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Fetuin mediates hepatic uptake of negatively charged nanoparticles via scavenger receptor

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Abstract

We tried to evaluate the possible involvement of fetuin in the scavenger receptors (SRs)-mediated hepatic uptake of polystyrene nanospheres with the size of 50 nm (NS-50), which has surface negative charge (zeta potential = -21.8 ± 2.3 mV). The liver perfusion studies in rats revealed that the hepatic uptake of NS-50 pre-coated with fetuin (NS-50-fetuin) was significantly inhibited by poly inosinic acid (poly I), a typical inhibitor of SRs, whereas that of plain NS-50 or NS-50 pre-coated with BSA (NS-50-BSA) was not. The uptake of NS-50-fetuin by cultured Kupffer cells was also significantly inhibited by poly I, and anti-class A scavenger receptors (SR-A) antibody, suggesting that fetuin on NS-50 mediated the recognition and internalization of NS-50 by Kupffer cells and at least SR-A would be responsible for the uptake. Taken that Western blot analysis confirmed that fetuin certainly adsorbed on the surface of NS-50 after the incubation of NS-50 with serum, the results obtained in the present study indicate that fetuin would be one of the serum proteins that were substantially involved in the hepatic uptake of NS-50 via SRs.

Keywords: Polystyrene nanosphere; Fetuin; Scavenger receptors; Hepatic uptake; Receptor-mediated phagocytosis

1. Introduction

Scavenger receptors (SRs), which were first reported by Brown and Goldstein (Goldstein et al., 1979), are a family of cell surface glycoproteins that are able to bind and internalize modified lipoproteins such as oxidized and acetylated low density lipoproteins (Greaves et al., 1998). In the past 20 years, SRs have been found to be able not only to recognize endogenous molecules such as collagen (McKeown et al., 1994) and altered-self such as apoptotic cells (Peiser and Gordon, 2001), but also to bind, internalize and degrade exogenous pathogen such as bacteria (Peiser et al., 2000). In addition, it was reported that these receptors play an important role to remove a variety of negatively charged substances such as dextran sulfate (Takakura et al., 1994), formaldehyde-treated albumin (Jansen et al., 1991) and liposomes containing negatively charged phospholipids (Rigotti et al., 1995).

Until now, it has been considered that the hepatic uptake of the negatively charged compounds including particles, e.g., phosphatidylserine-containing liposomes, via SRs is through the direct recognition of their negative charge (Kobzik, 1995). However, our previous study indicated that in addition to the surface negative charge, some serum proteins associated on the surface also play an important role in the SRs-mediated hepatic uptake of polystyrene nanospheres with the size of 50 nm (NS-50) which had surface negative charge (Furumoto et al., 2004). Therefore, it is important to identify serum proteins that are involved in the SRs-mediated hepatic uptake of negatively charged particles.

Fetuin and its human homologue (α_2-HS-glycoprotein) are acidic negative acute-phase glycoproteins (Lebreton et al., 1979; Green et al., 1988; Naseem et al., 2003). The normal serum level in adults is 0.3–0.6 mg/ml and falls significantly (30–50%) during injury and infection (Wang et al., 1998). Hepatocytes are the principal cell source of circulating fetuin (Dziegielewska et al., 1996). Although the biological roles of fetuin are not fully understood, there is accumulating knowledge on the function of this protein and it has been reported that fetuin strongly enhances phagocytosis of bacteria, DNA and apoptotic cells by peripheral

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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 GeI, 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):

$$C_b = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

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

$$AUC_0^t = \int_0^t C_b \, dt \quad (2)$$

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

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

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

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

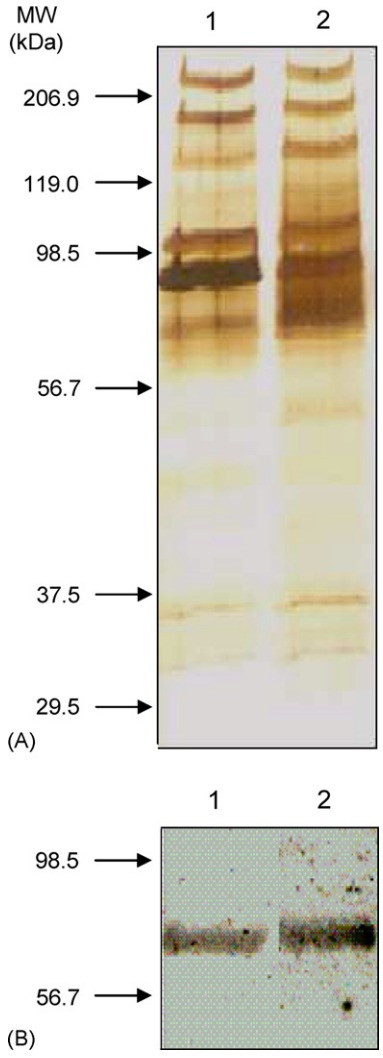

Fig. 1. Silver-stained SDS-PAGE of rat serum proteins adsorbed on the surface of NS-50 (A) and identification of fetuin by Western blot analysis (B). Lane 1, rat serum (loaded proteins A, 1 μg; B, 3 μg): lane 2, NS-50 pre-incubated with rat serum (A, 1 μg; B, 10 μg).

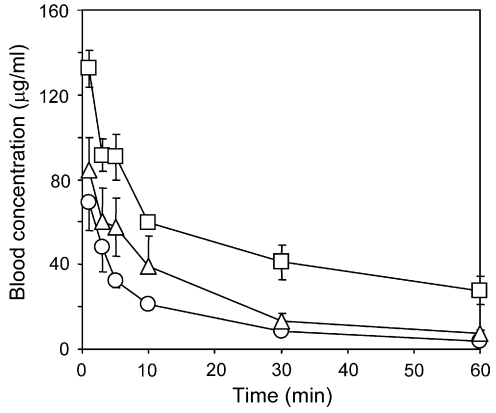

Fig. 2. Blood concentration–time profile of NS-50-fetuin after intravenous administration into rats at a dose of 12.5 mg/kg with or without pre-injection of poly I or poly C. Poly I or poly C was pre-injected at a dose of 10 mg/kg at 5 min before the injection of NS-50-fetuin. Results are expressed as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

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.

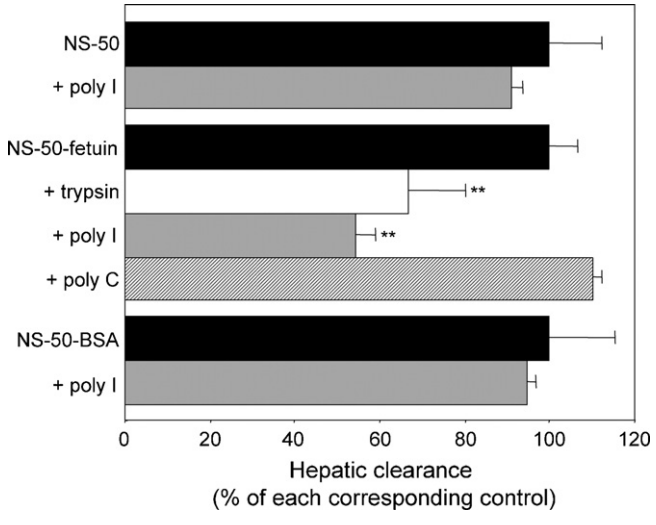

Fig. 3. Involvement of SRs in the hepatic uptake of NS-50-fetuin in the liver perfusion experiments. The prepared isolated liver was perfused with KRB buffer containing 25 μg/ml poly I or poly C before starting the perfusion of NS-50 or NS-50 pre-coated with fetuin or BSA. Results are expressed as the 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.

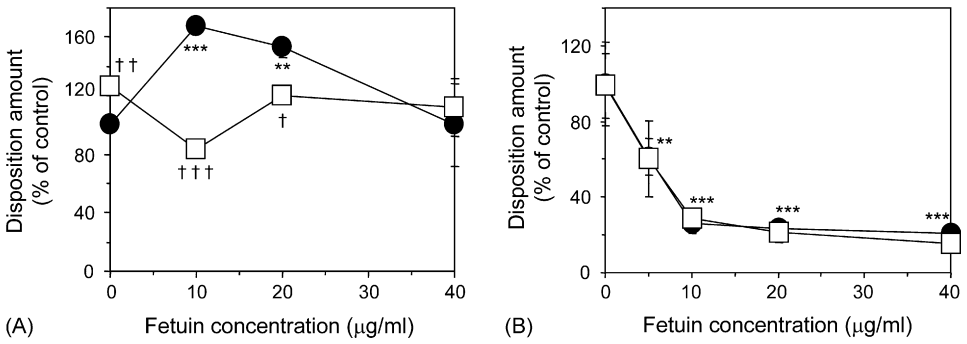

Fig. 4. Effect of trypsin-treatment of cell surface on the disposition of NS-50 to Kupffer cells (A) and hepatocytes (B) in the presence of various concentrations of fetuin. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were added to the cells. Before the addition of nanospheres, cells were incubated 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-

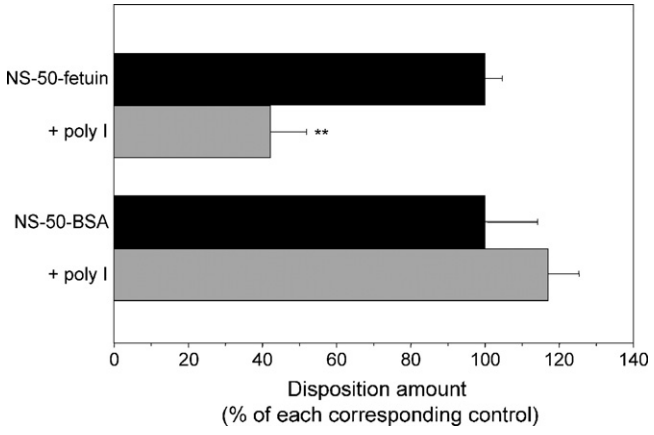

Fig. 5. Involvement of SRs in the uptake of NS-50-fetuin by Kupffer cells. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were added to the cells. Poly I (50 μg/ml) was added to the cells together with nanospheres. Results are expressed as the mean with a bar showing the S.D. ($n = 3$–5). $^{**}p < 0.01$, 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.

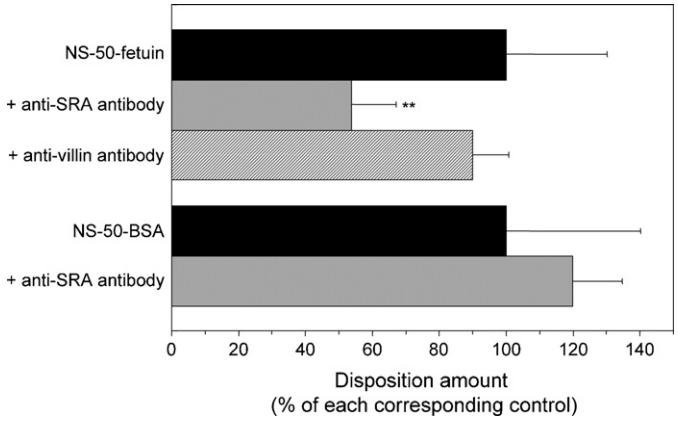

Fig. 6. Involvement of SR-A in the SRs-mediated uptake by Kupffer cells. Nanospheres (100 μg/ml) suspended with PBS (pH 7.4) were simultaneously added to the cells with 1 μg/ml anti-SR-A antibody or anti-villin antibody. The final concentration of antibody was 0.5 μg/ml. Results are expressed as the 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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