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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.

Keywords: Polystyrene nanosphere; Receptor-mediated uptake; Opsonins; Hepatic uptake; Kupffer cells

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 ($GdCl_3 \cdot 6H_2O$) 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% polyacrylamide gel (Ready GeI, 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^t_{liver} and AUC^t_0 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^t_{liver} was estimated by subtracting the remaining amount of LNS-50 in the reservoir from the total amount of dose. AUC^t_0 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-

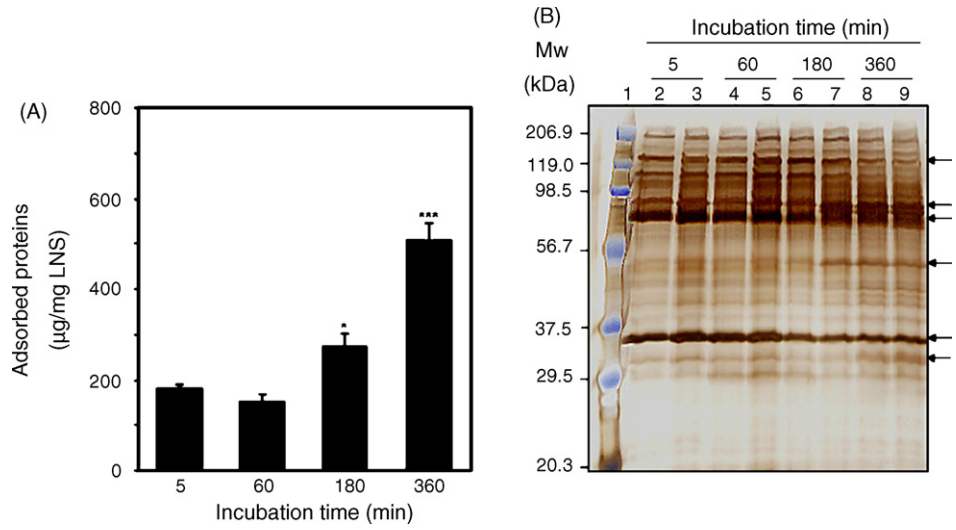

Fig. 1. Evaluation of total amount (A) and SDS-PAGE analysis (B) of serum proteins associated on the surface of LNS-50 after the incubation with serum for different periods of time. (A) After the incubation with serum, LNS-50 was separated and the total amount of serum proteins adsorbed was quantified. 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 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).

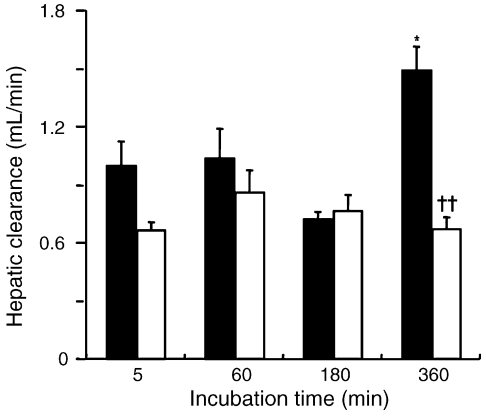

Fig. 2. Effect of pre-treatment of liver with trypsin on hepatic uptake of LNS-50 incubated with serum for different periods of time. The prepared isolated liver was perfused in the absence (■) or the presence (□) of trypsin in the perfusate before starting the perfusion of LNS-50. Results are expressed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

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

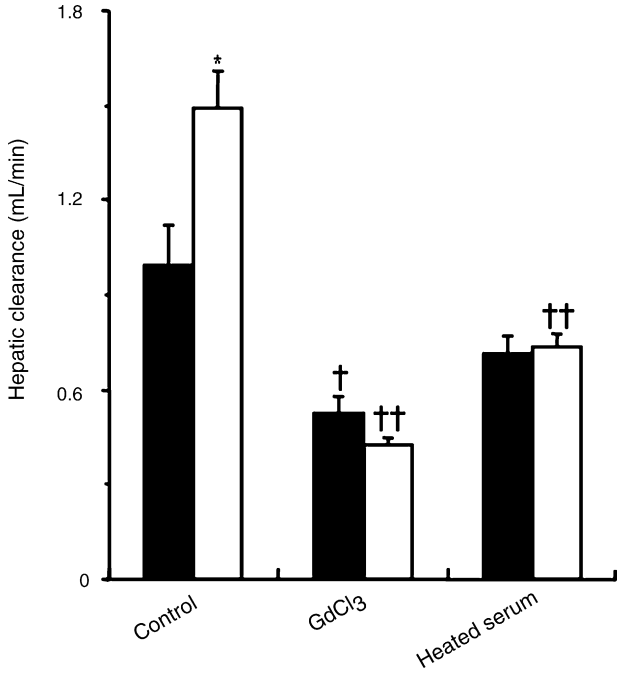

Fig. 3. Effect of pre-treatment of liver with gadolinium chloride and heated-treatment of serum on hepatic uptake of LNS-50 incubated with serum for 360 min. The prepared isolated liver was perfused with KRB buffer containing LNS-50 incubated in 5% (v/v) serum for 5 min (■) or 360 min (□). Results are 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. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, 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

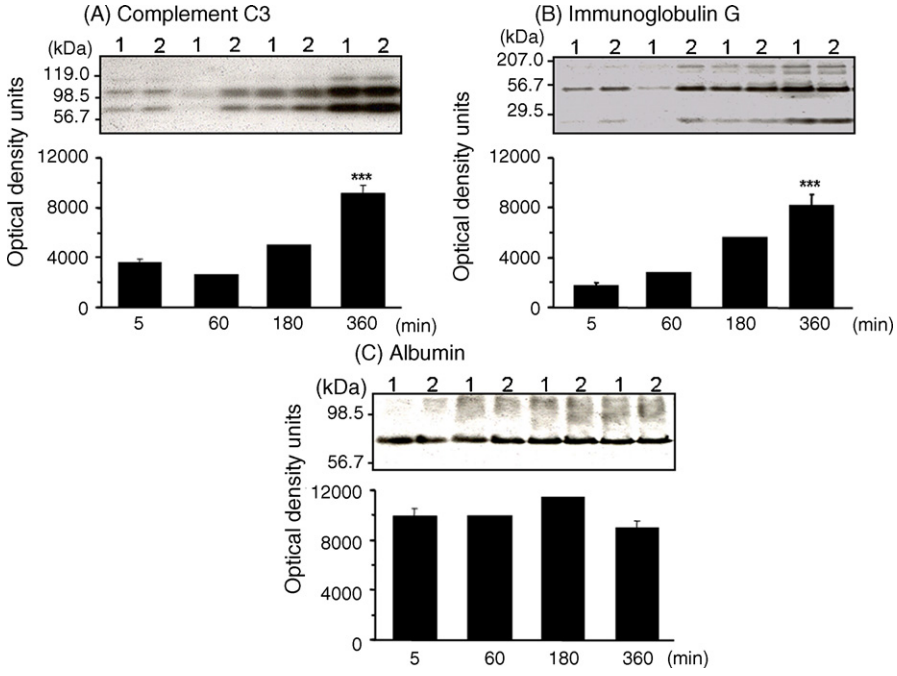

Fig. 4. Semi-quantification of complement C3 and immunoglobulin G associated on the surface of LNS-50 by Western blot analysis. Two samples for each incubation time were independently prepared and subjected to Western blot analysis. The same amount of protein (2.25 μg) was loaded on the gel. Results 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).

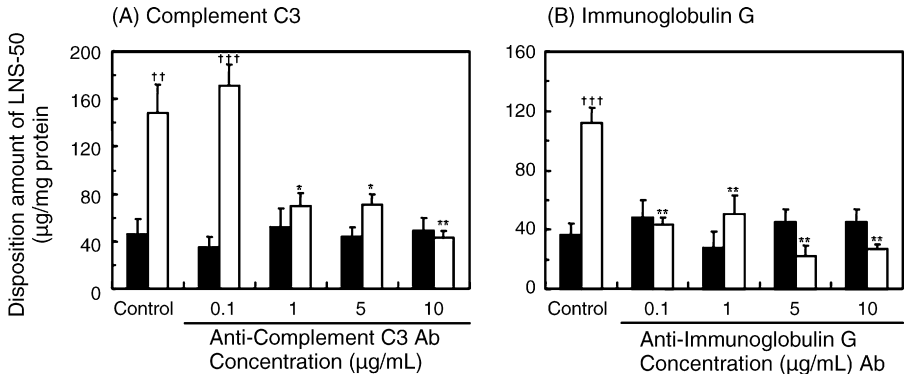

Fig. 5. Involvement of complement C3 and immunoglobulin G in the disposition of LNS-50 (360 min) to Kupffer cells. LNS-50 (100 μg/mL) suspended with PBS was simultaneously added to the cells with the various concentrations of anti-complement C3 antibody or anti-immunoglobulin G antibody. Results are 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. $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$, 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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