concentration of each tissue extract was determined by the modified Lowry method. Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

2.8. Tissue distribution of the [^{32}P]lipoplex

The [^{32}P]lipoplex was intravenously injected into mice. At predetermined time periods after injection, the blood was collected from the vena cava under ether anesthesia. The mice were then killed and the liver, spleen, kidney, lung, and heart were excised, rinsed with saline, and weighed. Tissue samples were digested in 0.7 ml Soluene-350 (Packard, Merdin, CT, USA) by overnight incubation at 45 °C. Then, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 M HCl, and, finally, 5 ml Clear-Sol I (Nacalai Tesque, Kyoto, Japan) were added to the digested samples. The radioactivity of the samples was assayed in a Beckman Model LS5000TA liquid scintillation counter (Beckman, Tokyo, Japan).

2.9. Intrahepatic distribution of the [^{32}P]lipoplex

Ten minutes after intravenous injection of the [^{32}P]lipoplex, the liver of each mouse was perfused with a buffer [16] and the liver cells were separated into parenchymal cells (PC) and nonparenchymal cells (NPC) by differential centrifugation. Then, the ^{32}P radioactivity of the cell suspensions was assayed as above.

2.10. Fractionation of liver homogenate

After intravenous injection of [^{111}In]Man-BSA, naked [^{111}In]pDNA or [^{111}In]both lipoplex into mice, the liver was sampled at intervals. HEPES–sucrose buffer (250 mM sucrose, 20 mM HEPES, 2 mM EDTA, pH 7.2) was added to the liver in a ratio of 3 μl buffer/mg tissue, then the liver was homogenized in a Potter-type homogenizer. The homogenate was centrifuged at 4 °C for 10 min at 800 × g to remove nuclei and debris. The supernatant was subsequently centrifuged at 4 °C for 30 min at 100,000 × g and the resulting supernatant was collected as the cytosol fraction. The pellet obtained was resuspended with 0.5 ml HEPES–sucrose buffer using a syringe and needle. Part of the suspension was mixed with HEPES–sucrose buffer and Percoll (Amersham Biosciences, Piscataway, NJ, USA) in a centrifuge tube. The final concentration of Percoll was adjusted to 35% (v/v). The sucrose solution (65% (w/v), 0.5 ml) was layered with a syringe and needle at the bottom of the centrifuge tube. The gradient was centrifuged at 4 °C for 25 min at 50,000 × g. Fractions (approximately 0.4 ml each) were collected from the bottom of the tube using a peristaltic pump and the radioactivity or enzyme activity of each fraction was measured.

2.11. Organelle marker detection

The activity of a fluid phase endocytosis marker, horseradish peroxidase (HRP), was detected using its substrate *o*-phenylenediamine dihydrochloride to identify the endosome fractions. Five minutes after injection of HRP, the liver was taken and fractionated by Percoll density gradient centrifugation as described above. Twenty microliters of each fraction was added to 150 μl 0.04% (w/v) *o*-phenylenediamine dihydrochloride, 0.012% (w/v) hydrogen peroxide in 0.1 M citrate–phosphate buffer (pH 5.0). The reaction was stopped by the addition of 20% (w/v) H_2SO_4 (50 μl). The activity of a lysosomal marker enzyme, β-hexosaminidase, was determined with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. Twenty microliters of each fraction was added to 50 μl 1.2 mM 4-methylumbelliferyl-β-D-galactoside solution and subsequently incubated at 37 °C for 30 min. The reaction was stopped by the addition of 150 μl 0.5 M glycine in 0.5 M carbonate buffer (pH 10.0). The fluorescence intensity of each sample was measured (excitation wavelength 365 nm; emission 460 nm).

2.12. PCR amplification of pDNA

pDNA in the subcellular fractions of the liver was purified by GenElute Plasmid Miniprep Kit (Sigma). Then, aregion of about 2.8 kbp of the pDNA containing the luciferase gene was amplified by PCR using a forward (5′-GTATCTGCTCCCTGCTTGTG-3′) and reverse (5′-TCCGCCTCAGAAGCCATAGA-3′) primer under standard conditions for 20 cycles.

3. Results

3.1. Physicochemical properties and gene expression of the lipoplex

The zeta potential of lipoplex and Man lipoplex was 9.78 ± 3.5 ($n=3$) and 12.5 ± 4.11 ($n=3$), respectively. The mean particle size of lipoplex and Man lipoplex was 287.2 ± 1.2 nm ($n=3$) and 285.4 ± 18.3 nm ($n=3$), respectively. Thus, physicochemical properties of both lipoplex were almost the same.

Fig. 1A demonstrates the gene expression after intravenous administration of Man lipoplex at 3 and 6 h. High gene expression was observed in the liver and spleen, which is expressed mannose receptor on cell surface. Fig. 1B shows the gene expression after intravenous administration of lipoplex at 6 h. Among these organs, the highest gene expression was observed in the lung.

3.2. Tissue distribution of the [^{32}P]lipoplex

Fig. 2 shows that the tissue radioactivity after intravenous administration of [^{32}P]lipoplex and Man lipoplex up to 6 h. Both [^{32}P]lipoplex rapidly disappeared from the blood circulation. [^{32}P]Lipoplex accumulated in the lung and liver, whereas [^{32}P]lipoplex accumulated largely in the liver.

3.3. Intrahepatic distribution of the [^{32}P]lipoplex between liver PC and NPC

After intravenous administration of the [^{32}P]Man lipoplex, the radioactivity in the liver was preferentially recovered from the NPC fractions, with the radioactivity ratio of NPC to PC (NPC/PC ratio on a cell-number basis) in the liver being approximately 9.6 (Fig. 3). On the other hand, the [^{32}P]lipoplex had an NPC/PC ratio of 1.9.

3.4. Subcellular localization of the [^{111}In]lipoplex

Fig. 4 shows the radioactivity of the cytosolic fractions in liver homogenate after intravenous administration of the [^{111}In]lipoplex and Man lipoplex up to 60 min. In both cases, the radioactivity gradually increased with time. Thirty and sixty minutes after intravenous administration, however, the cytosolic amount of the [^{111}In]Man lipoplex was always greater than that of the [^{111}In]lipoplex, suggesting that Man liposomes facilitate the release of pDNA from intracellular vehicles into the cytosol.

To characterize the gradient of liver homogenate using Percoll-gradient centrifugation, the enzymatic activity of β-hexosaminidase, a lysosome marker, was used for the determination of the endosomal fractions. As shown in Fig. 5A, β-hexosaminidase activity was predominantly detected in the lower

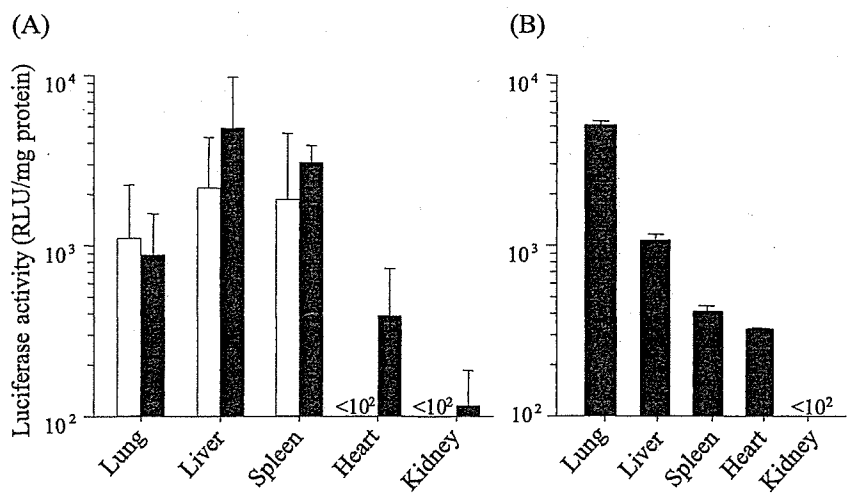

Fig. 1. Gene expression after intravenous administration at 3 (□) and 6 h (■) of Man lipoplex (A) and at 6 h of lipoplex (B) in mice. Each value represents the mean + S.D. values of three experiments.

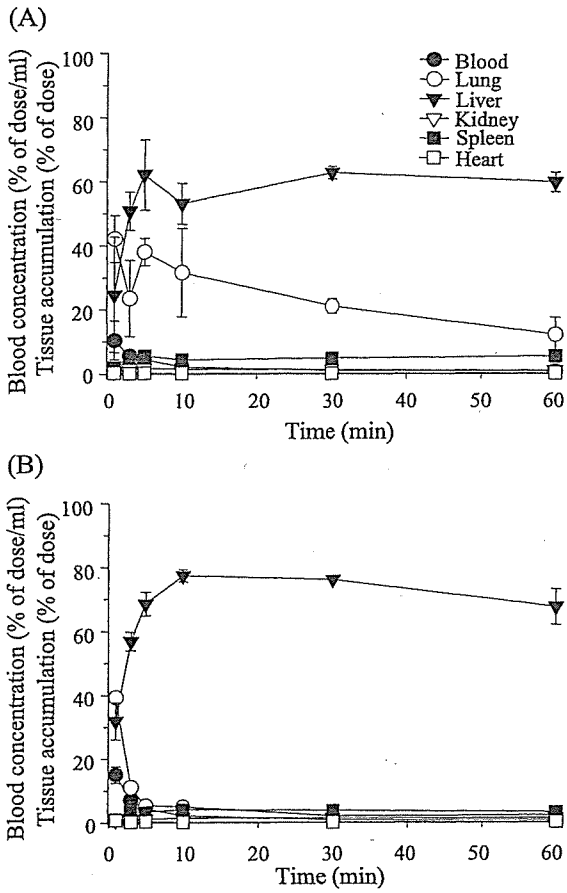

Fig. 2. Radioactivity in blood and tissues after intravenous administration of [^{32}P]lipoplex (A) or Man lipoplex (B) in mice. Each value represents the mean ± S.D. values of three experiments.

seven fractions. In a similar manner, the activity of horseradish peroxidase (HRP) injected intravenously into mice was used to confirm the lysosomal fractions. HRP was predominantly recovered in the upper seven fractions (data not shown). Therefore, the upper seven fractions and the lower seven fractions were identified as the endosomal and lysosomal fractions, respectively. To validate this system for the subcellular sorting of externally internalized compounds, the localization of [^{111}In]Man-BSA, a well-known ligand for mannose receptors [23], was then examined. At 15 min after intravenous administration, the radioactivity was largely recovered in the endosomal fractions, then the majority was transferred to the lysosomal fractions with time (Fig. 5B). These

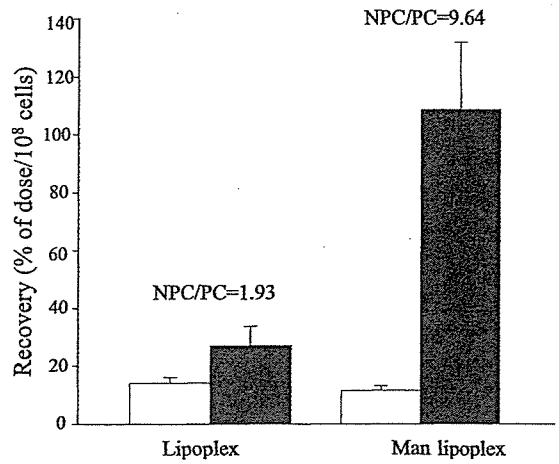

Fig. 3. Cellular localization of radioactivity between PC (□) and NPC (■) 30 min after intravenous administration of [^{32}P]lipoplex and Man lipoplex in mice. Each value represents the mean + S.D. values of three experiments.

results for [^{111}In]Man-BSA were in good agreement with previously reported characteristics of these ligands as far as mannose receptors [24] were concerned, indicating the validity of the detection method.

Fig. 6 shows the endosomal and lysosomal localization of radioactivity after intravenous admin-

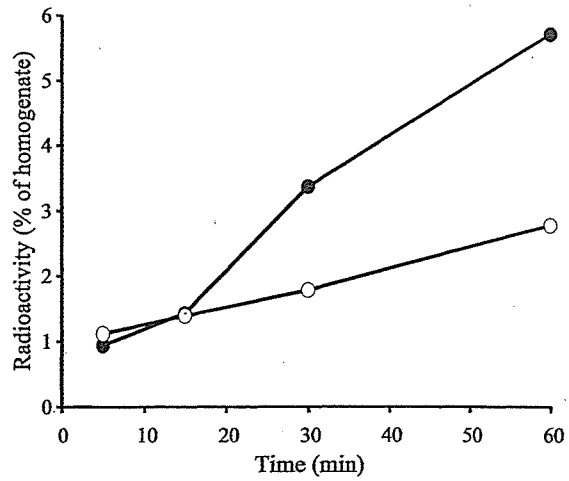

Fig. 4. Radioactivity in cytoplasmic fraction of mouse liver homogenates 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (○) or Man lipoplex (●) in mice. Similar results were obtained in two other independent runs.

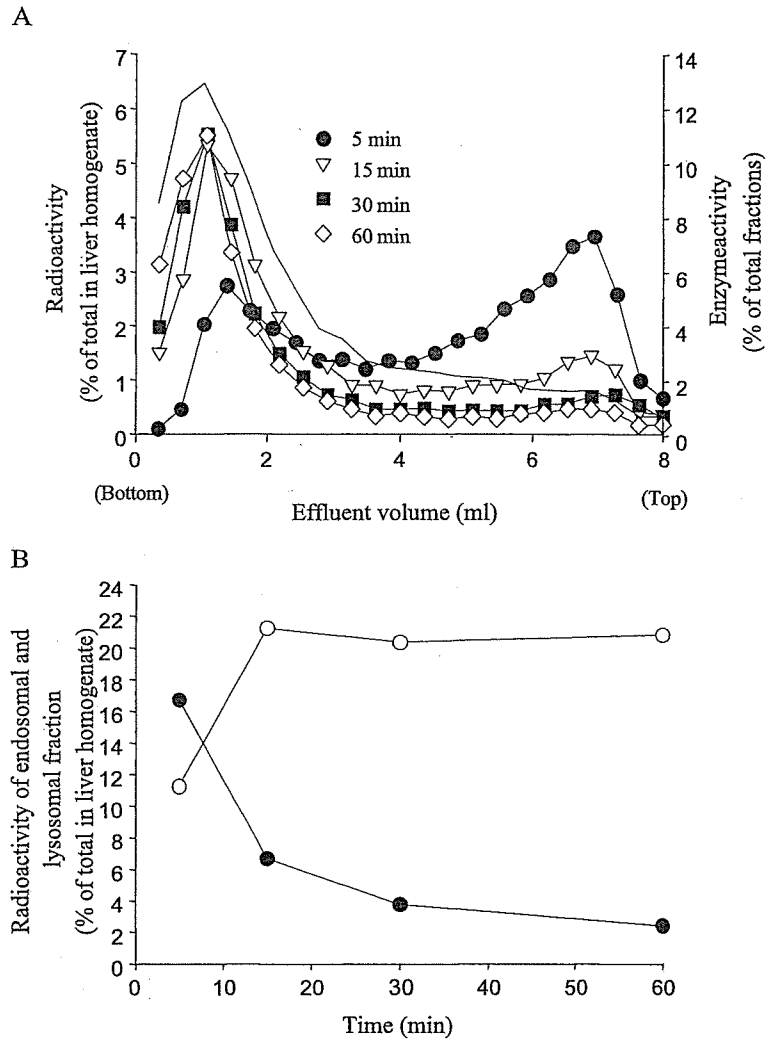

Fig. 5. Each fraction (A) and calculated endosomal (●) or lysosomal (○) localization (B) of radioactivity 5, 15, 30, and 60 min after intravenous administration of [^{111}In]mannosylated bovine serum albumin in mice. Liver homogenate was separated by Percoll density gradient centrifugation. Solid line in A represents the localization of β-hexosamidase activity. Similar results were obtained in two other independent runs.

istration of [^{111}In]lipoplex (B) or Man lipoplex (D). Sixty minutes after intravenous administration, the [^{111}In]Man lipoplex showed a larger distribution to the lysosome fractions than the [^{111}In]lipoplex. For comparison, naked [^{111}In]pDNA, which is extensively accumulated in the liver after intravenous administration but exhibits little gene expression [25], was also subjected to this assay (Fig. 6F). Naked [^{111}In]pDNA showed faster transfer to the lysosome fractions than both the [^{111}In]lipoplex and Man lipoplex.

3.5. PCR amplification

To determine whether pDNA within the subcellular fractions retained its structure, the luciferase sequence of pDNA was amplified by PCR (Fig. 7). When the Man lipoplex was intravenously adminis-

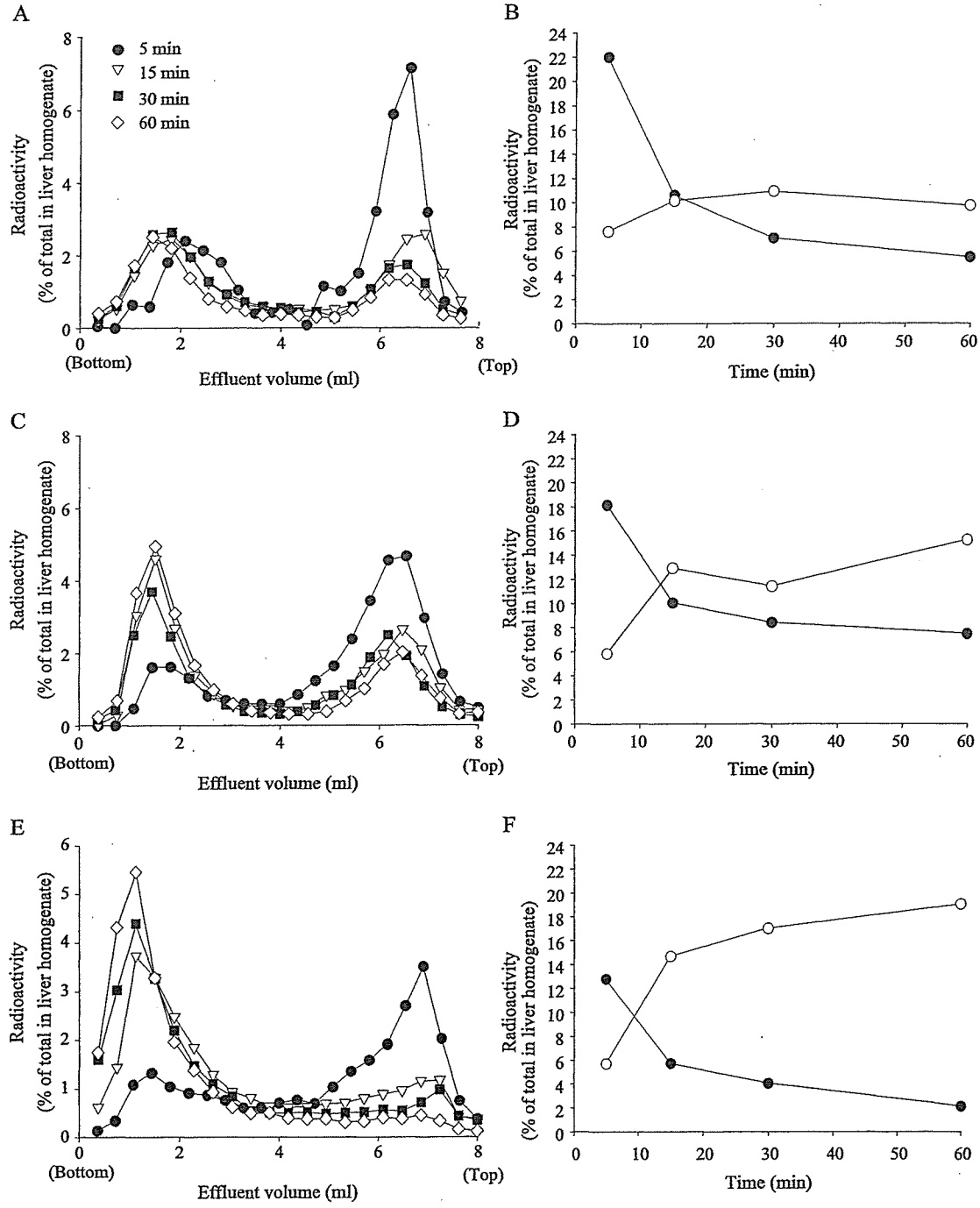

Fig. 6. Each fraction (A, C, and E) and calculated endosomal (●) and lysosomal (○) localization (B, D, and F) of radioactivity 5, 15, 30, and 6 min after intravenous administration of [^{111}In]lipoplex (A, B), Man lipoplex (C, D), or naked [^{111}In]pDNA (E, F) in mice. Liver homogena was separated by Percoll density gradient centrifugation. Similar results were obtained in two other independent runs.

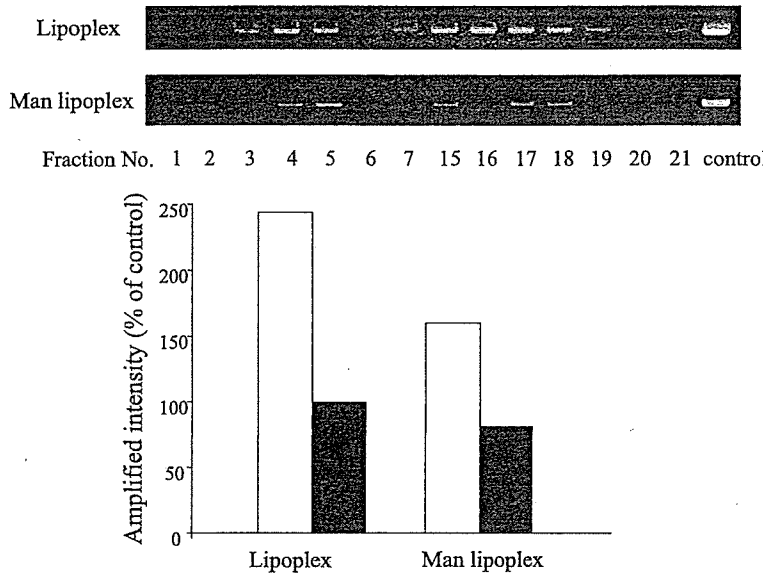

Fig. 7. Amplification of pDNA in endosomal (□) and lysosomal (■) fractions 30 min after intravenous administration of lipoplex and Man lipoplex in mice. Liver homogenate was separated by Percoll density gradient centrifugation. pDNA (1 ng/μl) was used as a control. Similar results were obtained in two other independent runs.

tered, the amounts of DNA amplified from the endosome and lysosome fractions were smaller than those after administration of the lipoplex.

4. Discussion

Transgene expression in target cells after intravenous administration of the Man lipoplex involves a number of distribution processes for pDNA: delivery to the target cells (tissue distribution), internalization, intracellular sorting, and nuclear entry [26]. In particular, the data presented in this study show the importance of intracellular sorting for efficient gene transfection of the Man lipoplex.

Since lipoplex was taken up the cell by the mechanism of endocytosis, pDNA needs to avoid degradation in lysosomes for improving the transfection efficiency. Thus, understanding of the intracellular fate of pDNA will help in the development of better transfection carrier systems. However, there are only a few studies that quantitatively investigate the intracellular fate of pDNA under in vivo conditions. Although so far several tracing methods of pDNA have been used such as ^{32}P-label by nick translation, however, radioactive metabolite, which are generated before and after the cellular uptake of radiolabeled pDNA, often make it extremely difficult to quantitative analyze the tissue distribution and pharmacokinetics of pDNA. In our preliminary experiment, when the fate of internalized [^{32}P]pDNA in liver homogenate was investigated, we found that [^{32}P]pDNA was not suitable for subcellular distribution studies because the radioactivity derived from [^{32}P]pDNA rapidly diminishes due to degradation during the preparation of subcellular fractions. More recently, we have developed [^{111}In]pDNA, an alternative radiolabeling method for pDNA [22], in a similar manner to the preparation of ^{111}In-labeled proteins [27,28], and demonstrated that the radioactivity of [^{111}In]pDNA is slowly released from cells after internalization. Therefore, [^{111}In]pDNA is considered the suitable method for analyzing the tissue and intrahepatic distribution of Man lipoplex. In fact, we observed that both [^{32}P]pDNA and [^{111}In]pDNA mainly accumulated in the liver after intravenous administration, but the radioactivity of ^{32}P gradually decreased (Fig. 2). In contrast, ^{111}In remained at a high level for 2 h after administration (data not shown). Such differences were explained by the poorer membrane permeability

of radioactive metabolites due to the attachment of DTPA for chelation of ^{111}In. Taking this into consideration, [^{111}In]pDNA was effective rebelling method for the subcellular distribution study of lipoplex.

As shown in Fig. 4, the cytoplasmic radioactivity was increased with time after intravenous injection of the [^{111}In]lipoplex and Man lipoplex; accordingly, pDNA is considered to be efficiently released into cytoplasm. On the other hand, the shift of radioactivity from the endosomal to the lysosomal fractions after intravenous administration of the [^{111}In]lipoplex and Man lipoplex suggests that both DC and Man liposomes promote pDNA transfer to lysosomes in the cell (Fig. 6). We previously reported that mannosylated proteins are internalized faster than cationic proteins, which are internalized by the liver via adsorptive endocytosis [16]. Receptor-mediated uptake of the Man lipoplex would explain its faster transport to the lysosome fractions. When naked [^{111}In]pDNA was injected intravenously, however, it showed more rapid transfer to lysosomes than both pDNA complexes. The lysosomes are where internalized substances are degraded, and it can be considered that lysosomal delivery is not suitable for transgene expression. Amplification of pDNA by PCR supported that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex (Fig. 7). Therefore, these results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

After administration into the blood circulation, the lipoplex interacts with various cells and molecules, such as serum proteins and erythrocytes [29,30]. The cationic nature of the lipoplex attracts negatively charged cells and molecules, which eventually leads to an alteration in the physicochemical properties of the complex. Generally, cellular uptake of a lipoplex is considered to be a nonspecific process based on the interaction of its excess positive charge and the negatively charged cell membrane. Thus, high accumulation of radioactivity was observed both in lung and liver after intravenous administration of the [^{32}P]lipoplex (Fig. 2). On the other hand, the [^{32}P]Man lipoplex did not accumulate in the lung to any great extent compared with the [^{32}P]lipoplex, suggesting that nonspecific interaction could be reduced by mannosylation of cationic liposomes. This distribution study may be partly supported by the fact that our previous observation involved the liver NPC selective gene transfection after intravenous administration of Man lipoplex [10].

In conclusion, the Man lipoplex showed specific accumulation in NPC and achieved higher gene expression than the lipoplex after intravenous administration. It was shown that pDNA delivered by Man liposomes, which is taken up by the mannose receptor, was more susceptible to intracellular degradation than that delivered by conventional cationic liposomes, and this would impair higher gene expression. Also, this observation leads us to believe that further carrier development studies are needed for improving the intracellular sorting of pDNA to avoid degradation.

Acknowledgements

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Effect of cationic charge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice

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The purpose of this study was to evaluate the effect of cationic charge of complexes after intravenous administration of cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)amino]butyl}formamide (Man-C4-Chol) containing cationic liposomes/pDNA complexes in mice. Transfection efficiency after intravenous administration of complex at a charge ratio (− : +) of 1.0 : 2.3 and/or 1.0 : 3.1 in liver and spleen expressing a mannose receptor on the cell surface were higher than those in lung. When complexes were formed at a charge ratio (− : +) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Although asialoglycoprotein receptors are expressed on hepatocytes, a liver-selective gene transfection was not achieved by the intravenous administration of pDNA complexed with cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)-amino]butyl}formamide (Gal-C4-Chol)/DOPE liposomes at a charge ratio (− : +) of 1.0 : 2.3. This information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery after intravenous administration.

1. Introduction

The success of *in vivo* gene therapy relies on the development of a vector that achieves target cell-specific, efficient, and prolonged transgene expression following its application. Non-viral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive for clinical application. Although the cationic liposome/plasmid DNA (pDNA) complex is a useful non-viral vector, it lacks specificity in delivery and transfection. Intravenous administration of pDNA/cationic liposome complexes expressed genes into various tissues, with highest gene expression in the lung (Templeton et al. 1997; Li et al. 1997). The attachment of a ligand that can be recognized by a specific mechanism would endow a vector with the ability to target a specific population of cells. In the search for cationic liposomes-based non-viral vectors, several ligands including asialofetuin (Hara et al. 1995), galactose (Kawakami et al. 1998; 2000a; Fumoto 2003), and mannose (Kawakami et al. 2000b; 2001; Sato et al. 2001) have been used to improve the delivery of pDNA to target cells. Therefore, the incorporation of such ligands into cationic liposomes would improve the cell-specificity of *in vivo* gene transfer by cationic liposome/pDNA complexes.

The mannose receptor is expressed on Kupffer cells, splenic, alveolar, peritoneal macrophages, monocyte-derived dendritic cells, and subsets of vascular and lymphatic endothelial cells (Weis et al. 1998). Recently, we designed cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)-amino]butyl}formamide (Man-C4-Chol) to prepare mannosylated cationic liposomes for mannose receptor-mediated gene delivery (Kawakami et al. 2000b). In the previous study, we have reported that the highest gene expression in the liver and spleen after intravenous injection was observed for Man-C4-Chol/dioleoylphosphatidylethanolamine (DOPE) liposome/pDNA complexes via mannose receptor-mediated endocytosis (Kawakami et al. 2000b). However, not only the nature of the ligands grafted to carriers but also the overall physicochemical properties of the complexes need to be optimized for the successful delivery of pDNA under systemic injection (Mahato et al. 1997; Kawakami et al. 2002). Although it is well known that a high cationic charge of cationic liposome/pDNA complexes enhances the transfection efficiency in the lung after intravenous injection because of the non-specific interaction between lung and complex (Templeton et al. 1997; Li et al. 1997), the effect of a cationic charge of ligands grafted cationic liposomes/pDNA complex on cell-selective transfection efficiency is not clear. Once the *in vivo* gene expression is linked with its physicochemical properties, it is then possible to design liposomes or a pDNA/liposome complex to enable cell-specific *in vivo* gene delivery.

In the present study, we tried to elucidate the effect of a cationic charge of complexes after intravenous injection of Man-C4-Chol containing a cationic liposome/pDNA complex in mice. Also, we evaluated the transfection efficiency of Man-C4-Chol containing a cationic liposome/pDNA complex using primary cultured mouse peritoneal

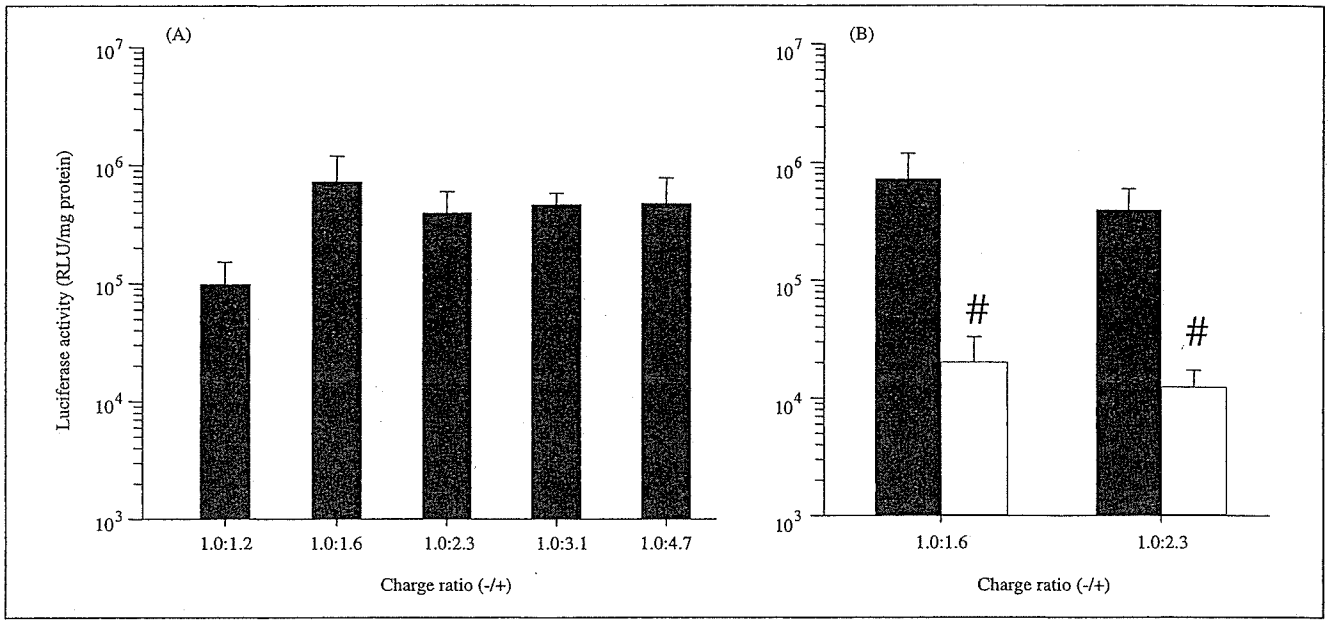

Fig. 1: Transfection activity at various charge ratio (− : +) (A) and the absence (■) or presence (□) of 1mg/ml mannan on transfection activity (B) of pDNA complexed with Man-C4-Chol/DOPE liposomes in cultured mouse peritoneal macrophages. pDNA concentration was fixed at 0.5 μg/ml in all experiments. Each value represents the mean ±S.D. values (n = 3). Stastical analysis was performed by analysis of variance ($^{\#}P < 0.05$)

macrophages which express the mannose receptor, to compare the results with *in vivo* application. pCMV-Luc was selected as a model pDNA because transfected luciferase is easy to detect by fluorescence.

2. Investigations, results and discussion

Complex formation between pDNA and Man-C4-Chol/DOPE complex at a charge ratio (− : +) of 1.0 : 1.2, 1.0 : 1.6, 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 was determined by agarose gel electrophoresis, followed by gel staining with ethidium bromide and photography under UV light. The addition of any Man-C4-Chol/DOPE liposome to pDNA at these charge ratios resulted in the formation of complexes that did not move towards the positive pole (data not shown).

First, we evaluated the transfection efficiency and its gene expression mechanism on transfection of pDNA complexed with Man-C4-Chol/DOPE liposome at various charge ratios (− : +) in cultured mouse peritoneal macrophages. As shown in Fig. 1, the transfection efficiency was almost the same at charge ratios (− : +) from 1.0 : 1.2 to 1.0 : 4.7. In the presence of 1 mg/ml mannan, the transfection efficiency of pDNA complexed with Man-C4-Chol/DOPE liposomes was significantly reduced. This result suggested that the mannose receptor-mediated endocytosis is involved in the transfection mechanism of pDNA complexed with Man-C4-Chol/DOPE liposomes.

Table: Mean particle sizes of liposome/pDNA (50 μg) complexes in 5% dextrose for *in vivo* experiments

Charge ratio (− : +)	Particle size (nm)
1.0 : 1.2	Aggregated
1.0 : 1.6	Aggregated
1.0 : 2.3	178.1 ± 13.1
1.0 : 3.1	118.6 ± 3.7
1.0 : 4.7	121.3 ± 8.5

Each value represents the mean ± S.D. values (n = 3).

Ogris et al. reported that mixing complexes at low ion strength prevents aggregation, although, large complexes resulting from aggregation showed high transfection efficiency *in vitro* in the case of DNA/transferrin-PEI complexes (Ogris et al. 1998). Referring to this report, the complexes were prepared with 5% dextrose solution. The Table 1 summarizes the particle sizes of Man-C4-Chol/DOPE liposome/pDNA complexes in 5% dextrose solution. When 50 μg of pDNA was mixed with Man-C4-Chol/DOPE liposomes, complexes at a charge ratio (− : +) of 1.0 : 1.2 and 1.0 : 1.6 were aggregated. In contrast, complexes at a charge ratio (− : +) from 1.0 : 2.3 to 1.0 : 4.7 can be prepared and their size is approximately 120–180 nm. The gene expression in mice following the intravenous injection with pDNA complexed with Man-C4-Chol/DOPE liposomes at charge ratio (− : +) of 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 is shown in Fig. 2. The transfection efficiency after intravenous administration of

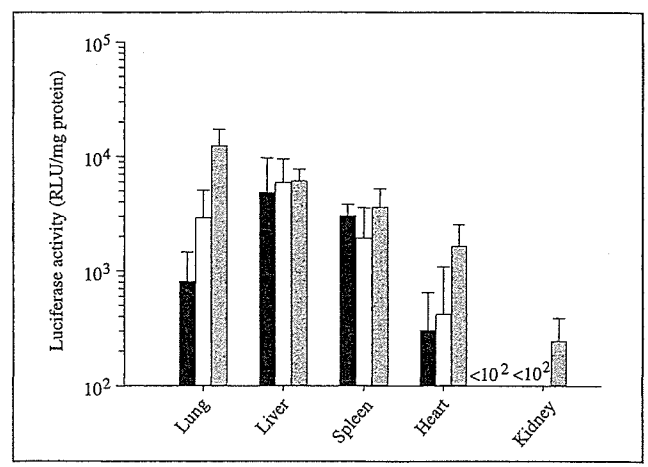

Fig. 2: Transfection activity of pDNA complexed with Man-C4-Chol/DOPE liposomes after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3 (■), 1.0 : 3.1 (□), and 1.0 : 4.7 (▨). Luciferase activity was determined 6 h post-injection in the lung, liver, spleen, heart, and kidney. Each value represents the mean ±S.D. values (n = 3)

complexes at charge ratios (− : +) of 1.0 : 2.3 and 1.0 : 3.1 in liver and spleen, expressing mannose receptors on the cell surface, were higher than those in lung. When the complex was formed at a charge ratio (− : +) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Also, these results are well congruent with our previous report concerning the asialoglycoprotein receptor-mediated gene transfection following the intraportal administration of pDNA complexed with DOTMA/Chol/cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)amino]butyl}formamide (Gal-C4-Chol) liposomes in mice (Kawakami et al. 2000a).

Intravenous gene delivery to the lung via pDNA/cationic liposome complexes has been reported. Several parameters have been identified to be important for achieving a high level of gene expression (Huang and Li 1997). Among them, a high cationic charge between a pDNA and cationic lipid is important for efficient intravenous gene delivery; however, these strategies of gene transfection include non-specific adsorption mediated endocytosis. In the present study, we demonstrated that liver and spleen selective gene transfection using mannose receptor mediated endocytosis is achieved by complexes prepared at a charge ratio (− : +) of 1.0 : 2.3 and/or 1.0 : 3.1. An excess cationic charge of complexes resulted in enhanced gene expression in the lung with non-specific interaction.

It has been reported that hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Referring to the results presented in Fig. 2, pDNA/galactosylated liposome complexes were prepared at a charge ratio (− : +) of 1.0 : 2.3. Sinusoids in the liver lobules are invested with a unique type of endothelial lining consisting of endothelial cells with flattened processes perforated by small fenestrae of about 200 nm in size. Therefore, pDNA/liposome complexes with a diameter less than this can readily pass through the fenestration into the Disse space and we prepared pDNA complexed with Gal-C4-Chol/DOPE liposomes having a size of approximately 150 nm in diameter for free to access to hepatocytes. Although the size of the liposomes was approximately 150 nm, selective gene targeting to the liver was not achieved (Fig. 3). For the target cells of mannosylated liposome/pDNA complexes, there is not need to pass through fenestrae; therefore, selective gene targeting in liver and spleen on macrophages may be achieved by intravenous administration.

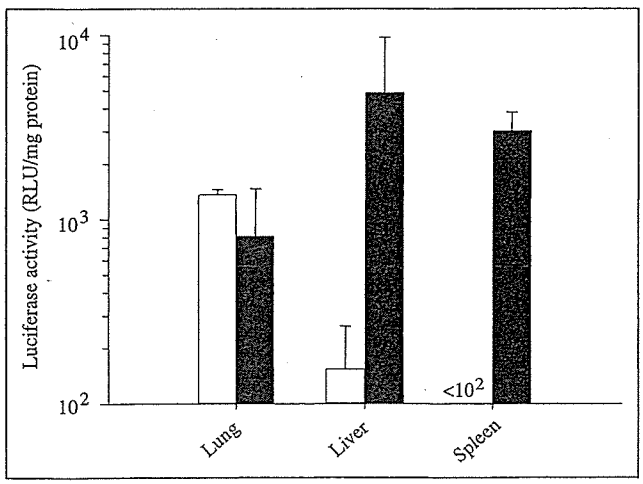

Fig. 3: Transfection activity of pDNA complexed with Gal-C4-Chol/DOPE liposomes (□) or Man-C4-Chol/DOPE liposomes (■) after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3. Luciferase activity was determined 6 h post-injection in the lung, liver, and spleen. Each value represents the mean ±S.D. values (n = 3)

The difference of transfection between galactosylated liposomes and mannosylated liposomes suggested that the size of complexes at a charge ratio (− : +) of 1.0 : 2.3 might be increased over 200 nm after intravenous administration. Thus, not only the introduction of a ligand to the cationic liposomes but also a controlled size of complexes is important for an efficient targeted gene delivery. We previously reported that the pDNA/cationic liposome complexes are interacting with erythrocytes after intravenous administration (Sakurai et al. 2001). Recently, Eliyahu et al. (2002) also characterized the interaction of blood components with pDNA/cationic liposome complexes under conditions relevant to *in vivo* intravenous administration. In their report, the selection of a medium (i.e. plasma and serum) and/or modification of the cationic liposomes with 1% polyethyleneglycol lipids reduced the aggregation of pDNA/cationic liposome complexes in the presence of erythrocytes; accordingly, such approaches may enhance the cell-specificity of pDNA complexed with galactosylated and mannosylated cationic liposomes. Further studies on the interaction with blood components and/or the synthesis of polyethyleneglycol-grafted glycosylated lipids for cell-selective gene delivery are required. These information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery under *in vivo* conditions.

3. Experimental

3.1. Materials

N-(4-Aminoethyl) carbamic acid *tert*-butyl ester and *N*-(4-aminobutyl) carbamic acid tert-butyl ester, *N*-(4-aminohexyl) carbamic acid tert-butyl ester were obtained from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate and DOPE were obtained from Sigma Chemicals (St. Louis, MO) and Avanti Polar-Lipids (Alabaster, AL), respectively. Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD). Opti-MEM I and other culture reagents were obtained from Gibco BRL (Grand Island, NY). All other chemicals were of the highest purity available.

3.2. Construction and preparation of pDNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA). pDNA was amplified in the E. coli strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

3.3. Synthesis of Man-C4-Chol and Gal-C4-Chol

As reported previously (Kawakami et al. 2000c), *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained from cholestery chloroformate and *N*-(4-aminobuthyl) carbamic acid tert-butyl ester. The product was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside or 2-imino-2-methoxyethyl-1-thiogalactoside (Lee et al. 1976) in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture *in vacuo*, the resultant material was suspended in water and dialyzed against water for 48 h. Then, the compounds were lyophilized.

3.4. Particle size measurements

The particle sizes of liposome/pDNA complexes were measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan).

3.5. Harvesting and culture of macrophages

Male ICR mice weighing 20–25 g were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Elicited macrophages were harvested from mice 4 days after intraperitoneal injection of 1 ml 2.9% thioglycolate medium (Nissui Pharmaceutical Co. LTD., Tokyo, Japan). The washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum

(FBS, Flow Laboratories, Irvine, U.K.), penicillin G (100 U/ml), and streptomycin (100 μg/ml) and were plated on 6- or 12-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) at a density of 3×10^5 cells/cm^2. After incubation for 24 h at 37 °C in 5% CO_2 – 95% air, non-adherent cells were washed off with culture medium and cells were cultivated for another 48 h.

3.6. Preparation of pDNA/liposome complexes for in vitro experiments

Man-C4-Chol was mixed with DOPE at a molar ratio of 3 : 2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 1 ml sterile 20 mM HEPES buffer (pH 7.8) in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 μM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (1 mg/ml) (Bartlett 1959). Cationic liposomes and pDNA (1.0 μg) in a 12 × 75 mm polystyrene tube were diluted with Opti-MEM at various charge ratios before carrying out the transfection experiment. The theoretical charge ratio of cationic lipid/pDNA was calculated as a molar ratio of Man-C4-Chol (monovalent) to a nucleotide unit (average molecular weight 330). Complex formation was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

3.7. In vitro transfection experiment

Macrophages were seeded in 10.5 cm^2 dishes at a density of 1.1×10^6 cells/cm^2 in RPMI 1640 supplemented with 10% fetal calf serum. After 3 days in culture, the culture medium was replaced with Opti-MEM I containing 0.5 μg/ml pDNA and cationic liposomes. Six hours later, the incubation medium was replaced again with RPMI 1640 supplemented with 10% FBS and incubated for an additional 18 h. Then, the cells were scraped and suspended in 200 μl pH 7.4 phosphate-buffered saline (PBS). One hundred microliters the cell suspension were subjected to three cycles of freezing (liquid N_2 for 3 min) and thawing (37 °C for 3 min), followed by centrifugation at 10,000 *g* for 3 min. The supernatants were stored at −20 °C until the luciferase assay was performed. Ten microliters of supernatant were mixed with 100 μl luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured in a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as the relative light units per mg protein. The protein content of the cell suspension in PBS was determined by the modified Lowry method using BSA as a standard (Wang and Smith 1975).

3.8. Preparation of pDNA/liposome complexes for in vivo experiments

Man-C4-Chol or Gal-C4-Chol was mixed with DOPE at a molar ratio of 3 : 2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 5 ml sterile 5% dextrose in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 μM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (3 mg/ml) (Bartlett 1959). Equal volumes of pDNA (50 μg) and stock liposome solution diluted with 5% dextrose solution to produce various ratios of pDNA/liposomes were mixed in 1.5 ml Eppendorf tubes at 4 °C. Then, the DNA solution was added rapidly to the surface of the liposome solution using a Pipetman and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip and was stored at 4 °C for 12 h.

3.9. In vivo transfection experiment

Five-week-old ICR mice were injected intravenenously with 300 μl of pDNA/liposome complexes using a 30-gauge syringe needle. Six hours after injection, mice were killed and lung, liver, kidney, spleen and heart were removed and assayed for gene expression. The organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1M Tris, pH 7.8). The lysis buffer was added in a weight ratio of 5 μl/mg for liver samples or 4 μl/mg for other organ samples. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C and 20 μl supernatant was analyzed to determine the luciferase activity using a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined by the modified Lowry method (Wang and Smith 1975). Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

3.10. Statistical analysis

Statistical comparisons were performed by analysis of variance. $P < 0.05$ was considered to be indicative of statistical significance.

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Note

Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells

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Abstract

The purpose of this study is to delineate uptake characteristics of mannosylated and fucosylated proteins in primary cultured sinusoidal endothelial cells and Kupffer cells. In cultured sinusoidal endothelial cells, uptake of mannosylated and fucosylated bovine serum albumin (BSA) was significantly inhibited by excess mannosylated and fucosylated BSAs but not by galactosylated BSA, suggesting that both glycosylated proteins might be primarily taken up via mannose receptors. In cultured Kupffer cells, uptake of fucosylated BSA was significantly inhibited by excess galactosylated BSA as well as mannosylated and fucosylated BSAs, although that of mannosylated BSA was inhibited only by mannosylated and fucosylated BSAs. This suggests that uptake of fucosylated BSA by Kupffer cells might be mediated by both Kupffer cell lectin (fucose receptor) and mannose receptor. On the other hand, in vivo hepatic uptake of fucosylated BSA was inhibited to a greater extent by $GdCl_3$ pretreatment than that of mannosylated BSA. Based on in vitro and in vivo experiments, it was concluded that fucosylated BSA is more Kupffer cell-selective because it exhibited a lower sinusoidal endothelial cell uptake than mannosylated BSA.

Keywords: Sinusoidal endothelial cells; Kupffer cells; Cell targeting; Drug delivery; Mannosylated BSA; Fucosylated BSA

1. Introduction

Receptor-mediated drug targeting is a promising approach to deliver therapeutic agents selectively to target cells and maximize their efficacy (Takakura

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and Hashida, 1996). We have demonstrated the usefulness of carbohydrate receptor-mediated drug delivery systems directed at both liver parenchymal (Nishikawa et al., 1995; Kawakami et al., 1998, 2000; Opanasopit et al., 2001a) and non-parenchymal (Ogawara et al., 1999; Opanasopit et al., 2001b) cells. Glycosylation of macromolecular (Nishikawa et al., 1993) and particulate (Kawakami et al., 1998; Ishida et al., 2004) carriers and proteinous drugs themselves (Fujita et al., 1992) with 2-imino-2-methoxyethyl-1-thioglycoside, which we have used so far, has several advantages including the simplicity of its structure, ease of synthesis, broad applicability, and satisfactory targetability.

In a series of investigations, we have demonstrated that mannosylated (Ogawara et al., 1999) and fucosylated (Opanasopit et al., 2001b) bovine serum albumins (BSAs) are efficiently taken up by liver non-parenchymal cells (NPC), mainly composed of sinusoidal endothelial cells and Kupffer cells, after intravenous injection. Similar results have been obtained with mannosylated liposomes (Opanasopit et al., 2001a), and fucosylated liposomes (Kawakami et al., 2000). Inhibition experiments (Opanasopit et al., 2001b) have shown that efficient NPC uptake of mannosylated and fucosylated delivery systems is primarily due to specialized sugar recognition systems.

However, the uptake by NPC via mannose and fucose receptors appears to be complicated. In vitro binding experiments using isolated receptor proteins have shown that the mannose receptor recognizes mannose and fucose but not galactose (Haltiwanger and Hill, 1986; Otter et al., 1992), and that the fucose receptor recognizes fucose and galactose but not mannose (Lehrman et al., 1986). It is also known that the mannose receptor is present on both Kupffer cells (Kuiper et al., 1994) and sinusoidal endothelial cells (Magnusson and Berg, 1993; Otter et al., 1992), and that the fucose receptor is presented on Kupffer cells (Lehrman and Hill, 1986; Haltiwanger et al., 1986). The multiplicities in substrate specificity and cellular localization of the receptors make it difficult to evaluate the contribution of each pathway to the overall in vivo NPC uptake. Further details of the mechanisms involved in NPC uptake are required to develop efficient drug delivery systems that can target sinusoidal endothelial cells and Kupffer cells. In order to obtain such information, it is necessary to elucidate the contribution of mannose receptor and fucose receptor on the uptake of mannosylated and fucosylated BSA using primary cultured Kupffer cells and sinusoidal endothelial cells.

This dearth of mechanistic information prompted us to investigate the uptake characteristics of mannosylated and fucosylated BSAs in rat Kupffer cells and sinusoidal endothelial cells. After both cell types were isolated from rat liver and cultivated, the cellular uptake characteristics of mannosylated and fucosylated BSAs were evaluated in the presence of inhibitors. In order to clarify the contribution of these cell types in vivo, we examined the hepatic uptake of mannosylated and fucosylated BSAs after intravenous administration of $GdCl_3$, a compound that down-regulates the function of Kupffer cells, to mice (Hardonk et al., 1992).

2. Materials and methods

2.1. Chemicals

BSA, collagenase (type I), dexamethasone, vascular endothelial growth factor (VEGF) and Minimum essential medium eagle Joklik modification for suspension culture were purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). D-Mannose, D-galactose, L-fucose, and chloramine-T were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Indium-111 chloride [^{111}In] was kindly supplied by Nihon Medi-Physics Co. Ltd. (Nishinomiya, Japan). Sodium iodide-125 [^{125}I] was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). RPMI1640 and Eagle's MEM 'Nissui' were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased from Dojindo Laboratory (Kumamoto, Japan). Rat tail collagen type I, insulin-transferin-selenious acid (ITS+ Premix), and human recombinant fibronectin were purchased from Becton Dickinson (Franklin Lakes, N.J., USA). All other chemicals were of the highest grade available.

2.2. Animals

Male ddY mice (5 weeks old) and male Wistar rats (200–250 g) were purchased from Shizuoka Agricul-

tural Co-operate Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by US National Institutes of Health and with the Guidelines of the Kyoto University Animal Experimentation Ethics Committee.

2.3. The synthesis of BSA derivatives with sugar moieties

Coupling of mannose and galactose moieties to BSA was carried out according to the method of Lee et al. (1976). Briefly, cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-D-mannoside was treated with 0.01 M sodium methoxide at room temperature for 24 h, and a syrup of 2-imino-2-methoxyethyl-1-thio-D-mannoside (IME-thiomannoside) was obtained after evaporation of the solvent. A quantity of the resultant syrup was added to BSA (100 mg) in 10 ml 50 mM borate buffer (pH 9.0). Coupling of fucose moieties to BSA was carried out by the method of Lee et al. (1976) with minor modifications (Kawakami et al., 2000). The molecular weight of the glycosylated proteins was determined by SDS-PAGE and the number of sugar residues was determined by the anthrone-sulfuric acid method using galactose, mannose and fucose as standards (Table 1).

2.4. Radiolabeling with indium-111 and iodine-125

^{111}In-labeled BSA was prepared using a bifunctional chelating agent, DTPA anhydride (Hnatowich et al., 1982). ^{125}I-labeling was performed by the chloramine-T method (Hunter and Greenwood, 1962).

2.5. Cell isolation and culture of sinusoidal endothelial cells and Kupffer cells

Liver sinusoidal endothelial cells and Kupffer cells from rat liver were isolated according to the method of Nagelkerke et al. (1983) with some modification. Endothelial cells were plated at 3.0×10^5 cells/cm^2 onto 24-well plates coated with rat tail collagen type I and human recombinant fibronectin, and cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 5 μg/ml vascular endothelial growth factor (VEGF), 1% (v/v) ITS+ Premix, 10 mg/ml amphotericin B, 10 μM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/ml L-glutamine for 3–4 days. Kupffer cells were cultured on 24-well plates (2.5×10^5 cells/cm^2) in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/ml L-glutamine, and 10 mg/ml amphotericin B for 24 h. Cells were cultured in a 5% CO_2–95% air incubator thermostatically controlled at 37 °C. The purity of the isolated endothelial cells and Kupffer cells was checked by factor VIII immunostaining and uptake by 4.5 μm latex beads, respectively.

2.6. Uptake of ^{125}I-glycosylated BSA in cultured liver sinusoidal endothelial cells and Kupffer cells

Prior to the uptake experiments, cells were equilibrated with serum-free RPMI medium containing 10 mM 2-[4-(2-hydoroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2% BSA at 37 °C for 20–30 min. Then, ^{125}I-glycosylated BSA (100,000 cpm/well) was added to the medium, followed by incubation at 37 °C for 2 h. In some groups, ^{125}I-glycosylated BSA was administered, together with different concentrations of mannosylated, fucosylated or galactosylated BSA. After 2 h incubation, the medium

Table 1
Physicochemical characteristics of synthesized glycosylated BSAs

Compounds	Molecular weight (Da)	Number of sugar residues (mol/mol BSA)
Mannosylated BSA (Man-BSA)	70800	36
Fucosylated BSA (Fuc-BSA)	70800	41
Galactosylated BSA (Gal-BSA)	68000	42

The number of molecular weight of glycosylated BSAs were estimated by SDS-PAGE. The number of sugar residue was determined by the anthron-sulfic method.

was collected, mixed with 45% trichloroacetic acid (TCA), and centrifuged to separate metabolites from intact glycosylated BSA. The radioactivity associated with metabolites in the supernatant was measured using a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan). In addition, the cells were washed three times with ice-cold phosphate-buffered saline and lysed with 0.25 ml of 0.3 M NaOH containing 0.1% Triton X-100. An aliquot of the lysate was taken for the determination of ^{125}I radioactivity and protein content. The radioactivity was counted by the scintillation counter while the protein content was measured by the modified Lowry method (Lowry et al., 1951) using BSA as a standard. The result of in vitro uptake experiment was expressed as total uptake amount of count of metabolites in the supernatant and of cell lysate.

2.7. *In vivo hepatic disposition in mice pretreated with $GdCl_3$*

An amount of 30 mg/kg $GdCl_3$ was intravenously injected into mice to down-regulate the functions of Kupffer cells (Hardonk et al., 1992). Twenty-four hours later, ^{111}In-mannosylated, ^{111}In-fucosylated or ^{111}In-galactosylated BSA (0.1 mg/kg) was administered by intravenous injection. At 5 min post injection, mice were killed under anesthesia and the liver was excised and plasma was collected. The radioactivity associated with these samples was determined in a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan).

3. Results

3.1. *Uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA by sinusoidal endothelial cells and Kupffer cells*

Fig. 1 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured sinusoidal endothelial cells. Without inhibitors, uptake of mannosylated BSA by sinusoidal endothelial cell was 1.4-fold higher than that of fucosylated BSA. In the presence of 50 mg/ml mannosylated or fucosylated BSA, the uptake of ^{125}I-mannosylated BSA was significantly inhibited (Fig. 1A). However, the uptake of ^{125}I-mannosylated BSA was not inhibited by the same concentration of galactosylated BSA (Fig. 1A). Similar inhibition patterns were observed with ^{125}I-fucosylated BSA (Fig. 1B).

Fig. 2 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured Kupffer cells. In the absence of inhibitors, ^{125}I-mannosylated and ^{125}I-fucosylated were similar with respect to the degree of uptake by Kupffer cells, and their uptake was significantly greater than that by cultured endothelial cells. The uptake of ^{125}I-mannosylated BSA was inhibited by mannosylated and fucosylated BSAs, but not by galactosylated BSA, similar to that observed in cultured endothelial cells. In contrast, the uptake of ^{125}I-fucosylated BSA was inhibited by galactosylated BSA, as well as mannosylated and fucosylated BSAs. Thus, fucosylated BSA exhibits different uptake characteristics from mannosylated BSA in Kupffer cells.

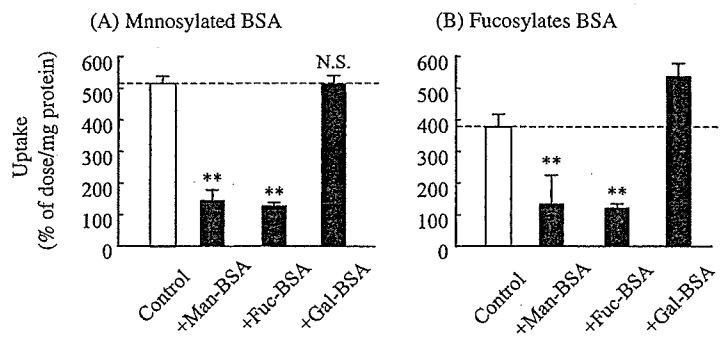

Fig. 1. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured endothelial cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$; N.S., not significant).

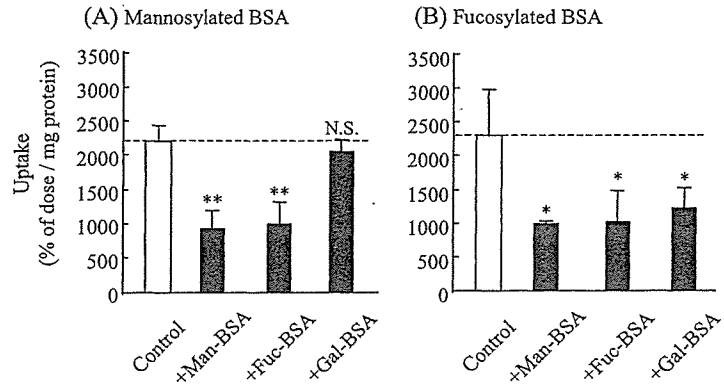

Fig. 2. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured Kupffer cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$; *, $P < 0.05$; N.S., not significant).

3.2. *Liver accumulation of ^{111}In-mannosylated and ^{111}In-fucosylated BSA with or without $GdCl_3$ pretreatment*

^{111}In-labeled BSA derivatives were used in the in vivo distribution studies, since ^{111}In is trapped in the cell interior after degradation so that the uptake rate can be evaluated precisely (Hnatowich et al., 1982). Fig. 3 shows the plasma concentration and hepatic uptake of ^{111}In-mannosylated and ^{111}In-fucosylated BSAs 5 min after intravenous injection. When mice were pretreated with $GdCl_3$, the plasma clearance and hepatic uptake of these proteins significantly decreased, and the inhibition effect was greater with fucosylated BSA than mannosylated BSA. Since $GdCl_3$ completely inhibits binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992), it appears that the contribution of Kupffer cells to uptake by whole liver is greater in the case of fucosylated BSA.

4. Discussion

Haltiwanger and Hill (1986) isolated macrophage mannose receptors from alveolar macrophages, and showed that these receptors recognize mannose, fucose, and *N*-acetylglucosamine but not galactose. Otter et al. (1992) found that mannose receptors

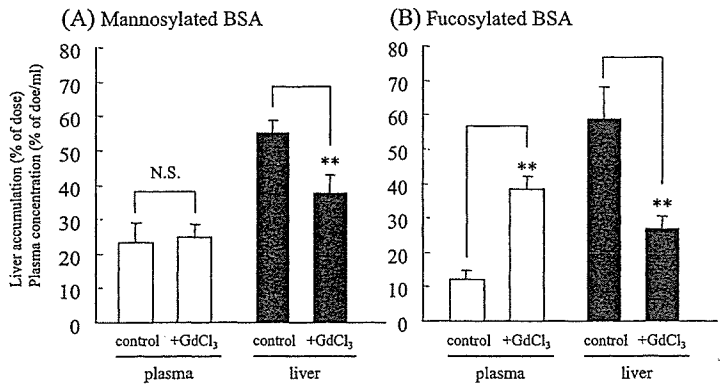

Fig. 3. Liver accumulation (■) and plasma concentration (□) of ^{111}In-mannosylated BSA (A), and ^{111}In-fucosylated BSA (B) (0.1 mg/kg) with or without pretreatment of $GdCl_3$ (30 mg/kg) at 5 min after intravenous injection into mice. $GdCl_3$ was injected 24 h before the experiments. Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$).

exhibiting the same substrate specificity are expressed in liver NPC. Magnusson and Berg (1993) reported that mannose receptors are expressed on isolated sinusoidal endothelial cells in binding experiments using an isolated sinusoidal endothelial cell suspension and ovalbumin, a mannose-terminated glycoprotein. In the present study, mannosylated and fucosylated BSAs were taken up efficiently by liver sinusoidal endothelial cells in primary culture, where the uptake was significantly inhibited by mannosylated and fucosylated BSAs but not by galactosylated BSA (Fig. 1). Therefore, it is likely that uptake of mannosylated and fucosylated BSAs by cultured sinusoidal endothelial cells is mediated primarily by mannose receptors.

Cultured Kupffer cells and sinusoidal endothelial cells exhibited similar inhibition profiles for mannosylated BSA, but not for fucosylated BSA: the uptake of fucosylated BSA was significantly inhibited by galactosylated BSA in Kupffer cells (Fig. 2B). Lehrman and co-workers isolated fucose receptors (Kupffer cell lectin) that are uniquely expressed in Kupffer cells by an immunohistological method (Lehrman and Hill, 1986), and demonstrated that binding of fucosylated BSA to these receptors can be inhibited by excess fucose or galactose, but not by mannose (Lehrman et al., 1986). In the present study, the uptake of fucosylated BSA was inhibited by galactosylated BSA (Fig. 2B), suggesting that fucose receptors might, at least in part, be responsible for the uptake of fucosylated BSA. In addition, mannose receptors also appear to be responsible for the uptake of fucosylated BSA, since the uptake of fucosylated BSA is inhibited by mannosylated BSA (Fig. 2B).

The present result for fucosylated BSA in Kupffer cells differed from that of Sarkar et al. (1996), who demonstrated that the uptake of fucose–human serum albumin (HSA) by isolated Kupffer cells was not inhibited by galactose–HSA. This difference might be due to the method of fucosylation: Sarkar et al. (1996) coupled *p*-aminophenyl-α-L-fucopyranoside and albumin with carbodiimide whereas we coupled albumin with 2-imino-2-methoxyethyl-1-thio-L-fucoside under weak alkaline conditions. Thus, it is likely that the difference in the structure at the 1-position of fucose might affect recognition of fucosylated albumin by Kupffer cell lectin that interacts with galactose. Glycosylation with 2-imino-2-methoxyethyl-1-thioglycoside is also characteristic in that the net charge of the proteins is unchanged since an imine is formed at the amino group modified with sugar. Jansen et al. (1991) demonstrated that para-aminophenyl mannose-terminated HSA was taken up by NPC via scavenger receptors, presumably due to an increase in negativity in accordance with sugar modification of the amino groups. However, they also showed that the liver uptake of mannosylated HSA synthesized using 2-imino-2-methoxyethyl-1-thiomannoside was not inhibited by formaldehyde-treated albumin, a typical scavenger receptor ligand (Jansen et al., 1991). Thus, the chosen method of sugar modification could play an important role in the design of glycosylated proteins.

We previously found that the cellular uptake of fucosylated BSA at 1 h post intravenous injection was 1.1, 6.2, and 21% of dose/10^8 cells for liver parenchymal cells, sinusoidal endothelial cells, and Kupffer cells, respectively (Opanasopit et al., 2001b), while that of mannosylated BSA was 0.841, 13.4, and 10.4% of dose/10^8 cells (Ogawara et al., 1999). To confirm the contribution of each cell type to the total hepatic uptake from a different point of view, we investigated the in vivo distribution of mannosylated and fucosylated BSAs in mice pretreated with $GdCl_3$ (Fig. 3). It is known that $GdCl_3$ completely inhibits the binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992). The reduction in the hepatic uptake produced by $GdCl_3$ pretreatment was greater for fucosylated BSA than mannosylated BSA (Fig. 3). This finding corresponded to previous results that fucosylated BSA was taken up more selectively by Kupffer cells than mannosylated BSA (Opanasopit et al., 2001b; Ogawara et al., 1999). The in vitro uptake experiment (Figs. 1 and 2) showed that the uptake by cultured sinusoidal endothelial cells was greater for mannosylated BSA than fucosylated BSA whereas the uptake by cultured Kupffer cells was similar for both proteins. Thus, the higher Kupffer cell-selectivity of fucosylated BSA might be due to lower uptake by the sinusoidal endothelium. When the data obtained following $GdCl_3$ pretreatment were compared with the data obtained from cell fractionation studies, account was taken of the fact that the number of sinusoidal endothelial cells was approximately twice that of Kupffer cells (Blomhoff et al., 1982; Pertoft and Smedsrod, 1986).

5. Concluding remarks

Different carbohydrate receptors with relatively broad substrate specificity are present in different hepatic cell types. Mannosylated BSA appears to be taken up via mannose receptors regardless of sinusoidal endothelial cells and Kupffer cells. However, it is likely that the uptake of fucosylated BSA by Kupffer cells is mediated by not only mannose receptors but fucose receptors while its uptake by sinusoidal endothelial cells is mediated primarily by mannose receptors. Moreover, fucosylated BSA is more Kupffer cell-selective because it exhibited a lower endothelial cell uptake than mannosylated BSA. This is potentially useful information for the development of carbohydrate receptor-mediated delivery strategies for biologically active proteins.

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