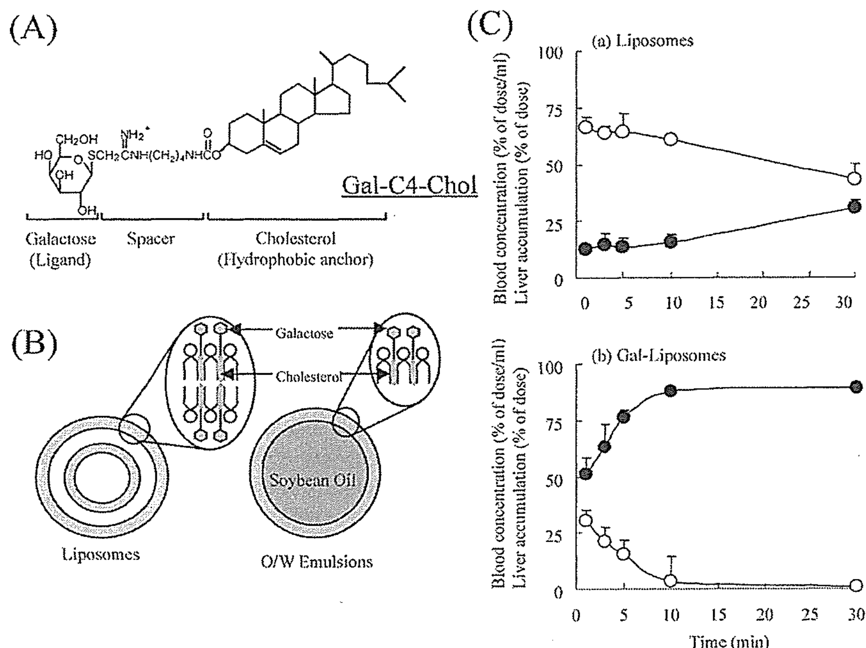

Fig. 3. (A) Structure of Gal-C4-Chol, (B) Scheme of Galactosylated Liposomes and Emulsions Using Gal-C4-Chol, and (C) Blood Concentration (○) and Liver Accumulation (●) of [^{3}H]Cholesteryl Hexadecyl Ether Labeled Liposomes (a) and Gal-Liposomes (b) Following the Intravenous Injection into Mice

Liposomes and Gal-liposomes were composed of DSPC, Chol, Gal-C4-Chol at a molar ratio of 12 : 8 and 12 : 7 : 1, and the lipid concentration was adjusted to 5 mg/ml. Each value represents the mean+S.D. of three experiments.

prisingly, a cationic liposome formulation, which was expected to have a longer retention in the tumor, showed a lower level of gene expression in these tumor models. Moreover, increasing the cationic charge of the lipoplex decreased the gene expression in the tumors. These results suggested that free pDNA might affect the gene expression following intratumoral injection.

4.3. Gene Expression Characteristics of pDNA and Its Complex with Cationic Liposomes after Intravitreal Injection To optimize the *in vivo* ocular transfection efficiency of pDNA/cationic liposome complexes, DOTMA/DOPE liposomes and DOTMA/cholesterol liposomes were prepared with varying amounts of pDNA.[44] pDNA/cationic liposome complexes were intravitreally injected in rabbits, and the luciferase activity in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body and retina were measured. In the case of intravitreal injection, the gene expression in ocular tissues of the lipoplex was markedly higher than those of naked pDNA. Taking the intratumoral injection results into consideration, the gene expression characteristics after local injection (*i.e.*, naked pDNA *vs.* lipoplex) differed from the injection site.

5. Liposomes for Drug Delivery Following Systemic Injection

Liposomes are an established example of a lipid carrier system that has been researched extensively. After intravenous injection, they are commonly retained in the blood circulation and removed by the RES. Thus, their application is mainly limited to persistent retention in the blood circulation and passive targeting to the RES or solid tumors with a highly permeable capillary endothelium. Since an ideal drug therapy has high therapeutic efficacy with few side effects, cell-specific targeting of liposomes is sometimes urgently required for a variety of clinical purposes.

5.1. Galactosylated Liposomes for Asialoglycoprotein Receptor-Mediated Drug Delivery to Hepatocytes Receptor-mediated drug delivery is a promising approach to site-selective drug delivery. Receptors for carbohydrates, such as the asialoglycoprotein receptor on hepatocytes (liver PC) and the mannose receptor on several macrophages and liver endothelial cells, recognize the corresponding sugars on the non-reducing terminal of sugar chains.[2] This mechanism would be an effective way to achieve hepatocyte targeting.

For the application of drug targeting systems to liposomes, we developed Gal-C4-Chol to modify liposomes with galactose moieties for hepatocyte-selective drug delivery (Figs. 3A, B).[45] As mentioned for Gal-emulsions, our strategy for the efficient targeting of lipid carriers by glycosylation is achieved by stable fixation of the sugar moiety on the surface of the liposomes under *in vivo* conditions. Since cationic charge enhances the non-specific interaction, galactosylated liposomes for drug delivery were prepared with by Gal-C4-Chol, neutral lipid (distearoylphosphatidylcholine) and cholesterol. Each formulation was labeled with [^{3}H]cholesteryl hexadecyl ether. After intravenous injection, galactosylated liposomes (Gal-liposome) rapidly disappeared from the blood and 85% of the dose had accumulated in the liver within 10 min, while the hepatic accumulation of bare liposomes was 12% (Fig. 3C). The liver was perfused with collagenase, and liver PC and NPC were separated by centrifugal differentiation to determine the cellular distribution. The PC/NPC ratios for Gal-liposomes and bare-liposomes were 15.1 and 1.1, respectively, indicating the PC-selectivity in Gal-liposomes. Furthermore, the hepatic uptake of Gal-liposome liposomes was significantly inhibited by the predosing of galactosylated bovine serum albumin, but not by that of bare-liposomes. These results indicated that Gal-liposomes

Table 1. Various Factors on *in Vivo* Gene Expression by Lipoplex

Factors	Effect on gene expression	Ref.
1. Lipoplex		
Charge	High cationic charge enhances gene expression	60, 61, 62, 63, 66
2. Cationic liposomes		
Helper lipid	Cholesterol containing liposomes enhance gene expression	58, 62, 64, 67
Size	Large sized liposomes enhance gene expression	62
3. pDNA		
Dose	High dose enhances gene expression	59, 60, 61, 63, 64, 66
CpG motif	CpG motif in pDNA induces the inflammatory cytokines;as a consequence, the terms of gene expression are decreased	81,82

are efficiently taken up by the asialoglycoprotein receptor on PC after intravenous injection.

These Gal-liposomes were able to effectively deliver prostaglandin E_1[46] and probucol[47] to hepatocytes *in vivo*, indicating that Gal-liposomes function as hepatocyte-selective drug carriers of lipophilic drugs. Moreover, the recognition by asialoglycoprotein receptors of Gal-liposomes *in vivo* may be affected by the cholesterol contents[48] and lipid compositions[47,49] in Gal-liposomes.

5.2. Mannose and Fucose Liposomes for Mannose and Fucose Receptor-Mediated Drug Delivery to Liver NPC After intravenous injection, we have demonstrated that mannosylated[50] and fucosylated[51] proteins are efficiently taken up by NPC, mainly composed of sinusoidal endothelial cells and Kupffer cells, and this uptake was mediated by mannose and fucose receptor mediated endocytosis.[52] Based on these observations, we synthesized two glycolipids, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl) formamide (Man-C4-Chol) and cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiofucosylethyl)-amino)butyl)formamide (Fuc-C4-Chol), to prepare the mannosylated (Man-) and fucosylated (Fuc-) liposomes for NPC-selective drug delivery *via* mannose and fucose receptor mediated uptake.[53,54] Furthermore, we have demonstrated that mannose-specific lectin in the serum, MBP, which binds to pathogens having mannose units on their surface, can bind to Man-liposomes, and these MBP-bound Man-liposomes are more efficiently recognized by macrophages.

6. Cationic Liposomes for Gene Delivery Following the Systemic Injection

In 1987, Felgner *et al.* reported that the use of cationic liposomes was more effective than either the calcium phosphate or the DEAE-dextran transfection technique in various cultured cells.[56] This technique is simple, highly reproducible and effective for both the transient and stable expression of transfected DNA. In 1993, Zhu *et al.* reported *in vivo* gene expression could be observed by the intravenous injection of pDNA complexed with DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride)/DOPE (dioleoylphosphatidylethanolamine) liposomes; however, its transfection efficacy seemed to relatively low.[57] In the late 1990s, several studies showed that various factors enhanced the gene expression *in vivo*.[58—67] These factors for *in vivo* gene expression due to lipoplex are listed in Table 1. After the intravenous administration of lipoplex, the lung shows the highest amount of gene expression among the various organs and the lung endothelial cells are the main contributor to transgene expression. We and others have confirmed that the gene expression level in the lung is 100—10000 times higher than that in the liver and spleen. Using these gene expression characteristics of the lung, lipoplex was applied to the prevention of lung cancer metastasis in mice.[68,69] Since the physicochemical properties of pDNA are almost the same, irrespective of the encoding cDNA for the therapy, targeting technologies are especially required for their broad clinical application.

For successful *in vivo* gene delivery using these carrier systems, there are many barriers to overcome.[70] Such factors include; i) the extent of DNA condensation, ii) particle size of the lipoplex, iii) interaction with endogenous components and tissues, iv) the route of administration, v) stability against nucleases, vi) controlled *in vivo* distribution, vii) binding to cell surface receptors and internalization, and viii) how intracellular trafficking affects *in vivo* gene delivery and expression.[71] Since many barriers from the injection site to the target cells exist for cell-selectivity with *in vivo* gene transfection, there are few *in vivo* reports compared with *in vitro* observation. In order to develop an effective cell-selective *in vivo* gene carrier, therefore, the distribution characteristics of gene medicine must be clarified.[72,73] Based on the pharmacokinetic information, we have developed novel cell-selective gene transfection carriers. In this section, we shall focus on the progress of research into targeted delivery systems of lipoplexes after systemic administration.

6.1. Distribution Characteristic of pDNA Following the Systemic Injection To develop a strategy for establishing liposomal carrier systems of pDNA, it is necessary to understand their *in vivo* distribution characteristics. Thus, the distribution characteristics of pDNA were analyzed by [^{32}P] labeled pDNA. After intravenous injection, [^{32}P] pDNA was rapidly eliminated from the plasma, involving extensive uptake by the liver.[74] Pharmacokinetic analysis demonstrated that the hepatic uptake clearance of pDNA is almost identical to the plasma flow rate in the liver, suggesting highly effective elimination by the liver. As for the uptake mechanism by the liver, a competitive inhibition study demonstrated that [^{32}P] pDNA is taken up preferentially by the liver NPC *via* a scavenger receptor-mediated process, in a manner specific for polyanions. The involvement of scavenger receptors in the hepatic uptake of pDNA has also been supported by a single-pass rat perfusion study[75] and an uptake study using primary cultured mouse peritoneal macrophages.[76,77]

6.2. Distribution and Gene Expression Characteristics of Lipoplexes Following Systemic Injection The distribution characteristics of lipoplexes at the early period is

Table 2. Cell-Selective Gene Delivery Carriers Using Ligands According to Their *in Vivo* Applications

Receptor	System	Results	Ref.
Asialoglycoprotein			
Wu, 1988	Asialoorosomucoid-polylysine	Expression in liver after i.v. injection	84
Perales, 1994	Galactose-polylysine	Expression in liver after i.v. injection	85
Hara, 1995	Asialofetuin-liposome	Expression in liver after intraportal injection	86
Kawakami, 2000	Galactose-liposome	Expression in hepatocytes after intraportal injection	87
Nishikawa, 2000	Galactose-polyornithine-HA2	Expression in hepatocytes after i.v. injection	88
Morimoto, 2003	Galactose-PEI	Expression in hepatocytes after intraportal injection	89
Fumoto, 2004	Galactose-liposome	Expression in hepatocytes after intraportal injection	90
Mannose			
Kawakami, 2000	Mannose-liposome	Expression in liver NPC after i.v. injection	91
Kawakami, 2004	Mannose-liposome	Expression in liver NPC after i.v. injection	92
Hattori, 2004	Mannose-liposome	Enhancement of immune responses by DNA vaccination	93
Transferrin			
Ogris, 1999	Transferrin-PEG-PEI (800 kDa)	Expression in cancer cells (s.c.) after i.v. injection	94
Kircheis, 2001	Transferrin-PEI (22 kDa)	Expression in cancer cells (s.c.) after i.v. injection	95
Shi, 2001	Transferrin-liposome	Expression in brain after i.v. injection	96
Kursa, 2003	Transferrin-PEG-PEI (22 kDa)	Expression in cancer cells (s.c.) after i.v. injection	97
Zhang, 2003	Transferrin-liposome	Expression in brain after i.v. injection	98
Folate			
Hofland, 2002	Folate-liposome	Expression in cancer cells (s.c.) after i.v. injection	99
Reddy, 2002	Folate-liposome	Efficient expression in intraperitoneal cancer cells after intraperitoneal injection	100

important for gene expression. In fact, Barron *et al.* recently demonstrated that lipoplex-mediated gene expression to the lung occurs within 60 min after intravenous injection.[78] We have emphasized the importance of distribution for the development of gene carriers; therefore, we evaluated the distribution characteristics of [^{32}P] lipoplex.[79,80] After the intravenous injection of a [^{32}P] lipoplex, a rapid clearance of pDNA from the circulation was observed with extensive accumulation in the lung and liver. As far as the type of liver cells involved was concerned, the [^{32}P] lipoplexes were predominantly taken up by liver NPC. As for the uptake mechanism by liver NPC, a competitive inhibition study demonstrated that the hepatic uptake of lipoplexes was significantly inhibited by the preceding administration of dextran sulfate, but not by poly [C] and poly [I], suggesting the involvement of a phagocytic process.

Recent studies have demonstrated that the intravenous administration of a lipoplex induced significant proinflammatory cytokine production in the blood and inhibited transgene expression in the pulmonary endothelial cells.[81,82] Even if gene expression is exhibited at a favorable level, a high toxicity would lead to failure in clinical application. We have demonstrated that tissue macrophages involving liver Kupffer cells and spleen macrophages are closely involved in TNF-α production.[83] This result corresponded with our previous distribution results that the [^{32}P] lipoplex was mainly distributed in the liver NPC.[84,85] Thus, it was suggested that avoiding lipoplex uptake and subsequent cytokine production by Kupffer cells and spleen macrophages would be a useful method of maintaining a high level of gene expression in the lung after repeated injections.

6.3. Galactosylated Cationic Liposomes for Asialoglycoprotein Receptor-Mediated Gene Delivery to Hepatocytes For cell-specific delivery, receptor-mediated endocytosis (RME) systems possessed by various cell types would be useful and a number of gene delivery systems have been developed to introduce foreign DNA into specific cells with RME. Table 2 summarizes the cell-selective *in vivo* gene delivery systems using ligand modified cationic liposomes or polymer.[84—100] Since there are many barriers under *in vivo* conditions, there are few reports about the cell-selective gene delivery using ligand-modified lipoplexes. In order to overcome these barriers, gene carrier systems must be developed by consideration of the effect of physicochemical properties on the *in vivo* distribution. In this section, we shall focus on the research into our targeted gene systems of lipoplexes using asialoglycoprotein receptors.

Hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Remy *et al.* reported the feasibility of using galactose-presenting lipopolyamine vectors for targeted gene transfer into hepatoma cells under *in vitro* conditions.[101] The inclusion of galactose residues in the electrically neutral complex increased the transgene expression approaching the level obtained with a large excess of cationic liposomes alone. For *in vivo* hepatocyte-selective gene transfection, we designed Gal-C4-Chol for the preparation of the galactosylated cationic liposomes.[39]

A distribution study demonstrated that the radioactivity in the liver from the [^{32}P] pDNA/Gal-C4-Chol incorporated complex (Gal-lipoplex) was about 75% of the dose, even 1 min after intraportal injection.[87] The hepatic gene expression of the Gal-lipoplex was more than 10-times greater than that of the pDNA complexed with conventional cationic liposomes. When the gene expression was examined by determining the intrahepatic cellular levels, the gene expression of liver PC of Gal-lipoplex was significantly higher than that of liver NPC. In contrast, there was little difference in the gene expression of PC and NPC of conventional cationic liposomes. In addition, an excess amount of galactosylated bovine serum albumin was intravenously injected prior to the injection of the Gal-lipoplex; the gene expression in the liver was significantly reduced, suggesting uptake via asialoglycoprotein receptor-mediated endocytosis.

However, the level of *in vivo* gene expression due to the

Gal-lipoplex was not as high as that expected from the *in vitro* results. There must be several barriers associated intrinsically with *in vivo* situations, such as convective blood flow in the liver, passage through the sinusoids and tissue interactions. To investigate these barrier processes, we studied the hepatic distribution profiles of Gal-lipoplexes using rat liver perfusion techniques[102] that allowed us to determine the uptake characteristics of various substances under different experimental conditions with the structure of the liver remaining intact.[103—105] In that study, we demonstrated that the penetration of the Gal-lipoplex through the hepatic fenestrated endothelium to the PC was greatly restricted in perfused rat liver in spite of the small size of the Gal-lipoplex (about 120 nm), as far as crossing the fenestrae was concerned.

In the next step, therefore, we tried to enhance the gene expression in the liver by preparing the novel stabilizing Gal-lipoplex.[96] Lipoplexes are often prepared in a nonionic solution due to their well-known tendency to aggregate out of solution as the salt concentration is increased.[106,107] Aggregation during lipoplex formation in ionic solution may be due to neutralization of the surface positive charge of the lipoplex intermediate by the associated counter-ion. Taking into account neutralization by the counter-ion, we hypothesized that the presence of an essential amount of sodium chloride (NaCl) during lipoplex formation might regulate the repulsion between cationic liposomes and thereby, the fusion of cationic liposomes in the lipoplex would be accelerated by the partial neutralization of the positive charge. Consequently, pDNA in the lipoplex could be largely covered by cationic lipids while retaining enough positive charge to prevent aggregate formation. Such types of lipoplex are expected to be more stable than the conventional lipoplex, which is prepared using a nonionic solution. After intraportal administration, the hepatic transfection activity of the Gal-SCR-lipoplex was approximately 10- to 20-times higher than that of the conventional galactosylated lipoplex in mice. The transfection activity in hepatocytes of the Gal-SCR-lipoplex was significantly higher than that of the conventional lipoplex, and pre-injection of asialoglycoprotein-receptor blocker markedly reduced the hepatic gene expression, suggesting that hepatocytes are responsible for high hepatic transgene expression of the Gal-SCR-lipoplex.

6.4. Mannosylated Cationic Liposomes for Mannose Receptor-Mediated Gene Delivery to Macrophages Macrophages are important targets for the gene therapy of a number of diseases, such as Gaucher's disease[108] and human immunodeficiency virus (HIV) infection,[109] but the process of gene transfection in such cases is not easy. The use of nonviral vectors is attractive for *in vivo* gene delivery because it is simpler than using viral systems and is free from some of the risks inherent in the latter. Erbacher *et al.* investigated the suitability of various glycosylated poly(L-lysine) derivatives for introducing pDNA into human monocyte-derived macrophages and found that mannosylated poly(L-lysine) exhibited high transfection activity.[110] However, they also reported that the transfection activity was markedly enhanced in the presence of chloroquine due to the prevention of endosomal and/or lysosomal degradation of pDNA after mannose receptor-mediated endocytosis; for this reason, their *in vivo* use remains limited.

Hence, a cationic liposome-based targeted gene delivery system is a better method under *in vivo* conditions. Recently, we synthesized Man-C4-Chol for mannose receptor-mediated gene transfection to macrophages,[91] which are known to express large numbers of mannose receptors on their surface. In primary cultured mouse peritoneal macrophages, a Man-C4-Chol containing lipoplex (Man-lipoplex) showed higher transfection activity than that of the conventional lipoplex. The presence of 20 mM mannose significantly inhibited the transfection efficiency of Man-lipoplex, suggesting that the mannosylated lipoplex is recognized and taken up by the mannose receptors on macrophages. To further enhance gene transfection, polyethylenimine (PEI) was incorporated into this liposome complex (DNA/Man-PEI-complexes), taking note of the pH-buffering capacity in endosomes and DNA-condensing activity of PEI.[111] It was demonstrated that multifunctional DNA/Man-PEI-complexes exhibit highly improved gene transfection in primary cultured macrophages *via* mannose receptor-mediated endocytosis.

After intravenous injection, the highest gene expression was observed in the liver after the intravenous injection of the Man-lipoplex in mice.[91] In addition, gene expression with Man-lipoplex in the liver was observed preferentially in the liver NPC and was significantly reduced by predosing with mannosylated bovine serum albumin. These results suggest that Man-lipoplex exhibits high transfection activity in NPC due to recognition by mannose receptors. Unlike the case of the Gal-lipoplex, cell-selective gene transfection can be achieved by the intravenous administration of the Man-lipoplex.[92] This phenomenon could be explained by the fact that in the liver and spleen, macrophages are present around endothelial cells; therefore, they are in contact with the lipoplex without passing through the sinusoids (100—200 nm). Hence, the Man-lipoplex is effective in an NPC-selective gene transfection system, even when administered intravenously. The same phenomenon may be achieved with the intraportally administered Man-lipoplex.[112] In order to obtain a theoretical strategy to develop an efficiently targetable gene carrier to the liver by mannosylation, we studied the tissue, intrahepatic distribution and subcellular localiza-

Table 3. Various Factors on *in Vivo* Cell-Selective Gene Expression by the Glycosylated Lipoplex

Factors	Effect on gene expression	Ref.
1. Glycosylated lipoplex		
Charge	Moderate cationic charge ratio (− : +), 1.0 : 2.3—1.0 : 3.1 is suitable	87, 92, 102
Size	Small and/or stabilized lipoplexes enhance gene expression	90
2. Glycosylated cationic liposomes		
Helper lipid	Depending on the administration routes	87, 112
3. pDNA		
Dose	High dose enhances gene expression	87, 90

tion of a [^{32}P]- or [^{111}In]-labeled Man-lipoplex after intravenous injection.[113] The radioactivity in the cytosolic fraction of liver homogenate of [^{111}In] Man-lipoplex was 2-times higher than that of the [^{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, the 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 the intracellular sorting may improve the transfection efficiency of the Man-lipoplex. Table 3 summarizes the various factors for *in vivo* cell-selective liposomal gene delivery obtained in our studies.

6.5. Application of Man-Lipoplex to Gene Therapy DNA vaccination, the administration of DNA-encoding antigen genes into the body, is of great interest in gene therapy for the immunotherapy of cancer and infectious diseases. Animal studies have shown that DNA immunization induces not only an antibody response but also a potent cell-mediated immune response against the encoding antigen.[114—116] This cell-mediated immune response plays a crucial role in the immune response against cancer and infectious diseases.[117] Recently, we showed that the targeted delivery of DNA vaccine by Man-C4-Chol liposomes is a potent method of DNA vaccine therapy.[93] Although further improvements in transfection efficacy are required, the targeted delivery of DNA vaccine to DCs may improve future *in vivo* DNA vaccine therapies.

7. Conclusions

Successful drug and gene therapy requires the development of a rational delivery technology that satisfies various requirements for each target disease. We developed various lipid carrier systems for targeted drug and/or gene delivery following local or systemic injection. This information will be of value for the future use, design, and development of drug and/or gene delivery systems based on lipid carriers.

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In Vivo Gene Transfer by Ligand-Modified Gene Carriers

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1 Introduction

Efficient in vivo gene transfer relies on the development of vectors that offer the efficient delivery of genes to target cells, high transfection efficiencies, and the persistence of transgene expression. Although the gene-transfer efficacy of the current non-viral vector systems is lower than that of viral vectors, for safety reasons, the former approach is useful for many applications. Furthermore, non-viral vectors have advantages over viral ones with respect to controlling the biodistribution of a gene, because the physicochemical properties that determine the tissue disposition of the gene carrier, such as the particle size, electric charge, and specific ligand, can be easily controlled in these vectors. Among the various types of non-viral vectors, cationic liposomes and cationic polymer-mediated gene transfection seem to be the most promising approaches because of the relatively high transfection efficiencies of such vectors and the ability to target gene delivery by their chemical modification (Mahato et al. 1997; Huang and Li 1997; Sagara and Kim 2002). Cationic carriers condense pDNA to form particles (100–200 nm) based on electrostatic interactions and protect it from degradation. In most cases, after intravenous injection of cationic carriers/pDNA complexes, the highest levels of gene expression occur in the lung because the lung capillaries are the first "traps" to be encountered. Therefore, the development of carrier systems that can escape from undesired tissue uptake and exhibit target-cell-specific gene expression are urgently required.

For cell-specific delivery, the receptor-mediated endocytosis (RME) systems of various cell types are potentially useful, and a number of gene delivery systems have been developed to introduce foreign DNA into specific cells by RME. In developing a strategy for efficient and safe in vivo gene deliver, however, a variety of aspects, such as the construction of sophisticated carrier systems, and the pharmacokinetics and physicochemical properties of pDNA, need to be considered (Takakura and Hashida

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1996). In this chapter, the pharmacokinetics of intravenously administered pDNA, with or without non-viral gene carriers, are discussed. This is followed by a review of the current receptor-mediated gene delivery systems developed for use in vivo.

2 Biodistribution Characteristics of Naked pDNA

It has been reported that intravenously administered naked pDNA does not lead to gene expression. Nonetheless, a strategy for establishing non-viral gene carrier systems, however, must consider the in vivo disposition characteristics of naked pDNA. Kawabata et al. (1995) demonstrated that intravenously injected ^{32}P-labeled-pDNA is rapidly eliminated from the plasma by a mechanism involving extensive uptake by the liver . Pharmacokinetic analysis demonstrated that hepatic clearance is almost identical to the plasma flow rate in the liver, suggesting highly effective elimination by this organ. In addition, pDNA is taken up preferentially by liver non-parenchymal cells via a scavenger-receptor-mediated process that is specific for polyanions. In vitro binding and uptake studies using cultured mouse peritoneal macrophages demonstrated that the binding of pDNA was significantly inhibited by polyinosinic acid (poly [I]) and dextran sulfate, which are substrates of the scavenger receptors, but not by polycytidylic acid (poly [C]), dextran, or EDTA, which are not substrates of this receptor. These data suggest that pDNA is taken up by macrophages via a mechanism mediated by a receptor like the macrophage scavenger receptor. Involvement of scavenger receptors (SRs) in the hepatic uptake of pDNA has also been supported by single-pass rat perfusion experiments using [^{32}P]-pDNA (Yoshida et al. 1996).

The class A scavenger receptor (SRA), the best characterized SR, recognizes a wide variety of anionic macromolecules based on their three-dimensional structure and seems likely to be responsible for pDNA uptake. Recently, Takakura et al. reported an in vitro study of [^{32}P]-pDNA binding and uptake using cultured CHO cells expressing SRA (CHO (SRA) cells) and peritoneal macrophages from SRA-knockout mice (Takakura et al. 1999). [^{32}P]-pDNA binding and uptake by CHO (SRA) cells were minimal and almost identical to that by wild-type CHO cells. Macrophages from the knockout mice showed pronounced pDNA binding and uptake, as did the control macrophages. In both types of macrophages, [^{32}P]-pDNA binding was significantly inhibited by pDNA, poly [I], and dextran sulfate, but not by poly [C] or Acetylated low density lipoprotein (Ac-LDL). These results provide direct evidence that SRA is not responsible for any significant binding and subsequent uptake of pDNA by mouse peritoneal macrophages. Instead, pDNA binding and uptake by mouse peritoneal macrophages are mediated by a specific mechanism involving defined polyanions. These findings form an important basis for further studies to elucidate the mechanisms of pDNA uptake by macrophages. It is clear, however, that the strong anionic charge of pDNA should be neutralized in order for it to escape being recognized by scavenger receptor-like mechanisms. For this reason, cationic liposomes and cationic polymers have been developed.

3 Biodistribution Characteristics of pDNA Complexed with Cationic Liposomes

Cationic liposome and polymers condense pDNA to form particles based on electrostatic interactions and protect it from degradation. For subsequent gene expression, the complex must contain an excess of cationic charges After intravenous administration of the pDNA complexes, the immediate effect of erythrocytes on is to induce aggregation of the complexes (Sakurai et al. 2001). Large aggregates (>400 nm) are readily entrapped in the lung capillaries. Mahato et al. (1995) demonstrated the disposition characteristics of [^{32}P]-pDNA-cationic liposome complex after intravenous administration in mice. Rapid clearance of [^{32}P]-pDNA from the circulation was observed, with extensive accumulation in the lung and liver. In addition, [^{32}P]-pDNA complexed with cationic liposomes was predominantly taken up by liver nonparenchymal cells and uptake was inhibited by the pre-administration of dextran sulfate, suggesting the involvement of a phagocytic process. 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. However, while intravenous administration of pDNA complexed with either cationic liposome or polymers led to gene expression in various tissues, the level of gene expression in the lung was extremely high (Zhu et al. 1993; Song et al. 1997; Goula et al. 1998; Uyechi et al. 2001). Therefore, in order to improve the delivery of pDNA to target cells, such as hepatocytes, macrophages, and tumors, several ligands have been used.

4 Biological Barriers to Gene Delivery

Many in vitro studies on gene delivery have not been predictive of in vivo functionality; therefore, most formulations are effective in vitro but fail to function in vivo. Cell culture models do not replicate many biological factors important for in vivo gene delivery. For example, under in vitro conditions of cellular proliferation, the charge of the complex, its size , and its interaction with erythrocytes are substantially different than under in vivo conditions. The following conditions must be taken into consideration in designing a gene delivery system for use in vivo: (1) The carrier should form a stable complex that can deliver intact DNA in the blood circulation; (2) the cationic nature of the complexes should be considered in order to allow escape from nonspecific biodistribution via electrostatic interactions; (3) the size of the complexes should be considered regarding access to target cells; (4) the carrier should contain appropriate ligands in order to ensure a high affinity for cellular receptors (Fig. 1).

4.1 Stability of pDNA Complexes in the Blood

When naked pDNA into administered intravenously to mice, it is rapidly degraded by DNAase and/or due to uptake by Kupffer cells; and thus gene expression is prevented. Both cationic liposomes and/or polymer improve the stability of pDNA by condensing it to particles of defined size by electrostatic interactions. Furthermore, condensed pDNA is protected from both degradation in the blood stream and nonspecific interaction with cell surfaces.

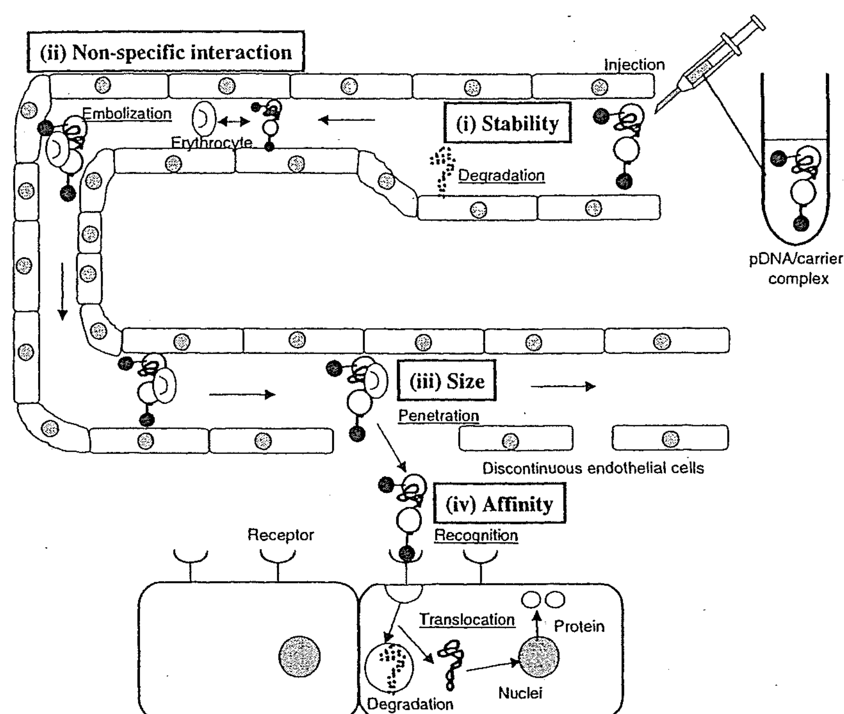

FIG. 1. Barriers to in vivo gene transfer by ligand-modified gene carriers

4.2 The Effect of Cationic Charge on In Vivo Gene Delivery

Yang and Huang (1997) reported that neutralization of the excess positive charge in the pDNA/cationic liposome complex by negatively charged serum proteins likely reduces the transfection efficiency in vitro. They demonstrated that this problem can be overcome by increasing the cationic charge ratio of pDNA/cationic liposome complexes, and that the optimal charge ratio (−:+) was 1.0:4.0 for efficient transfection, even in the presence of 20% serum. After intravenous administration, gene expression in the lung of pDNA/cationic liposome complexes carrying a higher cationic charge is enhanced due to electrostatic interactions. However, the cationic charge of pDNA/cationic liposome complexes can also result in non-specific biodistribution via electrostatic interactions.

We have evaluated the effect of cationic charge on asialoglycoprotein-receptor-mediated gene transfection systems using pDNA/galactosylated cationic liposome complexes administered intraportally (Kawakami et al. 2000a; Fumoto et al. 2003). When pDNA/galactosylated cationic liposome complexes were prepared at a charge ratio (−:+) of 1.0:2.3 and/or 1.0:3.1, selective gene expression in the liver was obtained, whereas at a charge ratio of 1.0:7.0, gene expression in the lung exceeded that in the liver, suggesting the highly non-specific interactions. Therefore, a cationic charge ratio (−:+) of pDNA to galactosylated cationic liposomes of 1.0:2.3 and/or 1.0:3.1 seems to be optimal for receptor-mediated in gene transfection.

We have also evaluated the effect of cationic charge on mannose receptor-mediated gene transfection systems using pDNA/mannosylated cationic liposome complexes administered intravenously (Kawakami et al. 2004). The transfection efficiencies in

liver and spleen, which express mannose receptors on their cell surfaces, after intravenous administration of complexes with a charge ratio (–:+) of 1.0:2.3 and/or 1.0:3.1 were higher than those in the lung. When complexes were formed at a charge ratio (–:+) of 1.0:4.7, the transfection efficiency in the lung was higher. These results confirm that complexes at a charge ratio (–:+) of 1.0:2.3 and/or 1.0:3.1 are optimized for receptor-mediated gene delivery systems using ligand-modified gene carriers.

4.3 The Effect of Size on In Vivo Gene Delivery

The size of the complexes is an important factor for controlling targeted gene delivery systems because the structure of the capillary wall varies greatly in different organs and tissues. Because of its large molecular weight, pDNA dose not effectively penetrate endothelial and epithelial barriers and can hardly extravasate from the vascular to the interstitial space. Following complex formation with cationic carriers, not only is pDNA condensed but its cellular uptake is also enhanced via electrostatic interactions.

pDNA/carrier complexes are often prepared in a non-ionic solution due to their well-known tendency to aggregate out of solution as the salt concentration is increased (Ogris et al. 1998). Aggregation during lipoplex formation in ionic solution might be due to neutralization of the surface positive charge of the lipoplex intermediates by the associated counter-ions. pDNA complexed with cationic liposomes at a charge ratio (–:+) of 1.0:2.3 is well condensed (100–200 nm) when the complexes are prepared in non-ionic solvent, such as dextrose and sucrose, compared with ionic solvent (Kawakami et al. 2000a).

5 In Vivo Receptor-Mediated Gene Delivery

The endothelium is a monolayer of metabolically active cells that mediate the bidirectional exchange of fluid between plasma and interstitial fluid. Thus, the endothelium has a profound influence on the extravasation of macromolecules. Discontinuous capillaries, also known as sinusoidal capillaries, are common in the liver, spleen, and bone marrow.

Tumor tissues are characterized by increased interstitial pressure, which may retard the extravasation of macromolecules. In addition, a lack of functional lymphatic drainage results in the passive accumulation of macromolecules. Capillary vessels in a human tumor inoculated into SCID mice are permeable even to liposomes up to 400 nm in diameter (Yuan et al. 1995). Hence, ligand-modified targeted gene delivery systems can be readily applied in liver, spleen, and tumors because of the size factor. In vivo receptor-mediated gene delivery systems are listed in Table 1.

5.1 Asialoglycoprotein Receptor-Mediated Gene Transfection

Hepatocytes exclusively express large numbers of high-affinity cell-surface receptors that bind and subsequently internalize asialoglycoproteins. In order to achieve liver-parenchymal-cell-specific gene transfection, a galactose moiety is introduced onto either cationic polymers or cationic liposomes.

TABLE 1. In vivo receptor-mediated gene delivery

Receptor	System	Results
Asialoglycoprotein		
Wu 1988	Asialoorosomucoid-polylysine	Expression in liver after i.v. injection
Perales 1994	Galactose-polylysine	Expression in liver after i.v. injection
Hara 1995	Asialofetuin-liposome	Expression in liver after intraportal injection
Kawakami 2000	Galactose-liposome	Expression in hepatocytes after intraportal injection
Nishikawa 2000	Galactose-polyornithine-HA2	Expression in hepatocytes after i.v. injection
Morimoto 2003	Galactose-PEI	Expression in hepatocytes after intraportal injection
Mannose		
Kawakami 2000	Mannose-liposome	Expression in non-parenchymal cells after i.v. injection
Kawakami 2004	Mannose-liposome	Expression in non-parenchymal cells after i.v. injection; effect of cationic charge
Hattori 2004	Mannose-liposome	Enhancement of immune responses by DNA vaccination
Transferrin		
Ogris 1999	Transferrin-PEG-PEI (800 kDa)	Expression in cancer cells (s.c.) after i.v. injection
Kircheis 2001	Transferrin-PEI (22 kDa)	Expression in cancer cells (s.c.) after i.v. injection
Kursa 2003	Transferrin-PEG-PEI(22 kDa)	Expression in cancer cells (s.c.) after i.v. injection
Folate		
Hofman 2002	Folate-liposome	Expression in cancer cells (s.c.) after i.v. injection
Reddy 2002	Folate-liposome	Efficient expression in intraperitoneal cancer cells after intraperitoneal injection

In the late 1980s, Wu et al. (1988) demonstrated successful in vivo gene transfer to liver using poly-L-lysine linked with asialo-orosomucoid (Chowdhury et al. 1993). Successful in vivo gene expression after intravenous injection has been also reported for glycosylated poly-L-lysine (Perales et al 1994). While these fusogenic peptides could be promising materials for enhancing in vivo gene expression, their transfection efficacy is low and must be improved for their successful use in gene therapy. Nishikawa et al. (2000) demonstrated that galactosylated poly-L-ornithine conjugated with a fusogenic peptide was very effective in improving the level of gene transfection after intravenous administration in mice.

In general, the transfection efficacy of cationic liposomes is higher than that of cationic polymers. Therefore, by using galactosylated cationic liposomes, effective hepatocyte targeting might be achieved. Liposomes can be galactosylated by coating them with either glycoproteins or galactose-conjugated synthetic lipids. As for targeted gene delivery by liposomes, Hara et al. reported that asialofetuin-labeled liposomes encapsulating pDNA were taken up by cultured hepatocytes via asialoglycoprotein receptor-mediated endocytosis and that the highest levels of hepatic gene expression were obtained after intraportal injection with a preload of EDTA (Hara

et al. 1995). However, the introduction of asialoglycoproteins to liposomes is complicated and there are a number of problems associated with the carriers, such as reproducibility and immunogenicity. Therefore, low-molecular-weight glycolipids appear to be more promising due to their low immunogenicity and high reproducibility. Remy et al. (1995) reported the feasibility of using galactose-presenting lipopolyamine vectors for targeted gene transfer into hepatoma cells under in vitro conditions. Inclusion of galactose residues in the electrically neutral complex increased transgene expression to nearly the value obtained with a large excess of cationic liposomes alone. The authors suggested that the galactose-presenting DNA particles avoid interacting with serum proteins because of their electrical neutrality.

Successful in vivo gene delivery systems require a thorough understanding of the pharmacokinetics and physicochemical properties of the complexes as well a theoretical design for galactosylated lipids as ligands for binding to target cells. Based on these considerations, we synthesized cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)alkyl)for mamide (Gal-C4-Chol), which possesses both the cationic charge necessary for pDNA binding and galactose residues as targetable ligands for binding to liver parenchymal cells. In vivo gene transfer was tested by optimizing the pharmacokinetics and physicochemical properties of the complexes (Kawakami et al. 2000a). The galactosylated lipid Gal-C4-Chol was specially designed as a modified cationic lipid because galactose residues can be stably fixed on liposomal membranes under in vivo conditions. The radioactivity in the liver from the Gal-C4-Chol liposome/[^{32}P] pDNA complexes was about 75% of the dose as early as 1 min after intraportal administration. Furthermore, hepatic gene expression of pDNA complexed with Gal-C4-Chol liposomes was more than a ten-fold greater than that of pDNA complexed with conventional cationic liposomes. When intrahepatic cellular levels of gene expression were examined, the expression by liver parenchymal cell (PC) of pDNA complexed with Gal-C4-Chol liposomes was significantly higher than that of liver non-parenchymal cells (NPC). By contrast, gene expression of PC and NPC of conventional cationic liposomes was almost the same. In addition, when an excess of galactosylated bovine serum albumin (Gal-BSA) was intravenously injected 5 min prior to injection of pDNA complexed with Gal-C4-Chol liposomes; gene expression in the liver was significantly reduced, suggesting that uptake occurred by asialoglycoprotein receptor-mediated endocytosis. Although the size of the Gal-C4-Chol liposome/pDNA complexes is about 120 nm, the highest level of gene expression was observed in the lung. We previously reported that intravenously administered pDNA/cationic liposome complexes interact with erythrocytes (Sakurai et al. 2001), which suggests that the Gal-C4-Chol liposome/pDNA complexes are aggregated by non-specific interaction with erythrocytes. Recently, Eliyahu et al. (2002) also reported that the medium (i.e. plasma and serum) and/or modification of cationic liposomes with 1% polyethyleneglycol lipids reduced the aggregation of pDNA/cationic liposome complexes in the presence of erythrocytes. As a consequence, polyethyleneglycol coating should enhance the cell-specificity of pDNA complexed with Gal-C4-Chol liposomes, even after intravenous administration. However, further studies on the interaction with blood components and on the synthesis of polyethyleneglycol-grafted glycosylated lipids for cell-selective gene delivery are needed.

5.2 Mannose Receptor-Mediated Gene Transfection

Macrophages are important targets for the gene therapy of diseases such as Gaucher's disease and human immunodeficiency virus (HIV) infection, but gene transfection of these cells is not easy. Non-viral vectors offer advantages for in vivo gene delivery because they are simpler and safer than viral systems. While the addition of DEAE-dextran is one method used for gene delivery to macrophages in vitro, this method is generally not suitable for in vivo use due to problems associated with cellular toxicity, low efficiency, and non-specific biodistribution. Erbacher et al. (1996) investigated the suitability of various glycosylated poly(L-lysine) derivatives for introducing pDNA into human-monocyte-derived macrophages and found that mannosylated poly(L-lysine) exhibited high transfection activity. The authors also reported that transfection activity was markedly enhanced in the presence of chloroquine due to the prevention of endosomal and/or lysosomal degradation of pDNA after mannose-receptor-mediated endocytosis. However, for in vivo application, it is difficult to use chloroquine, which limits the use of this approach.

One of the most promising non-viral gene delivery systems developed so far involves cationic liposomes because of their high in vivo transfection efficiency. Recently, we synthesized a novel mannosylated cholesterol derivative, Man-C4-Chol, for mannose receptor-mediated gene transfection of macrophages (Kawakami et al. 2000b, 2001), which express large numbers of mannose receptors on their surfaces. In primary cultured mouse peritoneal macrophages, pDNA complexed with Man-C4-Chol liposomes had a higher transfection activity than pDNA complexed with conventional cationic liposomes. The presence of 20 mM mannose significantly inhibited the transfection efficiency of the pDNA/Man-C4-Chol liposome complexes, suggesting that they are recognized and taken up by mannose receptors on macrophages. Gene transfection in macrophages was further enhanced by incorporating PEI into these complexes (pDNA/Man-C4-Chol-PEI-complexes), since it was observed that PEI has a pH-buffering effect in endosomes as well as DNA-condensing activity (Sato et al. 2001). In mouse peritoneal macrophages, the uptake and transfection activities of pDNA/Man-C4-Chol-PEI-complexes were, respectively, two- and six-fold higher than those of pDNA/ Man-C4-Chol liposome complexes. The presence of 1 mg mannan/ml significantly inhibited both the uptake and transfection efficiency of the complexes, suggesting a mechanism of mannose receptor-mediated endocytosis.

As for in vivo gene transfection, the highest level of gene expression was observed in the liver after intravenous injection of pDNA/Man-C4-Chol liposome complexes in mice. In the liver, expression was higher in NPC and was significantly reduced by pretreatment with mannosylated bovine serum albumin. These results suggest that pDNA complexed with mannosylated liposomes exhibits high transfection activity in liver NPC due to recognition by mannose receptors. In contrast to results obtained with Gal-C4-Chol liposome/pDNA complexes, cell-selective gene transfection could be achieved by the intravenous administration of Man-C4-Chol liposome/pDNA complexes. This phenomenon could be explained by the fact that the discontinuous capillaries in liver and spleen allow macrophages to come in contact with the complexes without passing through the sinusoids (100–200 nm). Hence, mannosylated gene carriers are effective for NPC-selective gene transfection.

Mannosylated liposomes also have potential applications in DNA vaccine therapy, because antigen-encoded pDNA must be efficiently transfected into dendritic cells, which express a large number of mannose receptors. Recently, Hattori et al. (2004) demonstrated the targeted delivery of DNA vaccine by Man-C4-Chol liposomes.

5.3 Transferrin Receptor-Mediated Gene Transfection

Transferrin, an iron-binding glycoprotein, is a well-studied ligand for tumor targeting. Iron-loaded transferrin is recognized by and binds to transferrin receptors on cell surfaces. In rapidly dividing cells, expression the receptor is elevated due to an increased cellular need for iron, while on the surfaces of malignant cells expression is often unregulated. Thus, transferrin has been used as a tumor-targeting ligand for several drug delivery systems.

Recently, a transferrin-linked polyethylenimine for tumor-selective gene transfection was developed (Ogris et al. 1999; Kircheis et al. 2001). In order to block undesired, non-specific interactions with blood components or non-target cells, the surface charge of the complexes was masked by either covalently attached hydrophilic polyethylene glycol or a higher density of attached transferrin. After intravenous injection, gene expression in the tumors was approximately 100-fold higher than in other tissues. More recently, the intravenous injection of PEG-PEI-transferrin containing pDNA encoding for tumor necrosis factor (TNF-α) was shown to inhibit tumor growth in murine tumor models (Kursa et al. 2003).

5.4 Folate Receptor-Mediated Gene Transfection

The folate receptor is overexpressed in a large fraction of human tumors, but is only minimally distributed in normal tissues. Therefore, this receptor has been used as a tumor-targeting ligand for several drug delivery systems. Recently, Hofland et al. (2002) synthesized folate-PEG-lipid derivatives for preparing folate-modified cationic liposomes. After intravenous injection of the folate-liposome complexes, gene expression in the tumors was not changed while that in the lungs was reduced compared with conventional complexes. After intraperitoneal injection into murine disseminated peritoneal tumors, however, the folate-liposome complex formulations produced an approximately ten-fold increase in tumor-associated gene expression, as compared with conventional complexes (Reddy et al. 2002).

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Evaluation of Gene Expression In Vivo After Intravenous and Intraportal Administration of Lipoplexes

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1 Introduction

Numerous kinds of synthetic compounds, such as cationic lipids and cationic polymers, have been developed as candidates for non-viral gene carrier molecules for use in gene therapy. Cationic compounds generally form positively charged complexes with DNA, and many of them have shown good results in in-vitro gene transfection experiments. However, in vivo, there are considerable effects of inactivation through non-specific interactions with plasma proteins (Dash et al. 1999, 2000; Tandia et al. 2003) and of rapid clearance by the reticuloendothelial system. Indeed, compounds that can be efficiently transfected into cultivated cells are not always necessarily applicable in vivo. Thus, new gene carriers, applicable to gene delivery in vivo, are needed.

Gene carrier molecules are required to have not only transfection ability but also therapeutic effects mediated by a transgene encoding therapeutic proteins, such as cytokines. A simple assay of in vivo transgene expression would allow the screening of effective gene carriers from a number of candidates. At present, the reporter gene assay is generally used to evaluate the transfection ability of gene carriers. In this system, firefly luciferase, β-galactosidase and green fluorescence protein are commonly used as reporter genes. The luciferase system is superior to the because it is easy to carry and does not require complicated techniques or expensive equipment. In addition, its simplicity enables the screening of a large number of samples, from various organs and a wide range of sample preparation methods.

This chapter focuses on the evaluation of transgene expression in vivo using firefly luciferase as the reporter gene after intravenous and intraportal injection. In addition, a method to separate parenchymal cells (PC) and non-parenchymal cells (NPC) from liver that facilitates the hepatic cellular localization of luciferase activity is introduced.

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2 Materials

2.1 Chemicals

N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), cholesteryl chloroformate, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Diphosphatidyl-ethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester and *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) were obtained from Tokyo Chemical Industry (Tokyo, Japan). The cationic cholesterol derivative, 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) was synthesized as previously reported (Gao and Huang 1991). All other chemicals were obtained commercially as reagent-grade products.

2.2 Preparation of Plasmid DNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN, Hilden, Germany). Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The DNA concentration was measured by UV absorption at 260 nm.

2.3 Preparation of Liposomes

1. A round-bottomed flask of appropriate size
2. Rotary evaporator
3. Vacuum desiccator
4. Bath-type and probe-type sonicator
5. Extruder (Liplex Biomembrane, Vancouver, Canada) or 0.45-μm pore size filter

2.4 Evaluation of Transgene Expression in Mice

1. Lysis buffer; 0.1 M Tris-HCl, 0.05% Triton X-100, 2 mM EDTA, pH 7.8
2. Homogenizer; Omni-Mixer TH (Omni International., Marietta, GA, USA)
3. Pica gene luminescence kit (TOYO B-Net, Tokyo, Japan)
4. Luminometer; Lumat LB 9507 (EG & G Berthold, Bad Wildbad, Germany)
5. Protein Quantification Kit (DOJINDO LABOLATORIES, Kumamoto, Japan)

2.5 Separation of Parenchymal and Non-parenchymal Cells from Liver

1. Ca^{2+}-, Mg^{2+}-free perfusion buffer; 10 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, and 0.4 mM Na_2HPO_4, pH 7.2
2. Ca^{2+}- and collagenase-supplemented perfusion buffer; Ca^{2+}-, Mg^{2+}-free perfusion buffer supplemented with 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I), pH 7.5

3. Hank's-HEPES buffer; 10 mM HEPES, 137 mM NaCl, 0.4 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, 3 mM $NaHCO_3$, 5.6 mM glucose, pH 7.3
4. Peristaltic pump SJ-1211 (ATTO, Tokyo, Japan)

3 Methods

3.1 Synthesis of Sugar-Modified Cholesterol Derivatives

Galactosylated and mannosylated cholesterol derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosyl-ethyl)amino)butyl) formamide (Gal-C4-chol) and cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosyl-ethyl)amino)butyl) formamide (Man-C4-Chol), respectively, were synthesized as previously described (Kawakami et al. 1998, 2000c). Briefly, cholesteryl chloroformate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 4 h at 4°C. The C4-Chol was reacted with five equivalents of 2-imino-2-methoxyethyl-1-thiogalactoside or 2-imino-2-methoxyethyl-1-thiomannoside, which were synthesized as previously described (Lee et al. 1976), in pyridine containing 1.1 equivalents of triethylamine for 24 h at room temperature. After evaporation of the reaction mixture in vacuo, the resultant material was suspended in water and dialyzed against water for 48 h using a dialysis membrane with a 12-kDa cut-off). After the dialyzate was lyophilized, the crude product was purified by re-crystallization three times in ethyl acetate.

3.2 Preparation of Liposomes

1. Dissolve lipids and/or cholesterol derivatives in chloroform at appropriate ratio.
2. Evaporate the mixture to dryness in a round-bottomed flask by rotary evaporator. The lipid film should be as thin and uniform as possible.
3. Vacuum-desiccate the resultant lipid film for overnight. Chloroform should be removed completely, since residual chloroform affects hydration of the lipid film and the formation of liposomes.
4. Hydrate the lipid film with sterile 5% dextrose solution.
5. Sonicate the dispersion for 5 min in a bath-type sonicator to form liposome.
6. Sonicate the liposome for 3 min on ice by using a probe-type sonicator.
7. Extrude the liposome ten times through a double-stacked 100-nm pore size polycarbonate membrane filter at 60°C by using an extruder. Alternatively, filter the liposomes with a 0.45-μm pore size filter.

3.3 Preparation of Lipoplexes

The liposome/DNA complexes (lipoplexes) for in vivo experiments were prepared as described by Templeton et al (1997). Briefly, equal volumes of DNA and stock liposome solution were diluted with 5% dextrose to produce various ratios of DNA/liposomes and then mixed in a microtube at room temperature.

1. Dilute DNA and liposome stock solution with 5% dextrose.
2. Add DNA solution rapidly to the surface of the liposome solution using a Pipetman pipette.