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Molecular Weight-Dependent Gene Transfection Activity of Unmodified and Galactosylated Polyethyleneimine on Hepatoma Cells and Mouse Liver

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To optimize a receptor-mediated and cell-selective gene transfer with polyethyleneimine (PEI)-based vector, we synthesized three galactosylated PEIs (Gal-PEI) with different molecular weights (PEI_{1800}, $PEI_{10,000}$, and $PEI_{70,000}$) and investigated their potential as a targetable vector to asialoglycoprotein receptor-positive cells. All PEI derivatives formed complexes with plasmid DNA (pDNA), whereas the particle size of the complex became smaller on increasing the molecular weight of PEI. Transfection efficiency in HepG2 cells with PEI was highest with PEI_{1800}; efficiency was next highest with $PEI_{10,000}$, although the cellular association was similar. After galactosylation, Gal_{19}-$PEI_{10,000}$/pDNA and Gal_{120}-$PEI_{70,000}$/pDNA showed considerable agglutination with a galactose-recognizing lectin, but Gal_9-PEI_{1800} did not, suggesting that galactose units on the Gal_9-PEI_{1800}-pDNA complex are not sufficiently available for recognition. Gal_{19}-$PEI_{10,000}$-pDNA and Gal_{120}-$PEI_{70,000}$-pDNA complexes showed galactose-inhibitable transgene expression in HepG2 cells. Transfection efficiency was greatest with Gal_{19}-$PEI_{10,000}$/pDNA, a result that highlights the importance of obtaining a balance between the cytotoxicity and the transfection activity, both of which are found to be a function of the molecular weight of PEI. After intraportal injection, however, Gal_{153}-$PEI_{70,000}$/pDNA having a low N/P ratio was most effective, suggesting that additional variables, such as the size of the complex, are important for *in vivo* gene transfer to hepatocytes.

Key Words: polyethyleneimine, asialoglycoprotein receptor, galactose, liver, hepatocyte, molecular weight, endocytosis

Introduction

Efficient *in vivo* gene transfer relies on the development of vectors that offer an efficient delivery of a gene to the target cell, high transfection efficiency, and persistence of transgene expression. Nonviral vectors have advantages over viral ones in controlling the tissue disposition of a gene, because the physicochemical properties that determine the tissue disposition of the drug or gene carrier [1,2], such as the particle size, electric charge, and specific ligand, can be easily controlled in nonviral vectors. Of the vectors reported so far, polyethyleneimine (PEI) is one of the most effective polymers in transferring plasmid DNA (pDNA) into cells *in vitro* and *in vivo* [3–6]. PEI is believed to enter the cell by endocytosis, and to possess a buffering capacity and an ability to swell when protonated [5,7]. Therefore, at low pH values, it is believed that PEI prevents acidification of the endosome and induces a large inflow of ions and water, subsequently leading to rupture of the endosomal or lysosomal membrane so that the PEI-pDNA complex is delivered to the cytoplasmic space [8,9]. Because the lysosomal degradation is a critical hurdle for transgene expression by nonviral delivery of pDNA, PEI has a relatively high transfection efficiency among polymeric nonviral vectors such as poly-L-lysine (PLL). PEI is also reported to undergo nuclear localization while retaining an ordered structure, once endocytosed [7].

Because of the cationic charge-mediated transfection, PEI introduces genetic materials into cells in a nonspecific manner. As for other cationic pDNA complexes [10], the PEI-pDNA complex transfects mainly the vascular endothelial cells of the lung after intravenous injection [11], and this could be an obstacle to *in vivo* gene transfer to other types of cells. Targeted delivery of pDNA using

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ligand-conjugated vectors is one solution for target cell-selective gene transfer. Various ligands have been studied to achieve target cell-selective transgene expression both *in vivo* and *in vitro* [2]. Among them, galactose is the most extensively studied ligand that is recognized by asialoglycoprotein receptors on hepatocytes. In previous studies, we synthesized several galactosylated poly-L-lysine (Gal-PLL) derivatives of different molecular weights and with different numbers of galactose residues [12]. Using these complexes, we reported that the tissue disposition and subsequent transfection efficiency of the Gal-PLL/pDNA complex were dependent on the physicochemical properties of Gal-PLL and on the complex formed. Increasing the number of galactose units per polymer would increase the affinity for the receptors [13], but too many galactose residues might reduce the ability of the polymer to interact with pDNA [12]. Therefore, a very precise design is required to develop an effective vector that achieves target cell-selective *in vivo* gene transfer. Various types of PEI have been used for the introduction of pDNA into cells in terms of their molecular weight and structure (linear or branched) [14–17], and the physicochemical properties of PEI or targetable PEI have been suggested to affect the transfection efficiency. However, the requirements for efficient gene transfer with PEI-pDNA complexes reported so far vary depending on the experimental conditions. Modification of PEI with a targeting ligand such as galactose could also be another variable in designing efficient PEI derivatives [18,19].

In the present study, therefore, we selected PEIs with average molecular weights of 1800 (PEI_{1800}), 10,000 ($PEI_{10,000}$), and 70,000 ($PEI_{70,000}$), and modified them with galactose (Gal-PEI). We first investigated the transfection efficiency of unmodified and galactosylated PEIs in HepG2 cells, a hepatoma cell line with asialoglycoprotein receptors, after complex formation with pDNA encoding a reporter gene. The results are discussed in relation to the physicochemical properties of the complex, particle size, and electric charge. Then, we examined *in vivo* gene transfection to mouse liver after injection of pDNA complex into the portal vein. We found that the properties of PEI that are required for efficient transgene expression differ between PEI and Gal-PEI in transfection to HepG2 cells, and also differ between *in vitro* and *in vivo* gene transfer using Gal-PEI.

Results

Complex Formation

We used the N/P ratio, the ratio of the concentration of total nitrogen atoms in PEI to the phosphate group (P) in pDNA, as an index of the complex formation. We prepared pDNA complexes at various N/P ratios and analyzed them by agarose gel electrophoresis (data not shown). Unmodified PEI easily formed a complex with pDNA at an N/P ratio > 3, irrespective of the molecular weight of PEI.

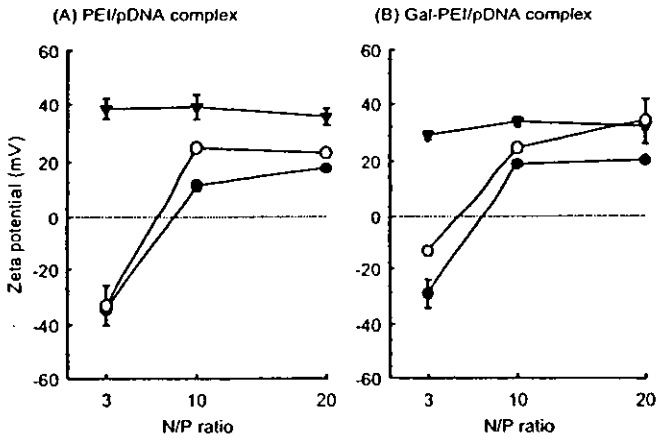

FIG. 1. Zeta potential of PEI-pDNA complex. pDNA was mixed with each PEI derivative at different N/P ratios. (A) PEI-pDNA complex with PEI_{1800} (filled circles), $PEI_{10,000}$ (open circles), or $PEI_{70,000}$ (filled triangles). (B) Gal-PEI/pDNA complex with Gal_9-PEI_{1800} (filled circles), Gal_{19}-$PEI_{10,000}$ (open circles), or Gal_{120}-$PEI_{70,000}$ (filled triangles). Results are the mean ± SD of three measurements.

In the case of Gal_9-PEI_{1800}, a greater amount of Gal-PEI was needed to induce disappearance of bands of free pDNA. For larger Gal-PEIs, their electrophoretic profiles on agarose gel were comparable to those of native PEIs.

Zeta Potential and Particle Size

Figure 1 shows the zeta potential of a pDNA complex prepared at different N/P ratios. The $PEI_{70,000}$-pDNA complex was positively charged even at an N/P ratio of 3, and the zeta potential was hardly affected by additional PEI. In contrast, complexes with $PEI_{10,000}$ or PEI_{1800} were negatively charged at an N/P ratio of 3, and increasing the ratio to 10 or 20 resulted in the formation of a positively charged complex. Modification of PEI with galactose hardly affected the overall zeta potential of the pDNA complex. The increase in the N/P ratio from 10 to 20 hardly changed the zeta potential of the complexes, indicating the possibility of the presence of free polymers in the mixtures.

At the low N/P ratio of 3, the PEI-pDNA complex was > 500 nm in diameter (Fig. 2A). A larger PEI gave a smaller sized complex at higher N/P ratios. With the $PEI_{70,000}$-pDNA complex, the particle size of the complex appeared to be reduced to ~ 100 nm as the N/P ratio increased. For Gal_{19}-$PEI_{10,000}$/pDNA and Gal_{120}-$PEI_{70,000}$/pDNA complexes, the particle size was smaller than that of the corresponding PEI-pDNA complex, indicating that galactose residues interfere to some degree with aggregation of the complex. The particle sizes of PEI_{1800}-pDNA and Gal_9-PEI_{1800}/pDNA complexes were > 1000 nm, even at high N/P ratios.

Lectin-Induced Agglutination

To investigate the accessibility of the galactose residues on the complex to galactose-recognizing lectins, we eval-

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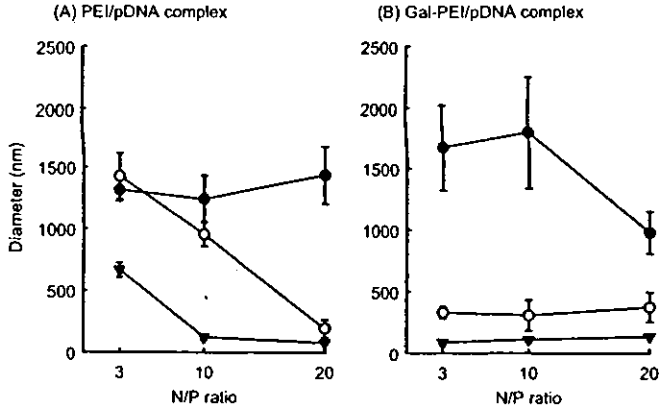

FIG. 2. Particle size of PEI-pDNA complex. pDNA was mixed with each PEI derivative at different N/P ratios. The particle size was measured by dynamic light scattering spectrophotometry. (A) PEI-pDNA complex with PEI_{1800} (filled circles), $PEI_{10,000}$ (open circles), or $PEI_{70,000}$ (filled triangles). (B) Gal-PEI-pDNA complex with Gal_9-PEI_{1800} (filled circles), Gal_{19}-$PEI_{10,000}$ (open circles), or Gal_{120}-$PEI_{70,000}$ (filled triangles). Results are the mean ± SD of three measurements.

uated the binding of Gal-PEI/pDNA complex to *Ricinus communis* agglutinin-120 (RCA120), a galactose-specific plant lectin, using turbidometric assay. Complexes with native PEI hardly interact with the lectin, whereas those with Gal-PEI showed markedly increased turbidity (Fig. 3). The interaction became striking with the increase in the molecular size of PEI. The absorbance was reduced by the addition of excess galactose, suggesting that there was a reversible interaction between the complex and the lectin. However, the addition of galactose only partially reduced the turbidity when Gal_{120}-$PEI_{70,000}$/pDNA was used. Despite possessing galactose residues, the Gal_9-

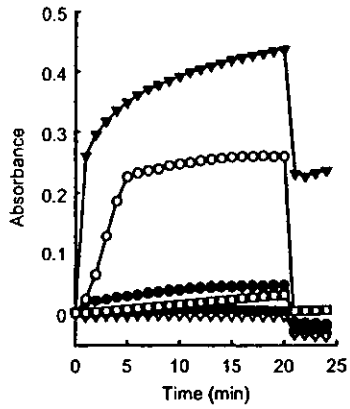

FIG. 3. Agglutination of PEI-pDNA complex induced by *Ricinus communis* agglutinin-120. The absorbance at 450 nm of PEI-pDNA complex (N/P ratio of 20) was continuously measured for 20 minutes, then D-galactose was added to the mixture. PEI-pDNA complexes: PEI_{1800} (open triangles), $PEI_{10,000}$ (filled squares), $PEI_{70,000}$ (open squares). Gal-PEI/pDNA complexes: Gal_9-PEI_{1800} (filled circles), Gal_{19}-$PEI_{10,000}$ (open circles), Gal_{120}-$PEI_{70,000}$ (filled triangles).

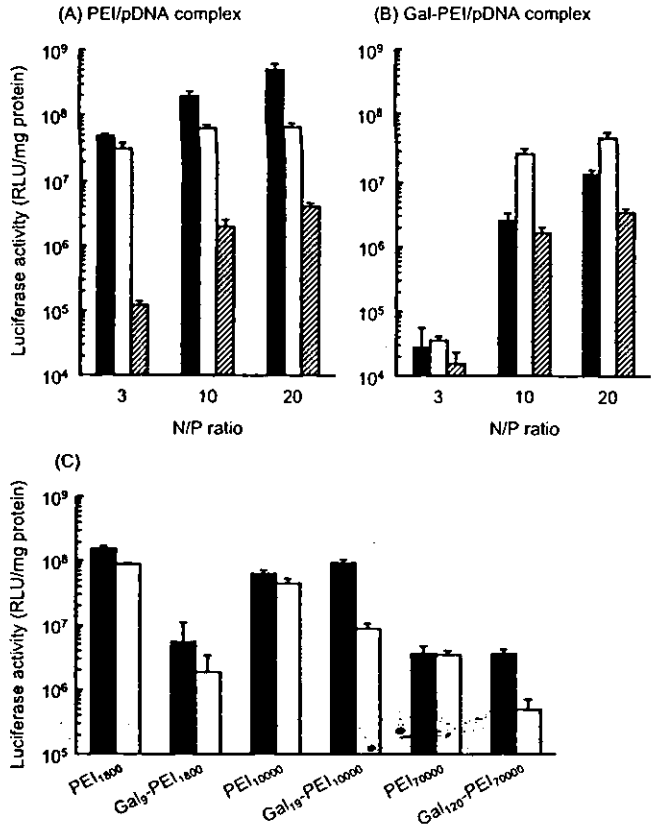

FIG. 4. Transfection efficiency of PEI-pDNA complex in HepG2 cells. The luciferase activity in the supernatant of cell homogenate was assayed 48 hours after transfection. (A) Transfection with PEI-pDNA complex at different N/P ratios as follows: PEI_{1800} (closed bars), $PEI_{10,000}$ (open bars), $PEI_{70,000}$ (hatched bars). (B) Transfection with Gal-PEI/pDNA complex at different N/P ratios as follows: Gal_9-PEI_{1800} (closed bars), Gal_{19}-$PEI_{10,000}$ (open bars), Gal_{120}-$PEI_{70,000}$ (hatched bars). (C) Effect of addition of D-galactose on transfection efficiency. Control (closed bars); addition of 20 mM D-galactose (open bars). Results are the mean + SD of at least three wells.

PEI_{1800}/pDNA complex did not undergo any substantial interaction with the lectin.

Transfection to HepG2 Cells

Any PEI-pDNA complex showed substantial transgene expression in HepG2 cells (Fig. 4A), but the amount was dependent on both the molecular size of PEI and the N/P ratio. The smallest one, PEI_{1800}, showed the highest transgene expression at any N/P ratio, followed by $PEI_{10,000}$ and $PEI_{70,000}$ in that order. Increasing the N/P ratio from 3 to 20 significantly increased transfection efficiency. (N/P vs N/P20, $P < 0.01$ for $PEI_{10,000}$ and $P < 0.001$ for $PEI_{1,000}$ and $PEI_{70,000}$.)

Transgene expression by Gal-PEI/pDNA complex was also observed in HepG2 cells (Fig. 4B). With any complex used, the efficiency was dependent on the N/P ratio, and the highest ratio of 20 gave the greatest transgene expression. However, unlike PEI-pDNA complexes, the interme-

diate Gal_{19}-$PEI_{10,000}$/pDNA showed significantly greater transgene expression than the other two Gal-PEI/pDNA complexes (Gal_{19} – $PEI_{10,000}$ vs Gal_{19} – $PEI_{1,800}$ or Gal_{120} – $PEI_{90,000}$, $P < 0.0001$ at N/P ratios of 10 and 20). The luciferase activity of Gal_9-PEI_{1800}/pDNA complexes was markedly lower compared with that of PEI_{1800}-pDNA complexes, whereas the transfection activity of Gal_{19}-$PEI_{10,000}$/pDNA and Gal_{120}-$PEI_{70,000}$/pDNA was equivalent to that of $PEI_{10,000}$-pDNA and $PEI_{70,000}$-pDNA complexes, respectively. When 20 mM galactose was added, there was only a slight reduction in the luciferase activity of Gal_9-PEI_{1800}/pDNA complexes (Fig. 4C). In contrast, we observed a significant reduction in transfection activity of Gal_{19}-$PEI_{10,000}$/pDNA and Gal_{120}-$PEI_{70,000}$/pDNA complexes following the addition of free galactose ($P < 0.01$). This result agrees well with the results using RCA120 (Fig. 3), and indicates that high-molecular-weight Gal-PEI/pDNA complexes are more easily taken up by hepatic cells through asialoglycoprotein receptors and, therefore, can deliver genetic material into hepatocytes more efficiently than lower molecular weight Gal-PEI/pDNA complexes. However, the transfection efficiency of Gal_9-PEI_{1800}/pDNA was lower than that of PEI_{1800}/pDNA at any N/P ratio. This result indicates that, subsequent to the introduction of galactose moieties into PEI, the mechanism of transfection may shift from a nonspecific one to a hepatocyte-specific one.

Cytotoxicity of PEI-pDNA Complex

The addition of any PEI-pDNA complex prepared at an N/P ratio of 3 reduced the viability of HepG2 cells to ~ 80% of the control value (Fig. 5). There were no statistically significant differences between any two of the complexes with different PEI at this N/P ratio. The viability of the cells was hardly affected by a change in the N/P ratio

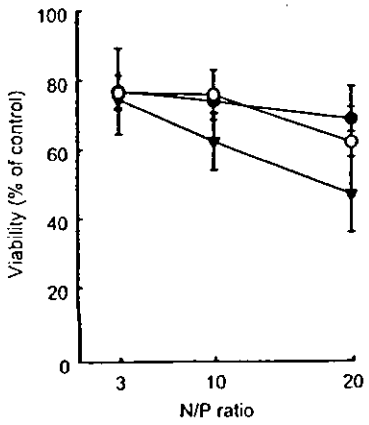

FIG. 5. Viability of HepG2 cells after addition of PEI-pDNA complex. After a 6-hour treatment of the cells with each complex, the viability was measured by MTT assay (see Materials and Methods). Results are the mean ± SD of at least three wells. PEI_{1800} (filled circles), $PEI_{10,000}$ (open circles), $PEI_{70,000}$ (filled triangles).

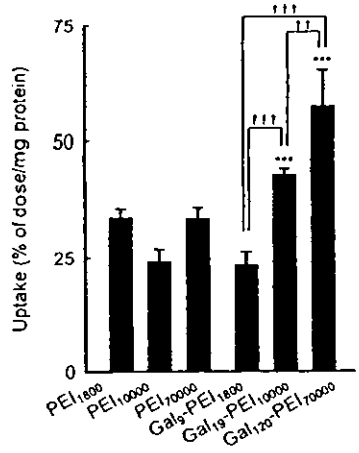

FIG. 6. Uptake of radioactivity in HepG2 cells at 37 °C after addition of PEI-[^{32}P]pDNA complex. Radioactivity in the cells was measured at 30 minutes after the addition of [^{32}P]pDNA complex (N/P ratio of 20) to the cells. Results are the mean + SD of at least three wells. Statistical analysis included analysis of variance (ANOVA), followed by the Student-Newmann-Keuls multiple comparison test; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$; $^{***}P < 0.001$, compared with the corresponding PEI-pDNA complex ($PEI_{10,000}$ vs. Gal_{19}-$PEI_{10,000}$ and $PEI_{70,000}$ vs.Gal_{153}-$PEI_{70,000}$).

of PEI_{1800}-pDNA complex, whereas it was reduced on increasing the N/P ratio with $PEI_{70,000}$-pDNA.

Cellular Association of [^{32}P]pDNA Complex

Figure 6 shows the amount of radioactivity in HepG2 cells at 30 minutes after the addition of [^{32}P]pDNA complex. There were no significant differences in the association of PEI-[^{32}P]pDNA by the cells. However, in the case of the Gal-PEI/pDNA complex, Gal_{120}-$PEI_{70,000}$/[^{32}P]pDNA showed the highest association, followed by Gal_{19}-$PEI_{10,000}$/[^{32}P]pDNA. The amounts of the radioactivity in the cells after the addition of Gal_{19}-$PEI_{10,000}$/[^{32}P]pDNA and Gal_{120}-$PEI_{70,000}$/[^{32}P]pDNA were significantly ($P < 0.001$) greater than those with $PEI_{10,000}$/[^{32}P]pDNA and $PEI_{70,000}$/[^{32}P]pDNA, respectively.

Transgene Expression in the Liver After Intraportal Injection

When injected into a tail vein, $PEI_{10,000}$/pDNA or $PEI_{70,000}$/pDNA complex achieved a high transgene expression in the lung, unlike either Gal_{19}-$PEI_{10,000}$/pDNA or Gal_{153}-$PEI_{70,000}$/pDNA complex (data not shown). In contrast, the injection of these Gal-PEI/pDNA complexes into the portal vein resulted in a relatively high transgene expression in the liver, compared to PEI-pDNA complexes (Fig. 7). The luciferase activity in the liver was higher than that in the lung for both Gal-PEI/pDNAs (data not shown). At any N/P ratio, the Gal_{153}-$PEI_{70,000}$/pDNA complex showed higher transgene expression in the liver than Gal_{19}-$PEI_{10,000}$/pDNA. Gal_{153}-$PEI_{70,000}$/pDNA with an N/P ratio of 3 resulted in the greatest transgene expression among the complexes prepared at different ratios. These

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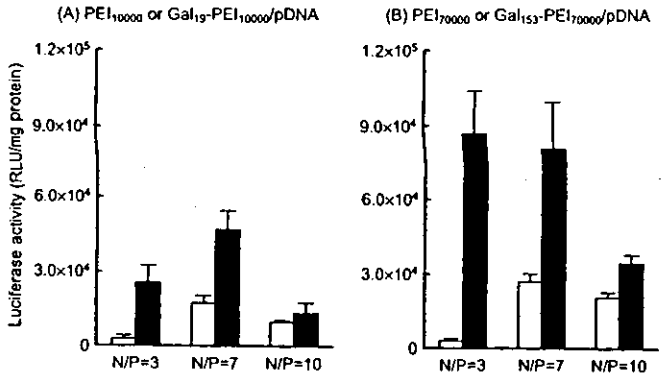

FIG. 7. Transgene expression in mouse liver after injection of PEI-pDNA or Gal-PEI/pDNA complex into the portal vein. (A) $PEI_{10,000}$-pDNA (open bars); Gal_{19}-$PEI_{10,000}$/pDNA (closed bars). (B) $PEI_{70,000}$-pDNA (open bars); Gal_{153}-$PEI_{70,000}$/pDNA (closed bars). At 6 hours after the injection of PEI-pDNA complex containing 50 μg pDNA, the liver was excised and the luciferase activity was measured: PEI (open bars), Gal-PEI (closed bars). Results are the mean + SD of at least three mice.

in vivo results did not correspond to those obtained with cultured HepG2 cells (Fig. 4B).

When we separated the liver cells into hepatocytes (the target cells) and other liver cells (nonparenchymal cells, NPC), $PEI_{70,000}$-pDNA brought about no significant differences in transgene expression (given in relative light units, RLU) in these cells (1.47×10^5 RLU/10^8 cells for hepatocytes, and 1.29×10^5 RLU/cells for NPC). In contrast, Gal_{153}-$PEI_{70,000}$/pDNA resulted in higher transgene expression in hepatocytes (2.67×10^5 RLU/10^8 cells) than in NPC (1.36×10^5 RLU/10^8 cells).

Discussion

A number of papers describe the usefulness of PEI for *in vitro* and *in vivo* gene transfer after complex formation with pDNA. However, controversial results have sometimes been reported with regard to the optimal characteristics of the PEI-pDNA complex for efficient gene transfer. This is probably because the requirements for effective transgene expression by a PEI-pDNA complex varies depending on the experimental situation, that is, *in vivo* or *in vitro*, cell type, and administration method. *In vitro* experiments can provide more detailed information on the requirements for transfection with a PEI-pDNA complex. However, they lack many of the key variables that are important for *in vivo* gene transfer, such as the interaction with blood components and passage through blood vessels. In contrast, *in vivo* results may involve various interactions between the PEI-pDNA complex and biological components, and precise events occurring *in vivo* are difficult to interpret. Because nonviral vectors such as PEI are designed for *in vivo* application, their usefulness should be ultimately examined by *in vivo* experiments. In the present study, based on these considerations, we first tried to clarify the importance of the physicochemical properties of PEI and Gal-PEI on transfection to HepG2 cells *in vitro*, then applied some formulations to *in vivo* studies. We selected three types of branched PEIs with different molecular weights as vector backbones, because several studies reported that the molecular weight of PEI is an important variable in gene transfer efficiency. Fischer *et al.* [15] reported that a smaller PEI was more potent in transgene expression in cultured cells.

When PEI is used as a targetable vector after being modified with a tissue- or cell-specific ligand, other influences should be considered, such as the affinity of a modified PEI for pDNA as well as the affinity of the complex for the target cell. In previous papers, we have reported that the physicochemical properties of cationic polymers are important for the final transgene expression when using polymer-pDNA complexes [12,20,21]. These previous studies indicate that the small polymer was not able to form a rigid complex with pDNA, resulting in less transfection efficiency. Zanta *et al.* [22] have already reported that lactosylated PEI is able to deliver gene to hepatocytes *in vitro*, but the effects of the molecular weight on receptor-mediated transfection with glycosylated PEI have hardly been examined yet. In this study, therefore, we selected three PEIs with varying molecular weights and modified them with galactose, and we systematically examined the transfection activity of PEI and Gal-PEI *in vitro* using a hepatoma cell line. Then, we demonstrated *in vivo* gene transfer by the targetable vector in mice.

No detectable level of transgene expression was obtained when a cationic liposome-pDNA complex was injected into the nucleus [23], indicating that the release of pDNA from the complex was highly important for transgene expression. On the other hand, Pollard *et al.* [6] showed that, unlike the cationic liposome-pDNA complex, the PEI-pDNA complex leads to transgene expression even after being injected into the nucleus. Recently, it has been reported that linear PEI improves nuclear delivery of pDNA relative to other systems, such as branched PEI and a cationic liposome [24]. These observations would indicate that PEI can be a good vector that efficiently transports pDNA to the nucleus, as long as the PEI complex reaches the cytoplasm of the target cell. Assuming that the cellular entry of the nonviral vector-pDNA complex is largely mediated by endocytosis, we can say that the cellular uptake and release from endocytotic vesicles of the complex correlate with the transfection efficiency of the PEI-pDNA complex. It is suggested that destabilization of the endosomal membrane is important for the release of pDNA from the cells [25]. Using cationic liposome-pDNA complexes, we earlier showed that the transfection efficiency in cultured cells is correlated with the ability of the complex to release pDNA when mixed with anionic liposomes, a model of endosomes [26].

Although PEI_{1800} failed to form a pDNA complex of

small particle size, it showed the highest transfection efficiency among the unmodified PEI complexes studied. These results correspond with a previous report [15], and a possible reason for the high efficiency is that it is less toxic to cells. However, when converted into the galactosylation form, Gal_9-PEI_{1800} showed significantly less transfection efficiency than Gal_{19}-$PEI_{10,000}$ ($P < 0.01$ at N/P ratios of 10 and 20). The small number of galactose units per PEI molecule might not be the reason, because the number of galactose units per total weight of the complex was greatest for the Gal_9-PEI_{1800} complex at a fixed N/P ratio. The calculated ratios of the number of galactose and phosphate groups of pDNA were 4.3, 1.6, and 1.5 for Gal_9-PEI_{1800}, Gal_{19}-$PEI_{10,000}$, and Gal_{120}-$PEI_{70,000}$, respectively, at an N/P ratio of 20. The Gal_9-PEI_{1800}/pDNA complex hardly aggregated with the addition of RCA120, and showed less uptake by HepG2 cells than other Gal-PEI/pDNA complexes. These results indicate that the lectins do not efficiently recognize galactose moieties on the Gal_9-PEI_{1800}/pDNA complex. Therefore, galactosylation reduced the transfection efficiency of PEI_{1800}. In addition, galactosylation of PEI may reduce its ability to rupture endosomal/lysosomal membranes. Modification of a cationic vector with sugar sometimes reduces the transfection efficiency of the vector in cells lacking the corresponding receptor [27]. In the present study, Gal-PEI/pDNA complexes resulted in less transfection efficiency in CT26 colon adenocarcinoma cells than PEI-pDNA complexes (data not shown).

As the N/P ratio increases, free PEI or Gal-PEI could exist along with formed pDNA complex in the test tube. Such free polymer could have an effect on many aspects of nonviral vector-mediated gene transfer. The cytotoxicity of the PEI-pDNA complexes increased with the increase in the N/P ratio from 3 to 20, suggesting that free PEI could be toxic to cells as are other positively charged polymers [28]. Free polymers might also inhibit the cellular uptake of the complex. However, charge-mediated uptake, or adsorptive endocytosis, has a large capacity with regard to cellular uptake and might not be saturated at the concentrations used in the present study. In contrast, the receptor-mediated uptake of Gal-PEI/pDNA complex would be inhibited by free Gal-PEI because of the limited number of asialoglycoprotein receptors on HepG2 cells or hepatocytes. In this case, however, the affinity of ligands for the receptors should be important in determining the effects of free Gal-PEI. We have earlier studied the hepatic uptake clearance of various galactosylated polymers in mice, and concluded that the surface density of galactose units on macromolecules determines the affinity for the asialoglycoprotein receptors [29]. Linear galactosylated polymers were less efficiently recognized by the receptors than globular galactosylated proteins [30]. The effective density of galactose should be greater with condensed Gal-PEI/pDNA complex than with free, extended Gal-PEI. Therefore, although the precise effects of free Gal-PEI on the uptake of Gal-PEI/pDNA complex needs to be examined, the inhibitory effects of free Gal-PEI seem not to be important for the cellular uptake of Gal-PEI/pDNA complex.

Larger Gal-PEIs showed substantial agglutination when mixed with RCA120, indicating that the galactose moieties on these derivatives are directed outwards in the pDNA complex. The addition of galactose to the mixture of Gal_{120}-$PEI_{70,000}$/pDNA and lectin reduced the turbidity to only half that of the peak value. These results suggest that the interaction between the complex and lectin is too strong to be fully dissociated with galactose monomer, because a multivalent interaction can increase the binding affinity of galactosylated ligand for the asialoglycoprotein receptor, a galactose-recognizing animal lectin specifically expressed on hepatocytes. Gal-PEI/pDNA complexes with larger Gal-PEIs showed greater association in HepG2 cells than the complex with Gal_9-PEI_{1800}, and association as well as transfection was inhibited by the addition of 20 mM galactose. These results indicate that Gal_{19}-$PEI_{10,000}$ and Gal_{120}-$PEI_{70,000}$ can deliver pDNA by complex formation to asialoglycoprotein receptor-positive cells by receptor-mediated endocytosis, as observed with galactosylated polymers and liposomes [12,20,27,31]. Gal_{120}-$PEI_{70,000}$ showed a significantly higher association in HepG2 cells ($P < 0.01$), but resulted in significantly less transfection in the cells than Gal_{19}-$PEI_{10,000}$ ($P < 0.01$ at N/P ratios of 10 and 20). This might be due to the difference in the cytotoxicity of these Gal-PEI/pDNA complexes. In addition, as observed with cationic liposome complexes [26], differences in the release of pDNA from the complex could also explain the lower transfection efficiency with Gal_{120}-$PEI_{70,000}$/pDNA complex. The smaller particle size of the complex (Fig. 2) would indicate its stronger affinity with pDNA, which might result in reduced release of pDNA after cellular uptake by endocytosis. The incomplete dissociation of Gal_{120}-$PEI_{70,000}$/pDNA complex from the lectin by the addition of galactose (Fig. 3) would also support this hypothesis.

In vivo gene transfer to hepatocytes requires not only the ability of a vector to introduce gene into the cells but also an efficient delivery of gene to the vicinity of the cells. After administration into the blood circulation, the pDNA complex interacts with various cells and molecules, such as serum proteins [32,33] and erythrocytes [34,35]. The cationic nature of the complex attracts negatively charged cells and molecules that alter the physicochemical properties of the complex. In this study, we failed to obtain efficient gene transfer in the liver by intravenous injection of Gal-PEI/pDNA complex. However, when injected into the portal vein of mice, Gal_{19}-$PEI_{10,000}$/pDNA and Gal_{153}-$PEI_{70,000}$/pDNA complexes showed a relatively high, liver-selective transgene expression compared with unmodified PEI-pDNA complexes. In this situation, however, Gal_{153}-$PEI_{70,000}$/pDNA complex showed greater

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transgene expression than Gal_{19}-$PEI_{10,000}$/pDNA, which was the best for cultured HepG2 cells among the Gal-PEI/pDNA complexes tested. Although this discrepancy needs to be examined, the differences in the particle size of these complexes (Fig. 2) should affect the *in vivo* transgene expression, because the capillaries in the liver possess pores and intercellular junctions of ~ 200 nm or less [36]. Separation of liver cells into hepatocytes and NPC clearly indicated that Gal_{153}-$PEI_{70,000}$/pDNA could deliver pDNA into hepatocytes, something not observed with unmodified $PEI_{70,000}$/pDNA. These results indicate that Gal-PEI is effective in introducing pDNA into cells *in vivo* when the complex can reach the target cell.

In summary, we have shown that the molecular weight of PEI greatly affects the transfection efficiency of both unmodified and galactosylated PEI. A small PEI molecule is advantageous for nonspecific transfection to cells, probably because it readily releases pDNA within cells, but after galactosylation it is not effective because of the poor availability on the molecule of galactose for cellular recognition. In contrast, a large PEI molecule is less effective in transgene expression, but asialoglycoprotein receptor-mediated recognition is considerable after galactosylation. Because of the need to balance these opposite effects of the molecular weight, the medium PEI was the most effective vector for asialoglycoprotein receptor-positive cells following galactosylation. When injected into mice, however, other variables such as the particle size of the complex should be involved in the final degree of transgene expression, and we found the largest Gal-PEI to be the most potent vector although it was not the best for *in vitro* transfection.

Materials and Methods

Chemicals. The branched PEI derivatives PEI_{1800}, $PEI_{10,000}$, and $PEI_{70,000}$ were purchased from Polysciences, Inc. (Warrington, PA). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Opti-MEM I was obtained from Gibco BRL (Grand Island, NY). Dulbecco's modified Eagle's minimum essential medium (DMEM) and other reagents for cell culture were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest grade available. pDNA encoding firefly luciferase cDNA fragment was prepared as described [20]. The plasmid was labeled with [γ-^{32}P]dCTP by nick translation. The specific activity of [^{32}P]pDNA was about 7×10^7 cpm/μg pDNA.

Synthesis of Gal-PEI. Galactosylation of PEI was carried out using 2-imino-2-methoxyethyl 1-thiogalactoside according to the method reported by Lee *et al.* [37]. The number of galactose units on PEI was controlled by the molar ratio of the starting materials. The Gal-PEI obtained was purified by dialysis and freeze-dried. The number of galactose units was determined by measurement of the galactose content by the anthrone-sulfuric acid method. Gal_9-PEI_{1800} (PEI_{1800} with 9 galactose units), Gal_{19}-$PEI_{10,000}$, Gal_{120}-$PEI_{70,000}$, and Gal_{153}-$PEI_{70,000}$ were obtained. Galactosylation increased the molecular weights of PEI through the attachment of galactose moieties, and the estimated molecular weights of Gal-PEIs were: 3900 (Gal_9-PEI_{1800}), 14,500 (Gal_{19}-$PEI_{10,000}$), 98,200 (Gal_{120}-$PEI_{70,000}$), and 106,000 (Gal_{153}-$PEI_{70,000}$). Because the method used for galactosylation hardly alters the electric charge of the modified molecules [37], the final amine concentration in the conjugates was calculated on the assumption that the effective number of amine groups in PEI is not altered by galactosylation.

Complex formation. Unless otherwise indicated, pDNA was mixed with varying amounts of PEI or Gal-PEI at a final concentration of 2 μg pDNA/ml in 5% dextrose solution, and left at room temperature for 30 minutes. The mixture obtained was subjected to 1% agarose gel electrophoresis to check the complex formation. The gel was stained with ethidium bromide and visualized on a transilluminator.

Zeta potential and particle size measurement. pDNA and PEI or Gal-PEI were mixed in 5% dextrose containing 1 mM NaCl to give a final concentration of 2 μg pDNA/ml. Sodium chloride was added to the solution for conductivity. After 30 minutes, the zeta potential of the pDNA complex was measured electrophoretically using a zeta potential analyzer (LEZA-500T, Otsuka Electronics, Osaka, Japan). Separately, pDNA was mixed with PEI or Gal-PEI in 5% dextrose at a final concentration of 5 μg pDNA/ml, and the complex obtained was incubated at room temperature for 30 minutes. Then, the particle size of the pDNA complex was measured by dynamic light-scattering spectrophotometry (LS-900, Otsuka Electronics, Osaka, Japan).

Lectin-induced agglutination. pDNA complex (20 μg/ml of pDNA, N/P ratio of 20) was incubated in the presence of RCA120 (90 μg/ml) in 500 μl PBS for 20 minutes. Then, galactose (100 μl of a 10 mg/ml solution) was added. Agglutination induced by RCA120 was continually determined by measuring the turbidity of the solution at 450 nm [15].

Transfection to HepG2 cells. pDNA and a PEI derivative, both of which were dissolved in 5% dextrose, were separately diluted with Opti-MEM I to a final concentration required, then the two were mixed to obtain pDNA complex. HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FBS at 37 °C under an atmosphere of 5% CO_2 in air. Cells were plated on a six-well cluster dish at a density of 2×10^5 cells/well (10.5 cm^2) and cultivated in 2 ml DMEM supplemented with 10% FBS. After 24 hours, the culture medium was replaced with Opti-MEM I containing pDNA (2.0 μg) complex. The medium was replaced again 6 hours later, with DMEM supplemented with 10% FBS, and incubated for an additional 42 hours. Then, the cells were scraped off and suspended in 150 μl PBS. The cell suspension was subjected to three cycles of freezing and thawing, and then centrifuged at 10,000g for 3 minutes. Then, 10 μl of the supernatant was mixed with 100 μl luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan), and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as the relative light units (RLU) per mg protein of cells. The protein content of the cell suspension was determined by a modified Lowry method using BSA as a standard. To examine the effect of galactose on transfection, it was added to the medium at a final concentration of 20 mM during the incubation of the cells with pDNA complex. The cells were treated as above, and the luciferase activity of the cell lysate was measured 48 hours after the start of a 6-hour incubation with pDNA complex.

MTT assay. HepG2 cells were seeded onto 96-well plates at a density of 1×10^4 cells/well (0.32 cm^2) in 100 μl DMEM supplemented with 10% FBS, and incubated at 37 °C for 24 hours. Then, the culture medium was replaced with 100 μl Opti-MEM I containing pDNA complex and incubated for an additional 6 hours. The cell viability was then measured by MTT assay. Initially, the cells were incubated with 100 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in Opti-MEM I for 4 hours. Then, after adding 100 μl of a 10% SDS solution was added, the mixture was incubated at 37 °C overnight. The absorbance at test and reference wavelengths of 550 and 655 nm, respectively, were measured.

Association of pDNA complex with HepG2 cells. Before complex formation, [^{32}P]pDNA was diluted with nonradiolabeled pDNA to give a final radioactivity of 5×10^4 cpm/well, with the ratio of unlabeled pDNA and [^{32}P]pDNA being ~ 350:1. pDNA and a PEI derivative in 5% dextrose solution were separately diluted with Hank's balanced salt solution to a

final concentration required, then the two were mixed to obtain pDNA complex. [^{32}P]pDNA complex (N/P ratio of 20) was added to HepG2 cells at a final concentration of 0.5 μg pDNA/ml, and incubated at 37 °C. At 30 minutes, the medium was removed and the cells were washed five times with PBS. The cells were solubilized with 0.5 ml NaOH (1 M) overnight and then neutralized with 0.1 ml HCl (5 M). ^{32}P radioactivity was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan) after addition of 5 ml Clear-Sol I (Nacalai tesque, Kyoto, Japan). The protein content of the samples was measured by the method described earlier.

In vivo gene transfer. ICR mice (female, 20–23 g) were purchased from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan) and maintained on standard food and water under conventional housing conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University. Mice were anesthetized with a peritoneal injection of pentobarbital sodium (50 mg/kg). An incision was made in the abdomen, and the portal vein was exposed. PEI or Gal-PEI/pDNA complex was prepared in 5% dextrose at N/P ratios of 3, 7, and 10, and incubated for 30 minutes. Each complex in 200 μl 5% dextrose was injected into the portal vein at a dose of 50 μg pDNA/mouse, and the abdomen was closed by clips. At 6 hours after injection, the liver and other organs were excised, homogenized, and assayed for luciferase activity. Transgene expression in hepatocytes and other liver cells (nonparenchymal cells including Kupffer cells and sinusoidal endothelial cells) was also measured in different mice. $PEI_{70,000}$/pDNA or Gal_{153}-$PEI_{70,000}$/pDNA complex was injected into mice as just described at a dose of 50 μg pDNA/mouse. Then, 6 hours after injection, the liver cells were separated into hepatocytes and other liver cells (nonparenchymal cells including Kupffer cells and endothelial cells) as reported earlier [20]. In brief, the liver was perfused with HEPES buffer (pH 7.5) containing collagenase. The dispersed cells were separated into hepatocyte and nonparenchymal cell fractions by differential centrifugation. Then, the cell suspensions were subjected to three cycles of freezing (liquid N_2 for 3 minutes) and thawing (37 °C for 3 minutes), and then centrifuged at 10,000g for 3 minutes. Finally, the luciferase activity of the supernatant was assayed as above.

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References

1. Nishikawa, M., Takakura, Y., and Hashida, M. (1996). Pharmacokinetic evaluation of polymeric carriers. *Adv. Drug Deliv. Rev.* 21: 135–155.
2. Nishikawa, M., and Huang, L. (2001). Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum. Gene Ther.* 12: 861–870.
3. Boussif, O., *et al.* (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* 92: 7297–7301.
4. Abdallah, B., *et al.* (1996). A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain: polyethylenimine. *Hum. Gene Ther.* 7: 1947–1954.
5. Boletta, A., *et al.* (1997). Nonviral gene delivery to the rat kidney with polyethylenimine. *Hum. Gene Ther.* 8: 1243–1251.
6. Pollard, H., *et al.* (1998). Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J. Biol. Chem.* 273: 7507–7511.
7. Godbey, W. T., Wu, K. K., and Mikos, A. G. (1999). Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. USA* 96: 5177–5181.
8. Wagner, E. (1998). Effects of membrane-active agents in gene delivery. *J. Cont. Rel.* 53: 155–158.
9. Klemm, A. R., Young, D., and Lloyd, J. B. (1998). Effects of polyethyleneimine on endocytosis and lysosome stability. *Biochem. Pharmacol.* 56: 41–46.
10. Liu, Y., *et al.* (1997). Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol.* 15: 167–173.
11. Goula, D., *et al.* (1998). Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* 5: 1291–1295.
12. Nishikawa, M., Takemura, S., Takakura, Y., and Hashida, M. (1998). Targeted delivery of plasmid DNA to hepatocytes *in vivo*: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. *J. Pharmacol. Exp. Ther.* 287: 408–415.
13. Hirabayashi, H., Nishikawa, M., Takakura, Y., and Hashida, M. (1996). Development and pharmacokinetics of galactosylated poly-L-glutamic acid as a biodegradable carrier for liver-specific drug delivery. *Pharm. Res.* 13: 880–884.
14. Tang, M. X., and Szoka, F. C. (1997). The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther.* 4: 823–832.
15. Fischer, D., Bieber, T., Li, Y., Elsasser, H. P., and Kissel, T. (1999). A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* 16: 1273–1279.
16. Zou, S. M., Erbacher, P., Remy, J. S, and Behr, J. P. (2000). Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* 2: 128–134.
17. Wightman, L., *et al.* (2001). Different behavior of branched and linear polyethylenimine for gene delivery *in vitro* and *in vivo*. *J. Gene Med.* 3: 362–372.
18. Ogris, M., *et al.* (1998). The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.* 5: 1425–1433.
19. Bettinger, T., Remy, J. S., and Erbacher, P. (1999). Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. *Bioconjug. Chem.* 10: 558–561.
20. Nishikawa, M., *et al.* (2000). Hepatocyte-targeted *in vivo* gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. *Gene Ther.* 7: 548–555.
21. Nishikawa, M., *et al.* (2000). Pharmacokinetics and *in vivo* gene transfer of plasmid DNA complexed with mannosylated poly(L-lysine) in mice. *J. Drug Target.* 8: 29–38.
22. Zanta, M. A., Boussif, O., Adib, A., and Behr, J. P. (1997). *In vitro* gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjug. Chem.* 8: 839–844.
23. Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., and Welsh, M. J. (1995). Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* 270: 18997–19007.
24. Brunner, S., Furtbauer, E., Sauer, T., Kursa, M., and Wagner, E. (2002). Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation. *Mol. Ther.* 5: 80–86, doi:101006/mthe.2001.0509.
25. Xu, Y., and Szoka, F. C. Jr. (1996). Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 35: 5616–5623.
26. Sakurai, F., *et al.* (2000). Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. *J. Cont. Rel.* 66: 255–269.
27. Kawakami, S., Fumoto, S., Nishikawa, M., Yamashita, F., and Hashida, M. (2000). *In vivo* gene delivery to the liver using novel galactosylated cationic liposomes. *Pharm. Res.* 17: 306–313.
28. Choksakulnimitr, S., Masuda, S., Tokuda, H., Takakura, Y., and Hashida, M. (1995). *In vitro* cytotoxicity of macromolecules in different cell culture systems. *J. Cont. Rel.* 34: 233–241.
29. Nishikawa, M., Miyazaki, C., Yamashita, F., Takakura, Y., and Hashida, M. (1995). Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. *Am. J. Physiol.* 268: G849–G856.
30. Hashida, M., Hirabayashi, H., Nishikawa, M., and Takakura, Y. (1997). Targeted delivery of drugs and proteins to the liver via receptor-mediated endocytosis. *J. Cont. Rel.* 46: 129–137.
31. Kawakami, S., Yamashita, F., Nishikawa, M., Takakura, Y., and Hashida, M. (1998). Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. *Biochem. Biophys. Res. Commun.* 252: 78–83, doi:101006/bbrc.1998.9602.
32. Barron, L. G., Kathleen, B., Meyer, B., Francis, C., and Szoka, F. C. Jr. (1998). Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. *Hum. Gene Ther.* 9: 315–323.
33. Li, S., *et al.* (1999). Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther.* 6: 585–594.
34. Sakurai, F., Nishioka, T., Yamashita, F., Takakura, Y., and Hashida, M. (2001). Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system. *Eur. J. Pharm. Biopharm.* 52: 165–172.
35. Sakurai, F., *et al.* (2001). Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Ther.* 8: 677–686.
36. Bennett, H. S., Luft, J. H., and Hampton, J. C. (1959). Morphological classification of vertebrate blood capillaries. *Am. J. Physiol.* 196: 381–390.
37. Lee, Y. C., Stowell, C. P., and Krantz, M. J. (1976). 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. *Biochemistry* 15: 3956–3963.

Control of hepatic disposition of mannosylated liposomes by PEGylation: effect of the molecular weight of PEG and the density of PEG and mannose

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To achieve a sustained and targeted delivery of liposomes to liver non-parenchymal cells, we modified distearoyl phosphatidylcholine and cholesterol liposomes (DSPC/Chol liposomes) with a mannosylated cholesterol derivative (Man-C4-Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol (PEG-DSPE). Each liposome formulation was adjusted to an approximate size of 70-90 nm, labelled with [^{3}H]cholesteryl hexadecyl ether, and injected intravenously into mice, to investigate the tissue distribution characteristics. The addition of Man-C4-Chol to the DSPC/Chol liposomes resulted in their rapid uptake by the liver. Then, the Man liposomes were combined with PEG-DSPE, reducing the hepatic uptake of the Man liposomes, depending on the molecular weight of the PEG. For example, PEG5000- or PEG2000-DSPE completely inhibited the mannose-mediated hepatic uptake of the Man liposomes, whereas PEG350-Man liposomes were gradually delivered to the liver. These results indicate that the delivery rate of Man liposomes can be controlled by altering the ratio of Man-C4-Chol and PEG350-DSPE.

Key words: Mannosylated liposomes – Mannose receptor – Polyethylene glycol – PEGylation – Sustained delivery – Kupffer cell – Sinusoidal endothelial cell.

Receptor-mediated drug targeting is a promising approach to the achievement of cell-specific delivery of pharmaceuticals. Liposomes having mannose units on their surface have been developed for targeting mannose receptor-positive cells such as macrophages, and have been used for the delivery of drugs, genes, antigens and immunomodulators [1-3]. In previous studies, we have synthesized a mannosylated cholesterol derivative, cholesten-5-yloxy-N-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol), and formulated mannosylated liposomes to achieve targeted delivery of genes and drugs to liver non-parenchymal cells [3-5]. The mannosylated liposomes were found to be delivered to the cells via mannose receptor-mediated endocytosis.

Since the recognition of mannosylated liposomes by the receptors is very efficient, the liposomes can be rapidly delivered to the cells. Such an efficient uptake might lead to a rapid reduction in drug (liposome) concentration in the systemic circulation, which, in turn, would result in short drug action duration [6]. Therefore, to avoid frequent repeated administrations, the drug delivery rate to the target needs to be controlled on some occasions.

Polyethylene glycol (PEG) is a popular polymer that is used to modify particulate drug carriers in order to avoid their uptake by cells in the mononuclear phagocyte system (MPS). In general, the modification of particulate carriers using PEG-containing molecules results in a prolongation of their blood circulation time [7, 8]. For liposomal delivery systems, the addition of PEG lipid results in a significant increase in the circulation time of the liposomes [9]. This effect is correlated with the ability of the polymer to reduce the binding of plasma proteins to the liposomes that is followed by reduced removal by the MPS [10]. The hydrophilicity and flexibility of the polymer makes liposomes sterically stable [11]. The effects of PEG lipid on the surface properties of liposomes depend on the molecular weight of the PEG chain and the concentration of PEG lipid in the formulation. Most studies using PEG liposomes have focused on preventing the surface of the liposomes binding to plasma proteins and subsequent MPS uptake [12-15], while the use of PEG lipid to control the delivery rate of actively targeted liposomes has received little attention.

The aim of this paper is to demonstrate the possible use of PEG lipid for the development of a sustained and targeted drug delivery system. To this end, we selected mannosylated liposomes as model delivery systems to liver non-parenchymal cells with the recognition by mannose receptors, and modified them with a PEG lipid. Various liposome formulations were prepared, and their tissue distribution was investigated in mice, after intravenous injection. The results obtained in the present study highlight the importance of the molecular weight of PEG, the density of PEG and mannose, in controlling the tissue distribution of PEGylated and mannosylated liposomes.

I. MATERIALS AND METHODS

1. Materials

Distearoyl phosphatidylcholine (DSPC) was purchased from Sigma Chemical Co. (St. Louis, MO, United States). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol (PEGx-DSPE), where x represents the average molecular weight of PEG (5000, 2000 or 350), was obtained from Avanti Polar Lipids (Alabaster, AL, United States). Cholesterol (Chol) was obtained from Nacalai Tesque (Kyoto, Japan). [^{3}H]Cholesteryl hexadecyl ether (CHE) was purchased from NEN Life Science Products (Boston, MA, United States). Man-C4-Chol was synthesized as reported previously [4]. All other chemicals were reagent grade products obtained commercially.

2. Animals

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

3. Preparation of liposomes

Liposomes with various lipid compositions were prepared as reported previously [4], using the following lipids: Man-C4-Chol, Chol, DSPC, and PEG-DSPE. The mixing molar ratios of each lipid in the formulations are summarized in *Table I*. Lipids with a trace amount of [^{3}H]CHE were dissolved in chloroform and evaporated to dryness in a round-bottom flask. The dried lipid film was then desiccated with a vacuum, for at least 4 h, to remove any residual organic solvent, before being hydrated in phosphate buffered saline (PBS, pH 7.4). The suspension was sonicated then extruded through a polycarbonate membrane (100 nm pore size) at 65°C. The mean vesicle diameters were determined using a laser light scattering particle size analyzer (LS-900, Otsuka Electronics, Osaka, Japan). The mean particle size of the liposome formulations is shown in *Table I*.

4. *In vivo* distribution experiment

Each type of [^{3}H]liposomes was injected intravenously into male ddY mice (25-28 g) via a lateral tail vein. At predetermined time periods (1, 3, 5, 10, 30, 60 min, 3, 5, 8, 24 and 48 h) following the injection, blood was collected from the *vena cava* under ether anæsthesia. The mice were then sacrificed 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, Meriden, CT, United States) with an overnight incubation at 45°C. 0.2 ml isopropanol, 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).

Table I - Lipid composition and the mean particle size of the liposomes.

Liposome	Lipid composition (DSPC/PEG-DSPE/ Chol/Man-C4-Chol, molar ratio)	Particle size (nm)*
DSPC/Chol liposomes	60/0/40/0	89.4 ± 5.7
Man (5)-liposomes	60/0/35/5	94.5 ± 10.9
PEG5000 (0.8)-liposomes	59.2/0.8/40/0	72.8 ± 20.6
PEG5000 (0.8)-Man (5)-liposomes	59.2/0.8/35/5	70.8 ± 12.8
PEG2000 (0.8)-liposomes	59.2/0.8/40/0	75.9 ± 15.7
PEG2000 (0.8)-Man (5)-liposomes	59.2/0.8/35/5	74.2 ± 19.0
PEG350 (0.8)-liposomes	59.2/0.8/40/0	88.4 ± 4.3
PEG350 (0.8)-Man (5)-liposomes	59.2/0.8/35/5	86.8 ± 6.9
PEG350 (0.8)-Man (7.5)-liposomes	59.2/0.8/32.5/7.5	84.5 ± 10.9
PEG350 (0.8)-Man (20)-liposomes	59.2/0.8/20/20	89.5 ± 11.8
PEG350 (1.6)-Man (7.5)-liposomes	58.4/1.6/32.5/7.5	77.5 ± 12.1

*The mean particle size of the liposomes was measured using a laser light scattering particle size analyzer. Results are expressed as the mean ± SD of three determinations.

5. Calculation of pharmacokinetic parameters

The distribution data of [^{3}H]liposomes were analyzed in terms of organ uptake clearance (CL_{org}) [16]. After injection of [^{3}H]liposomes, the change in the amount of radioactivity in a tissue with time can be described as:

$$(dX_i/dt) = CL_{org}\, C_p - K_{efflux,\,i}\, X_i \qquad \textbf{Eq. 1}$$

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

$$(dX_i/dt) = CL_{org}\, C_p \qquad \textbf{Eq. 2}$$

When *Equation 2* is integrated from time 0 to t, CL_{org} can be expressed as:

$$CL_{org} = \frac{X_{i,t}}{\int_0^t C_p dt} = \frac{X_{i,t}}{AUC_t} \qquad \textbf{Eq. 3}$$

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

II. RESULTS

1. Physicochemical properties of liposomes

The mean particle sizes of the liposomes were almost identical for all formulations (*Table I*). The size of each liposome preparation was less than 100 nm in diameter and remained stable for at least one month.

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

Figure 1 shows the blood concentration and liver accumulation time courses of [^{3}H]DSPC/Chol liposomes and [^{3}H]Man(5) liposomes at a dose of 25 mg lipid/kg. [^{3}H]DSPC/Chol liposomes showed a prolonged circulation in plasma, whereas [^{3}H]Man(5) liposomes, prepared by replacing 5 mol% of Chol in the DSPC/Chol liposomes with Man-C4-Chol, were rapidly delivered to the liver and the uptake reached about 75% of the injected dose within the first 10 min, as previously observed [4, 5]. Except for the liver, none of the other tissues exhibited a significant uptake of either [^{3}H]DSPC/Chol liposomes or [^{3}H]Man(5) liposomes (data not shown).

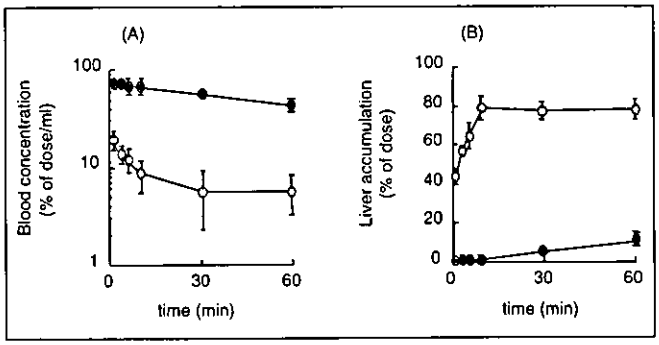

Figure 1 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (●) [^{3}H]DSPC/Chol liposomes (DSPC/Chol = 60/40); (○) [^{3}H]Man(5) liposomes (DSPC/Chol/Man-C4-Chol = 60/35/5). Results are expressed as the mean ± SD of three mice.

3. Effect of the molecular weight of PEG on the hepatic uptake of [^{3}H]liposomes

The blood concentration and liver accumulation time courses of [^{3}H]PEG(0.8) liposomes and [^{3}H]PEG(0.8)-Man(5) liposomes were examined (*Figures 2, 3 and 4* for PEG5000-, PEG2000-, or PEG350-DSPE-containing liposomes, respectively). In the case of PEG5000-, PEG2000- and PEG350-DSPE, PEGylation (the addition of PEG-DSPE to a liposome formulation) drastically reduced the hepatic uptake of [^{3}H]Man(5) liposomes. [^{3}H]PEG5000(0.8)-Man(5) liposomes and [^{3}H]PEG2000(0.8)-Man(5) liposomes showed similar distribution profiles to those of [^{3}H]PEG5000(0.8) liposomes and [^{3}H]PEG2000(0.8) liposomes, respectively, indicating that no significant hepatic uptake of [^{3}H]liposomes by mannose receptors took place even in the presence of mannose moieties on the surface of the liposomes. However, [^{3}H]PEG350(0.8)-Man(5) liposomes exhibited a different distribution profile from that of [^{3}H]PEG350(0.8) liposomes or [^{3}H]Man(5) liposomes. The hepatic uptake rate was intermediate between that of [^{3}H]PEG350(0.8) liposomes and [^{3}H]Man(5) liposomes.

Table II summarizes the AUC, CL_{total}, hepatic uptake clearance (CL_{liver}), and tissue uptake rate index (tissue uptake clearance per unit tissue weight) of representative tissues for the [^{3}H]liposomes. [^{3}H]Man(5) liposomes had a large CL_{liver} value (9.85 ml/h), whereas all three [^{3}H]PEG liposomes had a CL_{liver} value of about 1/200, or less, than that of [^{3}H]Man(5) liposomes. PEGylation of [^{3}H]Man(5) liposomes drastically reduced the CL_{liver} regardless of the length of the PEG chain on PEG-DSPE. Although the CL_{liver} values of [^{3}H]PEG5000(0.8)- and [^{3}H]PEG2000(0.8)-Man(5) liposomes were comparable with those of corresponding liposomes without Man-C4-Chol, that of [^{3}H]PEG350(0.8)-Man(5) liposomes (1.58 ml/h) was about 30-fold greater than that of [^{3}H]PEG350(0.8) liposomes. The CL_{liver} value of [^{3}H]PEG350(0.8)-Man(5) liposomes was found to be about one sixth that of [^{3}H]Man(5) liposomes.

4. Effect of the density of PEG and mannose on liposomes

Figure 5 shows the blood concentration and liver accumulation time courses of [^{3}H]PEG350(0.8)-Man liposomes at a dose of 25 mg lipid/kg. The molar ratio of Man-C4-Chol in the liposome formulations varied from 0 (PEG350(0.8) liposome), 5, 7.5 to 20 mol%, with a constant ratio of PEG content (0.8 mol%). Increasing the molar ratio of Man-C4-Chol led to a rapid and large accumulation of [^{3}H]liposomes in the liver.

Then, the content of PEG was doubled (1.6 mol%) in [^{3}H]PEG350-Man(7.5) liposomes (*Figure 6*). Increasing the PEG350-DSPE from 0.8 to 1.6 mol% of total lipids resulted in a reduced hepatic uptake of [^{3}H]liposomes.

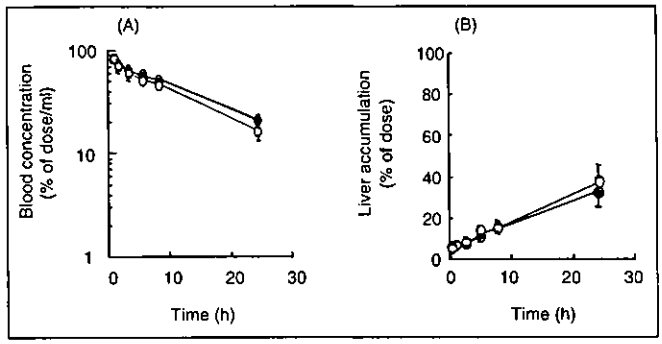

Figure 2 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]PEG5000 liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (●) [^{3}H]PEG5000(0.8) liposomes (DSPC/PEG5000-DSPE/Chol = 59.2/0.8/40); (○) [^{3}H]PEG5000(0.8)-Man(5) liposomes (DSPC/PEG5000-DSPE/Chol/Man-C4-Chol = 59.2/0.8/35/5). Results are expressed as the mean ± SD of three mice.

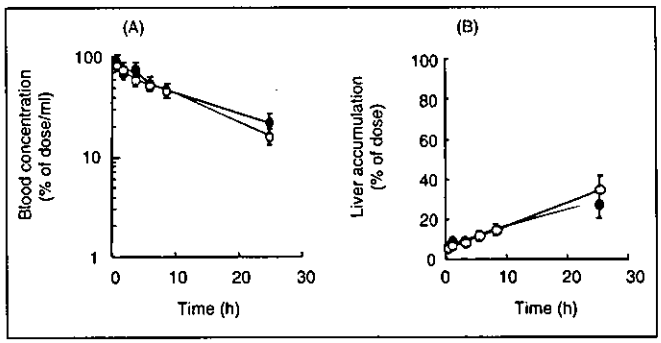

Figure 3 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]PEG2000 liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (●) [^{3}H]PEG2000(0.8) liposomes (DSPC/PEG2000-DSPE/Chol = 59.2/0.8/40); (○) [^{3}H]PEG2000(0.8)-Man(5) liposomes (DSPC/PEG2000-DSPE/Chol/Man-C4-Chol = 59.2/0.8/35/5). Results are expressed as the mean ± SD of three mice.

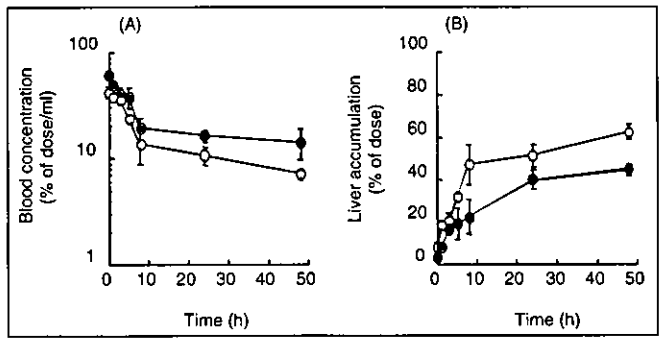

Figure 4 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]PEG350 liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (●) [^{3}H]PEG350(0.8) liposomes (DSPC/PEG350-DSPE/Chol = 59.2/0.8/40); (○) [^{3}H]PEG350(0.8)-Man(5) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 59.2/0.8/35/5). Results are expressed as the mean ± SD of three mice.

Table II - AUC, clearance, and tissue uptake rate index of [^{3}H] liposomes after intravenous injection in mice.

Liposome	AUC (% of dose h/ml)	Clearance (ml/h)		Tissue uptake rate index (ml/h/g)		
		Cl_{total}	Cl_{liver}	Liver	Kidney	Spleen
DSPC/Chol liposomes	76.3	0.80	0.15	0.12	0.01	0.65
Man(5) liposomes	10.2	10.2	9.85	8.20	0.20	8.40
PEG5000(0.8) liposomes	90.7	0.08	0.02	0.01	0.01	0.21
PEG5000(0.8)-Man(5) liposomes	77.4	0.09	0.03	0.02	0.01	0.04
PEG2000(0.8) liposomes	75.5	0.12	0.02	0.01	0.02	0.01
PEG2000(0.8)-Man(5) liposomes	68.9	0.12	0.04	0.02	0.03	0.01
PEG350(0.8) liposomes	78.1	0.13	0.05	0.03	0.02	0.16
PEG350(0.8)-Man(5) liposomes	24.5	1.89	1.58	3.07	0.03	1.20

The AUC, clearance and tissue uptake indexes calculated at 1 h after injection were shown.

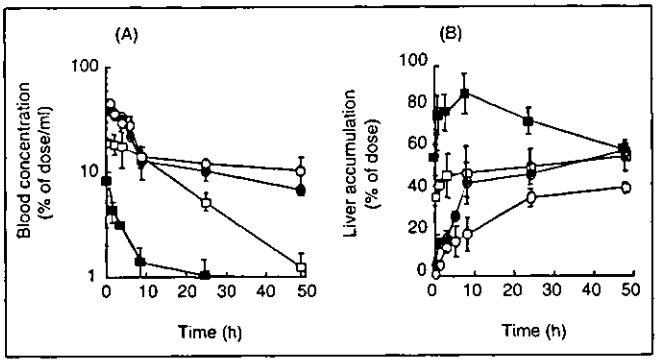

Figure 5 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]PEG350 liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (○) [^{3}H]PEG350(0.8) liposomes (DSPC/PEG350-DSPE/Chol = 59.2/0.8/40); (●) [^{3}H]PEG350(0.8)-Man(5) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 59.2/0.8/35/5).(□)[^{3}H]PEG350(0.8)-Man(7.5) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 59.2/0.8/32.5/7.5); (■) [^{3}H]PEG350(0.8)-Man(20) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 59.2/0.8/20/20). Results are expressed as the mean ± SD of three mice.

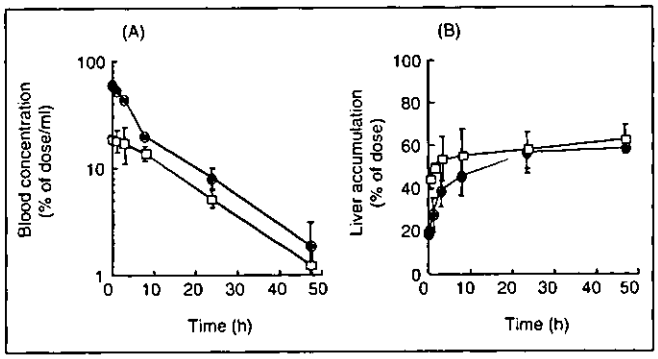

Figure 6 - Blood concentration (A) and liver accumulation time courses (B) of [^{3}H]PEG350-Man liposomes after intravenous injection into mice at a dose of 25 mg lipids/kg. (□) [^{3}H]PEG350(0.8)-Man(7.5) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 59.2/0.8/32.5/7.5); (●) [^{3}H]PEG350(1.6)-Man(7.5) liposomes (DSPC/PEG350-DSPE/Chol/Man-C4-Chol = 58.4/1.6/32.5/7.5). Results are expressed as the mean ± SD of three mice.

III. DISCUSSION

Targeted drug delivery to macrophages is an important strategy for achieving a variety of aims, such as the treatment of lysosomal storage diseases [17], the activation of macrophages with immunomodulators [18], cell or cell product depletion [19], and the blockade of macrophage functions [20]. To date, various liposomal formulations have been widely used for targeted drug delivery to macrophages [21]. Although liposomes are easily captured by macrophages following *in vivo* administration, active targeting of liposomes to the cells can increase the rate and extent of their uptake by the target cell population. Cell-specific targeting using the body's own carbohydrate recognition mechanisms has attracted great interest and we have reported the development of various polymeric or particulate carriers for this purpose [3, 22].

PEG lipids are commonly employed in liposomes and other lipid-based drug delivery systems to prolong their half-life within the systemic circulation by preventing rapid clearance by the MPS [23]. Liposomes with a sufficiently hydrophilic surface have been shown to have less protein, including opsonins, adsorbed onto their surface [24]. Vesicles containing varying amounts of PEG lipids with different molecular weight PEG chains have been reported to have different circulation times following an intravenous injection [25]. Synthetic lipid derivatives of PEG have been extensively tested for their ability to reduce the uptake of liposomes by MPS and to prolong the circulation half-life. A lipid derivative of PEG that has two saturated fatty acyl chains (PEG-DSPE) resulted in the longest circulation half-life among several PEG lipids [26]. A lipid derivative with only one saturated fatty acyl chain, such as PEG stearic acid, was even less effective in prolonging the circulation half-life of liposomes [27]. Therefore, in this study, PEG-DSPE was selected to control the hepatic uptake of Man-liposomes.

We have previously reported that DSPC/Chol liposomes modified with 5 mol% of Man-C4-Chol (Man liposomes) are mainly taken up by liver non-parenchymal cells, after an intravenous injection into mice [4]. Such liposomes exhibited an extensive hepatic uptake reaching about 75% of the injected dose at 10 min after injection. The uptake was significantly inhibited by pre-administration of an excess amount of mannosylated bovine serum albumin, suggesting that Man liposomes are taken up by liver non-parenchymal cells via mannose receptor-mediated endocytosis. In the present study, to control the hepatic uptake of the Man liposomes, various amounts of PEG-DSPE and Man-C4-Chol were incorporated into the liposomes. When 0.8 mol% of PEG5000-DSPE, PEG2000-DSPE or PEG350-DSPE was incorporated into

[^{3}H]Man(5) liposomes, the blood concentrations at 1 h following the intravenous injection were 65.7, 60.5 and 35.8% of dose/ml, respectively (*Figures 2-4*), whereas that of [^{3}H]Man liposomes lacking PEG-DSPE fell to less than 10% of the dose within the initial 5 min (*Figure 1*). The liver uptake clearance of [^{3}H]PEG(0.8)-Man(5) liposomes was proportional to the molecular weight of PEG and increasing the length of the PEG chain led to reduced liver uptake of the liposomes: PEG5000 ≈ PEG2000 < PEG350. However, when the PEG chain became too long (i.e., 5000 or 2000), the effects of liposome mannosylation completely disappeared. The liver accumulation of [^{3}H]PEG5000(0.8)-Man(5) or [^{3}H]PEG2000(0.8)-Man(5) liposomes was not significantly different from that of [^{3}H]PEG5000(0.8) or [^{3}H]PEG2000(0.8) liposomes (*Figures 2 and 3*). These results indicate that it is possible to control the hepatic uptake of Man liposomes by selecting a suitable chain length of PEG-DSPE, such as PEG350-DSPE. Klibanov *et al.* [28] have demonstrated liposome agglutination induced by the addition of streptavidin to PEG5000-containing liposomes incorporating a small amount of N-biotin-aminocaproyl-phosphatidyl ethanolamine and found that as little as 0.72 mol% PEG5000-PE in these liposomes completely abolished agglutination.

As PEG350-DSPE could control the hepatic uptake rate of Man liposomes, we then investigated the influence of the number (density) of Man-C4-Chol and PEG350-DSPE molecules on the hepatic uptake of PEG-Man liposomes. When the PEG content was constant at 0.8 mol% of total lipids, increasing the mannose density increased the hepatic uptake rate of the [^{3}H]PEG-Man liposomes (*Figure 5*). On the other hand, when the mannose content was fixed at 7.5 mol%, increasing the PEG350-DSPE density reduced the uptake rate (*Figure 6*). These results indicate that the delivery rate of liposomes to liver non-parenchymal cells can be controlled from 0.04 to 9.85 ml/h by adjusting the mixing ratio of Man-C4-Chol and PEG350-DSPE.

Thus, targeted and sustained delivery of liposomes to liver non-parenchymal cells was successfully achieved by optimizing the mixing ratio of Man-C4-Chol and PEG-DSPE and by choosing a PEG-DSPE with a short (350 average molecular weight) PEG chain. These findings provide basic information for achieving the rate-controlled targeted delivery of pharmaceuticals to cells using mannosylated liposomes.

REFERENCES

1. GARCON N., GREGORIADIS G., TAYLOR M., SUMMERFIELD J. - Mannose-mediated targeted immunoadjuvant action of liposomes. - Immunology, **64**, 743-745, 1988.
2. TODA S., ISHII N., OKADA E., KUSAKABE K., ARAI H., HAMAJIMA K., GORAI I., NISHIOKA K., OKUDA K. - HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-γ antibody. - Immunology, **92**, 111-117, 1997.
3. KAWAKAMI S., SATO A., NISHIKAWA M., YAMASHITA F., HASHIDA M. - Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. - Gene Ther., **7**, 292-299, 2000.
4. KAWAKAMI S., WONG J., SATO A., HATTORI Y., YAMASHITA F., HASHIDA M. - Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. - Biochim. Biophys. Acta, **1524**, 258-265, 2000.
5. OPANASOPIT P., SAKAI M., NISHIKAWA M., KAWAKAMI S., YAMASHITA F., HASHIDA M. - Inhibition of liver metastasis by targeting of immunomodulators using mannosylated liposome carriers. - J. Controlled Release, **80**, 283-294, 2002.
6. LEVY G. - Targeted drug delivery, Some pharmacokinetic considerations. - Pharm. Res., **4**, 3-4, 1987.
7. HARRIS J.M., MARTIN N.E., MODI M. - Pegylation: a novel process for modifying pharmacokinetics. - Clin. Pharmacokinet., **40**, 539-551, 2001.
8. BHADRA D., BHADRA S., JAIN P., JAIN N.K. - Pegnology: a review of PEG-ylated systems. - Pharmazie, **57**, 5-29, 2002.
9. WOODLE M.C. - Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation. - Chem. Phys. Lipids, **64**, 249-262, 1993.
10. OKU N., NAMBA Y. - Long-circulating liposomes. - Crit. Rev. Ther. Drug Carrier Syst., **11**, 231-270, 1994.
11. LASIC D.D., MARTIN F.J., GABIZON A., HUANG S.K., PAPAHADJOPOULOS D. - Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. - Biochim. Biophys. Acta, **1070**, 187-92, 1991.
12. MARUYAMA K. - *In vivo* targeting by liposomes. - Biol. Pharm. Bull., **23**, 791-799, 2000.
13. ISHIDA O., MARUYAMA K., TANAHASHI H., IWATSURU M., SASAKI K., ERIGUCHI M., YANAGIE H. - Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors *in vivo*. - Pharm. Res., **18**, 1042-1048, 2001.
14. WEBB M.S., SAXON D., WONG F.M., LIM H.J., WANG Z., BALLY M.B., CHOI L.S., CULLIS P.R., MAYER L.D. - Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine. - Biochim. Biophys. Acta, **1372**, 272-282, 1998.
15. REX S., ZUCKERMANN M.J., LAFLEUR M., SILVIUS J.R. - Experimental and Monte Carlo simulation studies of the thermodynamics of polyethyleneglycol chains grafted to lipid bilayers. - Biophys. J., **75**, 2900-2914, 1998.
16. NISHIKAWA M., OHTSUBO J., OHNO Y., FUJITA T., KOYAMA Y., YAMASHITA F., HASHIDA M., SEZAKI H. - Pharmacokinetics of receptor-mediated hepatic uptake of glycosylated albumin in mice. - Int. J. Pharm., **85**, 75-85, 1992.
17. FRIEDMAN B., VADDI K., PRESTON C., MAHON E., CATALDO J.R. - A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived β-glucocerebrosidase: implication for clinical efficacy in treatment of Gaucher disease. - Blood, **93**, 2807-2816, 1999.
18. KILLION J.J., FIDLER I.J. - Systemic targeting of liposome-encapsulated immunomodulators to macrophages for treatment of cancer metastasis. - Immunomethods, **4**, 273-279, 1994.
19. VAN ROOIJEN N.V., SANDERS A. - Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. - J. Immunol. Methods, **174**, 83-93, 1994.
20. GORDON S., RABINOWITZ S. - Macrophages as targets for drug delivery. - Adv. Drug Deliv. Rev., **4**, 27-47, 1989.
21. KANEDA Y. - Virosomes: evolution of the liposome as a targeted drug delivery system. - Adv. Drug Deliv. Rev., **43**, 197-205, 2000.
22. HASHIDA M., AKAMATSU K., NISHIKAWA M., YAMASHITA F., TAKAKURA Y. - Design of polymeric prodrugs of prostaglandin E1 having galactose residue for hepatocyte targeting. - J. Controlled Release, **62**, 253-262, 1999.
23. CULLIS P.R, CHONN A., SEMPLE S.C. - Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour *in vivo*. - Adv. Drug Deliv. Rev., **32**, 3-17, 1998.
24. DU H., CHANDAROY P., HUI S.W. - Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion. - Biochim. Biophys. Acta, **1326**, 236-248, 1997.

25. TORCHILIN V.P. - Polymer-coated long-circulating microparticulate pharmaceuticals. - J. Microencapsul., **15**, 1-19, 1998.
26. ALLEN T.M., HANSEN C., RUTLEDGE J. - Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. - Biochim. Biophys. Acta, **981**, 27-35, 1989.
27. ALLEN T.M., HANSEN C., MARTIN F., REDEMANN C., YAU-YOUNG A. -Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. - Biochim. Biophys. Acta, **1066**, 29-36, 1991.
28. KLIBANOV A.L., MARUYAMA K., BECKERLEG A.M., TORCHILIN V.P., HUANG L. - Activity of amphiphatic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. - Biochim. Biophys. Acta, **1062**, 142-148, 1991.

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GENE DELIVERY

Tissue and intrahepatic distribution and subcellular localization of a mannosylated lipoplex after intravenous administration in mice

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Abstract

We have previously reported that, unlike a lipoplex and mannosylated (Man) lipoplex underwent gene transfer to liver nonparenchymal cells (NPC) that possess mannose receptors after intravenous administration in mice. In this study, the tissue, intrahepatic distribution, and subcellular localization of the lipoplex after intravenous administration were investigated. DC-Chol liposome was selected as a cationic liposomes. After administration of lipoplex and Man lipoplex, the high gene expression was observed in the lung and liver, respectively. After administration of [^{32}P]Man lipoplex, about 80% of [^{32}P]plasmid DNA (pDNA) was accumulated in the liver. As for the intrahepatic distribution, the NPC/parenchymal cells (PC) ratio of [^{32}P]Man lipoplex was 9.64, whereas the NPC/PC ratio of [^{32}P]lipoplex was 1.93. The radioactivity in the cytosolic fraction of liver homogenate of [^{111}In]Man lipoplex was two-fold higher than that of [^{111}In]lipoplex, indicating that Man liposomes facilitate the release of pDNA into the cytosolic space. However, a rapid sorting of the radioactivity from endosomes to lysosomes was observed with the [^{111}In]Man lipoplex. Also, amplification of pDNA by PCR suggested that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex. These results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

Keywords: Gene delivery; Mannose receptor; Mannosylated liposomes; Cationic liposomes; Plasmid DNA

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. Nonviral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive vectors for clinical application. One of the most promising class of nonviral vectors developed so far is the cationic liposome-based transfection system. The lipoplex formation via electrostatic interaction of cationic liposomes and plasmid DNA (pDNA) facilitates the interaction of pDNA with cell membranes, leading to transgene expression in the cells [1]. In an attempt to increase the efficiency of transgene expression as well as to reduce cytotox-

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icity, several kinds of cationic lipids, such as quaternary ammonium detergents, cationic derivatives of cholesterol [2], diacylglycerol [1,3], and alkyl derivative of polyamines [4] have been developed. Among them, some lipoplex have been used in clinical trials for the treatment of cancer and cystic fibrosis [5,6].

The lipoplex is a useful nonviral vector, but it lacks specificity in delivery and transfection after systemic administration. Although the levels of gene expression vary from study to study, the lung invariably shows the highest gene expression. 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 macromolecule-based nonviral vectors, several ligands including galactose [7,8], mannose [9,10], transferrin [11], and antibodies [12] 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 lipoplex.

Mannose receptor-mediated targeting is a promising approach to achieve cell-specific delivery after systemic administration because (i) the expression of mannose receptors is restricted to the liver NPC and other macrophages, (ii) a complex entering the systemic circulation has easy access to the liver NPC, and (iii) the liver has a high blood flow. These physical and biological features give a mannosylated vector an opportunity to deliver pDNA to the liver NPC via mannose receptor-mediated endocytosis. Liver nonparenchymal cells (NPC), including sinusoidal endothelial cells and Kupffer cells, can be the targets for gene therapy because they have been implicated in a wide variety of diseases [13,14].

In a previous study, we developed a novel mannosylated derivative of cholesterol, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino) butyl)formamide (Man-C4-Chol), and used it to prepare a cationic liposome formulation (Man liposome) [10]. Man-C4-Chol possesses multi-functional properties, that is, (i) a lipophilic anchor moiety (cholesterol) for stable incorporation into liposomes, (ii) a mannose moiety for recognition by the mannose receptors, and (iii) an imino group for binding to pDNA via electrostatic interaction [15]. Furthermore, low-molecular-weight glycolipids are more promising due to their low immunogenicity, high reproducibility, and ease of mass production. Although, a high gene expression in the liver and spleen after intravenous injection was observed for Man lipoplex via mannose receptor-mediated endocytosis compared with the lung, its transfection efficiency was relatively low and, consequently, further improvements in the efficiency of transgene expression are required.

In order to obtain a theoretical strategy to develop an efficiently targetable gene carrier to the liver by mannosylation, therefore, detailed information on the distribution of a Man lipoplex needs to be obtained. In the present study, we studied the tissue, intrahepatic distribution, and subcellular localization of a [^{32}P]- or [^{111}In]-labeled Man lipoplex after intravenous administration. The results were compared with those for a 3β[*N*′,*N*′,*N*′-dimethylaminoethane]-carbamoyl]cholesterol liposomes (DC liposome), which is a cationic cholesterol derivative, based lipoplex [2].

2. Materials and methods

2.1. Chemicals

N-(4-Aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate was obtained from Sigma (St. Louis, MO, USA), dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar-Lipids (Alabaster, AL, USA). [α-^{32}P]dCTP was obtained from Amersham (Tokyo, Japan). ^{111}Indium chloride ([^{111}In]$InCl_3$) was supplied by Nihon Medi-Physics (Hyogo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride and 4-[*p*-azidosalicylamido]butylamine (ASBA) were purchased from Dojindo (Kumamoto, Japan) and Pierce Biotechnology (Rockford, IL, USA). Man-C4-Chol [10] and DC-Chol [2] were synthesized as reported previously. Mannosylated bovine serum albumin (Man-BSA) was synthesized and radiolabeled with [^{111}In]$InCl_3$ as reported previously [16]. All other chemicals were obtained commercially as reagent-grade products.

2.2. Construction and preparation of pDNA

pCMV-Luc was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from

pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the E coli strain DH5α, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Purity was confirmed by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

2.3. Animals

Female ICR mice (5-week-old, 20–25 g) were obtained from the Shizuoka Agricultural Co-operative 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 the US National Institutes of Health and with the Guidelines for Animal Experiments of Kyoto University.

2.4. Preparation of liposomes

Man-C4-Chol (8.1 μmol) or DC-Chol (10.0 μmol) was mixed with DOPE (5.4 μmol for Man-C4-Chol and 6.7 μmol for DC-Chol) in chloroform (5.0 ml) and evaporated to dryness in a round-bottomed flask. Then, the lipid film was vacuum desiccated to remove any residual organic solvent and resuspended in 5% (w/v) dextrose (2.5 ml). After hydration, the dispersion was sonicated for 5–10 min in a bath sonicator to produce liposomes. The liposome formulations were passed through a polycarbonate membrane filter (0.22 μm) for sterilization. The liposomal lipid concentration was determined by phosphorous analysis [17] and was adjusted to 3 mg/ml.

2.5. Lipoplex formation

The mixing ratio of liposomes with pDNA was expressed as a charge ratio, which is the molar ratio of the cationic lipids to the pDNA phosphate residues [18]. The charge ratio (+: −) of these liposomes and pDNA complex was adjusted to 2.3:1.0 for cell-selective gene transfection [19,20]. A solution of cationic liposomes (0.2 ml) was added to an equal volume of pDNA solution (0.2 ml and pDNA concentration ≅ 0.33 μg/μl) in a polyethylene tube. Then, the lipoplex was agitated rapidly by pipetting it up and down twice using a micropipette (PIPETMAN®, GILSON, Villier-le Bel, France) and left to stand for 30 min. The particle size and zeta potential of the lipoplex were measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan) and a laser electrophoresis zeta-potential analyzer (LEZA-500T, Otsuka Electronics), respectively.

2.6. pDNA radiolabeling methods

pDNA was radiolabeled with [α-^{32}P]dCTP by nick translation [21]. In a separate preparation, pDNA was radiolabeled with ^{111}In using a newly developed method [22]. Briefly, a dimethylsulfoxide solution of ASBA was added to DTPA anhydride under darkroom conditions, and the mixture was incubated at room temperature for 1 h. Then, pDNA solution was added to the mixture, and the mixture was immediately irradiated under an UV lamp at room temperature for 15 min to obtain DTPA-ASBA coupled pDNA (DTPA-ASBA-pDNA). The product was purified by precipitation twice with ethanol, and labeled with [^{111}In]$InCl_3$. The purity of each pDNA was checked by Sephadex G-25 column chromatography and 1% (w/v) agarose gel electrophoresis.

2.7. Gene expression experiments

Five-week-old ICR mice were injected intravenenously with 300 μl of lipoplex using a 30-gauge syringe needle. Three or 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, 0.1 M 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 and G Berthold, Bad Wildbad, Germany). The protein

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

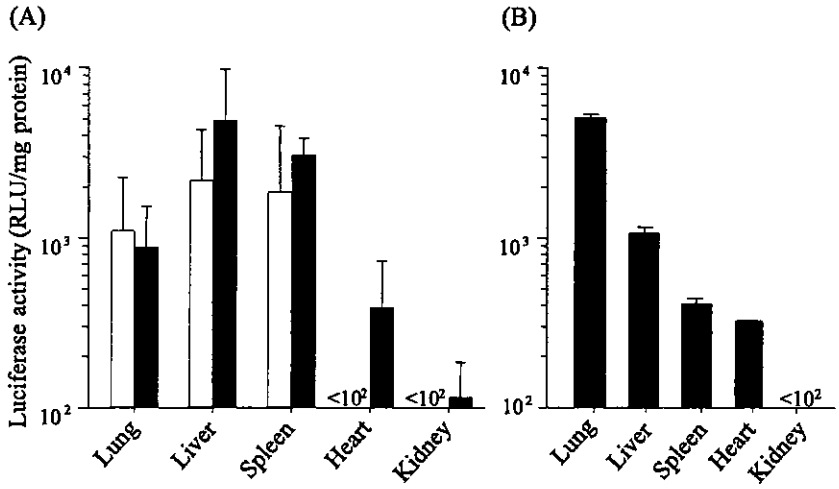

Fig. 1. Gene expression after intravenous administration at 3 (□) and 6 h (■) of Man lipoplex (A) and at 6 h of lipoplex (B) in mice. Each value represents the mean + S.D. values of three experiments.

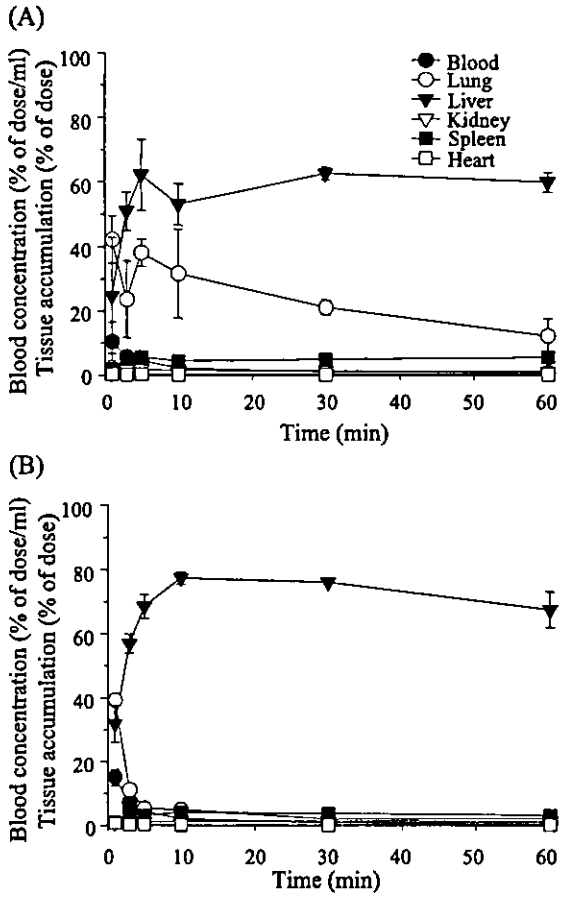

Fig. 2. Radioactivity in blood and tissues after intravenous administration of [^{32}P]lipoplex (A) or Man lipoplex (B) in mice. Each value represents the mean ± S.D. values of three experiments.

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

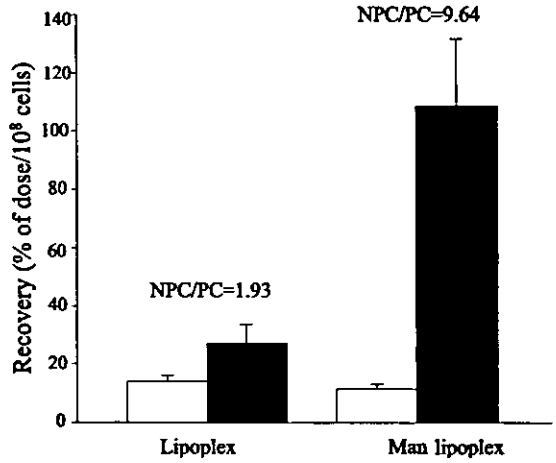

Fig. 3. Cellular localization of radioactivity between PC (□) and NPC (■) 30 min after intravenous administration of [^{32}P]lipoplex and Man lipoplex in mice. Each value represents the mean + S.D. values of three experiments.

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-

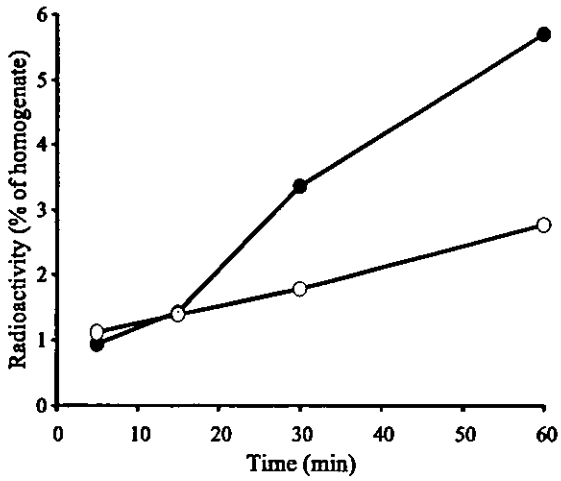

Fig. 4. Radioactivity in cytoplasmic fraction of mouse liver homogenates 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (○) or Man lipoplex (●) in mice. Similar results were obtained in two other independent runs.