytidylic acid (poly C), used as a negative control, on the in vivo disposition of NS-50-fetuin after intravenous administration into rats. Pharmacokinetic parameters are summarized in Table 1. Pre-injection of poly I significantly increased the
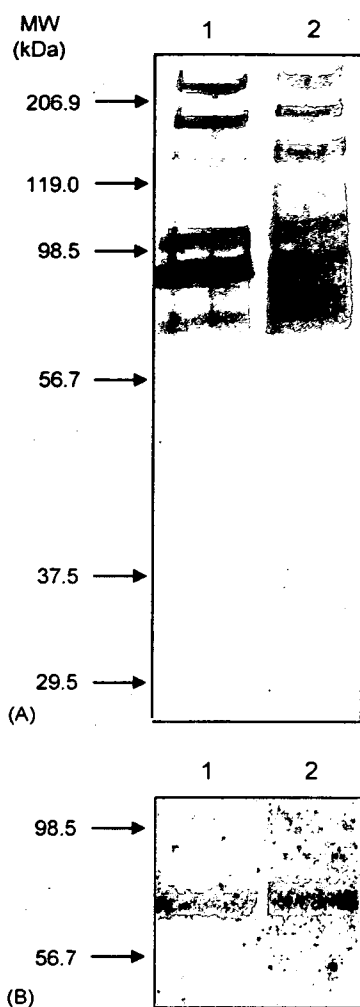
Fig. 1. Silver-stained SDS-PAGE of rat serum proteins adsorbed on the surface of NS-50 (A) and identification of fetuin by Western blot analysis (B). Lane 1, rat serum (loaded proteins A, 1 μg; B, 3 μg): lane 2, NS-50 pre-incubated with rat serum (A, 1 μg; B, 10 μg).

Table 1

Pharmacokinetic parameters of NS-50-fetuin after intravenous administration with pre-injection of poly I or poly C

Parameter	NS-50-fetuin	+ Poly I	+ Poly C
AUC_0^∞ (μg/ml min)	964 (271)	5346[**] (1325)	1656 (270)
CL_{total} (ml/min)	3.04 (0.52)	0.49[**] (0.12)	1.54[**] (0.26)
V_d (ml)	26.0 (3.56)	16.6 (2.00)	23.0 (6.63)
CL_{liver} (ml/min)	0.81 (0.14)	0.24[**] (0.04)	0.76 (0.08)
CL_{spleen} (ml/min)	0.042 (0.012)	0.056 (0.008)	0.021[*] (0.002)
CL_{lung} (ml/min)	0.020 (0.030)	0.002 (0.002)	0.002 (0.004)
CL_{kidney} (ml/min)	0.040 (0.056)	0.000 (0.000)	0.001 (0.001)

Results are expressed as the mean with S.D. values in parentheses ($n = 3$–6). $^*p < 0.05$; $^{**}p < 0.01$, compared with NS-50-fetuin.

AUC value and decreased the hepatic clearance value of NS-50-fetuin. This was not the case for the pre-injection of poly C where both AUC and hepatic clearance did not significantly change. These results indicate that SRs would be involved in the hepatic disposition of NS-50-fetuin in vivo. However, it is difficult to exclude out the possibility that other serum proteins associated on the NS-50-fetuin are involved in SRs-mediated hepatic uptake in the in vivo situation. We, therefore, performed liver perfusion experiments to more directly assess the possible involvement of SRs-mediated endocytosis in the hepatic uptake of NS-50-fetuin (Fig. 3). The hepatic clearance of NS-50-fetuin significantly reduced to 54% of the control by pre-treatment with poly I, but not with poly C. This was not the case for NS-50 or NS-50 pre-coated with BSA (NS-50-BSA) where poly I did not significantly influence their hepatic disposition. In addition, the pre-treatment of the liver with trypsin significantly reduced the hepatic clearance of NS-50-fetuin to 67% of the control, supporting the hepatic disposition of NS-50-fetuin via a receptor-mediated endocytosis.
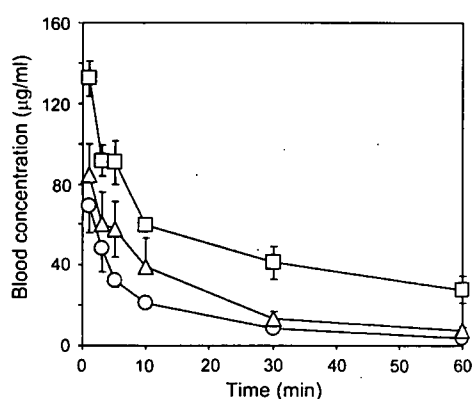

Fig. 2. Blood concentration–time profile of NS-50-fetuin after intravenous administration into rats at a dose of 12.5 mg/kg with or without pre-injection of poly I or poly C. Poly I or poly C was pre-injected at a dose of 10 mg/kg at 5 min before the injection of NS-50-fetuin. Results are expressed as the mean with a bar showing the S.D. ($n = 3$–6). Keys: (○) control; (□) pre-injection of poly I; (△) pre-injection of poly C.
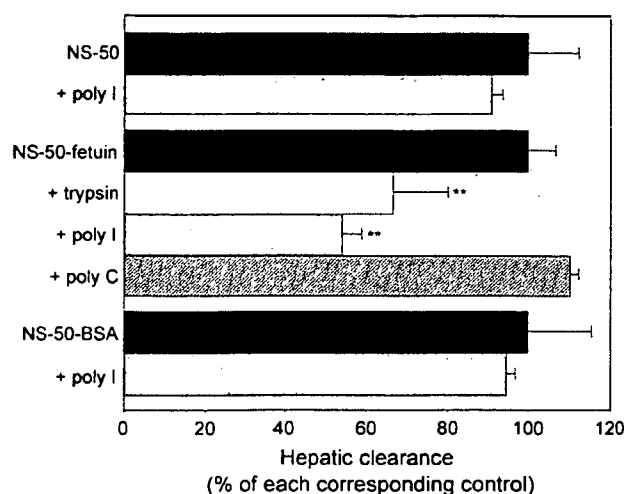

Fig. 3. Involvement of SRs in the hepatic uptake of NS-50-fetuin in the liver perfusion experiments. The prepared isolated liver was perfused with KRB buffer containing 25 μg/ml poly I or poly C before starting the perfusion of NS-50 or NS-50 pre-coated with fetuin or BSA. Results are expressed as the mean with a bar showing the S.D. ($n = 3$). $^{**}p < 0.01$, compared with corresponding control. The values of hepatic clearance for control were 5.77 ± 0.71, 2.15 ± 0.14 and 2.61 ± 0.41 ml/min for NS-50, NS-50-fetuin and NS-50-BSA, respectively.
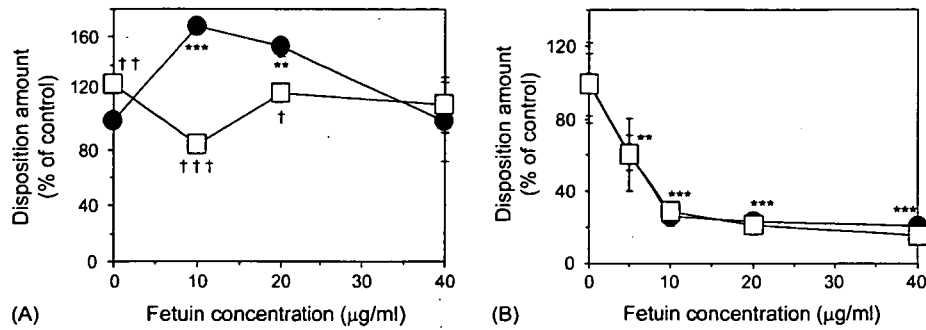
Fig. 4. Effect of trypsin-treatment of cell surface on the disposition of NS-50 to Kupffer cells (A) and hepatocytes (B) in the presence of various concentrations of fetuin. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were added to the cells. Before the addition of nanospheres, cells were incubated for 30 min at 37 °C with PBS alone (●) or PBS containing trypsin (□). Results are expressed as the mean with a bar showing the S.D. ($n = 3$–4). $**p < 0.01$; $***p < 0.001$, compared with each corresponding control (in the absence of fetuin). $\dagger p < 0.05$; $\dagger\dagger p < 0.01$; $\dagger\dagger\dagger p < 0.001$, compared with each corresponding control without trypsin treatment. Disposition amount for control was 131.5 ± 11.3 or 52.3 ± 11.5 μg particles/mg protein for Kupffer cells or hepatocytes, respectively.

We also performed the in vitro uptake study using primary cultured rat Kupffer cells and hepatocytes (Fig. 4). As shown in Fig. 4B, the disposition of NS-50 to hepatocytes was decreased in the presence of fetuin in the concentration-dependent manner. Pre-treatment of hepatocytes with trypsin did not affect the disposition amount of NS-50. On the contrary, the disposition of NS-50 to Kupffer cells was enhanced in the presence of fetuin (∼10 μg/ml) and this enhancing effect of fetuin decreased as the concentration of fetuin in the medium increased further (Fig. 4A). In addition, pre-treatment of Kupffer cells with trypsin tended to make these cells unresponsive to fetuin and the uptake amount of NS-50 was almost constant except for one experimental condition where the concentration of fetuin in the medium was 10 μg/ml. The presence of poly I in the medium also significantly reduced the uptake of NS-50-fetuin by Kupffer cells to 42% of the control, but not the uptake of NS-50-BSA (Fig. 5).

Furthermore, focusing on SR-A, we investigated whether SR-A was substantially responsible for the SRs-mediated uptake of NS-50-fetuin by Kupffer cells (Fig. 6). The presence of anti-

SR-A antibody in the incubation medium significantly reduced the uptake of NS-50-fetuin to 54% of the control. To confirm whether this phenomenon was specific for anti-SR-A antibody, we investigated the effect of anti-villin antibody, an irrelevant IgG, used as a negative control, on the uptake of NS-50-fetuin by Kupffer cells. It was found that anti-villin antibody did not influence the uptake of NS-50-fetuin by Kupffer cells. In contrast, the uptake of NS-50-BSA by Kupffer cells was not significantly influenced by the presence of anti-SR-A antibody.

4. Discussion

The hepatic uptake of particles has been believed to be dependent on various factors such as particle size, surface hydrophobicity and surface charge. However, we have clearly demonstrated that some serum proteins adsorbed on particles play an important role in their hepatic disposition by utilizing several kinds of polystyrene nanospheres (Ogawara et al., 1999; Furumoto et al., 2002). In the case of negatively charged par-
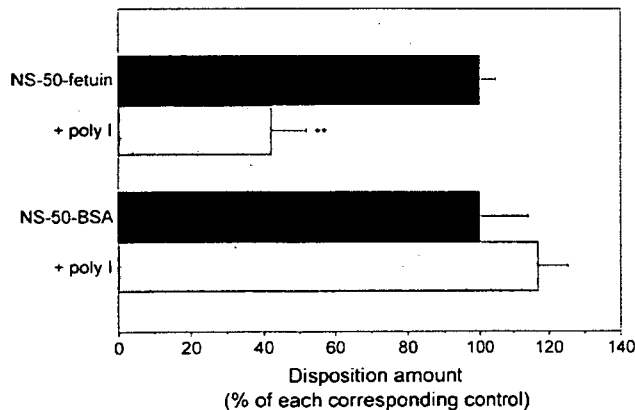

Fig. 5. Involvement of SRs in the uptake of NS-50-fetuin by Kupffer cells. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were added to the cells. Poly I (50 μg/ml) was added to the cells together with nanospheres. Results are expressed as the mean with a bar showing the S.D. ($n = 3$–5). $**p < 0.01$, compared with corresponding control. Disposition amount for control was 117.6 ± 8.3 or 66.8 ± 10.4 μg particles/mg protein for NS-50-fetuin or NS-50-BSA, respectively.
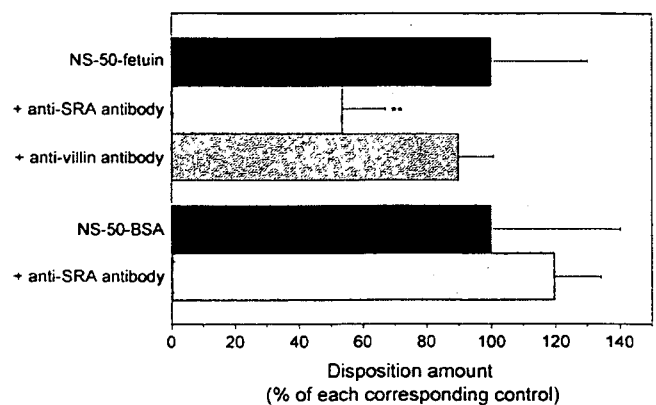

Fig. 6. Involvement of SR-A in the SRs-mediated uptake by Kupffer cells. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were simultaneously added to the cells with 1 μg/ml anti-SR-A antibody or anti-villin antibody. The final concentration of antibody was 0.5 μg/ml. Results are expressed as the mean with a bar showing the S.D. ($n = 4$–9). $**p < 0.01$, compared with corresponding control. Disposition amount for control was 159.2 ± 26.5 or 106.1 ± 20.3 μg particles/mg protein for NS-50-fetuin or NS-50-BSA, respectively.

ticles, it was reported that some blood components such as α_2-macroglobulin or apolipoprotein H were associated on the surface of negatively charged liposomes and were responsible for their rapid elimination from the systemic circulation after intravenous administration (Chonn et al., 1992, 1995). However, to date there is no report that describes substantial involvement of blood components in the SRs-mediated uptake of negatively charged nanoparticles by phagocytes.

Fetuin, an acidic glycoprotein in serum, was reported to strongly promote the phagocytosis of several substances through the terminal oligosaccharide residues highly rich in sialic acid, although it is still unknown whether a specific receptor for fetuin is responsible for the enhanced phagocytosis (Green et al., 1988; Wang et al., 1998; Jersmann et al., 2003). Therefore, in the present study, we focused on fetuin and tried to investigate the possible involvement of fetuin in SR-mediated hepatic uptake of NS-50 which had surface negative charge.

Hepatic clearance of NS-50-fetuin was significantly decreased by the pre-injection of poly I in the in vivo study (Table 1). Similar results were obtained with the pre-treatment of the liver with poly I in the liver perfusion experiments (Fig. 3). As poly I has been widely used as an inhibitor of SRs to confirm the possible involvement of SRs-mediated phagocytosis in the in vivo (Jansen et al., 1991; Terpstra and Berkel, 2000) and/or in the liver perfusion studies (Jansen et al., 1991), the present result strongly indicates that fetuin coated on the surface of NS-50 endowed NS-50 with the nature that could be recognized by SRs in the in vivo situation. Taken the results of Western blot analysis together (Fig. 1), these results would account for our previous finding that the hepatic uptake of NS-50 in the presence of serum was significantly inhibited by poly I but was not in the absence of serum (Furumoto et al., 2004).

Certain amount of fetuin significantly enhanced the disposition of NS-50 to Kupffer cells (Fig. 4A), but the disposition of NS-50 to hepatocytes decreased as the concentration of fetuin in the medium increased (Fig. 4B). The pre-treatment with trypsin did affect the disposition of NS-50 only in the case of Kupffer cells (Fig. 4A and B). Furthermore, taken that the disposition amount of NS-50-fetuin to the whole liver was significantly reduced by the trypsin treatment in the liver perfusion experiments (Fig. 3) where fetuin was applied in the perfusate at 10 μg/ml, Kupffer cells are mainly responsible for the hepatic disposition of NS-50 via a mechanism where fetuin is involved, and hepatocytes would lack in receptors contributing to the uptake of NS-50. Furthermore, as the hydrophobicity of NS-50 is decreased by the adsorption of serum proteins (Ogawara et al., 2001), the adsorption of fetuin would lower the hydrophobicity of NS-50, resulting in the decrease in the uptake of NS-50 by hepatocytes via hydrophobic interaction. The difference in the disposition characteristics of NS-50 between Kupffer cells and hepatocytes would be mainly attributed to the lack of the responsible SRs on hepatocytes which can recognize fetuin associated on the surface of NS-50. Although 40 μg/ml fetuin did not apparently change the disposition NS-50 to Kupffer cells (Fig. 4A), it might be because unbound fetuin competitively inhibited the receptor-mediated recognition of NS-50 associated with fetuin by Kupffer cells. In our preliminary experiment,

almost all the fetuin added into the medium was associated on the surface of NS-50 at 10 μg/ml, while 28% and 47% of fetuin was present as unbound at the concentration of 20 and 40 μg/ml, respectively (data not shown). Therefore, the increase in unbound fetuin in the medium would decrease the disposition amount of NS-50 to Kupffer cells (Fig. 4A). On the contrary, the in vivo study showed that hepatic clearance of NS-50-fetuin was drastically decreased by the pre-injection of poly I in spite of the presence of abundant amount of unbound or soluble form of fetuin in the serum (Table 1), suggesting that these unbound fetuin would not inhibit the hepatic uptake of NS-50-fetuin via SRs. It could be speculated that some components in serum might form the complex with unbound fetuin and be masking the recognition by phagocytes in the in vivo situation. The detailed mode of the fetuin recognition by phagocytes remains to be elucidated and will be the subject of our further study.

Similar to the liver perfusion experiments, the uptake of NS-50-fetuin to Kupffer cells was significantly inhibited with poly I (Fig. 5), suggesting that SRs-mediated endocytosis would be involved in the disposition of NS-50-fetuin to Kupffer cells. SRs are categorized into six groups, among which SR-A, SR-C, SR-D, SR-E and SR-F are known to be poly I-sensitive ones (Peiser and Gordon, 2001). As SR-A is mostly expressed on the surface of macrophages (Moghimi and Hunter, 2001), its possible involvement in the SRs-mediated uptake of NS-50-fetuin was investigated by utilizing anti-SR-A antibody (Fig. 6). The results indicated that SR-A was indeed responsible for the uptake of NS-50-fetuin by Kupffer cells. Possible involvement of other SRs in the uptake is underway and will be the subject of our further study.

In conclusion, we clearly demonstrated that fetuin, one of serum proteins associated on the surface of NS-50, played a substantial role in its uptake by Kupffer cells via SRs by utilizing NS-50-fetuin. Among SRs, at least SR-A would be responsible for the uptake. This is the first report demonstrating that at least class A scavenger receptors are recognizing fetuin associated onto the surface of the particles. Although it is not clarified yet whether fetuin is responsible for the uptake of other negatively charged particles, the present results will give an insight into the mechanisms behind the hepatic handling of the negatively charged particulate drug carrier.

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Effect of coupling of albumin onto surface of PEG liposome on its in vivo disposition

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Abstract

To evaluate the effect of coupling of albumin onto the surface of poly(ethylene glycol)-modified liposome (PEG liposome) on the in vivo disposition of liposome, pharmacokinetics and tissue distribution were examined after intravenous administration of rat serum albumin-modified PEG (RSA/PEG) liposome into rats. RSA/PEG liposome showed longer blood-circulating property than PEG liposome and the hepatic clearance for RSA/PEG liposome was significantly smaller than that for PEG liposome. Single-pass liver perfusion experiments also showed that the hepatic disposition of RSA/PEG liposome was much less than that of PEG liposome and that pre-treatment of liver with trypsin did not significantly reduce the hepatic disposition of RSA/PEG liposome, suggesting that RSA/PEG liposome could avoid the hepatic uptake via the receptor-mediated endocytosis. To unravel the mechanism behind the less affinity of RSA/PEG liposome to the liver, serum proteins associated on their surface were quantitatively and qualitatively assessed. The results showed that the coupling of albumin onto PEG liposome significantly reduced the total amount of serum proteins associated onto the surface, and SDS-PAGE revealed that the decrease in the association with liposomes for several serum proteins, which might have opsonic activity. From these findings, introduction of serum albumin onto PEG liposome could be useful to develop a new nanoparticulate formulation with a better pharmacokinetic property.
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Keywords: Liposomes; Poly(ethylene glycol); Rat serum albumin; Hepatic disposition; Serum opsonins

1. Introduction

Although liposomes are good candidates for efficient drug carriers, the rapid clearance by the reticuloendothelial system (RES) limits their application as drug carriers to other tissues and/or cells. Early studies demonstrated that several physico-chemical properties of liposomes such as size, lipid composition and surface charge, influence their pharmacokinetics after systemic administration (Allen et al., 1991a; Liu et al., 1995; Harashima et al., 1996; Li and Mitra, 1996). The serum proteins associated onto the surface of systematically administered liposomes were also suggested to influence their in vivo fate (Harashima et al., 1998; Semple et al., 1998). Among them, immunoglobulin G, complement C3 and fibronectin are well

known to function as opsonins to promote the hepatic disposition of liposomes (Hsu and Juliano, 1982; Derksen et al., 1987; Wassef and Alving, 1993). In addition, it was shown that some other serum proteins such as apolipoproteins and β_2-glycoprotein I, can function as opsonins depending on the lipid composition of the liposomes (Ivanov et al., 1985; Chonn et al., 1995). On the other hand, the existences of serum components that inhibit phagocytosis of pathogens or particles, dysopsonins, were also indicated (Park and Huang, 1993). Although dysopsonins for microorganisms such as immunoglobulin A and α_1-acid glycoprotein were already reported (van Oss et al., 1974; Absolom, 1986), there is no identified serum components with dysopsonic activity for liposomes so far.

Albumin is the most abundant protein in serum, of which the concentration is approximately 5% (w/v). The functions of albumin include the transport of both endogenous and exogenous ligands such as hormones and fatty acids to tissues, the maintenance of vascular integrity and transvascular oncotic pressure

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gradient (Peters, 1975). Although albumin is also known to be one of the most abundant proteins associated with both neutral and charged liposomes (Chonn et al., 1991; Gabizon and Papahadjopoulos, 1992; Du et al., 1997), there is little information on the substantial role of albumin in the in vivo behavior of liposomes after systemic administration (Torchilin et al., 1980).

In our previous study, the pre-coating of the surface of polystyrene nanospheres (50 nm in diameter) with albumin significantly decreased their hepatic disposition, especially the uptake by Kupffer cells, which resulted in their longer blood circulation time. The results suggested that albumin might provide dysopsonin-like activity by inhibiting the subsequent association of opsonins on the surface of polystyrene nanospheres (Ogawara et al., 2004). In the present study, therefore, we investigated the effect of coupling of albumin onto the surface of PEG-modified liposomes on their in vivo disposition after intravenous administration in rats.

2. Materials and methods

2.1. Chemicals

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and rat serum albumin (RSA) were purchased from Sigma (St. Louis, MO, USA). N-Hydroxysuccinimide (NHS) was obtained from Pierce (Rockford, IL, USA). Egg yolk phosphatidylcholine (EPC), cholesterol (Chol) and N-glutaryl phosphatidylethanolamine (NGPE) were purchased from ASAHI KASEI Chemicals Industry Inc. (Tokyo, Japan), Wako Pure Chemical Industry Inc. (Osaka, Japan) and Avanti Polar Lipids Inc. (Alabaster, AL, USA), respectively. Distearoyl phosphatidylethanolamine-N-[methoxy poly(ethylene glycol)-2000] (PEG-DSPE) was obtained from NOF Inc. (Tokyo). [³H]Cholesteryl hexadecyl ether ([³H]CHE) was purchased from Perkin-Elmer Life Science Inc. (Boston, MA, USA). All other chemicals were of the finest grade available.

2.2. Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25 °C and 55% of humidity, were allowed to free access to standard laboratory chow (Clea Japan, Tokyo) and water prior to the experiments. Rats weighing 220–240 g were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with "Interdisciplinary Principles and Guidelines of the Use of Animals in Research".

2.3. Liposome preparation

Liposomes were prepared according to the following procedures. EPC, Chol, NGPE and PEG-DSPE from stock solution were mixed at EPC:Chol:NGPE = 60:30:10 for non-PEG liposome or EPC:Chol:NGPE:PEG-DSPE = 55:30:10:5 for PEG liposomes with trace amount of [³H]CHE. Then, the lipid mixture was dried under reduced pressure and the resultant dried lipid was hydrated in phosphate buffer (pH 5.0) under mechani-

cal agitation. Then, the resulting multilamellar preparations were sized by repeated extrusion (15 times) through polycarbonate membrane filters (Millipore, Bedford, MA, USA) with pore size of 200 nm.

2.4. Preparation of RSA-modified PEG (RSA/PEG) liposomes

RSA was conjugated to liposomes by using carbodiimide as reported previously (Bogdanov et al., 1988; Holmberg et al., 1989; Nakajima and Ikada, 1995). To activate the carboxyl group of NGPE incorporated in liposomes, EDC (10 μmol/μmol total lipid (TL)) and NHS (10 μmol/μmol TL) were added to liposome suspension, and the mixture was further incubated for 15 min at room temperature. After 2-mercaptoethanol (5 mmol/μmol TL) was added, the mixture was applied to Sephadex G-25 (Sigma) column equilibrated with phosphate buffer (pH 5.0) and the liposome fractions were collected. RSA (0.01 μmol/μmol TL) was added to the liposome suspension and incubated for 18 h at 4 °C. Then, the liposome fractions were separated from unconjugated RSA by Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) column equilibrated with phosphate buffered saline (PBS, pH 7.4). Particle size of liposome was determined by dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Osaka), and particle sizes for non-PEG, PEG or RSA-modified PEG (RSA/PEG) liposomes in PBS (pH 7.4) were 208 ± 10, 215 ± 12 or 233 ± 15 nm, respectively. The particle size of each liposome in PBS containing rat serum (5%, v/v) was constant for up to 24 h. Quantity and quality of coupled RSA onto liposomes were evaluated by the method reported by Lowry et al. (1951) and SDS-PAGE, respectively, as described below. The amount of RSA conjugated onto the surface of PEG liposome was 19.3 ± 4.1 μg/μmol TL. SDS-PAGE under non-reducing condition revealed that RSA coupled onto the surface of liposome was exclusively in a monomeric form.

2.5. In vivo disposition experiments

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg). Liposomes were injected from femoral vein at a dose of 10 μmol TL/kg. Body temperature of rats was kept at 37 °C using a heat lamp during the experiment. Blood samples were withdrawn from the jugular vein at fixed time points, and then centrifuged immediately at 4000 × g. The obtained plasma was collected (100 μl) and scintillation medium (Clear-Sol II, Nacalai Tesque, Kyoto) was added.

For the tissue distribution study, organs (liver, spleen lung, kidney, heart, cerebrum, cerebellum, eyeball, muscle and lymph) were excised at 1 h after intravenous injection, and rinsed with PBS, and weighed. To solubilize organs, Soluene-350 (Packard Instrument Inc., Meriden, CT, USA) was added and incubated for 2 h at 50 °C before solubilized solution was neutralized by HCl. Scintillation medium was added to samples, and radioactivity was determined in a liquid scintillation counter (TRI-CARB® 2260XL, Packard Instrument Inc.).

Plasma concentrations of liposomes (C_p) versus time curves were analyzed by Eq. (1) using the non-linear least-squares regression program MULTI (Yamaoka et al., 1981).

$$C_p = A\, e^{-\alpha t} + B\, e^{-\beta t} \tag{1}$$

The area under the plasma concentration–time curve (AUC) was calculated by Eq. (2).

$$AUC_0^t = \int_0^t C_p\, dt \tag{2}$$

Tissue clearance (CL_{tissue}) were calculated by Eq. (3).

$$CL_{tissue} = \frac{X_{tissue}^t}{AUC_0^t} \quad (t = 1\,h) \tag{3}$$

where AUC_0^t means AUC value from 0 to time t, and X_{tissue}^t represents the amount of liposomes in a tissue at time t.

2.6. Rat liver single-pass constant infusion experiments

Liver perfusion experiments were performed under single-pass conditions at a flow rate of 13 ml/min. Perfused liver was prepared according to the method reported previously (Ogawara et al., 1999a). After a stabilization period of 13 min, liposomes were continuously infused from the portal vein for 20 min at the concentration of 0.5 nmol TL/ml in the presence of serum 1% (v/v). After a 5-min wash with Krebs–Ringer bicarbonate (KRB) buffer, the liver was excised and weighed, and the accumulated amount of liposomes in the liver was evaluated by measuring the radioactivity in the liver as described above. The serum was prepared just before use as follows: rat whole blood was collected from the carotid artery and allowed to clot at room temperature for 20 min, then centrifuged at 1500 × g for 20 min at 4 °C and the supernatant obtained was used.

2.7. Pre-treatment of liver with trypsin

Pre-treatment of the perfused liver with trypsin was conducted as reported previously (Ogawara et al., 1999b). In brief, after 5 min stabilization period of the perfused liver using KRB buffer as a perfusate, the liver was then perfused with KRB buffer containing trypsin (10 μg/ml) for 10 min. To wash the remaining trypsin in the vascular compartment, the liver was perfused with KRB buffer for another 5 min, then liposomes were perfused. It was already evidenced that the treatment with trypsin described above did affect only the receptor-mediated uptake of particles (Ogawara et al., 1999b).

2.8. Determination of amount of serum proteins associated with liposomes

Liposomes (2.5 μmol TL/ml) were incubated in rat serum (liposomes:serum = 1:1 v/v) for 20 min at 37 °C. Separation of liposomes from bulk serum proteins was achieved by Sepharose CL-4B gel filtration. Fractions of liposomes were collected, and the amount of serum proteins associated on the surface of liposomes were quantified by Lowry's method (Lowry et al., 1951)

and the amount of liposomes were quantified by measuring the radioactivity. The amount of serum proteins associated with RSA/PEG liposome was calculated by subtracting the amount of RSA coupled with liposomes from total protein amount measured.

2.9. SDS-polyacrylamide gel electrophoretic analysis of serum proteins associated with liposomes

Analysis of serum proteins associated with liposomes was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini Protean-II electrophoretic apparatus (Bio-Rad, Hercules, CA, USA) on 12.5% polyacrylamide gel (Ready Gel J, Bio-Rad). Prestained SDS-PAGE molecular weight standards (Bio-Rad) were used to estimate the molecular weights of the associated proteins. For the relative comparison of the proteins associated on the surface of different liposome preparations, the same amount of protein (3 μg) was loaded onto the gel. The detection of proteins was performed by a silver-stain procedure by using a silver-stain kit (Daiichi Pure Chemicals, Tokyo).

2.10. Statistical analysis

Statistical significance was evaluated by using Student's t-test or Dunnet's test for the single or multiple comparisons of experimental groups, respectively.

3. Results

The in vivo disposition of conventional liposome (non-PEG liposome), PEG liposome and RSA-modified PEG liposome (RSA/PEG liposome) was evaluated after the intravenous injection into rats (Fig. 1 and Table 1). The inclusion of PEG onto the surface of liposome significantly increased the AUC value about three times of conventional liposome. It was also revealed that the AUC value for RSA/PEG liposome was slightly larger than that of PEG liposome. Distribution vol-
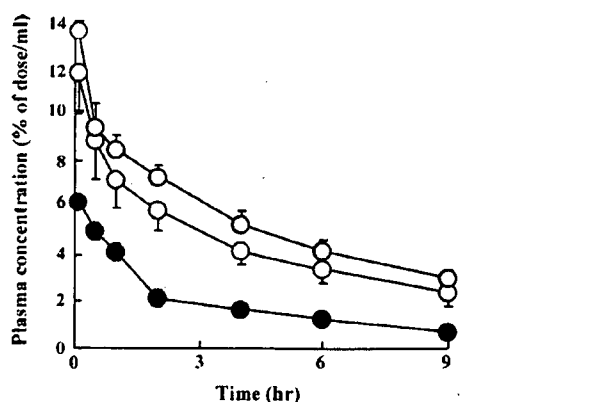

Fig. 1. Plasma concentration of conventional (non-PEG), PEG and RSA/PEG liposomes after intravenous injection into rats. Results are expressed as the mean with the bar showing the S.D. of three experiments. Keys: (●) non-PEG liposome; (○) PEG liposome; (⊘) RSA/PEG liposome.

Table 1

Pharmacokinetic parameters of conventional, PEG and RSA/PEG liposomes after intravenous injection into rats

Parameter (unit)	Conventional liposome	PEG liposome	PEG/RSA liposome
AUC (% of dose/ml h)	21.9 ± 1.3	63.9 ± 10.5*	84.9 ± 13.5*
CL_{total} (ml/h)	4.6 ± 0.3	1.6 ± 0.2***	1.2 ± 0.2***
V_d (ml)	14.8 ± 0.5	8.1 ± 1.1**	6.8 ± 0.3**
k_{el} (h^{-1})	0.31 ± 0.02	0.20 ± 0.01**	0.17 ± 0.03**

Results are expressed as the mean ± S.D. of three experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, compared with conventional liposome.

ume (V_d) and elimination rate constant (k_{el}) for PEG liposome significantly decreased compared with conventional liposome. RSA-modification tended to decrease both parameters further, although not significantly. Tissue distributions of these liposomes were also studied at 1 h after intravenous injection and it was found that liposomes mainly distributed to the liver and the spleen, and that the distribution of liposomes into other organs/tissues was negligible (less than 5% of dose). Tissue clearance calculated by Eq. (3) showed that PEG liposome had significantly smaller values both for the liver and the spleen than non-PEG liposome (Fig. 2). RSA modification further and significantly decreased the hepatic clearance of PEG liposome, but this was not the case for the spleen where both PEG liposome and RSA/PEG liposome had the similar clearances (Fig. 2).

Then, to elucidate the mechanisms behind the hepatic disposition of PEG liposome and RSA/PEG liposome, the effect of trypsin treatment on the hepatic disposition of these liposomes was investigated in the rat liver single-pass perfusion study utilizing the perfusate containing 1% serum (v/v) (Fig. 3). The hepatic accumulation of RSA/PEG liposome (2.1 ± 0.3 nmol total lipid (TL)) was found to be significantly less than that of PEG liposome (13.1 ± 0.9 nmol TL), suggesting that RSA/PEG

liposome has less affinity to the liver. Pre-treatment of the liver with trypsin drastically decreased the hepatic disposition of PEG liposome to 10% of control. However, this was not the case for RSA/PEG liposome where such a significant reduction was not observed. These results suggest that the hepatic disposition of PEG liposome can be mainly ascribed to the receptor-mediated uptake, but the further modification of liposome with RSA can avoid the hepatic disposition via the receptor-mediated endocytosis.

Since the recognition of surface-associated serum opsonins by their corresponding receptors is mainly a trigger for the receptor-mediated hepatic uptake of particles, the results shown in Fig. 3 also suggest that the amount of opsonins associated on the surface of PEG liposome would be reduced by RSA-modification. Therefore, we tried to evaluate the serum proteins associated on the surface of PEG liposome and RSA/PEG liposome quantitatively and qualitatively (Figs. 4 and 5). As shown in Fig. 4, it was revealed that the amount of serum proteins associated on the surface of RSA/PEG liposome was significantly smaller than that of PEG liposome. Furthermore, the serum proteins associated on these two liposomes were qualitatively analyzed by using SDS-PAGE (Fig. 5). Although a marked qualitative change in the surface-associated serum proteins by RSA modification was not observed, the relative amount of several serum proteins (e.g., around 200, 115 and 100 kDa in apparent molecular weight) was reduced by RSA modification.
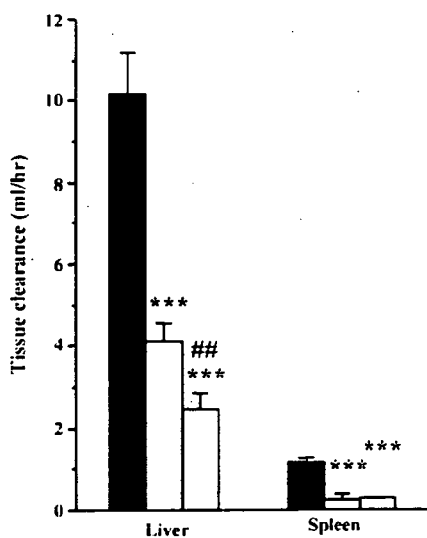

Fig. 2. Liver and spleen clearances of conventional (non-PEG), PEG and RSA/PEG liposomes. Results are expressed as the mean with a bar showing the S.D. of three experiments. (■) Non-PEG liposome; (□) PEG liposome; (□) PEG/RSA liposome. ***$p < 0.001$, compared with non-PEG liposome; ##$p < 0.01$, compared with PEG liposome.
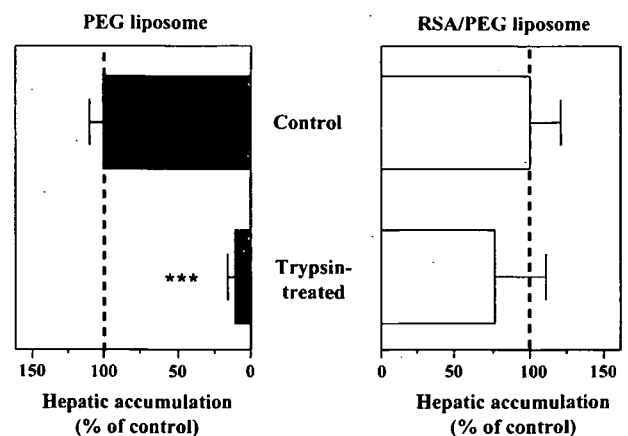

Fig. 3. Effect of pre-treatment with trypsin on the hepatic disposition of PEG liposome or RSA/PEG liposome in rat liver single-pass constant infusion experiments. Results are expressed as the mean with the bar showing the S.D. of three experiments. ***$p < 0.001$, compared with control.
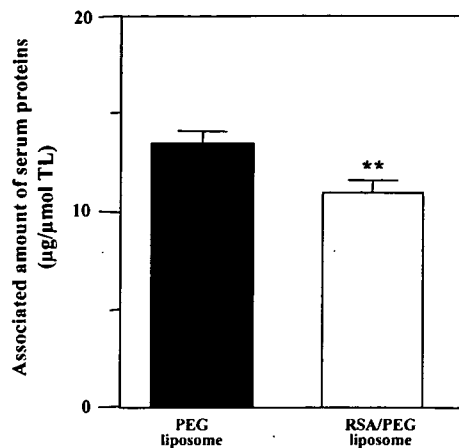
Fig. 4. Quantitative estimation of the rat serum proteins associated on the surface of PEG liposome and RSA/PEG liposome. The amount of serum proteins associated with RSA/PEG liposome was calculated by subtracting the amount of RSA coupled with liposomes from the total protein amount measured. Results are expressed as the mean with the bar showing the S.D. of three experiments. $**p < 0.01$, compared with PEG liposome.
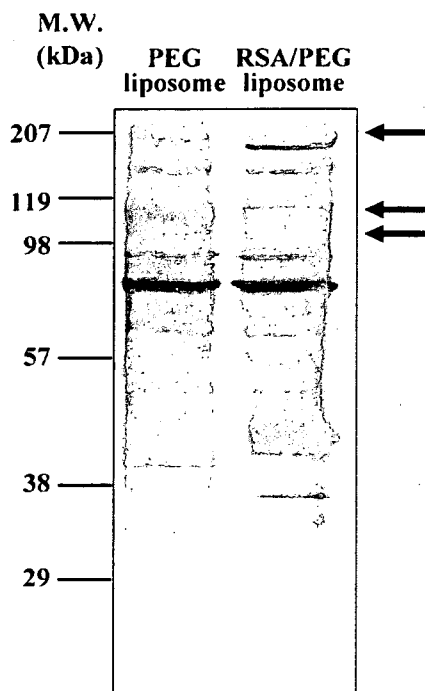

Fig. 5. Silver-stained SDS-PAGE of rat serum proteins associated on the surface of PEG liposome and RSA/PEG liposome. The same amount of protein (3 µg) was loaded onto each lane.

4. Discussion

Long-circulating particulate carriers can provide several advantages, for instance, for the development of artificial oxygen delivery or blood-pool imaging system (Weissig et al., 2000; Sakai and Tsuchi, 2006). Besides them, long-circulating particles are promising carriers for passive targeting of drugs into tumors or inflamed tissues where the integrity of the endothe-

lial barrier is perturbed (Gabizon, 1992; Jang et al., 2003). One of the most widely-used methods for enhancing the circulation properties of liposomes is the inclusion of PEG within the membrane surface of liposomes (Blime and Cevc, 1990; Klibanov et al., 1990; Allen et al., 1991b). PEG is considered to form a hydrated shell hindering the interaction of liposomes with serum proteins, thereby greatly reducing the opsonization and uptake by macrophages.

The purpose of our present study was to investigate the effect of coupling of RSA onto the surface of PEG liposome on the in vivo disposition. Our preliminary study showed that the RSA modification significantly increased the particle size of PEG liposome with 100 nm in diameter, but not the one with 200 nm in diameter. Therefore, we utilized the liposome with 200 nm in diameter to exclude the effect of the increase in the particle size of PEG liposome by RSA modification. As a result, the modification of PEG liposome surface with albumin significantly reduced the hepatic disposition of liposomes, resulting in further prolongation of their blood circulation time. The result clearly indicates that albumin conjugated on the surface substantially influenced the hepatic handling of PEG liposome.

Chonn et al. (1992) reported that the amount of serum proteins associated on the liposomes used was inversely related to their circulation half-lives. Our present findings also revealed that RSA/PEG liposome, onto which less amount of serum proteins associated (Fig. 4), showed more prolonged blood circulation time than PEG liposome (Fig. 1 and Table 1). It has been postulated that surface-grafted PEG would form either a mushroom or a brush conformation, depending on molecular weight and surface density of PEG on the liposomes (Needham et al., 1997; Nicholas et al., 2000; Johnstone et al., 2001). Originally, PEG incorporated into liposome forms the mushroom conformation, then PEG liposomes are thought to readily interact with serum proteins and with cell surface as well (Johnstone et al., 2001). On the other hand, a brush conformation, which is suggested to be induced by hydrostatic interactions between the surface of PEG liposome and serum proteins, can interfere with the subsequent interactions between PEG liposomes and phagocytes or other serum proteins (Mori et al., 1991; Johnstone et al., 2001). From these backgrounds, it is suggested that PEG molecules in RSA/PEG liposome would be in the brush conformation upon the introduction of RSA (Fig. 6). This could be the reason why the interaction of RSA/PEG liposome with serum proteins was significantly suppressed by the introduction of RSA (Fig. 4). Besides this speculation based on the change in the conformation of PEG molecule, other possible explanations for the reduced interaction of RSA/PEG liposome with serum proteins would be that (1) RSA introduced onto the surface of PEG liposome occupied the binding sites for the serum proteins and/or (2) RSA-modification further increased the apparent hydrophilicity of PEG liposome. However, the mechanisms behind the less association of RSA/PEG liposome with serum proteins remain to be elucidated and will be the subject of our further study.

Our previous study suggested that not only the amount but also the kind of serum proteins associated on the surface of polystyrene nanoparticles would be important determinants for their in vivo disposition (Ogawara et al., 2001; Furumoto et al.,
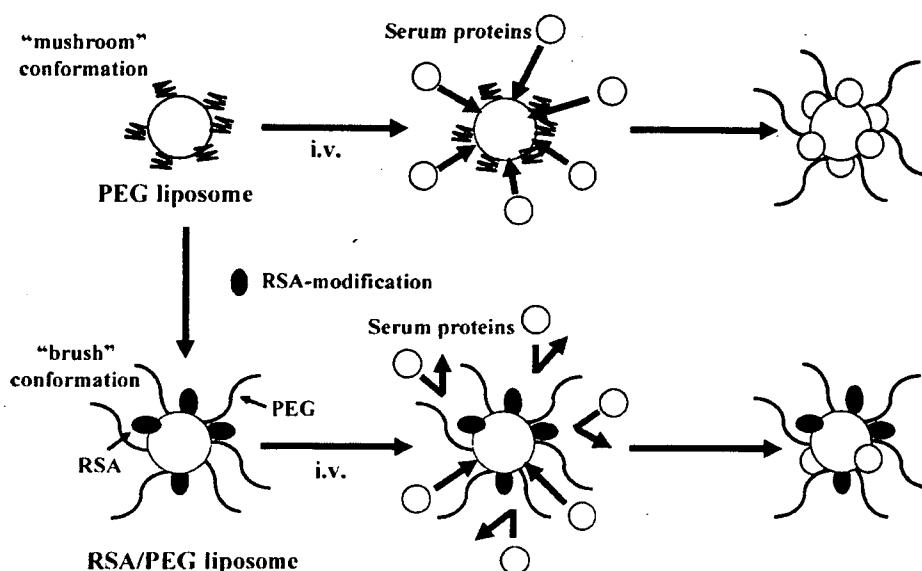
Fig. 6. Assumed mechanisms by which RSA-modification suppresses the association of serum proteins onto the surface of PEG liposome.

2002). Therefore, we performed SDS-PAGE analysis to qualitatively evaluate the serum proteins associated onto the surface of RSA/PEG or PEG liposome (Fig. 5). Although the modification of PEG liposome with RSA did not lead to a tremendous change in the kind of serum proteins associated compared with that of PEG liposomes, the association of some serum proteins (e.g., with apparent molecular weights of around 200, 115 and 100 kDa) was reduced (Fig. 5). Considering that the pretreatment of the liver with trypsin did not significantly decrease the hepatic disposition of RSA/PEG liposome (Fig. 3), some serum opsonins would be included in the proteins of which the amount adsorbed onto the surface was decreased. Western blotting will be useful to address the possible less-association of typical serum opsonins on RSA/PEG liposome and will be the subject of our further study.

In conclusion, the present study clearly indicated that the RSA modification on the surface of PEG liposome significantly decreased the hepatic disposition of liposomes, resulting in the more prolonged circulation time of PEG liposome after intravenous administration in vivo. This pharmacokinetic advantage of RSA/PEG liposome would be ascribed to the reduced association of the serum proteins including some given serum opsonins onto the surface.

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Time-dependent changes in opsonin amount associated on nanoparticles alter their hepatic uptake characteristics

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Abstract

The relationship between the time-dependent change in serum proteins adsorbed on nanoparticles and their disposition to the liver was investigated by employing lecithin-coated polystyrene nanosphere with a size of 50 nm (LNS-50) as a model nanoparticle in rats. The total amount of proteins adsorbed on LNS-50 increased and the qualitative profile of serum proteins adsorbed on LNS-50 changed during the incubation with serum up to 360 min. The liver perfusion study indicated that the hepatic uptake of LNS-50 incubated with serum for 360 min was significantly larger than those of LNS-50 incubated for shorter period. It was suggested that the increase in the hepatic uptake of LNS-50 with the increase in incubation time would be ascribed mainly to the increase in the opsonin-mediated uptake by Kupffer cells. Semi-quantification of major opsonins, complement C3 (C3) and immunoglobulin G (IgG), and in vitro uptake study in primary cultured Kupffer cells demonstrated that the increase in C3 and IgG amounts adsorbed on LNS-50 was directly reflected in the increased disposition of LNS-50 to Kupffer cells. These results indicate that the amounts of opsonins associated on nanoparticles would change over time and this process would be substantially reflected in the alteration of their hepatic disposition characteristics.

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1. Introduction

Utilization of nanoparticulate drug carriers such as liposomes and emulsions is considered to be one of the promising approaches to achieve the organ specific delivery of drugs and genes (Drummond et al., 1999; Moghimi et al., 2001; Andresen et al., 2005). Intravenously injected nanoparticles first contact and associate with blood components including opsonins which can be recognized by their specific receptors on macrophages in reticuloendothelial system (RES). This process, so-called opsonization, leads to the rapid elimination of nanoparticles from the blood circulation (Chonn et al., 1992; Oja et al., 1996; Scherphof and Kamps, 1998), which has limited the clinical application of nanoparticulate drug carriers so far. To understand and/or improve their in vivo disposition characteristics, the interaction of nanoparticles with plasma or serum proteins has been investigated intensively in in vitro studies. As a result,

it was demonstrated that the process of opsonization are largely affected by the physicochemical properties of nanoparticles such as size (Devine et al., 1994), charge (Gessner et al., 2002) and hydrophobicity (Gessner et al., 2000). Major opsonins such as immunoglobulins and complement-related proteins (Semple et al., 1998) and non-immune opsonins such as fibronectin, vitronectin and fibrinogen were also identified (Price et al., 2001). Furthermore, studies on the interaction of a single protein with the solid surface or studies on the recognition of the nanoparticles coated with a single protein by phagocytes have been extensively performed and have provided a lot of useful information including the identification of the corresponding receptor for each opsonin (Moghimi and Patel, 1998; Moghimi and Hunter, 2001; Scherphof and Kamps, 2001).

In spite of these numerous reports, it is still very difficult to fully understand or predict the in vivo behavior of injected nanoparticles. One of the main reasons for this would be that the adsorption of plasma or serum proteins on the surface of nanoparticles is highly complicated event due to the presence of protein–protein interaction (Devine and Marjan, 1997; Semple et al., 1998) and/or competitive adsorption processes among

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various proteins, so-called "Vromann-effect", where initially adsorbed proteins can be displaced by other proteins (Vroman et al., 1980; Vroman and Adams, 1986; Norman et al., 1993). It has been also reported that the incubation time is a crucial factor to determine the adsorption pattern of proteins on the solid surface (Blunk et al., 1996). Recently, Goppert and Muller indicated that a time-dependent change in the adsorption profiles on nanoparticles would occur in the in vivo situation as well (Goppert and Muller, 2005). Although there are many papers describing the relationship between the opsonin amounts associated on the surface of various particles and their affinity to the macrophages (Chonn et al., 1992; Oja et al., 1996; Scherphof and Kamps, 1998), there is no report investigating whether the time-dependent change in the adsorption profile of plasma or serum proteins including opsonins on nanoparticles substantially alters the recognition by macrophages or not.

Therefore, the objective of this study is to investigate the correlation between the time-dependent change in the adsorption pattern of serum proteins on lecithin-coated polystyrene nanoparticle with a size of 50 nm (LNS-50), used as a model nanoparticle, and its disposition characteristics to the liver. Furthermore, focusing on the representative opsonins such as complement C3 and immunoglobulin G, we also tried to evaluate the effect of the time-dependent change in the amount of these surface-bound opsonins on the uptake of LNS-50 to primary cultured Kupffer cells.

2. Materials and methods

2.1. Chemicals

Egg yolk lecithin was purchased from Sigma (St. Louis, MO). Gadolinium chloride (GdCl$_3$·6H$_2$O) was purchased from Nacalai Tesque, INC (Kyoto, Japan). Rabbit anti-rat immunoglobulin G (IgG) polyclonal antibody was purchased from Vector Laboratories (Burlingame, CA). Rabbit anti-rat serum albumin polyclonal antibody was purchased from Intercell Technologies (Hopewell, NJ). Sheep anti-rat complement C3 polyclonal antibody was purchased from Biogenesis (Poole, UK). All other chemical were of the finest grade available.

2.2. Animal

Male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 220–240 g were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with *Principles of Laboratory Animal Care* (*NIH publication #85-23*).

2.3. Preparation of rat serum

The serum was prepared just before use as follows: rat whole blood was collected from the carotid artery and allowed to clot at room temperature for 20 min, then centrifuged at 1500 × g for 20 min at 4 °C and the supernatant obtained was used as serum.

2.4. Preparation of nanospheres

Monodispersed, non-ionized polystyrene nanospheres (NS-50) covalently linked with fluorescein isothiocyanate, 50 nm in diameter, were used as received (Polysciences, Warrington, PA). For the preparation of lecithin-coated polystyrene nanosphere (LNS-50), the suspension of NS-50 was sonicated for 3 min with egg yolk lecithin (NS-50:lecithin = 1:4, w/w) on the ice by a probe type sonicator (Ohtake, Osaka, Japan). The diameters of LNS-50 were measured by a dynamic light scattering equipment (DLS-7000, Otsuka Electronics, Osaka) after incubated in KRB buffer containing rat serum (5% (v/v), pH 7.4). The diameter of LNS-50 did not significantly increase during the incubation up to 360 min and the diameters of LNS-50 after 5 and 360 min incubations were 77.4 ± 32.8 and 90.3 ± 35.9 nm, respectively. Subpopulations with larger diameter (>200 nm) were hardly observed (<5%) irrespective of the incubation time periods.

2.5. SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analysis

After the incubation of LNS-50 in KRB buffer containing rat serum (5% (v/v), pH 7.4) at 37 °C, LNS-50 was separated by ultracentrifugation using a Beckman Optima XL-90 (Beckman Instruments Inc., Palo Alto, CA) at 40,000 × g for 15 min at 4 °C and washed three times with KRB buffer (pH 7.4). After solubilizing with 10% SDS solution, the resulting protein solution was mixed with sample buffer composed of 0.1 M Tris–HCl, 4% SDS, 12% 2-mercaptoethanol and 20% glycerol. This mixture (protein solution:sample buffer = 3:1, v/v) was incubated for 30 min at 37 °C and was subjected to SDS-PAGE using 12.5% poly-acrylamide gel (Ready Gel, Bio-Rad, Hercules, CA). The detection of proteins was performed by a silver-stain procedure by using a silver-stain kit (Daiichi Pure Chemicals, Tokyo, Japan).

2.6. Liver perfusion experiments

Liver perfusion was carried out with KRB buffer (pH 7.4) following the recirculating perfusion procedure as reported previously (Furumoto et al., 2002). In brief, LNS-50 was suspended in the KRB buffer containing rat serum (5% (v/v), pH 7.4) and incubated for various time periods (5, 60, 180 or 360 min). Then, LNS-50, an initial concentration of 50 μg/mL, was recirculated in the isolated liver preparation at a flow rate of 13.0 mL/min for 50 min. The perfusate concentration of LNS-50 in the reservoir was fluorometrically determined (excitation maximum 458 nm, emission maximum 540 nm) (RF-540 Fluorescent Spectrometer, Shimadzu, Kyoto) until 50 min. Hepatic clearance (CL$_h$) was calculated according to Eq. (1):

$$CL_h = \frac{X^t_{liver}}{AUC^t_0} \quad (t = 50 \text{ min}) \tag{1}$$

where X_{liver}^{t} and AUC_{0}^{t} mean the amount of LNS-50 in the liver at time t and the area under the concentration of LNS-50 in the perfusate versus time curve from 0 to time t, respectively. X_{liver}^{t} was estimated by subtracting the remaining amount of LNS-50 in the reservoir from the total amount of dose. AUC_{0}^{t} was calculated according to the trapezoidal rule (Yamaoka et al., 1978).

2.7. Pre-treatment of liver with trypsin or gadolinium chloride

Pre-treatment of the perfused liver with trypsin was performed by following the method reported by Furumoto et al. (2002). Pre-treatment of rats with gadolinium chloride was performed as following the method reported by Lazar et al. (1989). In brief, gadolinium chloride (20 μmol/kg) was pre-injected from a femoral vein 24 h before the initiation of the liver perfusion experiments.

2.8. Western blot analysis

After SDS-PAGE was performed as described above, proteins were blotted on cellulose nitrate membranes (Advantec, Tokyo). For the detection of albumin, complement C3 and IgG, rabbit anti-rat albumin polyclonal antibody, sheep anti-rat complement C3 polyclonal antibody and rabbit anti-rat IgG polyclonal antibody were used at 1:250 dilution in blocking buffer. As second antibodies, peroxidase-linked anti-rabbit IgG polyclonal antibody (Chemicon International, Temecula, CA) and anti-sheep IgG polyclonal antibody (Kirkegaard and Perry Laboratories, Guildford, UK) were used at 1:5000 and 1:10,000 dilution in blocking buffer, respectively. The protein band was visualized with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the densitometric intensities of protein bands were quantified by Scion Image™ (Scion Corporation, Frederick, MD). Since SDS-PAGE was conducted under reducing condition where many small fragments can be generated from the protein of interest, the densitometric intensities of bands were integrated for each lane to semi-quantitatively evaluate the amount of C3 and IgG.

2.9. Isolation and culture of Kupffer cells

Kupffer cells were isolated after collagenase perfusion following the conventional procedures (Knook and Sleyster, 1976). Kupffer cells were grown in 24-well plates in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated FBS (Sigma), penicillin G (100 U/mL, Sigma) and streptomycin (100 μg/mL, Sigma). After the incubation for 2 h at 37 °C and 5% CO_2/95% air atmosphere, the medium was refreshed and then cultured at 37 °C and 5% CO_2/95% air atmosphere for 48 h before experiments. The purity of isolated Kupffer cells (over 95%) was checked morphologically and enzymatically by measuring peroxidase activity using 3,3-diaminobenzidine as a substrate.

2.10. Uptake experiments

LNS-50 was suspended in PBS containing rat serum (5% (v/v), pH 7.4) and incubated for 5 or 360 min. Then, cultured rat Kupffer cells were washed with FBS-free medium and uptake studies were started by adding LNS-50 (100 μg/mL) to the cells. In the case of inhibition study, LNS-50 was added to the cells together with different amounts of each antibody diluted with PBS (pH 7.4). The incubation with LNS-50 for the uptake was performed at 37 °C for 1 h. Then, the cells were washed with PBS and solubilized with 10% SDS solution for 12 h. Solubilized samples were lyophilized over 36 h, and the resulting dried samples were re-suspended in accurately measured volume of chloroform and mechanically shaken for 18 h. After the resulting suspension was filtered through a 0.22 μm solvent-resistant membrane filter (Millex HV Milopore, Bedford, MA), the fluorescence intensity of the filtrate was determined as described above.

2.11. Statistical analysis

Results are expressed as the mean ± S.E. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was evaluated by using Student's t-test or Dunnett's test for the single or multiple comparisons of experimental groups, respectively.

3. Results and discussion

To evaluate the relationship between the adsorption kinetics of serum proteins on the nanoparticle surface and the disposition characteristics of the particle to the liver, we used LNS-50 as a model nanoparticle, which exhibited extensively prolonged blood circulating property in vivo, compared with the non-coated one because of much less association of opsonins on the surface of LNS-50 (Ogawara et al., 2001). Furthermore, our preliminary studies showed that the liver accumulation of LNS-50 was lower than non-coated one in the early periods of time after injection but increased afterwards in the time-dependent fashion up to 24 h, resulting in the higher liver accumulation than non-coated one (data not shown). In the present study, first of all, we studied the adsorption kinetics of serum proteins on the surface of LNS-50 over time (Fig. 1). As shown in Fig. 1A, the total amount of serum proteins associated on LNS-50 did not change until 60 min, but then it significantly increased as the incubation time was prolonged. The amount of adsorbed proteins after 180- or 360-min incubation was 1.5- or 2.5-fold larger than that after 5-min incubation, respectively. SDS-PAGE analysis also showed that adsorption profile of serum proteins on LNS-50, especially the bands with arrows, was dramatically changed with the increase in incubation time (Fig. 1B). These results clearly indicated that not only the amount but also the kind of serum proteins associated on the surface of LNS-50 was changed in the incubation time-dependent fashion.

Then, the effect of the incubation time of LNS-50 with serum on the subsequent hepatic uptake of LNS-50 was inves-
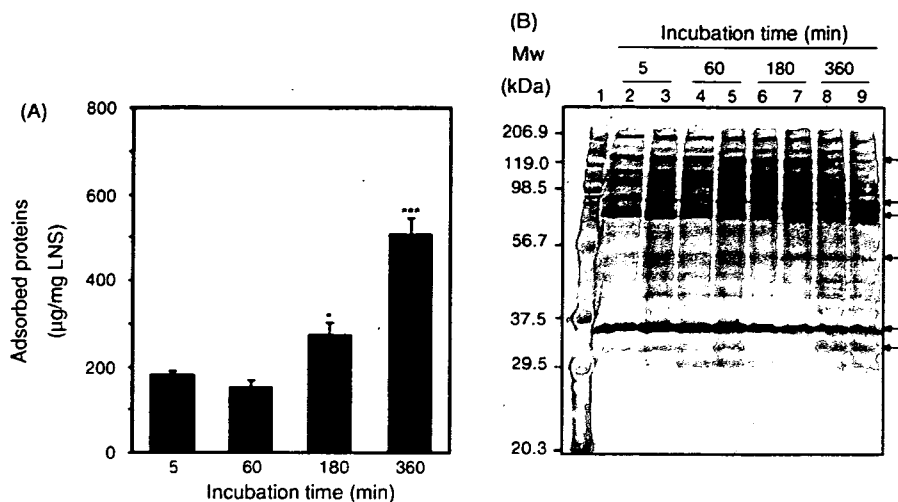
Fig. 1. Evaluation of total amount (A) and SDS-PAGE analysis (B) of serum proteins associated on the surface of LNS-50 after the incubation with serum for different periods of time. (A) After the incubation with serum, LNS-50 was separated and the total amount of serum proteins adsorbed was quantified. Results are expressed as the mean with a bar showing the S.E. ($n = 5$–14). $*p < 0.05$; $***p < 0.001$, compared with the value of 5-min incubation. (B) Two samples for each incubation time were independently prepared and subjected to SDS-PAGE analysis. The same amount of protein (1.75 μg) was loaded on the gel. Lane 1, molecular marker; Lanes 2 and 3, 5-min incubation; Lanes 4 and 5, 60-min incubation; Lanes 6 and 7, 180-min incubation; Lanes 8 and 9, 360-min incubation.

tigated in the liver perfusion experiments (Fig. 2). The results indicated that the hepatic clearance of LNS-50 incubated with serum for 360 min (LNS-50 (360 min)) was significantly larger than that of LNS-50 incubated with serum for 5 min (LNS-50 (5 min)), although statistically significant change in hepatic clearance was not observed for either LNS-50 (60 min) or LNS-50 (180 min). To unravel the mechanism behind the increase in hepatic uptake of LNS-50 by 360-min incubation, we applied trypsin-treatment technique (Furumoto et al., 2002) (Fig. 2). The results showed that the pre-treatment of the liver with trypsin significantly decreased the hepatic clearance of LNS-50 (360 min). The same treatment tended to decrease those of LNS-50 incubated for shorter period, but the decrease was not
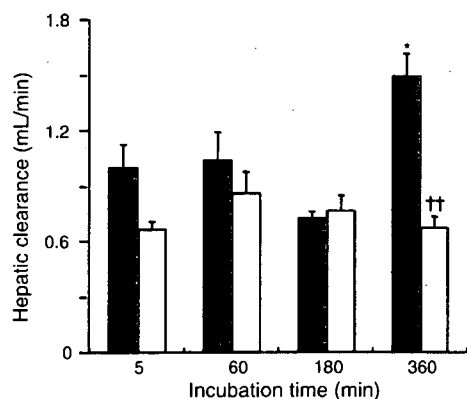

Fig. 2. Effect of pre-treatment of liver with trypsin on hepatic uptake of LNS-50 incubated with serum for different periods of time. The prepared isolated liver was perfused in the absence (■) or the presence (□) of trypsin in the perfusate before starting the perfusion of LNS-50. Results are expressed as the mean with a bar showing the S.E. ($n = 3$–6). $*p < 0.05$, compared with the value of 5-min incubation. $††p < 0.01$, compared with the corresponding group without trypsin treatment.

statistically significant. Of note, the trypsin treatment provided that the hepatic clearances of LNS-50 were almost the same irrespective of the incubation time, suggesting that this treatment effectively inhibited the hepatic uptake of LNS-50 via receptor-mediated mechanisms and that the increase in the hepatic uptake of LNS-50 by 360-min incubation would be ascribed mainly to the uptake via the receptor-mediated mechanisms. Therefore, we tried to further clarify the events underlying the increased receptor-mediated liver uptake of LNS-50 (360 min).

Gadolinium chloride, widely used to depress the phagocytotic activity of macrophage (Lazar et al., 1989; Hardonk et al., 1992; Vajdova et al., 2000; Lee et al., 2004), was utilized to investigate the involvement of Kupffer cells in the increased hepatic uptake of LNS-50 (360 min) in the liver perfusion study (Fig. 3). Although the pre-treatment with gadolinium chloride significantly decreased the hepatic clearance of both LNS-50 (360 min) and LNS-50 (5 min) compared with each corresponding control, the extent of the decrease in the hepatic clearance was 1.6-fold larger for LNS-50 (360 min) (55% of control) than that for LNS-50 (5 min) (34% of control). In addition, to investigate the involvement of the heat-labile opsonins such as complement components in the hepatic uptake of LNS-50 (360 min), LNS-50 was incubated with heated serum (56 °C, 30 min) for 5 or 360 min prior to the liver perfusion experiments (Fig. 3). The results showed that the hepatic clearance of LNS-50 incubated with heated serum for 360 min was significantly lower than that of LNS-50 (360 min), although there was no significant difference in the case of 5-min incubation. These results suggested that the increase in the hepatic uptake of LNS-50 by 360-min incubation would be ascribed mainly to the increase of the opsonin-mediated phagocytotic uptake by Kupffer cells. On the other hand, taken the patterns of serum proteins adsorbed on LNS-50 (Fig. 1), the incubation time-dependent increase in the amount of opsonins associated on the surface
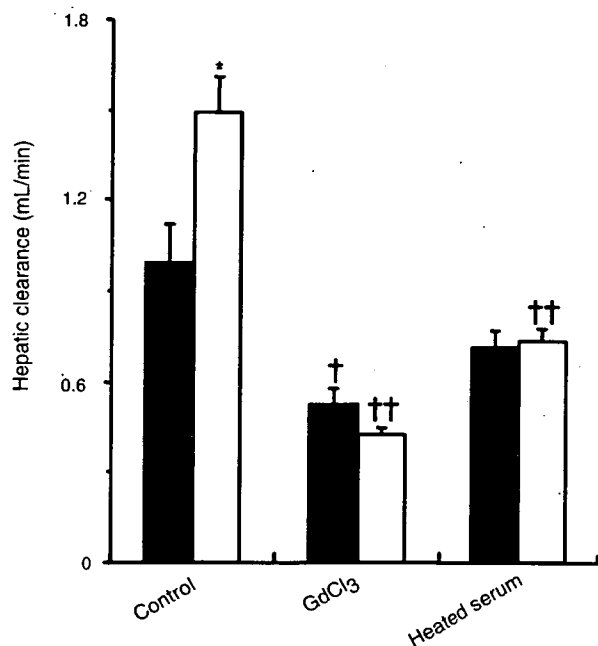
Fig. 3. Effect of pre-treatment of liver with gadolinium chloride and heated-treatment of serum on hepatic uptake of LNS-50 incubated with serum for 360 min. The prepared isolated liver was perfused with KRB buffer containing LNS-50 incubated in 5% (v/v) serum for 5 min (■) or 360 min (□). Results are expressed as the mean with a bar showing the S.E. ($n = 3–6$). *$p < 0.05$, compared with the value of 5-min incubation. †$p < 0.05$; ††$p < 0.01$, compared with each corresponding control value.

of LNS-50 would be reflected in the hepatic uptake of LNS-50 (360 min).

Among opsonins, complement C3 (C3) and immunoglobulin G are the most representative ones. C3 is cleaved in the process of complement activation cascade and generates C3b and iC3b, which strongly bind to each corresponding receptor expressed on the surface of Kupffer cells and promote the uptake of nanoparticles (Borchard and Kreuter, 1996; Luck et al., 1999; Ishida et al., 2000, 2001). IgG also strongly facilitates the uptake of nanoparticles to Kupffer cells via Fc receptor (Leroux et al., 1995; Moghimi and Patel, 1998; Semple et al., 1998). In addition, it has been already reported that the amounts of C3 and IgG on nanoparticles are time-dependently modulated during the incubation with serum or plasma (Allemann et al., 1997; Goppert and Muller, 2005). However, there is no report indicating the direct relationship between the time-dependent changes in the amount of a specific opsonin on nanoparticles and their opsonin-mediated phagocytotic uptake by macrophage. Therefore, focusing on C3 and IgG, the adsorption kinetics of both opsonins on LNS-50 was investigated by the incubation with serum (Fig. 4).

As shown in Fig. 4A and B, Western blot analysis was performed under the reducing condition and many fragments derived from C3 and IgG were detected due to the cleavage of disulfide bonds within the molecule. The semi-quantification of the densitometric intensities derived from C3 fragments and IgG fragments revealed that C3 and IgG adsorbed on LNS-50 significantly increased with the increase of incubation time, and
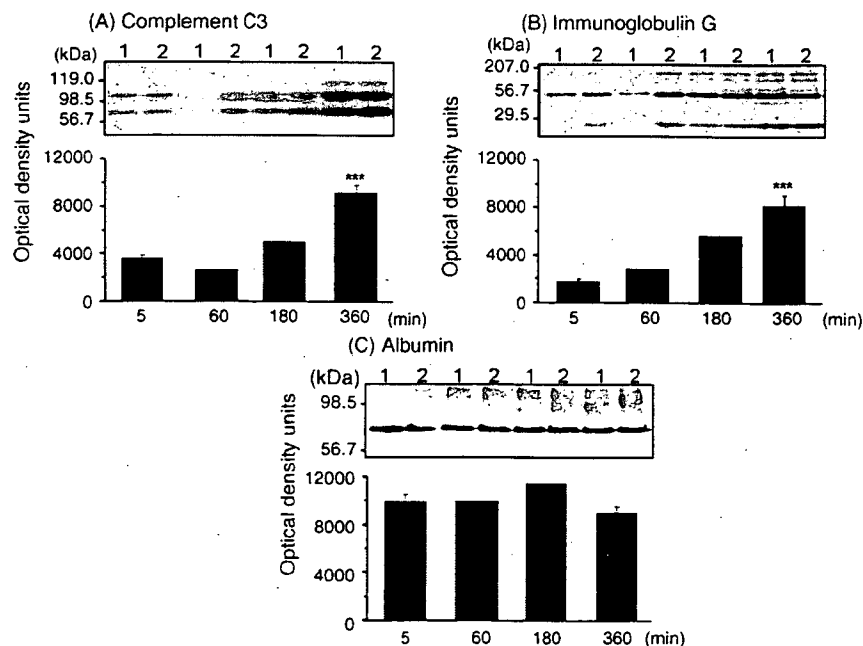

Fig. 4. Semi-quantification of complement C3 and immunoglobulin G associated on the surface of LNS-50 by Western blot analysis. Two samples for each incubation time were independently prepared and subjected to Western blot analysis. The same amount of protein (2.25 μg) was loaded on the gel. Results of semi-quantification for 5- or 360-min incubation are expressed as the mean with a bar showing the S.E. ($n = 6$). ***$p < 0.001$, compared with the corresponding value for 5-min incubation. In the cases for 60- or 180-min incubation, results are expressed as the average of two values as follows: complement C3 (60-min incubation, 1508 and 3542; 180-min incubation, 4269 and 5645), immunoglobulin G (60-min incubation, 591 and 5025; 180-min incubation, 4497 and 6634) and serum albumin (60-min incubation, 9489 and 10,217; 180-min incubation, 11,147 and 11,533).
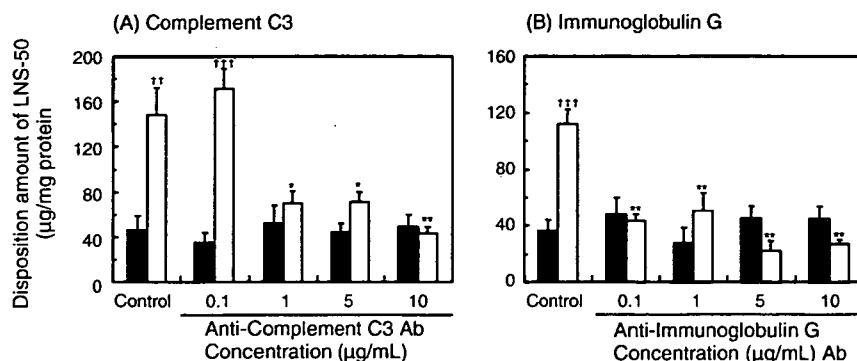
Fig. 5. Involvement of complement C3 and immunoglobulin G in the disposition of LNS-50 (360 min) to Kupffer cells. LNS-50 (100 μg/mL) suspended with PBS was simultaneously added to the cells with the various concentrations of anti-complement C3 antibody or anti-immunoglobulin G antibody. Results are expressed as the mean with a bar showing the S.E. $(n = 3–12)$. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with each corresponding control value. $^{\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$, compared with each corresponding value of LNS-50 (5 min). Keys ■, LNS-50 (5 min); □, LNS-50 (360 min).

that the amounts of C3 and IgG on LNS-50 (360 min) were 2.6- and 4.7-fold more than those on LNS-50 (5 min), respectively. On the other hand, albumin, having the dysopsonin-like activity (Ogawara et al., 2004), was not significantly changed in the amount adsorbed on LNS-50 throughout the incubation (Fig. 4C). Although the increase in the amount of C3 and IgG was observed for LNS-50 (180 min) and LNS-50 (360 min), the significant increase in the receptor-mediated hepatic uptake was found only for LNS-50 (360 min) (Fig. 2), suggesting that the density of these opsonins on LNS-50 (180 min) would be still insufficient to trigger the receptor-mediated hepatic uptake. Together with these studies, we also evaluated the uptake of LNS-50 (5 min) and LNS-50 (360 min) by Kupffer cells and the possible involvement of C3 and IgG in the uptake (Fig. 5). As shown in Fig. 5, the disposition amount of LNS-50 (360 min) to Kupffer cells was about three-fold larger than that of LNS-50 (5 min), which were similar to the tendency obtained in the liver perfusion study (Fig. 2). These results suggest that Kupffer cells play an important role in the hepatic uptake of LNS-50 (360 min). This high disposition of LNS-50 (360 min) to Kupffer cells was significantly decreased by the addition of either anti-complement C3 or anti-IgG antibody in a concentration-dependent manner. On the other hand, the disposition amount of LNS-50 (5 min) to Kupffer cells was not significantly influenced by the presence of these antibodies. These results clearly indicate that C3 and IgG were substantially functioning as opsonins in the disposition of LNS-50 (360 min) to Kupffer cells, but not in that of LNS-50 (5 min). Therefore, the high disposition of LNS-50 (360 min) to Kupffer cells could be ascribed to the C3 and IgG amounts on LNS-50 which was increased time-dependently during the incubation. IgG bound to exogenous materials is known to function either as a direct ligand for Fc receptor or as a trigger molecule to activate the complement cascade via classical pathway leading to the complement receptor-mediated uptake, thereby playing the central role in the self-defense system of the body (Absolom, 1986; Devine and Marjan, 1997). Therefore, anti-complement C3 antibody might inhibit partly the uptake of LNS-50 (360 min) via IgG-initiated complement receptor-mediated mechanism (Fig. 5). The reasons for the time-

dependent increase of IgG and C3 amounts on the surface of LNS-50 remain unclear, but the time-dependent increase in the amount of other proteins such as β2-glycoprotein I which has high affinity sites for the interaction with IgG in its molecules (Kertesz et al., 1995; Willems et al., 1996; Celli et al., 1999), might occur on the surface and this would lead to the time-dependent increase in the amount of IgG and C3 on the surface of LNS-50.

On the other hand, we also found that the amounts of other serum proteins such as apolipoprotein E and IgA on the surface of LNS-50, which are known to enhance the uptake of particles to hepatocytes (Sztul et al., 1985; Larkin et al., 1986; Yan et al., 2005), were time-dependently increased (data not shown). We are now investigating whether the increase in these proteins influences the uptake of LNS-50 by hepatocytes or not. The relative contribution of these proteins to the time-dependent increase in the uptake of LNS-50 by whole liver will also be the subject of our further study.

In conclusion, we clearly demonstrated that the amounts of opsonins such as C3 and IgG associated on LNS-50 were increased with the increase in incubation time with serum, and that this increase in their adsorbed amount was directly correlated with the increased uptake of LNS-50 by Kupffer cells. Although the mechanism behind the increase in the opsonins amounts adsorbed on LNS-50 and its physiological role remain to be elucidated, our present study suggests that the adsorption of opsonins on the nanoparticulate materials would time-dependently increase, and that it could lead to the increase in their affinity to the liver in the in vivo situation as well. Our finding will give deeper insight to understand the crucial role of serum proteins in the in vivo behavior of nanoparticulate drug carriers.

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